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GABA signaling affects motor function in the honey bee

Julie A Mustard[a], Lisa Jones[b] and Geraldine A Wright[b,c]
[a]University of Texas Rio Grande Valley, Brownsville TX USA 78520
[b]Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, United Kingdom
[c]Department of Zoology, University of Oxford, Oxford, OX1 3SZ

Corresponding author: Julie A Mustard, julie.mustard@utrgv.edu, 956 882 5869

Abstract

GABA is the most common inhibitory neurotransmitter in both vertebrate and invertebrate nervous systems. In insects, inhibition plays important roles at the neuromuscular junction, in the regulation of central pattern generators, and in the modulation of information in higher brain processing centers. Additionally, increasing our understanding of the functions of GABA is important since $GABA_A$ receptors are the targets of several classes of pesticides. To investigate the role of GABA in motor function, honey bee foragers were injected with GABA or with agonists or antagonists specific for either $GABA_A$ or $GABA_B$ receptors. Compounds that activated either type of GABA receptor decreased activity levels. Bees injected with the $GABA_A$ receptor antagonist picrotoxin lost the ability to right themselves, whereas blockade of $GABA_B$ receptors led to increases in grooming. Injection with antagonists of either $GABA_A$ or $GABA_B$ receptors resulted in an increase in extended wing behavior, during which bees kept their wings out at right angles to their body rather than folded along their back. These data suggest that the GABA receptor types play distinct roles in behavior and that GABA may affect behavior at several different levels.

Keywords: honeybee; locomotion; GABA receptor; righting reflex; grooming; RDL receptor

Introduction

gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter found at high concentrations in both vertebrate and invertebrate organisms. In insects, GABA is found throughout the central nervous system. In the honey bee brain, GABAergic neurons are found in the antennal lobes, optic lobes, mushroom bodies, central complex and the subesophageal ganglion (Bicker 1999; Schäfer and Bicker, 1986). Consistent with its wide expression, GABA is implicated in a number of behaviors. GABA signaling plays an important role in odor coding in the antennal lobe (Sachse and Galizia, 2002; Dupuis et al., 2010; Choudhary et al., 2012), and learning and memory in the mushroom bodies (Raccuglia and Mueller, 2014). Unilateral injection of GABA into the honey bee brain near the alpha lobe (now referred to as the vertical lobe; Ito et al., 2014) of the mushroom bodies initiated circling behavior contralateral to the side of the injection (Michelsen and Braun, 1987) suggesting that GABA is involved in the control of locomotor behavior. Similarly, in *Drosophila* inhibition of the GABA transporters, leading to increased synaptic GABA levels, decreased locomotor activity and led to a loss of the righting reflex (Leal and Neckameyer, 2002). As well as acting on structures in the brain to influence motor behavior, GABA may also act on central pattern generators (CPG) that underlie the generation of many rhythmic behaviors such as walking, breathing or flying. In the mollusk *Aplysia california*, neurons in the CPG that regulates feeding motor behaviors contain GABA (Diaz-Rios and Miller, 2005). GABA is also essential for coordinating the muscles required for movement of the nematode, *C. elegans* (McIntire et al., 1993; Shen et al., 2016). Additionally, unlike the vertebrate neuromuscular junction, which is innervated solely by excitatory motor neurons, the muscle fibers of arthropods are also regulated by inhibitory motor neurons so that GABA may control motor function by directly acting on muscles (Wolf, 2014).

In both mammals and invertebrates, GABA acts on target neurons via cell surface receptor proteins that fall into two general categories: (1) $GABA_A$ type receptors which are ionotropic members of the cys-loop ligand gated channel family, and (2) metabotropic $GABA_B$ type receptors, which are members of the G-protein coupled receptor family. Characterization of the $GABA_A$ receptors in the honey bee mushroom bodies and antennal lobe demonstrated that these receptors are activated by the agonist muscimol and blocked by the antagonist picrotoxin (Barbara et al., 2005; Dupuis et al., 2010; Grünewald and Wersing, 2008; Palmer and Harvey, 2014). These results are similar to the pharmacology observed for the *Drosophila* $GABA_A$ receptors (Gisselmann et al., 2004; Lee et al., 2003; Zhang et al., 1995). Pharmacological evidence suggests that $GABA_B$ receptors are also found in the honey bee brain (Froeser et al., 2014; Raccuglia and Mueller, 2014). However, the pharmacology of insect $GABA_B$ receptors is not well characterized. Baclofen, an effective agonist of vertebrate $GABA_B$ receptors (Bowery, 1993), has been reported to not be effective at *Drosophila* $GABA_B$ receptors expressed in *Xenopus* oocytes (Mezler et al., 2001). However, baclofen was found to mimic the effects of GABA on climbing behavior in *Drosophila* (Hillman et al., 2012) and it acts as an agonist on cockroach and crayfish $GABA_B$ receptors (Blankenburg et al., 2015; Golan and Grossman, 1996).

Understanding the actions of GABA in insects is of particular interest as the $GABA_A$ RDL (resistant to dieldrin) receptor is the target of both the cylcodiene class of pesticides, such as dieldrin, and the phenylpyrazoles, which includes the commonly used fipronil (see ffrench-Constant et al., 2016 for a recent review). The effects of GABA on insect behavior may also be relevant from an ecological perspective as it is found in the nectar of some plants, and may play a role in attracting specific insect pollinators (Bogo et al., 2019; Nepi, 2014; Petanidou et al., 2006). On the other hand, plants also upregulate GABA synthesis as a response to herbivory and feeding on plant tissue and increased GABA levels reduced larval growth (Scholz et al., 2015), suggesting that plants use GABA to reduce damage from pests. Thus, an understanding of the effects of GABA signaling on behavior is of interest from a perspective of understanding the impacts of pesticide use and may also provide insight into plant - pollinator interactions.

To examine the roles of GABA signaling on motor function in the honey bee, forager bees were injected with agonists or antagonists specific for either $GABA_A$ or $GABA_B$ receptors and the effects on the behavior of freely moving bees were analyzed. Modulation of GABA signaling produced significant effects on locomotor behavior, including changes in the amount of time spent walking and grooming, and the righting reflex. Activation or blocking of $GABA_A$ versus $GABA_B$ receptors produced specific effects on these behaviors. Furthermore, antagonists for either receptor type resulted in bees spending more time holding their wings in an extended position.

Methods

Honey bees

Honey bees (*Apis mellifera mellifera*) were originally obtained from the National Bee Unit, York, UK, and were subsequently maintained outdoors at Newcastle University. Forager bees were collected outside the hive in small plastic vials, then cold anaesthetised and transferred to a plastic rearing cage as in Paoli et al. (2014). The box was fitted with 2 x 2 ml feeding tubes containing 1 M sucrose solution, and bees were left overnight at RT and allowed to feed *ad libitum*.

Pharmacological treatment

Muscimol, baclofen, picrotoxin and CGP35348 of >99.5% purity were obtained from Tocris Bioscience. The compounds were dissolved in 1 M dimethyl sulfoxide (DMSO; Sigma Aldrich) to make the working concentrations of 1.0, 0.10 and 0.01 mM used for injection. These concentrations of ligands were used as this range of agonists and antagonists were effective for other neurotransmitter receptors (Fussnecker et al., 2006; Mustard et al., 2010). Individual bees were collected from the box into a plastic vial and cold anaesthetised prior to the injection procedure. Each bee was restrained in a metal harness, then a hole was made in the dorsal side of the thorax using a 19 gauge needle. A 10 µl Hamilton syringe was used to inject 1 µl of treatment solution. Control animals were injected with either 1 µl of double distilled water or 1 M DMSO; data from the water and DMSO injected animals was pooled for subsequent analyses, as there was no significant difference between the behaviors of bees in these groups.

Behavioral observations

After injection with the indicated substance, the bee was released directly into the observation arena (a 150 × 15 mm petri dish) and allowed to recover for 5 min prior to observation. Behaviors were live scored during a 15 min observation as described in Maze et al., 2006 using the Observer version 5.0 software from Noldus Information Technology. Five motor function behaviors were defined: walking, still (not engaged in any other behavior, but standing still, previously referred to as stopped behavior), upside down (unable to perform the righting reflex), grooming, and fanning of the wings. These behaviors were mutually exclusive state events, such that each behavior could only be replaced by a subsequent behavior. During initial studies, it was observed that the GABA receptor antagonists were effecting the position in which the bees held their wings. Therefore, two wing related behaviors were defined: wings held out from the sides of the body at a right angle in an extended position, or wings held in a folded position along the top of the abdomen. Wing position was selected simultaneously with other behaviors only to be replaced by the alternative wing position. Observations were done at the same time of day, and multiple treatment groups were done each time to randomize effects across groups.

Data analysis

The total percent of time spent performing each behavior was analysed as the dependent variable in all analyses. Data for the control groups (water or DMSO) was not significantly different (Mann-Whitney: walk: Z = -0.530, P = 0.596; still: Z = -0.381, P = 0.703; grooming: Z = -0.683, P = 0.495; upside down: Z = -0.077, P = 0.938; fanning: Z = -1.19, P = 0.232), so these data were pooled for all subsequent analyses. The dependent variables in this analysis were the time spent engaging in each behavior; because these are mutually-exclusive categories measured over a defined interval, they are co-linear. To account for collinear dependencies of the measured variables, we first subjected the entire data set to a factor analysis (principle components method), as in Hurst et al. (2015). The factor scores for each component generated by the analysis were used as the dependent variables in a multivariate analysis of variance (MANOVA) for a model with drug and drug concentration as the independent variables. (Note: In this two factor model, the control (no drug) treatment was not included, as we did not conduct separate control experiments for each drug). The data for each drug were separately analysed against the control in individual MANOVAs with concentration as the independent variable. *Post-hoc* pairwise comparisons were conducted using Dunnett's multiple comparisons against the control group and each concentration of each drug. A generalized linear model was used to analyse wing position, with time spent in each position entered as a linear scale variable. *Post hoc* pairwise comparisons were conducted using Sidak's multiple comparisons against the control.

Results

Honey bees spend most of their time walking

Individual bees in the control group spent the majority of their time engaged in active behaviors including walking (~60%), grooming (~10%) and fanning (less than 1%), and only 30% of their time still (Figure 1). Bees would fall onto their backs (upside down), usually while climbing on the side or top of the observation area; control bees would quickly right themselves (average time upside down was 1.0 ± 0.33 s), so that the total time spent upside down during the interval was very brief. This behavioral profile is consistent with control bees in previous studies using the same behavioral assay (Maze et al., 2006; Williamson et al., 2014), where bees were fed treatments, rather than being injected. These data suggest that injection itself did not have major impacts on behavior.

Our experiments revealed that GABAergic neurotransmission was essential for the regulation of motor function in honey bees. Factor analysis reduced the data to 3 significant components that accounted for 85% of the variation in the data (Table 1). Time spent walking was positively correlated to time spent fanning and negatively correlated to time spent standing still (Component 1, Table 1). Time spent upside down and time spent grooming were not strongly correlated to the performance of any other behavior (Table 1). In general, the drug injected and the concentrations we used influenced the amount of time spent walking, still, and fanning (Comp1, Table 2). The effect of drug concentration on the amount of time spent upside down or time spent grooming depended on which drug was injected (Table 2). To understand the impact of drug concentration on these behaviors, the data for each drug was analysed separately against the control treatment.

GABA did not significantly affect the time spent engaged in walking, time spent still, or fanning behaviors (Figure 2, Comp 1, MANOVA $F_{3,46} = 0.443$, $P = 0.723$). Time spent upside down was also not significantly affected by GABA injection (Comp 2, MANOVA, $F_{3,46} = 2.66$, $P = 0.059$). However, GABA injection affected grooming behavior in a dose-dependent way (Comp 3, MANOVA, $F_{3,46} = 3.64$, $P = 0.019$).

Activation of the $GABA_A$ receptor reduces walking behavior

Muscimol acts as an agonist at honey bee $GABA_A$ receptors (Grünewald and Wersing, 2008). Injection with muscimol reduced time spent walking and increased time spent still (Fig 3). The inverse relationship between walking and time spent still was dose-dependent, with the strongest effect for the 10^{-4} M concentration (Fig 3, Comp 1, MANOVA $F_{3,46} = 5.84$, $P = 0.002$). Neither time spent upside down (Comp 2, MANOVA $F_{3,46} = 2.18$, $P = 0.103$) or grooming (Comp 3, MANOVA $F_{3,46} = 0.552$, $P = 0.649$) were significantly affected by muscimol.

Blockade of the $GABA_A$ receptor effects the righting reflex

Picrotoxin blocks honey bee $GABA_A$ receptors (Grünewald and Wersing, 2008). Honey bees treated with the $GABA_A$ receptor antagonist picrotoxin showed dose-dependent deficits in the righting reflex, and spent a significantly larger portion of time upside down (Fig 4, Comp 2, MANOVA $F_{3,46} = 100$, $P < 0.001$). Bees treated with the highest concentration (10^{-3} M) were completely unable to right themselves and spent the entire 15 min observation period upside down. Bees injected with 10^{-4} M picrotoxin had difficulty righting themselves, but were still able to walk. Variation in the other behaviors was not significantly affected by picrotoxin (Comp 1, MANOVA $F_{3,46} = 0.783$, $P = 0.510$; Comp 3, MANOVA $F_{3,46} = 0.124$, $P = 0.305$).

Activation of $GABA_B$ receptors reduces time spent walking

Baclofen acts as a $GABA_B$ receptor agonist in *Drosophila* (Hillman et al., 2012) and cockroach (Blankenburg et al., 2015). As with activation of the $GABA_A$ receptors, treatment with the $GABA_B$ receptor agonist baclofen at concentrations $\leq 10^{-4}$ M resulted in bees spending significantly less time walking and more time still than control bees (Fig 5, Comp 1, MANOVA $F_{3,46} = 3.99$, $P = 0.013$). Bees injected with 10^{-5} M baclofen also spent more time upside down (Comp 2, MANOVA $F_{3,46} = 4.02$, $P = 0.013$). Baclofen did not significantly affect grooming behavior (Comp 3, MANOVA $F_{3,46} = 0.123$, $P = 0.310$).

Inhibition of $GABA_B$ receptors increases grooming behavior

CGP35348 is an antagonist at mammalian $GABA_B$ receptors, and has also been shown to be effective at crayfish neuromuscular synapses (Bowery, 1993; Golan and Grossman, 1996). Injection with CGP35348 produced significant increases in upside down behavior (Comp 2, MANOVA $F_{3,46} = 4.10$, $P = 0.012$) and in grooming behavior (Figure 6, Comp 3, MANOVA $F_{3,46} = 16.9$, $P < 0.001$). Injection with CGP35348 did not have a significant effect on walking, still, and fanning behavior (Comp 1, MANOVA $F_{3,46} = 2.33$, $P = 0.086$).

$GABA_A$ and $GABA_B$ act together to control wing position

Injections with specific concentrations of the $GABA_A$ receptor antagonist picrotoxin or the $GABA_B$ receptor antagonist CGP35348 lead to bees holding their wings in an extended position for significantly more time than control bees (Figure 7, GLZM, drug x concentration $\chi_8^2 = 34$, $P < 0.001$).

Discussion

Signaling via GABA has a significant effect on motor function in the honey bee. Activation of GABA pathways through injection of the $GABA_A$ receptor agonist muscimol or the $GABA_B$ receptor agonist baclofen resulted in a decrease in amount of time spent in walking and an increase in time spent still. These results are similar to those observed in *Drosophila*, where increases in synaptic GABA via blockade of the GABA reuptake transporters or activation of $GABA_B$ receptors lead to reduced locomotor activity (Leal and Neckameyer, 2002; Dzitoyeva et al., 2003). Conversely, treatment with the $GABA_A$ receptor blocker picrotoxin or the $GABA_B$ receptor antagonist CGP35348 did not increase time spent still. Instead, bees injected with picrotoxin could not right themselves using the righting reflex, suggesting $GABA_A$ receptors are essential for the coordination of limbs necessary for regaining postural control. Injection of the $GABA_B$ receptor antagonist had a distinct effect from that of picrotoxin and resulted in an increase in grooming behavior. Additionally, treatment with either picrotoxin or the $GABA_B$ receptor antagonist lead to an extended wing phenotype suggesting that both $GABA_A$ and $GABA_B$ receptors play an essential role in the control of wing position.

Pharmacological agents injected into the hemolymph of honey bees spread throughout the body, including the brain, abdomen and thorax (Barron et al., 2007). Therefore, the treatments used in this study could have targeted multiple tissues to affect behavior at several different levels including at the neuromuscular junction, the regulation of central pattern generators (CPG), and circuits in the brain that initiate different behaviors. Furthermore, although we included GABA as a 'positive control' we did not see a significant effect of the injection of GABA on behavior. We expect that this is because neurons and glial cells are efficient at taking up excess GABA in order to prevent overstimulation of GABA receptors (Olsen and DeLorey, 1999). The changes in behavior were dose-dependent. Picrotoxin and CGP35348 showed a linear dependence with dose in that higher doses showed an increase in the effects on behavior. On the other hand, muscimol and baclofen showed a dose dependency where the 10^{-4} and 10^{-5} treatments had more of an effect on behavior than the 10^{-3} M treatment. This non-linear response to dose is commonly observed in behavioral studies and may reflect the differing affinities of different receptor subtypes (for examples see: Fussnecker et al., 2006; Mustard et al., 2010; Scheiner et al., 2002).

Picrotoxin is a known $GABA_A$ antagonist in insects. However, in cultured honey bee antennal lobe neurons, it also affects acetylcholine and glutamate induced currents (Barbara et al, 2005). It should be noted, however, that partial blockade of the nicotinic acetylcholine receptor required the bath application of a relatively high concentration of picrotoxin (10^{-3} M). Even though concentrations up to 10^{-3} M of picrotoxin were injected into the hemolymph in our experiments, we expect that the 1 μl of treatment would be distributed throughout the bee resulting in much lower local concentrations (Barron et al., 2007). Studies from other insects *in vivo* suggest that picrotoxin is more effective at $GABA_A$ channels than glutamate gated channels (GluCl) (house fly, Eguchi et al.2006; locust, Janssen et al., 2007, 2010). Nevertheless, GluCl is expressed in the brain and muscles of the honey bee (El Hassani et al., 2012), therefore it is possible that some of the observed effects are due to the blockade of GluCl channels by picrotoxin. Histamine gated chloride channels are also found in the insect nervous system, and play an important role in the visual system (Hardie, 1989). Although only a few of these channels have been pharmacologically characterized, picrotoxin does not appear to be an effective antagonist at these receptors (Kita et al., 2017; Gisselman et al., 2002).

The increase in time with wings extended observed after injection with either the $GABA_A$ or $GABA_B$ antagonists, and the hyperextended limbs observed in *Drosophila* when GABA levels were manipulated (Leal and Neckameyer, 2002), appears to be specific to GABA signaling. A similar increase in wing extended behavior has not been observed in bees when the same behavioral assay was used to examine the effects other neuroactive substances such as octopamine and tyramine (Fussnecker et al., 2006), dopamine (Mustard et al., 2010) or compounds that activate the nicotinic acetylcholine receptor (Williamson et al., 2014). The presence of hyperextended limbs or extended wings was not reported when picrotoxin was injected into the brains of honey bees (Michelsen and Braun, 1987), suggesting that this effect is due to inhibition of GABA receptors on the thoracic ganglion or at the neuromuscular junction.

Bees injected with GABA receptor agonists spent more time standing still. This could be a result of increased inhibition at the neuromuscular junction. Unlike mammals, the insect neuromuscular junction contains both excitatory and inhibitory input, perhaps due to the need to control motor function with fewer muscle fibers and neurons (Wolf, 2014). Muscles in both the body wall and limbs receive inhibitory input in insects (Pearson & Bergman, 1969; Hale & Burrows, 1985). The presence of common inhibitory neurons that supply multiple leg muscles has been shown in insects including locust, cricket, cockroach and walking stick (Pearson & Bergman, 1969; Wolf, 2014). However, the presence of similar inhibitory neurons in the honey bee has not yet been confirmed and studies on honey bee muscle fibers isolated from the tibia did not reveal any changes in current in response to treatment with GABA (Collet and Belzunces, 2006). Nonetheless, given the prevalence of inhibitory neurons acting on muscles in other insects, it is most likely that they are also present in honey bees, and activation of GABA receptors on muscles would be expected to increase inhibition and reduce walking behavior.

Both of the activities significantly affected by GABA receptor antagonists, the righting reflex and grooming, are controlled by central pattern generators (Berkowitz and Laurent, 1996; Sherman et al., 1977; Zill 1986). GABA may act to modulate the activity of CPGs. In *Manduca sexta*, treatment of isolated nerve cords with picrotoxin induced motor patterns similar to those seen for flight (Vierk et al., 2010) suggesting blockade of $\mathrm{GABA_A}$ receptors releases the flight-like pattern generator from inhibition. Similarly, treatment with picrotoxin releases inhibition of the descending neurons in the antennal grooming circuit of *Drosophila* (Hampel et al., 2015). Although CPGs generate rhythmic behavior without the need for external signals, mechanosensory feedback is essential for coordination and selection of behavior (Song et al., 2007). For example, inhibition plays an essential role in proprioceptive feedback necessary for fine control of the femur-tibia joint in the stick insect (Sauer et al., 1997). A loss of fine motor control may interfere with the coordination of leg movements required for bees to right themselves, producing the large amounts of time bees injected with picrotoxin spend upside down. Therefore, GABA signaling could influence locomotor behavior by directly acting on CPGs or by affecting the sensory information involved in adapting CPGs to the environment.

Brain centers such as the mushroom bodies, central complex and subesophageal ganglion integrate information and regulate motor behavior, and each of these regions of the honey bee brain contains GABAergic neurons (Schäfer and Bicker, 1986). Injection of GABA or muscimol near the vertical lobe of the mushroom bodies on one side of the honey bee brain produced contralateral turning behavior, whereas injection of picrotoxin resulted in ipsilateral turning (Michelsen and Braun, 1987), suggesting that GABA is important for regulation of locomotion by the brain. Inhibition, which may be due to the actions of GABA or other inhibitory neurotransmitters, plays an important role in selecting and coordinating behaviors. For example, in the cockroach, the supraesophageal ganglion inhibits walking behavior while stimulating flight, whereas the subesophageal ganglion inhibits flying and excites walking (Gal and Libersat, 2006). Mechanosensory and other information regulating locomotion is sent from higher brain centers via descending neurons that pass through the neck connective to the thoracic ganglion that contains the CPGs (Namiki et al., 2018; Knebel et al., 2019). In *Drosophila*, more than a third of the descending neurons express GABA (Hsu and Bhandawat, 2016), and in the honey bee, GABAergic neurons are seen entering the cervical connection (Schäfer and Bicker, 1986). The presence of GABA in these neurons suggests that it plays a central role in communication between the brain and the neurons comprising CPGs or regulating the neuromuscular junction.

In mammals, ethanol has multiple effects on GABAergic signaling including influencing the release of GABA, directly affecting the activity of $\mathrm{GABA_A}$ receptors, and modifying the expression levels of both $\mathrm{GABA_A}$ and $\mathrm{GABA_B}$ receptors (see Roberto and Varodayan, 2017 for a review). A comparison of the GABA data with the influence of ethanol on motor function in honey bees (Maze et al. 2006) suggests that ethanol may also effect GABA signaling in insects. When bees were fed with solutions containing 10-25% ethanol, they spent more time grooming; a similar increase in grooming was observed when bees were injected with the $\mathrm{GABA_B}$ antagonist CPG35348. $\mathrm{GABA_B}$ receptors were also found to play a role in the behavioral effects of ethanol in *Drosophila* (Dzitoyeva et al. 2003). Ethanol may act at $\mathrm{GABA_A}$ receptors as well, as bees fed with higher concentrations (75-100%) could not perform the righting reflex, mimicking the effects of blocking $\mathrm{GABA_A}$ receptors with picrotoxin. More studies will be needed to verify the connection between GABA and ethanol signaling. Increased grooming and failure to perform the righting reflex may also indicate toxicosis; however, treatment with toxic substances also led to behaviors such as abdomen dragging and/or curling of the abdomen, neither of which were observed in this study (Alkassab and Kirchner, 2018; Hurst et al., 2014; Schneider et al., 2012). The specificity and dose dependency of the different effects of the GABA agonists and antagonists provide evidence that the behavioral changes are not due to toxicity or malaise caused by the compounds used here.

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Conflict of Interest: The authors declare that they have no conflict of interest.
Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figure Legends

Fig. 1 Behavioral profile of control honey bees. The percent time engaged in each behavior by control bees during the entire 15 min observation. Bars represent the mean ± SEM, N = 20

Fig. 2 Injection of bees with GABA did not produce significant shifts in walking, still, fanning, or upside down behavior. Injection of bees with GABA affected the amount of time spent grooming, in a dose-dependent manner (10^{-5} M, Dunnett's *post hoc*, $P < 0.05$). Bars represent the mean ± SEM; for the control group, N = 20, N = 10 for groups injected with each concentration of GABA.

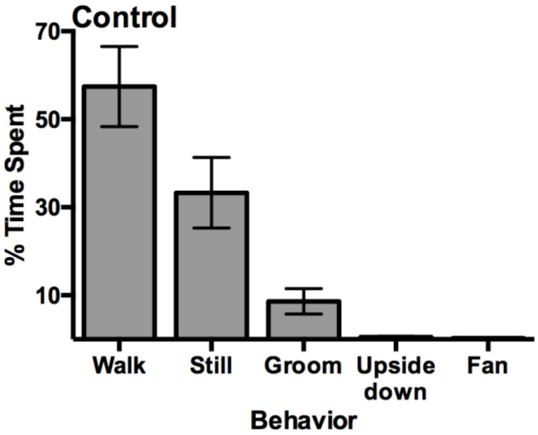
Fig. 3 Bees treated with the $\mathrm{GABA_A}$ receptor agonist muscimol significantly reduce the amount of time spent walking and spent more time standing still (10^{-4} M and 10^{-5} M, Dunnett's *post hoc*, $P < 0.05$). Bars represent the mean ± SEM; for the control group, N = 20, N = 10 for groups injected with each concentration of muscimol. Asterisks indicate *post hoc* < 0.05.

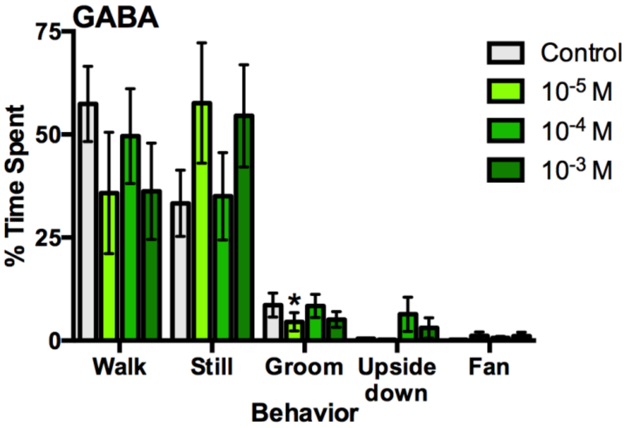
Fig. 4 Bees treated with the concentrations $\geq 10^{-4}$ M $\mathrm{GABA_A}$ receptor antagonist picrotoxin are unable to right themselves. Bars represent the mean ± SEM; for the control group, N = 20, N = 10 for groups injected with each concentration of picrotoxin. Asterisks indicate Dunnett's post hoc $P < 0.05$.

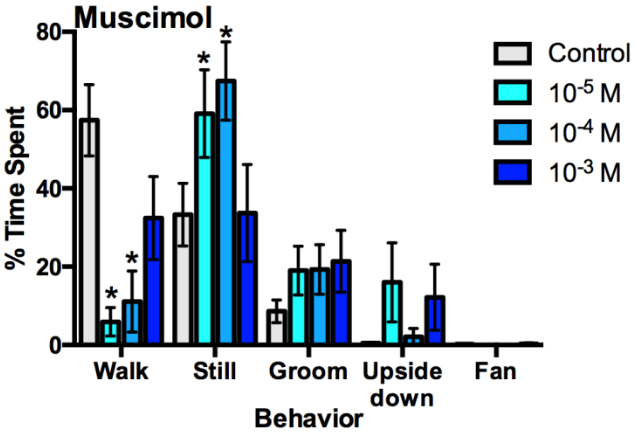
Fig. 5 Bees treated with the $\mathrm{GABA_B}$ receptor agonist baclofen significantly reduce the amount of time they spend walking and spent more time upside down. The effect on the behaviors depended on the dose of the drug. The 10^{-4} M baclofen had the strongest effect on walking and standing still (Dunnett's *post hoc* $P < 0.05$); the 10^{-5} M baclofen had the strongest effect on time spent upside down (Dunnett's *post hoc* $P < 0.05$). Bars represent the mean ± SEM; for the control group, N = 20, N = 10 for groups injected with each concentration of baclofen. Asterisks indicate Dunnett's post hoc $P < 0.05$.

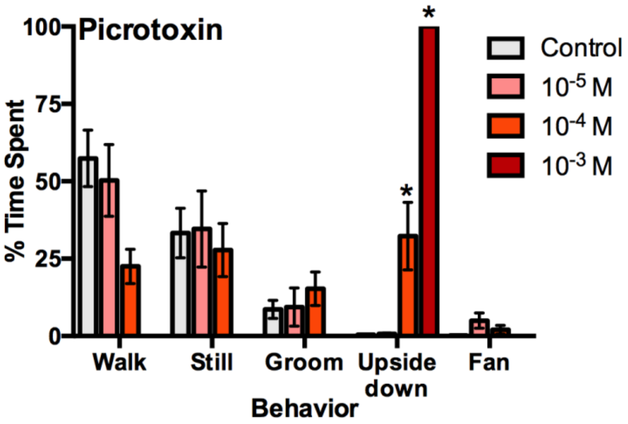
Fig. 6 Bees injected with the $\mathrm{GABA_B}$ receptor antagonist CGP35348 at concentrations $\geq 10^{-4}$ M displayed a striking increase in the time spent grooming. Bees injected with the 10^{-3} M CGP35348 spent more time upside down (Dunnett's *post hoc* $P < 0.05$). Bars represent the mean ± SEM; for the control group, N = 20, N = 10 for groups injected with each concentration of CGP35348. Asterisks indicate Dunnett's post hoc $P < 0.05$.

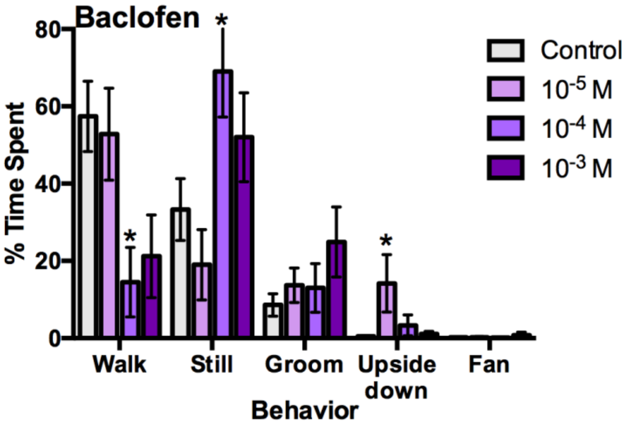
Fig. 7 Bees treated with the $\mathrm{GABA_B}$ receptor antagonist CGP35348 or the $\mathrm{GABA_A}$ receptor antagonist picrotoxin significantly increase the amount of time they spend holding their wings extended out from their body. Bars represent the mean ± SEM; for the control group, N = 20, N = 10 for all other treatment groups. Asterisks indicate groups that are significantly different from control bees ($p<0.05$). Sidak's *post hoc*; for picrotoxin 10^{-5} M $P = 0.025$; 10^{-3} M $P < 0.001$; for CGP35348 10^{-5} M $P = 0.01$, 10^{-4} M $P < 0.001$

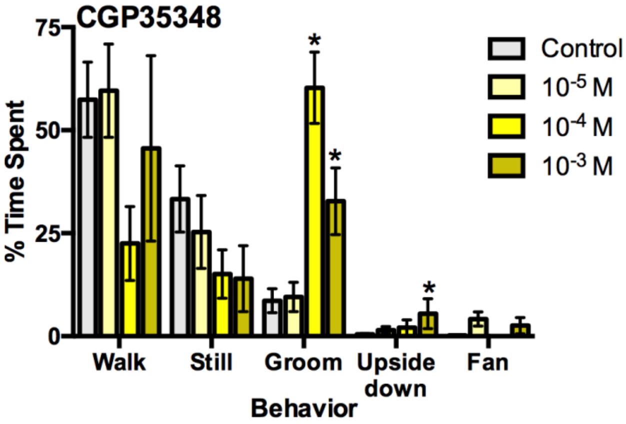

Control
70
50
30
10
% Time Spent
Walk
Still
Groom
Upside down
Fan
Behavior


GABA
% Time Spent
75
50
25
0
*
Walk
Still
Groom
Upside down
Fan
Behavior
Control
10^{-5} M
10^{-4} M
10^{-3} M


Muscimol
80
60
40
20
0
% Time Spent
*
*
*
*
Walk
Still
Groom
Upside down
Fan
Behavior
Control
10-5 M
10-4 M
10-3 M


Picrotoxin
% Time Spent
100
75
50
25
0
Walk
Still
Groom
Upside down
Fan
Behavior
*
*
Control
10^-5 M
10^-4 M
10^-3 M


Baclofen
80
60
40
20
0
% Time Spent
*
*
*
Walk
Still
Groom
Upside down
Fan
Behavior
Control
10-5 M
10-4 M
10-3 M


CGP35348
% Time Spent
75
50
25
0
*
*
*
Walk
Still
Groom
Upside down
Fan
Behavior
Control
10-5 M
10-4 M
10-3 M

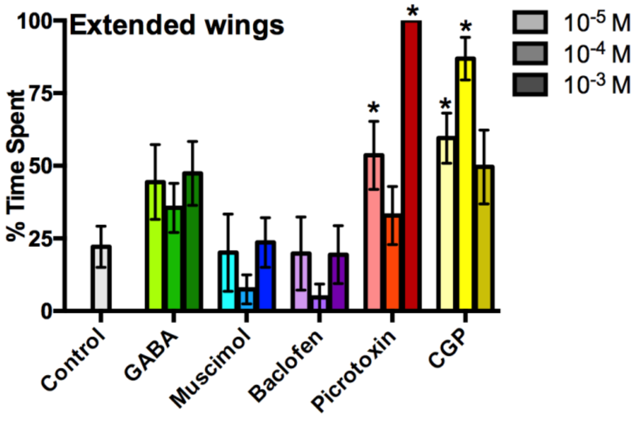

Extended wings
% Time Spent
100
75
50
25
0
Control
GABA
Muscimol
Baclofen
Picrotoxin
CGP
*
*
*
*
10^{-5} M
10^{-4} M
10^{-3} M

Table 1. Factor analysis of measured behaviors after injection with drugs that target GABA receptors.

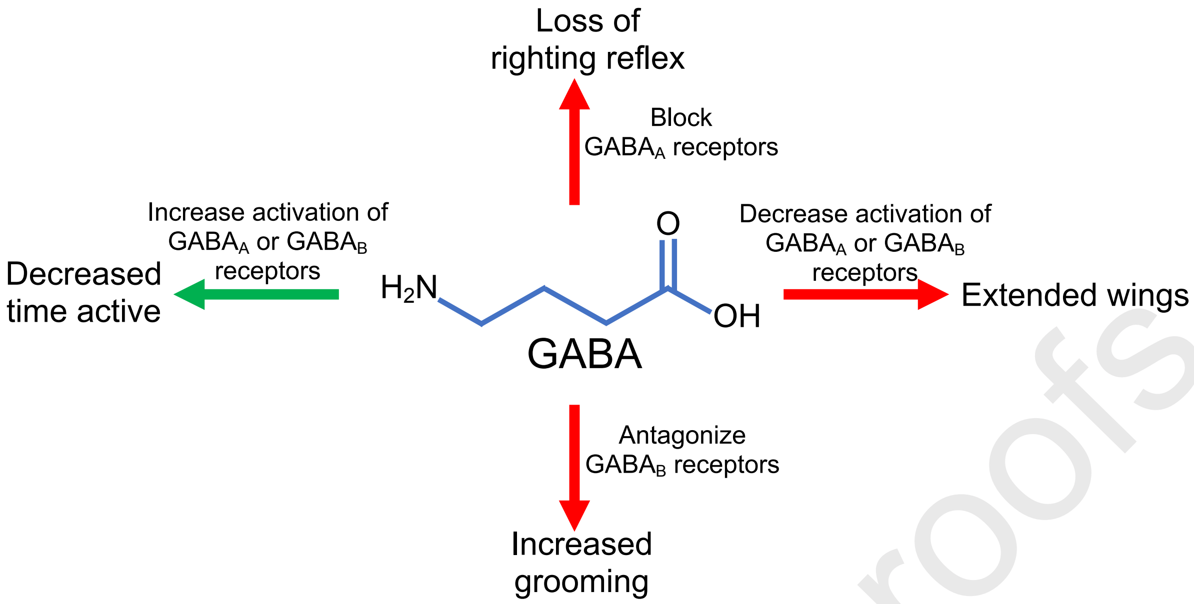
	Component		
	1	2	3
% of Variance	37.7	24.9	22.8
Walking	0.882	-0.295	0.083
Still	-0.788	-0.372	-0.489
Upside Down	0.073	0.984	-0.136
Grooming	-0.322	-0.087	0.901
Fanning	0.616	-0.214	-0.257

Grey highlighting indicates the component that best represents each variable.

Table 2. MANOVA for the factor scores representing the components from the factor analysis in Table 1.

Source	Dependent Variable	Type III Sum of Squares	df	F	P-value
Intercept	Component 1	0.24	1	0.281	0.597
	Component 2	0.379	1	1.06	0.305
	Component 3	0.013	1	0.017	0.898
Drug	Component 1	15.1	4	4.42	**0.002**
	Component 2	41.7	4	29.1	<0.001
	Component 3	31.2	4	10.3	<0.001
Concentration	Component 1	6.51	2	3.82	**0.024**
	Component 2	13.4	2	18.8	<0.001
	Component 3	5.3	2	3.49	0.033
Drug * Concentration	Component 1	12.2	8	1.79	0.084
	Component 2	62.1	8	21.7	**<0.001**
	Component 3	23.7	8	3.90	**<0.001**
Error	Component 1	115	135		
	Component 2	48.3	135		
	Component 3	102	135		
Total	Component 1	149	150		
	Component 2	166	150		
	Component 3	162	150		

Bold indicates highest order model term with P < 0.05.


Loss of
righting reflex
Block
GABA_A receptors
Increase activation of
GABA_A or GABA_B
receptors
Decreased
time active
H2N
O
OH
GABA
Decrease activation of
GABA_A or GABA_B
receptors
Extended wings
Antagonize
GABA_B receptors
Increased
grooming

Highlights

Signaling via $GABA_A$ or $GABA_B$ receptors show different effects on honey bee behavior

$GABA_A$ or $GABA_B$ receptor antagonists produced a distinctive extended wing behavior

Activation of either $GABA_A$ or $GABA_B$ receptors reduced activity levels

Blockade of $GABA_A$ receptors led to a loss of the righting reflex

Treatment with a $GABA_B$ receptor antagonist increased grooming behavior