Published in final edited form as:

Behav Brain Res. 2015 October 1; 292: 174–183. doi:10.1016/j.bbr.2015.06.019.

The effect of observers on behavior and the brain during aggressive encounters

Julie K. Desjardins, Lisa Becker, and Russell D. Fernald*

Abstract

What effect does an audience have on an animal's behavior and where is this influence registered in the brain? To answer these questions, we analyzed male cichlid fish fighting in the presence of audiences of various compositions and measured expression of immediate early genes in the brain as a proxy for neural activity. We hypothesized their behavior would change depending on who was watching them. We measured behavioral responses from both the "watchers" and the "watched" during aggressive encounters and found that males fighting in the presence of an audience were more aggressive than males fighting without an audience. Depending on the nature of the audience, immediate early gene expression in key brain nuclei was differentially influenced. Both when an audience of larger males watched fighting males, and when they were watching larger males fighting, nuclei in the brain considered homologous with mammalian nuclei known to be associated with anxiety showed increased activity. When males were in the presence of any audience or when males saw any other males fighting, nuclei in the brain known to be involved in reproduction and aggression were differentially activated relative to control animals. In all cases, there was a close relationship between patterns of brain gene expression between fighters and observers. This suggests that the network of brain regions known as the social behavior network, common across vertebrates, are activated not only in association with the expression of social behavior but also by the reception of social information.

Introduction

Animals need to identify and attract mates in a social context where conspecifics of different status compete. Their behavior requires communication over both long and short distances to attract females while avoiding competitors. When signaling over relatively larger distances, there can be multiple potential receivers as well as bystanders, resulting in a transient "communication network" [1-4]. In such networks, animals may use the behavior of others to acquire novel information through social learning. Such networks are thought to have influenced the evolution of social vertebrates by favoring individuals that successfully collect information to regulate their future behavior. For example, aggressive interactions between individuals might provide information about fighting ability, physical condition and

*Author for correspondence 371 Serra Mall, Department of Biology and Neuroscience Program, Gilbert Building, Rm. 316, Stanford University, Stanford, California, 94305-5020, rferald@stanford.edu.

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motivation that can be gathered at little risk to the individual, prior to actual physical interactions [2, 3]. Acting on social information that has been collected by observers has been shown for a number of vertebrate species including fish, birds and mammals [4-7].

The cichlid fish species studied here, *Astatotilapia burtoni*, has been shown to gather information as bystanders about fighting ability in pairs of individuals and using observation alone can construct a rank order amongst animals they have only seen in pairwise fights [8]. This skill, called transitive inference allows analysis of a social hierarchy from incomplete information based solely on observed interactions. Since visual social signals are readily accessible to bystanders, the presence of additional receivers as bystanders can have important effects on signaling behavior [9-12]. Siamese fighting fish (*Betta splendens*) males, for example, monitor aggressive interactions between neighboring males and use information about relative fighting ability in subsequent aggressive encounters [13-14].

To identify brain loci involved in processing social information, many studies have used expression of immediate early genes (IEGs) such as the transcription factors *cfos* and *egr-1* (also known as *zenk*, *zif268*, *krox-24*, *ngfi-a*, *tis8*), to mark active sites in the brain [15-16]. IEGs reflect the relationship between extracellular signals and changes in gene expression, so that measuring their expression levels can reveal how and where specific brain regions respond to novel stimuli. For example, male prairie voles (*Microtus ochrogaster*) that have recently pair-bonded show increases in IEG activity in the ventral forebrain [17]. In female prairie voles, forebrain IEG activity has been linked to maternal behavior including decreased partner preference and increased parental behavior [18-19].

Neuronal induction as reflected by IEG response can also result from species-specific stimuli. In mice, for example, the reception of pheromonal information results in neuronal induction activity throughout the brain including the olfactory nuclei but also the amygdala and other specific nuclei within the hypothalamus [20]. Similarly, in female zebra finches (*Taeniopygia guttata*), testing mate preference revealed increased IEG expression in telencephalic auditory areas in response to hearing a preferred male's song [21] and in female European starlings (*Sturnus vulgaris*), recent social interactions with males influence forebrain IEG expression in response to mate-choice cues [22].

In some fish species, social interactions have been traced to system-wide physiological and behavioral changes. For example, the presence of an audience in a cichlid fish (*Oreochromis mossambicus*) influences aggressive behavior and alters circulating androgen levels [23] and similarly, in male *B. splendens*, a fish specific androgen, 11-ketotestosterone, increases in the presence of a male audience and decreases in the presence of a female audience [24].

However there are fewer studies about the induction of IEG responses to social interactions in fish. In *A. burtoni* when females saw their preferred male win a fight, gene activity was increased in parts of the brain associated with reproduction, however, when females saw their preferred male lose a fight, gene activity was increased in the parts of the brain associated with stress and anxiety [25].

In the brains of all vertebrates, social information is processed by a “social behavior network” (SBN), first identified by [26] that is comprised of six brain nuclei – the medial

extended amygdala, the ventral nucleus of the ventral telencephalon (Vv) (septal formation in part), the pre-optic area, the anterior hypothalamus and the ventromedial hypothalamus of the midbrain. These six nuclei are characterized by their interconnectivity, the presence of steroid hormone receptors and their roles in social behavior including parental care, aggression, affiliation, pair-bonding and mating. This network was later mapped onto homologous brain regions in teleost fish and birds revealing the evolutionary conservation of brain nuclei associated with social behavior [27]. More recently, O'Connell and Hofmann [28] extended this social network to include the mesolimbic reward system suggesting a larger social-decision making (SDM) network involved in the neural control of social behavior.

The role of the SBN and the SDM in the production of a range of social behaviors has been established, but their relative importance in processing social information is less well studied. There have been numerous studies of social communication signals amongst animals interacting but fewer in which the effect(s) of information alone have been analyzed. Goodson et al. [29] showed that IEG expression in vasotocin expressing neurons differed in males depending on whether they were exposed to a female vs. a waterbath or nothing. To identify the role of social information alone, we previously showed that in *A. burtoni*, females seeing their preferred male win a fight, produced different patterns of induced IEG activity as opposed to seeing their preferred male lose a fight, as noted above [25].

Here, we asked what brain nuclei, including those in the SBN, were activated by social information by analyzing the effect an audience has on fighting among male fish. We used *Astatotilapia burtoni*, an African cichlid fish for these experiments because of its complex social interactions and responsivity to changing social and physical environments. *A. burtoni* live in colonies above a food resource in the shallow shore pools and river estuaries of Lake Tanganyika in the rift valley system of east Africa [30]. Males exist as two different, reversible phenotypes. Dominant (D) males are brightly colored (blue or yellow) with a black stripe through the eye (eye bar), an opercular black spot at the caudal tip of the gill cover, prominent egg-spots on the anal fin, and a red humeral patch on the body. These males comprise a small fraction of each population and vigorously defend territories against rival males where they actively court and spawn with females. It is important to note that dominant *A. burtoni* fight more or less continuously while they hold a territory and yet have very low levels of cortisol, the stress hormone known to reflect stress level [31]. In contrast, subordinate (non-dominant or ND) males are dull in coloration, do not hold territories or reproduce, school with females and other ND males, flee from the aggressive interactions of D males and have much higher levels of cortisol than D males. In this social system, males can and do switch between D and ND status depending on the outcome of male-male fights but only D males are reproductively active.

Since males must become D to reproduce, we wondered whether they attended to the status of other males when fighting, possibly as a part of assessing their chances for social ascent. ND males can quickly assume social dominance and become reproductively active as observed both in the field [30] and the lab [15]. Correspondingly, D males will quickly become ND after losing a social challenge. Thus, social information about neighbors is

potentially valuable for informing behavioral decisions of males and has been shown in *A. burtoni* [7]. We know that D males are capable of gathering complex information about other individuals and can use transitive inference to determine their likelihood of winning a fight [see above, [8] but it is not known whether the presence of an observer might influence aggressive behavior or how and where this information is represented in the brain.

We measured how territorial *A. burtoni* behaved in the presence of uninvolved bystanders during an aggressive encounter with a conspecific. We also measured whether and where the effect of fighting in the presence of an observer would influence brain activity of both the combatants and observers. We systematically varied the relative sizes of the observers and the fighters to discover whether the size of the observer influenced either behavior or its brain signature and measured quantitative mRNA expression of the immediate early gene *egr-1* as a proxy for brain activity in nine micro-dissected regions of the brain, comprising the SBN and three additional brain nuclei. We hypothesized that males viewing other males fighting would alter their behavior depending on who was watching whom and that combatants and observers would express distinct neural expression patterns depending on their relative sizes.

Methods

Subjects

All subjects were male descendants of wild caught *Astatotilapia burtoni* from Lake Tanganyika, Africa [30]. Prior to the experiments, fish were kept in aquaria under housing conditions that mimic their natural environment (28°C, pH 8, 12:12h light:dark cycle) and were fed each morning with cichlid pellets and flakes (AquaDine: Healdsburg, Ca). Procedures for maintaining, observing and sacrificing the fish were in accordance with Stanford's Administrative Panel for Laboratory Animal Care. D males were transferred to a 121L experimental tank 3 days prior to the start of trials to ensure that they had established a territory in the experimental tank. None of the fish in any trial were previously familiar with each other.

Behavioral trials

For behavioral trials, a 121 L, a three-chambered experimental fish tank was constructed (Figure 1) and the floor covered with crushed coral. Fixed clear barriers that were sealed to prevent chemical communication and removable opaque barriers separated each of the chambers. The opaque barriers were used to isolate fish between trials and removed to start trials. In each condition and in both rounds of the experiment, trials lasted 20-minutes and began when all opaque barriers were removed. Twenty minutes was chosen used based on a previous analysis of the time course of *egr-1* induction in *A. burtoni* [32]. Experiments were conducted in two rounds to allow comparison of gene expression across animals of slightly different size. In the first round, body length (e.g. standard length from mouth to base of tail) of fighting males (N=24) remained constant at (5.5cm $\pm$ 0.5cm) and body size of observing males varied according to the condition. In the size-matched condition, fighting males were 5.5cm $\pm$ 0.5cm, when the observer was smaller it was 3.5cm $\pm$ 0.5cm and when the observer was larger it was 8.5cm $\pm$ 0.5cm. None of the observers were reused between pairs of

fighting males. In control conditions, there were no males watching the fighting males so barriers were removed and revealed an empty tank. In this round of experiments, only the fighting males (N=24) were sacrificed from which we collected brain tissue. Behavioral and neural data from one randomly chosen fighting male per pair is included in analyses below.

In the second round, males watching the fights were all similar in size ($5.5\text{cm} \pm 0.5\text{cm}$) and fighting males varied according to the condition. When size matched, fighting males were $5.5\text{cm} \pm 0.5\text{cm}$, when the observer was smaller fighting males were $8.5\text{cm} \pm 0.5\text{cm}$ and when the observer was larger, fighting males were $3.5\text{cm} \pm 0.5\text{cm}$. In control conditions, there were no males fighting. In this round of experiments, only the observing males were sacrificed and we only collected brain tissue from these males. It is the neural data from these fighting males that is included in analyses below. These two rounds of experiments allow us to compare males that are all of $5.5\text{cm} \pm 0.5\text{cm}$ in size, independent of experimental condition.

In round 1, all of the fighting males were of the same size and the watching males varied in size. We used 4 conditions: 1) Observer smaller; 2) Observer larger; 3) Observer size matched; 4) No observer (control). In round 2, all of observing males were the same size and the fighting males varied in size. We again used 4 conditions: 1) Fighting males smaller; 2) Fighting males larger; 3) Fighting males size matched; 4) No fighting males (control). All trials were videotaped (Sony mini-DV HDR-HC5 Handycam; New York) and scored for aggression by an observer without knowledge of the experimental condition or any hypotheses. Aggressive interactions occurred across a clear barrier and aggressive behaviors counted included bites, rams, and lateral displays that are all typical cichlid aggressive behaviors [33]. Throughout the behavioral trials, observing males only very rarely interacted with the fighting males. To assure this, we increased distance between observing males and fighting males from 5mm (between fighting males) to 115mm (between fighting males and observers). On the few occasions when observing males did interact with fighting males, trials were promptly halted and not included in the experimental data for analysis.

Sample collection, microdissection and real time quantitative polymerase chain reaction

Fish were sacrificed immediately after the completion of each 20-minute trial by rapid cervical transection. Brain tissue was collected and mounted (Optimal cutting temperature, Neg 50; Thermo Scientific, Waltham, MA) and stored at -80°C until sectioning.

Frozen brain tissue was sliced in the coronal plane into $300\mu\text{m}$ thick sections using a cryostat (Microm HM550, Thermo Scientific, Waltham, MA) and mounted on glass microscope slides. Specimens were kept frozen throughout sectioning and completed slides were stored at -80°C for later microdissection [25, 34-36]. A frozen stage (BFS-30MP; Physitemp, Clifton, NJ) viewed through a dissection microscope was used to identify and microdissect specific brain regions. Microdissections were performed using a modified 27G needle with an internal diameter of $190\mu\text{m}$ as a punch. Brain atlases from *A. burtoni* [37, 38] and from other fish species [40-42] were used to target brain regions centered on the following nuclei where proposed putative mammalian homologues are given in parentheses. These homologues are based on a consensus from several reviews [27, 40-43]: lateral part of the dorsal telencephalon (Dl; sampled in ventral region, Dlv) (hippocampus, medial

pallium); medial part of the dorsal telencephalon (Dm) (pallial amygdala, ventral pallium); ventral nucleus of the ventral telencephalon (Vv) (septal formation in part); supracommissural nucleus of the ventral telencephalon (Vs; sampled in Vsm region) (extended amygdala: medial amygdala, central amygdala, bed nucleus of the stria terminalis); preoptic area (POA) (preoptic area); ventral tuberal nucleus (VTn) (anterior hypothalamus in part); anterior tuberal nucleus (ATn) (ventromedial hypothalamus in part); and corpus cerebellum (Ce) (sampled in the granule cell layer, CCeG). These brain regions include the SBN and others (DI, Ce), and were chosen to be consistent with previous studies [25]. We used previously published landmarks to locate the nuclei during brain punches [44]. Technical limitations of microdissection meant that tissue collection was centered on these nuclei and standardized across individuals, but some nuclei may also contain portions of surrounding regions. To minimize variability, we used animals closely matched in size and the majority of the tissue should include the target nuclei and the remainder would likely result in increased variation in our samples, rather than systematic error. Tissue was collected directly into lysis buffer (RNeasy Micro kit, Qiagen), frozen on dry ice, and stored at -80°C until RNA.

Total RNA was extracted from the micro-dissected brain areas (Qiagen RNeasy Plus Micro Kit: Alameda, CA), cDNA synthesis was conducted using oligo dT (Bio-Rad iScript cDNA; Hercules, CA), and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to quantify mRNA expression in each region of the brain. The qRT-PCR was based on procedures previously developed in our lab (Desjardins et al., 2010). Primers for *egr-1* and two housekeeping genes (*18s* and *GAPDH*) were designed according to published sequences [*egr-1*: 30 *18s* and *GAPDH*: 43]. The qRT-PCR was performed using 20 μl duplicate reactions (SsoFast EvaGreen Supermix; Bio-Rad, Hercules, CA) using an RT-PCR matching (Bio-Rad, CFX96; Bio-Rad, Hercules, CA).

Data Analysis

To analyze the behavior, an overall aggression score was calculated by summing all individual aggressive acts including bites, rams, and lateral displays. This score was used in statistical tests between males who fought in the presence of different audiences. Data analysis was conducted using JMP software (SAS institute; Cary, North Carolina).

For qRT-PCR plate readings, raw fluorescence data were analyzed using a curve-fitting algorithm to calculate the number of cycles required to read the threshold value of gene amplification [45]. The geometric mean of the two housekeeping gene levels (*18s* and *GAPDH*) served as the normalized standard for the expression of *egr-1*.

Prior to data analysis, behavior and mRNA expression levels were tested to see whether they met the assumptions of the parametric tests. All residuals of the means were normally distributed and groups had equal variances. To determine whether mRNA expression levels could explain the probability that males had been in the observer larger, observer small, size matched or control conditions, two logistic regressions were used. The first was on fighters alone and the second on observers alone with treatment group (observer larger, observer small, size matched, control) as the independent variables and the normalized expression of *egr-1* in each brain area as dependent variables. Based on the results, some variables were

removed from subsequent analysis because they did not contribute significantly to the differences between treatment groups. With the remaining variables, we conducted one-way ANOVAS and corrected (Bonferroni) post-hoc tests to determine specific effects within brain regions. Alpha levels for individual tests were adjusted so that overall alpha did not exceed 0.05.

To discover whether data from fighters and observers were related, another logistic regression was conducted with individual type (fighters and observers) as the independent variable and normalized *egr-1* expression in each brain area as the dependent variables. As described above, some variables were removed from subsequent analysis because they did not contribute significantly to the differences between treatment groups. With the remaining variables, we conducted one-way ANOVAS and Bonferroni corrected post-hoc tests. To further understand differences and similarities between fighters and observers, discriminant function and hierarchical cluster analyses using Ward's minimum variance method were conducted. Once the cluster analysis was conducted, χ^2 was used to test whether the observed presence within a cluster differed from the expected, being randomly distributed across clusters.

Results

Animals being watched

Overall, males who fought in the presence of an audience were significantly more aggressive than males that fought without an audience (Figure 2; $F_{3,60} = 28.35$; $p < 0.0001$). Males that fought in the presence of a size matched or a smaller observer were more aggressive during fights than when there was no observer or a larger observer ($p < 0.001$).

We used a logistic regression to identify which brain areas were activated during aggressive encounters under different conditions. Males that had fought in the presence of a larger observer, smaller observer, size matched observer and no observer (control) revealed that *egr-1* mRNA expression levels varied between treatments ($\chi^2 = 62.844$, $p = 0.0001$) and subsequent likelihood ratio tests revealed that expression in five brain areas Vv ($\chi^2 = 33.389$, $p = 0.0001$), Dm ($\chi^2 = 5.707$, $p = 0.016$), Cce ($\chi^2 = 4.161$, $p = 0.041$), PAG ($\chi^2 = 5.098$, $p = 0.023$) and POA ($\chi^2 = 9.776$, $p = 0.0018$) were significantly affected by observers. Subsequent additional statistical tests were only conducted with these 5 brain regions. In the cerebellum (Cce) and the medial part of the dorsal telencephalon (Dm; Figure 3a, b), *egr-1* activity was highest when males fought in the presence of an observer that was larger or smaller than themselves (Cce: $F_{2,41} = 7.884$, $p = 0.003$; Dm: $F_{2,41} = 2.608$, $p = 0.051$). In the pre-optic area (POA; Figure 3c, d), *egr-1* expression was higher when males fought in the presence of any audience (POA: $F_{2,41} = 3.333$, $p = 0.029$), ventral nucleus of the ventral telencephalon (Vv) (septal formation in part), *egr-1* expression was highest when males fighting were smaller than the observer (Figure 3e; $F_{2,41} = 52.145$, $p = 0.0001$).

Animals watching

To identify brain areas that were activated in males who watched fights between larger, smaller, and size matched fish and no fish (control), logistic regression revealed that *egr-1*

mRNA expression levels varied between treatments ($\chi^2=37.363$, $p=0.0058$) and subsequent likelihood ratio tests revealed that that expression in POA ($\chi^2=4.058$, $p=0.04$), Vm ($\chi^2=10.582$, $p=0.001$), Dm ($\chi^2=5.089$, $p=0.001$), and Vv ($\chi^2=14.957$, $p=0.0001$) were closely related to treatment type. Consequently, additional tests were only conducted with these 4 brain regions (Figure 4). In the ventromedial telencephalon (Vm; Figure 4a) and the medial part of the dorsal telencephalon (Dm; Figure 4b), *egr-1* activity was highest when the observer that was larger or smaller than the fighters while the size matched and control conditions were similar (Vm: $F_{2,21}=3.864$, $p=0.028$; Dm: $F_{2,21}=2.506$, $p=0.057$). Similar to the males fighting in the presence of any audience, in the pre-optic area (POA; Figure 4c), *egr-1* expression was higher when observers were in the presence of any audience (POA: $F_{2,21}=3.310$, $p=0.053$), finally in the Vv, *egr-1* expression was highest when observers were smaller than the fighters (Figure 4d; $F_{2,21}=12.347$, $p=0.001$).

Relationship between fighting and observing males

Differential expression of *egr-1* in the brain of fighters and observers varied systematically (logistic regression: $\chi^2=20.699$, $p=0.014$). However, stepwise regression revealed that only expression in PAG ($\chi^2=5.963$, $p=0.015$), Vm ($\chi^2=3.90$, $p=0.051$) and Dm ($\chi^2=3.299$, $p=0.060$) contributed to that difference. None of the other nine brain areas contributed to the difference between fighters and observers. In addition, discriminant function analysis of all brain areas using fighter or observer as a co-variate misclassified 29.6% of all individuals suggesting that while some differences in brain gene expression exist, there are significant similarities. Finally, a cluster analysis revealed that individuals fell into 3 main clusters of individuals and that fighters (figure 5), observers and controls (those who fought without any observers and observers who saw nothing) fell into clusters differently than expected. That is, our hypothesis that fighters and observers would have different responses was not supported as described more fully below.

Chi-Squared analysis of cluster membership revealed that fighters and observers did not cluster differently than expected ($\chi^2=10.410$, $p=0.1665$) however the treatments did differentially cluster ($\chi^2=36.079$, $p=0.021$) meaning that fighters and observers resemble each other more than either resemble controls. In addition, gene expression in the brain areas sampled clustered logically in that those nuclei in the telencephalon clustered closely together (POA, Dm, Dl, Vv), nuclei in the hypothalamus (AH, Vm) clustered closely and those in the hindbrain (PAG, R) clustered closely. As might be predicted, *egr-1* expression in the cerebellum (CCe) was not closely related to any other brain regions.

Discussion

In this experimental paradigm, we found that males fight more aggressively in the presence of an observer but do not modulate their aggression significantly based on the size of the observer. In naturally occurring border fights, the presence of an observer may signal the need to increase aggression and this could serve as a signal to neighboring male. Interestingly, brain gene expression patterns in fighting males do respond differently depending on observer sizes as measured by differential activation of specific brain regions.

We hypothesized that since the social brain network activity reflects coordinated functions of distinct brain regions it might also play a role in processing social information independent of behavior. Based on our data, we identified a “social information processing system” that includes the medial part of the dorsal telencephalon (Dm proposed to be homologous to the amygdala), the pre-optic area (POA) and the ventral nucleus of the ventral telencephalon (Vv) (septal formation in part) that are a subset of the SBN. All three of these nuclei expressed significantly changed immediate early gene expression for signalers across audience types and for audiences in the presence of different signalers. This is supported by data from Desjardins et al. [25] where females who had seen their preferred male either win or lose a fight had similar patterns of brain activity as found in this experiment in males seeing differentially sized individuals fighting. This suggests that the SBN could comprise a network central to processing social information as well as producing key behaviors. Specifically, in all vertebrates, the amygdala or its homologue is critically important for correct responses to social signals. For example, it has been shown that humans with amygdalar damage have impaired recognition of social emotions [46] and that amygdalar damage impairs the ability to process the “overall” emotional message but not the details of the task [47]. The amygdala also plays an essential role in neural systems that associate social stimuli (auditory, visual and olfactory) with value, and directs unconscious responses during social encounters, alerting us to relevant stimuli in our environment [48]. In these fish, key nuclei of the social behavior network are activated by the reception of social information alone, suggesting that they may play a role in planning future behavior.

In addition to the amygdala, the POA and the Vv show differential expression during the display and reception of social information. The POA is a region of the brain known to be associated with mating, aggression and motivation [49, 50]. In signalers, the increase in IEGs in the POA could be related to the act of aggression, however, in observers, it may be related to simply witnessing the act of aggression but could also reflect a priming effect of viewing aggressive encounters. This priming effect has been documented in a number of vertebrates including humans where researchers showed an increase in violent or aggressive thoughts and sentiments after exposure to aggressive videos [51]. The ventral nucleus of the ventral telencephalon (Vv) (septal formation in part) showed a similar pattern of IEG activation to the POA. The ventral nucleus of the ventral telencephalon (Vv) (septal formation in part) a likely homologue of the lateral septum in other vertebrates is a region of the brain conserved across all vertebrates known to be involved in anxiety via the histaminergic system [52]. Neurons from this area are connected with a number of limbic, diencephalic and midbrain regions known to play a role in the regulation of mood and motivation in vertebrates. While it is difficult to know whether the individuals involved in our trials experienced anxiety, it may be the case that being in the presence of two larger individuals fighting (in the case of observers) and being watched by a larger individual (in the case of signalers) could be fear inducing.

In previous research with *A. burtoni*, [8] showed that when forced to choose, adult males preferred to affiliate with weaker or less competitive males, suggesting they may be attempting to avoid aggression from a larger more competitive male. The ventral nucleus of the ventral telencephalon (Vv) (septal formation in part) is also involved in the reward

pathway, as has been shown by microinjections of GABA_A receptor agonists directly into the lateral septum that produced an antianxiety-like effect in rats whereas [53] injections of morphine had the opposite effect [54].

Cluster analysis revealed that observers (audiences) and signalers showed overlapping brain gene expression patterns distinct from those patterns in controls. Specifically, the POA and the ventral nucleus of the ventral telencephalon (Vv) (septal formation in part) showed similar responses in observers and signalers. This hints at the existence of a similar circuitry involved in the reception, integration and interpretation of social information.

What molecular actors might play a role in mediating the expression of immediate early genes in response to social information. In vertebrates, recent observations relating neuropeptides to social behavior has focused on members of the oxytocin/vasopressin family. Homologs of oxytocin and vasopressin existed at least 700 million years ago and have been identified in such diverse organisms as hydra, worms, insects, and vertebrates. Among these distant taxa, oxytocin and vasopressin related peptides play a general role in the modulation of social and reproductive behavior. Isotocin and vasotocin are expressed in the hypothalamus. Even though both oxytocin and vasopressin have a conserved role in modulating social behavior in general, the specific behaviors they influence vary substantially among different species. One consistent pattern is the role of oxytocin and vasopressin in influencing individual differences in parenting, social recognition and affiliative behaviors [55]. In signalers, the ventromedial hypothalamus (Vm) showed differential activation in signalers displaying in the presence of smaller observers. In our signalers, it may be possible that increases in oxytocin and vasopressin may mediate aggression and specifically, aggression in the presence of an audience. In adult male Wistar rats, microdialysis of AVP to the lateral septum and the bed nucleus of the stria terminalis stimulated aggression towards an intruder male, respectively [56]. The lateral septum in mammals considered homologous to the ventral nucleus of the ventral telencephalon (Vv) (septal formation in part) and is known to play an important role in regulating motivation and mood [57]. This nucleus is also important in regulating fear, possibly via the hypothalamus.

Interestingly, a number of nuclei of the SBN were not activated throughout these social challenges. Specifically, in both signalers and observers the anterior hypothalamus (AH) was not activated in either condition. The role AH has been well established in the display of aggressive behavior in a number of vertebrates [58-60] specifically in hamsters blocking of vasopressin receptors in the AH resulted in a reduction of aggressive behavior [61]. In the case of these fish, it is possible that the AH is not involved in the differential expression of aggression across social contexts but may be implicated in the regulation of aggression under territorial or other contexts. Future experiments will include female observers since our previous studies have shown that some of their responses in mate choice depend on their reproductive state [62].

Our data suggest that a subset of the SBN nuclei are not only important for controlling social behavior but also play a key role in social cognition. Some combination of nodes in the SBN including significant contribution from the reward pathway appear to be regulating the reception and display of social information in the aggressive context studied here. Social

decisions are related to the reward circuitry whose central brain nucleus, the POA, depends critically on the dopaminergic neurotransmitter system [28]. Our results show that a network of brain nuclei respond to social information alone, suggesting a “social information processing system” with a role for behavioral planning and anticipation. Since we know that the reward pathway depends critically on anticipation perhaps the nuclei revealed here are linked to future behavioral reward or punishment.

Acknowledgements

This research was supported by NSF IOS -0923588 and NIH NS034950 grants to RDF. We thank members of the Fernald laboratory for useful comments on earlier drafts of the manuscript.

References

- [1]. Dabelsteen, T. Interactive playback: a finely tuned response. In: McGregor, PK., editor. Playback and studies of animal communication. Plenum Press; NewYork: 1992. p. 97-110.
- [2]. McGregor PK. Signalling in territorial systems: a context for individual identification, ranging and eavesdropping. Phil. Trans. R. Soc. Lond. B. 1993; 340:237–244.
- [3]. McGregor, PK.; Dabelsteen, T. Communication networks. In: Kroodsmma, DE.; Miller, EH., editors. The ecology and evolution of acoustic communication in birds. Comstock; Cornell, Ithaca, NY: 1996. p. 409-425.
- [4]. McGregor, PK. Animal Communication Networks. Cambridge University Press; Cambridge U.K.: 2005. p. 567
- [5]. Eason PK, Stamps JA. An early warning system for detecting intruders in a territorial animal. Anim Behav. 1993; 46:1105–1109.
- [6]. Pinto A, Oates J, Grutter A, Bshary R. Cleaner wrasses *Labroides dimidiatus* are more cooperative in the presence of an audience. Curr Biol. 2011; 21:1140–1144. [PubMed: 21700458]
- [7]. Desjardins JK, Hofmann HA, Fernald RD. Social context influences aggressive and courtship behavior in a cichlid fish. PLoS One. 2012; 7:e32781. [PubMed: 22807996]
- [8]. Grosenick L, Clement TS, Fernald RD. Fish can infer social rank by observation alone. Nature. 2007; 445:429–432. [PubMed: 17251980]
- [9]. Gyger M, Karakashian SJ, Marler P. Avian alarm calling: is there an audience effect? Anim. Behav. 1986; 34:1570–1572.
- [10]. Karakashian SJ, Gyger M, Marler P. Audience effects on alarm calling in chickens (*Gallus gallus*). J. Comp. Psychol. 1988; 102:129–135. [PubMed: 3396311]
- [11]. Gyger M. Audience effects on alarm calling. Ethol. Ecol. Evol. 1990; 2:227–232.
- [12]. Bierbach D, Girndt A, Hamfler S, Klein M, Muchsch F, Penshorn M, Schwinn M, Zimmer C, Schlupp I, Streit B, Plath M. Male fish use prior knowledge about rivals to adjust their mate choice. Biol Lett. 2011; 7:349–351. [PubMed: 21208944]
- [13]. Oliveira RF, McGregor PK, Latruffe C. Know thine enemy: fighting fish gather information from observing conspecific interactions. Proc Roy Soc. B. 1998; 265:1045–1049.
- [14]. Oliveira, RF. Hormones, social context and animal communication. In: McGregor, PK., editor. Animal Communication Networks. Cambridge Univ Press; 2005. p. 481-520.
- [15]. Burmeister SS, Jarvis ED, Fernald RD. Rapid Behavioral and Genomic Responses to Social Opportunity. PLoS Biol. 2005; 3(11):e363. [PubMed: 16216088]
- [16]. Robinson GE, Fernald RD, Clayton DF. Genes and social behavior. Science. 2008; 322(5903): 896–900. Epub 2008/11/08. doi: 322/5903/896 [pii]10.1126/science.1159277. [PubMed: 18988841]
- [17]. Cushing BS, Mogekwu N, Le WW, Hoffman GE, Carter CS. Cohabitation induced Fos immunoreactivity in the monogamous prairie vole. Brain Res. 2003; 965(1-2):203–11. [PubMed: 12591139]

- [18]. Cushing BS, Martin JO, Young LJ, Carter CS. The effects of peptides on partner preference formation are predicted by habitat in prairie voles. *Horm Behav.* 2001; 39:48–58. [PubMed: 11161883]
- [19]. Ross HE, Cole CD, Smith Y, Neumann ID, Landgraf R, Murphy AZ, Young LJ. Characterization of the oxytocin system regulating affiliative behavior in female prairie voles. *Neurosci.* 2009; 162:892–903.
- [20]. Dulac C, Torello AT. Molecular detection of pheromone signals in mammals: from genes to behavior. *Nat Rev Neuro.* 2003; 4:551–562.
- [21]. Woolley SC, Doupe AJ. Social context-induced song variation affects female behavior and gene expression. *PLoS Biol.* 2008; 6:e62–e67. [PubMed: 18351801]
- [22]. Sockman KW, Gentner TQ, Ball GF. Recent experience modulates forebrain gene-expression in response to mate-choice cues in European starlings. *Proc Biol Sci.* 2002; 269:2479–2485. [PubMed: 12495492]
- [23]. Oliveira RF. Social behavior in context: Hormonal modulation of behavioral plasticity and social competence. *Int Comp Biol.* 2009; 49:423–440.
- [24]. Dzieweczynski TL, Bessler AM, Shelton DS, Rowland WJ. Effect of a dummy audience on male-male interactions in Siamese fighting fish, *Betta splendens*. *Ethol.* 2006; 112:127–133.
- [25]. Desjardins JK, Klausner JQ, Fernald RD. Female genomic response to mate information. *PNAS.* 2010; 107:21176–21180. [PubMed: 21106763]
- [26]. Newman SW. The medial extended amygdala in male reproductive behavior: A node in the mammalian social behavior network. *Ann NY Acad Sci.* 1999; 877:242–257. [PubMed: 10415653]
- [27]. Goodson JL. The vertebrate social behavior network: Evolutionary themes and variations. *Horm Behav.* 2005; 48:11–22. [PubMed: 15885690]
- [28]. O'Connell LA, Hofmann HA. The vertebrate mesolimbic reward system and the social behavior network: A comparative synthesis. *J comp neurol.* 2011; 519:3599–3639. [PubMed: 21800319]
- [29]. Goodson JL, Rinaldi J, Kelly AM. Vasotocin Neurons in the Bed Nucleus of the Stria Terminalis Preferentially Process Social Information and Exhibit Properties that Dichotomize Courting and Non-Courting Phenotypes. *Horm Behav.* 2009; 55(1):197–202. [PubMed: 19013174]
- [30]. Fernald RD, Hirata NR. Field study of *Haplochromis burtoni*: Quantitative behavioural observations. *Anim Behav.* 1977; 25:964–975.
- [31]. Fox HE, White SA, Kao MHF, Fernald RD. Stress and dominance in a social fish. *J Neuro.* 1997; 17:6463–6469.
- [32]. Burmeister SS, Fernald RD. Evolutionary conservation of the *egr-1* immediate-early gene response in a teleost. *J Comp Neurol.* 2005; 481(2):220–32. [PubMed: 15562507]
- [33]. Fernald RD. Quantitative behavioural observations of *Haplochromis burtoni* under semi-natural conditions. *Anim Behav.* 1977; 25:643–653.
- [34]. Wood LS, Desjardins JK, Fernald RD. Effects of stress and motivation on performing a spatial task. *Neurobiol Learn Mem.* 2011; 95:277–285. [PubMed: 21145980]
- [35]. Korzan WJ, Summers TR, Summers CH. Monoaminergic activities of limbic regions are elevated during aggression: influence of sympathetic social signaling. *Brain Res.* 2000; 870:170–178. [PubMed: 10869515]
- [36]. Desjardins JK, Fernald RD. What do fish make of mirror images? *Biol Lett.* 2010; 123:456–459.
- [37]. Fernald RD, Shelton LC. The organization of the diencephalons and the pretectum in the cichlid fish, *Haplochromis burtoni*. *J.Comp.Neurol.* 1985; 238:202–217. [PubMed: 4044911]
- [38]. Burmeister SS, Munshi RG, Fernald RD. Cytoarchitecture of a cichlid fish telencephalon. *Brain Behav. Evol.* 2009; 74:110–120. [PubMed: 19729898]
- [39]. Munoz-Cueto, JA.; Sarasquete, C.; Zohar, AH.; Kah, O. An atlas of the brain of the gilthead seabream (*Sparus aurata*). Maryland Sea Grant; College Park: 2001.
- [40]. Maler L, Sas E, Johnston S, Ellis W. An atlas of the brain of the electric fish *Apteronotus leptorhynchus*. *J Chem Neuroanat.* 1991; 4:1–38. [PubMed: 2012682]
- [41]. Reiner A, Northcutt RG. An immunohisto- chemical study of the telencephalon of the Senegal bichir (*Polypterus senegalus*). *J. Comp. Neurol.* 1992; 319:359–386. [PubMed: 1351063]

- [42]. Wullimann MF, Mueller T. Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. *J Comp Neurol*. 2004; 475(2):143–62. [PubMed: 15211457]
- [43]. Lima, Maximino M.G.; Araujo, J.; Oliveira, KRM.; Herculano, AM.; Stewart, AM.; Kyzar, EJ.; Cachat, J.; Kalueff, AV. The Serotonergic System of Zebrafish: Genomics, Neuroanatomy and Neuropharmacology. In: Hall, S., editor. *Serotonin: Biosynthesis, Regulation and Health Implications*. Nova Science; NY: 2013.
- [44]. Maruska KP, Zhang A, Neboori A, Fernald RD. Social opportunity causes rapid transcriptional changes in the social behaviour network of the brain in an African cichlid fish. *J Neuroendocrinology*. 2013; 25(2):145–57. [PubMed: 22958303]
- [45]. Zhao S, Fernald RD. Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J Comp Biol*. 2005; 12:1047–1064.
- [46]. Adolphs R, Baron-Cohen S, Tranel D. Impaired recognition of social emotions following amygdala damage. *J Cog Neurosci*. 2002; 14:1264–1274.
- [47]. Adolphs R, Gosselin F, Buchanan TW, Tranel D, Schyns P, Damasio AR. A mechanism for impaired fear recognition after amygdala damage. *Nature*. 2005; 433:68–72. [PubMed: 15635411]
- [48]. Skuse DH, Gallagher L. Genetic influences on social cognition. *Pediatric Res*. 2011; 69:85–91.
- [49]. Hull EM, Dominguez JM. Getting his act together: Roles of glutamate, nitric oxide, and dopamine in the medial preoptic area. *Brain Res*. 2006; 1126:66–75. [PubMed: 16963001]
- [50]. Ikemoto S. Brain reward circuitry beyond the mesolimbic dopamine system: A neurobiological theory. *Neurosci Biobehav Rev*. 2010; 1:23–29.
- [51]. Anderson CA. Effects of violent movies and trait hostility on hostile feelings and aggressive thoughts. *Agg behave*. 1997; 23:161–178.
- [52]. Degroot A, Kashluba S, Treit D. Septal GABAergic and hippocampal cholinergic systems modulate anxiety in the plus-maze and shock-probe tests. *Pharmacol Biochem Behav*. 2001; 69:391–399. [PubMed: 11509196]
- [53]. Zarrindast MR, Babapoor-Farrokhran S, Babapoor-Farrokhran S, Rezayof A. Involvement of opioidergic system of the ventral hippocampus, the nucleus accumbens or the central amygdala in anxiety-related behavior. *Life Sci*. 2008; 82:1175–1181. [PubMed: 18456284]
- [54]. Le Merrer J, Gavello-Baudy S, Galey D, Cazala P. Morphine self-administration into the lateral septum depends on dopaminergic mechanisms: Evidence from pharmacology and Fos neuroimaging. *Behav Brain Res*. 2006; 180:203–217. [PubMed: 17467070]
- [55]. Donaldson ZR, Young LJ. Oxytocin, Vasopressin and the neurogenetics of sociality. *Science*. 2008; 322:900–904. [PubMed: 18988842]
- [56]. Veenema AH, Beiderbeck DI, Lukas M, Neumann ID. Distinct correlations of vasopressin release within the lateral septum and the bed nucleus of the stria terminalis with the display of intermale aggression. *Horm Behav*. 2010; 58:273–281. [PubMed: 20298693]
- [57]. Sheehan TP, Chambers RW, Russell DS. Regulation of affect by the lateral septum: implications for neuropsychiatry. *Brain Rees. Rev*. 2004; 46:71–117.
- [58]. Fuchs SAG, Edinger HM, Siegel A. The role of the anterior hypothalamus in affective defense behavior elicited from the ventromedial hypothalamus of the cat. *Brain Res*. 1985; 330:93–107. [PubMed: 4039214]
- [59]. Edwards DA, Nahai FR, Wright P. Pathways linking the olfactory bulbs with the medial preoptic anterior hypothalamus are important for intermale aggression in mice. *Physiol Behav*. 1993; 53:611–615. [PubMed: 8451331]
- [60]. Larson ET, O'Malley DM, Melloni RH Jr. Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. *Behav Brain Res*. 2006; 167:94–102. [PubMed: 16213035]
- [61]. Ferris CF, Potegal M. Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters. *Physiol Behav*. 1988; 44:235–239. [PubMed: 2853382]
- [62]. Clement TS, Grens KE, Fernald RD. Female affiliative preference depends on reproductive state in the African cichlid fish, *Astatotilapia burtoni*. *Behavioral Ecology*. 2005; 16:83–88.

Highlights

- Males fighting in the presence of an audience are more aggressive than those without an audience;
- Brain nuclei activated in fighters and observers were similar;
- When males were watched by larger males, brain nuclei associated with anxiety were activated;
- The social behavior network, common to vertebrates is also active in receiving social information.

Figure 1.

Experimental tanks used for all experiments. Compartments marked “A” housed individuals that were fighters, were engaged in an aggressive encounter. Bystanders or observers were housed in the compartment labeled “B”. Between all individuals there were fixed clear barriers (solid lines) and removable opaque barriers (dashed lines).

Figure 2.

Mean total aggressive acts ($\pm$ SE) of fighting animals in the presence of a larger observer, a smaller observer, a size matched observer and nothing (control). Total aggression was the sum of all aggressive behaviors displayed in a 20-minute interaction. Different letters above the bars represent statistically significant differences (N=24).

Figure 3.

Mean normalized *egr-1* expression ($\pm$ SE) as a function of audience and brain area in signalers. a) cerebellum (Ce), b) medial part of the dorsal telencephalon (Dm), c) periaqueductal gray, d) pre-optic area, e) ventral nucleus of the ventral telencephalon (Vv) (septal formation in part). Different letters above the bars represent statistically significant differences (N=41). .

Figure 4.

Mean normalized *egr-1* expression (±SE) as a function of audience and brain area in observers. a) ventromedial hypothalamus b) medial part of the dorsal telencephalon (Dm), c) periaqueductal gray, d) ventral nucleus of the ventral telencephalon (Vv) (septal formation in part). Different letters above the bars represent statistically significant differences (N=23).

Figure 5.

Cluster analysis and gray scale map of normalized brain gene expression levels in signalers (s), observers (o) and controls (c) based on gene expression in the cerebellum (Cce), the raphe nucleus (R), the periaqueductal gray (PAG), the anterior hypothalamus (Ah), the ventromedial hypothalamus (Vm), the pre-optic area (POA), the lateral part of the dorsal telencephalon (Dl), the medial part of the dorsal telencephalon (Dm) and the ventral nucleus of the ventral telencephalon (Vv) (septal formation in part). The gray scale map shows gray cells for each individual brain region for each individual. Values plotted as a color on a gradient from light gray to black where lighter cells are assigned to small values of brain gene expression and darker cells are assigned to larger values of brain gene expression. This map allows for visualization of the magnitudes of brain gene expression relative to the clusters of individuals. The three main clusters within the data are identified by the boxes and numerals on the left of the panel. The relationship in gene expression between brain nuclei is shown at the bottom of the figure.