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A Role for Oxytocin-Like Receptor in Social Habituation in a Teleost

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Hippocampus · Preoptic area · Immediate early genes · Social bonding · Social habituation · Gene expression · Teleost

Abstract
Oxytocin (OT) mediates social habituation in rodent model systems, but its role in mediating this effect in other vertebrates is unknown. We used males of the African cichlid fish, *Astatotilapia burtoni*, to investigate two aspects of isotocin (IT; an OT homolog) signaling in social habituation. First, we examined the expression of IT receptor 2 (ITR2) as well as two immediate early genes in brain regions implicated in social recognition. Next, we examined IT neuron activity using immunohistochemistry. Patterns of gene expression in homologs of the amygdala and hippocampus implicate IT signaling in these regions in social habituation to a territorial neighbor. In the preoptic area, the expression of the ITR2 subtype and IT neuron activity respond to the presence of a male, independent of familiarity. Our results implicate IT in mediating social habituation in a teleost.

Introduction

Oxytocin (OT) is an evolutionarily ancient neuropeptide, produced in neurosecretory cells in the brain in lineages from mammals to segmented worms [Tessmar-Raible et al., 2007; Donaldson and Young, 2008]. OT has been implicated in a variety of social behaviors, including social recognition, learning and memory, and the modulation of aggression, fear, and anxiety [Choleris et al., 2004; Lee et al., 2009]. It has been well-studied for its role in mediating monogamous pair bonds in voles and finches [Klatt and Goodson, 2013; Johnson and Young, 2015], same-sex social bonds in mammals [Beery and Zucker, 2010; Crockford et al., 2013; Romero et al., 2014], and paternal care in mammals [Gordon et al., 2010] and cichlid fishes [O'Connell et al., 2012]. Interestingly, OT may alter social attention by suppressing vigilance toward threatening social stimuli, which explains many of its observed prosocial effects [Ebitz et al., 2013; Ebitz and Platt, 2014]. In summary, OT appears to have a role in social habituation processes, particularly in mammals, whereby an individual decreases their response to a social stimulus after repeated exposure [Heyes, 1994].

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The habituation/dishabituation procedure has provided a wealth of information regarding the effects of OT and related signaling molecules on social habituation and recognition in rodent model systems [Gheusi et al., 1994; Choleris et al., 2006]. Importantly, behavioral ecologists have intensively studied a related phenomenon across species within the context of the "dear enemy" effect, whereby territorial individuals show reduced aggression to familiar neighbors [Temeles, 1994]. We consider social habituation to be the main process mediating reduced aggressive behavior between neighboring conspecifics [Owen and Perrill, 1998; Bee and Gerhardt, 2001, reviewed in Peeke and Peeke, 1973]. Given that neighbors are not always in sight, discrimination between individual conspecifics is necessary and can occur via individual recognition and/or based on location or situation. Despite the common occurrence of social habituation across species, surprisingly, the role of nonmammalian OT homologs in social habituation has not yet been examined. Interestingly, though the role of these nonapeptides in social behavior has been well documented, the specific effect of OT homologs on social behavior remains unclear [Godwin and Thompson, 2012]. Furthermore, little is known about the location(s) of action of OT homologs in the brain during social habituation processes.

Here, we use the highly social African cichlid fish, *Astatotilapia burtoni*, a model system in social neuroscience [Hofmann, 2003; Fernald and Maruska, 2012], to test the hypothesis that the anamniote OT homolog (ortholog), isotocin (IT), plays a role in mediating social habituation. Territorial *A. burtoni* males aggressively defend their spawning territory from other males, but they exhibit a robust dear enemy effect with their familiar neighbors via reduced aggression over time [Weitekamp and Hofmann, 2017]. In order to investigate the mechanisms of social habituation, an important component of the dear enemy effect, we examined 2 forms of IT signaling. In teleosts, IT is produced in the preoptic area (POA), and IT receptors (ITRs) are widely distributed throughout the brain [Huffman et al., 2012]. We chose to examine the POA as well as the homologs of the amygdala (AMY) and hippocampus (HIP), as these regions contain ITRs [Huffman et al., 2012] and are known to mediate social cognition in mammals [Popik and van Ree, 1991; Adolphs, 2010; Spreng and Mar, 2012], possibly also in teleosts [Demski, 2013]. Furthermore, their large relative size and location at the perimeter of the brain make them amenable to molecular studies.

The goal of these experiments was to identify neural correlates of social habituation by comparing territorial males that were either repeatedly exposed to another territorial male (familiar neighbor; FN), repeatedly exposed to a social control (no neighbor; NN), or exposed to a novel territorial male (novel male; NM) (Fig. 1). Given that the study species, *A. burtoni*, is capable of individual recognition [Grosenick et al., 2007; Desjardins et al., 2010], it seems likely that repeated exposure to another male results in familiarity. In the first experiment, we examined the gene expression of ITR2 (*itr2*) and 2 immediate early genes (IEGs) commonly used as markers of neuronal activity, *c-fos* and *egr-1* [Herdegen and Leah, 1998], in putative teleost homologs of the mammalian AMY, POA, and HIP [O'Connell and Hofmann, 2011]. We predicted that the FN treatment would result in an upregulation of ITR in each brain region. In the second experiment, we examined colocalization of IT with c-Fos in the POA, the primary source of IT in teleost fishes, to examine the role of IT neuron activation in social habituation, predicting there would be more IT neuron activity with the FN treatment.

Materials and Methods

Behavioral Paradigm

The original research reported here was performed under guidelines established by the Institutional Animal Care and Use Committee at the University of Texas at Austin, TX, USA. All fish were first maintained in naturalistic communities comprising adult females and territorial and nonterritorial males. The experiments were performed in adjacent 35-L experimental tanks, separated by an opaque divider. Each tank contained terracotta shards to serve as a bower for the focal male or a refuge for nonreproductive females. These females were taken from stable-community tanks shortly after spawning and after removing their brood, so as to provide a social environment of neutral valence for the males. *A. burtoni* females require approximately 3 weeks after spawning before again becoming gravid and, thus, attractive to males [Kidd et al., 2013].

For the FN and NM treatments, size-matched males (within 1 mm of standard length) originating from separate community tanks were placed in adjacent tanks (Fig. 1). For NN, a male was placed in 1 tank while the adjacent tank contained an additional nonreproductive female and no males. The opaque divider remained in place between the paired tanks for 1 week to allow the fish to acclimate to the experimental setup. Following the acclimation period for the FN and NN treatments, we removed the opaque divider for 1 h twice daily, at 10:00 and 15:00 h, for 4 continuous days. For the NM, we removed the opaque divider only on day 4 at 10:00 h. Fish readily interact socially across the adjacent tanks [O'Connell et al., 2013].

Following 1 h of exposure on day 4, all males were killed by rapid cervical transection. Their brains were treated in 1 of 2 ways. For experiment 1, they were immediately embedded in OCT (Tissue-Tek, Fisher Scientific, Pittsburgh, PA, USA), and frozen on dry

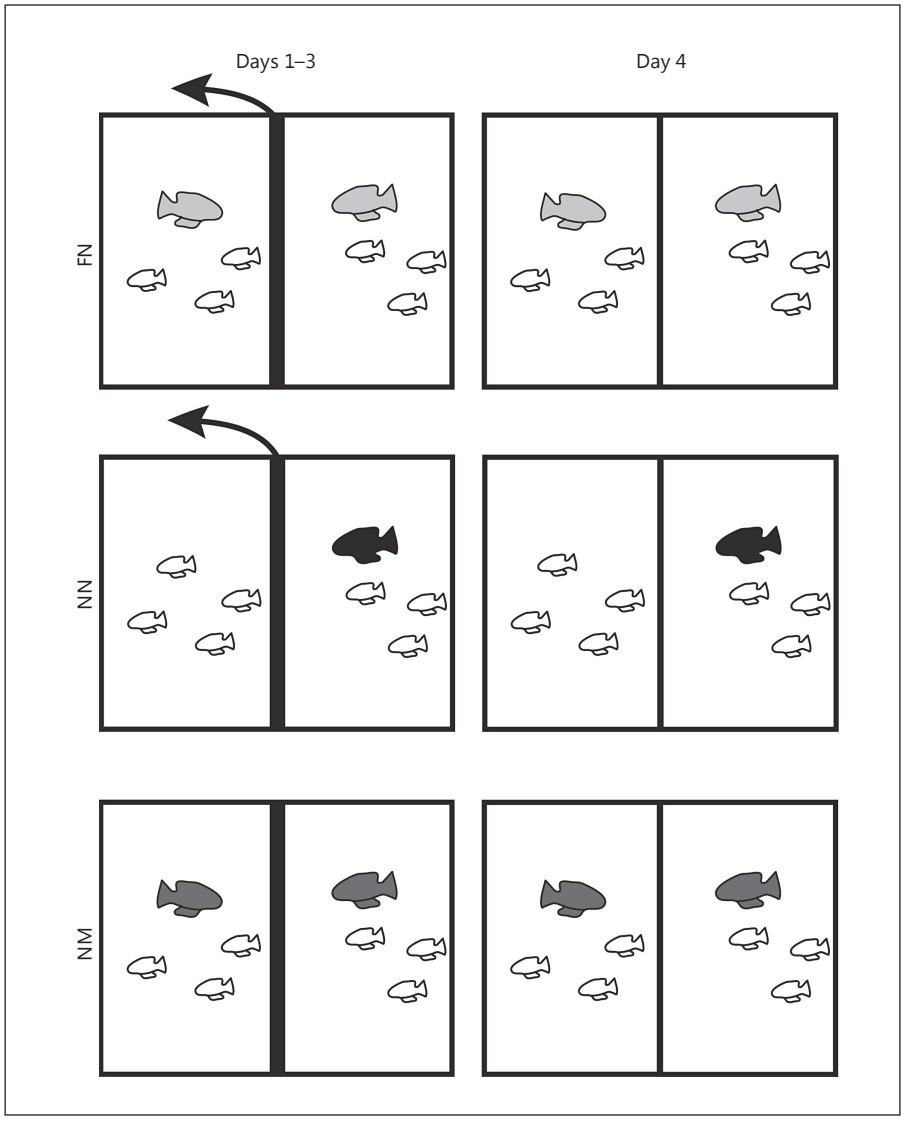

Fig. 1. Experimental design. The visual barrier separating adjacent tanks was removed on days 1–3 for the familiar neighbor (FN) and no neighbor (NN) treatments, allowing males to habituate to a male and 3 nonreproductive females, or to 4 nonreproductive females, respectively. For the novel male (NM) treatment, the divider remained in place on days 1–3. On day 4, the dividers were removed for all treatment groups.

ice. For experiment 2, the brains were placed in chilled 4% paraformaldehyde and fixed overnight at 4°C. They were then rinsed in 1× PBS and cryoprotected in 30% sucrose at 4°C overnight. Finally, they were embedded in OCT and frozen on dry ice. All brains were stored at –80°C until further processing.

The males in experiment 1 were older and (due to indeterminate growth) larger (57.4 mm on average; range 52–63 mm) than in experiment 2 (50 mm on average; range 45–56 mm). In this species, older males are less aggressive [Weitekamp et al., 2017]. However, because there were no differences within the groups for each experiment, the treatment group comparisons should not be affected by size.

We recorded behavior on day 1 (FN only) and day 4 (FN and NM) immediately following the removal of the opaque divider. The behavior of the focal resident male was scored using JWatcher v1 [Blumstein and Daniel, 2007] for a 10-min duration 20 min after the removal of the divider. We quantified forward and lateral displays toward the adjacent male, as well as chases and reproductive displays toward the females. Forward and lateral displays were summed as "aggressive displays." Female directed displays occurred at a very low frequency, consistent across groups and, as such, were not examined further.

Experiment 1: Candidate Gene Expression

Quantitative PCR

Brains were sliced on a cryostat in the coronal plane at 300 µm. A 300-µm-diameter sample corer tool (Fine Science Tools, Foster City, CA, USA) was used to microdissect the Dm-3 (a subdivision of the medial zone of the dorsal telencephalon; putative AMY homolog [O'Connell and Hofmann, 2011]), the POA, and the Dlv (the ventral subdivision of the lateral zone of the dorsal telencephalon; putative HIP homolog [O'Connell and Hofmann, 2011]). For the POA, we aimed to consistently include the portion that contains IT neurons, although adjacent regions were likely included as well. Two microdissected punches (left and right hemisphere) per region were taken and stored in DNA/RNA Shield (Zymo Re-

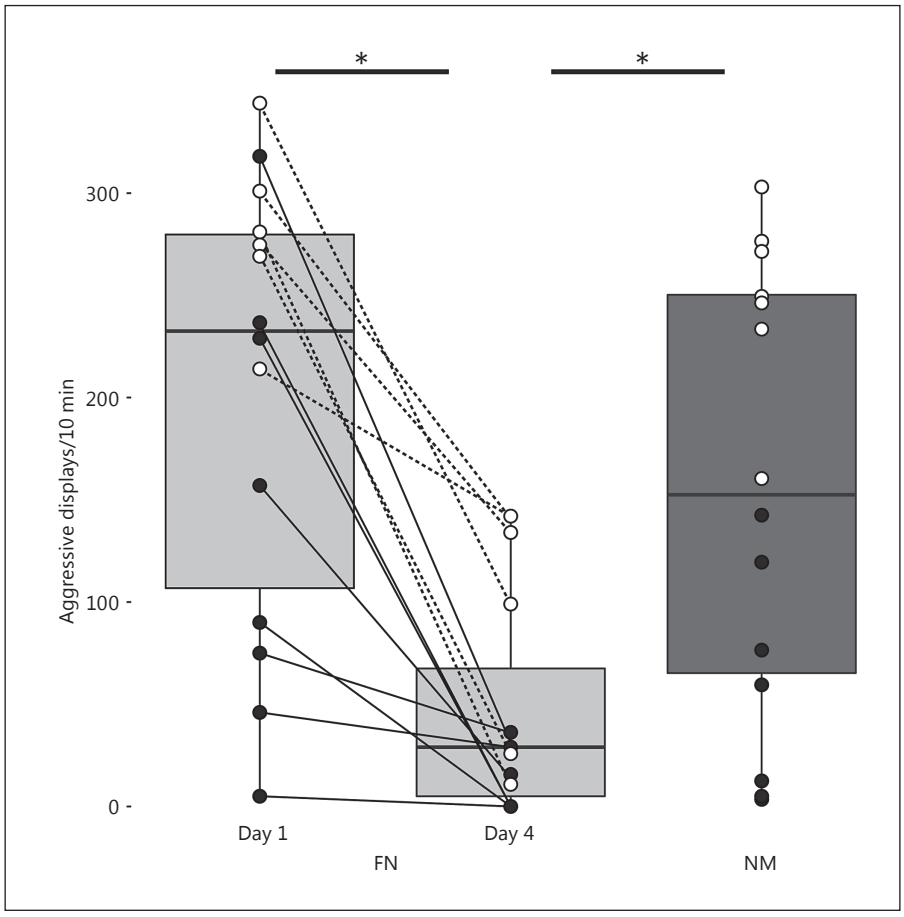

Fig. 2. Male-directed aggressive displays in the familiar neighbor (FN) and novel male (NM) treatment groups. In FN, aggressive displays toward the adjacent male are significantly lower on day 4 than on day 1 (lines show repeated measures; paired *t* test). Similarly, aggressive displays toward the adjacent male are significantly lower in FN (day 4) than in the NM group (the Welch *t* test). The no neighbor (NN) group is not included because there is no adjacent male and thus no male-directed aggression is displayed by the focal animal. $n = 14$ per group. The box plots show the median, and upper and lower quartiles, and range. Data from experiments 1 (qPCR; older males; filled circles, solid lines) and 2 (IHC; younger males; open circles, dashed lines) are indicated for clarity. * $\alpha = 0.05$ indicates significance.

search, Irvine, CA, USA) at –80°C until processing. To homogenize tissue prior to RNA extraction, ZR BashingBeads (Zymo Research) were added to samples suspended in DNA/RNA Shield, and the tubes were vortexed. To further lyse tissue, proteinase K digestion was done for 2 h at 55°C. Total RNA was extracted in accordance with the protocol for the Quick-RNA MicroPrep kit (Zymo Research). To prevent genomic DNA contamination, RNA samples were treated with DNase (Zymo Research) during the isolation procedure. RNA was reverse-transcribed to cDNA using the GoScript reverse transcription system (Promega Corp., Madison, WI, USA).

qPCR was used to measure the mRNA levels of *itr2*, *egr-1*, and *c-fos*. Many teleosts [Ocampo Daza et al., 2012], including cichlids [O'Connor et al., 2015], have 2 ITRs, likely a result of a whole-genome duplication that occurred in the common ancestor of all extant teleosts [Meyer and Van de Peer, 2005]. As the presence of 2 ITRs in cichlids was shown only recently, very little is known about their respective functions. Here, we examine ITR2, because its distribution has previously been characterized in the brain of *A. burtoni* [Huffman et al., 2012]. For each sample, target gene expression was measured in triplicate in the ViiA™ 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.

Experiment 2: IT Neuron Activity

Immunohistochemistry

Brains were sectioned (30 μm) on a cryostat and thaw mounted on Super Frost Plus slides (Fisher Scientific) into 4 series. Sections were incubated in a mix of 1:1,000 guinea pig-anti-OT polyclonal antibody (Millipore, AB15704) and 1:500 rabbit anti-c-Fos primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; cat. No. sc-253) in 1× PBS with 2% normal goat serum (NGS) and 0.3% Triton X-100 at room temperature overnight. Slides were washed twice in 1× PBS and then incubated in a mix of 1:200 anti-rabbit Texas Red (Invitrogen, T2767) and 1:800 anti-guinea pig Alexa Fluor 488 (Invitrogen, A11073) in a 2% NGS and 0.3% Triton X-100 in 1× PBS solution. After rinsing, slides were coverslipped using Vectashield hardset mounting media with DAPI (Vector Laboratories). The specificity of both antibodies was confirmed previously [O'Connell et al., 2012, 2013; IT and c-Fos, respectively].

Fluorescence Detection of c-Fos and IT

A fluorescence signal was detected using a Zeiss Axio Imager. A1 microscope (×10) equipped with FITC and TRITC filters to allow visualization of the immunoreactivity of IT with the FITC channel and of c-Fos with the TRITC channel. Photographs were taken with a digital camera (AxioCam MRc, Zeiss; ×20) using the AxioVision (Zeiss) image acquisition and processing software.

The observer was blinded to treatment. The number of neurons exhibiting colocalization of both IT and c-Fos in the POA was quantified by superimposing images generated by FITC and TRITC fluorescence filters. IT-positive neurons were visually identified by Alexa Fluor 488 staining in the cell bodies, and c-Fos-positive neurons were visually identified by Texas Red staining in the nucleus. Double-labeled neurons were identified by toggling between the IT image and the merged image (IT and c-Fos). The total number of neurons positive for IT as well as the number of IT and c-Fos colocalized neurons was determined by averaging the data from all the sections containing IT neurons (approx. 2/individual). The resulting data are presented as the proportion of IT neurons labeled with c-Fos.

Statistics

All statistical tests were performed using R v3.2.5. To demonstrate that males habituate in their aggressive response in the FN treatment, we used the paired Student *t* test on aggressive behavior on days 1 and 4, for which there were videos ($n = 14$). To examine for differences in aggressive behavior between the FN and NM treatments on day 4, we used the Welch 2-sample *t* test (FN: $n = 15$; NM: $n = 14$). In experiment 1, to determine the relative gene expression of each sample, the R package MCMC.qPCR was used [Matz et al., 2013]. This method is preferable to more traditional methods because it accounts for differences in efficiencies between genes. The package uses a Poisson-log normal generalized linear mixed model, based on raw cycle threshold values, to infer fold changes in target genes in response to fixed factors while accounting for random variation between replicates and samples. The model-fitting process employs a Bayesian Markov Chain Monte Carlo algorithm that can include control genes as priors. We included the treatment group as a fixed factor and used the control gene 18S as a prior in the models to further account for variation in initial RNA levels. The disadvantage to this approach is that normalized expression values cannot be extracted. In order to investigate correlations between gene expression and behavior, we also analyzed the data using the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001]. To examine associations between gene expression and behavior and IT neuron activity and behavior, we used linear regression analysis. The data from experiment 2 were analyzed using the Kruskal-Wallis test, and the Dunn test was used for post hoc comparisons. To adjust for multiple-hypothesis testing, we used the procedure of Benjamini and Hochberg [1995].

Results

With the FN treatment, aggressive behavior was lower on day 4 than on day 1 ($t = -5.93$, $p = 4.96 \times 10^{-5}$). Similarly, on day 4, aggressive behavior was lower with FN than with NM treatment ($t = 3.31$, $p = 3.77 \times 10^{-3}$) (Fig. 2).

Experiment 1: Candidate Gene Expression

In the Dm-3 (putative AMY homolog; Fig. 3), we found that *itr2* was significantly higher with FN than with both NN ($p = 2.64 \times 10^{-4}$) and NM ($p = 2.24 \times 10^{-3}$) treatment, but that *egr-1* and *c-fos* expression did not differ across treatments. In the Dlv (putative HIP homolog), *itr2* was significantly lower with NM than with both FN ($p = 0.030$) and NN ($p = 0.016$) treatment; *c-fos* was higher with NM than with both FN ($p = 6.31 \times 10^{-4}$) and NN ($p = 6.89 \times 10^{-5}$) treatment; *egr-1* was significantly higher with NM than with NN ($p = 0.042$) treatment and borderline significant with FN ($p = 0.056$) treatment. In the POA, *itr2* was higher with NN than NM ($p = 5.15 \times 10^{-3}$) treatment but was similar with FN, NN, and NM treatment; *egr-1* was lower with NN than with both FN ($p = 0.012$) and NM ($p = 0.022$) treatment; *c-fos* did not differ across treatments. Notably, *itr2* expression appeared to be higher in the POA than in the other 2 regions. The relationship between gene expression and aggressive behavior was nonsignificant in all 3 regions examined.

Experiment 2: IT Neuron Activity

There was a significant effect of treatment on the proportion of Fos-positive IT neurons in the POA (Kruskal-Wallis $\chi^2 = 6.67$, $p = 0.036$; Fig. 4a). The proportion of double-labeled neurons was higher with NN than with both FN ($p = 0.021$) and NM ($p = 0.0267$) treatment; FN and NM treatments did not differ ($p = 0.40$). There was a weak nonsignificant positive relationship between the proportion of double-labeled neurons and aggressive behavior ($R^2 = 0.224$, $p = 0.087$; Fig. 4c).

Discussion

Social habituation is common across a wide range of taxa, yet the neural mechanisms underlying this behavior have not been investigated outside of the habituation/dishabituation paradigm in rodents [Choleris et al., 2006]. In this study, we examined 2 forms of IT signaling, to test the hypothesis that the anamniote homolog of OT serves a role in mediating social habituation, an important component of the dear enemy effect [Peeke and Peeke, 1973]. We found evidence that *itr2* in the Dm, the putative AMY homolog, may play an important role in social habituation in teleosts (Fig. 3a). These data are consistent with the role of OT in mammalian social habituation. In rodents, the OT receptor in the medial AMY is critical for social recognition [Ferguson et al., 2001; Gur et al., 2014] and OT in the central AMY has anxiolytic effects [Bale et al., 2001]. In humans, the AMY is necessary for OT-facilitated socially reinforced learning [Hurlemann et al., 2010]. Together, this suggests a conserved role for IT/OT acting in the AMY in behaviors involved in social habituation.

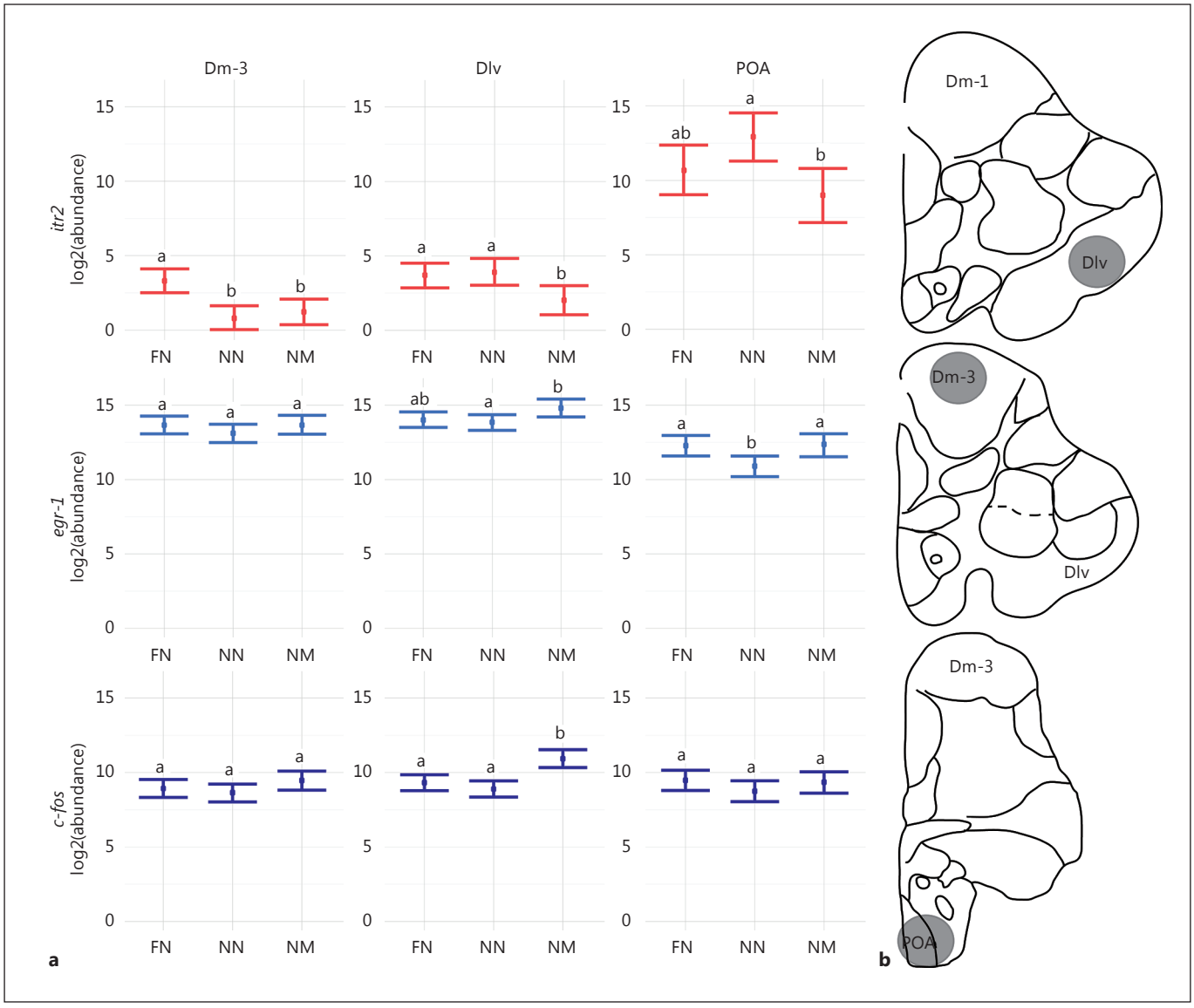

Fig. 3. a Transcript abundances of selected genes across treatment groups in Dm-3 (putative AMY homolog), POA, and Dlv (HIP homolog). The points are posterior means, the whiskers denote 95% credible intervals. Letters denote homogeneous subgroups (α = 0.05). *n* = 8 per group. **b** Representative coronal slices from which brain regions were microdissected, including the Dlv, Dm-3, and POA. Dm-3, medial zone of the dorsal telencephalon; Dlv, ventral part of the lateral zone of the dorsal telencephalon; POA, preoptic area.

Importantly, the homology relationships between amygdaloid nuclei in mammals and the corresponding pallial and subpallial regions in teleosts are still unclear [Maximino et al., 2013]. The tetrapod AMY itself has been notoriously difficult to classify as a coherent functional unit [Swanson and Petrovich, 1998]. Lesions to the Dm in goldfish, as opposed to in the Dlv, suggest that this region is part of an emotional learning and memory system, consistent with the mammalian AMY [Portavella et al., 2004b, 2004a]. Based on other functional, chemical, anatomical, and hodological evidence, the Dm has been proposed specifically to be homologous to the mammalian basolateral/lateral AMY [O'Connell and Hofmann, 2011; Maximino et al., 2013]. These homologies are not exact, however. For example, there does not appear to be a medial AMY in teleosts [Maximino et al., 2013]. Here, we show that *itr2* in the Dm responded to social habituation independent of olfactory input. This basolateral/lat-

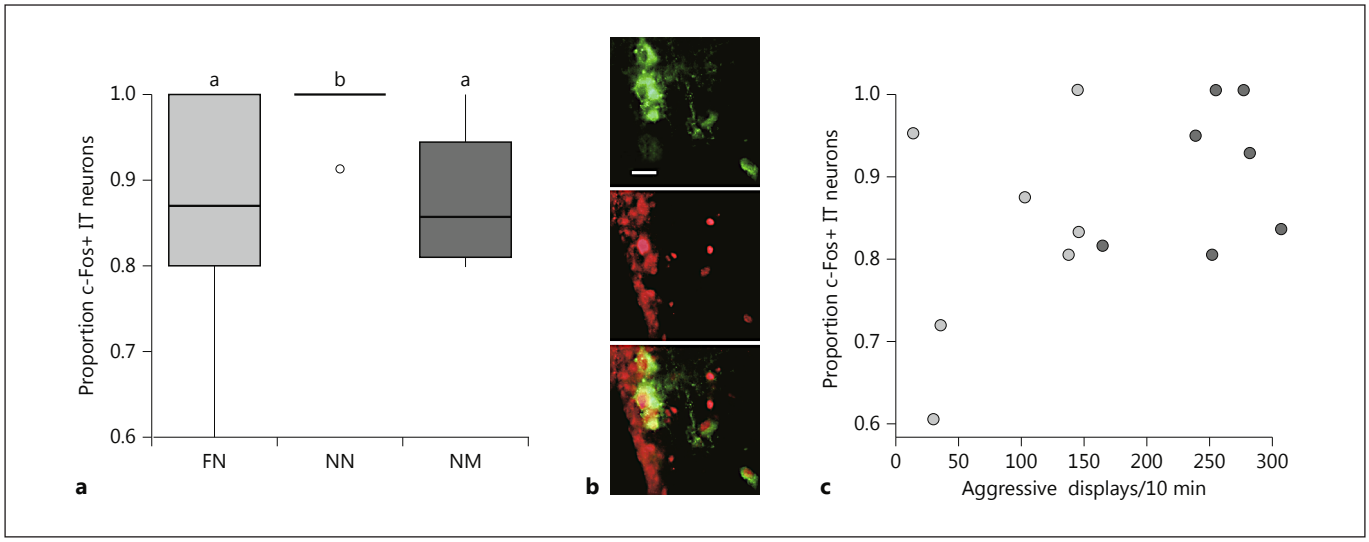

Fig. 4. Colocalization of IT and c-Fos in the POA. **a** The proportion of colocalized IT neurons is higher in the NN ($n = 7$) than in the FN ($n = 12$) and NM ($n = 9$) groups. Letters denote homogeneous subgroups ($\alpha = 0.05$; the Dunn test). The box plots show the median, upper and lower quartiles, and range. The circle shown in NN is an outlier. **b** Representative micrographs of IT (green cytoplasm; top), c-Fos (red nucleus; middle), and the merged image (bottom; ×40). The image is from the FN group and has been brightness enhanced. **c** There is a nonsignificant relationship ($\alpha = 0.05$) between the proportion of colocalized neurons and aggressive behavior (light grey circles, FN; dark grey circles, NM). $n = 7$ per group. FN, familiar neighbor; NM, novel male; NN, no neighbor; POA, preoptic area.

eral AMY-like nucleus in teleosts may function similarly to the medial AMY in social recognition processes.

In the HIP homolog, we found that the IEG *c-fos* was higher after exposure to an NM than with FN and NN exposure, with *egr-1* showing a similar pattern (Fig. 3a). IEGs may decrease with social habituation through repeated exposure to the NM stimulus. The IEGs serve important functions in learning and long-term memory [Tischmeyer and Grimm, 1999]. For example, mutant mice lacking *egr-1* fail to exhibit late long-term potentiation in the HIP and show impaired long-term memory, while the short-term memory remains intact [Jones et al., 2001]. Consistent with our data, in a region of the auditory telencephalon, the caudomedial neostriatum of the zebra finch (*Taeniopygia guttata*), *egr-1* expression is induced following the presentation of a novel song, but rapidly declines upon repeated exposure to the same song [Mello et al., 1995]. A similar pattern of *egr-1* expression is observed in the HIP homolog in the weakly electric fish, *Apteronotus leptorhynchus*, when males are presented with signals from familiar conspecifics [Harvey-Girard et al., 2010]. We also found that *itr2* expression in the HIP homolog decreased during exposure to an NM (Fig. 3a), which may interact with the IEG pathways in memory formation. It would be interesting to examine whether social habituation to an NM elicits different patterns of gene expression than with a less salient or nonsocial novel stimulus, which our experiment did not explicitly test.

In the POA, we found that the FN and NM contexts elicited similar response patterns, both in terms of gene expression and IT neuron activity. This region appears to respond to the presence of a male, independent of behavior or familiarity. Surprisingly, we found that a large majority of IT neurons are activated in all 3 social contexts (Fig. 4). In the NN condition, nearly 100% of the IT neurons were c-Fos-positive. In contrast, in the FN and NM contexts, a small subset (approx. 15%) of these cells were inactive, suggesting that the presence of a male results in the inhibition of some of these neurons. Furthermore, aggressive behavior did not correlate with IT neuron activity, suggesting that IT activity in the POA does not mediate aggressive behavior per se, but rather reflects social context. Interestingly, IEG induction in the POA showed an opposite pattern to IT neuron activity, in that *egr-1* expression levels are higher in the presence of a male. The *egr-1* mRNA may thus be part of ≥1 different functional pathways with different downstream targets, and is not involved in the cellular activation of IT neurons. Examining the change in expression of preoptic *it* mRNA in the social context studied here may contribute further insight

into the role of IT and the POA in mediating social habituation.

Interestingly, OT has been implicated in several processes underlying social habituation including social recognition, learning and memory, and the modulation of aggression, fear, and anxiety [Choleris et al., 2004]. It will be important to design studies that allow us to dissociate each of these individual processes in order to examine how the precise action of OT in the brain is coordinated to ultimately result in the adaptive response of the social habituation examined here. Furthermore, given that natural variation in OT receptor expression can influence behavior [Ophir et al., 2009], it would be interesting to examine whether the increase in ITR expression in response to having an FN affects the behavioral response to other social stimuli. Similarly, as it appears that most teleosts have 2 ITRs, it will be interesting to investigate whether gene expression of the other receptor (ITR1) shares the same spatial and temporal dynamics in response to social habituation. Lastly, the attenuation in aggression toward familiar male partners as a consequence of social habituation likely sets the stage for cooperative interactions, such as through the formation of defense coalitions against conspecific intruders [Getty, 1987; Weitekamp and Hofmann, 2017]. The role of OT and its homologs in mediating these types of cooperative behavior should be examined, as OT has been implicated in cooperation between human individuals [De Dreu, 2012; Rilling et al., 2012].

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Disclosure Statement

There were no conflicts of interest.

Author Contributions

C.A.W., B.M.N., and H.A.H. designed the experiments. C.A.W. and B.M.N. collected the brains for experiment 1. C.A.W. performed qPCR. P.V. and T.K.S.-L. collected the brains for experiment 2. Z.T. performed IHC and cell counting. C.A.W. analyzed the data. C.A.W. and H.A.H. wrote the paper with input from T.K.S.-L.

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