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Whole brain monoamine detection and manipulation in a stalk-eyed fly

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HIGHLIGHTS

- We developed a sample preparation method for whole brain for detection of monoamines from single small-bodied insects.
- The method involves a freeze-thaw sample preparation step and a modification of HPLC with ED protocols.
- We elevated 5-HT levels ~8-fold that of control levels in stalk-eyed fly brains by feeding the 5-HT precursor 5-HTP.
- We found elevated 5-HT altered the probability of winning a contest over a food resource.
- This method can be used to test roles of monoamines in modulating aggression in stalk-eyed flies and other small insects.

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ABSTRACT

Understanding the physiological mechanisms that influence conflict resolution is of great importance because the outcome of contests over limited resources such as mates, territories, and food has significant fitness consequences. Male stalk-eyed flies (*Teleopsis dalmanni*) compete over territory and mates and provide an excellent model system to study aggression. To investigate potential effects of serotonin (5-HT) on aggressive behavior in these flies, we developed a dissection and sample preparation method sufficiently sensitive to measure monoamine concentrations from whole brain samples of small insects. This new method allows the detection of monoamines from a single fly brain using high performance liquid chromatography with electrochemical detection. The method allows for the detection and quantification of octopamine (OA), 5-hydroxytryptophan (5-HTP), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), tyramine (TA), and serotonin (5-HT) and provides a means for assessing changes in stalk-eyed fly brain monoamine concentrations in response to drug administration in food media. We successfully elevated 5-HT levels approximately 8-fold that of control levels in stalk-eyed fly brains by oral administration of the 5-HT precursor 5-HTP. Furthermore, in size-matched competitions for a food resource, flies that had elevated 5-HT in response to 5-HTP pretreatment exhibited a high probability of winning the contests. These results suggest that 5-HT enhances aggression in the stalk-eyed fly and highlight the potential of our method for testing putative roles of monoamines in modulating self and rival assessment in conflict resolution.

1. Introduction

Biogenic amines mediate behavioral patterns in both vertebrates and invertebrates (Libersat and Pflueger, 2004; Huber, 2005; Nichols, 2006). Classical monoamines, such as dopamine and serotonin, are involved in a variety of physiological processes by acting as important neurotransmitters, neuromodulators, and neurohormones across both taxa (Evans, 1980; Farmer et al., 1996; Huber et al., 1997; Kravitz and Huber, 2003; Cnaani et al., 2003; Cornil et al., 2005; Stevenson et al., 2005; Summers and Winberg, 2006; Øverli et al., 2007; Chen et al., 2008). Typically thought of as primarily invertebrate neurotransmitters, the trace amines octopamine (OA) and tyramine (TA) have gained interest as potentially important vertebrate neurotransmitters after the discovery of the vertebrate trace-amine-associated receptors (TAARs) (Hardie and Hirsh, 2006; Roeder, 2005). In contrast to vertebrates, OA and TA are abundant in the nervous system of invertebrates, including insects, which makes the stalk-eyed fly an excellent study system for testing the physiological and behavioral roles of these trace amines (Evans, 1980). In addition, norepinephrine (NE), dopamine (DA) and serotonin (5-HT) are also readily found in the invertebrate CNS (reviewed in Roeder, 2005). A recent study suggests that

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pharmacological responses and metabolic pathways for 5-HT in insects are similar to those characterized for vertebrates including reserpine-induced release and synthesis from tryptophan (Vieira et al., 2007). The use of the stalk-eyed fly as a model organism for monoamine research will enable investigation into the neurochemical mechanisms underlying different behaviors such as aggression.

The stalk-eyed fly, *Teleopsis dalmanni* (Fig. 1), provides an exciting model for investigating ultimate and proximate mechanisms underlying the expression of aggressive behaviors because males display their eye stalks as aggressive signals and their behavioral repertoire has been well characterized (Egge et al., 2011). In intra-specific competitions for mates and food resources, males exhibit behaviors that are easily scored and predictable—since the aggressive encounters follow a stereotypical progression (Egge et al., 2011). The outcome of these types of contests over limited resources has profound fitness implications for many organisms, including stalk-eyed flies (Burkhardt et al., 1994; Cotton et al., 2010; Small et al., 2009). The full development of stalk-eyed flies as a model to study the physiological mechanisms underlying aggression have, up to this point, been limited because of the difficulty in measuring monoamine concentrations in the brain of a single fly. The ability to assess changes and differences in monoamine concentrations in conspecifics will allow us to investigate the mechanisms and determinants of this important phenomenon. Furthermore, manipulating concentrations of key monoamines in the CNS of the stalk-eyed fly will provide greater insight into their functions as behavioral modifiers for aggression and other physiological responses. In this study, we focus on 5-HT because heightened levels of neural 5-HT in several invertebrates, such as *Drosophila* and *Larinioides*, has been linked to the amplification of aggressive (Dierick and Greenspan, 2007; Stevenson et al., 2005) and antipredatory (Jones et al., 2011) behaviors, demonstrating its role as an important neuromodulator for specific behavioral patterns.

In order to study the potential roles of monoaminergic neurotransmission in regulating stereotypical patterns of aggressive escalation using the stalk-eyed fly as a model, we modified an earlier method for the detection of monoamines from rat tissue punches by high performance liquid chromatography with electrochemical detection (Renner and Luine, 1984, 1986) by increasing the concentrations of methanol and the ion-pairing agent octanesulfonic acid and increasing the potential applied to the electrochemical cell. In order to apply the method to measure monoamines in a single whole brain from the stalk-eyed fly, which typically has a mass of less than 0.3 mg (Ribak and Swallow, 2007), we tested the feasibility of extracting the monoamines from preparations in which the head exoskeleton was spilt and from samples in which the brain was removed from the exoskeleton by freeze-thawing the brain in buffer to minimize monoamine loses during sample preparation. Using this method, we were able to reproducibly detect and quantify OA, the 5-HT precursor, 5-hydroxytryptophan (5-HTP), DA, TA, and 5-HT. In addition, the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) was detectable in some samples. Although the focus of this study was the detection and manipulation of 5-HT in single stalk-eyed fly brains to study aggressive behavior, the ability to detect DA, OA and TA in the same samples provides future opportunities to test for neurotransmitter interactions as well as direct assessments of pharmacological manipulations targeting the respective amines that could potentially impact behavior. As noted in earlier work, OA and TA are typically thought of as difficult biogenic amines to quantify, due to the high-oxidation potential required and the presence of co-eluding electroactive compounds (Hardie and Hirsh, 2006). Both OA and TA are clearly resolved along with the other monoamines in a single assay using the current method.

To validate our method as a means of determining a functional link between monoamines and behavior, we manipulated brain 5-HT concentrations by oral administration of the 5-HT precursor, 5-HTP. We were able to increase 5-HT concentrations in the brain of a stalk-eyed fly in a dose-dependent manner while simultaneously monitoring other monoamines. Furthermore, we determined the duration of augmented brain 5-HT concentrations in response to oral administration of 5-HTP and applied this information to assess the effects of increased 5-HT on aggressive behavior in competitions for a food resource.

Fig. 1. Males of the sexually dimorphic *Teleopsis dalmanni* engage in ritualized aggressive behavior in contests over feeding and roosting sites.

2. Materials and methods

2.1. Food preparation

The flies were fed sterilized, pureed whole ear sweet corn in which various concentrations of 5-hydroxy-L-tryptophan were applied (H9772; Sigma, St. Louis, MO). Four separate mixtures of food media (100 ml) containing 5-HTP or control were prepared as follows: 0 g/100 ml, 0.3 g/100 ml, 1 g/100 ml, and 3 g/100 ml. Additionally, a concentration of 1 g/100 ml tyramine/food media was administered (T90344; Sigma, St. Louis, MO) to flies involved in experiments to test the feasibility of increasing brain TA concentrations for future studies. All food mixtures also included 25 mg of ascorbic acid as a stabilizer (Dierick and Greenspan, 2007), 1 ml of the mold inhibitor methylparaben (Wilkinson, 1993) and a drop of food coloring to ensure sufficient mixing.

2.2. Subjects and drug administration

The species used in this experiment was *T. dalmanni*, a stalk-eyed fly native to Malaysia. The eyes of stalk-eyed flies (Diptera; Diopsidae) are positioned at the end of rigid peduncles that project laterally from the head. In dimorphic species, such as *T. dalmanni* (Fig. 1), the average eye-stalk length of males exceeds that of females and can even exceed body length. Based on morphological measurements of flies reared under conditions similar to those reported here, the average body mass of male flies is 7.12 mg, with the head, including eye stalks and bulbs, comprising approximately 9% of body mass (Ribak and Swallow, 2007). All subjects were descendants of pupae obtained from Dr. Gerald Wilkinson at the University of Maryland-College Park. The male participants

were taken from a large population (~100 individuals) housed in cages (45 cm × 22 cm × 19 cm) with strings attached to the roof, simulating rootlets to encourage breeding and natural competitive behavior. All subjects had equal access to food, water, and mates and were kept on a 12-h light/dark cycle between 22 and 24 °C at ~85% humidity. These males were transferred to smaller cages (14 cm × 14 cm × 14 cm) containing 10 individuals each and were fed the respective food mixtures from a communal dish of food. For evaluation of dose-dependent changes in brain 5-HT concentrations, each group was allowed to feed for 4 days from media containing vehicle or the respective 5-HTP concentrations before the brain removal and analysis. Similarly, flies involved in the TA experiment were allowed to feed for 4 days at the 1 g/100 ml concentration before immediate brain removal. The time course for increases in brain 5-HT was determined by allowing the flies to feed on the 3 g 5-HTP food mixture for 4 days followed by removing access to the food for 6, 12, and 24 h. Males were at least 3 weeks post-eclosion but less than 2 months post-eclosion to provide similar age groups and to avoid possible aging effects.

2.3. Behavioral analysis

After a four-day period during which the flies were fed either 3 g 5-HTP/100 ml or control media, the flies were briefly CO_2 anesthetized, placed on a stage with the thoracic spines down and photographed with a digital camera under X15-20 magnification. Scion Image (National Institutes of Health) was used to measure eye span and body length to the nearest 0.01 mm from digital images (Ribak et al., 2009), and the thorax was painted for visual identification (Egge et al., 2011). Size-matched pairs were transferred to a fighting arena (11 cm × 6.5 cm × 5 cm) containing moist filter paper as a floor and a removable central barrier separating the individuals following a slightly modified laboratory protocol (Egge et al., 2011; Egge and Swallow, 2011). Rather than a 24 h starvation period, the two flies remained separated in the arena for a starvation period of 12 h. This phase was to encourage aggressive behavior once the barrier was removed and a single drop of corn (~4 mm in diameter) was dropped in the center of the arena. After removal of the barrier, control and 5-HTP pretreated flies were evaluated in a 10-min forced-fight paradigm, which consisted of several aggressive interactions within that time frame. After 10 min of behavioral interactions, flies were aspirated out of the arena and their brains were rapidly dissected and stored as described below. The interactions were digitally recorded and behaviors were scored, blind with regard to treatment, using the free event recorder JWatcher (Blumstein et al., 2007). Contests occurred within each trial when one fly approached another or the pair lined up eye stalks in a parallel manner and ended when the flies were at least one body length apart and/or not engage with each other for a period of 3 s. Over the course of the 10-min trial, pairs of flies could engage in multiple contests. A fly was determined to be a loser if it turned away to retreat or if it quickly ran away from its paired conspecific more often than the rival fly over the course of the 10-min interaction (Egge et al., 2011; Panhuis and Wilkinson, 1999). Conversely, the fly that showed fewer retreat behaviors than its rival was scored as the winner.

2.4. Dissection

Using an aspirator, flies were removed from their cages and anesthetized by administration of CO_2 gas. Heads were removed near the frontal area of the neck using micro-scissors. The eye stalks and eye bulbs, which contain tissue, photopigments, and nerve terminals (Buschbeck and Hoy, 1998) that could interfere with the analysis, were removed approximately ¾ of the way down; the other ¼ remained attached to the head. Pilot trials indicated that removing the eye stalks resulted in a much cleaner chromatographic sample without compromising the desired monoamine measurements (data not shown). The mouthparts were also removed to avoid contamination by food particles, which may have been trapped, from entering the sample and artificially elevating 5-HTP. Micro-tweezers were inserted into the cavity, which once held the mouthparts, and the exoskeleton was split to expose the brain. The brain and remaining exoskeleton was submerged in 60 μl acetate buffer (pH 5.0) containing the internal standard α-methyldopamine (Merck) and stored at −80 °C. The use of acetate buffer as an extraction medium rather than the antioxidant perchloric acid was based on earlier findings that low concentrations of 5-HT and 5-HIAA extracted from small tissue samples are stable in acetate buffer (Renner and Luine, 1986) but not in perchloric acid (Renner and Luine, 1984). In addition, to determine the contribution of the exoskeleton to monoamine concentrations, samples in which the brain was completely removed from the exoskeleton were also prepared and stored in the same manner.

2.5. Data acquisition

Norepinephrine (NE), octopamine (OA), 5-hydroxytryptophan (5-HTP), dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), tyramine (TA), and serotonin (5-HT) (all standards were obtained from Sigma–Aldridge, St. Louis, MO) were detected by use of high performance liquid chromatography with electrochemical detection following modification of a previously described method (Renner and Luine, 1984) by markedly increasing the concentration of octanyl sulfonic acid, using methanol instead of acetylnitrate as the organic modifier and increasing the applied potential to +0.9 V relative to an Ag/AgCl reference electrode for the oxidation of OA and TA. The frozen brain samples were thawed and centrifuged at 17,000 rpms. Simple freeze-thawing of small tissue volumes has been shown to be effective for extracting monoamines with recoveries typically exceeding 90% (Renner and Luine, 1984), an important consideration when attempting to analyze insect brain monoamine concentrations. The supernatant was removed and 45 μl of the sample was injected into the chromatographic system. The amines were separated with a C_{18} 4 μm NOVA-PAK radial compression column (Waters Associates Inc., Milford, MA). Electrochemical detection of the monoamines was carried out using an LC 4 potentiostat and a glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN). The applied potential was set at +0.9 V with respect to an Ag/AgCl reference electrode set at a sensitivity of either 0.5 or 1 nA/V. The initial mobile phase consisted of 8.6 g sodium acetate, 250 mg EDTA, 11 g citric acid, 330 mg octylsulfonic acid, 160 ml methanol, pH 3.7 (all chemicals were obtained from Sigma–Aldridge, St. Louis, MO) in 1 L of distilled water. Small additional increments of octylsulfonic acid and methanol were then added to the mobile phase until the desired separation was achieved. Under these conditions, retention times for the standards were: NE 3.58 min; OA 4.44 min; 5-HTP 5.16 min; DOPAC 5.69 min, DA 7.1 min, 5-HIAA 9.23 min, TA 10.34 min, alpha-methyl DA (internal standard) 13.78 min, 5-HT 16.84. Identity of peaks in the brain samples were tested by comparing the sample peak retention times to standard runs in response to changing mobile phase conditions (pH, octylsulfonic acid concentrations), changes in peak heights obtained by varying the applied potential and, in the case of 5-HT, pharmacological treatment. After removal of the supernatant, the tissue pellet was dissolved in 60 μl of 0.4 M NaOH and analyzed for protein (Bradford, 1976). The sample monoamines and respective metabolite concentrations were calculated from peak height values obtained from standard runs using the CSW32 data program (DataApex Ltd., Czech Republic) set in the internal standard mode. Appropriate corrections for injection volume vs. preparation volume were made and the resulting

Table 1
Concentrations of monoamines detected in single brain analysis of the stalk-eyed fly prepared from partial exoskeleton (Mean ± SEM).

Compound	Concentrations expressed as pg/μg protein	Total tissue content (pg)
Octopamine (n = 10)	387 ± 28.5	2833.9 ± 229.7
5-Hydroxytryptophan (n = 10)	21.7 ± 2.5	135.6 ± 24.7
Dopamine (n = 10)	667.5 ± 46.9	4703.4 ± 194.7
Tyramine (n = 6)	29.2 ± 4.9	157.9 ± 17.7
Serotonin (n = 8)	22.9 ± 3.4	165.6 ± 21.4

amine concentrations were divided by μg protein in the sample to yield pg amine/μg protein.

2.6. Statistical analysis

Statistical significance between differing 5-HTP treatments and duration time was determined using a Kruskal–Wallis One Way Analysis on Ranks, and post hoc analysis was conducted using the Dunn's method to test for differences between treatments. Statistical significance between control and TA treated flies was determined using a student t-test. Results for monoamine analyses are expressed as the mean pg amine/μg protein ± SEM. An exact binomial test was performed to examine whether individuals with higher neural 5-HT levels were more likely to win a contest than their size-matched counterpart with lower 5-HT. Results are expressed as the probability of success with a 95% confidence interval.

3. Results

Concentrations of monoamines detected from the analysis of single brains from stalk-eyed flies are summarized in Table 1. The mean 5-HT concentration for whole brain analysis was 22.9 ± 3.44 (pg/μg protein) for control insects. The neurotransmitter DA was, by far, the most abundant monoamine in the CNS of untreated flies. In addition, relatively high concentrations of OA were detected, accompanied by comparatively low, but detectable, concentrations of TA. 5-Hydroxytryptophan concentrations were also low in control flies. The 5-HT metabolite, 5-HIAA was not included in the table. Although 5-HIAA concentrations were quantifiable in some brain samples, the metabolite was present in low concentrations and often did not exceed 2:1 signal to noise ratio.

To test for potential monoamine contributions from the exoskeleton, hand dissected brain tissue was quantified and compared to the partial head preparations described above. In brain samples that excluded the exoskeleton, DA concentrations were still very high and represented the most abundant amine detected in an isolated stalk-eyed fly brain. Total brain concentrations of 5-HT were similar in samples prepared from isolated brain and partial exoskeleton dissections (180.2 ± 16.6 vs. 165.6 ± 21.4 pg, respectively). However, when 5-HT was quantified with respect to μg protein (Table 2), 5-HT concentrations were higher in samples prepared from isolated brain.

Table 2
Serotonin (5-HT) and dopamine (DA) content in samples prepared from partial exoskeleton and isolated brain dissections (total content, pg ± SEM) and tissue concentrations (pg/μg protein ± SEM).

	Partial exoskeleton (n = 10)	Whole isolated brain (n = 9)
5-HT (pg/μg protein)	22.9 ± 3.4	69.3 ± 7.3
Total content (pg)	165.6 ± 21.4	180.2 ± 16.6
DA (pg/μg protein)	667.5 ± 46.9	178.2 ± 15.2
Total content (pg)	4703.4 ± 194.7	479.8 ± 58.1

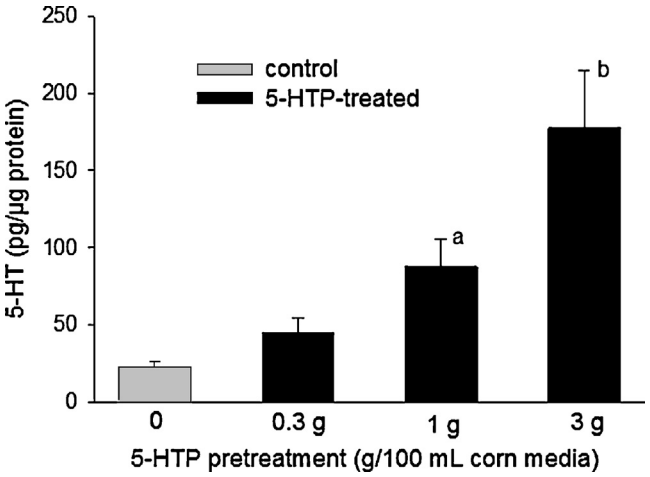

Fig. 2. Pretreatment with food media containing 0, 0.3 g, 1 g, and 3 g of the 5-HT precursor 5-HTP dose-dependently increased single whole brain/partial head 5-HT concentrations (mean ± SEM). [a] $p < 0.05$ vs. control; [b] $p < 0.05$ vs. control, 0.3 g.

Application of this monoamine analysis method to study the effects of oral administration of the 5-HT precursor, 5-HTP, on brain 5-HT concentrations showed that 5-HT can be increased in a dose-dependent manner (Fig. 2). Brain concentrations of the 5-HT precursor, 5-HTP, increased following treatments of the 5-HTP/food mixture as expected. The 3 g/100 ml 5-HTP/food mixture resulted in approximately an 8-fold increase of 5-HT relative to control concentrations (177 ± 37.7 vs. 22.9 ± 3.44 pg/μg protein, respectively; Fig. 2). Similarly, application of this monoamine analysis method to study the feasibility of using of oral administration of TA indicated that this approach can be used to selectively increase TA. Although TA is a precursor to OA (Roeder, 2005), this treatment did not significantly affect brain OA concentrations (control vs. 1 g TA: 365.8 ± 27.7; 300.6 ± 52.4 pg/μg protein, respectively; $p > 0.25$). Single brain levels of TA increased dramatically following oral administration of 1 g/100 ml TA/food (24.3 ± 6.2 vs. 781.9 ± 114 pg/μg protein; Fig. 3).

The time-course for the duration of increased brain 5-HT following removal of the 3 g 5-HTP/100 ml food source are summarized in Fig. 4. The increase in 5-HT levels obtained using the 3 g 5-HTP/100 ml were maintained for at least 12 h following removal of the food source (Fig. 4). This end point is important because using a competition for a food source to monitor aggression in stalk-eyed

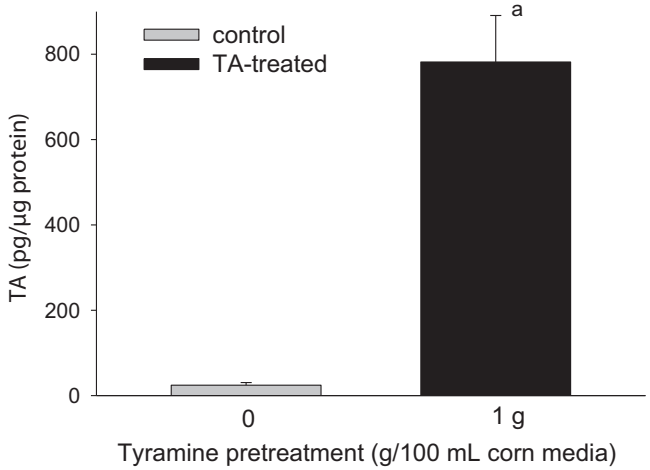

Fig. 3. Pretreatment with food media containing 0, and 1 g of the OA precursor TA increased single whole brain/partial head 5-HT concentrations (mean ± SEM). [a] $p < 0.002$ vs. control.

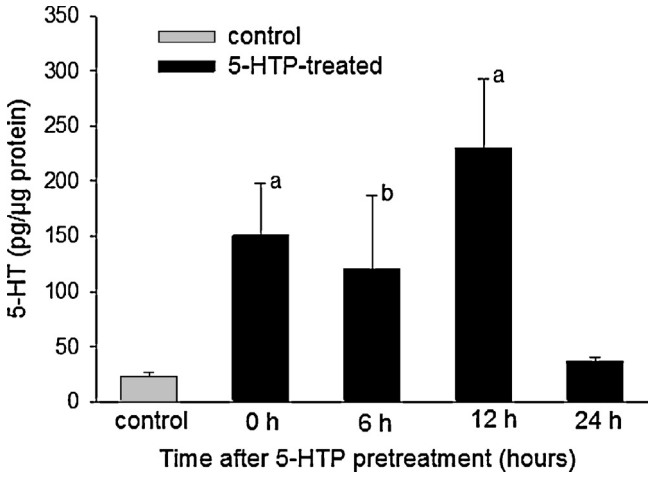

Fig. 4. Increased 5-HT concentrations (mean ± SEM) in the brain, following the removal of the 3 g 5-HTP/100 ml media and subsequent food deprivation, were maintained for at least 12 h and returned to baseline concentrations by 24 h. [a] $p < 0.05$ vs. control, 24 h; [b] $p < 0.05$ vs. control.

flies requires a period of starvation (Egge et al., 2011; Egge and Swallow, 2011; Panhuis and Wilkinson, 1999). Baseline concentrations of 5-HT were restored by 24 h following removal of the food source.

Finally, we tested if manipulation of 5-HT levels might impact the expression of aggressive behavior. We observed a total of 47 contests in the 20 trials with an average of 2.35 ± 2.21 (mean ± standard deviation) contests per trial (range 1–10). In all 20 contests we identified a clear winner and loser. Of the 47 trials, the eventual winner only lost in 3 individual contests. In 18 of the 20 trials, the trial winner won all individual contests. When combined with the brain analysis of 5-HT, results showed that males with pharmacologically increased brain 5-HT concentrations have an increased probability of winning in size-matched intraspecific contests over a food resource. Males with higher concentrations of 5-HT won 17 out of 20 bouts (exact binomial test $p = 0.0026$; 95% confidence interval = 0.621–0.968; Fig. 5) while males with lower concentrations of 5-HT won only 3 out of 20 bouts (exact binomial test $p = 0.0026$; 95% confidence interval = 0.032–0.038; Fig. 5). Experiments designed to decrease CNS 5-HT levels by oral

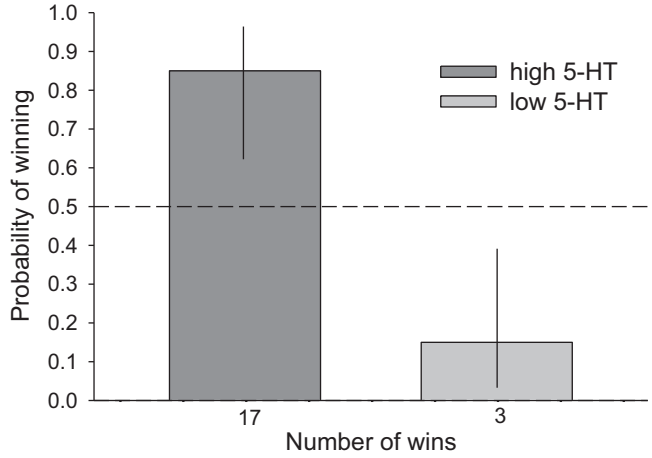

Fig. 5. Stalk-eyed flies with higher brain 5-HT concentrations (dark bar) won a high percentage of aggressive encounters in competition over a food resource ($p < 0.003$; $n = 20$ contests). The dashed line represented the hypothesized probability of success without a treatment effect.

administration of α-methyl-tryptophan (Dierick and Greenspan, 2007; Rillich et al., 2011) are currently in progress.

4. Discussion

The stalk-eyed fly *T. dalmanni* (Diptera; Diopsidae) represents an excellent model for investigating the potential roles of monoamines in modulating aggressive behaviors. Males of this sexually dimorphic species use their eye stalks as critical aggressive signals in different contexts crucial for fitness (Wilkinson and Johns, 2005). Male flies aggressively defend both diurnal feeding sites (de la Motte and Burkhardt, 1983; Panhuis and Wilkinson, 1999) and nocturnal roosting sites that allow mating access to females (Burkhardt and de la Motte, 1985, 1987; Small et al., 2009). Larger males with broader eye spans exclude smaller rivals and thus gain access to the limiting resources of food and mates (Burkhardt and de la Motte, 1988; Burkhardt et al., 1994; Cotton et al., 2010). Agonistic encounters between males follow a stereotyped escalated progression that is terminated when one of the rivals capitulates and departs (Egge and Swallow, 2011; Egge et al., 2011). Interestingly, stalk-eyed flies exhibit many of the components of the aggressive display used in intrasexual competition when threatened by a predator; males are more likely to use aggressive components, including rearing up on their hind legs, prominently displaying their fore legs parallel to their eye stalks and even jabbing, than are females (Worthington and Swallow, 2010, 2011).

The analytical method described above provides a means to study the effects of monoamines on aggressive responsiveness in this model by providing the means to successfully detect multiple monoamines including OA, 5-HTP, DA, TA, and 5-HT in a whole-brain sample from a single stalk-eyed fly (Table 1). In addition, low concentrations of the 5-HT metabolite, 5-HIAA were detected in a percentage of the samples. Studies using *Drosophila* to test behavioral effects of altering brain monoamine concentrations require pooling brains and conclusions are based on mean population responses (Hardie and Hirsh, 2006). The ability to take measurements from a single brain allows for a more direct assessment of individual responses in studies investigating monoaminergic influences on behavioral outcomes. While a similar approach has been used to test the role of OA in modifying the response to olfactory stimulation and labor division in honey bees (Barron and Robinson, 2005), honey bees are significantly larger than stalk-eyed flies. For comparison, the head of a honey bee (9.97 mg; Cooper et al., 1985) is approximately 37 times larger than the head of a stalk-eyed fly (0.27 mg; Ribak and Swallow, 2007). To establish *T. dalmanni* as a model organism for studying effects of monoamines on aggression in insects, we needed to confirm that we would be able to not only detect the amines in a much smaller individual brain but also alter the amines, in this case 5-HT, and demonstrate the ability to measure a behavioral response.

In vertebrates, monoamines such as 5-HT and DA are phylogenetically conserved and are present in multiple species including insects (Evans, 1980; Chen et al., 2008). Amines such as TA and OA, which are abundant in insects, are only present in trace amounts in mammals (Evans, 1980; Roeder, 2005). Octopamine in insects is believed to function as the invertebrate equivalent of NE (Verlinden et al., 2010). We were not able to consistently detect NE in samples prepared from the partial exoskeleton or isolated brain dissections of the stalk-eyed fly. The presence of NE has been reported in worker honey bee (Sasaki and Nagao, 2001) and house cricket (*Achea*; Pyza et al., 1991) brains. However, NE is not believed to function as a neurotransmitter in insects (Roeder, 1999) and it may represent a dopamine metabolite in these organisms. We were also not able to detect epinephrine or the mammalian DA metabolites DOPAC and homovanillic acid in the stalk-eyed fly brain.

In comparing our results obtained from samples prepared from partially dissected exoskeleton preparations to isolated brain tissue, it is evident that the presence of the exoskeleton contributed to the high DA concentrations. This result is consistent with an earlier study, in which the exoskeleton was found to contribute to the high levels of DA in whole head samples of *Drosophila*, possibly due to its role as an intermediate in the sclerotization process of the cuticle (Hardie and Hirsh, 2006). Similar to their results, we found lower DA levels in the dissected brain relative to DA levels in partial head samples, which contained portions of the exoskeleton (Table 2). However, DA was still the most abundant amine in isolated brain samples. Total brain content of the other amines were similar when the two sample preparation methods were compared. However, with the exception of DA, amine levels from isolated brain were consistently higher relative to partial head levels when quantitated with respect to μg protein. The most likely explanation for this result is the presence of higher protein content in samples containing the exoskeleton and associated musculature. To avoid potential complications in data quantification, we recommend analysis of dissected brain that excludes the exoskeleton in future studies.

In order to study the potential effects of brain 5-HT on aggressive behavior in stalk-eyed flies, we first tested the validity of using the food medium as a vehicle to systemically deliver the 5-HT precursor, 5-HTP as a means of augmenting brain 5-HT concentrations. The results indicate that 5-HTP pretreatment delivered in food media produces a robust dose-dependent increase in brain 5-HT concentrations. Since administration of 5-HTP was oral, the variation in 5-HT concentrations can be partially attributable to the last time ingestion took place as well as to the amount of food ingested. Nevertheless, the results clearly demonstrate that fairly reliable increases in stalk-eyed fly brain 5-HT can be obtained with 5-HTP loading and can be applied to test the effects of the manipulation on the expression of behaviors. This approach has the advantage of being a non-invasive pharmacological manipulation. However, since 5-HTP is available systemically and presumably, increases in 5-HT are not limited to the CNS, potential side effects of the manipulations on other organ systems must be considered in interpreting results. In our study, there was no indication of physical impairment from the various treatments but we were unable to evaluate other physiological parameters such as feeding and metabolic rates. Orally administering TA results in significant neural increases of the monoamine. However, despite the large concentration increase of TA, brain OA concentrations in the TA treatment group did not significantly differ from controls. The reason TA fails to increase OA levels in the stalk-eyed fly brain is unclear. We anticipated that this treatment, in addition to increasing TA, would also increase OA since octopaminergic neurons in several insect species express octopamine transporters that have high affinity for TA (Lange, 2009).

Our behavioral paradigm used to study male aggressive competition over a food resource requires that the stalk-eyed flies be starved prior to initiating the behavioral test (Egge et al., 2011). Thus, it was important to determine the duration for which increased brain concentrations of 5-HT were present after 5-HTP preloading. For this test we chose the highest 5-HTP concentration (3 g/100 ml media). Our results indicate that brain 5-HT concentrations remain elevated for at least 12 h after food deprivation. The ability to measure 5-HT in individual fly brains eliminates the potential confounds of amount consumed or time of last ingestion by allowing for direct assessment between 5-HT and behavioral outcomes. In previous studies (Egge et al., 2011; Egge and Swallow, 2011), we had used a 24 h starvation period. However, because 5-HT levels in treated flies did not differ significantly from control flies after a 24 h period, we used the shorter 12 h starvation period. In the current study, we found that flies engaged in 2.35 contests per trial compared to 6.1 contests per trial as reported previously (Egge et al., 2011). These results indicate that while this food deprivation protocol was sufficient to elicit aggressive competition over the introduced food resource, it may have reduced, to some degree, the incentive to fight.

Much of the work on the expression of behaviors in arthropods has focused on the roles of 5-HT (reviewed in Blenau and Thamm, 2011) and OA, a noradrenergic analog (reviewed in Roeder, 2005). The effects of the respective amines in modulating invertebrate aggression are variable. In several species of crustaceans, increased 5-HT concentrations are linked to increased aggression while increases in OA have the opposite effect (Antonsen and Paul, 1997; Huber et al., 1997; Livingstone et al., 1980; Pedetta et al., 2010). Similarly, 5-HT and OA appear to have opposing behavioral effects in orb-weaving spiders (Jones et al., 2011). In contrast to crustaceans, exogenously administered 5-HT decreases aggressiveness while OA appears to increase aggressiveness in the spider based on the duration of antipredator huddling. In insects, activation of both 5-HT and OA systems can enhance aggressiveness. For example, OA enhances aggression in both *Drosophila* (Hoyer et al., 2008; Zhou et al., 2008) and field crickets (*Gryllus bimaculatus*) (Rillich and Stevenson, 2011; Rillich et al., 2011) and OA neurons have been identified in the *Drosophila* subesophageal ganglion that drive the behavior (Zhou et al., 2008). The effects of manipulating 5-HT on insect aggression are less clear. Early reports indicated that 5-HT had no effect on aggression in *Drosophila* (Baier et al., 2002). More recently, both pharmacological and genetic manipulations that elevate 5-HT have been shown to increase *Drosophila* aggression (Alekseyenko et al., 2010; Dierick and Greenspan, 2007). We conducted behavioral trials using our stalk-eyed model to determine if increasing brain 5-HT impacted aggression in stalk-eyed flies competing for a food resource. Our initial trials showed that individuals with higher brain 5-HT won 85% (17 of 20) of the contests indicating that flies with higher CNS 5-HT than their opponent have an increased probability of winning contests in size-matched intra-specific aggressive interactions (Fig. 5). These data are consistent with previous work that found *Drosophila melanogaster* exhibited increased fighting frequency when pooled brain 5-HT levels were increased approximately 15- to 20-fold relative to controls (Dierick and Greenspan, 2007) and that genetic manipulations that elevate 5-HT increase *Drosophila* aggression (Alekseyenko et al., 2010).

5. Conclusion

In both vertebrates and invertebrates, monoamines play important roles in modulating physiological processes and behaviors (Evans, 1980; Farmer et al., 1996; Huber et al., 1997; Kravitz and Huber, 2003; Cnaani et al., 2003; Libersat and Pflueger, 2004; Cornil et al., 2005; Huber, 2005; Stevenson et al., 2005; Nichols, 2006; Summers and Winberg, 2006; Øverli et al., 2007; Chen et al., 2008). The sexually dimorphic stalk-eyed fly, *T. dalmanni*, provides an ideal model system to investigate the role of monoamines in aggression because males use their exaggerated head morphology in dramatic ritualized aggressive displays in a number of behavioral contexts. In this study, we describe a technique for the detection and quantification of a variety of different biogenic amines in a single whole brain sample of a stalk-eyed fly and apply the method to characterize pharmacological manipulations of 5-HT concentrations in the brain and subsequent behavioral effects of the manipulation. Our results suggest increases in 5-HT enhances aggressive behavior and the likelihood of winning a competition over a food resource.

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