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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.



## 1. 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 (Andretic et al., 2005; Kume et al., 2005). Thus, dopamine may act at multiple sites to influence locomotor 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,

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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.

## 2. Materials and methods

### 2.1. 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 flupentixol 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.

### 2.2. 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 $NaH_2PO_4$, 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 15 min after injection.

### 2.3. 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 h 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.

### 2.4. Behavioral observations

Honey bees were released from their harnesses into a 150 mm × 15 mm Petri dish (Fisher) which served as the observation arena. Bees were allowed to acclimate to the arena for 5 min 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.

### 2.5. 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 AmEF1α (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-AGCCGCTCACCACCATCCAGCACA-3BHQ1. For EF1α 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 EF1α