Video Article

Neuropharmacological Manipulation of Restrained and Free-flying Honey Bees, *Apis mellifera*

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Abstract

Honey bees demonstrate astonishing learning abilities and advanced social behavior and communication. In addition, their brain is small, easy to visualize and to study. Therefore, bees have long been a favored model amongst neurobiologists and neuroethologists for studying the neural basis of social and natural behavior. It is important, however, that the experimental techniques used to study bees do not interfere with the behaviors being studied. Because of this, it has been necessary to develop a range of techniques for pharmacological manipulation of honey bees. In this paper we demonstrate methods for treating restrained or free-flying honey bees with a wide range of pharmacological agents. These include both noninvasive methods such as oral and topical treatments, as well as more invasive methods that allow for precise drug delivery in either systemic or localized fashion. Finally, we discuss the advantages and disadvantages of each method and describe common hurdles and how to best overcome them. We conclude with a discussion on the importance of adapting the experimental method to the biological questions rather than the other way around.

Video Link

The video component of this article can be found at http://www.jove.com/video/54695/

Introduction

Since Karl von Frisch elucidated their dance language[1], honey bees have remained a popular study species for researchers in animal behavior and neurobiology. In recent years a myriad of new disciplines have emerged at the intersection of these two fields, and several other disciplines (*e.g.,* molecular biology, genomics, and computer science) have arisen alongside them. This has led to rapid development of new theories and models for understanding how behavior results from activity within nervous systems. Because of the unique lifestyle, rich behavioral repertoire, and ease of experimental and pharmacological manipulation, bees have remained at the forefront of this revolution.

Honey bees are being used to study basic neurobiological questions such as those underlying learning and memory[2,3], decision making[4], olfactory[5], or visual processing[6]. In recent years, the honey bee has even been used as a model for studying topics generally reserved for medical research, such as the effects of addictive drugs[7-11], sleep[12], ageing[13], or the mechanisms underlying anaesthesia[14].

Unlike for the classical genetic model organisms (*e.g.*, *D. melanogaster*, *C. elegans*, *M. musculus*), there are very few genetic tools available for manipulating neural functions in honey bees, although this is currently changing[15]. Instead, honey bee studies have primarily relied on pharmacological manipulations. This has been very successful; however, the diversity of bee research is such that a range of methods for pharmacological administration are needed. Research with honey bees addresses highly diverse questions, is studied by researchers from different disciplines and backgrounds, and uses a variety of experimental approaches. Many research questions require bees to either be free-flying, freely interacting in their colony, or both. This can make it difficult to keep track of individual experimental animals, and makes restraint or cannulation unfeasible.

To accommodate the diversity of honey bee research, a variety of drug delivery methods are needed, allowing for robust and flexible administration while ensuring that the pharmacokinetic and pharmacodynamic profiles, invasiveness of the method, and its reliability, suit the paradigm in question. Because of these diverse needs, most research groups have developed their own unique drug administration methods. So far, this has been a strength of the bee research community; it has led to the development of arrays of methods allowing for administration of the same drug in different circumstances. Our goal here is not to develop a single standardized method for pharmacological manipulations of bees,

but rather to highlight methods that have proven to be particularly successful, and help researchers adopt these. We discuss the basic principles of how they work, as well as their advantages and disadvantages.

Protocol

1. Drug Administration for Harnessed Bees

1. **Oral treatment**
    1. Prepare 1.5 M sucrose solution by mixing 257 g of sucrose with 500 ml of water (it is easier to dissolve this amount of sucrose in boiling water). Store sucrose solution at 4 °C until use.
       NOTE: Sucrose solution provides a very hospitable environment for certain microorganisms, and thus easily becomes contaminated and unpalatable to bees. Bulk sucrose solution can be aliquoted and stored at -20 °C until use.
    2. Decide on an appropriate drug dose (how to achieve it, is addressed in the discussion section below), and prepare a solution such that the preferred drug dose is dissolved in 20 µL sucrose solution (*e.g.*, to deliver 20 µg, dilute drug at a ratio of 1 mg/ml). Harness bees according to Felsenberg et al. (2011)[16]. Do this step at least 12 hr before drug treatment to ensure that bees are no longer stressed out from harnessing when the drug solution is presented.
       NOTE: For more consistent results, it is best to starve bees (by placing harnessed bees in an incubator at 34 °C and 70% humidity) O/N.
    3. Using a micropipette, touch a drop of 1.5 M sucrose water to the antenna of a harnessed bee. When the proboscis is extended, touch a 20 µl droplet of 1.5 M sucrose containing the drug directly to the proboscis of the bee. Make sure the bee consumes everything. As vehicle control use 1.5 M sucrose solution without added drugs.
       NOTE: The amount of sucrose solution might need to be adjusted based on experimental plans. If appetitive conditioning is intended, feeding the bees just prior to training will interfere with bees' responsiveness.
    4. Discard or set aside bees that do not consume all of the sucrose.
       NOTE: If a large number of bees fail to drink the sucrose solution, the feeding schedule might need to be adjusted.

2. **Injection into the thorax**
    1. Prepare drug in honey bee Ringer[17] as follows:
        1. Mix and autoclave 7.45 g NaCl, 0.448 g KCl, 0.812 g $MgCl_2$, 0.735 g $CaCl_2$, 54.72 g sucrose, 4.95 g D-glucose, and 2.48 g HEPES in 1,000 ml of water. Be careful when storing Ringer as it is easily contaminated. Aliquot and store Ringer at -20 °C until use.
        2. Dissolve the drug in Ringer solution and then dilute so that the desired amount is present in 5 µl. As an example, if bees are to be treated with 5 µg of a drug, 1 g can initially be dissolved in 1 ml in Ringer, before being diluted 1:1,000 in Ringer for a final solution of 1 µg/µl.
           NOTE: Alternatively, commercially available PBS (Phosphate-buffered Salin) can be used instead of Ringer solution.
    2. Make a microscalpel by breaking off the corner of a double-edged razor blade with a blade holder. Attach the blade fragment to a blade holder so that it makes a nice blade with a sharp end point.
    3. Under a stereomicroscope, carefully use the microscalpel to cut a 2 mm hole just above the scutellum, next to the posterior wing process of a bee's thorax. Avoid cutting too deep as this might injure flight muscles, and be careful to avoid the wing hinges. Ideally, only cut three sides, so that the flap of cuticle can later be folded back to close the site of injury.
    4. Using a micropipette, deposit 5 µlL Ringer (or PBS) containing the drug on top of the hole in the thorax. Carefully monitor under microscope to ensure the entire drop is absorbed into the hemolymph. Use Ringer (or PBS) as a vehicle control.
    5. If possible, move the cuticle flap back over the hole. After 5-10 hr, it will reattach and seal.
       NOTE: As an alternative to this technique, inject 1 µl directly into the thorax using a glass syringe, after opening a small hole in the middle of the frenum (transverse line in the posterior region of the scutellum) with a syringe needle (diameter: 0.6 mm, G: 23). This circumvents the need to first cut the thorax with a scalpel and the injection site is smaller, but this method will leave the injection site exposed.

3. **Ocellus injection**
   NOTE: This is a method suitable for delivering molecules throughout the head capsule, into the hemolymph.
    1. Prepare drugs as in 1.2.1, but adjust drug concentrations such that the desired dose will be contained in 1 µl of Ringer or PBS (less volume can be absorbed through the ocellus hole than through the thorax).
    2. Prepare a microscalpel as in 1.2.2. Under a stereomicroscope, lock the head of a harnessed bee in place by filling the neck crevice with wax. Use low-temperature melting wax (*e.g.,* dental wax) in order to avoid damaging antennal olfactory receptors or other cells that may be important for assessing behavior (*e.g.,* olfactory learning). Then carefully remove the lens of the median ocellus by inserting the tip of the microscalpel under the lens and gently break the lens free from the head capsule.
       NOTE: It is also possible to place wax carefully over the antennae to prevent movement.
    3. Carefully pipette drug onto the ocellus hole. Wait until all is taken into the head capsule. Remove dental wax from the antennae and allow the bee to rest for a while before continuing the experimental procedure. Use Ringer (or PBS) as a vehicle control.

4. **Injection into the ocellar tract**
   NOTE: The ocellar tract contains large fibers, connecting to most regions of the central brain[18]. This treatment method enables applying compounds to the brain only, but not targeting specific subregions of the brain.
    1. Prepare bee as in 1.3.2. and remove the lens of the median ocellus with the tip of a microscalpel as in 1.3.3.
       NOTE: This can be done up to 2 hr before the injection. Based on our experience, fed bees are better able to cope with this surgery than starved bees
    2. Fill a 10 µl glass syringe equipped with a small gauge (*e.g.,* 33, diameter: 210 µm) needle with drug solution prepared as in 1.3.1.

3. Using a manual micromanipulator, insert the syringe tip through the ocellar retina into the head capsule to a depth of 50 µm and inject 250 nl of solution.
4. After use, rinse the syringe 3 times with distilled water, then 3 times with 75% ethanol.

5. **Microinjection into particular brain structures**
NOTE: In addition to the systematic treatments mentioned above, it is possible to perform microinjections into particular brain structures. This allows for pharmacological manipulation of one or more brain regions, while leaving others unaffected. This works best with brain regions that are easy to recognize from the anterior brain surface (*e.g.*, antennal lobes, mushroom body calyces or vertical lobes, or the optic lobes), but other regions have been targeted. Please note that the orientation (anterior/posterior, dorsal/ventral) refers to the body axis, rather, than to the neuraxis[19].
    1. Prepare the drug in Ringer or PBS in the same manner as in 1.2.1, adding a fluorescent (*e.g.,* 0.5 mg/ml Dextran, Alexa 546 or 568 fluor) or nonfluorescent dye (*e.g.*, 1 mM methylene blue).
    NOTE: The addition of a fluorescent dye will allow verification of the injection location after the experiment is over (using confocal microscopy, following brain dissection), whereas nonfluorescent dyes allow direct monitoring during the experiment.
    2. To make glass pipettes for injection, insert glass capillaries of the correct diameter into holder clamps of an electrode puller (1.0 mm for the standard holder included for the microinjector mentioned in the materials list). Adjust pull and heat settings to produce an approximately 0.5 cm long tip (settings will be different for every puller, even if the same model is used).
    NOTE: Ideally, the two pipettes pulled from one glass should have the same length and shape, so that both can be used.
    3. Under a stereomicroscope, break the tips to obtain an outer diameter of about 10-15 µm, based on visual estimation using a scale on a graticule inserted into the ocular. The steps on the scale are defined by the manufacturer and can be corrected for the magnification used.
    4. Then, fill the glass pipettes with the solution to inject. If glass capillaries with filaments are used, fill the pipette by placing the back side into the drug solution, otherwise fill tip using microloader tips.
    5. Insert the filled glass pipette into the capillary holder of a microinjector, which is controlled by a manual or electronic micromanipulator.
    6. Calibrate the microinjector to inject the desired volume (0.5-2 nl, depending on the size of the brain structure targeted). For this, inject directly into a small Petri dish containing mineral oil and measure the diameter of the droplet with the graticule. Change settings until the desired volume is reached.
    7. Fix the head of a harnessed bee using soft dental wax as in 1.3.2, before cutting an opening into the anterior part of the head capsule, using a microscalpel, with three cuts: one just below the median ocellus (ventral), one at the border of the right or left eye and one above the antenna stems (dorsal). Use a piece of dental wax to hold the opened flap in place.
    8. Carefully push glands and trachea lying on top of the brain aside using fine forceps, then make a small rupture into the neurilemma (very thin membrane around the brain) above the targeted brain structure.
    NOTE: If many bees are to be treated at once, this procedure can be performed earlier; however, be careful to not leave bees in this state too long (no more than 30 min), as their brains might desiccate.
    9. Insert the tip into the desired brain region, and adjust depth perpendicular to the brain surface (*e.g.,* 60 µm for mushroom body calyces). Inject the preset volume. For lateral brain regions, inject bilaterally (*i.e.* do one injection to each hemisphere). If a nonfluorescent dye is used, ensure the injection occurred in the right region upon observation while injecting. If a fluorescent dye is used do the same under fluorescent light using a stereomicroscope with a fluorescence viewing system.
    10. Afterwards, place the open flap back over the bee's head. Melt a crystal of eicosane, which is approximately 1 mm in diameter, using a thin wire wrapped around the tip of a micro soldering iron (melting temperature is 35-37°C) and seal the cuts. This will greatly reduce mortality.
    11. Release the bee from the harness for behavioral analysis (but see discussion), or keep in the harness for experiments on restrained bees – *e.g.,* proboscis extension reflex (PER) testing[20].
    12. If a fluorescent dye was used, ensure that the injection hit the area of interest after the experiment is over using a confocal laser scanning microscope (**Figure. 1**).
    NOTE: This is particularly useful when targeting deeper brain areas (where it would be hard to see nonfluorescent dye during the injection phase).

2. Drug Administration Methods for Free-flying Bees

1. **Oral treatment**
    1. Prepare drug in the same way as in steps 1.1.1-1.1.2. Add drug solution to a feeder and place in refrigerator for storage.
    NOTE: Any feeder will do, such as an upside-down bottle cap or a jar inverted on tissue paper.
    2. Train bees to a gravity feeder containing 1 M or 0.5 M sucrose solution by placing a feeder close to the hive. Once bees start foraging at the feeder, gradually move it further away until it is at a comfortable distance to avoid being stung (minimum 5 m).
    3. Paint-mark bees in order to keep track of individual honey bees. Make a list of all color combinations that will be used. When a bee lands at the feeder, carefully mark its abdomen with two colors, and make a note on the list that the combination is taken.
    4. Swap the gravity feeder for a feeder containing the drug/sucrose solution. Take note of the marked bees that visit the feeder. Catch any unmarked bee visiting the feeder as bees are prolific recruiters, and the numbers of bees visiting the drugged feeder can quickly get out of control. This is especially problematic if the same experiment is to be performed on successive days, as naïve bees might no longer be naïve.
    NOTE: As an alternative to training individual bees to a feeder, previous authors have successfully fed drug-laced sucrose water to an entire hive[21-23].

2. **Topical treatment**
NOTE: The objective is to dissolve the compound of interest in a solvent that can penetrate the waxy insect cuticle. Different solvents can be used for this purpose. The most commonly used include acetone, dimethylformamide (DMF) and dimethylsulphoxide (DMSO).

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1. Evaluate which solvent works best for the compound at hand. If a strong phenotype is expected from an overdose (*e.g.,* paralysis or death), treat bees (step 2.2.2) with a high dose (*e.g.,* 20 µg cocaine[7]) dissolved in each of the different solvents and carefully monitor time until paralysis or death.
2. Using a 1 µL microcapillary (or a microsyringe, which can be fitted on an appropriate repeating dispenser) and microcapillary holder, draw 1 µl of the drug solution (*e.g.,* 3 µg/µl of cocaine) into the capillary. Expel the drop, and carefully paint it onto the thorax of a marked bee. Cover as large of an area a possible with the solution, rather than leaving a solid drop, as the bee is then likely to groom it off. Be careful not to allow the compound to contact the wing hinges, or this can draw it off the thorax and along the wings where it will evaporate without being absorbed into the hemolymph.
NOTE: Depending on the research goal, this method can also be used to administer drugs to the bee's abdomen. However, drugs reach the CNS quicker and in larger quantities when applied to the thorax[24].This method works equally well with harnessed as with free-flying bees.

3. **Volatilized treatment**
    1. Dissolve drugs (previously this method has been used to deliver cocaine to honey bees[10]) in 100 % ethanol. To ensure solubility, do not use a hydrochloride or other salt forms of the drug if possible. When making a dilution prepare it so that the amount to be delivered to a bee is present in 100 µl. Use pure ethanol as a vehicle control.
    2. To create a filament, use the same procedure as McClung and Hirsh[25].
        1. Briefly explained: wind up nichrome wire tightly around a nail and attach to two electrical wires (one on each end of the filament). Remove the nail. The remaining nichrome coil is referred to as the filament.
        2. Thread the two wires through carefully drilled holes in the lid of a 50 ml centrifuge tube, which should be resistant to the temperature chosen. Glue the wires in place with liquid silicone.
        NOTE: This will make the tube airtight. This is essential to avoid secondary exposure to the experimenter and ensure that bees are treated with the appropriate dose.
    3. Attach the wires leading to the filament to a power source. Using a thermocouple to measure the temperature of the filament, experiment with different voltage/current combinations until one that results in an appropriate temperature profile for the drug in question, ideally, one that allows for 10 sec of heating or less. This is very important, refer to relevant literature (*e.g.,* in order for cocaine to volatilize it needs to be heated to at least 200 °C, but at temperatures over 350 °C it is broken down into secondary compounds[26]).
    4. Carefully pipette 100 µl of drug containing ethanol solution onto the filament. Spread the liquid over as much filament surface as possible as this will increase evaporation efficiency. Leave the filament exposed at room temperature until all the ethanol has evaporated.
    NOTE: If the ethanol is not sufficiently evaporated, bees will be treated with both the drug of choice and ethanol. Bees are extremely sensitive to ethanol, and some drugs have synergistic interactions with ethanol, which will bias experimental results.
    5. Once the ethanol has completely evaporated (drug precipitate can usually be seen on the dry filament under a microscope), catch a free-flying bee in a 50 ml tube. Carefully close the lid containing the filament.
    6. Turn on the power for 10 sec, turn the power off and wait another 50 sec (to allow the volatilized compound to cool and thereby condense or deposit). Release the bee.
    NOTE: While this treatment method works excellently for free-flying bees, it can be used just as effectively with harnessed bees. Simply attach the harnessed bee inside a 50 ml tube. Reload the filament as described in 2.3.4 between bees. For higher throughput, several filaments can be used in parallel.

Representative Results

A selection of representative results for the methods described above are shown, primarily to demonstrate that the methods allow pharmacological agents to reach the brain and affect honey bee behavior.

Specific effects on brain processes can be easily obtained following thorax injection.

Because pharmacological agents injected through the thorax may act on multiple targets in the body, and get diluted into the body before reaching the brain, this technique may raise possible specificity concerns. Nevertheless, it has been used widely in the literature to interfere with cognitive processes, without the necessity to use very high doses that might yield major secondary effects. For example, blockers of transcription have been administered using this technique, in order to identify phases of memory that require gene expression. Thorax injection of such molecules is compatible with survival for several days[27], which means that their potential toxic action on other targets can be limited, provided the concentration is well chosen. In such conditions, selective and time-dependent effects on memory can be obtained, thus showing efficient targeting of the brain (**Figure. 2**).

Diffusion of molecules into the head hemolymph leads to quick, dose-dependent effects.

Ocellus injection is a way to enable a quick diffusion of molecules of interest into the whole head through the hemolymph, especially if they may have many widespread targets in the brain. This method was used to administrate allatostatins, neuropeptides that may also act as neurohormones[28]). As a consequence, a reduced performance was observed in an olfactory learning assay, consistent with the suggested presence of allatostatin receptors in different brain regions involved in olfactory processing and learning[28]. A dose-dependent curve for this effect could be established, by injecting different concentrations to independent groups run in parallel (**Figure. 3**).

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Different ways of administration can yield to similar effects on brain function.

Emetine, a blocker of protein synthesis, is used to impair the formation of early olfactory memory long-term memory, which is typically expressed 1-2 days after conditioning. In most published studies it has been injected into the thorax[29]. We showed that similar effects could be obtained by administering it directly to the brain through the ocellar tract (**Figure. 4**): providing an adjustment of injection parameters (smaller volume, higher concentration and shorter delay before conditioning), we obtained a decrease (~20%) similar to that found in the literature using the same drug amount (10 nM) – compare with **Figure 4** in Stollhoff *et al.*, 2005[29].

The effects of localized injections are confined in time and space

To test the spatial and temporal properties of drugs microinjected into specific brain regions, harnessed bees were trained in an olfactory PER conditioning paradigm, and then injected bilaterally with 0.5 nl of 740 mM procaine (an anesthetic) in the mushroom body calyces or vertical lobes (saline was used as a control). When bees were successively tested for recall 1, 2, and 3 hr after injection, performance was only impaired in bees with bilateral injections into the lobes (**Figure. 5**). Intact neural output from the lobes, but not from the calyces, is known to be necessary for olfactory memory retrieval, so this suggests that procaine remained localized to the lobe in which it had been injected for at least 3 hr. It also shows that, when injected into the calyces, diffusion into the nearby lobes was limited over the same period, since a calycal injection of procaine did not lead to blockade of the lobes.

Behavioral phenotypes following drug administration are often context-dependent

Previous experiments have shown that after treatment with cocaine bees over-estimate the quality of a sucrose solution[10,30]. To see if this effect was dependent on context (here, baseline sucrose quality), free-flying honey bees were treated with volatilized cocaine. Individually marked free-flying honey bees were allowed to forage at a feeder containing 1 M sucrose solution. At the feeder, bees were gently captured in a 50 ml centrifuge tube as they were about to alight from the feeder. Bees were treated with either 100 µg of freebase cocaine or vehicle control (evaporated ethanol). After treatment, the sucrose feeder was either replaced by a 0.5 M or a 2.0 M sucrose feeder, and the rate foragers returned to the feeder was recorded. Using this paradigm, cocaine-treated bees increased their foraging effort at the 0.5 M feeder, but not at the 2.0 M feeder (**Figure 6**). The difference in effect seen with the two sucrose concentrations nicely demonstrates the importance of taking environmental cues into account when studying bee behavior.

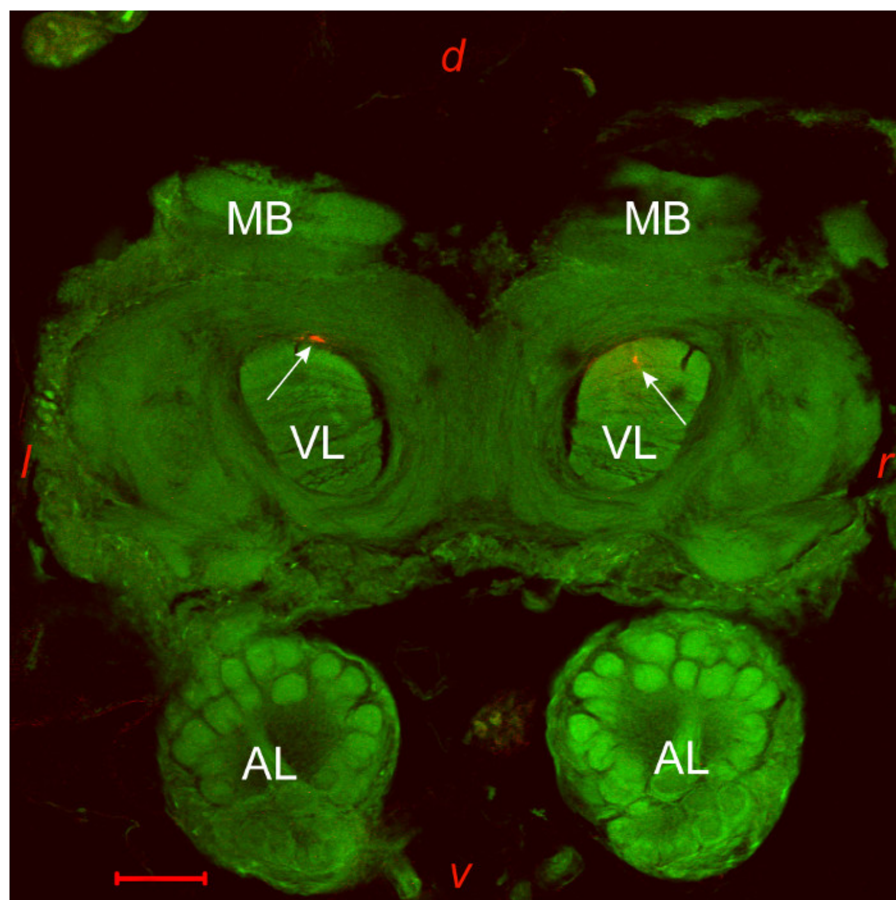

Figure 1: Confocal Laser scanning Image of the Injection Site. Alexa 546-labelled dextran is injected together with the drug solution (red). To identify the neuropils a counter-staining with DAPI is added (green). In the right hemisphere the injection site was located in the vertical lobe (VL), shown as an example for a successful injection. In the left hemisphere the injection site was located dorsal of the vertical lobe in the ring neuropil, shown as an example for an unsuccessful injection. Scale bar = 100 µm, MB: Mushroom Bodies, AL: Antennal Lobes, *d*: dorsal, *v*: ventral, *l*: left, *r*: right. Please click here to view a larger version of this figure.

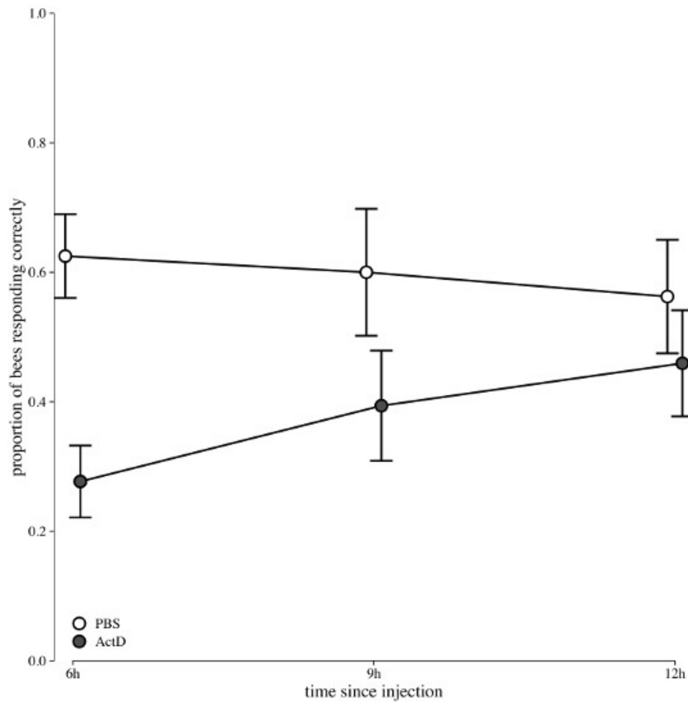

Figure 2: Time-dependent Effect of Actinomycin D (Transcription Blocker) on Long-term Memory, when Injected into the Thorax. At different delays following appetitive olfactory conditioning (6, 9 or 12 h), 1 µL actinomycin D (1.5 mM in PBS) was injected into the thorax. Long-term Memory (LTM) retrieval was assessed 3 d after conditioning (n = 25 - 65). Memory performance was reduced in a time-dependent fashion, as compared to that of PBS-treated controls: the effect was significant when injection too place 6 h after conditioning (χ^2= 18.04, p <0.005), but not at longer delays (9 h: χ^2= 0.95; 12 h: χ^2= 0.47), suggesting that LTM formation requires a wave of transcription that takes place during a defined time window after conditioning. Error bars represent standard errors. Data was previously published[27] and is recreated here with permission. Please click here to view a larger version of this figure.

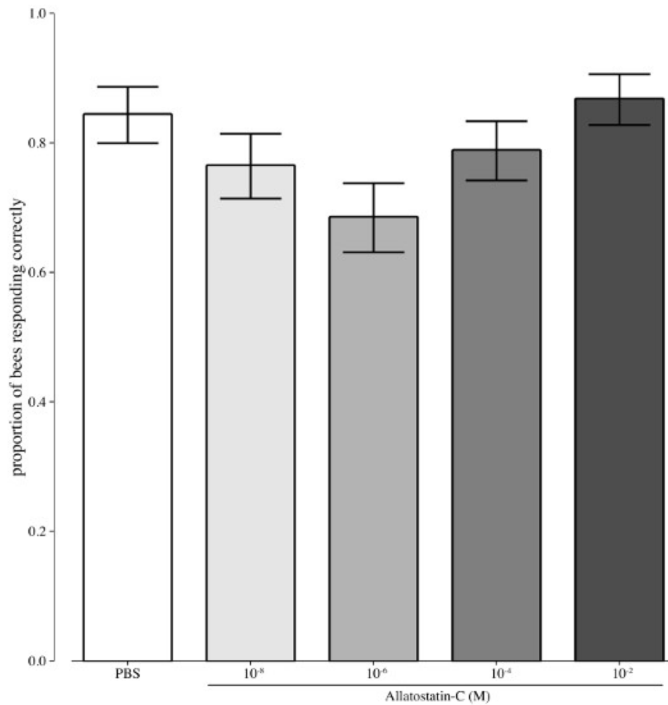

Figure 3: Dose-dependent Inhibition of Learning Performance Following Ocellar Injection of a Neuropeptide. The neuropeptide allatostatin C was injected into the head hemolymph (200 nl in PBS), through the median ocellus, 1 hr before olfactory conditioning. Independent groups of animals injected with different concentrations (or PBS for controls) were trained. Allatostatin C treatment led to a decrease in the learning performance, as assessed by the percentage of conditioned responses in the last conditioning, in a dose-dependent manner following a U-shape curve (n = 70-78). This decrease was significant at 10^{-6} M but not at other concentrations. Error bars represent standard errors. Data was previously published[28], and is adapted here with permission. Please click here to view a larger version of this figure.

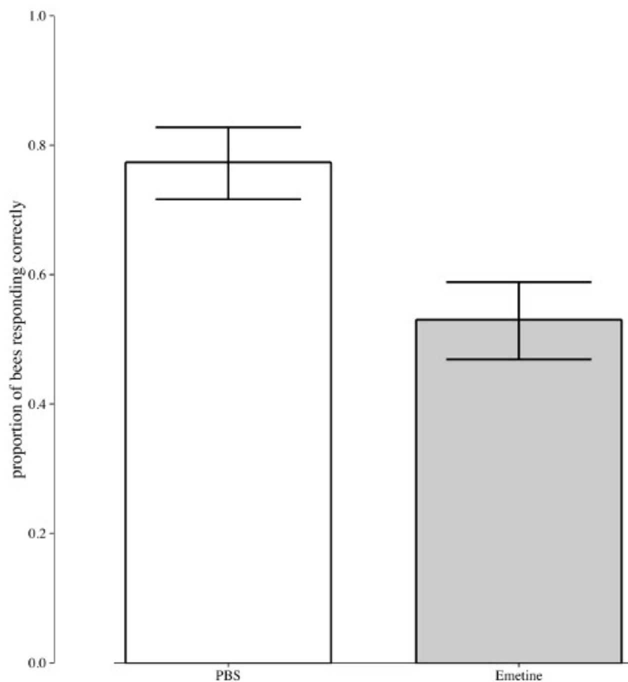

Figure 4: Blockade of 1 Day Memory Following Injection of Emetine (Translation Inhibitor) through the Ocellar Tract. The protein synthesis inhibitor emetine (50 mM in PBS, 200 nl) was injected into the brain, through the ocellar tract, 20 min before olfactory conditioning. Memory was then tested 24 h later. The treatment significantly impaired memory retention ($\chi^2 = 7.03$, $p < 0.01$) as compared to PBS-treated controls (n = 57-70). Error bars represent standard errors. JM Devaud, unpublished data. Please click here to view a larger version of this figure.

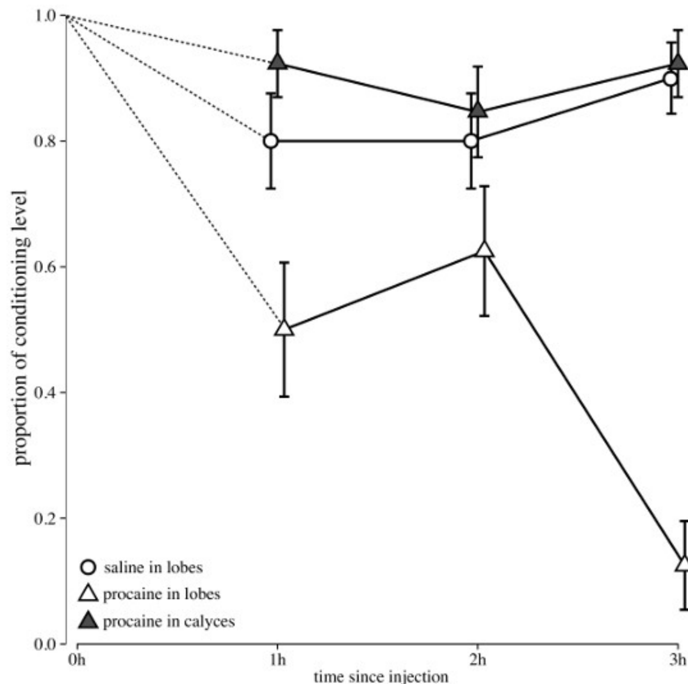

Figure 5: Anatomical and Temporal Specificity of Microinjections. Following appetitive olfactory conditioning, procaine was injected bilaterally into either the mushroom body calyces or vertical lobes. Memory retrieval was assessed 1 hr after injection and was only affected by procaine injections into the lobes (1 hr after treatment: vs. saline: $\chi^2 = 10.00$, $p < 0.005$; vs. procaine to calyces: $\chi^2 = 32.92$, $p < 0.005$). The effect could still be seen 2 hr ($\chi^2 = 6.65$, $p < 0.01$) and 3 ($\chi^2 = 27.22$, $p < 0.005$) after injection, and was still location-specific (2 hr: $\chi^2 = 8.60$, $p < 0.05$; 3 hr: $\chi^2 = 17.15$, $p < 0.0001$), suggesting that only the injected area was affected by procaine. Proportions are relative to conditioning level during the last conditioning trial. Error bars represent standard errors (n = 23-28). Data was previously published[31], and is recreated here with permission. Please click here to view a larger version of this figure.

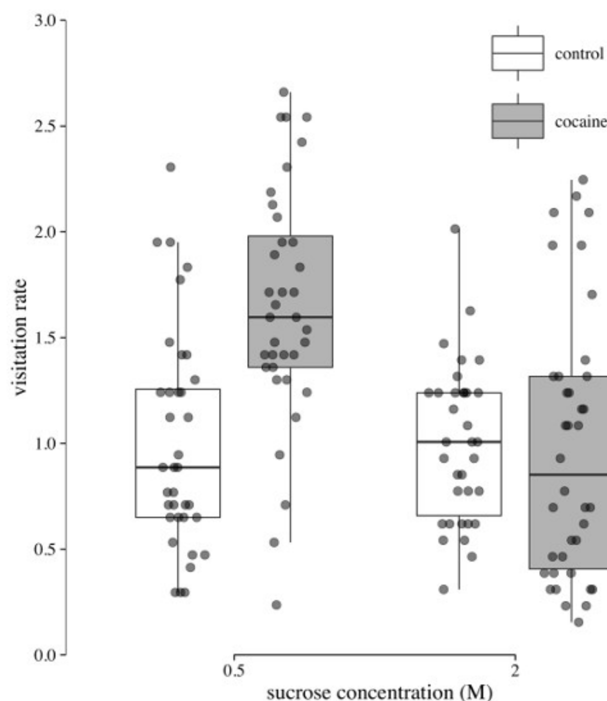

Figure 6: Effects of Cocaine on Free-flying Bees. Visitation rate (number of visits by a given bee/average visits for all bees during test period) was increased following volatilized cocaine treatment at a low quality source (0.5M : $t_{70} = 5.0710$, $p = 0.00003$), but not at a high quality source (2M: $t_{70} = -0.2087$, $p = 0.8353$). The boxes represent 1st and 3rd quartiles with the midline showing the median. The whiskers extend to 1.5x the interquartile range. Outliers are not plotted as all individual data points are superimposed. Data was previously published[10], and is recreated here with permission. Please click here to view a larger version of this figure.

Treatment	Can be done with free- flying bees?	Pros	Cons
Oral treatment	Yes.	Easy, minimally invasive.	Bee digestion is not straightforward
Topical treatment	Yes.	Easy, minimally invasive, quick.	Repeated treatments can be problematic.
Injection into the thorax	Complicated, affects bees flying abilities	Consistent and robust.	Somewhat invasive. Potential to harm/stress bee.
Injection into the median ocellus	Not recommended.	Consistent and robust, somewhat localized.	Somewhat invasive. Potential to harm/stress bee.
Injection into the ocellar tract	Not recommended.	Very localized	Very invasive. Potential to harm/ stress bee.
Micro-injection into brain regions	Not recommended.	Very localized	Very invasive, hard to perform. Potential to harm/stress bee.
Volatilized drug delivery	Yes.	Easy, minimally invasive, quick.	Does not work for all drugs.

Table 1: Comparison of the Different Treatment Methods and Their Properties.

Discussion

The methods outlined above allow simple, effective and robust treatment of either free-flying or harnessed honey bees. These methods are compatible with many experimental paradigms and biological questions (**Table 1**). All of the free-flying methods can easily be applied to harnessed bees. The reverse is less successful, however, since temporary restraint and invasive treatment methods can often compromise bees' flying ability.

The methods have been presented from a brain-centric perspective. This is not due to inherent limitations of the techniques, but rather because of the authors' personal interests. There is no reason why these methods cannot be used for studying other organs. However, small modifications might be needed to make the method more suitable to other organ systems. For example, while topical treatment intended to reach the brain is typically applied to the thorax, it might be better to apply this to the abdomen if the intended target is the ovaries. Similarly, injections can easily be applied to other areas than the thorax or head (*e.g.,* abdominal organs can be targeted by injecting between the abdominal sclerites).

In terms of which compounds can be administered to bees, there really are no limits. Typically, people have administered pharmacological compounds such as signal molecules[21] or their antagonists[32], and custom-made peptides[28]. However, there has been a recent increase in administering to bees compounds with applied questions in mind, such as pesticides[33] and anthropogenic contaminants[34]. Recently, compounds administered have started to include RNA molecules that interfere with gene expression directly, such as dsRNA activating the RNA interference pathway[35] or even microRNAs[36] and antagomiRs[37]. Not all methods work equally well for all compounds. This is perhaps best illustrated by bitter or sour compounds that make sugar water unpalatable to bees, thus preventing them from consuming it. Fragile molecules, such as RNAs or certain polypeptides, are broken down when heated during a volatilization procedure or placed in a harsh solvent like DMF. It is therefore important to understand the chemistry of what is being administered to ensure it survives the treatment procedure.

Getting a pharmacological agent into a bee is the easy part, but there are three big concerns that should never be taken lightly when performing pharmacological experiments. The first is figuring out a good dose for the experiment in question. Depending on the drug, there might already be published literature available, but for the most part, this will have to be resolved by a mixture of literature searches, informed guesswork, and dose-response curves. Depending on how complicated the experimental protocol is, it might be useful to first generate a dose-response curve in a simpler bioassay (*e.g.,* quantifying overall movement or survival) to get a better idea of a dose-range worth trying in a more elaborate bioassay. In our laboratory, a starting dose is either found in the bee literature or by doing a mg/kg conversion based on data from the rodent literature. From this starting point, bees are treated with the starting dose, plus 2 or 3 doses 10 times larger and smaller than the starting dose (*e.g.,* if the starting dose is 1 mg, 0.01, 0.1, 10, and 100 mg would also be used), and of course an appropriate vehicle control.

The second problem is slightly more finicky: drug specificity. Most drugs were not developed with honey bees, or any other insect, in mind. Because of this, off-target effects are common (*e.g.,* mianserin, a vertebrate serotonin receptor antagonist[38], was long thought to be an insect octopaminergic receptor antagonist, but recent findings show that in bees it is also a dopaminergic receptor antagonist[39]). A common solution to this problem is, rather than relying on only one drug, to repeat the same experiment with a suite of drugs known to have the target of interest in common. Basically, if several drugs are known to block a certain target, observing similar results across different drugs should give greater confidence that the drug has the expected effect, since different drugs often have unique off-target profiles.

The last issue involves ensuring that the drug is acting where it is supposed to be acting. In this regard, there will always be a trade-off between specificity and invasiveness. Systematic treatment methods are generally the least invasive, but there is no control of where in the bee body the drug is having its effect. Even for microinjection of targeted tissues drugs may travel with the hemolymph to other parts of the bee body. How this issue is addressed needs to be informed by the questions asked. For certain experiments anatomical location is irrelevant, whereas for others this is the only question of importance. The best way to address this is to start with systemic treatments and gradually narrow down to an anatomical location by using increasingly more specific methods. If the behavior being studied is particularly incompatible with invasive treatment methods, it might be worth trying to deconstruct it into simpler components before doing a whole series of experiments with very specific pharmacological treatments.

This problem of drug leakage is even more exaggerated with oral treatment of free-flying bees, where drugs can affect non-target bees. Forager honey bees collect nectar in the field to bring back to their colony. They will offload the majority of their sucrose solution in the hive upon returning rather than absorb it. In the hive it is packed in cells, dehydrated, and stored as honey. Because of this, drugs can potentially affect non-target bees. With more specific methods (such as microinjections) this problem is minimized.

With these caveats in mind, and addressed properly, neuropharmacological manipulation of honey bees can be a very powerful tool. While transgenic tools are being developed for honey bees[15], because of their social lifestyle it is unlikely that transgenics will ever be an easy and reliable way to conduct these kinds of experiments. It is therefore likely that pharmacology will continue to be an important element of bee research in the future. While some bee researchers have made calls for standardized experimental methods[40], in this case this would be a mistake. Part of the power of the bee system has always been the diversity of experimental approaches, and how techniques have been developed with real biological questions in mind rather than the other way around. It is nevertheless important that we ensure usage of the most appropriate method for the question at hand. If comparisons to previous studies are key, standardized protocols must be followed strictly. However, utilizing established protocol for the sake of using standardized methods must not be allowed to stand in the way of the development of novel methods that can open new experimental possibilities.

Disclosures

The authors have nothing to disclose.

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