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Modulation of motor behavior by dopamine and the D1-like dopamine receptor AmDOP2 in the honey bee

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Abstract

Determining the specific molecular pathways through which dopamine affects behavior has been complicated by the presence of multiple dopamine receptor subtypes that couple to different second messenger pathways. The observation of freely moving adult bees in an arena was used to investigate the role of dopamine signaling in regulating the behavior of the honey bee. Dopamine or the dopamine receptor antagonist flupenthixol was injected into the hemolymph of worker honey bees. Significant differences between treated and control bees were seen for all behaviors (walking, stopped, upside down, grooming, flying and fanning), and behavioral shifts were dependent on drug dosage and time after injection. To examine the role of dopamine signaling through a specific dopamine receptor in the brain, RNA interference was used to reduce expression levels of a D1-like receptor, AmDOP2. Injection of *Amdop2* dsRNA into the mushroom bodies reduced the levels of *Amdop2* mRNA and produced significant changes in the amount of time honey bees spent performing specific behaviors with reductions in time spent walking offset by increases in grooming or time spent stopped. Taken together these results establish that dopamine plays an important role in regulating motor behavior of the honey bee.

Keywords

Apis mellifera; locomotor activity; grooming; mushroom body; RNAi

Introduction

In invertebrates, numerous studies have shown that dopamine signaling affects locomotion (Chase et al., 2004; Draper et al., 2007; Lima and Miesenbock, 2005; Pendleton et al., 2002; Sawin et al., 2000; Yellman et al., 1997). Furthermore, dopamine affects locomotor behavior on several levels including modulation of the neuromuscular junction (Cooper and Neckameyer, 1999; Dasari and Cooper, 2004), regulation of central pattern generators (Puhl and Mesce, 2008; Svensson et al., 2001) and setting general arousal levels (Andreatic et al., 2005; Kume et al., 2005). Thus, dopamine may act at multiple sites to influence locomotor

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behavior by affecting sensory information in the periphery, the regulation of central pattern generators and/or higher order processing of information in the brain.

Although the role of dopamine in modulating behavior has been studied extensively, the distinct molecular pathways through which dopamine acts to affect different behaviors are still not well understood. Both, vertebrates and invertebrates, have two distinct classes of dopamine receptors: D1-like receptors that increase intracellular cAMP levels when activated and D2-like receptors which cause a decrease in cAMP levels in the presence of dopamine (Mustard et al., 2005; Neve et al., 2004). In the past, establishing the role of specific dopamine receptors in behavior was difficult due to the lack of pharmacological agents that are selective for specific invertebrate dopamine receptor subtypes. In addition, dopamine signaling plays an important role during development (Neckameyer, 1996), suggesting that animals with mutations in their dopamine receptors may show changes in behavior due to developmental defects.

In this study, we use both pharmacological and molecular approaches to examine the role of dopamine signaling in regulating the behavior of freely moving adult honey bees. The role of dopamine in modulating behavior in honey bees was characterized via injections of dopamine or the general dopamine receptor antagonist flupenthixol into the hemolymph leading to global perturbations in dopamine signaling. In the honey bee, three distinct dopamine receptors, two D1-like receptors, AmDOP1 and AmDOP2 (Blenau et al., 1998; Humphries et al., 2003), and one D2-like receptor, AmDOP3 (Beggs et al., 2005) have been cloned and characterized. Each receptor has a unique expression pattern; however, each receptor is expressed in the mushroom bodies of the brain of adult workers (Beggs et al., 2005; Blenau et al., 1998; Humphries et al., 2003; Kurshan et al., 2003). In insects, the mushroom bodies of the brain have been implicated as regions that control and coordinate locomotor activity (Besson and Martin, 2005; Martin et al., 1998; Mizunami et al., 1998) making the role of dopamine signaling in the mushroom bodies of particular interest. The role of a D1-like dopamine receptor, AmDOP2, in the brain was investigated using RNAi mediated knockdown of expression. AmDOP2 is the ortholog of the *Drosophila* DAMB dopamine receptor, also known as DopR99B (Feng et al., 1996; Han et al., 1996). Injection of dsRNA corresponding to the sequence of the *Amdop2* receptor gene into the mushroom bodies reduced *Amdop2* mRNA levels and affected a subset of the behaviors influenced by treatment with the dopamine receptor antagonist flupenthixol. These results provide insight into the specific contributions of this receptor to motor behavior and show that the combination of pharmacological treatments with RNAi is a useful strategy for revealing the roles of biogenic amines in behavior.

Materials and methods

Subjects

Honey bees used in this study were from the New World Carniolan population maintained at the Rothenbuhler Honey Bee Research Laboratory at Ohio State University. Pollen foragers were used exclusively in this study as biogenic amine levels vary in the brains of worker honey bees depending on their age and the behavioral task in which they specialize (Schulz and Robinson, 1999; Taylor et al., 1992; Wagener-Hulme et al., 1999). Therefore, using bees in one specific task group should minimize the natural variation in endogenous dopamine levels between individual bees in the experiment. Individual worker bees returning to the colony were captured in small glass vials and placed at 4 °C until motionless. Bees to be treated with dopamine or flupenthixol were placed in a harness and restrained with a strip of tape placed between the head and thorax. They were then fed 18 µl of 2 M sucrose, left overnight at room temperature and used for experiments the next day. Bees to be injected with dsRNA were positioned in 1.5 ml microcentrifuge tubes with the lids and the ends cut off, restrained with tape and a small drop of dental wax was used to anneal each bee's head to the side of the tube. Injection of dsRNA (see below) was done on the same day as they were captured.

Pharmacological Treatment

Bees were treated with dopamine hydrochloride or cis-(Z)-flupenthixol dihydrochloride (Sigma Aldrich, St Louis, MO) diluted in injection buffer (5 mM KCl, 10 mM NaH₂PO₄, pH 7.8). Subjects were fed 9 µl of 1 M sucrose immediately prior to injection. Treatment consisted of 2 µl of buffer containing the indicated concentration of drug, or buffer alone as a control, injected under the cuticle between the second and third abdominal segments using a 10 µl syringe (Hamilton Company, Reno, NV). Observations began fifteen minutes after injection.

dsRNA Treatment

dsRNA was synthesized using the PCR template method (Kennerdell and Carthew, 1998) using T7 RNAP promotor linked oligonucleotides. PCR primers specific for *Amdop2* were AGAGATTTTCGTAGAGCGTTCG and GAGGGTGTCGTATTGTCCAAC. The sequence of the entire synthesized dsRNA fragment was BLASTed against the sequence of the honey bee genome. Besides the *Amdop2* gene, the dsRNA fragment did not show the level of homology (runs of at least 19 nt of identical sequence) necessary to produce RNA interference at any other region of the genome (Kulkarni et al., 2006). As a control, dsRNA was also synthesized corresponding to the *Drosophila friend of echinoid (fred)* gene. *fred* is a paralog of *echinoid* that is expressed in the central nervous system and interacts with the Notch signaling pathway. Disruption of *fred* via the same RNAi construct used in this work has been shown to have significant effects on sensory organ precursor cells during *Drosophila* development (Chandra et al., 2003), and yet this construct does not contain the level of sequence homology necessary to induce RNAi in honey bee. Therefore, *Dmfred* dsRNA acts as a control for the nonspecific effects of dsRNA treatment. PCR primers used to produce the *Dmfred* template were ATGGTGACATTGGAAATACACAG and CCTCTTATGCTGTCCAAAGGAT. dsRNA was synthesized in vitro from the PCR templates using the Maxiscript kit (Ambion), ethanol precipitated, quantitated and diluted to 125 ng/µl in injection buffer.

A small window was cut in the cuticle of the head capsule exposing the brain. 4 nL of injection buffer or 4 nL of injection buffer containing 125 ng/ µl of *Amdop2* or *Dmfred* dsRNA was injected into each calyx of the mushroom bodies. (Four injections per brain.) Injections were done using a Picospritzer II (Parker Hannifin corporation). Bees were kept in their harnesses in a humidified box at room temperature for either 24 or 48 hrs before observation and fed 18 µl of 2 M sucrose daily. Bees were fed 9 µl of 1 M sucrose prior to beginning the observation.

Behavioral Observations

Honey bees were released from their harnesses into a 150 × 15 mm Petri dish (Fisher) which served as the observation arena. Bees were allowed to acclimate to the arena for five minutes before observations began. Initial studies determined six mutually exclusive behaviors in which worker bees engage in the observation arena: walking, stopped, upside down, grooming, fanning or flying. These behaviors are also observed in natural contexts such as at the colony entrance. Upside down behavior was seen when bees walking on the lid or side of the arena fell off onto their backs so that they were lying on their dorsal surface with their legs in the air. Normally, bees are able to quickly right themselves. Any situation in which the bee used its legs to rub other parts of its body was considered grooming. Fanning consisted of stationary bees with raised abdomens that would rapidly beat their wings. Since the observation arenas are covered with a lid to prevent the subject from flying away, flying behavior consisted of short flying hops. A “stopped” bee was not engaged in any other behavior, but was simply standing still. A behavioral profile was determined by measuring how much time a bee spent engaged in each behavior; since each behavior was mutually exclusive, the total percent time spent in each behavior adds to 100%. Behavioral data were collected using The Observer (Noldus Information Technology) software. All observations were done by one individual

(PMP). Experimental observations were done at approximately the same time every day (afternoon/early evening) with multiple treatment groups in a randomized order.

Quantification of *Amdop2* expression

Total RNA was isolated from dissected brains using Trizol (Invitrogen) as described by the manufacturer. cDNA was synthesized using the iScript kit (Bio-Rad). The levels of *Amdop2* and *AmEF1 α* (NCBI accession number X52884) expression were determined using quantitative real time PCR with Taqman probes. For *Amdop2* quantification, the PCR primers are: CCGAGGACCTCCAGGATCTC and TCTTCTCCTTGGCGAACTTGG, and the probe sequence is FAM-AGCCGCTCACCACCATCCAGCAC-3BHQ1. For *EF1 α* the PCR primers are AAGATGGTAACGCTGACGGAAA and GAAGAGCCTTATCGGTAGGTCTG, and the probe is VIC-CTGATCGAAGCTCCCGAC-MGBNFQ. All primers and probes were from Integrated DNA Technologies (Coralville, IA), except for the *EF1 α* probe, which was from Applied Biosystems (Foster City, CA). Regions of *Amdop2* amplified for quantitative analysis did not overlap with the region encoded in the dsRNA used for RNAi. PCR reactions were done using Applied Biosystems 2X master mix on an Applied Biosystems 7500 Real Time PCR instrument. Levels of expression were determined using the relative standard curve method (ABI User's Guide #2).

Data Analysis

The behavioral profiles of the bees were expressed as the percent time spent engaged in each behavior during a 5 min interval. For statistical analyses of the behavioral data, the data were transformed by taking the arcsine to produce a normal distribution (Sokal and Rohlf, 1981). Data were analyzed using the general linear modeling procedure in the JMP version 5.1 statistical analysis software (SAS Institute Incorporated, Cary, NC, USA). We evaluated the effect of different drug dosages or dsRNA injection on the frequencies of different behaviors within each of the defined time intervals. The main significance test of interest was the interaction of behavior frequencies with treatment. A significant interaction indicates that the amount of time spent in each behavior within that time interval depends on treatment. For the pharmacological experiments, if the interaction term was significant, *post hoc* t tests were performed to compare the time bees spent in each activity for each concentration of treatment with the time spent in that activity by buffer injected control subjects. For the RNAi experiments, the buffer injected and *Amdop2* dsRNA injected bees were compared with the *Dmfred* injected bees. A significant *post hoc* test ($p \leq 0.05$) is indicated by asterisks within columns in the figures.

Results

Analysis of the behavioral profile of the control (buffer injected) bees revealed differences in the amount of time spent in each behavior during the course of the observation (time spent in each behavior versus interval after injection, $F_{25,25} = 4.17$, $p < 0.0001$). For example, compare the behavior of the buffer bees at the 20–25 min interval, when they spent the majority of their time walking, with their behavior at 35–40 min, when they spent less time walking and more time grooming. *Post hoc* analysis showed control bees significantly changed the amount of time spent walking, stopped, grooming and upside down during different intervals. Thus, behavioral profiles of bees receiving different treatments were compared within 5 min time intervals.

Effects of injection with dopamine

Bees injected with dopamine showed shifts in their behavioral profiles that were both time and dose dependent when compared with control bees (Figure 1). Observation at the earliest time point, 15–20 min after injection, did not reveal significant differences in behavior ($F_{20,20} =$

1.46, $p = 0.09$). However, the behavioral profiles of bees in different treatment groups were significantly different 20–25 min ($F_{20,20} = 1.75$, $p = 0.02$), 25–30 min ($F_{20,20} = 2.70$, $p < 0.0001$) and 30–35 min ($F_{20,20} = 2.77$, $p < 0.0001$) after injection. For example, at 25–30 min after injection, comparison of the behavioral profile bar for control and 5×10^{-4} M dopamine treated bees reveals that dopamine injected bees spent less time walking than control bees, but more time upside down or fanning. Although the trends in behavior, such as increases in stopped behavior for the 5×10^{-5} M dopamine treated bees, continued, the behavioral profiles of the bees at later time points, 35–40 min ($F_{20,20} = 1.11$, $p = 0.3$) and 40–45 min ($F_{20,20} = 1.28$, $p = 0.1$) after injection, were not significantly different for the different treatments. Interestingly, this lack of difference may be due to a change in the behavior of the control (buffer) bees later in the observation period as well as changes in the effects of the exogenous dopamine.

There was also a dose dependent effect of dopamine treatment. For example, comparing the 20–25, 25–30 and 30–35 min observation periods, bees injected with 5×10^{-4} M dopamine spent significantly more time fanning or upside down, and thus, less time walking than control bees. Bees treated with the lowest dose, 5×10^{-5} M dopamine, also spent less time walking than control bees, however, this was due to increases in grooming, fanning and stopped behavior. Furthermore, bees treated with the highest concentration of dopamine, 5×10^{-2} M, offset a reduction in walking with a significant increase in grooming behavior at 30–35 min after injection. Although there was a general trend for 5×10^{-5} M dopamine to decrease flying behavior, there were no significant differences in flying for dopamine treated versus control bees.

Effects of injection with a dopamine receptor antagonist

The synthetic compound flupenthixol has been shown to be a potent antagonist of both D1-like (Mustard et al., 2003) and D2-like (Hearn et al., 2002) insect dopamine receptors. Injection of flupenthixol into the hemolymph had a significant impact on the behavior of honey bees for each interval over the entire observation period (Figure 2; 15–20 min ($F_{20,20} = 2.95$, $p < 0.0001$), 20–25 min ($F_{20,20} = 4.40$, $p < 0.0001$), 25–30 min ($F_{20,20} = 4.60$, $p < 0.0001$), 30–35 min ($F_{20,20} = 4.33$, $p < 0.0001$), 35–40 min ($F_{20,20} = 2.59$, $p = 0.0003$), and 40–45 min ($F_{20,20} = 2.07$, $p = 0.004$)). The effects of treatment with flupenthixol were, in general, decreases in time spent walking or flying offset by increases in time spent stopped or upside down. As with dopamine treatment, the changes in behavior were time and dose dependent. For example, while bees treated with 5×10^{-6} M flupenthixol showed significant increases in stopped behavior at every interval, during the first two intervals significant increases in time spent grooming were also observed. In contrast, bees injected with 5×10^{-4} M flupenthixol spent significantly more time upside down than control bees. Furthermore, bees treated with 5×10^{-5} M flupenthixol, an intermediate concentration, did not show any significant differences in behavior compared with control bees.

Effects of AmDOP2 dopamine receptor knockdown

To investigate the role of a specific dopamine receptor, AmDOP2, in the mushroom bodies in regulating motor behavior, we knocked down expression of the *Amdop2* gene using RNAi (Figure 3). Significant differences in behavioral profiles between buffer injected bees, *Dmfred* dsRNA injected bees and bees treated with *Amdop2* dsRNA were observed for all intervals of the observation period both 24 hrs (0–5 min, $F_{10,10} = 4.52$, $p < 0.0001$; 5–10 min, $F_{10,10} = 4.71$, $p < 0.0001$; 10–15 min, $F_{10,10} = 2.76$, $p = 0.002$; 15–20 min, $F_{10,10} = 3.95$, $p < 0.0001$) and 48 hrs after injection (0–5 min, $F_{10,10} = 5.15$, $p < 0.0001$; 5–10 min, $F_{10,10} = 3.66$, $p = 0.0001$; 10–15 min, $F_{10,10} = 2.70$, $p = 0.003$; 15–20 min, $F_{10,10} = 4.04$, $p < 0.0001$). As the amount of time spent engaged in each behavior was significantly affected by treatment for all observations, post-hoc tests were used to examine which behaviors differed between which treatments. The only significant difference in behavior between buffer and *Dmfred* dsRNA

treated bees occurred 48 hrs after injection in the 15 – 20 min interval when *Dmfred* bees showed a reduction in grooming compared with buffer bees. Grooming behavior for *Amdop2* dsRNA treated bees did not differ from either buffer or *Dmfred* bees during this interval. Given that buffer injected and *Dmfred* dsRNA treated bees exhibited essentially the same behavior, we focused on differences between *Dmfred* and *Amdop2* dsRNA injected bees.

Bees injected with *Amdop2* dsRNA showed a significant reduction in time spent walking in all intervals compared with *Dmfred* dsRNA treated controls. For observations at both 24 and 48 h after injection the general trend was for the reduction in walking to be offset by increases in grooming behavior early in the observation period (0 – 10 min), and then later by increases in time spent stopped (0 – 20). *Amdop2* dsRNA treatment did not significantly affect the amount of time bees spent flying, fanning or upside down.

Quantitation of the effects of dsRNA injection on *Amdop2* expression

After each bee was observed to assess its behavior, its brain was removed and the levels of *Amdop2* were determined using reverse transcription followed by quantitative real time PCR (Figure 4). Injection of *Amdop2* but not *Dmfred* dsRNA into the mushroom bodies significantly reduced the level of *Amdop2* transcript 48 hours after injection of the dsRNA.

Discussion

Injection of dopamine or the dopamine receptor antagonist flupenthixol into the hemolymph had significant effects on motor behavior. Bees treated with dopamine or flupenthixol show a dose dependent U-shaped effect, where the behavioral effects are not linear with concentration. This non-linear effect of dose and response has been observed in other studies investigating the effects of biogenic amines on honey bee behavior (Barron et al., 2007; Fussnecker et al., 2006; Scheiner et al., 2002). Furthermore, the observed changes in behavior are probably not simply due to the treatments making the bees feel sick as larger effects were often observed at lower drug concentrations.

Flupenthixol is a potent antagonist for invertebrate dopamine receptors that acts to block signaling through both D1-like and D2-like dopamine receptors (Hearn et al., 2002; Mustard et al., 2003). Flupenthixol has been reported to act as a weak antagonist at octopamine receptors in the cockroach (Gole et al., 1987). However, flupenthixol injection has a distinct (and much more profound) effect on the behavioral profile of honey bees than injection of the octopamine and tyramine receptor antagonists mianserin and yohimbine did in an experimental protocol identical to the one presented here (Fussnecker et al., 2006). In addition, binding studies on honey bee brain homogenate revealed that flupenthixol has very low affinity for serotonin receptors (Blenau et al., 1995). Flupenthixol may act at other biogenic amine receptor types, especially at higher concentrations, however the contributions from blocking the types of receptors are probably minimal compared to the effect of flupenthixol on dopamine receptors.

Although dopamine and flupenthixol treatment both reduced the amount of time spent walking, the changes in the behavioral profiles overall were distinct for the two drugs. By observing the entire behavioral profile, rather than simply using a line crossing type assay as a measure of locomotor activity, it was clear that reductions in walking behavior due to injection with dopamine were offset by increases in other active behaviors such as grooming or fanning and a trend for increases in flying (although this was not significant for any one interval). In contrast, the overall trend for injection with flupenthixol was a greater reduction in time spent in active behaviors such as walking and flying, and more time spent grooming or stopped. Interestingly, other studies examining the effects of biogenic amines on walking in honey bees have also shown that treatment with both the receptor agonist and the antagonist resulted in a reduction in walking (Bloch and Meshi, 2007; Fussnecker et al., 2006).

Injection of dopamine into the hemolymph has been shown to lead to significant increases in dopamine in the brain and thoracic ganglia 30 min after injection (Linn et al., 1994). This result suggests that dopamine or flupenthixol injected into the hemolymph could be affecting dopamine receptors in both the central nervous system and/or the periphery. Although little is known about the distribution of dopamine receptors in tissues other than the brain in the honey bee, work on *Drosophila* and *C. elegans* suggests that dopamine receptors are present in a number of other tissues (Chase et al., 2004; Draper et al., 2007; Kim et al., 2003; Tsalik et al., 2003). Rhythmic behaviors, such as walking, flying and grooming, are controlled by central pattern generators (CPGs) that are usually located in the thoracic ganglion of insects. Dopamine acts directly on CPGs to produce specific behaviors and modulate the properties of the CPG (Harris-Warrick et al., 1995; Puhl and Mesce, 2008; Svensson et al., 2001). In addition, dopamine signaling works at the periphery to regulate motor circuits (Cooper and Neckameyer, 1999; Dasari and Cooper, 2004). Thus, dopamine or flupenthixol in the hemolymph may affect locomotion by acting directly on dopamine receptors in the thoracic ganglion, at the periphery or in the brain.

In order to examine the role of a specific dopamine receptor in the brain in regulating behavior, the expression level of *Amdop2*, was down regulated via RNAi. In invertebrates, dopamine acts through at least three different receptors that all have distinct functional properties (Mustard et al., 2005). *Amdop2* shows a relatively restricted expression pattern in the worker bee brain with high levels of expression in the Kenyon cells of the mushroom bodies (Humphries et al., 2003; Kurshan et al., 2003). Injection of dsRNA into the mushroom bodies would not be expected to affect expression of receptors at the periphery or in the thoracic ganglion. Although the dsRNA was injected into the calyces of the mushroom bodies, it is also possible that the RNAi effect spread to the central complex region of the brain. The central complex has also been implicated in the control of locomotion (Martin et al., 1999; Strauss and Heisenberg, 1993); however the level of expression of *Amdop2* in this structure has not been thoroughly investigated. There were significant differences in the behavioral profiles of bees injected with *Amdop2* dsRNA compared to the control (*Dmfred* dsRNA) bees for each observation interval. Bees receiving the *Amdop2* dsRNA treatment were observed to spend less time engaged in walking behavior which was offset by more time spent grooming or stopped. Fewer behaviors were significantly affected by *Amdop2* knockdown than by pharmacological treatment.

In comparison with bees injected with flupenthixol or dopamine, bees with reduced *Amdop2* expression did not show increases in upside down behavior suggesting that the coordination of leg movements necessary for the righting reflex was not affected. It is possible that dopamine signaling at the periphery or in the thoracic ganglion is important for leg coordination rather than regulation of the righting reflex by the brain. It is also possible that signaling through other dopamine receptors may play an important role in the regulation of the righting reflex so that a reduction in *Amdop2* transcript levels did not affect this behavior. The significant reduction in flying behavior observed in the flupenthixol treated bees was also not observed in *Amdop2* knockdown bees, suggesting that flying does not require signaling via AmDOP2 in the mushroom bodies.

Increases in grooming behavior were observed for bees receiving either pharmacological treatment or in the *Amdop2* knockdown experiment. Dopamine signaling has been shown to play a role in grooming behavior in insects (Chang et al., 2006; Weisel-Eichler et al., 1999; Yellman et al., 1997). Furthermore, an ethological analysis of spontaneous behavior in D_{1A} receptor null mice revealed a very similar pattern of behavior to *Amdop2* knockdown bees. Mice lacking the D_{1A} receptor engaged in increased levels of grooming when placed in a novel environment, and this increase in grooming was reduced to wild type levels over time (Clifford et al., 1998). Although grooming is important for removal of debris and parasites, grooming

also occurs in situations where it is not related to skin maintenance. For example, in vertebrates, grooming may have a role in stress reduction or “dearousal” (Delius, 1988; Komorowska and Pellis, 2004; Spruijt et al., 1992). Recent work shows that dopamine is involved in regulating the general level of arousal in *Drosophila* (Andreatic et al., 2005; Kume et al., 2005); therefore, the observed increase in grooming may be an attempt to regulate arousal.

Early in the observation period, *Amdop2* knockdown bees showed increases in the frequency of grooming behavior; however, a shift to significant increases in time spent stopped was observed later (10 to 20 min into the observation). Treatment with flupenthixol also resulted in significant increases in time spent stopped. The large increase in time spent stopped where bees are not engaged in any activity was surprising as the buffer and *Dmfred* control bees spent very little time stopped during this period. Since the mushroom bodies receive multimodal sensory information (Fahrbach, 2006; Farris, 2005), it is possible that disrupting dopamine signaling prevents bees from being able to decide which activity to pursue. Recent work supports this possibility by showing that dopamine signaling in the mushroom bodies is necessary for *Drosophila* to make decisions requiring the comparison of positional and color cues (Zhang et al., 2007). Thus, the significant increase in stopped behavior observed with flupenthixol treatment or knockdown of *Amdop2* expression may be due to a defect in the processing of sensory information necessary for the bee to decide what action to take resulting in the bee simply standing still. Alternatively, a recent report from Andreatic et al. (2005) showed that blocking dopamine synthesis produced sleep-like behavior in *Drosophila*. Therefore, it is also possible that the stopped behavior observed in flupenthixol treated and *Amdop2* knockdown bees could be due to bees entering into a sleep-like state. Sleep behavior in honey bees has been carefully evaluated (Eban-Rothschild and Bloch, 2008), so future investigations including a detailed study of the stopped behavior could determine if disrupting dopamine signaling in bees results in increased time sleeping.

This study shows that dopamine signaling is important for determining the behavioral profile of the honey bee. In general, increases in dopamine lead to a reduction in walking, but increases in other active behaviors such as fanning and grooming. Blocking dopamine signaling via injection of flupenthixol resulted in less time spent in active behaviors such as walking and flying and increases in stopped or grooming behavior. This difference in activity levels of bees exhibiting reduced walking suggests that future studies on the modulation of behavior by biogenic amines would benefit from examining other behaviors as well as using traditional line-crossing assays as measures of activity. The RNAi mediated knockdown of a specific dopamine receptor, *Amdop2*, in the mushroom bodies of the brain also reduced walking behavior while increasing time spent stopped or grooming. These data suggest that *Amdop2* in the brain is involved in regulating shifts between walking, grooming and stopped behavior but not in regulating the righting reflex (upside down behavior) or flying behavior. This study provides a foundation for future work examining the importance of dopamine signaling in regulating distinct behaviors and elucidating the roles of specific dopamine receptors.

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Figure 1.

The effects of dopamine injection on honey bee behavioral profiles. The percent of time bees engaged in each behavior during each interval is shown for treatment with each concentration of dopamine. The interval time refers to the time after injection of the indicated treatment. Observations started 5 min after bees were released into the arena. Data shown are the mean with error bars illustrating the s.e.m. (N buffer = 20, N for all other treatment groups = 10). Behavioral profiles are significantly different among bees in the different treatment groups at the 20–25, 25–30 and the 30–35 min intervals. For intervals in which significant differences in behavioral profiles occurred, asterisks indicate significant differences in time spent in that

behavior by bees treated with the indicated concentration of dopamine versus buffer controls (t test, $p \leq 0.05$).

Figure 2.

The effects of flupenthixol on honey bee behavioral profiles. The percent of time bees engaged in each behavior during each interval is shown for treatment with each concentration of flupenthixol. The interval time refers to the time after injection of the indicated treatment. Observations started 5 min after bees were released into the arena. Data shown are the mean with error bars illustrating the s.e.m. (N buffer = 20, N for all other treatment groups = 10). The behavioral profiles for bees in each treatment group are significantly different at every interval. Asterisks indicate significant differences in time spent in that behavior by bees treated with the indicated concentration of dopamine versus buffer controls (t test, $p \leq 0.05$). The

asterisks on the top of the “other behaviors” bars indicate that treatment had a significant effect on flying behavior.

Figure 3.

The effects of AmDOP2 receptor knockdown in the brain via RNAi on honey bee behavioral profiles. The percent of time subjects spent engaged in each behavior is shown for each treatment. The interval of time elapsed since the beginning of the observation is indicated. Observations started 5 min after bees were released into the arena. Significant effects on the behavioral profiles of bees injected with *Amdop2* dsRNA compared with those injected with buffer or *Dmfred* (control) dsRNA were observed either **A**) 24 hours ($N_{\text{buffer}} = 15$, $N_{\text{fred}} = 15$, $N_{\text{dop2}} = 21$) or **B**) 48 hours after injection ($N_{\text{buffer}} = 13$, $N_{\text{fred}} = 14$, $N_{\text{dop2}} = 19$). For intervals in which significant differences in behavioral profiles occurred, asterisks indicate significant differences in time spent in that behavior by bees treated with *Amdop2* versus *Dmfred* controls (t test, $p \leq 0.05$). Error bars show the s.e.m.

Figure 4.

Levels of *Amdop2* expression in honey bee brains treated with buffer or dsRNA. The levels of *Amdop2* transcript normalized to transcript levels of the honey bee elongation factor EF1 α are shown. Error bars represent the s.e.m. Levels are shown for brains dissected at either 24 or 48 hrs after injection, just after the behavioral assay. 48 h after injection, brains injected with *Amdop2* dsRNA show a significant reduction in *Amdop2* transcripts compared with bees injected with buffer or *Dmfred* dsRNA. Columns that share the same letter are not significantly different (ANOVA, $p = 0.001$; followed by Tukey's Multiple Comparison post hoc test for a $p < 0.5$).