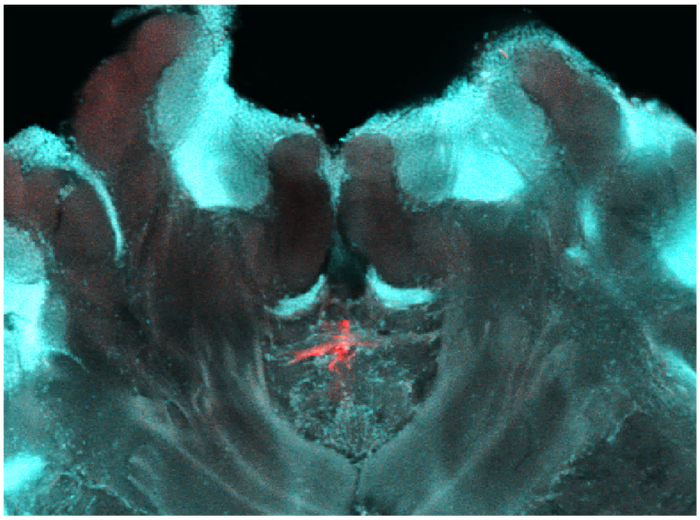
Neuroethological analysis of visually oriented behavior in honey bees



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A thesis submitted in partial fulfillment of the requirements for the degree of

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Summary

The honey bee is an excellent navigator and visual learner, but we know little how and why it performs so well. Two regions of the honey bee brain are crucial for learning and memory and in orientation in space – the mushroom bodies (MBs) and the central complex (CX). Both regions process major sensory input of different modalities. The mushroom bodies are key regions for associative learning. The CX plays a major role in processing visual input to generate a representation of orientation in relation to the environment and regulates motor output. My aim is to understand the role the MBs, the CX and adjacent regions of the protocerebrum play in visual learning, locomotion and orientation in the honey bee. I present how neuropharmacological manipulation in free-moving and restrained bees can be used to investigate behaviour. The key method for the studies described in this thesis was microinjection of the local and reversible anaesthetic procaine into the investigated brain regions. In the first experimental study, I explored the role of the mushroom bodies and the central complex in an aversive visual learning assay. I concluded that the mushroom bodies and the central complex both contributed to the behavioural response to a learned visual stimulus. In the second study, I investigated what role the MBs and the CX play in modifying locomotion and orientation to a visual stimulus. I found that reducing neural activity in one MB calyx by procaine-injections led to lower walking speed and a lower number of walking bouts compared to controls. Injections with procaine into the CX and the adjacent protocerebrum led to an increase in turning in dark conditions compared to controls. Using a new visual sequence learning assay, I present that honey bees can anticipate an upcoming light in a light sequence of three lights with experience. This behaviour was impaired after procaine-injections into the CX and the adjacent protocerebrum. In my final review chapter I discuss how recent research corroborates the CX as key structure for generation and reading of the waggle dance. Finally, I discuss how my findings contribute to understanding of how visual information is processed and integrated by the insect brain to generate the appropriate motor response.

Declaration

I hereby certify that the work in this thesis entitled '**Neuroethological analysis of visually oriented behavior in honey bees**' has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University and to the University of Konstanz under the cotutelle agreement.

I also certify that the thesis is an original piece of research and it has been written by me. Any help or assistance that I have received in my research work and the preparation of the thesis itself has been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

Jenny A. Plath (43000029)

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List of Original Publications

This thesis is based on the following original publications:

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Chapter III:

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Chapter V (earlier version):

Barron, A.B.*., and **Plath, J.A.*** (2017). The evolution of honey bee dance communication: a mechanistic perspective. *J. Exp. Biol.* 220**,** 4439 -4346 doi: 10.1242/jeb.142778

http://jeb.biologists.org/content/220/23/4339

*equal contributions

List of Contributions

Unpublished chapters and original publications have been included in this thesis with permission of all co-authors and the publishers.

Andrew B. Barron: AB

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Nicholas H. Kirkerud: NK

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	Chapter I	**Chapter II**	**Chapter III**	**Chapter IV**	**Chapter V**	**Chapter VI**
Conception & Design	**JP**, AB	ES, **JP**, AB	**JP**, BE, NK	**JP**, GG	**JP**, GG	**JP**, AB
Planning & Implementation	**JP**	ES, **JP**	**JP**, BE	**JP**	**JP**	AB, **JP**
Data collection	-	ES, **JP**, AB, JD	**JP**, BE, US	**JP**, BE	**JP**	-
Analysis & Interpretation	-	ES, **JP**, AB, JD	**JP**, NK	**JP**, AB	**JP**, AB	-
Writing	**JP**, AB	ES, **JP**, AB, JD	**JP**, BE, AB, NK, GG, US	**JP**, AB, GG	**JP**, AB, GG	**JP**, AB
Overall responsibility	**JP**	ES, **JP**	**JP**, BE	**JP**	**JP**	**JP**, AB

Abbreviations

CBU upper division of the central body

CX central complex

CB central body

CBL lower division of the central body

EB ellipsoid body

FB fan-shaped body

PB protocerebral bridge

NO noduli

MB mushroom body

MBC mushroom body calyx

HL horizontal lobes

VL vertical lobes

TB neuron tangential neuron projecting into the protocerebral bridge

TL neuron tangential neuron projecting into the lower division of the central body

TU neuron tangential neuron projecting into the upper division of the central body

TN neuron tangential neuron projecting into the noduli

LAL lateral accessory lobe

Introduction

The ability to find and remember where good food sources are located is crucial for survival across the animal kingdom. In the past decades, numerous studies have demonstrated the remarkable ability of insects to navigate in known and unknown terrain (Collett and Collett, 2000; Wehner et al., 1996) and to associate olfactory, chemical or visual properties of a food source with its value (Giurfa, 2007). Insects use polarization information from the sky, the azimuth of the celestial body and visual landmarks to orientate in their environment and to navigate to a goal (Menzel et al., 2006; Menzel et al., 1998; von Frisch, 1967; Wehner, 1984; Wehner, 2003; Wehner et al., 1996). Central place foragers, such as honey bees, are able to find and follow efficient foraging routes (Menzel et al., 1998; von Frisch, 1967) and even novel short-cuts between food sites or when returning to the hive (Menzel et al., 2012). Furthermore, honey bees associate odor, color and shape of a flower after only one rewarding visit (Giurfa, 2007). Even though we know much about the behaviors, we still know little about the underlying neural processes and substrates controlling and regulating visual learning, spatial orientation and navigation. Honey bees can be used as a model to investigate what is happening in the brain during associative learning, spatial orientation and spatial learning.

Overview of the honey bee brain

The honey bee brain has a volume of 0.4 – 0.6 mm^2 and contains approximately 1 million neurons. This is about ten times as many neurons as the fruit fly and about 100,000 times less than a human brain. The honey bee brain is compartmentalized into sensory neuropils processing second order visual, olfactory or tactile information, which is passed on to higher order processing areas. Higher-order sensory processing happens in two prominent structures in the honey brain: The mushroom bodies (MBs) and the central complex (CX). The MBs are large paired neuropils, which comprise about a third of the total neuron count. Sensory inputs are received and integrated by neurons called Kenyon cells which are organized in four cup-like structures called the MB calyces (MBCs) (Mobbs, 1982). The Kenyon-cells send axons through the peduncle at the base of the cup and divide into the horizontal lobes (HLs) and the vertical lobes (VLs). Here, the Kenyon cells project onto a

small number of output neurons, which form the efferent pathways of the MB (Mobbs, 1982; Rybak and Menzel, 1993; Strausfeld, 2002). In the fruit fly, the lobes receive modulatory input from dopaminergic neurons (Waddell, 2013), which carry information about the external context and the behavioral state (e.g. whether the animal is flying) (Cohn et al., 2015). To my knowledge, this exact pathway has not been found in the honey bee, but stainings for dopamine-like immunoactivity have been found in the same regions of MB extrinsic neurons in bees (Schäfer and Rehder, 1989). This suggests, that dopaminergic neurons could also modulate MB output according to the behavioral state in honey bees.

The second higher-order processing center is the CX. The CX comprises a group of interconnected neuropils: the elongated bar-shaped protocerebral bridge (PB), the kidney-shaped upper and lower divisions of the central body (CBU and CBL) and the round noduli (NO). The CX is a midline spanning structure that connects the two brain hemispheres. The CX is not directly connected to the sensory neuropils, but rather receives processed sensory information (Pfeiffer and Homberg, 2014). Processing in the CX is dominated by visual information, but CX neurons in the honey bee (Homberg, 1985; Milde, 1988) or other insects (Pfeiffer and Homberg, 2014) also respond to olfactory or mechanical stimuli.

Anatomical studies in different insects have shown, that the CX receives input via the lateral accessory lobes (LALs) and the superior medial protocerebrum (SMP) (Pfeiffer and Homberg, 2014). The SMP is a part of the unstructured protocerebrum, which does not receive direct sensory input. Rather, it receives mechanosensory information from other areas of the brain, but is also connected to the MBs (Ito et al., 1998; Strausfeld, 2002). In locusts, CX output neurons connect to descending motor pathways to the thoracic ganglia in the LALs (Heinze and Homberg, 2008).

Using insects to investigate the neural basis of visual learning and spatial orientation

The remarkable learning abilities of honey bees have been used to develop the Proboscis Extension Response (PER) assay, which pairs a sucrose reward with an odor stimulus (Bitterman et al., 1983; Felsenberg et al., 2011; Giurfa and Sandoz, 2012). Pharmacological interference, for example by feeding a drug, made it possible to investigate which

neurotransmitters, proteins or processes play a role in learning and memory. For example, the different memory phases after olfactory learning were investigated with the help of translation inhibitors (Stollhoff et al., 2005) or the role of acetylcholine in retention of an olfactory memory was tested by injecting a nicotinic acetylcholine antagonist (Lozano et al., 1996). Since honey bees are harnessed in the PER assay, this can be combined with a number of techniques to explore what is happening in the brain directly when a bee processes sensory information. For example, how odors are represented by activity changes in the odor processing parts of the honey bee brain, was investigated using calcium imaging techniques (e.g., Galizia et al., 1999).

While great progress has been made towards understanding the mechanisms of olfactory learning in the brain, very little research is available on associative learning of colors and shapes. This is mainly due to difficulties in establishing high learning rates in a PER assay with visual conditioned stimuli (Balamurali et al., 2015; Dobrin and Fahrbach, 2012; Hori et al., 2006; Hori et al., 2007; Kuwabara, 1957; Niggebrugge et al., 2009). To overcome these difficulties, Kirkerud et al. (2017) developed a chamber to condition free-walking honey bees with colored light fields and electric shocks. This conditioning assay was used in this thesis to investigate the functional roles of different brain regions in aversive color learning (Chapter III).

Uncovering the neural mechanisms and processes underlying navigation remains a great challenge, since studying what happens in the brain of a moving animals is very difficult. However, great progress has been made when focusing on smaller behavioral tasks that contribute towards navigation. While many studies corroborate a role of the MBs in sensory association and learning (Heisenberg, 1998; Menzel, 2012; Zars, 2000), the MBs have also been implicated in locomotor regulation and control (Zars, 2000) and context-dependent regulation of motor output (Cohn et al., 2015). Thus, the functions of the MBs are still far from understood. The CX has a strong role in spatial orientation, spatial learning and in providing a representation of body orientation by integrating external and internal information (Turner-Evans and Jayaraman, 2016; Varga et al., 2017). Furthermore, inhibiting or ablating the CBU in the CX impairs spatial memory (Ofstad et al., 2011). Most of this research used flies, cockroaches and locusts with only little focus on central place

foragers such as honey bees (for discussion, see Chapter I). The work presented here aims to contribute to the functional roles of different brain regions towards regulating locomotion, orientation in space (Chapter IV) and in spatial learning (Chapter V) in insects by using honey bees as a model.

Prospectus

In my thesis, I present a series of chapters around the topic of how the insect brain integrates visual information to initiate appropriate motor responses and to create spatial memories. Chapters are presented as review articles and as research papers to examine the functional roles of the MBs, the CX and associated regions. The chapters are written as papers, which have been published or are prepared ready for submission.

Research on the functions of the CX has recently gained rapid momentum. In **Chapter I**, I provide a comprehensive overview of recent findings and present perspectives for future research. I highlight roles of the CX in processing of polarized light, motion and spatial information processing, and spatial memory. I stress the importance of more research in central place foragers, such as bees, which have been underrepresented in CX research in comparison to other insects.

I used neuropharmacological manipulation to investigate behavior as a key technique throughout the experimental chapters of this thesis. In **Chapter II**, I give background and detailed descriptions of neuropharmacological methods used in ethological research in honey bees, including the microinjection technique used in Chapters III – V. Local drug injections can be used to investigate functional roles of the targeted brain region. The protocols are backed up with representative results and I discuss the advantages and disadvantages of each method.

To associate stimuli with known positive or negative stimuli is crucial for an animal's survival. In **Chapter III**, to explore how different brain regions are involved in visual learning, I microinjected a local anesthetic into different parts of the MBs and into the CX and tested their behavior in a visual learning assay in which color stimuli are paired with electric shocks. While the MBs were crucial for color learning, the CX had a role in

initiating the behavioral response to the learned stimulus.

To initiate and regulate locomotion in response to external stimuli is one of the most essential roles of the insect brain. In **Chapter IV**, I explored how different brain regions are involved in locomotor control with and without visual stimuli. I found reduced speeds as well as a reduced number of walking bouts in the dark, after anesthetizing in the MB input regions but not after anesthetizing the MB output regions. When the CX (including the input regions in the protocerebrum) were anesthetized, animals turned considerably more compared to controls in dark conditions. I additionally investigated the role of the CX in a orientation and orientation learning to distinct visual stimuli. I showed that control animals were able to anticipate an upcoming visual stimulus in a regular sequence of visual stimuli with experience. Anticipation behavior was impaired when the CX (including the input regions in the protocerebrum) was anesthetized. This demonstrates that the CX plays an important role in learning complex spatial relationships of visual stimuli in honey bees. This corroborates a role of regulation of walking speed and activity by the MBs and a role of orientation and turning by the CX.

Honey bees transfer information gathered during a foraging flight to nestmates via the famous waggle dance. In **Chapter V**, I illustrate the differences in dance communication across different bee species. The role of the CX in navigation and spatial orientation has been established in numerous studies. It makes sense, that the CX is also involved in the waggle dance, and I present supporting evidence for this hypothesis. I introduce experimental approaches to investigate the neural basis underlying bee dance and discuss the challenges involved.

In a final discussion section, I incorporate my findings into the current research to create a functional information flow model in the insect brain. I provide an outlook into future experiments and directions of neuroethological research in honey bees.

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Chapter I

Current progress in understanding the functions of the insect central complex

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Abstract

The central complex is a group of neuropils in the center of the insect brain which performs higher sensory integration. This region is involved in diverse vital behavioral processes including visual processing, motor coordination, orientation and navigation. Little is known of the circuit organization and properties within this region, and we here review recent progress toward a functional understanding of the central complex. Since central complex research is increasingly limited to just a few model systems, we argue that studies of the central complex in species with broad behavioral repertoires and strong navigational capabilities such as bees and ants will aid in determining the functions of this region.

Introduction

The central complex (CX) spans across the midline connecting both hemispheres of the insect brain, and is highly interconnected with the surrounding protocerebrum [1]. Exciting new studies include analyses of CX network structures and properties and explore the involvement of the CX in processing of polarized light, motion processing, spatial memory and motor control [2••]. In this review, we focus on these behavioral functions.

Research is presently dominated by a few insect species: the discoid cockroach *Blaberus discoidalis*, the monarch butterfly *Danaus plexippus*, the fruit fly *Drosophila melanogaster* and the desert locust *Schistocerca gregaria*. The CX is conserved across insects and other closely related arthropod groups [1,3,4]; hence many functions are most likely to be quite generalizable across other insects as well. We discuss future directions for CX research.

Brief anatomy and connections of the central complex

All parts of the CX are interconnected. The CX neuropils are the protocerebral bridge (PB), the central body (CB) and two noduli (Figure 1a). The CB is divided into the upper unit (CBU) and the lower unit (CBL); also termed fan-shaped body (FB) and ellipsoid body (EB) respectively in the fruit fly CX literature [5•]. The PB and the CB are structured in columns created by the distinct arborization pattern of the columnar neurons [2••] (Figure 1b,c).

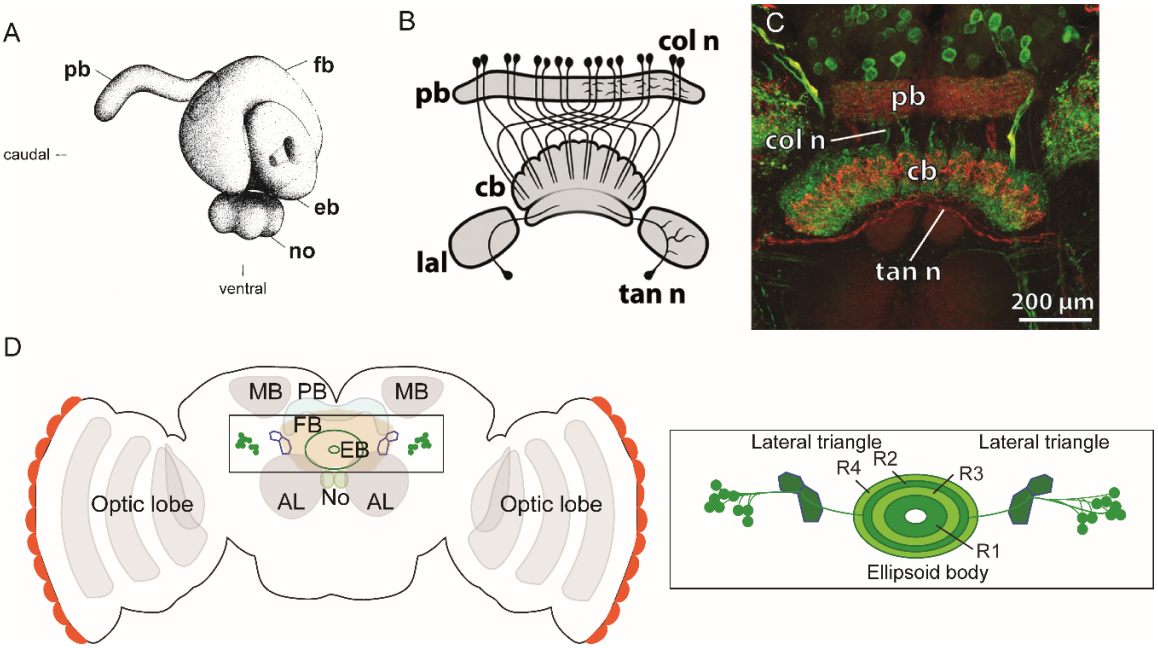


Figure 1: Central complex structure and architecture. **(a)** Central complex neuropils in the fruit fly: protocerebral bridge (pb), fan-shaped body (fb) and ellipsoid body (eb), which combined are termed central body, and the noduli (no). **(b)** Schematic drawing of connections between the central complex neuropils in the cockroach. Columnar neurons (col n) connect between the protocerebral bridge (pb) and the central body (cb). Tangential neurons (tan n) provide input and output connections to the adjacent lateral accessory lobe (lal). **(c)** Original staining of the central complex neuropils and neuronal connections in the cockroach *Periplaneta americana* (red: allatostatin-like immunoreactivity, green: tachykinin-like immunoreactivity). **(d)** Architecture of the central brain of the fruit fly showing the mushroom bodies (MB), the antennal lobes (AL), the noduli (NO), the ellipsoid body (EB) the fan-shaped body (FB) and the protocerebral bridge (PB). Marked in green are the ring neuron groups R1–R4 and in blue the lateral triangle. Originals: (a) modified from [55] with permission of Springer-Verlag; (b) by Carsten Heuer from [56]; (c) from [56] as modified from [3] with the permission of Elsevier; (d) reprinted from [24] by permission from Macmillian Publishers Ltd: *Nature*, copyright (2013).

Three large fiber tracts lead from and to the CX: the anterior bundles, the isthmus tracts and fibers connecting to the PB [2••]. The CX does not seem to have direct connections to the mushroom bodies (MB) [2••,6], except for a recently discovered neuron in the butterfly brain [7•].

The CX mainly receives indirect visual input [2••], and probably indirect mechanosensory and olfactory input [8– 10]. Two parallel visual pathways have been identified in locusts, bees and butterflies [7•,11–14]. The anterior pathway originates in the visual neuropils and does not directly enter the CBL, but enters indirectly via the anterior lobe of the lobula, the anterior optic tubercle and the median and lateral bulb (Figure 2b). In the locust polarized light input is conveyed to the CX via this pathway, and this is assumed to be the case for bees and butterflies as well [7•,12,13].

Functions of the central complex

During their daily foraging activities insects have to find their way to food sources and back to their nests or hiding places in known and unknown terrain. To be successful the animal needs navigation and orientation skills, spatial memory and a quickly updated visual working memory. The animal needs to select and initiate the most appropriate motor outputs to affect locomotion and foraging. The CX is involved in all these processes (Table 1) and recent progress has been made to determine how.

Processing of polarized light

Many insects navigate with the help of celestial cues including the position of the sun, the pattern of polarized light and the chromatic gradient of the sky, for example [7•,15].

Scattering in the atmosphere results in a linear polarization of sun light (Rayleigh scattering, [16,17]). A property of polarized light is the electric field vector (*E*-vector), which indicates the orientation of polarization. Different *E*-vectors are arranged in a concentric pattern around the sun's position (Figure 2a). This is used by many insects to orientate and navigate, even when the sun is blocked by clouds.

In locusts, polarized light information enters the CX via the anterior visual pathway (Figure 2b). Neurons in the PB columns are specific in their response to *E*-vector orientation and

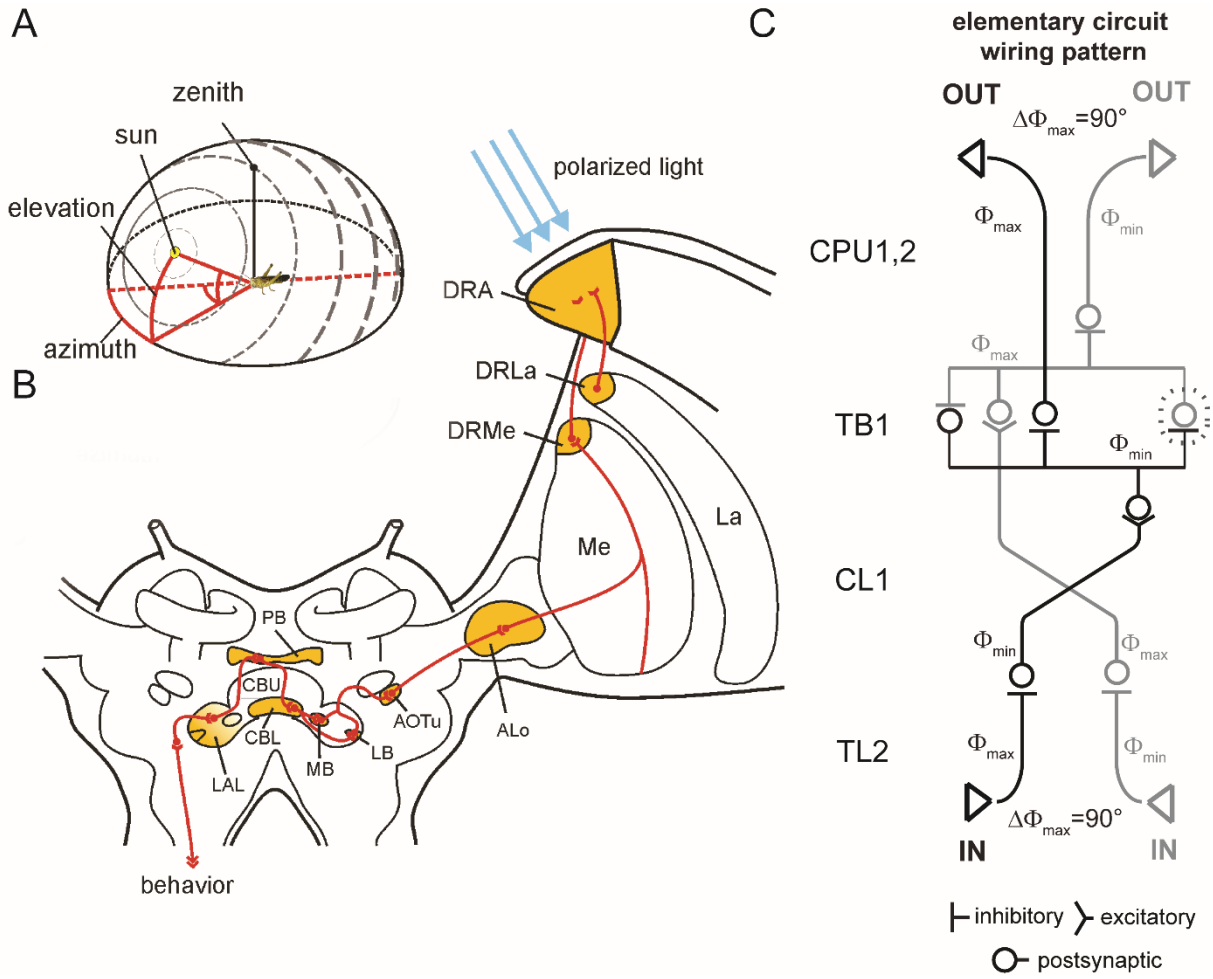
differ in their peak activity from one column to the next, spanning over 180° across the entire PB [2••,18]. Thus, the PB network provides a central polarotopic representation of the sky polarization pattern. It has therefore been suggested that the CX is the main neuropil to process celestial compass information [2••,15,19]; but how does this processing work?

The representation of a specific *E*-vector angle in the individual columns in the PB likely arises from an antagonistic integration of different input paths [20••]. As illustrated in Figure 2c, information about the preferred *E*-vector enters the CX via tangential neurons (TL2). There is a strong indication that the information is passed on inverted via an inhibitory synapse to columnar neurons (CL1). This reduces the activity in the CL1 neurons and the downstream tangential neurons in the PB (TB1). The model suggests that a pair of TB1 neurons integrates information coming from two TL2-CL1-TB1 networks: one TB1 neuron being inhibited and one TB1 neuron being disinhibited by the same *E*-vector angle. The preferred *E*-vector angles of the paired TB1 neurons are 90° apart so that when one TB1 neuron is excited it inhibits its paired partner. Each TB1 neuron displays robust antagonistic responses to the preferred and to the antipreferred angle (perpendicular to preferred angle) as a result (Figure 2c).

Furthermore, some tangential neurons have two activity peaks at different solar azimuths for certain solar elevations [21], one being at the solar and one being at the antisolar position. Since the activity maxima at different solar azimuths differ between units, the locust can identify the correct position of the sun at certain elevations solely based on *E*-vector information without requiring other celestial compass information.

Additionally, animals seem to use polarized light information to stay on course [20••]: neurons downstream of the TL2 neurons exhibited adapting responses when stationary polarization input was given and nonadapting responses when rotating polarization input was given, providing a possible simple neural mechanism for maintaining a constant heading relative to the *E*-vector.

Figure 2: Processing of polarization in the central complex. **(a)** *E*-vectors of polarized light are arranged in concentric circles around the sun and can be used by insects to navigate. The sun's position is determined by the azimuth and the elevation. **(b)** The anterior polarization pathway originates in the dorsal rim areas of the compound eye (DRA: dorsal rim area) and the visual neuropils; (DRLa, DRMe: dorsal rim areas of lamina and medulla). Information enters the central body lower unit (CBL) via the anterior lobe of the lobula (ALo), the anterior optic tubercle (AOTu), the lateral bulb (LB) and the medial bulb (MB). In the central complex, the information is relayed via the central body upper unit (CBU) and the protocerebral bridge (PB) to be processed and generate behavioral output via the lateral accessory lobe (LAL). **(c)** Proposed circuit for processing of polarized light. Information about the preferred *E*-vector angle (Φ_{max}) enters the central body by TL2 neurons and is passed inverted on via an inhibitory synapse to CL1 neurons and further to the TB1 neurons in the PB. Two TB1 neurons integrate information coming from two such networks which are tuned antagonistically to the same *E*-vector. The information subsequently leaves the PB via the CPU1 and CPU2 neurons. Originals: (a) from [21] as modified from [57] with permission of John Wiley and Sons and Elsevier, (b) from [21] with permission of Elsevier; (c) modified from [20••] with permission of the American Physiological Society


A
zenith
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AOTu
ALo
behavior
C
elementary circuit
wiring pattern
OUT
OUT
ΔΦmax=90°
Φmax
Φmin
CPU1,2
TB1
CL1
TL2
IN
IN
inhibitory
excitatory
postsynaptic

Table 1: Functions of the central complex in different insects

Insect species investigated	Function of the central complex	Part of central complex involved	Method used	Ref.
Schistocerca gregaria, possibly *Drosophila melanogaster* and *Danaus plexippus*	Processing of polarized light by *E*-Vector tuning of tangential neurons and antagonistic coding in tangential-columnar neuron networks	PB, CBL	Intracellular recordings	[7,20,25]
Schistocerca gregaria	Determination of sun position by different activity peaks at solar and antisolar position for different solar elevations	CBL	Intracellular recordings	[21]
Schistocerca gregaria	Processing of looming stimuli	PB, CBU, CBL	Intracellular recordings	[22]
Schistocerca gregaria, Drosophila melanogaster	Processing of translational movement	PB, CBU (FB), CBL	Intracellular recordings, calcium imaging	[22,23]
Drosophila melanogaster	Activity modulation by behavioral state	CBU (FB), CBL (EB)	Intracellular recordings, calcium imaging	[23,24]
Drosophila melanogaster	Visual working memory for spatial orientation depends on different molecular mechanisms of the ring neurons in the EB.	CBL (EB)	Calcium imaging, histology and behavioral analysis using an LED arena	[28,29]
Drosophila melanogaster	Visual place memory relying on visual patterns	CBL	Behavioral analysis using an LED arena	[30]
Blaberus discoidalis, Gryllus bimaculatus	Neural activity correlated with walking activity and turning	CBL, CBU or not specified	Extracellular multichannel recording in tethered or free-walking animals, Procaine injections	[32-35]
Drosophila melanogaster	Groups of dendrites from columnar neurons encode for the fly's position in relation to a visual landmark, which continues when no visual cues are present during walking and when the animal has stopped.	CBL (EB)	Calcium imaging in tethered animals on a track ball	[24,31]

Motion and spatial information processing

During flight it is imperative for the animal to react to approaching objects such as obstacles or predators. Antagonistic responses to opposite stimuli in the CX also seems to play a role here: In the locust many CX units showed excitation to a looming stimulus displayed to one eye and inhibition when displayed to the other eye [22]. Similar response patterns were found in the fruit fly when forward motion versus backward motion was perceived. [23]. Interestingly, the animal's state influenced motion processing: neuronal responses to visual stimuli were measured during flight but not during rest [23]. Seelig *et al.* [24] focused on responses of the dendritic arborization of EB neurons (ring neurons) in the fruit fly [24] (Figure 1d). The dendrites form condensations (microglomeruli) in the lateral triangle (lateral bulb, Figures 1d and 2b) and receive visual input. Here, responses to visual stimuli were diminished during flight but not during walking. Thus, it is argued that responses of the ring neuron dendrites relate to a modulation of motor output and providing behaviorally relevant visual information than to direct motor control [24]. Furthermore, the response patterns indicated that the microglomeruli in this region were arranged as a spatial map relating to the visual field of the fly [24]. Lin *et al.* [25•] suggest that several such topographical maps may occur in the CX. Whether these topographical arrangements are organized by similar networks as those that have been found for the polarization pathways in the PB remains to be investigated.

Spatial memory

An important aspect of orientation and navigation is a quickly updated visual working memory (VWM) as well as visual and spatial memory. The detour paradigm has been developed as a lab assay to test VWM in fruit flies [26]. This assay makes use of the Buridan's paradigm in which the fly walks between two opposing black stripes (Figure 3a). In the detour paradigm, the stripes disappear and a new stripe appears perpendicularly (Figure 3b). After a successful orientation toward the new stripe it is removed so that the fly is left without visual cues (Figure 3c). In 80% of the cases the fly will turn toward its original heading using idiothetic (use of internal cues when navigating) information of the initial path. Several recent studies have shown that different sets of ring neurons in the EB are needed for intact VWM function in the detour paradigm [26,28,29]. The EB is also involved in visual place learning [30] in the fruit fly (Figure 3d). Ofstad *et al.* [30] presented an assay

in which the fly had to find a cool tile in a heated arena in relation to a visual pattern projected on the walls. The time the flies needed to find the tile decreased over successive learning trials, but when EB neurons were silenced learning was impaired [30].

Seelig and Jayaraman [31••] investigated neuronal activity during landmark orientation in tethered but walking fruit flies. Groups of columnar neurons originating in the EB encode for the fruit fly's orientation in relation to a landmark. Intriguingly, the EB activity profile was maintained in the absence of visual cues when the fly was walking as well as when the fly stopped, indicating a formation of visual working memory or short-term memory [31••]. Hence, this network provides a possible basis for navigation relying on path integration by maintaining a representation of the animal's position even when visual landmarks are no longer available.

Sensory information processing for motor control

Whether the CX directly initiates and controls motor output remains an open question. Newly developed techniques that allow recording of neural activity while a cockroach or cricket is walking are great advances toward clarifying this matter [32,33].

In cockroaches, various activity patterns in CB units were found in response to wide-field visual motion stimuli which elicit visually guided behavior [34]. Walking was diminished when parts of the CX were anesthetized, thus showing that CX activity is necessary for initiation of locomotion. Furthermore, in both, crickets and cockroaches, activity changes of different CX units were correlated with specific directions of turns, while other units were attuned to walking activity regardless of turning direction, or were attuned to turning activity in general [33,35]. The majority of recorded activity changes preceded locomotion. This evidence favors a more direct role of the CX in initiation of locomotion. Some of the neurons, however, changed their firing rate after the locomotion onset or change. This might indicate a feedback pathway to the CX. Kai and Okada [33] suggest that the ongoing activity during walking could arise from reafferent control for mechanosensory inputs, and from exteroceptive and proprioceptive inputs due to movement of body parts.

Interestingly, in cockroaches only a few neurons responded to antennal stimulation when it occurred after an active movement of the antenna to a rod [35]. In contrast, many neurons

responded to imposed antenna stimulation. The authors suggest that the neurons responding to self-generated antennal contact might also be arranged in a map-like representation similar to the *E*-vector representation in the PB.

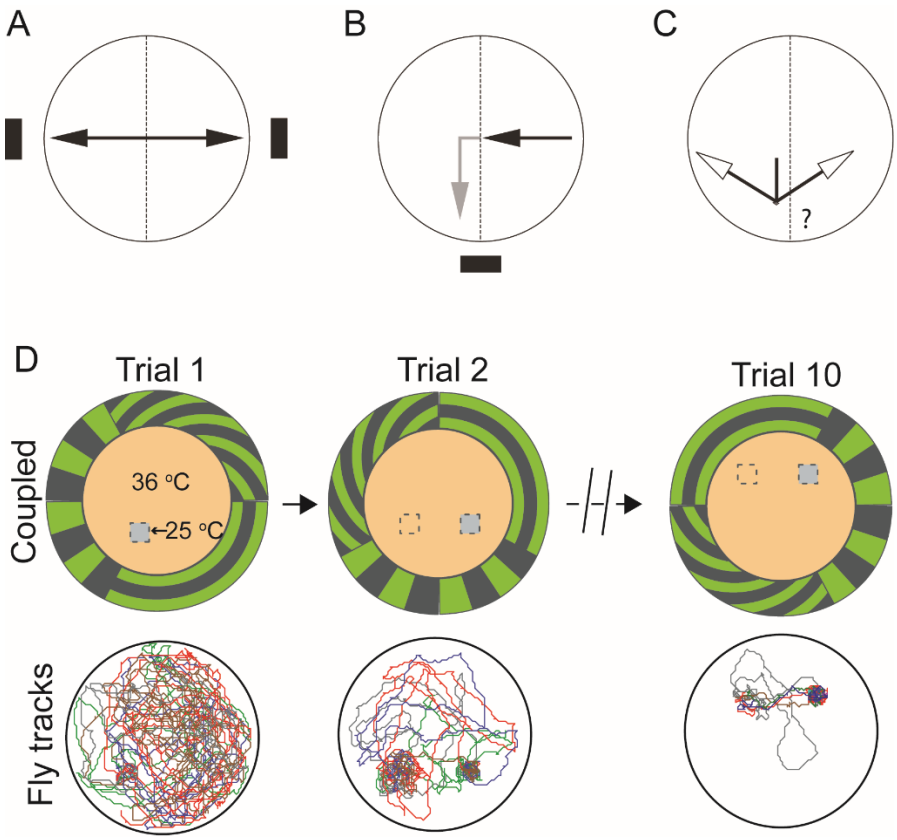

Figure 3: Spatial learning paradigms used to test CX function in fruit flies. (a–c) Detour paradigm: (a) when black bars are displayed on the walls of the arena the fly walks between them. (b) When the fly has successfully oriented toward one of the black bars, the two black bars disappear and one black bar appears perpendicularly. (c) When the fly has successfully oriented toward the black bar, the bar disappears and the fly is left without visual cues and memory of the previously visible bars can be tested. (d) Visual place memory: the fly has to find the cooled tile in a heated arena. Time to find the cool tile in relation to the visual pattern displayed on the arena walls decreases with number of trials. Originals: (a–c) reprinted from [26] by permission from Macmillian Publishers Ltd: Nature, copyright (2008); (d) reprinted from [30] by permission from Macmillian Publishers Ltd: Nature, copyright (2011).

Toward an understanding of the central complex functions

Recent studies of the CX have begun analyzing the neuronal architecture of the CX neuropils [7•,25•,36]. They confirm that the CX comprises a network for complex information processing and integration. Features of CX neural systems include neurons with symmetrical morphologies and connections on both brain sides, converging and diverging pathways and numerous parallel pathways. Network architectures include tiling of neurons, which is the spreading of neighboring arborization without overlap to increase innervation surface and to minimize functional redundancy in the innervated area [37].

A model for horizontal and vertical signal propagation in the CX used the new network information from the fruit fly [38]. In horizontal propagation, the signal passes from an input node to many output nodes. In vertical propagation, the signal is passed from an input node to an output node. Remarkably, the pattern of the CX network indicated a high efficiency in horizontal as well as vertical signal propagation, which was mainly related to the inclusion of hubs in the network — these being highly interconnected clusters of neurons.

Interestingly, Lin *et al.* identify two loops in the CX network, which could be related to a reverberation function [25•]. Reverberation is defined as the persistence of neural activity in a circuit network beyond the stimulus [39], and is associated with consolidating memories during sleep in mammals [40], or to working memory [41]. It remains to be investigated if reverberation in the CX is connected to similar processes. However, the CX has been implicated in both reverberation and sleep in insects: Donlea and colleagues showed that sleep could be induced by activation of neurons connecting to the FB, which were also shown to be crucial for sleep homeostasis [42]. Further, when sleep was induced after massed training (short interval between training trials) long-term memory was formed [43], while massed training alone did not lead to long-term memory formation.

Conclusions and future prospects

The studies reviewed here show great progress toward uncovering the functional roles of the CX (Table 1). New techniques such as recording from free-walking insects and advanced neuronal tracing technologies will help to further map the numerous functions the CX is

associated with.

However, neuroethological studies on the CX are increasingly involving only a few insect species. The current functions localized to the CX are all important for movement and navigation. It is therefore unfortunate that the CX has been barely explored in central-place foraging ants and bees for which navigation is so important and well developed. In recent years, new techniques to investigate navigation, spatial orientation and visually guided behavior in bees and ants have been developed or improved [44–50]. They include tracking in the field with harmonic radar [49] and radio frequency identification tags [48], and 3D reconstruction of an insect's environment [50]. This makes it possible to study navigational and visual orientation in great detail and in large numbers. Further, the honey bee has been established as a powerful model system for learning and memory using free-flying bees as well as harnessed honey bees [51–53]. We propose that ant and bee species could be ideal for further study of the role of the CX in orientation and navigation and would help to complete a comparative analysis of the CX functions.

It is still a mystery how insects with a much smaller brain compared to vertebrates can solve similar complex navigational tasks [54]. Representation of body orientation in reference to a visual landmark in the fruit fly EB [31••] is an exciting finding which provides a vital starting point from which to further uncover the underlying mechanisms.

Analysis of the CX is gaining momentum rapidly, and more knowledge of this region will fill a critical gap in our comprehension of the insect's brain.

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•• of outstanding interest

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Chapter II

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 has 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 Saline) 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 µl 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 hrs., 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.

3. 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 non-fluorescent 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 non-fluorescent 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 non-fluorescent dye during the injection phase).

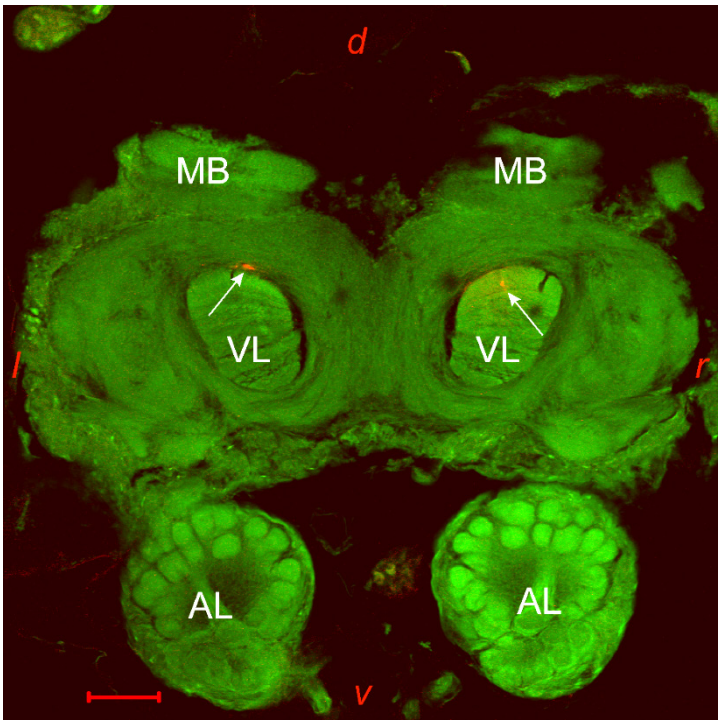

Figure 1: Confocal laser canning Image of the Injection Site. Alexa 546-labelled dextran is injected together with the drug solution (red). To identify the neuropils a counter-staining with DAPI is added (green). In the right hemisphere, the injection site was located in the vertical lobe (VL), shown 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.

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).

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 secs 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 secs, turn the power off and wait another 50 secs (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**).

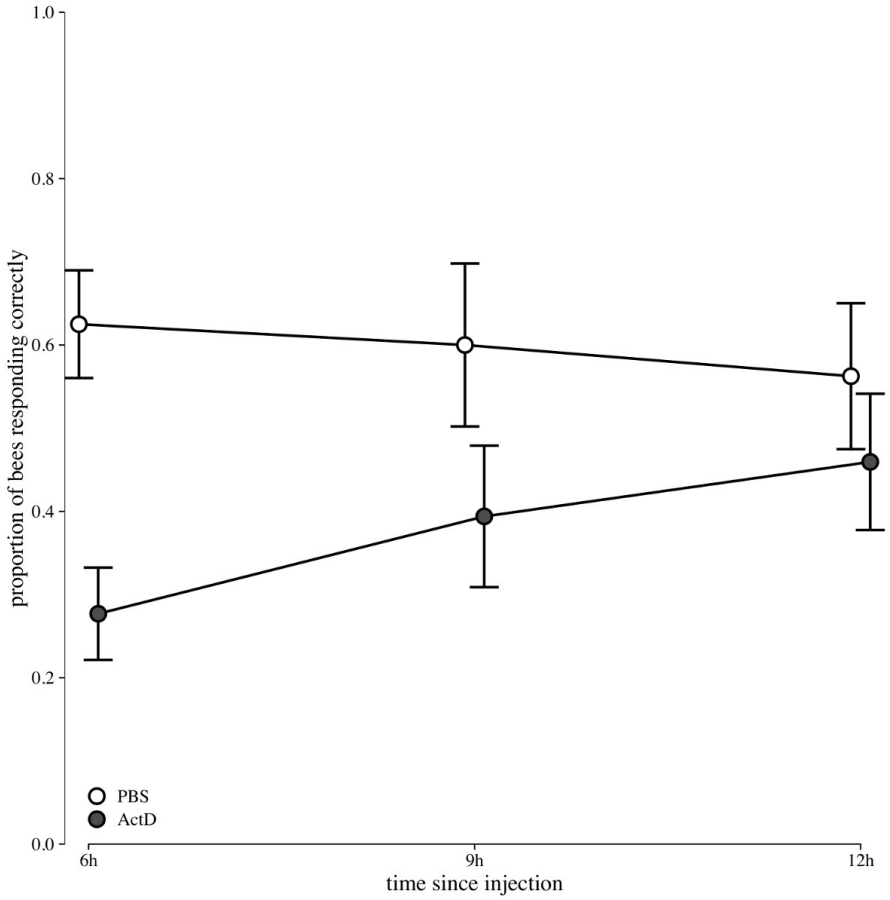

Figure 2: Time-dependent Effect of Actinomycin D (Transcription Blocker) on Long-term Memory, when Injected into the Thorax. At different delays following appetitive olfactory conditioning (6, 9 or 12 h), 1 µL actinomycin D (1.5 mM in PBS) was injected into the thorax. Long-term Memory (LTM) retrieval 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.

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**).

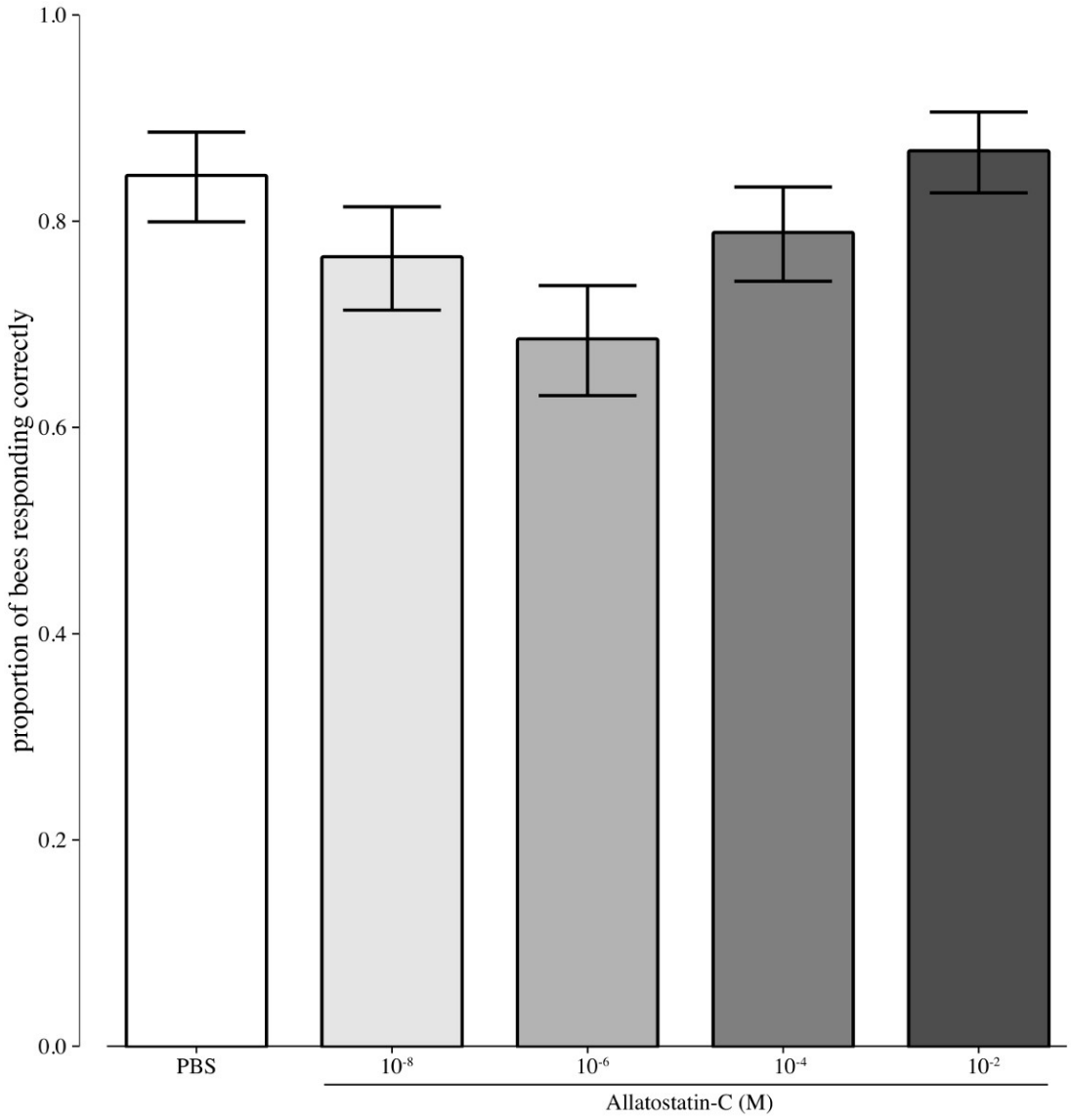

Figure 3: Dose-dependent Inhibition of Learning Performance Following Ocellar Injection of a Neuropeptide. The neuropeptide Allatostatin C was injected into the head hemolymph (200 nl in PBS), through the median ocellus, 1 hr before olfactory conditioning. Independent groups of animals injected with 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.

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 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].

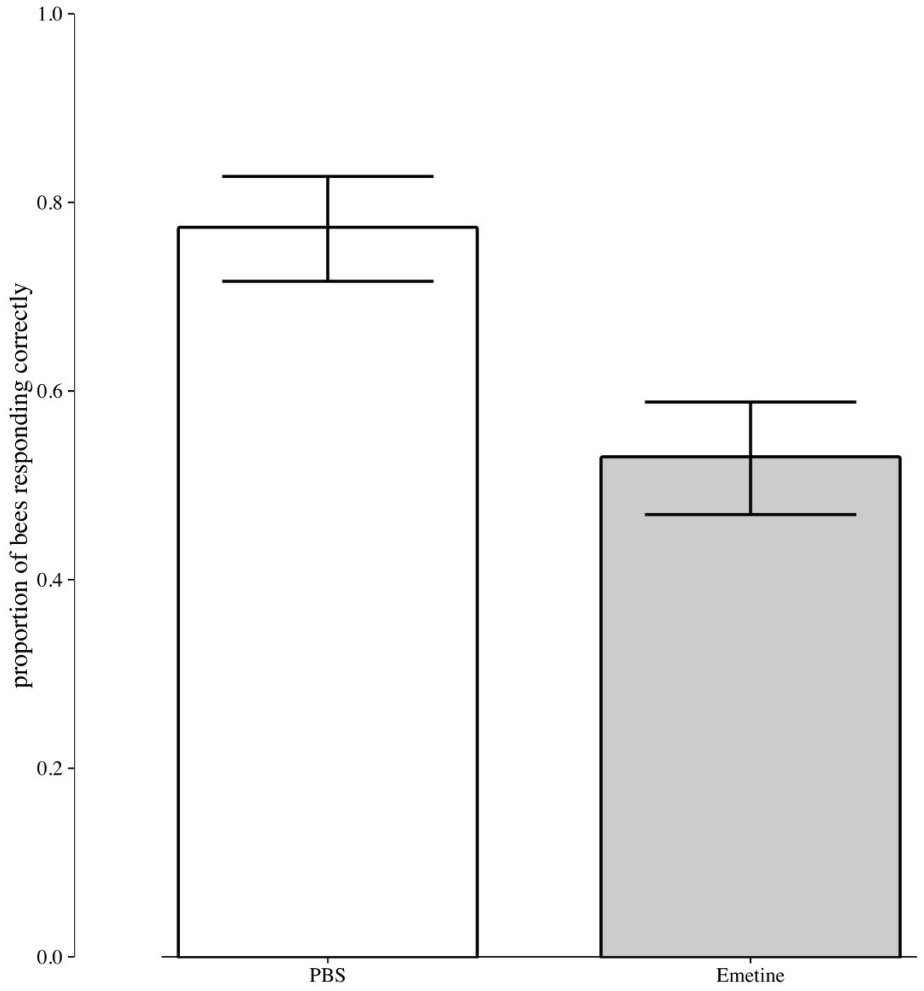

Figure 4: Blockade of 1 Day Memory Following Injection of Emetine (Translation Inhibitor) through the Ocellar Tract. The protein synthesis inhibitor emetine (50 mM in PBS, 200 nl) was injected into the brain, through the ocellar tract, 20 min before olfactory conditioning. Memory was then tested 24 h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

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 hrs. 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.

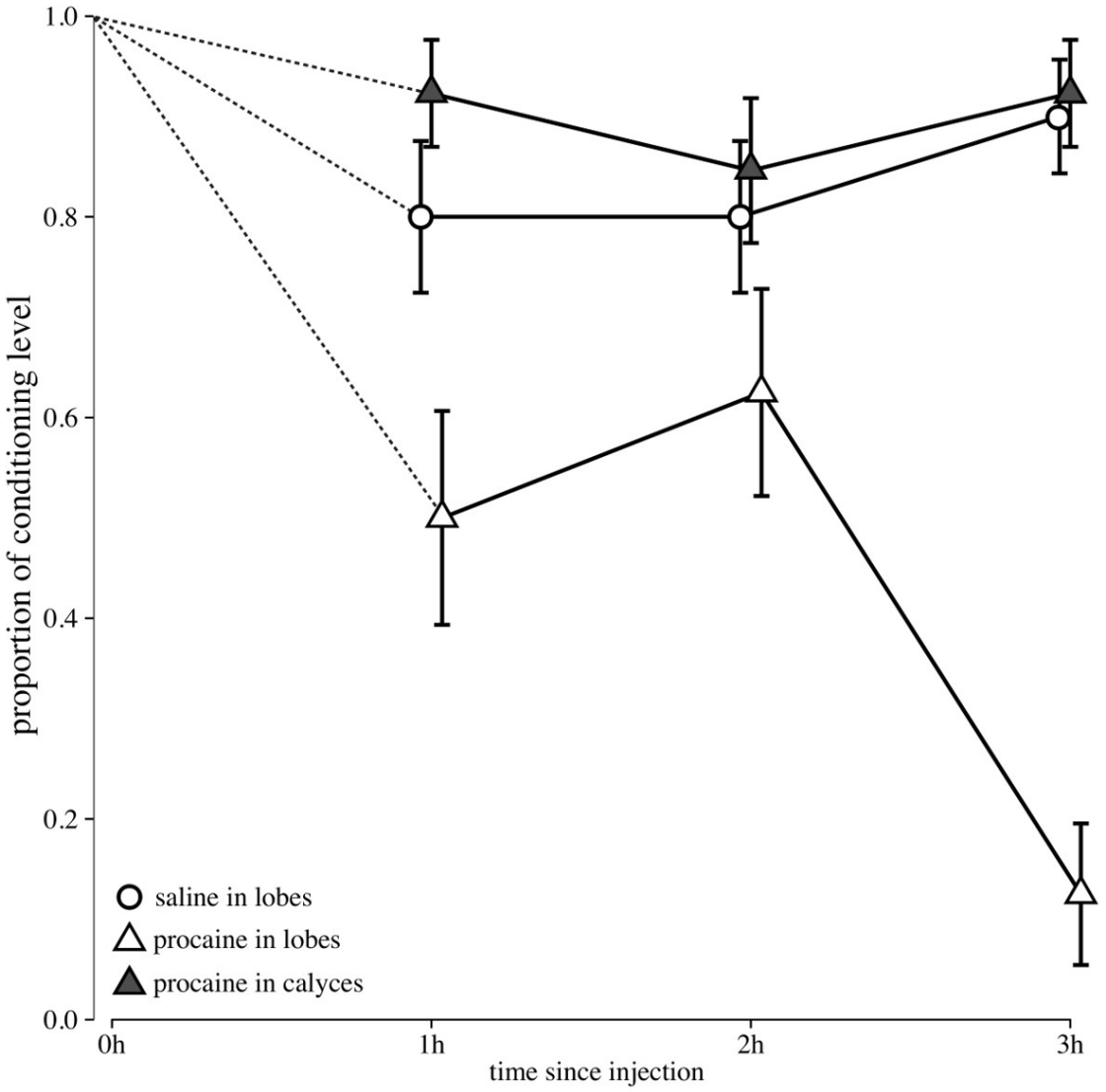

Figure 5: Anatomical and Temporal Specificity of Microinjections. Following appetitive olfactory conditioning, procaine was injected bilaterally into either the mushroom body calyces or vertical lobes. Memory retrieval was assessed 1 hr after injection and was only affected by procaine injections into 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 hrs. ($\chi^2 = 6.65$, $p < 0.01$) and 3 hrs. ($\chi^2 = 27.22$, $p < 0.005$) after injection, and was still location-specific (2 hrs.: $\chi^2 = 8.60$, $p < 0.05$; 3 hrs.: $\chi^2 = 17.15$, $p < 0.0001$), 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.

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.

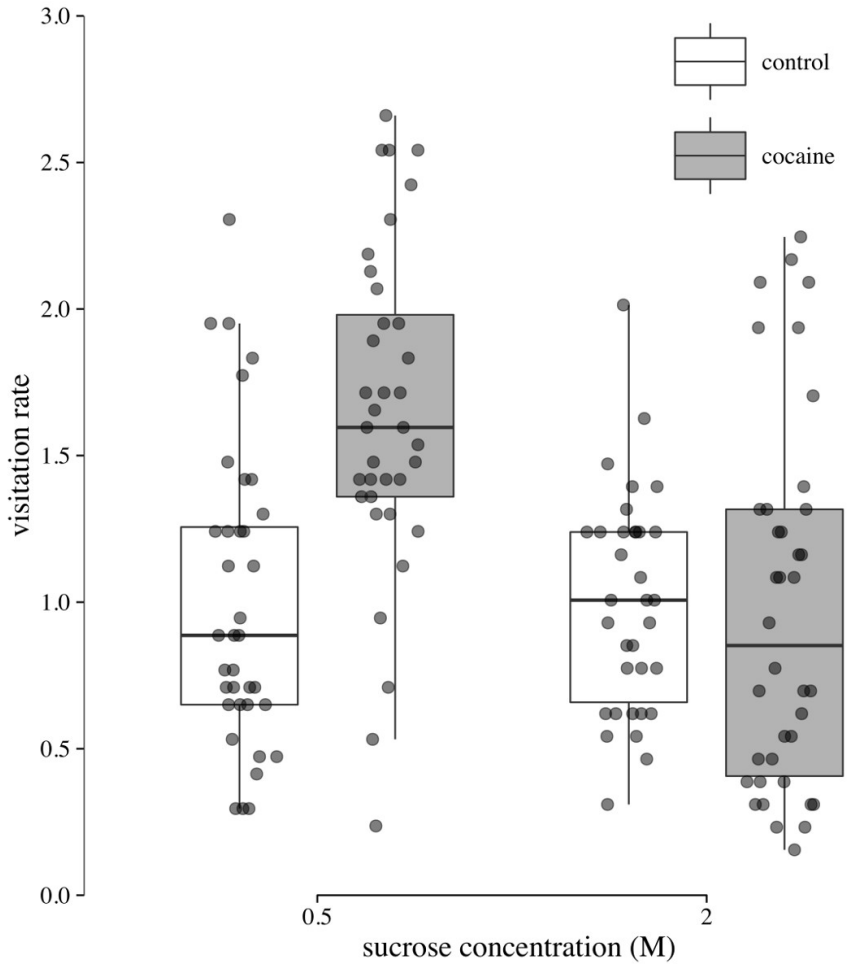

Figure 6: Effects of Cocaine on Free-flying Bees. Visitation rate (number of visits by a given bee/average visits for all bees during test period) was increased following volatilized cocaine treatment at a low quality source (0.5 M: $t_{70} = 5.0710$, $p = 0.00003$), but not at a high quality source (2 M: $t_{70} = -0.2087$, $p = 0.8353$). 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.

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

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.

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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Chapter III

Different Roles for Honey Bee Mushroom Bodies and Central Complex in Visual Learning of Colored Lights in an Aversive Conditioning Assay

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Abstract

The honey bee is an excellent visual learner, but we know little about how and why it performs so well, or how visual information is learned by the bee brain. Here we examined the different roles of two key integrative regions of the brain in visual learning: the mushroom bodies and the central complex. We tested bees' learning performance in a new assay of color learning that used electric shock as punishment. In this assay, a light field was paired with electric shock. The other half of the conditioning chamber was illuminated with light of a different wavelength and not paired with shocks. The unrestrained bee could run away from the light stimulus and thereby associate one wavelength with punishment, and the other with safety. We compared learning performance of bees in which either the central complex or mushroom bodies had been transiently inactivated by microinjection of the reversible anesthetic procaine. Control bees learned to escape the shock-paired light field and to spend more time in the safe light field after a few trials. When ventral lobe neurons of the mushroom bodies were silenced, bees were no longer able to associate one light field with shock. By contrast, silencing of one collar region of the mushroom body calyx did not alter behavior in the learning assay in comparison to control treatment. Bees with silenced central complex neurons did not leave the shock-paired light field in the middle trials of training, even after a few seconds of being shocked. We discussed how mushroom bodies and the central complex both contribute to aversive visual learning with an operant component.

Keywords: visual learning, operant learning, mushroom bodies, central complex, honey bees, procaine

Introduction

Learning of a predictive relationship between a stimulus or an action and a certain outcome is essential for an animal's survival. Honey bees are excellent learners, quickly forming association between stimuli of different sensory modalities and meaningful appetitive and aversive stimuli (Giurfa, 2007). Over the past decades, research has been dedicated to uncover the neural mechanisms and processes underlying learning in bees, and honey bees have been established as a powerful model to investigate learning and memory (Menzel, 1999, 2001, 2012; Giurfa, 2003, 2007). Learning assays are typically performed with free-flying bees as well as harnessed bees (Menzel, 1999, 2001; Giurfa, 2003, 2007; Menzel, 2012). Free-flying bees readily learn olfactory as well as visual stimuli. Appetitive learning and memory dynamics have been studied extensively using odors and colors or shapes paired with sucrose rewards.

Harnessed bees have been used in the proboscis extension response (PER) assay, in which the conditioned stimulus (CS) is paired with a sucrose reward (unconditioned stimulus: US) which leads to an extension of the proboscis (Bitterman et al., 1983; Felsenberg et al., 2011; Giurfa and Sandoz, 2012). Olfactory conditioning is easily studied with this assay since 50–60% of the trained bees already respond to an odor after one CS-US pairing (Bitterman et al., 1983; Felsenberg et al., 2011). It has proven difficult, however, to achieve successful conditioning of color stimuli with rewards or punishment in harnessed honey bees. Differential conditioning with a reward-paired color stimulus and a non-rewarded color stimulus resulted in moderate learning rates when the antennae were ablated (Kuwabara, 1957; Hori et al., 2006, 2007; Niggebrugge et al., 2009), when the bee was able to turn her head easily (Dobrin and Fahrbach, 2012) or when the color stimulus was combined with movement (Balamurali et al., 2015). Colored light, however, has been used successfully as a context for olfactory learning in PER when presented as an occasion-setter (Mota et al., 2011) or in a reinstatement paradigm (Plath et al., 2012). The difficulty in establishing robust visual learning in the PER assay has inhibited functional analyses of roles of different brain regions in visual learning in bees.

Here we used a recently developed aversive visual conditioning assay: the Automated Performance Index System (APIS) (Kirkerud et al., 2017) to analyze the roles of central

processing regions of the bee brain in visual learning. This system was an adapted version of the one used for aversive olfactory conditioning (Kirkerud et al., 2013; Schott et al., 2015; Wehmann et al., 2015). In the APIS assay bees are able to move freely in a conditioning chamber, which is equipped with LEDs to provide visual stimuli of different wavelengths and intensities. Visual stimuli can be paired with low voltage electric shocks. Tracking of the animal's position is fully automated thanks to infrared sensors in the chamber. The chamber can be used to investigate differential learning presenting light in half of the chamber and light with different properties in the other half. One light field is paired with electric shock, so that the bee needs to cross over to the other half of the chamber to avoid being shocked. The assay has been extensively tested with different light stimuli including light of different wavelengths and intensities (Kirkerud et al., 2017). Bees easily learn to associate 465 nm light (blue for humans) and 590 nm light (yellow for humans) but not 525 nm light (green for humans; in the following, we use the human colors instead of the wavelengths for simplicity) with the aversive shock stimulus. In this study, we paired blue light with shocks in one half of the chamber and illuminated the "safe" part of the chamber with green light. Bees can be treated pharmacologically and then their behavior can be assessed in the APIS chamber. Here, we investigated the role the mushroom bodies (MBs) and the central complex (CX) in visual learning.

MBs and the CX are considered the main integrative centers in the insect brain, and both regions could be involved in learning an appropriate behavioral response to a visual stimulus. We investigated the behavioral consequence of silencing of the input region of the MBs, the collar region in the mushroom body calyces (MBC), and the vertical lobes (VL) as the output region of the MBs. The collar region receives direct visual input from the lobula and medulla in honey bees (Ehmer and Gronenberg, 2002; Gronenberg and Lopez-Riquelme, 2004). A recent study has found two types of Kenyon cells in the fruit fly MBC that respond to either light intensity or wavelength (color) information relayed from the optic neuropils (Vogt et al., 2016). Interestingly, in flies both types of neurons are required for learning and memory in an aversive differential conditioning, either testing different intensities or different wavelengths. The output of the collar region in the mushroom bodies terminates in an inner layer of the vertical lobes in honey bees (Strausfeld, 2002). It has been repeatedly shown that the vertical lobes play a crucial role for different forms of

olfactory learning and memory formation in honey bees (Menzel, 1999, 2012) and fruit flies (Heisenberg, 2003; Keene and Waddell, 2007; Busto et al., 2010; Davis, 2011), but visual learning has only been investigated sparsley so far.

The CX comprises a group of unpaired neuropils in the center of the insect brain. One important role of the CX is generation of motor outputs according to processed internal and external stimuli (Pfeiffer and Homberg, 2014; Plath and Barron, 2015). The CX is essential for the initiation and termination of walking, turning and climbing behavior in fruit flies (Strauss and Heisenberg, 1993; Martin et al., 1999; Strauss, 2002; Poeck et al., 2008; Triphan et al., 2010), cockroaches (Guo and Ritzmann, 2013; Guo et al., 2014; Martin et al., 2015) and crickets (Kai and Okada, 2013) and is considered as site for action selection and goal-directed behavior (Libersat and Gal, 2013; Strausfeld and Hirth, 2013; Barron et al., 2015; Fiore et al., 2015; Barron and Klein, 2016). A role of the CX in visual learning of patterns and spatial features has been shown in various behavioral assays using fruit flies (Liu et al., 2006; Neuser et al., 2008; Wang et al., 2008; Pan et al., 2009; Hou et al., 2011; Ofstad et al., 2011; Kuntz et al., 2012, 2017).

In this study, we used the transient and local anesthetic procaine to selectively silence neural activity in these three brain regions. Procaine is a reversible blocker of voltage-gated Na+-channels and other voltage-gated channels to a lesser degree and has been established as a means to study olfactory learning and memory in honey bees (Muller et al., 2003; Devaud et al., 2007, 2015). Procaine has also been utilized to show that silencing the central body reduces spontaneous walking and optomotor responses (Kathman et al., 2014; Kaiser and Libersat, 2015). Our expectation was that mushroom bodies are needed for this form of visual conditioning with a strong operant component. This allowed the bee to learn from consequences of her behavior and not only from a stimulus-stimulus pairing. Interrupting processing in the collar region and blocking the further processing in the output regions of the mushroom bodies could lead to an impairment in performance in aversive visual learning which can be measured in the APIS assay. We hypothesized further that learning of the stimulus-shock pairing would remain intact when the central complex was anesthetized but the reaction of running away from the stimulus would be impaired. We discuss how our results will contribute to uncovering mechanisms underlying visual learning in insects.

Materials and Methods

Animals and Surgical Procedure

For all experiments, honey bees were collected from two established queen-right colonies at Macquarie University in Sydney, Australia. Foragers were collected at the hive entrance while leaving for a foraging bout. Bees were immobilized on ice and harnessed in PER tubes (Bitterman et al., 1983; Felsenberg et al., 2011). To prepare the animals for injections, the bee's neck was filled with soft dental wax to prevent movement of the head. A stripe of wax was positioned loosely over the antennae to prevent their movement during the operation.

For MBC injections, we entered through the ocellar tract. The lens of the median ocellus was carefully pushed outwards with the tip of a micro-scalpel and a small incision was made into the neurilemma sheath covering the brain to ease entering of the micropipette.

To access the brain for intracerebral injections (VL and CX), a window was cut into the head capsule with three cuts: One above the antennal stems (dorsal), one below the median ocellus (ventral), and one at the border of the right eye (Devaud et al., 2007). The created flap was opened and held in place with soft dental wax. The glands and trachea above the brain were carefully moved aside and a small incision was made into the neurilemma above the target structure to enable a smooth entry of the micropipette during injections. After injections, the flap was carefully released to close the window and sealed with a drop of eicosane (Sigma-Aldrich Australia) melted at ~35°C). For detailed demonstration of the procedure please refer to Søvik et al. (2016).

Injections

In the following study four different treatment groups were compared: procaine-injected animals (procaine/proc), saline-injected animals (vehicle/veh), animals that underwent the operation and injection procedure without having any solution injected into the brain (sham), and non-treated animals (NT), which were directly transferred to the chamber after catching.

To locally and temporarily inhibit neural activity, the drug procaine was used. In the honey bee procaine reduces Na^+- and K^+-currents and spiking activity in mushroom body neurons (Devaud et al., 2007). Procaine HCl (Sigma-Aldrich Australia) was dissolved in

physiological saline (7.54 g/L NaCl, 0.448 g/L KCl, 0.872 g/L $MgCl_2 \times 6\ H_20$, 0.735 g/L $CaCl_2 \times 2H_20$, 54.72 g/L Sucrose, 4.95 g/L D-glucose, and 2.38 g/L HEPES, pH = 6.7, 500 mOsm, Sigma-Aldrich Australia, see Burger et al., 2013) as a stock solution of 40% (w/v). On the day of the experiment, the solution was diluted with additional saline to create a 20% (w/v) procaine solution. Physiological saline was also used as a control solution. To identify the injection site afterwards, both solutions contained 0.5 mg/ml dextran Alexa fluor 546 or dextran Alexa fluor 568 (10.000 MW, Molecular probes by Life technologies, Carlsbad, CA, USA). Microinjections were performed with a microinjector (Eppendorf, Hamburg, Germany) and an electronic micromanipulator (Luigs & Neumann Feinmechanik und Elektrotechnik, Ratingen, Germany). Micropipettes were pulled from glass capillaries (World Precisions Instruments, Sarasota, FL, USA) using an electrode puller (Scientific & Research Instruments, Karnataka, India). The tips were broken to an outer diameter of 10–15 µm. The injection volume was adjusted and rechecked both before and after every animal by measuring a droplet injected into mineral oil.

Injections into the MBC occurred via the ocellar tract of the median ocellus. The micropipette was brought to the opening of the removed lens and then finely adjusted until the micropipette was just above the incision made earlier. The micropipette wasthen inserted to a maximum injection depth of ~215 µm and a volume of ~2 nL was injected. The micropipette was removed and the bee was quickly transferred into the conditioning chamber (**Figure 1A**).

To target the center of the VL, ~1 nL of solution was injected into each lobe at a depth of ~60 µm and at an angle of 68– 70° relative to the brain surface. A stereomicroscope fluorescent adapter was then used to visualize the injection site (Green-Light and Filter Set; NIGHTSEA, Lexington, MA, USA). Successful injections were identified by spreading of the fluorescent dye throughout the VL. To target the CX, ~0.5 nL of solution was injected at a depth of ~330 µm and at an angle of 68–75° relative to the brain surface; entering at the midline between the VLs. Successful injections were identified using laser scanning confocal microscopy (see below).

Behavioral Assay

Honey bees were conditioned in the APIS chamber, designed and manufactured at the

University of Konstanz, Germany with an aversive visual conditioning paradigm established in (Kirkerud et al., 2017). Tracking of the bee and delivery of stimuli in APIS are fully automated which eliminates human error or bias. Due to the design of the chamber, bees can only move in almost straight lines, either toward or away from a stimulus, and any turn made by the animal is tracked as a complete reversal by the sensors. Shock and light stimuli were controlled with a script loaded into the system software. The program utilizes sensor feedback to determine the bee's location and initiates stimuli at specified time points. The operation of the chamber and the assay used are similar to methods used earlier in flies (Zars et al., 2000; Claridge-Chang et al., 2009).

Following injection, the bee was quickly placed into the chamber and allowed to acclimate for 15 min while freely moving around in the dark. The conditioning protocol consisted of one unreinforced preference test followed by nine reinforced training trials (**Figure 1B**), and ending with four unreinforced test trials (**Figure 1C**). In each trial, a blue light field (λ^B = 465 nm, Luminous intensity: 105 mcd) was switched on in the half of the chamber where the bee was situated and a green light field (λ^G = 525 nm, Luminous intensity: 119 mcd) illuminated the opposite half. All trials lasted 14 s and were presented at regular intervals of 44 s (from onset to onset). For the training trials, electric shock pulses (10 V, 4 Hz, 100 ms) were activated 3 s after light onset. These shock pulses were delivered to the feet of the bee through the metal grid as long as movement sensors on the blue side were triggered. This meant that the bee could either escape the shocks by crossing from the shock-predicting blue side to the safe green side or potentially avoid them completely by escaping within 3 s and remain on the green side until the end of the trial. Since bees were always located on the blue half at trial onset (Figure S3), there was an inherent bias in the calculated preference toward this side. Once the behavioral assay was complete, the bee was quickly placed onto ice and anesthetized for dissection.

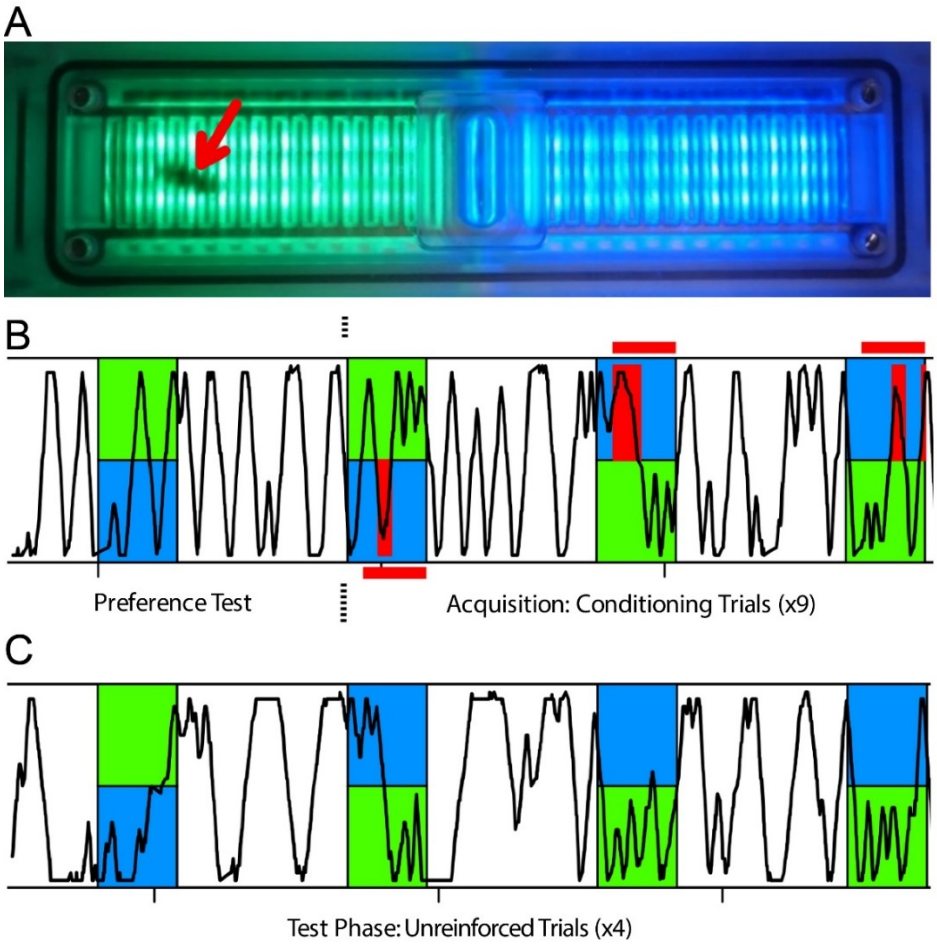


Figure 1: APIS learning assay used in this study. (A) The APIS chamber can be illuminated with two different light fields of varying wavelengths and intensities; in this case light appearing green to humans and light appearing blue to humans. The chamber is equipped with an electrifiable grid to deliver 10 V shocks to the bee's feet and with infrared sensors to automatically track the bee's movement. A bee in the chamber (red arrow) could only move in a straight line, either toward or away from a stimulus, and turns were scored as a reversal of direction as detected by the infrared sensors. **(B, C)** Typical running trace of a bee in the chamber. Blue and green indicate illumination wavelength and red indicates when shocks were available (red horizontal bars) or delivered (red vertical bars) to the bee. Blue light was always illuminating the half of the chamber in which the bee was located at light-onset. **(B)** After an acclimatization period of 15 min post-injection, the bee was exposed to 14 s of both green and blue illumination as a preference test. The bee was then subjected to nine conditioning trials in which, after 3 s of illumination, the bee experienced shocks on the blue side for another 11 s, but not on the green side. **(C)** Subsequently, the bee was tested four times with 14 s of illumination without shocks to determine the post-training response to blue and green light fields.

Histology and Imaging

Once anesthetized, the bee's head capsule was opened and the brain was removed in 0.1 M PBS (Sigma-Aldrich Australia) using forceps and a fresh breaker-blade piece. Whole brains were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hattfield, PA, USA) in 0.1 M PBS overnight in a chilled room (16°C). Brains were then washed in 0.1 M PBS (3 × 10 min) at room temperature (22°C) and stored in the fridge (4°C). Samples were either washed daily with fresh 0.1 M PBS or they were processed immediately for histology.

Whole brains were incubated in 250 μL DAPI (2 μg/ml, Sigma-Aldrich Australia) in 0.1 M PBS and 0.2% Triton-X 100 (Sigma-Aldrich Australia) overnight. Brains were then washed in 0.1 M PBS (3 × 10 min) followed by an ethanol dehydration series (i.e., 50, 70, 90, 98, 100, 100% 10–30 min each step) and cleared in methylsalicylate (Sigma-Aldrich Australia).

Brains were then mounted on previously prepared slides with a cavity well. Wells were created with glass cover slips (Marienfeld-Superior, Lauda-Koeningshofen, Germany) and custom-made aluminum slides (manufactured at the University of Konstanz, Germany) secured together using DPX mounting medium (Sigma-Aldrich Australia). Cleared brains were mounted in the well using DPX mounting medium and sealed with another cover slip.

Samples were imaged (4.77 μm slice) using an Olympus Fluoview inverted confocal microscope (FV-1000 IX81) located at Macquarie University in Sydney, Australia. DAPI staining and auto-fluorescence of the tissue was used to identify the neuropils and determine the location of the injection site marked by the Alexa dye (**Figure 2)**.

All injections in the CX group were located in the central body (**Figures 2E, F**). One injection in the vehicle group **(Figure 2E**, red dot with black border), and one injection in the procaine group (**Figure 2F**, red dot with black border), was located at the border of the lower division of the central body and some dye was also found in the noduli; indicating that those areas were possibly affected as well. Since the performance in APIS was very similar for both injection sites, results were presented for all combined CX injections.

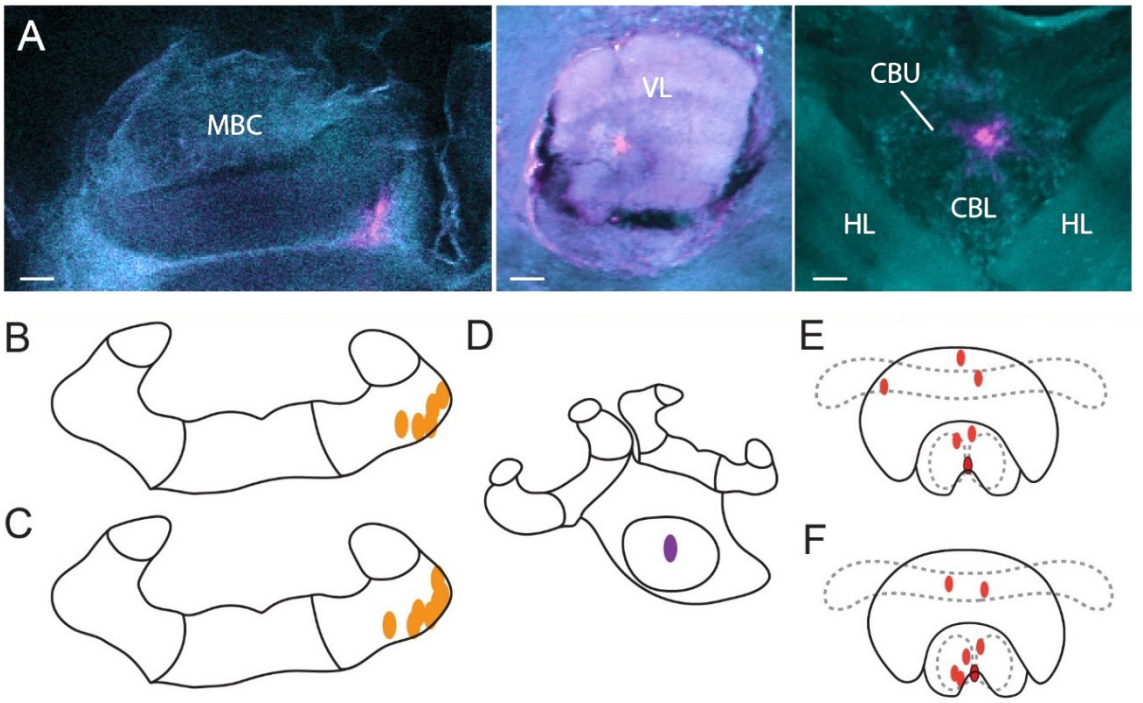


Figure 2: Injection sites. (A) Alexa dye injections are shown in magenta (false color) in the MBC (left), VL (middle) and the CX (right). A DAPI-counterstain and auto-fluorescence of the brain tissue (false colored in cyan) allowed us to identify brain neuropils. Orientation of all three scans was aligned with rostral (neuraxis) facing upwards. Injections of vehicle **(B)** and of procaine solution **(C)** into the MBC as identified by the CLSM scans. Injections into the VL **(D)** were identified visually with fluorescent light and were all located in the center. Injections of vehicle **(E)** and of procaine solution **(F)** into the central body (red dots) and injections located at the border of the lower division of the central body with spread into the noduli (red dots with black border). MBC, mushroom body calyces; VL, ventral lobes; HL, horizontal lobes; CBU, upper division of the central body; CBL lower division of the central body; Scale bar = 30 µm.

Data Analysis

The data was analyzed and graphed using R 3.3.2 (R Core Team, Vienna, Austria) and RStudio 1.0.136 (RStudio Inc., Boston, MA, USA) with a custom written script. As a measurement for learning, the Performance Index (PI) was calculated: difference between time spent on the green side of the chamber and time spent on the blue (shocked) side of the chamber divided by the total trial time:

$$\boldsymbol{PI = \frac{t(green) - t(blue)}{t(green) + t(blue)}}$$

This resulted in a variable ranging from –1 to 1, where positive values indicate that the bee spent more time on the safe side than on the shocked side, negative values the opposite. A bee that had learnt to associate the blue light with shock would run away from the blue side shortly after light-onset and avoid returning to the blue side. As a consequence, the relative time spent on the green side increased leading to higher PI-values (**Figure 3A**). A bee that had not learnt, spent equal amounts of time on each side or more time on the blue side. A bee that had not learnt, would be expected to have lower PI-values (**Figure 3B**).

To investigate the movement pattern of the bee in more detail we further analyzed how many reversals of direction were performed in the chamber. We analyzed the total number of reversals per trial and the Reversing Difference: number of reversals performed on the blue side subtracted from the number of reversals performed on the green side of the chamber divided by the total number of reversals:

$$\boldsymbol{Reversing\ Difference = \frac{reversals(green) - reversals(blue)}{reversals(green) + reversals(blue)}}$$

A bee that had learnt to avoid returning to the blue side typically ran back and forth on the green side (**Figure 3A**). If a bee had not learnt to avoid the blue side, we found two patterns: either she was running back and forth in the whole chamber (**Figure 3C**) or she was running back and forth on the blue side (**Figure 3D**). In the former case, the number of reversals performed would be equal for both sides (Reversing Difference close to zero). In the latter case, the number of reversals performed was higher on the blue side than on the green side (negative Reversing Difference).

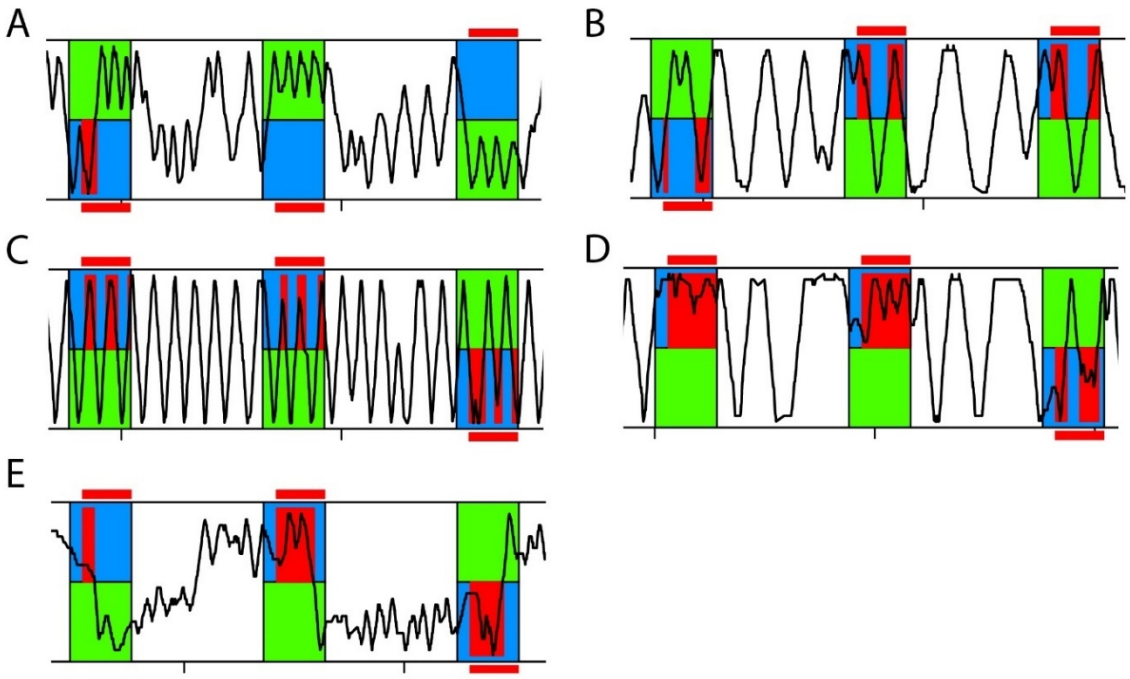


Figure 3: Representative running traces of individual bees in APIS. Three training trials are shown. The bee was exposed to 14 s of blue and green light fields. After a 3 s delay, the bee experienced shock when located on the blue side (red). **(A)** Typical running trace of a bee spending more time on the green side than on the blue side, thus achieving high Performance Indices (PIs). **(B)** Typical running trace of a bee spending more time on the blue side than on the green side, thus achieving low PIs. **(C)** Typical running trace of a bee with an equal number of reversals on the green and blue side, thus achieving a Reversing Difference close to zero. **(D)** Representative running trace of a bee reversing more often on the blue side than on the green side, thus achieving a negative Reversing Difference. **(E)** Typical running trace of a slowly responding bee taking a long time to cross over to the green side at the beginning of each trial and after light-onset, thus achieving a high Crossing Latency.

As another parameter for learning performance as well as to evaluate the reaction to the shock-paired light, we analyzed how fast an animal would cross over to the green side after light-onset (Crossing Latency). If the bee managed to cross over under 3 s, she could completely avoid being shocked due to the delay of the shock-onset after light-onset, assuming she would not then return to the blue side (**Figure 3A**, second and third trial shown). If Crossing Latency was higher than 3 s she would experience shocks on the blue side (**Figure 3E**).

For statistical analysis of PI, Speed, Reversing Difference, Crossing Latency and Position in Chamber (at light-onset), the calculated data were fitted to linear mixed models with trial and treatment (procaine, vehicle, sham, NT) as fixed effects and bee identity as a random effect to correct for repeated measurements in the training, as well as the test phase (lme function in the R nlme package, Pinheiro et al., 2016). For statistical analysis of Reverses per Trial the calculated data were fitted to generalized linear mixed models (Poisson distribution) with trial and treatment (procaine, vehicle, sham, NT) as fixed effects and bee identity as a random effect to correct for repeated measurements in the training, as well as the test phase (glmer function in the R lme4 package, Bates et al., 2015). Statistical differences were determined *post-hoc* with the Tukey's range test using the R multcomp package (Hothorn et al., 2008). Since bees with lower speeds could not perform well in this assay in which performance is based on movement, animals with lower speeds than 2.1 cm/s were excluded from the analysis (Figure S1).

Results

Control Animals Learned to Remain on the Green Side

In this study, we investigated color learning and how the animal's behavior in response to a learned stimulus changed. We first studied the behavior of the non-treated (NT) and sham-treated control groups. NT and sham-treated bees both developed a preference for the safe green side after few trials of color-shock conditioning (**Figure 4**). For both control groups PIs increased over the course of training (**Figure 4A**). PIs corresponded to around 39% of the first trial spent on the green side which increased to 61% (NT) and 72% (sham) in last trial. Increase of PIs from the first to the last trial was significant for both, NT animals (paired *t*-test, df = 25, $t = -2.682$, $p = 0.013$) and for sham-treated animals (paired *t*-test,

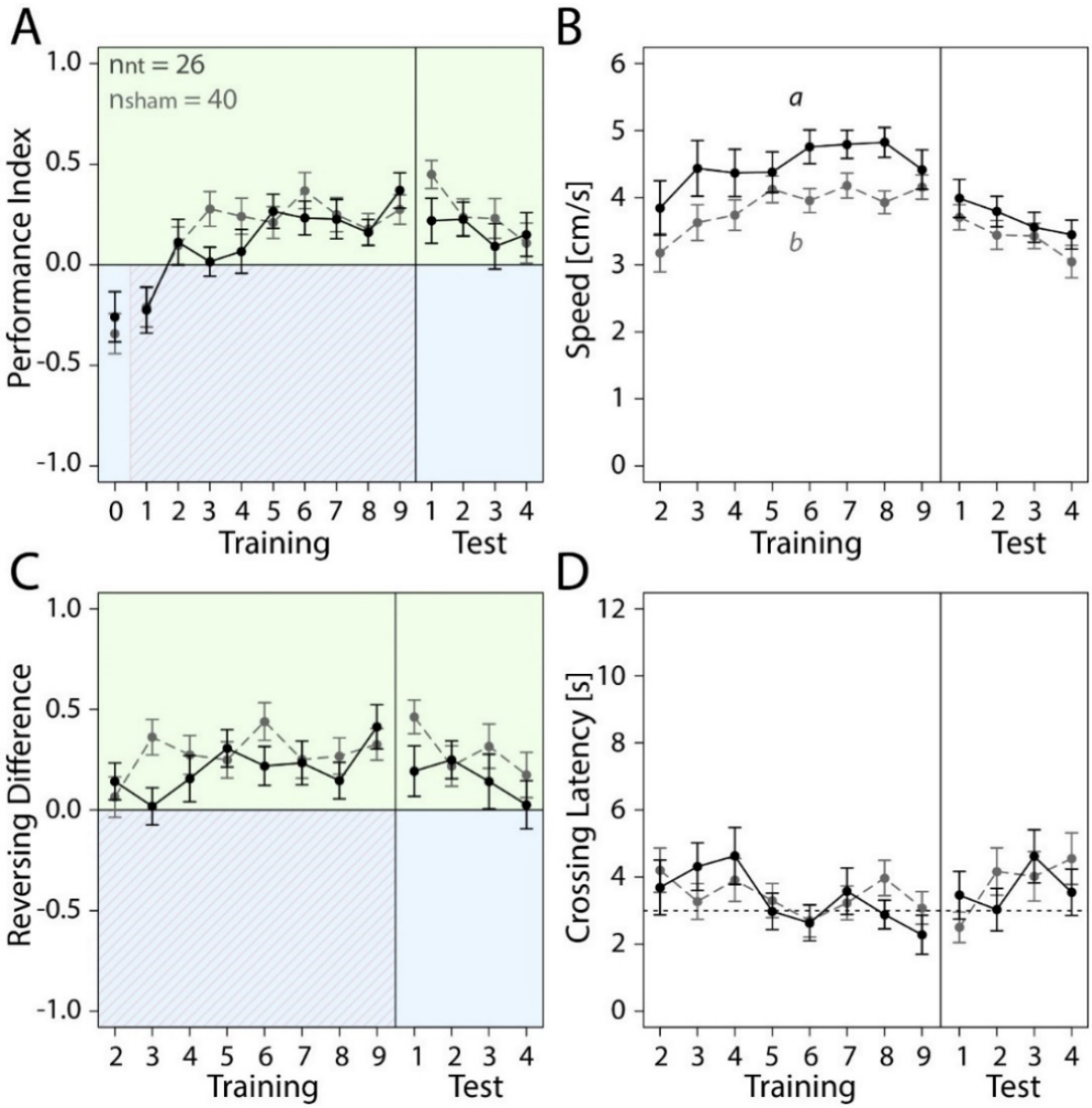
df = 39, $t = -5.4861$, $p < 0.001$). In the test phase both groups continued to spend more time on the green side (**Figure 4A**).

We further explored how running and reversing in the chamber changed in response to the first light-shock pairing. Sham-treated animals were slower than NT-animals in the training but not in the test phase (**Figure 4B**). After five conditioning trials both groups performed on average three to five more reversals on the green side (**Figure 4C**). The total number of reversals performed in the chamber remained constant in that period (Figure S2A). Both groups crossed over to the green side after 2 to 4 s into the trial (**Figure 4D**). In the last training trial 20 out of 26 NT-animals and 21 out of 40 sham-treated animals crossed over under 3 s (data not shown). Taken together, after learning to associate blue light with shock the control bees ran away from the blue side before or shortly after shock-onset and thereafter ran back and forth on the green side.

Procaine Injections into the MBC Did Not Impair Performance in the Visual Learning Paradigm

We then examined how silencing of neurons of a collar region in the MBC with procaine injections changed the bees' behavior in the APIS assay (**Figure 5**). Procaine- and vehicle-injected animals were compared to sham-treated animals which were operated on in the same way. Overall, we observed no impairment of the bees' performance in the learning assay due to the injections. All bees were able to avoid the blue side after a few trials and moved normally. Curiously, we found a difference between PIs for all three groups in the preference test (**Figure 5A**). However, this did not seem to have an effect on the training where all groups performed similarly. Neither speed (**Figure 5B**), Reversing Differences (**Figure 5C**), Reversals per Trial (Figure S2B) or Crossing Latencies (**Figure 5D**) after the second trial were affected by injections (Table S1).

Figure 4: With training, bees of sham and NT control groups learned to spend more time on the safe green side than the shocked blue side. Means ± SEM are plotted for all variables. Non-treated animals (NT) are shown in black, sham-treated animals (sham) in gray. No effect of the different injection methods used for the different regions on any of the four variables shown was found (ANOVA, $p > 0.05$). Sham-treated animals were therefore pooled into one group to compare with NT animals. Significant treatment effects determined with an LMM ($p < 0.05$, Table S1) are indicated with letters a and b. Bees were subjected to one preference test (0) nine training trials and four test trials. Control animals spent more time on the green side and avoided the shock-paired blue side (shocked period indicated by red diagonal lines) after a few trials. **(A)** No effect of treatment on Performance Index was found in training or in the test phase (Table S1). **(B)** An LMM indicated a significant effect of treatment on speed (Table S1). After one conditioning trial, speed was lower in sham-treated animals than in NT-animals in the training (post-hoc Tukey HSD, $z = -2.188$, $p = 0.03$), but no significant effect of treatment on speed was found in the test phase (Table S1) **(C)** Number of reversals on the green side was higher after one conditioning trial. No significant effect of treatment was found in training or in the test phase (Table S1). **(D)** Crossing Latency approached the 3-s threshold (horizontal dashed line) over the course of training, which corresponds to the delay between light-onset and shock-onset. **(A)** No significant effect of treatment on Crossing Latency was found for training or in the test phase (Table S1).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Training
Test
B
a
b
Speed [cm/s]
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5
4
3
2
1
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2 3 4 5 6 7 8 9 1 2 3 4
Training
Test
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Reversing Difference
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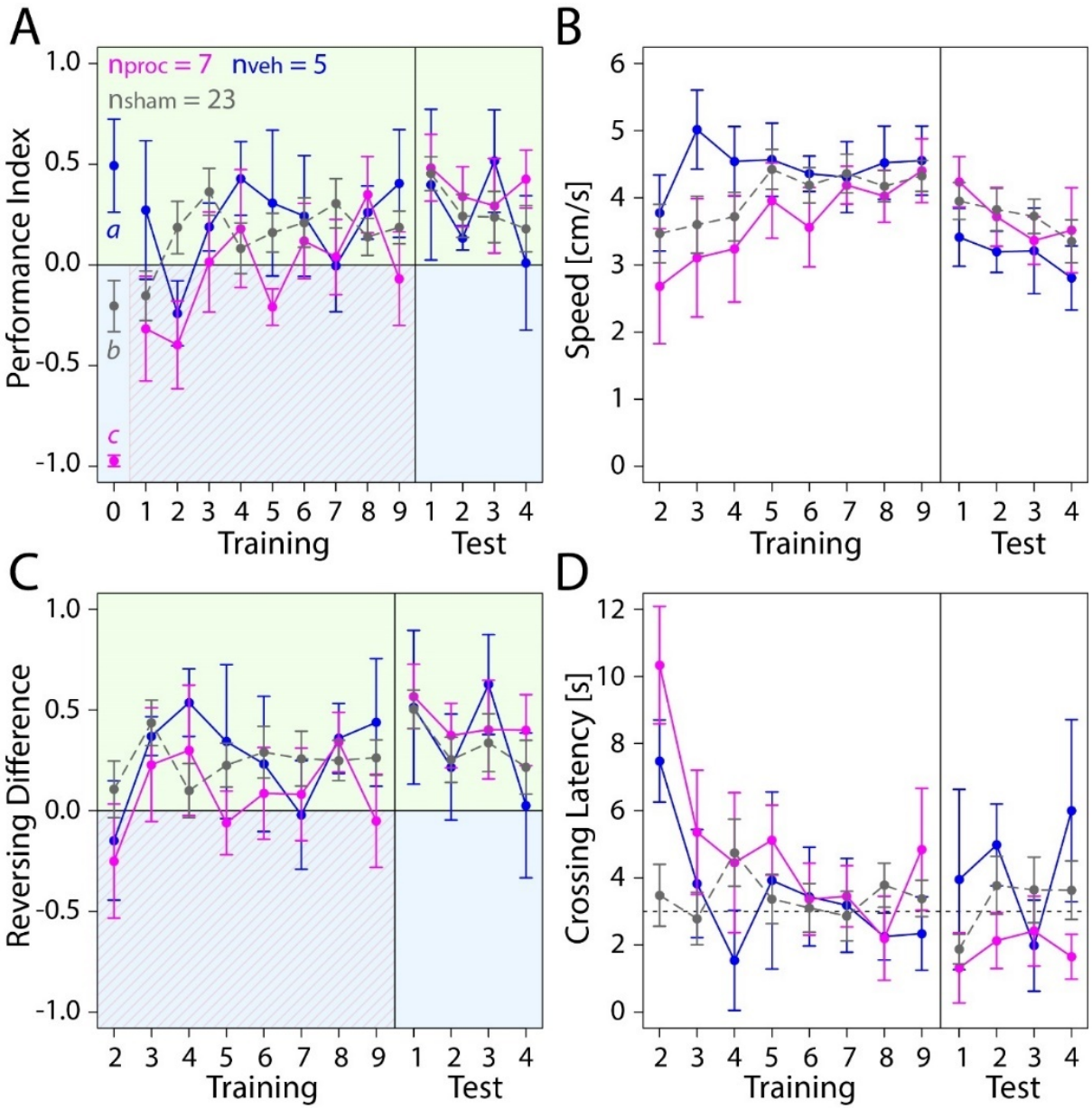
2 3 4 5 6 7 8 9 1 2 3 4
Training
Test
D
Crossing Latency [s]
12
10
8
6
4
2
0
2 3 4 5 6 7 8 9 1 2 3 4
Training
Test

Figure 5: Comparison of behavior in the APIS assay for bees injected with the vehicle (blue) or procaine solution (magenta) into the MBC, or sham-treated bees (gray). All groups learned to spend more time on the green side. Means ± SEM are plotted for all variables. Significant treatment effects determined with an LMM ($p < 0.05$, Table S1) are indicated with letters a, b, and c. Bees were subjected to one preference test (0) nine training trials and four test trials. **(A)** An LMM indicated an effect of treatment on Performance Index (PI) in the preference test (Table S1). Treatment comparison with a Tukey HSD post-hoc test revealed differences in PIs of vehicle and sham groups ($z = 2.631$, $p = 0.02$), PIs of procaine and sham groups ($z = -3.310$, $p = 0.003$) and PIs of procaine and vehicle groups ($z = -4.657$, $p < 0.001$). An LMM indicated a significant difference between PIs of procaine and sham groups in training (Table S1), but a Tukey post-hoc test, which corrects for multiple testing indicated no difference between PIs of these groups ($z = 2.080$, $p = 0.09$). No effect of treatment on PIs was found for the test phase (LMM, Table S1). All bees spent more time on the green side and avoided the shock-paired blue side (shocks indicated by diagonal lines) after a few trials. **(B)** Speed did not differ between experimental groups (LMM, Table S1). **(C)** Number of reversals on the green side was higher after one conditioning trial. No effect of treatment on Reversing Differences was found in training or in the test phase (Table S1). **(D)** Crossing Latency approached the 3-s threshold (horizontal dashed line) over the course of training, which corresponds to the delay between light-onset and shock-onset. No significant effect of treatment on Crossing Latency was found for training or in the test phase (Table S1).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b
c
Training
Test
B
Speed [cm/s]
Training
Test
C
Reversing Difference
Training
Test
D
Crossing Latency [s]
Training
Test

Procaine and Vehicle Injections into the VLs Impaired Performance in the Visual Learning Assay

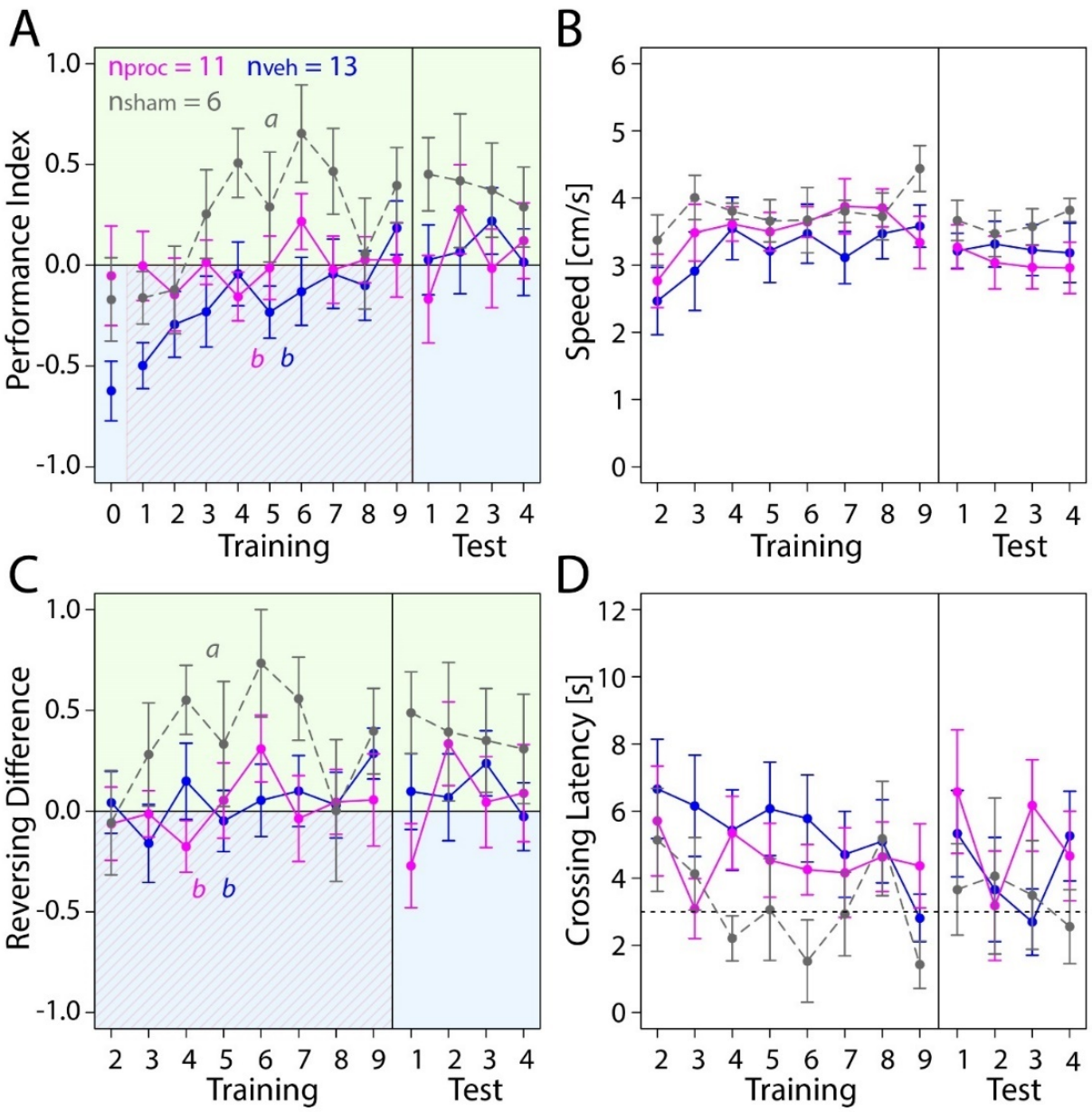
Next, we investigated which role the VL as part of the MB output played in visual learning (**Figure 6**). Surprisingly, injections into the VL with either, procaine or vehicle solution resulted in impairment of color learning. Both groups achieved mean PI-values around zero, indicating that they spent equal amount of time on both sides (**Figure 6A**). This was not the case in sham-treated animals, which preferred the safe green side after two trials. Thus, injection of the vehicle (with or without procaine), but not the insertion of the micropipette itself impaired learning of the light-shock pairing. Lower PIs in vehicle and procaine groups were not the result of impaired locomotion, since speed (**Figure 6B**) was not affected by treatment (Table S1). Furthermore, vehicle and procaine groups with injections into the VLs showed equal number of turns on the green side as on the blue side (**Figure 6C**), while Reversals per Trial (Figure S2C) remained unaffected. This indicated that the bees were either running back and forth from one side of the chamber to the other or were spending equal amounts of time running back and forth on each side. However, Crossing Latencies (**Figure 6D**) were found not to be significantly different (Table S1). Thus, vehicle- and procaine-treated bees ran away from the shocks after a similar delay as sham-treated bees in most trials.

Procaine Injections into the CX Changed Behavioral Responses in the Visual Learning Paradigm

Lastly, we explored how an animal's performance in the APIS-chamber was changed by silencing neural activity in the CX with procaine (**Figure 7**). Procaine-treated animals did not show a preference for the green side in the middle trials of the training. Rather, they remained on the shock-paired blue side longer than vehicle- and sham-treated animals. PIs were lower in procaine-treated animals in the training (**Figure 7A**). In fact, these bees spent 60–70% of the trial duration on the blue side in the middle of the training. Hence, the animals either did not leave the blue side or returned to the blue side more often. This behavior was not due to an impairment in locomotion since we found no differences in speed (**Figure 7B**) in the training (Table S1). However, toward the end of the training and in the test phase procaine-treated bees preferred the green side and PIs were similar to those found for vehicle- or sham-treated bees. We further explored if the ability to reverse in the

chamber might have been affected. Procaine-treated bees did not reverse in the chamber less often than vehicle-or sham-treated bees (Figure S2D) (Table S1). But they performed on average three to four more reversals on the blue side than on the green side in the middle trials of training (**Figure 7C**). In contrast, vehicle- and sham-treated bees performed on average three to five more reversals on the green side in the same trials. Additionally, Crossing Latency was found to be on average 6 to 8 s in the middle trials for procaine-treated bees (**Figure 7D**). This was about twice as long as Crossing Latencies found for vehicle-treated and sham-treated bees and around 40–60% of the trial duration. Thus, procaine-treated bees did not leave the blue side even when the shocks were delivered for more than 3 s. Differences in Crossing Latencies were not due to different starting positions at light-onset in the training (Figure S3D) (Table S1).

Figure 6: Comparison of behavior in APIS for bees injected with vehicle (blue) or procaine solution (magenta) into the VLs, or sham-treated bees (gray). Learning to differentiate the shock-paired blue side and the safe green side was impaired in procaine and vehicle groups. Means ± SEM are plotted for all variables. Significant treatment effects determined with an LMM ($p < 0.05$) are indicated with letters a and b. Bees were subjected to one preference test (0) nine training trials and four test trials. **(A)** An LMM indicated an effect of treatment on Performance Index (PI) in the training but not in the test phase (Table S1). Treatment comparison with a Tukey HSD post-hoc test showed differences in PIs of vehicle and sham groups ($z = -4.217$, $p < 0.001$) and PIs of procaine and sham groups ($z = -2.638$, $p = 0.02$). **(B)** Speed did not differ between experimental groups (LMM, Table S1). **(C)** Reversing Differences were affected by treatment in the training but not in the test phase (LMM, Table S1). Treatment comparison with a Tukey HSD post-hoc test showed differences in Reversing Difference of vehicle and sham groups ($z = -3.107$, $p = 0.005$) and Revering Differences of procaine and sham groups ($z = -3.567$, $p = 0.001$). (D) Crossing Latency approached the 3-s threshold (horizontal dashed line) over the course of training, which corresponds to the delay between light-onset and shock-onset. No significant effect of treatment on Crossing Latency was found for training or in the test phase (Table S1).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Training
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Reversing Difference
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Crossing Latency [s]
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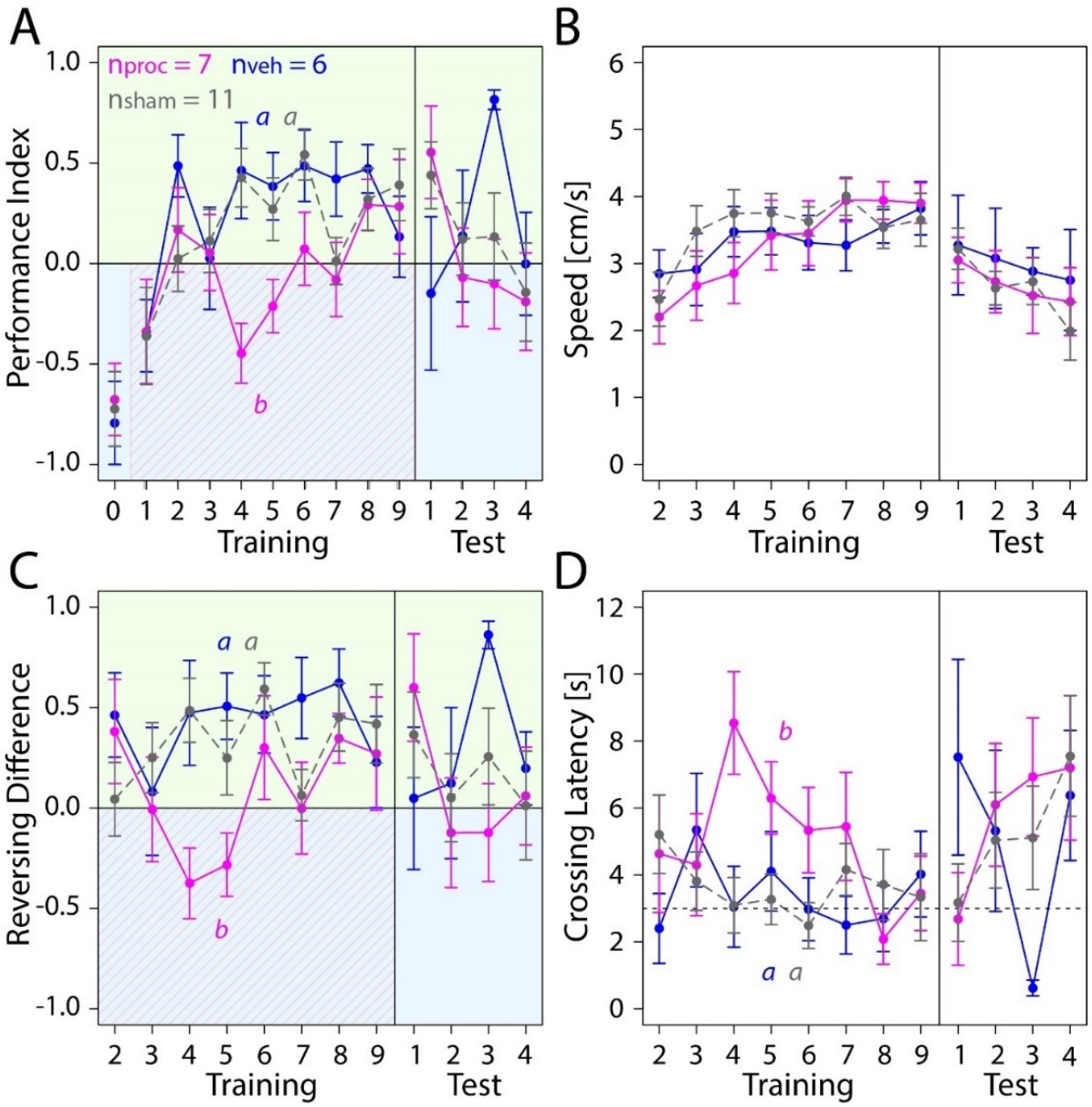
Figure 7: Comparison of behavior in APIS for bees injected with vehicle (blue) or procaine solution (magenta) into the CX, or sham-treated bees (gray). Bees injected with procaine into the CX did not run away from the shock-paired blue side. Means ± SEM are plotted for all variables. Significant treatment effects determined with an LMM ($p < 0.05$, Table S1) are indicated with letters a and b. Bees were subjected to one preference test (0) nine training trials and four test trials. **(A)** Performance Indices (PIs) were affected by treatment in the training but not in the test phase (LMM, Table S1). Treatment comparison with a Tukey HSD post-hoc test showed differences in PIs of procaine and sham groups ($z = -2.512$, $p = 0.03$) and PIs of procaine and vehicle groups ($z = -3.052$, $p = 0.006$). **(B)** Speed did not differ between experimental groups (LMM, Table S1). **(C)** An LMM indicated an effect of treatment on Reversing Differences in the training but not in the test phase (Table S1). Treatment comparison with a Tukey HSD post-hoc test revealed differences in Reversing Difference of procaine and sham groups ($z = -2.629$, $p = 0.02$) and Reversing Differences of procaine and vehicle groups ($z = -2.995$, $p = 0.008$). **(D)** In vehicle and sham groups Crossing Latency approached the 3-s threshold (horizontal dashed line) over the course of training, which corresponds to the delay between light-onset and shock-onset. An LMM revealed an effect of treatment on Crossing Latency in the training but not in the test phase (Table S1). Treatment comparison with a Tukey HSD post-hoc test showed differences in Crossing Latencies of procaine and sham groups ($z = 2.467$, $p = 0.04$) and Crossing Latencies of procaine and vehicle groups ($z = 2.532$, $p = 0.03$).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Discussion

About a decade ago the MBs were believed to process mainly olfactory information to generate meaningful associations to other stimuli. The CX was believed to primarily process visual and spatial information. Amongst other recent studies this study has shown this division might not necessarily be so clear. Our data indicate that the VLs as part of the MB output as well as the CX are involved in differential visual learning in the APIS assay.

Mushroom Body Function Was Required for Visual Learning with a Choice Component

Control bees escaped the shock-paired light field and avoided returning to it after only a few conditioning trials (**Figure 4**). These results were congruent with data obtained from untreated forager bees conditioned in the same assay in Konstanz, Germany (Kirkerud et al., 2017), and confirms the robustness of the paradigm across continents. While the operation and injection is an invasive procedure, we found that sham-treated animals recovered well and showed no deficits in learning performance compared to NT animals. In contrast, bees with silenced VLs escaped the shock-paired light field but failed to remain in the safe light field (**Figure 6**). Instead, they ran back and forth in the chamber resulting in lower PIs. This behavior indicated that they most likely failed to associate one light field with danger and the other light field with safety. We found a similar behavior in bees injected with the vehicle only. A similar phenomenon was found when injections of PBS into the MB lobes led to a reduced performance in olfactory reversal learning in comparison to injections into the calyces (Boitard et al., 2015). However, no effect of the vehicle was found when observing neural activity changes due to injections using calcium imaging (Girardin et al., 2013).

When targeting one collar region of the MBC with procaine we found no deficits in performance (**Figure 5**). But since the honey bee collar region receives color input (Ehmer and Gronenberg, 2002; Gronenberg and Lopez-Riquelme, 2004) and the VLs were clearly involved in visual learning in APIS, it is possible that silencing neurons in only one of the eight collar regions in all MBCs might not have been sufficient to impair performance in the APIS assay. Further studies impacting all collar regions are necessary to clarify, but technically this would be extremely tricky to do.

In freely moving fruit flies, MB function was required for a visual paradigm with color stimuli and aversive reinforcement (Vogt et al., 2014, 2016). Similar to the paradigm presented here, blue and green light fields were presented simultaneously rather than sequentially. These findings stand in contrast to other studies implicating no involvement of the MBs in visual learning. Mutant flies (Drosophila melanogaster) with severely underdeveloped MBs and interrupted MB input were either conditioned by being shaken while illuminated with one color (Heisenberg et al., 1985) or trained with heat stimuli in a differential visual assay while being tethered in a flight simulator (Wolf et al., 1998). In both cases, mutant flies showed no learning deficits. In the latter case, the fly was able to terminate the heat stimulus by turning left or right until the adjacent 90◦-quadrant of the arena was faced and the arena was then illuminated with light of a different color. This suggests that the MBs are involved in color learning which includes a choice situation rather than learning of sequentially presented color stimuli in a differential paradigm. Indeed, it has been shown that MBs are required to make a choice of responding to conflicting information of color and shape or color and position based on saliency (Tang and Guo, 2001; Zhang et al., 2007).

In both, bees and flies the dominant input to the MBs is olfactory, but it appears that MBs are also crucial for learning of visual information in bees in a binary-choice assay. Strausfeld (2012) and Farris (2015) argue that processing of visual information in the MB in insects is largely driven by the ecological relevance in the animal's life and the nature of visual input received. Large MBs with developed calyces are therefore not limited to species which rely predominately on olfactory information to navigate in their environment. They can also be found in aquatic beetle species which navigate mainly by vision (Lin and Strausfeld, 2012). It remains to be investigated if the MBs play a role in visual learning in other insect orders as well.

Silencing Neurons in the Central Complex Affected the Behavioral Response

We also found that silencing of neurons in the CX led to a change in behavior (**Figure 7**). Procaine-treated bees spent more time in the safe light field than on the shock-paired light field in the second and third trials and in the end of the training. This indicates, that learning of the light-shock pairing might still have been present. In the middle of the

training period, however, procaine-treated bees remained on the shock-paired side of the chamber even after several seconds of shocks being delivered. This was not a result of an impaired ability to initiate reversals or an inability to walk in a straight line (Figure S2D). Nor was it caused by a major deficit in locomotion since speed was not found to be affected by procaine-injections, and rather bees appeared unable to execute an avoidance of the shocked light field.

But why was the effect not visible in the first learning trials? It seems very unlikely that procaine was only active in the middle trials of the training. Cockroaches with central bodies silenced by procaine showed deficits in locomotion and optomotor responses immediately after injections (Kathman et al., 2014; Kaiser and Libersat, 2015). Another explanation is that the response in the first trials might have mainly been driven by a direct reaction to the shocks, resulting in a short-lasting reflex-like escape maneuver. Initial responses to the shock could have been initiated by more direct and faster-processing "escape-pathways" generating a quick behavioral response to an obnoxious stimulus without involving the CX. Various escape reactions in insects have been proposed that bypass the higher processing centers of the brain (Horridge, 1962; Card, 2012). Is it possible that silencing of the CX only interfered with coordinating a motor response to a learned visual stimulus, but not an escape response from an aversive stimulus? In this case, a learned response to the blue light field would have been impaired but not the response to the shock itself. Toward the end of the training the procaine-effect seemed to have worn off, since the bees rapidly increased the proportion of time spent on the safe green side.

The CX has been implicated as the site to generate goal-directed behavior and to modulate movement in insects (Strausfeld and Hirth, 2013; Barron et al., 2015; Plath and Barron, 2015). Various studies have shown that the CX is crucial for spatial orientation memory (Neuser et al., 2008; Kuntz et al., 2012, 2017), visual pattern memory (Liu et al., 2006; Hou et al., 2011) and visual place learning (Ofstad et al., 2011) in fruit flies. A recent study has shown that a group of neurons in the ellipsoid body (part of the CX in the fruit fly) represents the orientation of the animals in relation to a visual stimulus (Seelig and Jayaraman, 2015). Taken together, the CX clearly has a role in visual learning and memory involving spatial orientation of the cues in fruit flies and possibly in other insects. We propose that the CX might also initiate the appropriate responses to learned stimuli which

are processed by the MBs such as color stimuli.

Information about a Learned Stimulus Might Be Conveyed Indirectly to the Central Complex

Taken together, we showed that both, the MBs and the CX contributed to the behavioral response to a learned light stimulus. We propose the MBs integrated the coinciding shock and light information and the CX initiated the escape from the light field. We summarized the information flow between the different brain regions with the addition of other findings from different insect orders (**Figure 8**). To integrate coinciding shock and light information, both stimuli need to be received by the MBs. In the fruit fly γ lobe (part of the VL), a descending Kenyon cell carrying olfactory information forms synapses along the axon with a set of MB output neurons. Dopaminergic neurons modulate these individual compartments in relation to the internal state of the animal (Cohn et al., 2015). In flies, a group of these dopaminergic neurons (PPL1 cluster) carry information of aversive stimuli such as electric shocks (Waddell, 2013; Kaun and Rothenfluh, 2017). It needs to be studied, however, if this process is also found in other insect orders. In fruit flies, olfactory short-term memory is formed in the γ lobes which transitions into long-term memory to α and β lobes via the α' and β' lobes. Kenyon cells which convey wavelength and intensity information to the collar (Vogt et al., 2016) descend into the γ lobes in fruit flies. It remains to be investigated where exactly visual memories relating to color information are formed and where they transition from short-term to long-term memories.

A great question remains, whether there is a connection between the MBs and the CX. A direct connection between the MBs and the CX has not been found so far, with the exception of a single neuron recently discovered in the monarch butterfly (Heinze et al., 2013). An indirect connection could be found in the superior medial protocerebrum (Strausfeld and Hirth, 2013), which comprises outputs from the MBs carrying visual information in fruit flies (Ito et al., 1998) as a well as inputs to the upper division of the central body found in different insects (Strausfeld and Hirth, 2013; Pfeiffer and Homberg, 2014). It is therefore possible that information about the learned sensory association generated by the MBs is passed on indirectly to the CX in order to produce the conditioned response. Evidence for a connection between the MBs and CX manifesting in behavior was found when a sensory preconditioning paradigm involving cross-modal stimuli was

investigated (Zhang et al., 2013). Here, an olfactory stimulus and a visual stimulus based on elevation were pre-conditioned. Then one stimulus was paired with reinforcement. A subsequent test of the other stimulus produced a response, even though it was never reinforced. Tested individually, blocking part of the MBs abolished olfactory memory and blocking part of the ellipsoid body (part of the CX in the fruit fly) abolished visual elevation memory. Remarkably, when the olfactory stimulus was reinforced after pre-conditioning and MBs were blocked, animals responded to the visual elevation stimulus. Thus, an association of the two CSs must have occurred in the pre-conditioning.

To explore the connection between the MBs and the CX will be a challenge in the future. The vast knowledge gained about learning and memory in the honey bee field in combination with pharmacological techniques (Felsenberg et al., 2011; Søvik et al., 2016) and assays such as APIS could provide a powerful tool to uncover how the different brain regions interact.

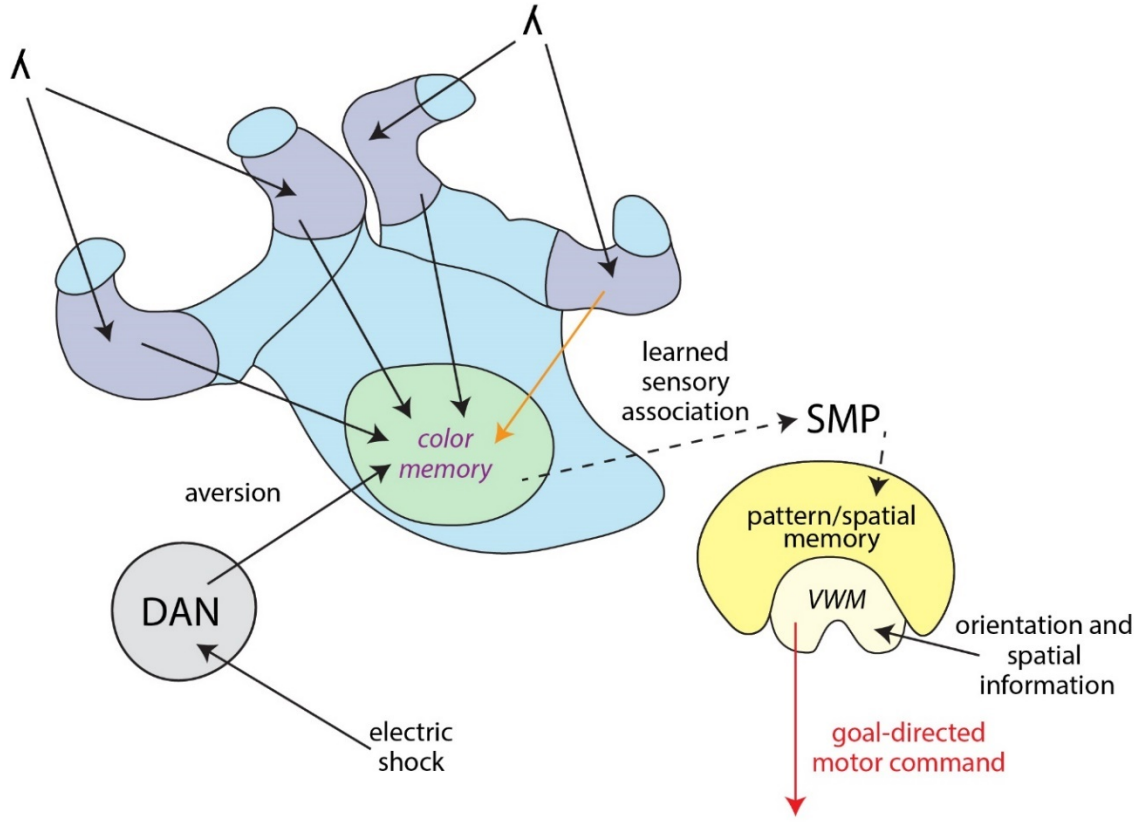

Figure 8: Information flow model for differential color learning in a binary choice assay. Information about the light wavelength (λ) enters the collar region (dark blue) of the MBC from the optic neuropils. Visual information is passed on from the collar region to the VL (light green) via Kenyon cells. This process was partially disrupted by a procaine injection into one collar region (orange arrow). Electric shock information is passed on from the ventral nerve cord to dopaminergic neurons (DAN, gray) which modulate MB output. In the VL wavelength information is associated with aversion and most likely color memories are formed here. This process was disrupted by procaine-injections into the VL (marked in purple). Information about the learned sensory association might be passed on indirectly to the CX (yellow) via the superior medial protocerebrum (SMP). The CX receives orientation and spatial information and processes how the animal is orientated in relation to its environment using visual working memory (VWM). The CX initiates a goal-directed motor response, possibly modified in regards to the learned sensory association. This process was disrupted by procaine-injections into the CX (red arrow).

Author Contributions

All authors (JP, BE, NK, US, CG, and AB) contributed substantially to the design and conception of the experiments, analysis and interpretation of the data and revision of the manuscript. JP, BE, and US contributed to acquisition and analysis of the data. JP and NK contributed to R analysis and statistical analysis of the data. JP and BE drafted the manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2017.00098/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

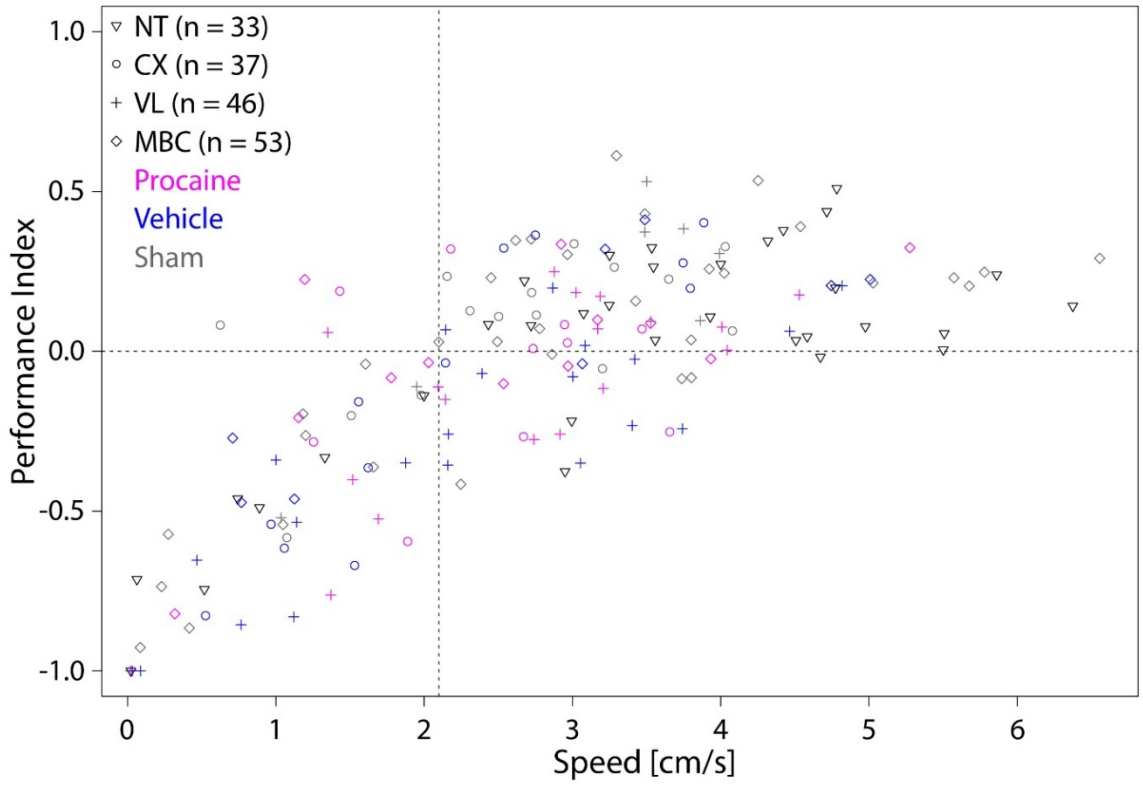

Figure S1: Correlation of Performance Index (PI) with speed in the chamber. Mean speed and PIs across all nine training trials and all four test trials are shown for each individual animal. Bees with a mean speed lower than 2.1 cm/s (dashed vertical line) rarely achieve a positive PI (above horizontal dashed line) which indicates learning, and were excluded from further analyses. 7 out of 33 NT animals, 6 out of 12 vehicle animals injected into the CX, 3 out of 10 procaine animals injected into the CX, 4 out of 15 sham animals injected into the CX, 8 out of 21 vehicle animals injected into the VLs, 6 out of 17 procaine animals injected into the VLs, 2 out of 8 sham animals injected into the VLs, 3 out of 8 vehicle animals injected into the MBC, 5 out of 12 procaine animals injected into the MBC, and 10 out of 33 sham animals injected into the MBC were excluded.

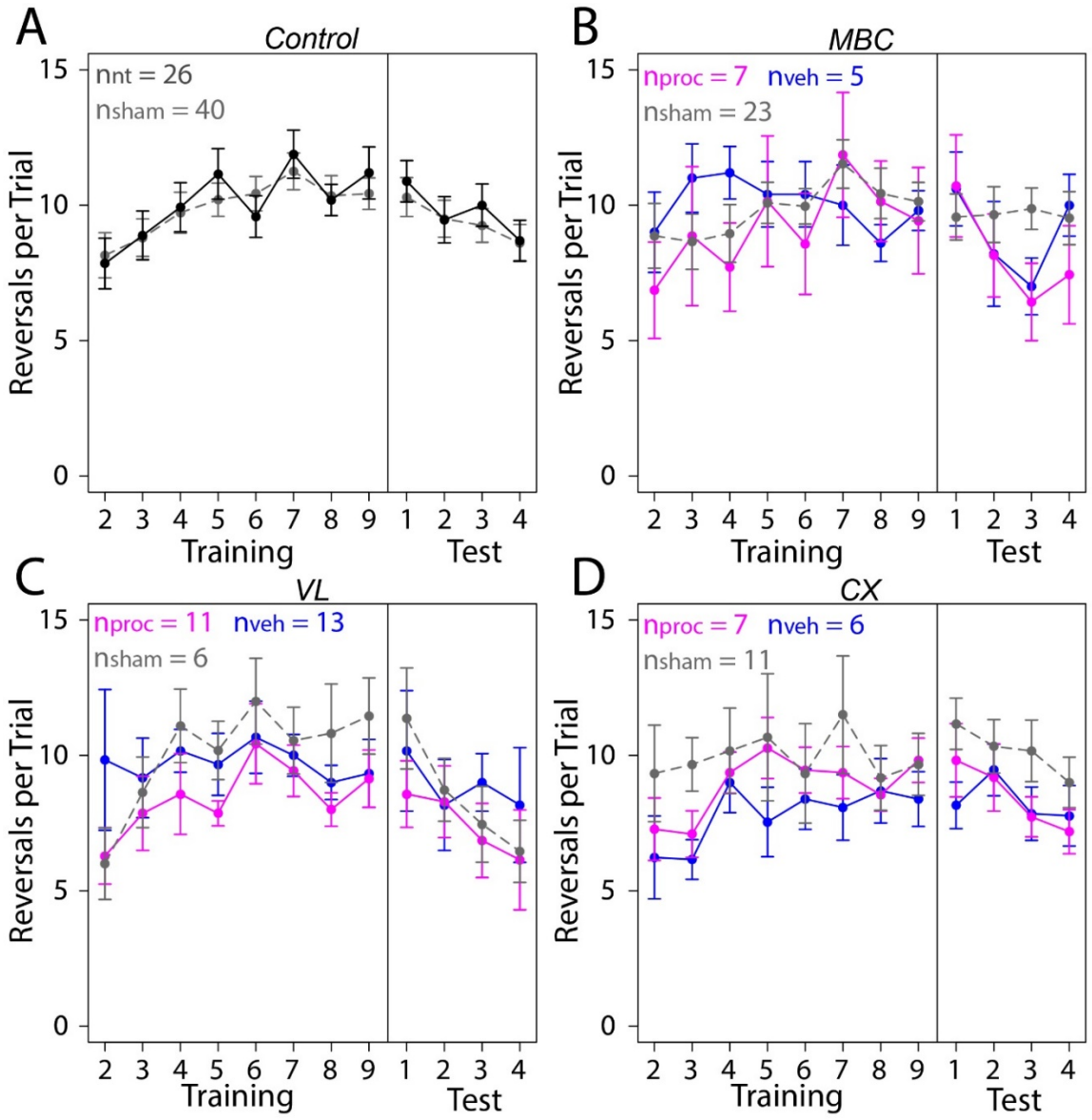

Figure S2: Number of Reversals per Trial after one light-shock pairing. NT is shown in black, sham in grey, vehicle in blue and procaine in magenta. Mean number of reversals (± SEM) are shown for each trial for control animals **(A)**, for animals injected into the collar region of the MBC **(B)**, for animals injected into the VLs **(C)** and for animals injected into the CX **(D)**. LMMs indicated no effects off treatment on Reversals per Trial for any of the four variables (Table S1).

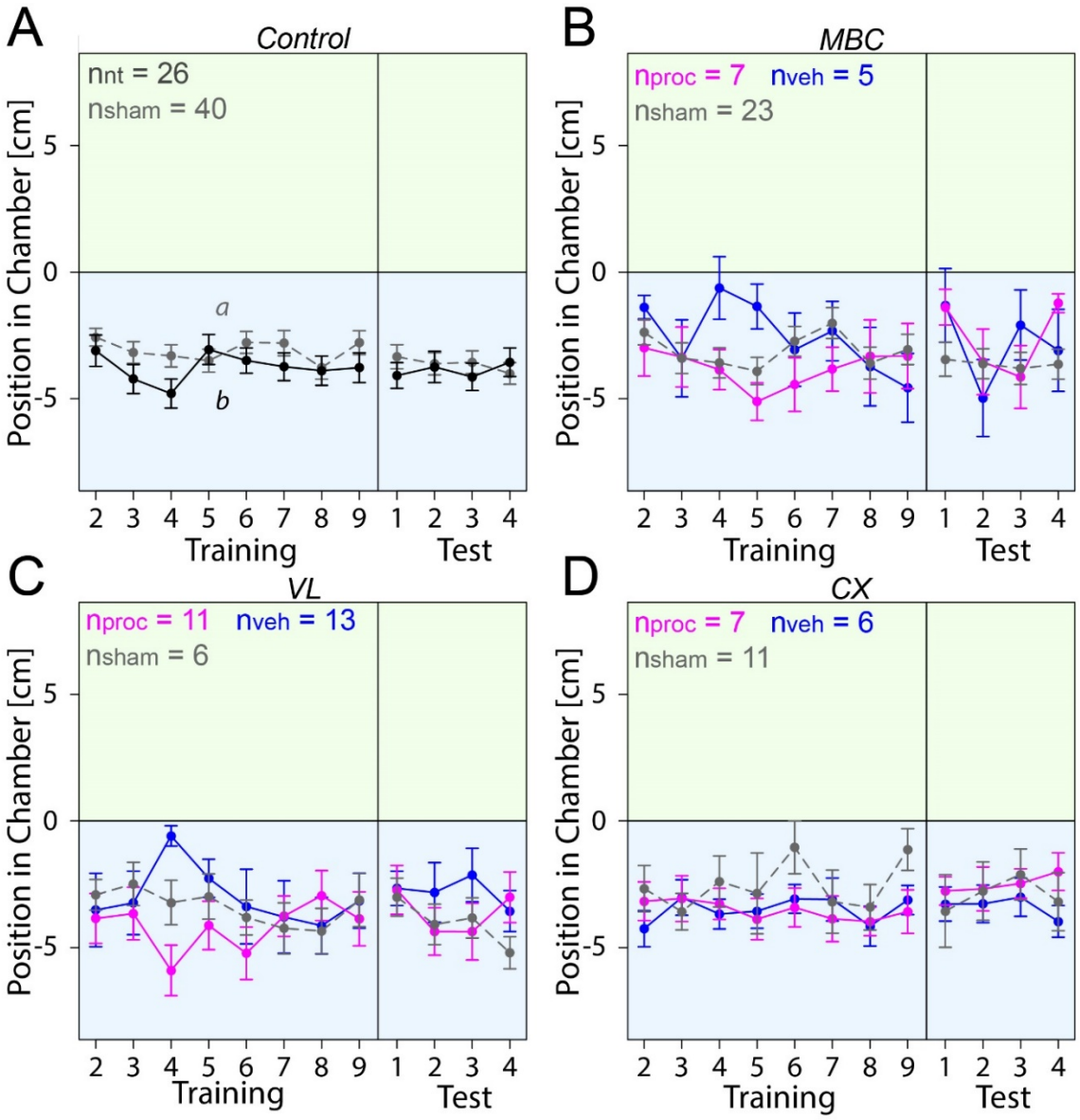

Figure S3: Position at light-onset after first trial. NT is shown in black, sham in grey, vehicle in blue and procaine in magenta. Mean position (± SEM) is shown for each trial for control animals **(A)**, for animals injected into the collar region of the MBC **(B)**, for animals injected into the VLs **(C)** and for animals injected into the CX **(D)**. LMMs indicated no effects of treatment on position for animals injected into the MBC, the VLs or the CX (Table S1). An LMM revealed an effect of treatment on position for the control group (Table S1) as indicated by the letters a and b.

Table S1: Summary of Linear Mixed Model (PI, Speed, Reversing Difference, Crossing Latency, Position in Chamber) and Generalized Linear Mixed Model (Reverses per Trial) results for effect of treatment on variable. LMM or GLMM testing sham against NT (Control) and vehicle (veh) or procaine (proc) against sham after injections into the ventral lobes (VL), central complex (CX) or the mushroom body calyx (MBC). Degrees of Freedom (DF); Estimate (Est); Standard Error (SE).

				Training				Test			
			DF	Est	SE	t/z	p	Est	SE	t/z	p
Control	*PI*	sham	32	0.051	0.052	0.975	0.33	0.084	0.086	0.978	0.33
	Speed			-0.616	0.282	-2.188	0.03	-0.290	0.265	-1.097	0.28
	Reversing Difference			0.330	0.624	0.529	0.60	1.253	0.890	1.407	0.16
	Crossing Latency			0.086	0.409	0.210	0.83	0.148	0.649	0.227	0.82
	Reverses per Trial			-0.012	0.077	-0.154	0.88	-0.041	0.094	-0.433	0.67
	Position in Chamber			0.673	0.332	2.029	0.047	0.250	0.356	0.703	0.48
MBC	*PI*	veh	32	0.042	0.108	0.392	0.70	-0.014	0.168	-0.082	0.94
		proc		-0.197	0.095	-2.080	0.046	0.107	0.147	0.729	0.47
	Speed	veh		0.361	0.622	0.581	0.57	-0.558	0.494	-1.130	0.27
		proc		-0.505	0.544	-0.929	0.36	-0.005	0.432	-0.011	0.99
	Reversing Difference	veh		0.680	1.256	0.542	0.59	-1.004	1.621	-0.612	0.54
		proc		-0.816	1.098	-0.743	0.46	0.517	0.418	0.365	0.72
	Crossing Latency	veh		0.058	0.890	0.065	0.95	1.000	1.191	0.837	0.41
		proc		1.451	0.779	1.863	0.07	-1.353	1.042	-1.298	0.20
	Reverses per Trial	veh		0.050	0.141	0.354	0.72	-0.056	0.177	-0.314	0.75
		proc		-0.086	0.125	-0.690	0.49	-0.171	0.157	-1.092	0.28
	Position in Chamber	veh		0.524	0.594	0.882	0.38	0.760	0.770	0.987	0.33
		proc		-0.697	0.519	-1.341	0.19	1.051	0.674	1.509	1.28

	Preference Test						
			DF	Estimate	SE	t	p
MBC	*PI*	veh	32	0.698	0.265	2.631	0.01
		proc		-0.767	0.232	-3.31	> 0.01

Table S1 *continued*

				Training				Test			
			DF	Est	SE	t	p	Est	SE	t	p
VL	*PI*	veh	27	-0.414	0.098	-4.217	< 0.001	-0.301	0.233	-1.292	0.21
		proc		-0.266	0.101	-2.638	0.01	-0.329	0.240	-1.375	0.18
	Speed	veh		-0.624	0.397	-1.571	0.13	-0.398	0.528	-0.754	0.46
		proc		-0.363	0.108	-0.888	0.38	-0.573	0.543	-1.055	0.30
	Reversing Difference	veh		-3.396	1.093	-3.107	< 0.01	-2.615	1.991	-1.314	0.20
		proc		-4.009	1.124	-3.567	< 0.01	-3.500	2.047	-1.701	0.10
	Crossing Latency	veh		2.138	1.044	2.047	0.05	0.790	1.695	0.466	0.65
		proc		1.312	1.074	1.221	0.23	1.700	1.743	0.975	0.34
	Reverses per Trial	veh		-0.259	0.144	-1.800	0.07	-0.226	0.154	-1.467	0.14
		proc		-0.098	0.147	-0.668	0.50	-0.194	0.159	-1.222	0.22
	Position in Chamber	veh		-0.963	0.663	-1.453	0.16	-0.475	0.666	-0.713	0.48
		proc		-0.994	0.682	-1.457	0.16	0.428	0.685	0.624	0.54
CX	*PI*	veh	21	0.086	0.090	0.953	0.35	0.062	0.229	0.272	0.79
		proc		-0.216	0.086	-2.512	0.02	-0.090	0.218	-0.412	0.68
	Speed	veh		-0.043	0.313	-0.137	0.89	0.353	0.592	0.596	0.56
		proc		-1.870	0.298	-0.628	0.54	0.039	0.564	0.069	0.95
	Reversing Difference	veh		0.818	1.046	0.780	0.44	0.295	2.053	0.144	0.89
		proc		-2.628	1.000	-2.626	0.02	-1.380	1.956	-0.707	0.49
	Crossing Latency	veh		-0.249	0.586	-0.425	0.68	-0.258	1.757	-0.147	0.88
		proc		1.376	0.558	2.467	0.02	0.510	1.674	0.304	0.76
	Reverses per Trial	veh		-0.009	0.127	-0.071	0.94	0.042	0.219	0.192	0.85
		proc		-0.160	0.122	-1.302	0.19	-0.090	0.210	-0.430	0.67
	Position in Chamber	veh		0.384	0.829	0.463	0.65	1.124	0.696	1.780	0.09
		proc		-0.775	0.790	-0.981	0.34	0.414	0.663	0.625	0.54

Chapter IV

Different central brain regions regulate locomotion, spatial orientation and orientation learning in honey bees

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Abstract

Locomotion is one of the most fundamental behaviors in the animal kingdom. In insects, two structures which process higher-order sensory information are also involved in control of walking activity: the mushroom bodies (MBs) and the central complex (CX). While the MBs are crucial for associative learning and memory, the CX is important for orientation in space and spatial learning. The CX receives strong input from the surrounding protocerebrum, including the superior medial protocerebrum (SMP). In this study, we used microinjections of the local anesthetic procaine to temporarily inactivate parts of the MBs and the CX including the adjacent SMP (CX/SMP). We found that injections with procaine into the mushroom body calyx, but not the ventral lobes of the MBs reduced speed and number of walking bouts in a dark rectangular chamber within 15 minutes after injections. Ventral lobe injections with vehicle solution led to a decrease in speed over time. Walking activity was affected after vehicle or procaine-injections into the CX/SMP region, and we found an increase in turning when animals were allowed to move freely in a round arena in dark conditions. Phases with dark conditions were followed by phases with three lights activated in a consistent sequence. Between any two light activations bees experienced a two-second long dark period. In the dark periods between the lights, sham-injected and vehicle injected animals increasingly oriented away from the light that had just deactivated and oriented towards the locations of the other lights in the sequence. When moving towards the locations of the other lights, angular speed dropped, indicating a targeted orientation towards the position of the light in the dark. Bees also improved in their orientation toward an active light over experience of multiple presentations of the sequence. Procaine-injected animals showed a marked reduction in the ability to orient towards the locations of the lights in the dark period. We discuss the different roles of the MBs and CX in locomotor control and how the CX/SMP region could be involved in this form of spatial learning.

Introduction

Locomotion in insects is the result of the complex interplay of decentralized movement of the limbs on the level of the ganglia in the insect's body and a central control by the brain (Buschges, 2005; Buschges et al., 2008; Pearson, 1993). To initiate and regulate locomotion, the insect's brain has to receive and integrate external sensory information and sensory feedback from the limbs (Huston and Jayaraman, 2011; Pearson, 1993). The insect brain receives visual, olfactory, chemosensory and mechanosensory input and integrates these different sensory stimuli to produce an appropriate behavioral response (Huston and Jayaraman, 2011; Wessnitzer and Webb, 2006). The main structures in the insect brain responsible for processing integration of sensory stimuli of different modalities are the mushroom bodies (MBs) and the central complex (CX). In honey bees, the MBs are comprised of four cup-like structures called the mushroom body calyces (MBCs), which predominantly receive olfactory, but also visual and mechanosensory inputs (Ehmer and Gronenberg, 2002; Gronenberg and Lopez-Riquelme, 2004; Mobbs, 1982). Outputs from the MBCs run through the peduncle and divide into the horizontal lobes and vertical lobes (VLs) (Rybak and Menzel, 1993; Strausfeld, 2002). These MB output structures are strongly involved in associative learning and memory (Giurfa, 2007; Menzel, 1999; Menzel, 2001; Menzel, 2012), including olfactory, visual and tactile learning. Moreover, MBs play a role in place learning in cockroaches (*Periplaneta americana*) (Mizunami et al., 1998b).

The MBs are also directly involved in control of locomotion in fruit flies (*Drosophila melanogaster*) (Helfrich-Forster et al., 2002; Martin et al., 1998; Serway et al., 2009), crickets (*Gryllus campestris*, *Acheta domesticus*) (Huber, 1960) and cockroaches (Kaiser and Libersat, 2015). When processing in the MBs was disrupted by a structural defect in fruit flies or by drug injections in cockroaches, walking activity was enhanced. This is interpreted as meaning that the MBs normally suppress walking activity. This may be true for longer-term walking activity only, however, since MBs seem to enhance walking activity in an initial period of 15 minutes in fruit flies (Serway et al., 2009). Serway et al. (2009) reported that free-walking flies with ablated MBs showed reduced walking activity when provided with black bars as visual stimuli. As pointed out by the authors, in most groups of flies with ablated MBs the initial walking activity was also lower compared to

controls in Martin et al. (1998). After 10 – 20 minutes, this relationship reversed in the study by Martin et al. (1998) and walking activity was higher in flies with ablated MBs compared to controls. At a closer look, it seems that the control flies had a very high activity peak at the beginning of the experiment which rapidly dropped over the time course of three hours, while walking activity of flies with ablated MBs was on a lower level at the beginning of the experiment but decreased less over three hours.

The CX comprises different interconnected neuropils: the upper division of the central body (CBU), the lower division of the central body (CBL), the protocerebral bridge (PB) and the noduli (NO) (Pfeiffer and Homberg, 2014; Plath and Barron, 2015). The CX is strongly involved in representation of an insects' orientation in space (Green et al., 2017; Heinze, 2015; Homberg et al., 2011; Kim et al., 2017; Seelig and Jayaraman, 2015; Turner-Evans et al., 2017; Varga et al., 2017; Varga and Ritzmann, 2016) and visual memory of spatial features (Kuntz et al., 2012; Kuntz et al., 2017; Liu et al., 2006; Neuser et al., 2008; Pan et al., 2009; Thran et al., 2013; Wang et al., 2008). In many insects, the CX receives polarized light input, which is used for orientation and navigation guided by celestial cues (Heinze and Homberg, 2007; Homberg et al., 2011). Additionally, the CX initiates and modifies walking activity (Strauss, 2002), e.g. in form of turning behavior in cockroaches (Guo and Ritzmann, 2013a; Martin et al., 2015; Ridgel et al., 2007), crickets (Kai and Okada, 2013) or fruit flies (Strauss, 2002). For example, when neurons in the CX were stimulated, the free-walking cockroach (*Blaberus discoidalis*) initiated a turn (Martin et al., 2015). In contrast to the MBs, walking activity in fruit flies (Martin et al., 1999; Pielage et al., 2002; Poeck et al., 2008; Strauss, 2002; Strauss et al., 1992; Strauss and Heisenberg, 1993) or cockroaches (Kaiser and Libersat, 2015) was reduced, or lost completely when processing in the CX was disrupted by anesthesia or ablations.

The CX does not receive direct input from sensory lobes in the brain, but is strongly connected to the surrounding protocerebrum (Pfeiffer and Homberg, 2014), including the adjacent superior medial protocerebrum (SMP) (Hanesch et al., 1989; Phillips-Portillo and Strausfeld, 2012; Young and Armstrong, 2010). The SMP does not receive direct sensory input, but rather processed input from other areas of the brain. The SMP receives output from the MBs (Ito et al., 1998; Strausfeld, 2002) and mechanosensory input from other brain

regions (Ignell et al., 2005; Strausfeld, 1976). Notably, the SMP includes many descending neurons which provide motor-related information to the ganglia (Hedwig, 2000; Hsu and Bhandawat, 2016; Okada et al., 2003; Zorovic and Hedwig, 2011). Connections to both, the CX and the MBs, make the SMP to a possible area for indirect connections between the two main higher-order sensory processing centers in the insect brain (Phillips-Portillo and Strausfeld, 2012; Strausfeld, 2012; Strausfeld and Hirth, 2013).

Recent studies have shown that the CX encodes for orientation and direction of the animal in relation to external visual landmarks (Green et al., 2017; Kim et al., 2017; Seelig and Jayaraman, 2013; Seelig and Jayaraman, 2015; Turner-Evans et al., 2017; Varga and Ritzmann, 2016) by integrating orientation angles and angular velocity (Green et al., 2017; Turner-Evans et al., 2017). The animal's orientation is represented by activity of one group of ring neurons in the EB (ellipsoid body, CBL in fruit flies (*Drosophila melanogaster*), which moves around the EB according to changing orientations (Seelig and Jayaraman, 2015). This activity bump was still visible beyond presentation of the visual landmarks (Seelig and Jayaraman, 2015), which points towards a form of visual working memory. Single- and multi-unit recordings in the cockroach revealed that individual cells in the CX code for head direction (Varga and Ritzmann, 2016). A great advance in uncovering the mechanisms underlying visual working memory in the fruit fly EB has been made recently: nitric oxide encodes a short visual working memory trace by modulating the opening of cGMP-regulated ion channels (Kuntz et al., 2017). The authors propose that ring neurons in one segment of the EB receive coinciding visual and idiothetic information from the body. This leads to an increase of nitric oxide production, causing an opening of the cGMP-regulated ion channels. The resulting Ca^{2+} influx into the neuron would encode for a visual memory trace lasting for several seconds. However, how longer-lasting visual and spatial memories are encoded in the CX remains to be investigated.

Additionally, the CX is strongly involved in visual pattern learning (Li et al., 2009; Liu et al., 2006; Pan et al., 2009; Wang et al., 2008), visual orientation memory (Kuntz et al., 2012; Kuntz et al., 2017; Neuser et al., 2008; Thran et al., 2013) and spatial learning of visual features (Ofstad et al., 2011) in fruit flies. Visual working memory has been investigated in flies with the detour paradigm (Kuntz et al., 2012; Kuntz et al., 2017; Neuser et al., 2008;

Thran et al., 2013). In this paradigm, flies with clipped wings walk in an open arena, which is surrounded by water. Two opposing dark bars beyond the water cause the flies to walk back and forth, trying to reach the two bars. After a while, the two bars are deactivated and instead a third bar appears in a 90° angle. As soon as the fly has oriented towards the new bar, it disappears, which leads to an orientation back to the initial path in most flies. How well a fly can remember spatial features was also investigated (Ofstad et al., 2011). Here, a fly had to find a cool area in a hot arena by remembering the spatial relationship of the cool area and visual landmarks. Both processes, visual working memory and spatial memory, were impaired when parts of the CX were disrupted.

Here, we investigated the roles of the MBs and the CX in structuring walking activity and the role of the CX including the surrounding SMP (CX/SMP) in orientation in space and spatial learning. To analyze the roles of the different brain regions in these behaviors, we microinjected the anesthetic procaine. Procaine was previously used to analyze learning in honey bees (Devaud et al., 2007; Devaud et al., 2015; Muller et al., 2003; Plath et al., 2017) and orientation and locomotion in cockroaches (Kaiser and Libersat, 2015; Kathman et al., 2014). Procaine-injected animals were compared to vehicle- or sham-injected animals. Walking activity was tested in a dark narrow chamber for 15 minutes after injections into the MBC, VLs or the CX. Orientation in dark and light conditions was studied in animals in a round arena for 30 minutes after injections into the CX/SMP. The assay was structured as phases with low-light conditions (dark phases) and phases with distinct visual stimuli displayed in a regular sequence (light phases). As visual stimuli, we used small LED lights, which were not visible to the bee when deactivated. The lights were arranged in a triangle covering one half of a circle (isosceles triangle). In contrast to the detour paradigm, only one light was active at a time and the three lights were activated in a regular sequence (i.e. Light 1, dark period, Light 2, dark period, Light 3, dark period, Light 1, ...). Between two light presentations the bee experienced a dark period, which allowed us to investigate if the bee remembered the positions of the lights. With this assay, we could explore if bees can anticipate the next light in the sequence due to learning the sequence of light positions. It should be noted, that 'learning' and 'anticipation' are used as phenomenal terms only, and do not imply an underlying psychological mechanism. We expected that orientation

behavior and visual working memory would be impaired in procaine-injected animals.

Methods and Materials

Animals and surgical procedure

Honey bee foragers were collected from the hive entrances when leaving the colonies to forage. Their Colonies were located at Macquarie University in Sydney, Australia and at the University of Konstanz, Germany. To prepare bees for injections, they were immobilized by placing them briefly on ice and harnessed in PER tubes for further treatment (Bitterman et al., 1983; Felsenberg et al., 2011). Movement of the head and the antennae was blocked by placing a piece of soft dental wax in the bees' neck and another piece loosely over the antennae.

To target the MBC, the lens of the median ocellus was removed and a small incision was made into the neurilemma sheath covering the brain to ease entering with the micropipette. To inject into the VLs and the ventral parts of the CX and adjacent SMP (CX/SMP), a window was cut into the anterior head capsule to expose the brain. Three cuts were made to create a cuticle flap: one above the antennal stems (dorsal), one below the median ocellus (ventral), and one at the border of the right eye (Devaud et al., 2007; Sovik et al., 2016). To access the dorsal parts of the CX/SMP, three cuts were made into the posterior part of the head capsule: one above the neck hole (dorsal), one below the lateral ocelli (ventral) and one at the border of the right eye. A piece of soft wax was positioned over the opened flap to keep it in place. To access the brain, glands and trachea were carefully moved to the side with fine forceps. A small rupture was made into the neurilemma sheath above the targeted structure. After injections, the window was closed and the cuts were sealed with a drop of melted eicosane (Sigma-Aldrich, Germany/Australia). Detailed instructions for the procedure are given in Sovik et al. (2016).

Injections

Control groups included sham-injected animals (sham) and animals injected with saline (vehicle/veh). These were compared to procaine-injected animals (procaine/proc). Procaine reversibly inactivates neurons by reducing Na^+- and K^+-currents and spiking activity in

honey bees (Devaud et al., 2007). A 20 % (w/v) procaine solution was prepared from a 40 % stock solution on the morning of the experiment. The stock solution contained Procaine HCl (Sigma-Aldrich Australia/Germany) dissolved in physiological saline (7.54 g/L NaCl, 0.448 g/L KCl, 0.872 g/L $MgCl_2$ x 6 H_20, 0.735 g/L $CaCl_2$ x $2H_20$, 54.72 g/L Sucrose, 4.95 g/L D-glucose, and 2.38 g/L HEPES, pH = 6.7, 500 mOsm, Sigma-Aldrich Australia/Germany, (see Burger et al., 2013)). The procaine solution and the saline solution for the vehicle injections contained 0.5 mg/ml dextran Alexa fluor 546 or dextran Alexa fluor 568 (10.000 MW, Molecular probes by Life technologies, Carlsbad, CA, USA) to identify the injection sites using confocal laser scanning microscopy (see below). Before the experiments, glass capillaries (World Precisions Instruments, Sarasota, FL, USA) were pulled into micropipettes with an electrode puller (Sutter Instrument, Novato, CA 94949, USA or Scientific & Research Instruments, Karnataka, India). The tips were broken under a microscope to create an outer diameter of 10 – 15 µm. The micropipette was filled with the solution and inserted into a microinjector (Eppendorf, Hamburg, Germany) connected to an electronic micromanipulator (Luigs & Neumann Feinmechanik und Elektrotechnik, Ratingen, Germany) to perform injections.

For all injections, the micropipette was inserted into the incision made earlier. The MBC was injected with a volume of ~2 nL at a depth of ~215 µm. The VLs were injected with ~1 nL at a depth of ~60 µm. The CX and SMP were injected with ~0.5 nL at various depths. Injections were performed under fluorescent light using a stereomicroscope fluorescent adapter (Green- Light and Filter Set; NIGHTSEA, Lexington, MA, USA) or a fluorescent stereomicroscope (Leica MZ 16FA, Leica Microsystems, Wetzlar, Germany) to identify the injection site (VL) and/or to guide the insertion of the micropipette into the brain surface (MBC, CX/SMP).

Behavioral assays

Locomotion and orientation/spatial learning were investigated using two different behavioral assays with independent groups of bees.

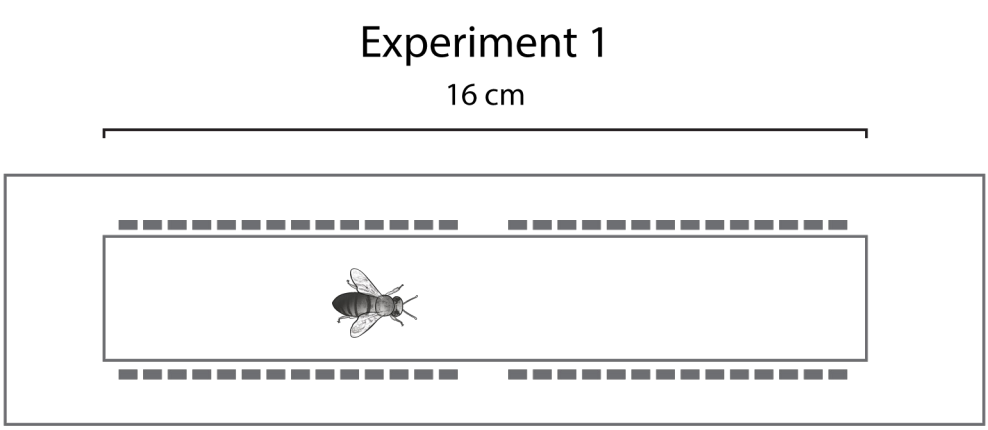
Experiment 1: After injections into the MBC, VLs or CX/SMP, bees were transferred into a dark rectangular chamber to investigate straight walking (**Figure 1A**). The rectangular

chamber was originally created for olfactory and visual conditioning (Kirkerud et al., 2017; Kirkerud et al., 2013) and manufactured at the University of Konstanz, Germany. A bee can freely walk back and forth in the chamber, while tracking is fully automated by infrared sensors and a custom designed system software. Only full turns resulting in a reversal of direction and straight movement are registered by the software. After injections, activity was tracked for 15 minutes in total darkness.

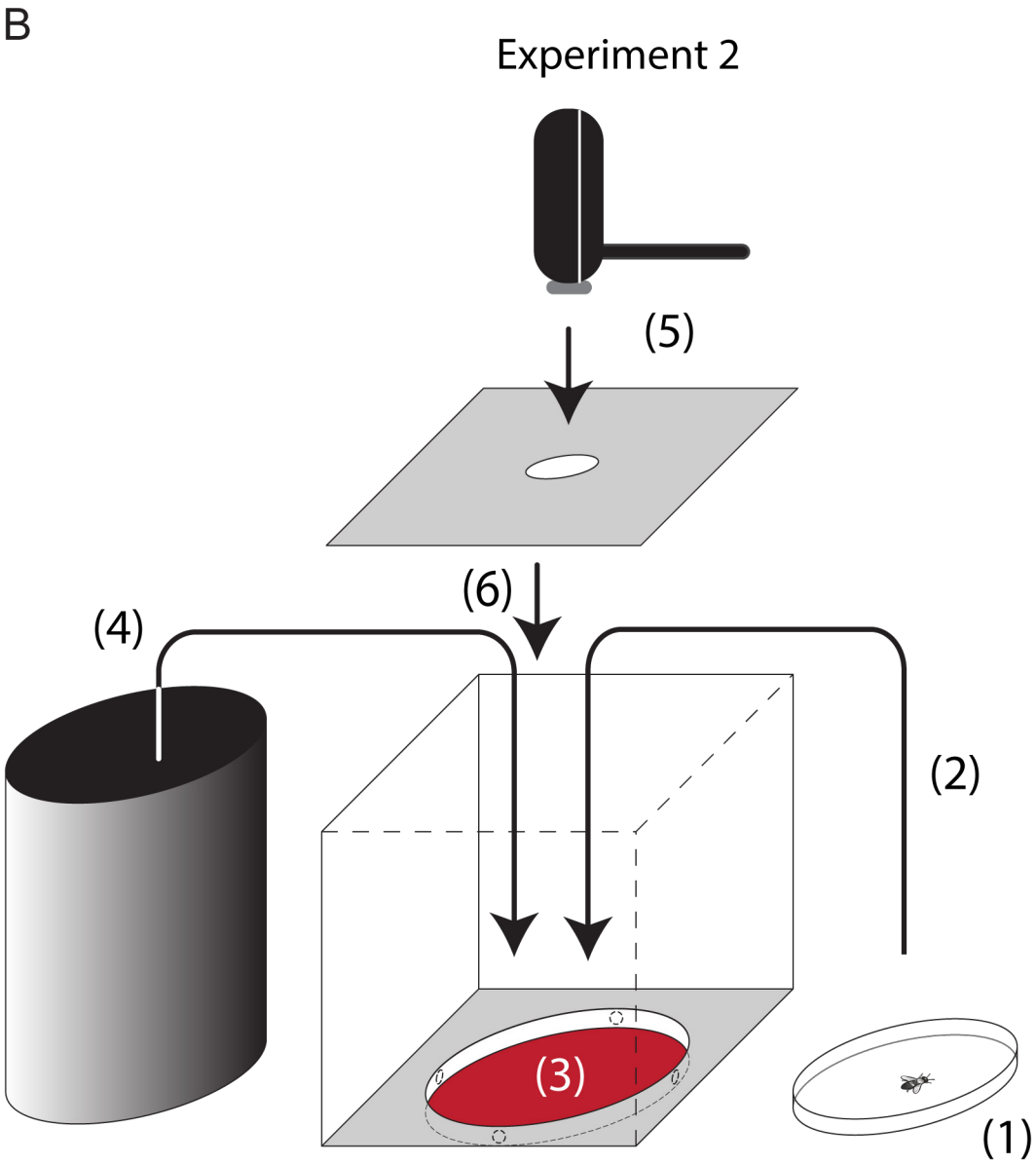
Experiment 2: After injections into the CX/SMP, bees were transferred into a 140 mm round clear plastic dish (**Figure 1B**). If the bee was too agitated (e.g. buzzing and struggling in the harness) it was briefly calmed down on ice. The dish was placed into a wooden box sitting on a LCD screen to provide background illumination (**Figure 1B**). The background was red corresponding to a wavelength of 650 nm (RGB 255/0/0), which is less visible to honey bees (Chittka and Waser, 1997). A black cylinder was placed around the arena to conceal the corners of the wooden box which could serve as visual landmarks. White LED lights were positioned at 0°, 90°,180° and 270° behind a white plastic ribbon and only visible to the bee when activated.

Figure 1 Experimental setups. (A) Experiment 1: Rectangular chamber to assess straight walking in total darkness. The chamber is equipped with infrared sensors (grey rectangles) to track the bee's movement automatically. **(B)** Experiment 2: Round arena to investigate walking behavior and orientation towards lights. The bee was transferred into a round covered dish (1). The dish was placed into a round arena in a wooden box (2). The wooden box was positioned on a flat LCD screen providing red background illumination for the camera and surrounded a white opaque plastic stripe (3). Hidden behind the white stripe were four white LED lights, which became only visible in the arena when activated (3, dashed small circles). A black cardboard cylinder was placed around the arena to conceal the corners of the wooden box (4). A camcorder was placed into a hole in the lid for filming (5). The lid covered the box completely creating low light conditions for the bee, unless one of the lights was activated (6).

A



B



After being place into the arena, bees could move around freely in the round dish for 5 min in darkness (dark phase) (**Figure 2A**). In minutes 6 – 10 (light phase), the lights were switched on for two seconds with a two second break in a clockwise circling sequence (0°, 180°, 270°) (**Figure 2B**) or a counter-clockwise circling sequence (0°, 180°, 90°) (**Figure 2C**). The circling direction of the sequence was consistent for each bee. The light phase was followed by a dark phase in minutes 11 – 15, a light phase in minutes 16 – 20, another dark phase in minutes 21 – 25 and another light phase in minutes 26 – 30. Over the course of one light phase the bee was subjected to a block of 75 trials each (block 1, 2, 3 corresponding to each light phase), which means that each light was activated 25 times within 5 minutes. The assay was filmed with a camcorder (Panasonic HC-V10, Panasonic, Kadoma, Japan) mounted on top of the wooden box (**Figure 1B**).

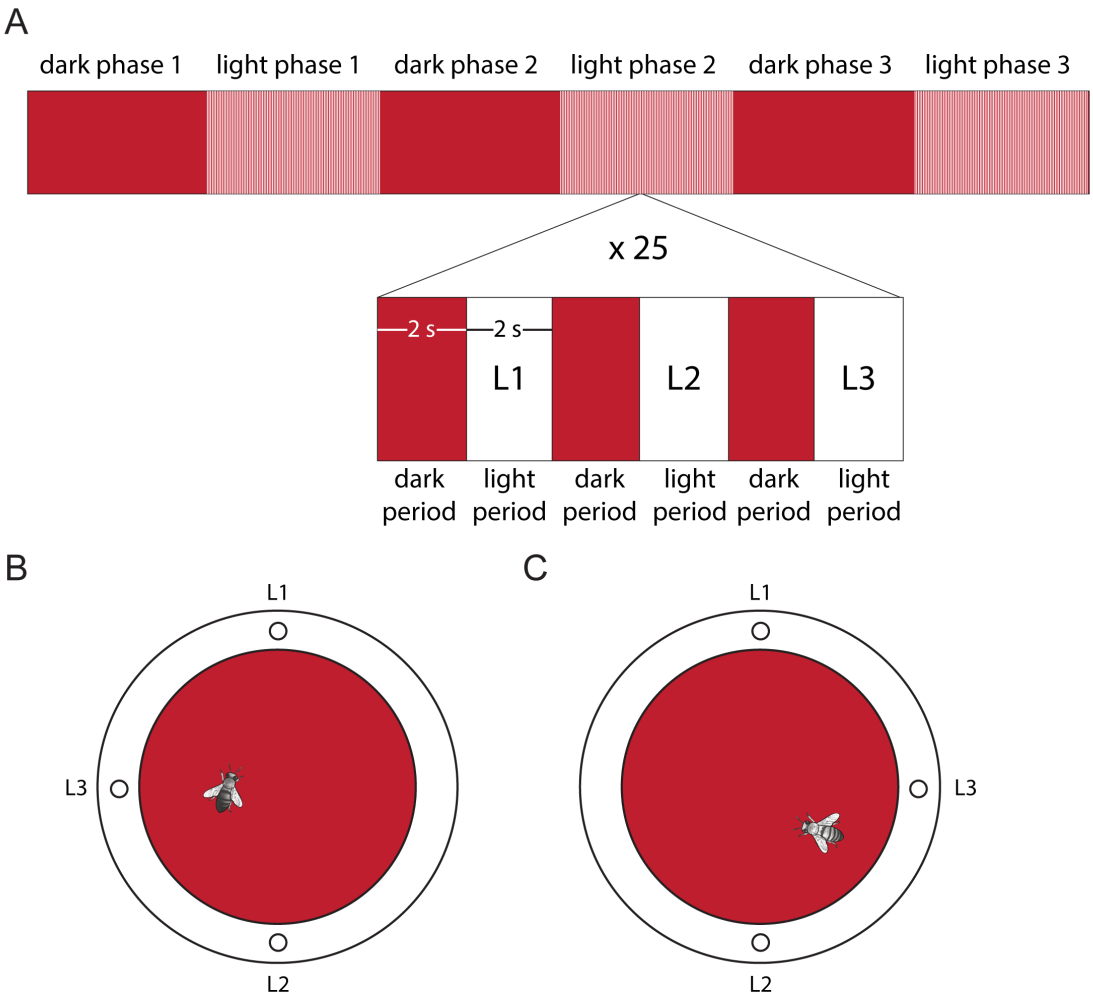


Figure 2: Experimental design for Experiment 2. (A) After injections, the bees could walk freely in the arena for 5 minutes in low-light conditions (dark phase). Next, the bee was subjected to a light phase with visual stimuli. This was repeated twice, making a total of three dark phases and three light phases in 30 minutes. In each light phase, three white LED lights (L1 – L3) around the arena were activated individually in a sequence for two seconds each (light period). Between two light presentations, the bees experienced two seconds of darkness (dark period). Each light phase consisted of 75 light presentations. The lights were activated in a clockwise **(B)** or counter-clockwise **(C)** sequence and at no timepoint was more than one light activated. This sequence was consistent for each bee.

Histology and Imaging

After the behavioral assay, bees were anesthetized on ice and the head capsule was removed. The brain was carefully dissected out from the head capsule in 0.1 M PBS (Sigma-Aldrich Australia). For fixation of the tissue, 4 % paraformaldehyde (Electron Microscopy Sciences, Hattfield, PA, USA) in 0.1 M PBS (Sigma-Aldrich Australia) was used (overnight). Brains were washed several times with PBS and stored in the fridge or processed directly. Stored samples were placed into fresh PBS every day.

To create a background staining, brains were incubated in 250 µL DAPI (2 µg/ml, Sigma-Aldrich Australia) in 0.1 M PBS and 0.2 % Triton-X 100 (Sigma-Aldrich Australia) overnight and washed in PBS afterwards. Brains were then dehydrated with an ethanol series (i.e. 50 %, 70 %, 90 %, 98 %, 100 %, 100 %; 10-30 min each step) and cleared in methyl salicylate (Sigma-Aldrich Australia) or xylene (Sigma-Aldrich Germany).

For mounting, well plates were created with a custom-made aluminum slide (manufactured at the University of Konstanz, Germany) and a glass cover slip (Marienfeld-Superior, Lauda-Koeningshofen, Germany), which were glued together with DPX mounting medium (Sigma-Aldrich Australia/Germany). Brains were placed into fresh DPX mounting medium in the well and covered with another cover slip. Samples were scanned with a confocal laser scanning microscope located at Macquarie University in Sydney (Olympus, Tokio, Japan) or at the University of Konstanz, Germany (Carl Zeiss Microscopy, Jena, Germany). The outline of the neuropils was easily identifiable by the DAPI background staining and auto-fluorescence of the tissue. For animals transferred into the chamber for locomotion analysis, injection locations are shown in **Figure 3**. Dye was found in input regions for the CX in the SMP with some dye in the CX or vice versa for the CX/SMP group (**Figure 3D – G, Figure 4)**.

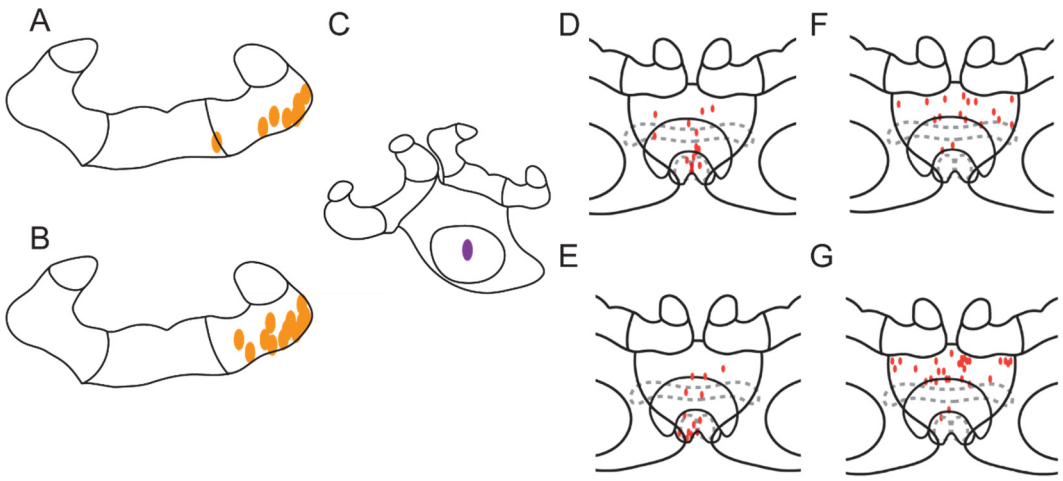


Figure 3: Injection loci in the brain. Injections with vehicle solution **(A)** and procaine solutions **(B)** located in the MBC. **(C)** Injections into the VLs were checked directly under fluorescent light while injecting, and were all located in the center of the VL (depth: ~ 60 µm). Injections with vehicle solution **(D, F)** and procaine solution **(E, G)** in the CX/SMP region. After injections into the CX/SMP area, the animals were transferred into the rectangular chamber **(D, E)** or into the round arena **(F, G).**

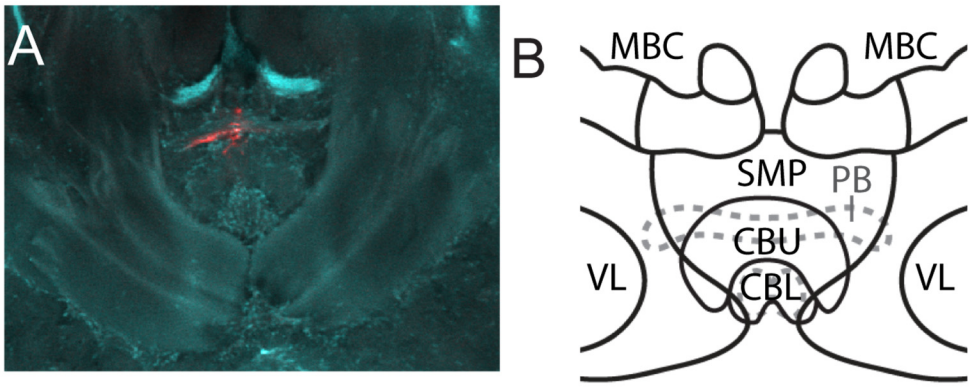


Figure 4:. Injection sites. (A) CLSM image of injections site in the honey bee brain. Loci of injections were identified by adding fluorescent dye to the injection solution (red). A DAPI-counterstain and auto-fluorescence was used to identify the neuropils (cyan). **(B)** Injection sites were found in the CX and SMP. The CX comprises the CBU, CBL and PB located in the center of the honey bee brain between the MBs. CBU: upper division of the central body, CBL: lower division of the central body, PB: protocerebral bridge, SMP: superior medial protocerebrum, MBC: mushroom body calyx, VL: ventral lobes

Data analysis

To investigate movement and orientation behavior in the second assay, bees were tracked using Caltech Multiple Fly Tracker (v. 0.5.6, http://ctrax.sourceforge.net/). Bees that died during the experiment were excluded from data analysis. Data were analyzed and graphed with a custom written R script with R 3.3.2 (R Core Team, Vienna, Austria) and RStudio 1.0.136 (RStudio Inc., Boston, MA, USA). To determine significant effects Linear Mixed Models (R package nlme (Pinheiro et al., 2016)) or Generalized Linear Mixed Models were performed (R package lme4, (Bates et al., 2015)). Model selection was based on visual analysis of homogeneity and normality of the residuals.

To analyze walking activity, speed per minute, number of bout and bout duration was analyzed. A bout was defined as ongoing activity which the walking speed was at least 0.3 cm/s. For the analysis of orientation learning, a subset of animals was used. Bees that did not move over the whole experiment (speed < 0.3 cm/s) rarely oriented toward the lights and were therefore excluded from further analysis. Bees that died during the experiment, which showed circling behavior **(see Figure S 1)** or groomed in all three trial blocks were also excluded.

To analyze orientation of the bee with respect to one of the lights, the angle between the direction of the light and the rostro-caudal body axis was calculated and plotted. For an individual bee, which showed anticipation of next light the sequence this is shown in (**Figure 5)**. An angle of zero radians means that the bee directly faced the light. If a bee turned away from a light, the angle became larger and if it turned towards a light the angle became smaller. To visualize orientation over one dark period and one light period the angle over one light period was plotted (**Figure 5B**). Since the bees used in this experiment were positively phototactic, they usually oriented towards a light when it was active (***L(on)***, e.g. Light 3). In the dark period between two light presentations, we distinguish four different behaviors: The bee could remain oriented towards the light which just deactivated (***L(0)***, e.g. Light 3). The bee could also turn away from the L0 and orient towards the light which was activated in the sequence before (***L(-1)***, e.g. Light 2). Or the bee could orient towards the light which would be activated next in the sequence (***L(+1)***, e.g. Light 1). In other words, the L(+1) in the dark period is identical to L(on) in the following light period. The fourth

option is that the bee oriented towards the empty side of the arena. Since each dark period lasted for two seconds, a moving bee could change its orientation over time and for example orient towards the L(-1) first (angle for L(-1) decreases), then turn away from the L(-1) (angle for L(-1) increases) and instead orient towards the L(+1). To visualize this over time, we plotted the angle in relation to the three positions of the lights (L(-1), L(0), L(+1)) in the dark period and in the light period (two deactivated lights, L-on). If a bee anticipated the next light before it was activated, the angle towards L(+1) would decrease in the dark period, as can be seen in **Figure 3B** for three consecutive trials.

We further investigated how much a bee turned over time (angular speed). A high number of turns resulted in a high average angular speed (**Figure 5A**). A straight walking bee produced a low average angular speed. To visualize how turning behavior changed over time, we plotted angular speed over one dark period and one light period.

Since it would be difficult to see changes over 225 individual trials (3 blocks), we pooled the mean of angle towards a light (or mean angular speed respectively) for all bees over one block (75 trials) (**Figure 6**). For each block, angle in respect to L(-1), L(0), and L(+1) are shown across one dark period and in the following light period.

To analyze how the angle or angular speed changed between an early and a late stage of the dark period, angles to light or angular speed were binned by 20 frames. Angle difference or angular speed difference was calculated by subtracting the mean value first bin from the mean value in the fourth bin (Figure 6B):

$$angle\ difference = mean(angle)_{bin\ 4} - mean(angle)_{bin\ 1}$$

$$angular\ speed\ difference = mean(angular\ speed)_{bin\ 4} - mean(angular\ speed)_{bin\ 1}$$

The same was done for the light period (difference between ninth and sixth bin). If the mean angle for bin 4 was smaller than in bin 1, angle difference became negative **(Figure 6E)**. If the mean angle for bin 4 was larger than for bin 1, angle difference became positive **(Figure 6E)**. Consequently, a negative angle difference showed a turn towards the light's position and a positive angle difference a turn away from the light's position. Analogous to angle difference and angular speed difference, we also analyzed distance difference and speed difference.

Figure 5: Example of orientation of a single bee anticipating the next light over three subsequent trials in a later phase of the experiment. (A) The running track of the bee is shown in black and orientation is shown as colored arrows. Each color corresponds to orientations in one dark period and one light period. After the first dark period (D1), the first light (colored in red) was active for one light period (L1). After the second dark period (D2), the second light (colored in blue) activated (L3), followed by another dark period (D3) and the activation of the third light (colored in grey) in the light period (L3). Black squares indicate sections of low angular speed, which means that the bee walked straightly. Black circles indicate sections of high angular speed, which shows that the bee turned frequently. **(B)** Angle with respect to the position of the first light (colored in red), the position of the second light (colored in blue) and then the position of the third light (colored in grey) over one sequence of three trials. An angle of zero radians means that the bee directly faced the respective light. **(A, B)** In the first dark period the bee oriented and walked towards the first light (colored in red), which was activated in the following light period. This bee had already started towards the next light (colored in blue) when the first light was still active and continued to stay oriented towards the second light in the following dark period. After the second light was deactivated, the bee started towards the third light (colored in grey) and then turned away. When the third light was active, the bee oriented and walked towards the light. In all three trials the bee anticipated the next light by turning towards it before it activated.

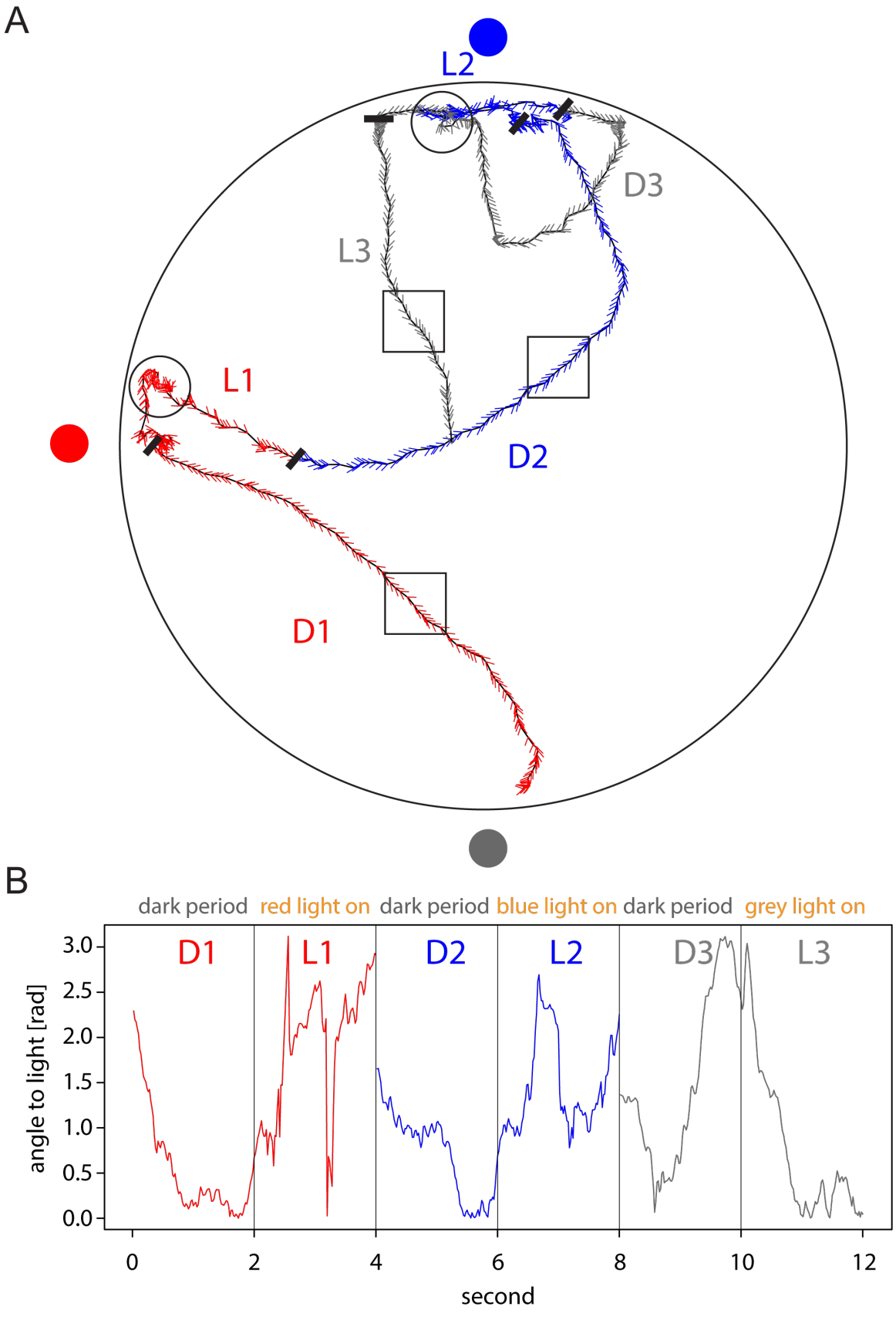
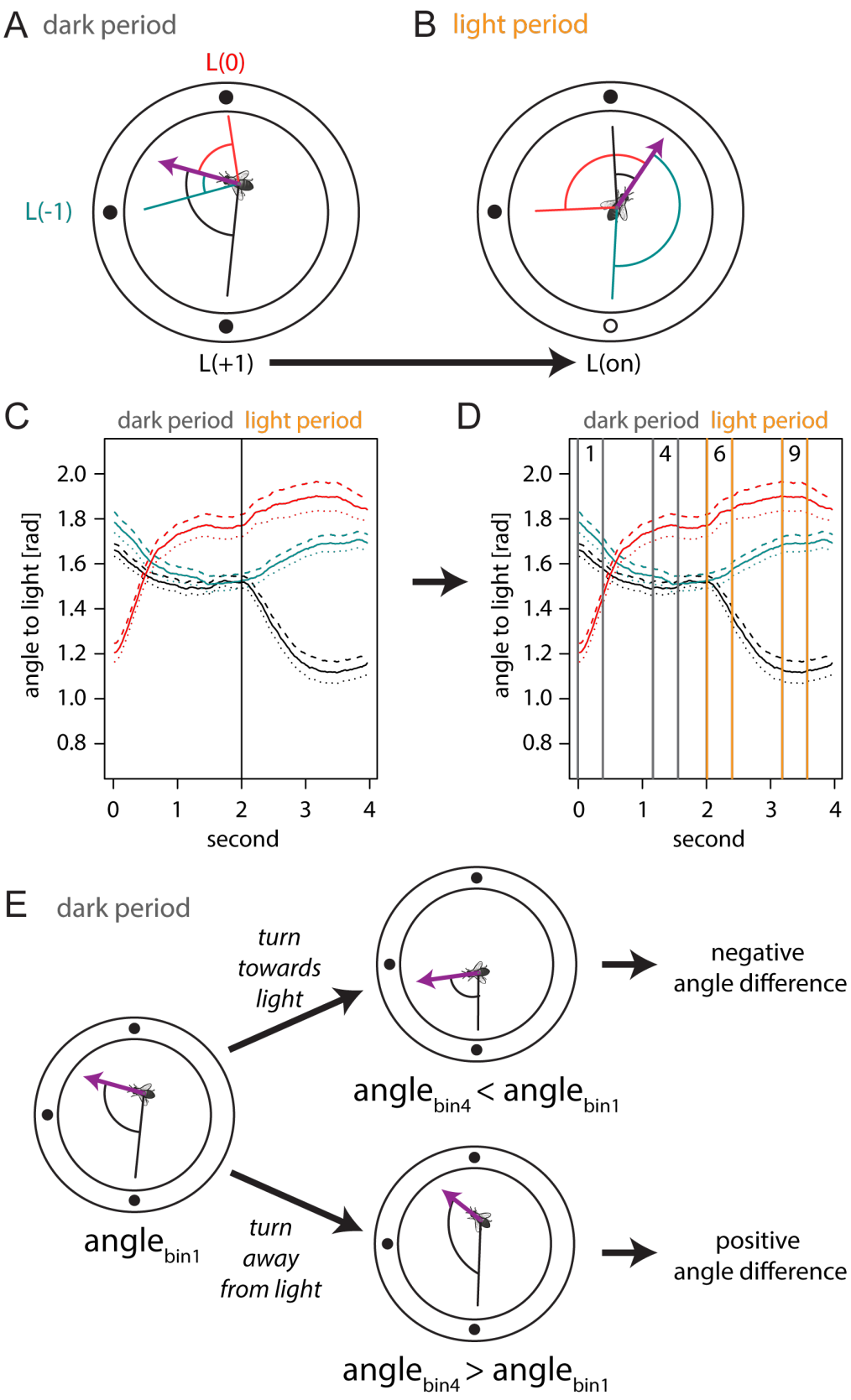

A
L2
D3
L3
L1
D2
D1
B
dark period
red light on
dark period
blue light on
dark period
grey light on
D1
L1
D2
L2
D3
L3
angle to light [rad]
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
second

Figure 6: Analysis of orientation angle. (A, B) Orientation of the bee was defined as the rostro-caudal body axis with the head facing forwards (violet arrows). At each timepoint during the experiment the directions of relative to the deactivated lights' positions (filled circles) in relation to the bee could be determined (red angle, black angle, cyan angle). The angle to a light was defined as the angle between the direction of the light and the orientation of the bee. **(A)** In the dark period, angles to the deactivated lights were determined: to the light which just deactivated (L(0), red), to the light which would be activated next (L(+1), black) and to the light which was activated prior to the L(0)-light (L(-1), cyan). **(B)** In the light period the L(+1) became activate and was now defined as the L(on) (black). The red and cyan angle indicated the direction of the other two deactivated lights. **(C)** Mean angle towards the three light positions was pooled for each block over the dark period (all lights off) and the light period (one light on). **(D)** For further analysis, angles to lights were binned by 20 frames over one dark period and one light period. To determine if bees turned towards or away from the position of the deactivated lights in the dark period, the difference between the fourth bin and the first bin was calculated (angle difference). To determine if bees turned towards or away from the activated light in the light period, the difference between the ninth bin and the sixth bin was calculated (angle difference). **(E)** If the mean angle to light was smaller in bin 4 compared to bin 1, the bees had turned towards the respective light's position. If the mean angle to light was larger in bin 4 compared to bin 1, the bees had turned away from the respective light's position.


A dark period
B light period
L(0)
L(-1)
L(+1)
L(on)
C
dark period light period
angle to light [rad]
second
D
dark period light period
1
4
6
9
angle to light [rad]
second
E dark period
turn towards light
negative angle difference
angle bin4 < angle bin1
angle bin1
turn away from light
positive angle difference
angle bin4 > angle bin1

Results

Procaine-injections into the MBC reduced walking activity

In this study, we investigated walking behavior in dark and light conditions and the orientation to a light source. First, we explored walking in a narrow, rectangular chamber in complete darkness **(Figure 7, Figure 8)**. We compared locomotion of animals injected with procaine, vehicle to sham-injected animals over a duration of 15 minutes post-injection. After injections into the MBC with procaine, animals were significantly slower in the beginning of the experiment compared to sham group (**Figure 7A**, Table S 1). This was not the case for the vehicle group (**Figure 7A**, Table S 1). Both, vehicle and procaine-treated animals, however, showed significantly higher rates of change for speed over time. Sham-injected and vehicle-injected bees were active for around 50 short bouts of about 0.45 - 0.5 seconds over the 15 minutes (**Figure 8A)**. Bout duration did not change significantly over time in the sham group and no significant effects were found for the vehicle treatment (**Figure 8A,** Table S 1). A significant effect of the procaine treatment on bout duration was found, as well as a significantly different rate of change (negative slope). The number of bouts change significantly over time for the sham group (**Figure 8A,** Table S 1) and a significant effect of both, the vehicle- and the procaine-treatment on bout number was found (**Figure 8A,** Table S 1). Additionally, both groups showed significantly higher rates of change for bout number over time (**Figure 8A,** Table S 1). This suggests that both, the vehicle the procaine group show a lower walking activity compared to the sham group in the beginning of the experiment, but while the vehicle seem to recover towards the end, the procaine group remained less active towards the end and walking activity was structured into longer but less bouts compared to the sham group.

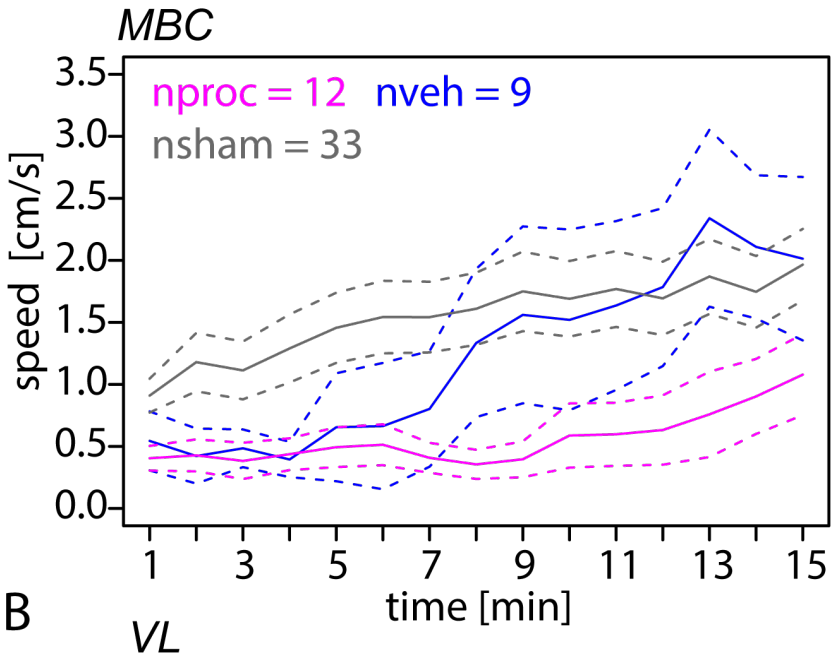
For animals injected into the VLs, a significant increase of speed over time was found for the sham group (**Figure 7B**, Table S 1). Curiously, a significantly different rate of change was found for the vehicle group, but not the procaine group (**Figure 7B**, Table S 1). Instead of increasing speeds over time, speeds were decreasing on average in the vehicle group. Bout duration did not change significantly over time for the sham group (**Figure 8B**, Table S 1). No effects were found for the vehicle treatment on bout duration, but the procaine group showed a significantly lower rate of change (negative slope). Bout number increased significantly over time for the sham group (**Figure 8B**, Table S 1). In the vehicle group bout number decreased over time, while bout numbers increased significantly more in the procaine group compared to the sham group (**Figure 8B**, Table S 1).

After injections into the CX/SMP, the sham group showed a significant decrease of speed over time (**Figure 7C**, Table S 1) and no change of bout duration over time (**Figure 8C**, Table S 1). No effects of treatment on speed or on bout duration were found (**Figure 8C**, Table S 1) Bout number increased significantly over time for the sham group and a higher rate of change was found for the vehicle and the procaine group (**Figure 8C**, Table S 1).

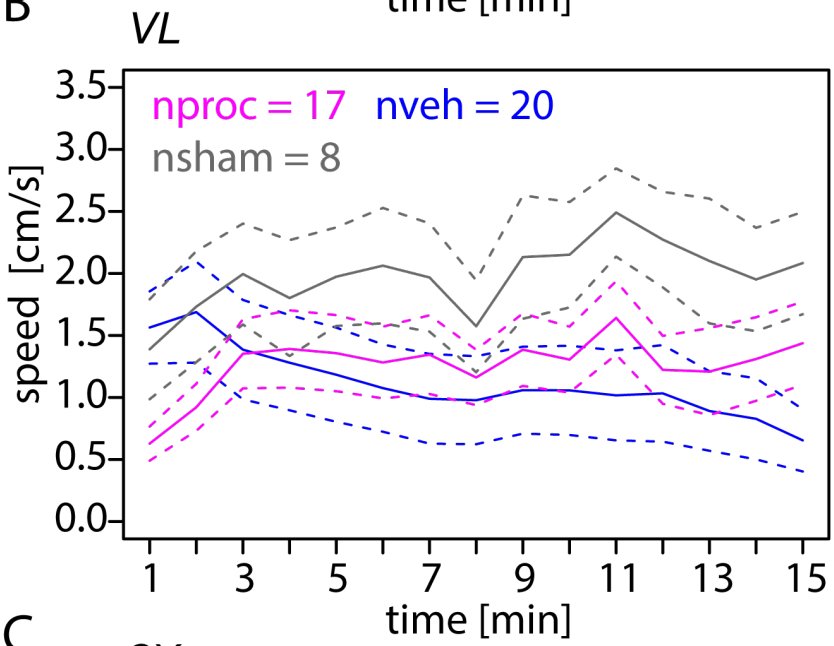
Additionally, we found a different rate of change for speed over time in the sham group after injections into the MBC and the VL in comparison to the sham group after injections into the CX (Table S 1). This was due to a higher number of bouts, rather than a difference in bout duration between the sham groups (Table S 1).

Figure 7: Walking activity in a dark rectangular chamber for animals injected into the MBC, the VLs and the CX/SMP. Means ± SEM are plotted for all variables. Sham-treated animals (sham) are shown in grey, vehicle-injected animals in blue and procaine-injected animals in magenta. Significant were effects determined with GLMMs ($p < 0.05$). **(A)** Speed in the chamber for animals injected into the MBC. A GLMM indicated a significant increase speed over time for the sham group. A significant effect of the procaine treatment on speed and a significantly different rate of change for speed over time was found for procaine and vehicle groups (Table S1). **(B)** Speed in the chamber for animals injected into the VL. A GLMM indicated a significant increase of speed over time for the sham group. A significantly different rate of change for speed over time was found for the vehicle group, but no significant effects of procaine treatment on speed were found (Table S1) **(C**) Speed in the chamber for animals injected into the CX. A GLMM indicated a significant increase of speed over time for the sham group. No significant effects of treatment on speed were found (Table S1).

A


MBC
nproc = 12
nveh = 9
nsham = 33
speed [cm/s]
time [min]

B


VL
nproc = 17
nveh = 20
nsham = 8
speed [cm/s]
time [min]

C

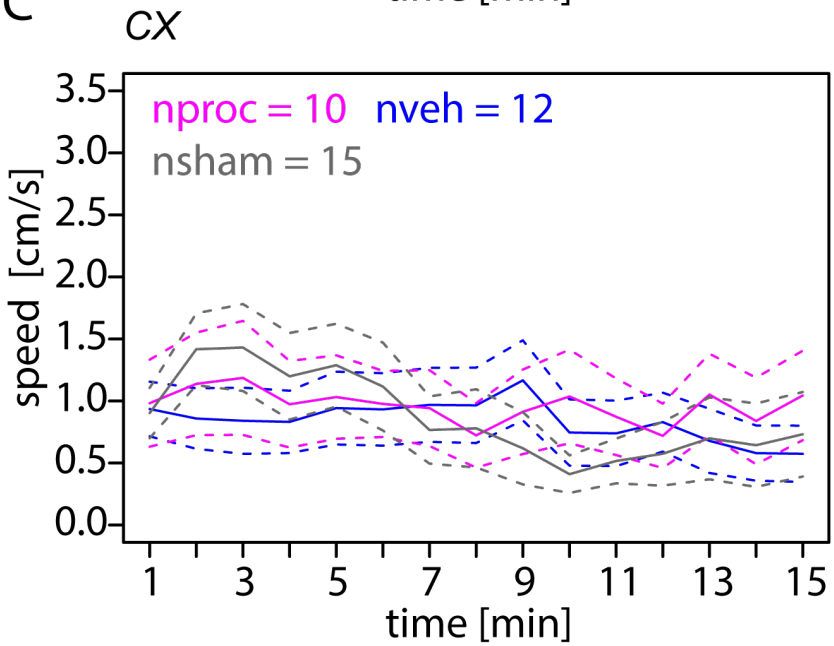
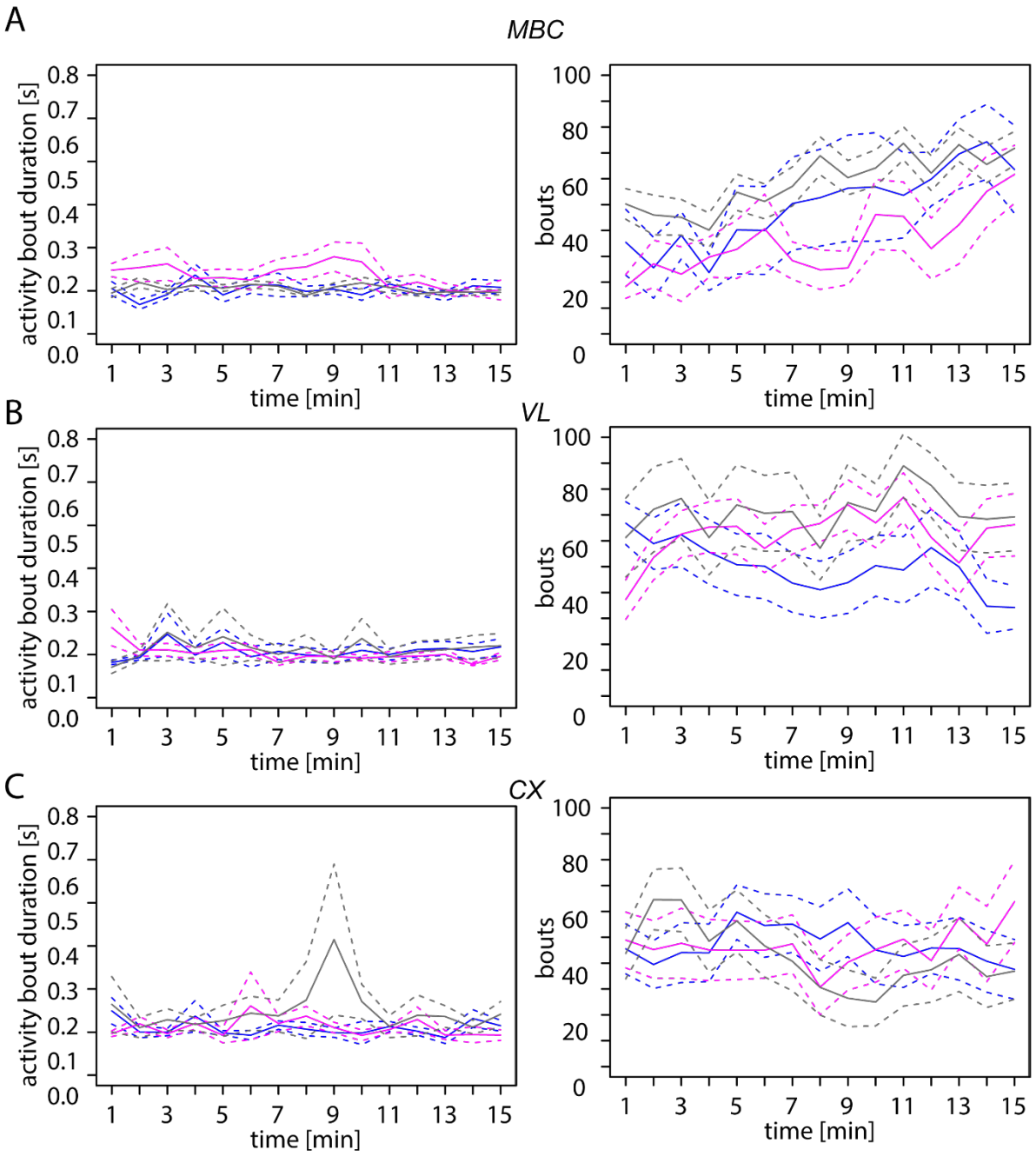

CX
nproc = 10
nveh = 12
nsham = 15
speed [cm/s]
time [min]

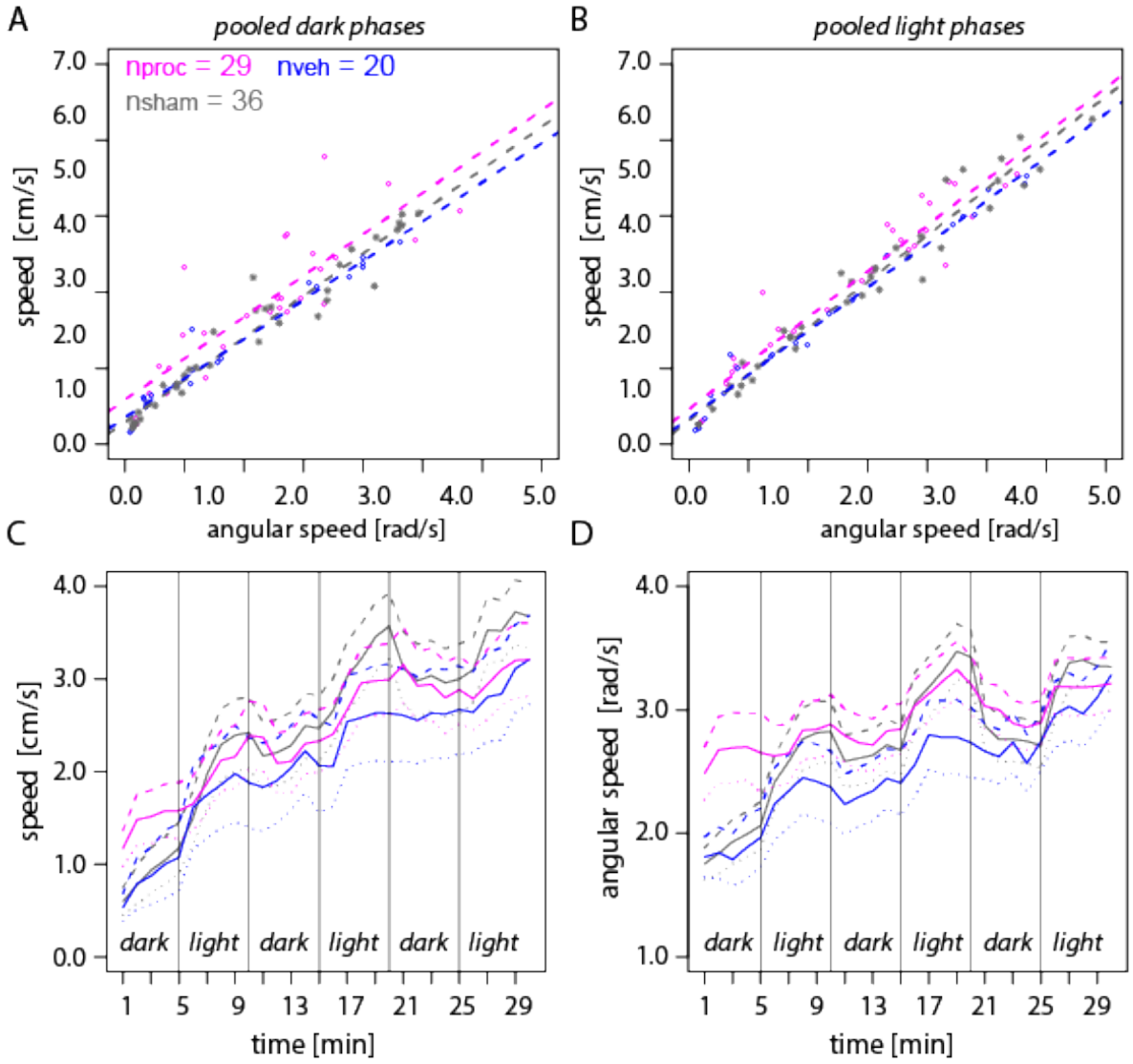
Figure 8: Activity bout duration and number of bouts performed in a dark rectangular chamber for animals injected into the MBC, the VLs and the CX/SMP. Means ± SEM are plotted for all variables. Sham-treated animals (sham) are shown in grey, vehicle-injected animals in blue and procaine-injected animals in magenta. Significant were effects determined with GLMMs ($p < 0.05$). **(A)** Activity bout duration (left) and number of bouts (right) for animals injected into the MBC. A GLMM indicated a significant effect of procaine treatment on activity bout duration and a significantly different rate of change for activity duration over time for the procaine group (Table S1). A GLMM indicated a significant increase of number of bouts over time for the sham group and a significantly different rate of change and intercept for bout number over time for the vehicle group and the procaine group (Table S 1). **(B)** Activity bout duration (left) and number of bouts (right) for animals injected into the VL. A GLMM indicated a significantly lower rate of activity duration over time for the procaine group (Table S 1). A GLMM indicated a significantly different rate of change for bout number over time for the vehicle group and the procaine group (Table S1). **(C**) Activity bout duration (left) and number of bouts (right) for animals injected into the CX. No significant effects were indicated by a GLMM for effects of treatment on activity bout duration (Table S 1). A GLMM indicated a significant decrease of number of bouts over time for the sham group and a significantly different rate of change for bout number over time for the vehicle group and the procaine group (Table S 1).


A
MBC
activity bout duration [s]
bouts
time [min]
B
VL
C
CX

Procaine-injections into the CX/SMP region resulted in high angular speeds in the dark

Next, we investigated locomotion and orientation towards lights in a round arena. Here, we compared animals injected with procaine into the CX/SMP area with vehicle and sham groups. First, we investigated the relationship of speed and angular speed. With increasing speeds, angular speed increased in the sham group (Table S 2). This was true for the dark phases (**Figure 9A**), as well as for the light phases with lights presented sequentially (**Figure 9B**). A significant effect and a different rate of change was found for the procaine group (Table S 2), but not for the vehicle group. Hence, procaine-treated animals show higher angular speed for similar speeds in comparison to the sham group. This was not the case for the light phase – here, no treatment effects were found.

Figure 9: Speed and angular speed in a round arena for animals injected into the CX/SMP with procaine (magenta), vehicle (blue) or sham-injected animals (grey). Means ± SEM are plotted for all variables. Significant effects were determined with GLMMs ($p < 0.05$). **(A)** Speed – angular speed relationship pooled for all three dark phases (1 – 5 min, 11 – 15 min, 21 – 25 min) in the round arena. A GLMM indicated a significant increase of angular speed with increasing speeds for the sham group. The rate of change was significantly different in procaine (Table S2) **(B)** Speed – angular speed relationship pooled for all three light phases (6 – 10 min, 16 – 20 min, 26 – 30 min) in the round arena. A GLMM indicated a significant increase of angular speed with increasing speeds for the sham group, but no effects of treatments on the rate of change were detected. **(C)** A GLMM indicated a significant increase of speed over time and a significant effect of the light phase on speed for the sham group (Table S2). The rate of change and the effect of the light phase on speed was significantly different in the vehicle and the procaine group (Table S2). In the dark phase, the procaine group displayed significantly higher speeds (Table S2) **(D)** A GLMM indicated a significant increase of angular speed over time and a significant effect of the light phase on angular speed for the sham group (Table S2). The rate of change was significantly different in the procaine group and the effect of the light phase on angular speed was significantly different in the vehicle and the procaine group (Table S2). In the dark phase, the procaine group displayed significantly higher speeds (Table S2)


A
pooled dark phases
nproc = 29
nveh = 20
nsham = 36
speed [cm/s]
angular speed [rad/s]
B
pooled light phases
speed [cm/s]
angular speed [rad/s]
C
speed [cm/s]
dark
light
dark
light
dark
light
time [min]
D
angular speed [rad/s]
dark
light
dark
light
dark
light
time [min]

Overall, speeds and angular speeds increased over all six phases for the sham group (**Figure 9C, D,** Table S 2). Additionally, speeds and angular speeds were higher in light phases compared to dark phases in the sham group. The procaine group exhibited higher speeds and angular speeds, but a lower rate of change for angular speeds and speeds. The latter was found for the vehicle group as well (**Figure 9C**, Table S 2). Both, procaine and vehicle groups exhibited a significantly lower effect of changing the phase from dark to light compared to the sham group for speeds and angular speeds (Table S 2).

In 4 out of 29 cases procaine-treated bees were not able to orient and walk properly and turned continuously in circles. Three bees with very lateral injections into the SMP circled very fast and turned into the contra-lateral direction of the injection site (Figure S 1). One bee with a more central injection changed turning sides and seemed to be walking backwards. These bees were able to walk straighter when a light appeared, but reverted back to circling after the light was switched off. Activity bout duration increased while the number of activity bouts decreased over the six phases of the experiment for the sham group **(Figure 10)**. A significant effect of the procaine-treatment on bout duration and on the rate of change (Table S 2). Bout number decreased less in vehicle and procaine groups (Table S 2).

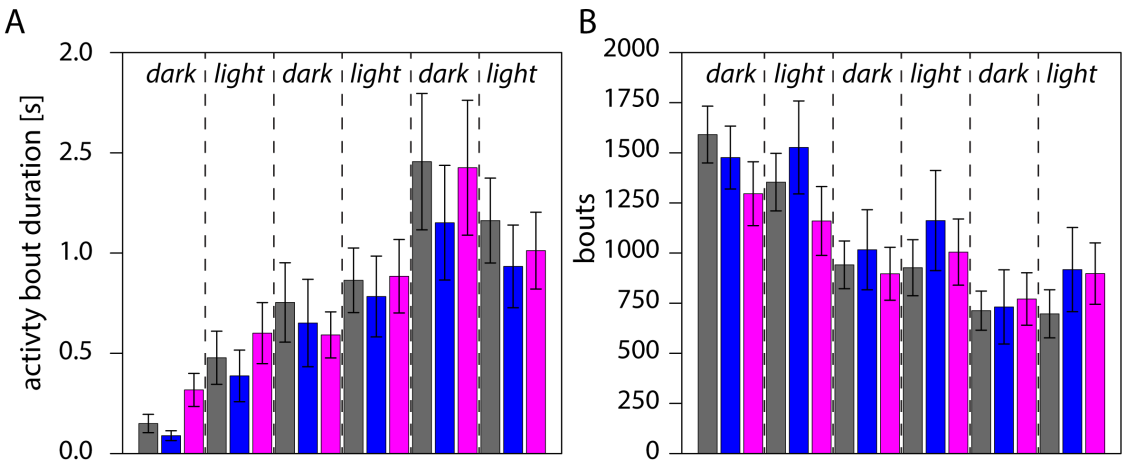


Figure 10: Activity in a round dish for animals injected into the CX/SMP with procaine (magenta) or vehicle solution(blue) or for sham-injected animals (grey). Means ± SEM are plotted for all variables. Significant effects were determined with GLMMs ($p < 0.05$) **(A)** A GLMM indicated a significant increase of bout duration over time for the sham group and a significant effect of the procaine treatment on bout duration (Table S 2). Bout duration and change of bout duration over time was found to be significantly different in the procaine group. **(B)** A GLMM indicated a significant increase of bout number over time for the sham group (Table S 2). Change of bout number over time was found to be significantly different in the vehicle and procaine group.

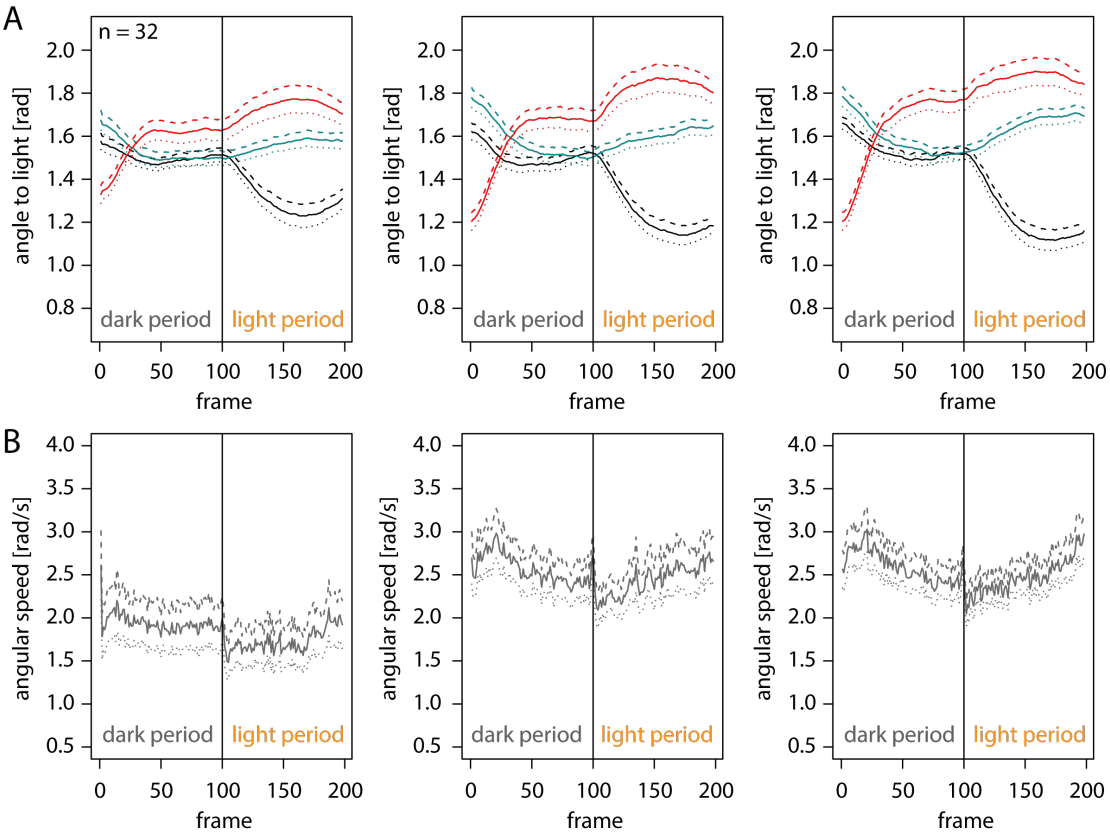
Control bees but not procaine-treated bees orient increasingly towards L(-1) and L(+1) in the sequence

After analyzing walking activity over the whole experiment and in the dark and light phases, we investigated orientation towards lights presented in a sequence in the three light phases. For this analysis, animals with very low movement speeds and very high angular speed (circling bees) were excluded. In the light phases, three LED lights were activated individually in a regular sequence (light period) with breaks in between (dark period). Initial observations of individual bees, led us to test whether a bee could remember L(-1) and/or anticipate L(+1). Since the main effect caused by the procaine-treatment was a change in angular speed (orientation), we first analyzed how the bees' orientation changed over the dark period and the light period. To illustrate of the orientation behavior, we plotted angle with respect to the three lights over one dark period and one light period. We pooled mean angles for each block of 75 trials **(Figure 11, Figure 12, Figure 13)** and quantified the observed effects (see below and **Figure 14**). The typical behavior of a sham-treated animal in this assay was to turn away from the light which just deactivated (L(0) in the dark period. (**Figure 11**). If a bee remembered L(-1), the angle to L(-1) should decrease, indicating a turn towards L(-1). If a bee anticipated L(+1), the angle to L(+1) should decrease, indicating a turn towards L(+1).

In the first block, bees initially turned towards L(-1) and L(+1) in the dark period, but did not continue to orient towards (L-1) or L(+1), which resulted in rather flat curves (**Figure 11**). In the last block, the bees continued to turn towards L(-1) and L(+1), resulting in a stronger decrease in angle (**Figure 11**, panel 3). In the light period, the bees turned towards the light which was active (L(on)). The strongest change seemed to occur between the first and the fourth bin of the dark period and the sixth and the ninth bin of the light period, when dark and light period were divided into five bins. When performing and LMM for this part of the curve, we found that angle of light changed over time in the dark period of block 1 for L(0), L(-1) and L(+1) (Table S 3). This was also true for the L(on) in the light period. We further found an effect of block and a significantly different rate of change for L(0), L(-1) and L(+1) in the dark period and of L(on) in the light period of block 2 and 3.(Table S 3)

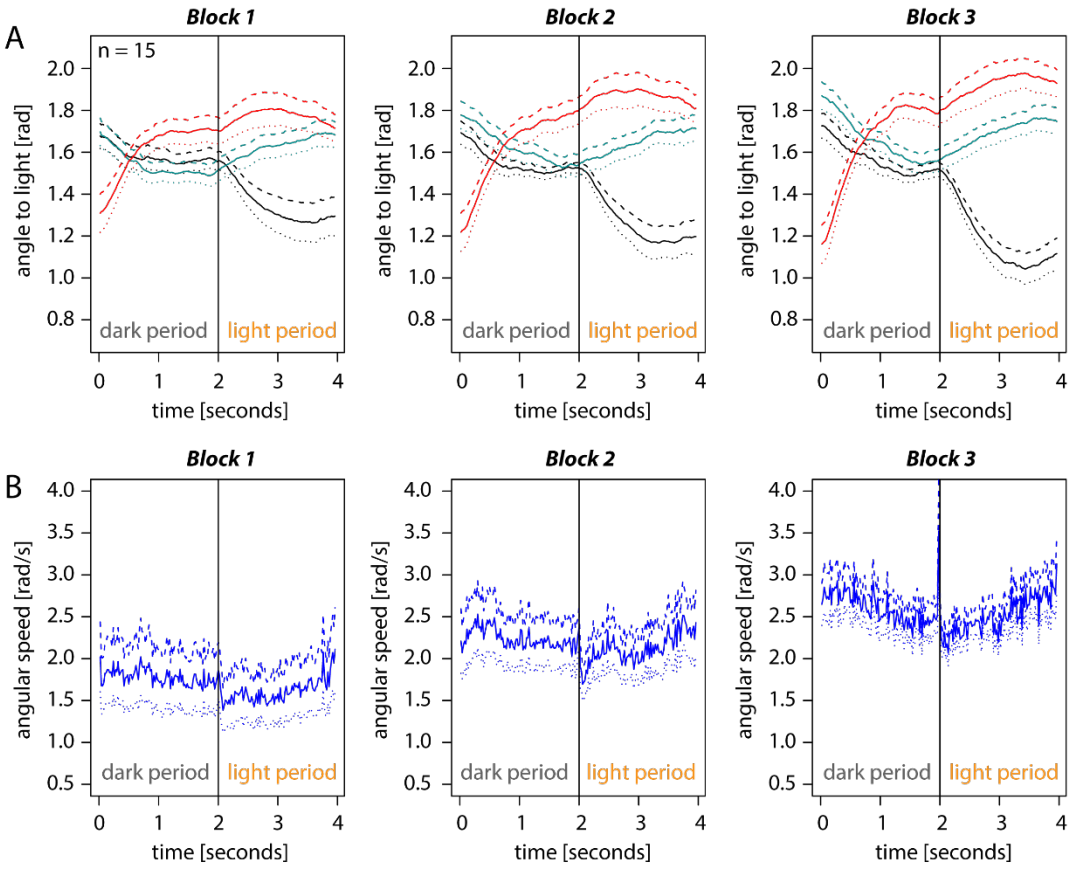
We observed, that the bees showed a high turn rate at the beginning of the dark period, after the light was just deactivated. This resulted in high angular speeds **(Figure 11 B)**. After the initial turn, most bees seem to walk straight into a certain direction, which resulted in a decreasing angular speed **(Figure 11 B)**. An LMM indicated a significant change of angular speed over time for the initial part of the curve (first to fourth bin). We found an effect of block and a different rate of change for block 2 and 3 (Table S 3). Here, we found significantly lower angular speeds for the dark period between light 1 and light 2, which were 180° apart (Table S 3).

Figure 11: Angle to the three light positions and angular speed over one dark and one light period pooled for each block of trials for sham-treated bees. Mean angle (A) or angular speed (B) ± SEM was pooled for each block (75 trials each). Significant effects were determined with LMMs ($p < 0.05$) **(A)** L(0) (red), L(+1) (black) or L(-1) (cyan) were deactivated in the dark period and the angle in respect to their positions is shown. A decrease in angle indicates a turn towards the respective light's position and an increase a turn away from the respective light's position. In the dark period, bees turned away (increasing angle to light) from L(0) and turned towards L(+1) and/or L(-1). An LMM performed on the initial of the curve (between 0 and 1.6 seconds in the dark period) indicated a significant effect of block and a significantly different rate of change for L(0), L(+1) and L(-1) in block 2 and 3 (Table S 3). In the next light period L(+1) was activated and became the L(on) (continues as black line). An LMM performed on the linear part of the curve (between 2 and 3.6 seconds in the light period) indicated a significant decrease of the angle over time for block 1 (Table S 3). The rate of change was significantly different in block 2 and block 3. **(B)** Bees showed high angular speeds in the beginning of the dark period decreasing over the dark period, which corresponds to less turning and a straighter walking path. An LMM performed on the initial part of the curve (between 0 and 1.6 seconds in the dark period) indicated a significant change of the angular speed over time for the block 1 (Table S 3). An effect of block on angular speed was found for block 2 and 3 (Table S 3).


A
n = 32
angle to light [rad]
2.0
1.8
1.6
1.4
1.2
1.0
0.8
dark period
light period
0
50
100
150
200
frame
B
angular speed [rad/s]
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5

We further investigated orientation behavior of the vehicle group (**Figure 12A**). Similar to the sham group, the vehicle group turned away from L(0) in the dark period and turned towards L(-1) and/or L(+1). For L(0) and L(-1) we found a significantly higher intercept in block 2 and 3, while for L(+1) we found this effect only in block 3 (Table S 3). Angle changed over time all lights in the dark period and different rates of change were found for block 2 and 3 (Table S 3). Similarly, angle to the activated light L(on) in the light period decreased over time and the change rate was different in block 2 and 3 (Table S 3), but an effect of block on angle was found for block 3, but not for block 2 (Table S 3). This suggests, vehicle-treated bees rather turn towards L(-1) in the initial phase of the experiment and that turning behavior to L(+1) in the dark period, which is subsequently activated in the light period, becomes more pronounced in block 3. Angular speed in the vehicle group decreased over time in the dark period (**Figure 12B**) (Table S 3). We found that angular speed was higher in block 2 and 3 (Table S 3). Additionally, the drop in angular speed was more pronounced in block 2 and 3 (Table S 3).

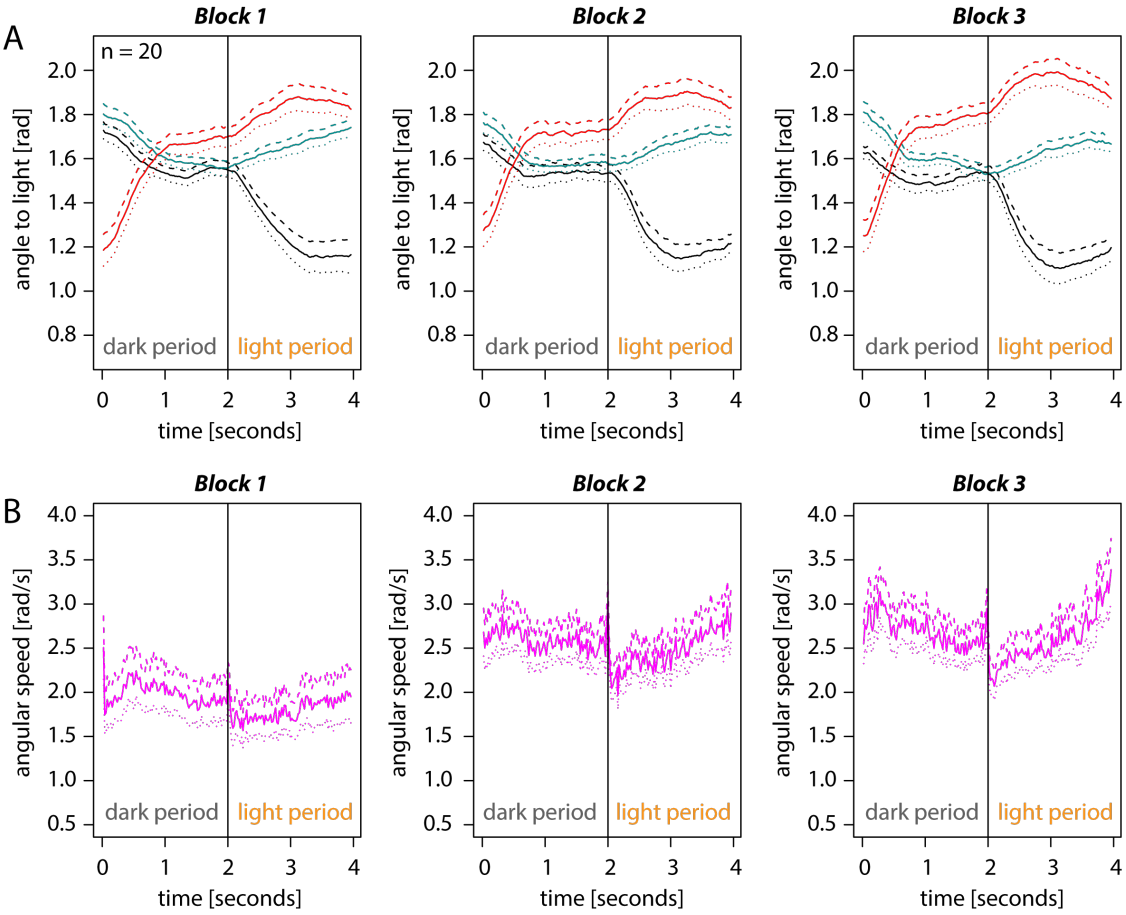
Figure 12: Orientation and angular speed of vehicle-injected animals. Mean angle (A) or angular speed (B) ± SEM was pooled for each block (75 trials each). Significant effects were determined with LMMs ($p < 0.05$). **(A)** L(0) (red), L(+1) (black) or L(-1) (cyan) were deactivated in the dark period and the angle in respect to their positions is shown. A decrease in angle indicates a turn towards the respective light's position and an increase a turn away from the respective light's position. In the dark period, bees turned away (increasing angle to light) from L(0) and turned towards L(+1) and/or L(-1). An LMM performed on the initial of the curve (between 0 and 1.6 seconds in the dark period) indicated a significant effect of block on angle was found for L(0) and L(-1), while for L(+1) a significant effect of block on angle was found for block 2 but not for block 3 (Table S 3). A significantly different rate of change for L(0), L(+1) and L(-1) in block 2 and 3. In the next light period L(+1) was activated and became the L(on) (continues as black line) . An LMM performed on the linear part of the curve (between 2 and 3.6 seconds in the light period) indicated a significant decrease of the angle over time for block 1 (Table S 3). A significant effect of block on angle was found for block 2 but not for block 3 (Table S 3). **(B)** Bees showed high angular speeds in the beginning of the dark period decreasing over the dark period, which corresponds to less turning and a straighter walking path. An LMM performed on the initial part of the curve (between 0 and 1.6 seconds in the dark period) indicated a significant change of the angular speed over time for the block 1 (Table S 3). The rate of change was significantly different in block 2 and block 3. (Table S 3).


A
Block 1
Block 2
Block 3
n = 15
angle to light [rad]
dark period
light period
time [seconds]
B
angular speed [rad/s]

When animals were injected with procaine, the turns away from L(0) and towards L(-1) and L(+1) were strongest in block 1 (**Figure 13A**). In contrast sham and vehicle groups, intercepts were higher for L(0) and lower for L(-1) and L(+1) and the rate of change was lower for all three lights in block 2 and 3 (Table S 3). This suggests, that rather than performing larger turns away from L(0) and towards L(-1)/L(+1), procaine-treated bees performed smaller turns. Additionally, angles were found to be higher for the dark period between lights 1 and 2 which were 180° apart for L(+1) and lower for the dark period between lights 2 and 3, which were 90° apart (Table S 3) A similar effect was found in the light period, where bees performed larger turns towards the activated light in block 1 compared to blocks 2 and 3 (Table S 3) (**Figure 13B**). Angular speeds in the dark period were found to be higher in blocks 2 and 3 compared to block 1 and a drop in angular speed was found in block 1. The rate of change was not different in block 2, but in block 3.

This suggests, that the procaine groups showed high changes in orientation in response to the light sequence in block 1. Rather than becoming more pronounced orientation towards and away the positions of the light in the dark period and the activated light in the light period as it was found for control bees, orientation became less pronounced in the procaine group.

Figure 13: Orientation and angular speed of procaine-injected animals. Mean angle (A) or angular speed (B) ± SEM was pooled for each block (75 trials each). Significant effects were determined with LMMs ($p < 0.05$). **(A)** L(0) (red), L(+1) (black) or L(-1) (cyan) were deactivated in the dark period and the angle in respect to their positions is shown. A decrease in angle indicates a turn towards the respective light's position and an increase a turn away from the respective light's position. In the dark period, bees turned away (increasing angle to light) from L(0) and turned towards L(+1) and/or L(-1) in block 1. An LMM performed on the initial of the curve (between 0 and 1.6 seconds in the dark period) indicated a significant effect of block on angle was found for L(0), L(-1) and L(+1) (Table S 3) A significantly different rate of change for L(0), L(+1) and L(-1) in block 2 and 3. In the next light period L(+1) was activated and became the L(on) (continues as black line). An LMM performed on the linear part of the curve (between 2 and 3.6 seconds in the light period) indicated a significant decrease of the angle over time for block 1 (Table S 3). A significant effect of block on angle was found for block 2 but not for block 3. The rate of change was significantly different in block 2 and block 3 (Table S 3). **(B)** Bees showed high angular speeds in the beginning of the dark period decreasing over the dark period, which corresponds to less turning and a straighter walking path. An LMM performed on the initial part of the curve (between 0 and 1.6 seconds in the dark period) indicated a significant change of the angular speed over time for the block 1 (Table S 3). The rate of change was significantly different in block 2 and block 3 (Table S 3)


A
Block 1
Block 2
Block 3
n = 20
angle to light [rad]
dark period
light period
time [seconds]
B
angular speed [rad/s]

Control animals but not procaine-treated animals turned towards L(+1)

To quantify how orientation in regard to the different lights' positions changed and to compare the treatment groups, we analyzed angle difference and angular speed difference (see **Figure 6**). We calculated the difference between the mean angle for the lowest part of the curve (bin 4) and the angle at the beginning of the dark period (bin 1). This was done for the light period similarly (difference between bin 9 and bin 6). Difference showed how the angle towards a light's position or the amount of turning changed from the initial dark or light period to the later part of the dark or light period.

Angle differences and angular speed differences were determined for all treatment groups **(Figure S 2, Figure S 3, Figure S 4)** and effects were analyzed with a GLMM (Table S 4). Regression lines were compared between treatments **(Figure 14)**. The control groups, sham- and vehicle-treated animals, increasingly turned towards the light L(on), when it was activated in the light periods (Table S 4) **(Figure 14A)**. This was not the case for the procaine group, which did not show any change in angle difference over light periods (Table S 4) **(Figure 14A)**. This means, that procaine-treated animals did not change their orientation behavior to the activated light over the course of the experiment. Interestingly, larger turns towards light 2 were found in comparison to light 1 the beginning of the experiment (Table S 4). This was not the case for light 3. Light 2 was located 180° in relation to light 1.

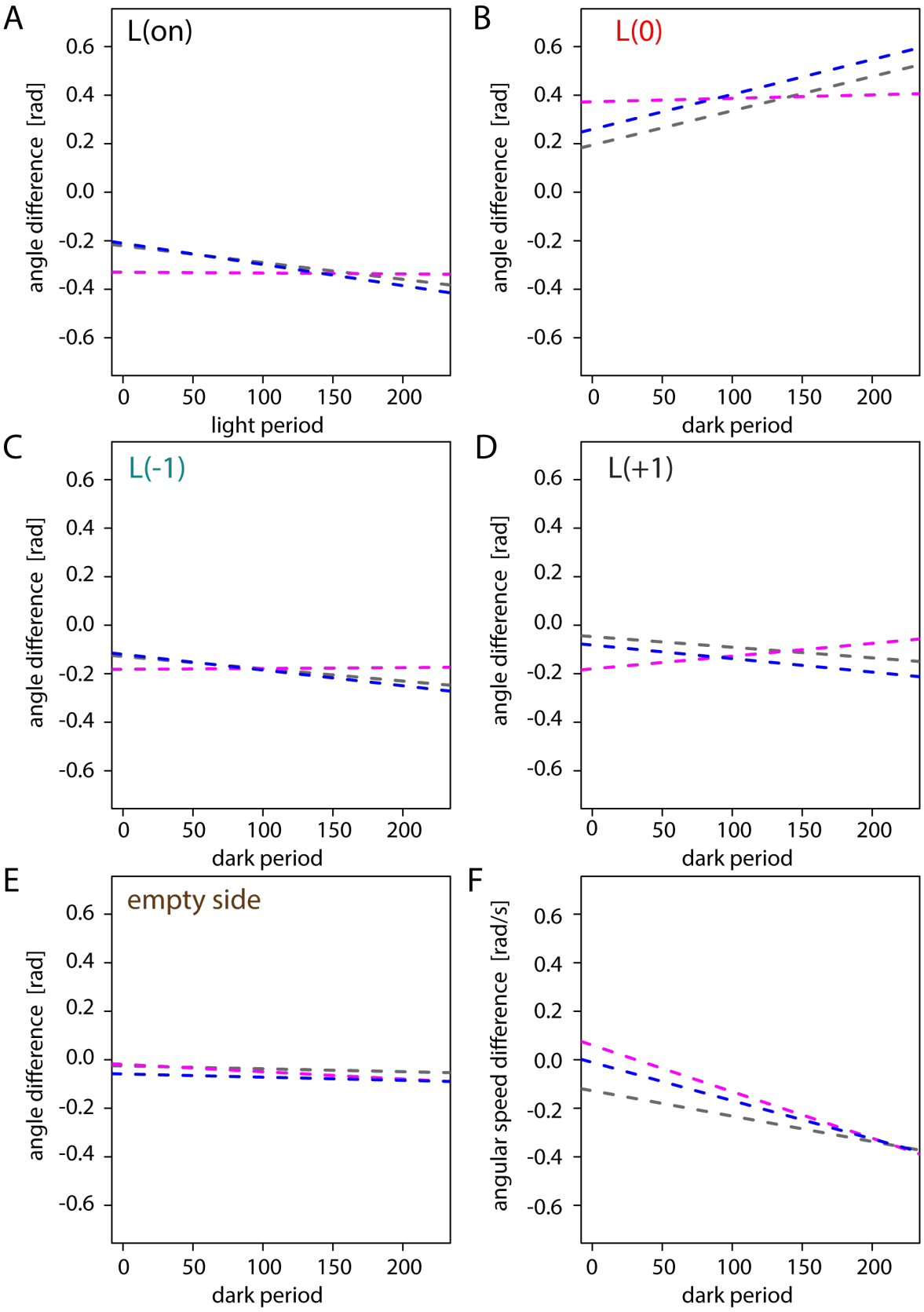
In the dark periods, control animals increasingly turned away from L(0). The procaine group, in contrast, showed higher angle differences (larger turns) at the beginning of the experiments, and a lower rate of change over dark periods (Table S 4) **(Figure 14B)**. When turning away from L(0), control bees increasingly turned towards L(-1) and/or L(+1) over dark periods (Table S 4) **(Figure 14C, D)**. This behavior was not observed in the procaine group, which instead did not increase turns towards L(-1) and decreased turns towards L(+1). Angle differences were different in the beginning of the experiment for the procaine group in comparison to the sham group (Table S 4) **(Figure 14D)**.

We found significantly larger turns away from the position of light 2 (when light 3 was activated next) early in the experiment (Table S 4). Additionally, we found larger turns towards the position of light 1, when it was the preceding light in the sequence (L(-1)) early in the experiment and to the position of light 2 when it was the next light in the sequence (L(+1)) early in the experiment. This suggests, that the bees tended to orient towards and away from the position of light 2 in the early dark periods. None of the treatment groups increased turns towards the empty side of the arena, where no light was activated at any time (Table S 4) **(Figure 14E)**. At the beginning of the experiment, however, the bees tended to orient towards the empty side more often when light 2 or light 3 was activated next.

Next, we found that the drop in angular speed which was shown in previous analyses did increase in all treatment groups over dark periods (Table S 4) **(Figure 14F)**. Taken together, this suggests that control animals were able to change orientation behavior with experience, while procaine animals showed overall larger turns in the beginning of the experiment, but did not change orientation behavior over the course of the experiment. The drop in angular speed was less pronounced in the beginning of the experiment when light 2 or light 3 were activated next in comparison to light 1.

The effects described above seemed to be quite consistent between individuals, even in individual which showed very low activity over most of the experiment (Figure S 5 - Figure S 13**).** Interestingly, these individuals display the same trends of orientation which can be seen in very active individuals. This suggests that the animals might learn the positions of the light, even when they are sitting still.

Figure 14: With experience, control animals increased orientation towards L(-1) or L(+1). Regression lines determined with GLMMs comparing mean angle differences **(A-E)** or mean angular differences over dark periods **(B-F)** or light periods **(A)** for the sham group (grey), the vehicle group (blue) and the procaine group (magenta). Significant effects were determined with GLMMs ($p < 0.05$). **(A)** Sham and vehicle groups increasingly turn towards the activated light L(on) (negative angle differences). Change of angle difference over light periods was significant (Table S 4). Change of angle difference over light periods was significantly different in the procaine group and close to zero. **(B)** Sham and vehicle groups increasingly turned away from L(0) (Table S 4) (positive angle difference). Angle difference were higher in the beginning of experiment for the procaine group in comparison to the sham group (Table S 4). Change of angle difference over light periods was significantly different in the procaine group (Table **S 4**). **(C)** Sham and vehicle groups increasingly turn towards the L(-1) (negative angle differences). Change of angle difference over light periods was significant (Table S 4). Change of angle difference over light periods was significantly different in the procaine group and close to zero **(D)** Sham and vehicle groups increasingly turn towards the L(+1) (negative angle differences). Change of angle difference over light periods was significant (Table S 4). Angle difference were higher in the beginning of experiment for the procaine group in comparison to the sham group (Table S 4). Change of angle difference over light periods was significantly different in the procaine group with a positive slope **(E)** Angle differences did not change significantly for the empty side of the arena in sham, vehicle and procaine groups (Table S 4). **(F)** Sham and vehicle and procaine groups showed increasing drops in angular speeds over dark periods (negative angular speed differences). Change of angular difference over dark periods was significant (Table S 4).


A
L(on)
angle difference [rad]
light period
B
L(0)
angle difference [rad]
dark period
C
L(-1)
angle difference [rad]
dark period
D
L(+1)
angle difference [rad]
dark period
E
empty side
angle difference [rad]
dark period
F
angular speed difference [rad/s]
dark period

Bees in the arena approach L(+1) independent of treatment

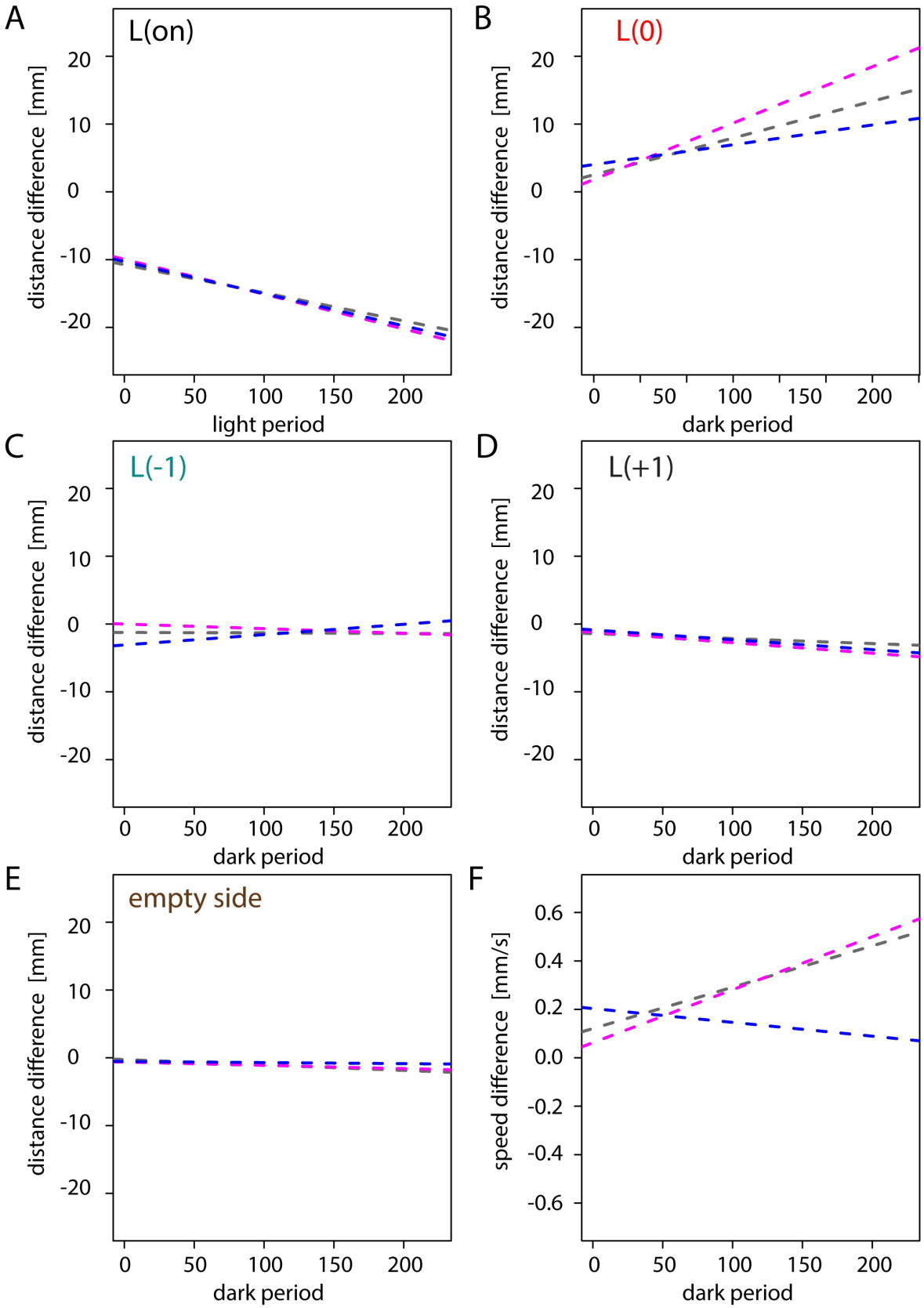
Analogous to the angle and angular speed analyses, we determined distance differences and speed differences **(Figure 15)**. A negative distance difference indicates that the bee approached the position of the light and a positive distance difference that the bee moved away from the light.

Interestingly, we found very few treatment effects for mean distance difference over light or dark periods (Table S 5). Independent of treatment, the animals increased approaches to the activated light L(on) **(Figure 15 A)**. Approaches of light 2 were more pronounced in the beginning of the experiment, compared to light 1 (Table S 5). This was not the case for light 3.

Over dark periods, the bees walked away from L(0) **(Figure 15 B)**and increasingly approached L(+1) **(Figure 15 D)**. When light 3 was activated next, however, the change of distance away from light 2 was not as high as for light 3. Also, the light 3 was approached less, when it was activated next in the sequence (L(+1)) in the beginning of the experiment. No change in distance difference over dark periods for L(-1) was found in the sham and procaine group, but the rate of change was significantly different in the vehicle group (positive slope) **(Figure 15 D)**. When light 3 was activated next, however, bees tended to approach the preceding light 1 (L(-1) in the sequence in the beginning of the experiment. This suggests, that bees in the arena tend to approach the position of light 1 more in the beginning of the experiment.

Curiously, we found a significant change of mean distance difference over dark periods for the empty side (Table S 5) **(Figure 15 E)**. Approaches to the empty side of the arena were more pronounced, when light 2 or light 3 was activated next (Table S 5). We found an increase in speed in the dark phase for all treatment groups (Table S 5) **(Figure 15 F).** This increase in speed became larger over dark periods for the sham and procaine groups (Table S 5) **(Figure 15 F)**. For the vehicle group, however, this increase in speed became smaller over dark periods.

Figure 15: With experience, animals were closer to L(+1) in the dark periods.
Regression lines determined with GLMMs comparing mean distance differences **(A-E)** or mean speed differences over dark periods **(B-F)** or light periods **(A)** for the sham group (grey), the vehicle group (blue) and the procaine group (magenta). Significant effects were determined with GLMMs ($p < 0.05$). **(A)** The Sham group showed larger distance changes when the light L(on) was activated (negative distance differences). This behavior was not influenced by treatment (Table S 5). Over dark periods, all groups walked further away from L(0) **(B)** and increasingly approached L (+1) **(D)**, but not L(-1) **(C). (E)** Over dark periods, all groups increasingly approached the empty side of the arena (Table S 5). **(F)**The sham and procaine groups increased speeds in the initial dark period. This increase became more pronounced over dark periods. In the vehicle group the speed increase became less pronounced over dark periods (Table S 5).


A
L(on)
distance difference [mm]
light period
B
L(0)
distance difference [mm]
dark period
C
L(-1)
distance difference [mm]
dark period
D
L(+1)
distance difference [mm]
dark period
E
empty side
distance difference [mm]
dark period
F
speed difference [mm/s]
dark period

Discussion

Here, we explored how the mushroom bodies, the central complex and the adjacent superior medial protocerebrum are involved in regulating walking activity and turning under different light conditions.

The MBCs are involved in regulating walking activity

Injections of procaine into the MBC, but not the VLs led to reduced speed, when bees were walking in a dark chamber (**Figure 7A, B**). In both, the procaine and the vehicle group, the increase of speed over time was larger than in the sham group (**Figure 7A**). The vehicle group displayed slightly lower speeds (trend) in the beginning of the experiment in the vehicle group, but the bees seemed to have recovered quickly and displayed similar speeds compared to the sham group in the second half of the experiment (**Figure 7A**). Interestingly, we found a higher duration of activity bouts in the procaine groups injected to the MBC **(Figure 8A)**. Injection of vehicle and procaine solutions into the MBC, led to a reduction in bout number in the beginning of the experiment **(Figure 8A)** and a higher increase of number of bouts over time compared to the sham group. This suggests, that injection of a solution itself decreases the frequency of walking activity, but while the vehicle group seems to recover quickly, injection of the procaine solution result in longer bouts of lower speeds over the course of the experiment.

In animals injected into the VLs we found increasing speeds over time for the sham group (**Figure 7B**), but a decrease in speed over time for the vehicle group. In this case no effect of the procaine treatment on speed was found. Interestingly, procaine injections into the VLs led to a decrease in activity bout duration over time **(Figure 8B)**. While no effect of treatment on the number of bouts was found, number of bouts increased over time for the procaine group and decreased over time for the vehicle group **(Figure 8B)**. This suggests, that vehicle injections into the VLs by itself impaired walking activity. The addition of procaine seems to increase walking activity to a degree, leading to shorter bouts of higher activity.

A subgroup of the animals analyzed here was used to study color learning after sham, vehicle and procaine injections (Plath et al., 2017). Our findings showed that vehicle and

procaine-injections into this area of the VLs led to an impairment of aversive learning of colored lights, when speeds were similar in all groups (Plath et al., 2017). This suggest, that even though deficits in walking activity were not detected they could still have been the cause for the reduced performance in the learning assay for the vehicle group. Further large-scale analyses of individual effects could clarify this.

The effects found for the procaine group seemed to be reduced in the final minutes of the experiment. This could indicate that the procaine effect started to wear off towards the end of the experiment due to dilution or break-down of the drug. The effect duration of procaine was similar in cockroaches, which showed reduced optomotor responses for approximately 15 minutes after injections into the CX (Kathman et al., 2014).

There is evidence from multiple electrophysiological studies of different insects, that the VLs process mechanosensory and tactile stimuli (Li and Strausfeld, 1997), which could be important to an insect walking in dark conditions. Studies in free-walking crickets have shown that activity of some neurons found in the MB lobes correlated with walking activity in cockroaches (Mizunami et al., 1998a). This units could have been affected by the injections, however, why and if the addition of procaine counteracts the vehicle effect remains to be investigated. It is possible, but highly speculative, that the injection of ions with the vehicle solution imbalances neural activity in this region, while procaine injection would lead to a general decrease of neural activity in the region.

Findings obtained with fruit flies with ablated MBs also showed reduced walking activity in an initial walking period of 15 minutes (Serway et al., 2009), when flies were stimulated to walk with vertical bars as visual stimuli. These and our findings stand in contrast to other findings in fruit flies (Helfrich-Forster et al., 2002; Martin et al., 1998) and cockroaches (Kaiser and Libersat, 2015), which have shown that disruption of MBs increased long-term walking activity. At a closer look, most groups of flies with ablated or disturbed Kenyon cells in the MBs showed a lower walking activity in the first 10 – 15 minutes compared to controls (Martin et al., 1998). But while walking activity decreased rapidly in controls, walking activity in flies with ablated or disturbed MBs remained rather constant or only decreased slowly. Similar to findings in this study, walking bout duration was also higher

in MB-disturbed flies compared to controls. However, while the average duration of bouts in this study was determined as less than one second, flies continuously walked for hundreds of seconds in one bout (Martin et al., 1998). Since the body to chamber ratio was similar in this study and in the study presented by Martin et al. (1998), this could either be due to the difference in measurement techniques or due to differences in the species' ecology. While Martin et al. (1998) measured walking activity by crossing of a single infrared beam in the center of the chamber, we were able to monitor walking activity throughout the chamber. Thus, one large bout of "continuous activity" recorded by crossing a single beam in the chamber by in the flies could in fact be a cluster of very short bouts of walking and pausing, which were not registered by the single infrared beam.

As Martin et al. (1998) point out, the initial peak of activity in control flies could be due to handling and/or the novel situation in the experimental chamber and activity decreases over time. We propose, structuring of activity and resting periods as well as walking speed in response to the surroundings seem to be regulated by the Kenyon cells in the MBs. In this study, a change in activity was already seen in procaine-treated bees, when only the medial collar region of one out of four MBCs was targeted. This region receives visual input in the honey bee (Ehmer and Gronenberg, 2002; Gronenberg and Lopez-Riquelme, 2004). Since no visual input was given to the animals in this study, why should this region be involved in locomotor control? In flies, the MBs are required for generalization of different visual contexts provided by background illumination with lights of different wavelengths (Liu et al., 1999). The authors propose that this process relies on ongoing analysis of incoming sensory stimuli. This suggests, that the absence of visual stimuli could also serve as a context for the animal and that this process was disrupted by procaine-injections into the MBC in this study.

The CB and the SMP are involved in orientation in dark and light conditions

We did not find an effect of treatment on walking speeds in the dark rectangular chamber for animals injected with procaine into the CX/SMP (**Figure 7C**). However, sham injections into the CX region led a decrease in speed and bout number over time, while sham injections into the MBC and into the VLs led to an increase in speed bout number over time.

This suggests, that inserting the injection pipette itself might already cause an impairment in walking activity. As the CX integrates major connections between to hemispheres to modify motor output (Plath and Barron, 2007), even small disruptions to the network might cause an impairment in motor control.

We further tested walking activity and orientation, as well as orientation learning in a round arena with alternating dark and light phases. In the light phase, three distinct visual stimuli (LEDs) were activated in a sequence. We first analyzed walking activity in the arena (**Figure 9**). We found that speed and angular speed increased over time and higher speeds in the light phases compared to the dark phases for the sham group. After vehicle injections, we found a lower increase of speed, but not for angular speed over time. Additionally, the effect of the light phase on speed and angular speed was lower in the vehicle group. The same effects were found for the procaine group, but the procaine group additionally displayed higher speeds and angular speeds in the dark phase and a significantly lower increase of angular speed over time. Higher speeds were a result of higher bout durations in the procaine group **(Figure 10A)**. This suggests that procaine injections mainly affect the orientation of the animals and structure of walking activity, while walking speed was affected by vehicle as well as procaine injections. It seems, already small disturbances to the region, such injecting a vehicle solution impair locomotion, which again suggests, that this region is particular sensitive to any kind of disruptions.

This stands in contrast to results obtained with fruit flies with a disturbed or ablated CX (Poeck et al., 2008; Strauss, 2002; Strauss et al., 1992; Strauss and Heisenberg, 1993) or cockroaches which were injected with considerably larger volumes of procaine solution (Kaiser and Libersat, 2015): walking activity of animals with a disturbed CX was considerably reduced compared to controls. In all these studies, connectivity across the midline was disrupted. It has been suggested that left-right balancing of input in the CX is crucial for control of locomotion (Strausfeld, 1999; Strauss, 2002). It is therefore possible that injections of smaller volumes such as used here did not disrupt these processes in a degree to affect walking speed. This provides an advantage for the study of motor patterns or responses to stimuli that rely on locomotion. Indeed, we found that procaine-injections into the CX led to an impaired response to a learned visual stimulus in free-walking bees

(Plath et al., 2017).

When analyzing the relationship of angular speed and speed, we found that procaine-injected animals displayed higher angular speeds at similar sham-injected animals in the dark phases, but not the light phases (**Figure 9**). The effect on turning behavior was strongest in four procaine-injected animals, which circled continuously **(Figure S 1)**. Circling behavior was also observed in the fruit fly CX-mutant *C31* (Strauss, 2002) and in flies lacking expression of the transcription factor Single minded (Sim) (Pielage et al., 2002). In these flies the midline of the CBU and CBL (*C31)*, or the CBU and the PB are partially disrupted. Circling and abnormal turning behavior was also observed in cockroaches with off-center lesions around the CB, cutting off input from the surrounding protocerebrum (Ridgel et al., 2007). This suggests, that a disruption of hemisphere crossing input or a unilateral disturbance of the CX and the adjacent SMP causes a continuous turning behavior.

But why were the circling bees able to walk straighter when a visual target was visible? Visual input is mainly relayed to the CX via the lateral accessory lobes (Pfeiffer and Homberg, 2014), which is integrated to a representation of the animals heading and orientation in relation to external landmarks (Green et al., 2017; Kim et al., 2017; Seelig and Jayaraman, 2015; Turner-Evans et al., 2017; Varga and Ritzmann, 2016). The underlying network in the PB and the CBL integrates and accumulates body angle to shift the neural representation of the orientation angle (Green et al., 2017; Turner-Evans et al., 2017). Neuronal activity in units connecting the PB and the CBL correlated with angular velocity (Green et al., 2017; Turner-Evans et al., 2017). Turner-Evans et al. (2017) and Green et al. (2017) propose that heading direction coding in the CX requires integration of visual stimuli and proprioceptive feedback. The SMP receives mechanosensory inputs and proprioceptive inputs from other regions of the brain (Ignell et al., 2005; Strausfeld, 1976), including the antennal mechanosensory and motor center. Since the antennae play an important role in course control and orientation in insects (Staudacher et al., 2005), the SMP could receive feedback information about head and antennae positions. Taken together, this suggests that the SMP could provide proprioceptive information to the CX to be integrated with visual stimuli, which was disrupted by procaine-injection into the CX/SMP. Further anatomical

and electrophysiological studies could clarify the role of different neurons in the SMP.

Control bees learned positions of the preceding and upcoming light in a sequence

We further analyzed spatial learning and memory in the light phases of the experiment, where three lights around an arena were displayed in a sequence. In the dark period between two lights, we found that with increasing experience of the sequence sham-injected as well as vehicle-injected bees increasingly oriented away from last light to be on (L(0)) and instead oriented towards the preceding light's position (L(-1)) or the upcoming light's position (L(+1)) **(Figure 14 B-D)**. This was not true for the empty side of the arena, which showed that these findings were not caused by a general increase of turns into random directions **(Figure 14 E)**. Additionally, we found a drop in angular speed in the dark period **(Figure 14 F)**. This indicates, that the decrease in orientation angle towards the L-1) or L(+1) was caused by a rapid turn away from L(0) and a stable orientation towards a remembered target. Orientation towards the active light (L(on)) also increased over time. This could be due to the stronger orientation towards the light before it was activated **(Figure 14 A)**. In the beginning of the experiment, bees performed larger turns away from light 2 (L(0)) and towards light 1 (L(-1)), when light 3 was activated next. Bees performed larger turns towards light 2, however, when it activated next. This suggests, that initially only the positions of the light could be remembered, but not the sequence.

We further analyzed how the distance to the lights' positions changed in the dark and light periods. Interestingly, sham and vehicle groups increased approaches to (decreased their distance) L(+1) over time, but not to L(-1) **(Figure 15 C,D)**. Additionally, vehicle-treated animals reduced speed in the dark period in contrast to sham-treated animals, which increased speed. As discussed above, the vehicle injections had an effect on speed, but not angular speed. But vehicle-treated were still able to increase turns towards the next light in the sequence. This suggests, that learning abilities were still intact after vehicle injections into the CX/SMP. Curiously, we also found an increase in turns towards the empty side of the arena. This could be due to bees walking via the empty side towards a different light's position, while following the border of the arena.

At this point, we cannot say if bees truly anticipate L(+1) in a sequence, since turning

towards L(+1) as well as L(-1) improved over time. Also, the distance difference effects observed were overall not very strong. The behavior might have been affected by the background light of the LCD screen, which could have been slightly variable between individual assays. Since the results were quite similar between individuals, however, this was likely no confound. Also, the results clearly show that the bees learned about the positions of the lights even when they were off. Turning towards L(-1) could be merely a result of spatial working memory similar to that observed in the detour paradigm in flies (Neuser et al., 2008). However, since the orientation behaviour towards L(-1) as well as L(+1) improved over trials, the observed behaviour was most likely due to learning of the positions and/or the spatial relationship of the lights. This is further supported by the findings of the distance analyses, since bees clearly increased approaches the next light in the sequence only and by the finding that bees did not increase turning towards the empty side of the arena on average.

It is unlikely that this was only due to olfactory cues followed by the bee, since the bees were located at different positions in the dish in every trial. However, this could be tested in future experiments: the bee could be removed from the dish after undergoing the sequence assay and be placed into a new dish to test for retrieval of the spatial memory at different time points after the assay. This would (A) eliminate the possibility of any odor cues and (B) test if memory retrieval is possible and if this changes over time.

Honey bees are successful in different forms of elemental and non-elemental forms of learning, where a stimulus is given together with a reward or punishment (Giurfa, 2003; Giurfa, 2007). It is remarkable, that the bees improved orientation towards L(-1) and L(+1), since no overt reinforcement was presented here. This suggests, that the internal drive (phototaxis) to approach a light is very strong and might be self-reinforcing. Alternatively, reaching the light without being able to escape the dish could serve as a punishment.

Phototaxis changes with the behavioral development of honey bees (Ben-Shahar, 2005; Ben-Shahar et al., 2003). In contrast to foragers, nurses are only weakly phototactic (Southwick and Moritz, 1987). Recent findings in fruit flies indicate that phototaxis in insects is not a hard-wired process, but can be influenced by behavioral states (Gorostiza et

al., 2016). The authors found a change in phototactic behavior in flies with missing wing utility and concluded that phototactic behavior is flexible and responsive to internal and external changes. In future experiments, it would be interesting to investigate how nurses or insects with other ecologies perform in the sequence assay. This could also show if anticipation of the upcoming light depends on positive phototaxis.

Procaine injections impaired the ability to remember the light positions

We found that procaine-injections into the CX/SMP region resulted in an impairment in spatial learning. Procaine-treated bees were unable to orient towards the upcoming light (L(+1)) in the sequence **(Figure 14).** Overall, larger turns were found in the early phase of the experiment compared to the sham group with little changes over time **(Figure 14)**. Nevertheless, procaine-treated bees still produced a drop in angular speed. This could indicate that they were still able stay oriented towards a certain direction, but might not able to anticipate were the next light would be activated. This behavior was quite consistent between individuals **(**Figure S 11**,** Figure S 12**,** Figure S 13**),** which suggests that the behavioral effect caused by the procaine-injections into the CX/SMP region were quite reliable. Interestingly, procaine-treated bees still approached L(+1) more often towards the end of the experiment, however this effect was overall quite small in all groups. Because this effect was so subtle, it is possible that differences could not be detected.

Procaine effects on behavior in insects can last from 20 – 25 minutes after injections when optomotor responses were investigated (Kathman et al., 2014) after injections into the CX or at least 90 minutes in olfactory learning assays after injections into the antennal lobes (Devaud et al., 2007). This suggests, that the duration of the procaine effect depends on the injection locus and/or on the process affected. This could be tested by injecting at different time points in relation to the sequence assay, e.g. at 15 minute-increments up to two hours before the assay.

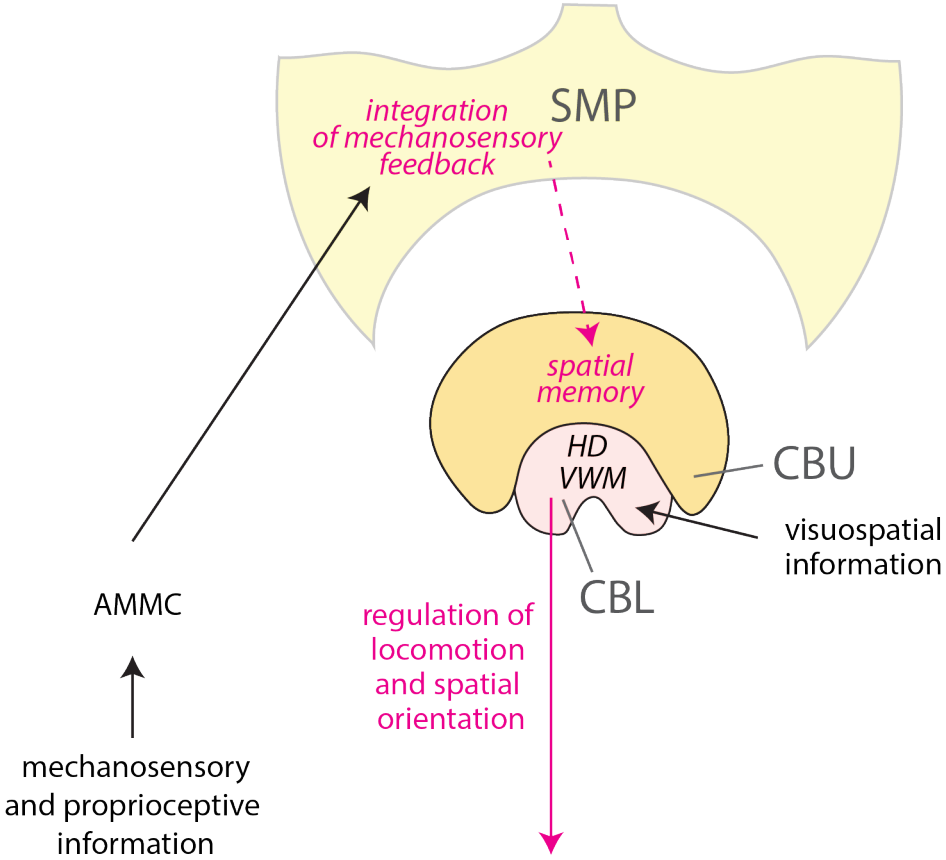
There are several possible explanations for which processes were disrupted by the procaine-injections. Firstly, the ability to initiate a turn towards a target could be disrupted. Activity in different CX neurons precedes and correlates with turning behavior (Bender et al., 2010; Guo and Ritzmann, 2013b; Martin et al., 2015). However, turning behavior in

procaine-treated animals was similar to the control. This is supported by our findings discussed above, which showed that angular speed in the presence of visual stimuli or in the dark periods between visual stimuli was not influenced by treatment. A second possibility is that the bee could not relate its body orientation to the visual surrounding. The CX plays a major role in spatial orientation (Varga et al., 2017), and it is possible that these processes were disrupted by procaine-injections. Finally, it is equally likely that procaine-injections disrupted the ability to transfer the visual working memory of the light into a more stable spatial memory, since there is strong evidence supporting a role of the CX in visual pattern learning (Li et al., 2009; Liu et al., 2006; Wang et al., 2008), spatial orientation memory (Kuntz et al., 2012; Kuntz et al., 2017; Neuser et al., 2008; Thran et al., 2013) and spatial learning.

At this point we cannot conclude if blocking sensory input from the SMP to the CX or if blocking of motor output from the CX to the SMP was affected. Some neurons, which are involved in visual pattern memory and which connect the SMP and CBU have presynapses as well as postsynapses in the SMP (Li et al., 2009; Young and Armstrong, 2010). Phillips-Portillo (2012) suggested that these neurons could be involved in computations and not only in relaying sensory information. Further histological studies could provide suggestions towards functionality of neurons connecting SMP and CX.

To summarize, we found that the mushroom bodies as well as the CX/SMP region play a crucial role in structuring and regulating walking activity. Procaine-injections into the CX/SMP resulted in higher angular speeds in the dark phases and an impaired ability to orient towards the next light in regular light sequence in the light phases. Hence, disruptions of the CX/SMP region impaired processes which are crucial for orientation in dark conditions as well as orientation learning of distinct visual targets. This could be due to interruptions to sensory information needed for these processes, which most likely includes mechanosensory information **(Figure 16)**.

Figure 16: Information flow model for spatial orientation and spatial memory. Mechanosensory and proprioceptive information are conveyed to the SMP from the AMMC (antennal mechanosensory and motor center). Since many connections are found between the SMP and the CX, mechanosensory feedback information could be passed on from the SMP to the CX. The CBU is involved in different forms of spatial learning, which could rely on mechanosensory feedback information of the insect's body. The CBL processes visual information and proprioceptive information to encode HD (head direction) to create VWMs (visual working memories) needed for orientation in space. CX: central complex, CBU: upper division of the central body, CBL: lower division of the central body, SMP: superior medial protocerebrum, AMMC: antennal mechanosensory and motor center


integration
of mechanosensory
feedback
SMP
spatial
memory
HD
VWM
CBU
visuospatial
information
CBL
AMMC
regulation of
locomotion
and spatial
orientation
mechanosensory
and proprioceptive
information

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Supplemental Material

Table S 1: Summary of Generalized Linear Mixed Model results for walking activity in a dark chamber after injections. Effects of treatment, time and interaction of time and treatment were analyzed on speed (family: gaussian, link: log), bout duration (family: Gamma, link: log), and bout number (family: poisson, link: log). Sham-treated animals were compared with vehicle-injected (veh) or procaine-injected (proc) animals after injections into the mushroom body calyces (MBC) the ventral lobes (VL) or the central complex (CX). Effects of injection site (CX, MBC, VL) on speed, bout duration and bout number was compared between sham-treated groups. Degrees of Freedom (DF); Estimate (Est); Standard Error (SE).

group	dependent variable	variable	treatment	DF	Est	SE	t	p
MBC	speed	intercept	sham	52	-0.435	0.213	-2.047	0.04
		time	sham		0.390	0.037	10.624	< 0.001
		treatment	veh		-0.823	0.484	-1.703	0.09
			proc		-1.845	0.522	-3.536	< 0.01
		treatment * time	veh		0.561	0.101	5.564	< 0.01
			proc		0.771	0.225	3.429	< 0.01
	bout duration	intercept	sham		-0.613	0.055	-11.241	< 0.001
		time	sham		-0.053	0.041	-1.297	0.19
		treatment	veh		-0.089	0.126	-0.708	0.48
			proc		0.410	0.106	3.863	< 0.01
		treatment * time	veh		0.108	0.093	1.160	0.25
			proc		-0.199	0.080	-2.473	0.01
	bout number	intercept	sham		3.572	0.137	26.161	< 0.001
		time	sham		0.234	0.014	16.930	< 0.001
		treatment	veh		-0.745	0.310	-2.401	0.02
			proc		-0.993	0.266	-3.730	< 0.01
		treatment * time	veh		0.424	0.035	12.288	< 0.001
			proc		0.299	0.033	8.988	< 0.001

Table S 1 cont.

group	dependent variable	variable	treatment	DF	Est	SE	t	p
VL	speed	intercept	sham	45	0.358	0.362	0.989	0.32
		time	sham		-0.751	0.440	-1.708	0.09
		treatment	veh		-0.609	0.446	-1.367	0.17
			proc		0.155	0.066	2.364	0.02
		treatment * time	veh		-0.372	0.083	-4.486	< 0.01
			proc		0.101	0.096	1.047	0.30
	bout duration	intercept	sham		-0.743	0.154	-4.817	< 0.01
		time	sham		-0.025	0.182	-0.137	0.89
		treatment	veh		0.229	0.187	1.225	0.22
			proc		0.042	0.076	0.560	0.58
		treatment * time	veh		0.040	0.093	0.427	0.67
			proc		-0.231	0.093	-2.477	0.01
	bout number	intercept	sham		4.072	0.293	13.899	< 0.001
		time	sham		-0.521	0.347	-1.499	0.13
		treatment	veh		-0.237	0.355	-0.667	0.50
			proc		0.058	0.023	2.593	0.01
		treatment * time	veh		-0.360	0.030	-11.984	< 0.001
			proc		0.087	0.029	3.040	< 0.01

Table S 1 cont.

group	dependent variable	variable	treatment	DF	Est	SE	t	p
CX	speed	intercept	sham	36	-0.378	0.305	-1.239	0.22
		time	sham		-0.271	0.085	-3.188	< 0.01
		treatment	veh		-0.106	0.448	-0.236	0.81
			proc		-0.054	0.472	-0.114	0.91
		treatment * time	veh		0.169	0.129	1.312	0.19
			proc		0.099	0.128	0.769	0.44
	bout duration	intercept	sham		-0.578	0.133	-4.339	< 0.01
		time	sham		0.098	0.072	1.353	0.18
		treatment	veh		0.013	0.200	0.067	0.95
			proc		0.004	0.211	0.018	0.99
		treatment * time	veh		-0.175	0.104	-1.680	0.09
			proc		-0.153	0.112	-1.365	0.17
	bout number	intercept	sham		3.556	0.262	13.563	< 0.001
		time	sham		-0.468	0.024	-19.439	< 0.001
		treatment	veh		-0.099	0.377	-0.264	0.79
			proc		-0.109	0.384	-0.282	0.78
		treatment * time	veh		0.413	0.033	12.466	< 0.001
			proc		0.366	0.034	10.889	< 0.001

Table S 1 cont.

group	dependent variable	variable	treatment	DF	Est	SE	t	p
sham	speed	intercept	CX	36	2.592	0.618	4.192	< 0.01
		time	CX		0.310	0.126	2.448	0.01
		treatment	MBC		-0.588	0.694	-0.847	0.40
			VL		-1.775	0.950	-1.870	0.06
		treatment * time	MBC		-0.430	0.136	-3.167	0.00
			VL		-0.368	0.155	-2.369	0.02
	bout duration	intercept	CX		-0.570	0.118	-4.811	0.00
		time	CX		0.089	0.058	1.553	0.12
		treatment	MBC		-0.052	0.143	-0.365	0.72
			VL		-0.167	0.203	-0.823	0.41
		treatment * time	MBC		-0.134	0.069	-1.942	0.05
			VL		-0.051	0.089	-0.574	0.57
	bout number	intercept	CX		3.509	0.212	16.585	< 0.001
		time	CX		-0.430	0.022	-19.433	< 0.001
		treatment	MBC		0.089	0.255	0.348	0.73
			VL		0.570	0.357	1.596	0.11
		treatment * time	MBC		0.641	0.025	25.233	< 0.001
			VL		0.483	0.030	16.101	< 0.001

Table S 2: Summary of Generalized Linear Mixed Model results for walking activity in a round arena after injections. Effects of speed, treatment, time and interaction of treatment and speed on angular speed (family: Gamma, link: inverse) in the dark phase in the light phase. Effects of treatment, time and interaction of time and treatment were analyzed on speed (family: Gamma, link: log), bout duration (family: Gamma, link: log), and bout number (family: poisson, link: log). Sham-treated animals were compared with vehicle-injected (veh) or procaine-injected (proc) animals after injections into the CX/SMP region. Degrees of Freedom (DF)

dependent variable	fixed variable	Estimate	standard error	t	p
angular speed (dark phase)	Intercept	1.4896	0.1391	10.707	< 0.001
	treatment - vehicle	0.0766	0.2386	0.321	0.75
	treatment - procaine	-0.5400	0.1716	-3.146	< 0.01
	speed	-0.2578	0.0335	-7.701	< 0.01
	treatment - vehicle:speed	-0.0274	0.0606	-0.452	0.65
	treatment - procaine:speed	0.1206	0.0417	2.890	< 0.01
angular speed (light phase)	Intercept	0.9465	0.0868	10.909	< 0.001
	treatment - vehicle	0.3047	0.1743	1.748	0.08
	treatment - procaine	0.0931	0.1423	0.654	0.51
	speed	-0.1215	0.0165	-7.386	< 0.01
	treatment - vehicle:speed	-0.0626	0.0349	-1.796	0.07
	treatment - procaine:speed	-0.0370	0.0302	-1.225	0.22
angular speed	Intercept	-0.3377	0.1371	-2.463	0.01
	time	0.5779	0.0384	15.047	< 0.001
	treatment - vehicle	-0.0719	0.2291	-0.314	0.75
	treatment - procaine	0.6088	0.2050	2.970	< 0.01
	phase - light	0.2887	0.0372	7.762	< 0.01
	time:treatment - vehicle	-0.0553	0.0634	-0.872	0.38
	time:treatment - procaine	-0.4103	0.0568	-7.224	< 0.01
	treatment - vehicle:phase - light	-0.1792	0.0621	-2.886	< 0.01
	treatment - procaine:phase - light	-0.2439	0.0557	-4.381	< 0.01

Table S 2 cont.

dependent variable	fixed variable	Estimate	standard error	t	p
speed	Intercept	-0.5396	0.1810	-2.982	< 0.01
	time	0.9945	0.0515	19.324	< 0.001
	treatment - vehicle	-0.0239	0.3028	-0.079	0.937
	treatment - procaine	0.6937	0.2705	2.565	0.01
	phase - light	0.2883	0.0482	5.984	< 0.01
	time:treatment - vehicle	-0.2137	0.0864	-2.475	0.01
	time:treatment - procaine	-0.5961	0.0757	-7.873	< 0.01
	treatment - vehicle:phase - light	-0.1687	0.0797	-2.117	0.03
	treatment - procaine:phase - light	-0.2634	0.0718	-3.666	< 0.01
bout duration	Intercept	1.2780	0.2642	4.837	< 0.01
	treatment - vehicle	-0.0004	0.4432	-0.001	1.00
	treatment - procaine	0.8877	0.3946	2.250	0.02
	phase	1.6171	0.1265	12.782	< 0.001
	treatment - vehicle:phase	-0.2492	0.2153	-1.157	0.25
	treatment - procaine:phase	-0.8510	0.1871	-4.548	< 0.01
bout number	Intercept	7.3539	0.1208	60.860	< 0.001
	treatment - vehicle	-0.1232	0.2054	-0.600	0.55
	treatment - procaine	-0.3664	0.1903	-1.930	0.05
	phase	-0.7646	0.0052	-147.290	< 0.001
	treatment - vehicle:phase	0.1153	0.0089	12.910	< 0.001
	treatment - procaine:phase	0.3788	0.0081	46.550	< 0.001

Table S 3: Summary of Linear Mixed Model results for angle and angular speed over three blocks in a round arena. Effects of time, block, light identity and interaction of time and block were analyzed on angle and angular speed with sham-, vehicle- and procaine-treated animals. Degrees of Freedom (DF)

group	light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
sham	L(0)	angle	Intercept	1.4177	0.0474	22651	29.934	< 0.001
			time	0.0036	0.0002	22651	21.985	< 0.001
			block 2	-0.1036	0.0107	22651	-9.653	< 0.001
			block 3	-0.1023	0.0107	22651	-9.530	< 0.001
			light 2	0.0219	0.0616	62	0.356	0.72
			light 3	-0.0542	0.0616	62	-0.881	0.38
			time:block 2	0.0027	0.0002	22651	11.541	< 0.001
			time:block 3	0.0038	0.0002	22651	16.350	< 0.001
	L(-1)		Intercept	1.6150	0.0436	22651	37.045	< 0.001
			time	-0.0021	0.0002	22651	-13.615	< 0.001
			block 2	0.1217	0.0103	22651	11.874	< 0.001
			block 3	0.1191	0.0103	22651	11.622	< 0.001
			light 2	0.0053	0.0604	62	0.088	0.93
			light 3	0.0175	0.0604	62	0.290	0.77
			time:block 2	-0.0015	0.0002	22651	-6.822	< 0.001
			time:block 3	-0.0013	0.0002	22651	-5.736	< 0.001
	L(+1)		Intercept)	1.4980	0.0426	22651	35.147	< 0.001
			time	-0.0009	0.0001	22651	-6.413	< 0.001
			block 2	0.0317	0.0095	22651	3.333	< 0.01
			block 3	0.0829	0.0095	22651	8.717	< 0.001
			light 2	0.0489	0.0596	62	0.821	0.42
			light 3	0.0724	0.0596	62	1.215	0.23
			time:block 2	-0.0007	0.0002	22651	-3.538	< 0.001
			time:block 3	-0.0012	0.0002	22651	-5.568	< 0.001

Table S 3 cont.

group	light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
sham	L(on)	angle	Intercept	1.4980	0.0426	22651	35.147	< 0.001
			time	-0.0009	0.0001	22651	-6.413	< 0.001
			block 2	0.0317	0.0095	22651	3.333	< 0.01
			block 3	0.0829	0.0095	22651	8.717	< 0.001
			light 2	0.0489	0.0596	62	0.821	0.42
			light 3	0.0724	0.0596	62	1.215	0.23
			time:block 2	-0.0007	0.0002	22651	-3.538	< 0.001
			time:block 3	-0.0012	0.0002	22651	-5.568	< 0.001
		angular speed	Intercept)	2.0842	0.1907	22651	10.931	< 0.001
			time	-0.0025	0.0005	22651	-4.828	< 0.001
			block 2	0.7527	0.0342	22651	22.028	< 0.001
			block 3	0.8286	0.0342	22651	24.249	< 0.001
			light 2	-0.0816	0.0309	62	-2.639	0.011
			light 3	-0.0259	0.0309	62	-0.839	0.40
			time:block 2	-0.0023	0.0007	22651	-3.130	< 0.01
			time:block 3	-0.0034	0.0007	22651	-4.587	< 0.001

Table S 3 cont.

group	light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
vehicle	L(0)	angle	Intercept	1.3667	0.0547	10615	24.978	< 0.001
			time	0.0051	0.0002	10615	22.634	< 0.001
			block 2	-0.0839	0.0147	10615	-5.714	< 0.001
			block 3	-0.1206	0.0147	10615	-8.218	< 0.001
			light 2	0.0317	0.0746	28	0.424	0.68
			light 3	0.0206	0.0746	28	0.276	0.78
			time:block 2	0.0019	0.0003	10615	6.014	< 0.001
			time:block 3	0.0036	0.0003	10615	11.209	< 0.001
	L(-1)		Intercept	1.6587	0.0550	10615	30.174	< 0.001
			time	-0.0024	0.0002	10615	-11.950	< 0.001
			block 2	0.1226	0.0129	10615	9.471	< 0.001
			block 3	0.2088	0.0129	10615	16.127	< 0.001
			light 2	-0.0654	0.0724	28	-0.904	0.37
			light 3	0.0287	0.0724	28	0.397	0.69
			time:block 2	-0.0006	0.0003	10615	-2.270	0.023
			time:block 3	-0.0018	0.0003	10615	-6.370	< 0.001
	L(+1)		Intercept)	1.5852	0.0577	10615	27.459	< 0.001
			time	-0.0015	0.0002	10615	-7.992	< 0.001
			block 2	0.0089	0.0122	10615	0.730	0.47
			block 3	0.0602	0.0122	10615	4.942	< 0.001
			light 2	0.1375	0.0796	28	1.728	0.10
			light 3	0.0395	0.0796	28	0.496	0.62
			time:block 2	-0.0009	0.0003	10615	-3.394	< 0.01
			time:block 3	-0.0016	0.0003	10615	-6.147	< 0.001

Table S 3 cont.

group	light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
vehicle	L(on)	angle	Intercept	1.5852	0.0577	10615	27.459	< 0.001
			time	-0.0015	0.0002	10615	-7.992	< 0.001
			block 2	0.0089	0.0122	10615	0.730	0.47
			block 3	0.0602	0.0122	10615	4.942	< 0.001
			light 2	0.1375	0.0796	28	1.728	0.10
			light 3	0.0395	0.0796	28	0.496	0.62
			time:block 2	-0.0009	0.0003	10615	-3.394	< 0.01
			time:block 3	-0.0016	0.0003	10615	-6.147	< 0.001
		angular speed	Intercept)	1.8656	0.2706	10615	6.893	< 0.001
			time	-0.0018	0.0006	10615	-2.956	< 0.01
			block 2	0.4886	0.0393	10615	12.418	< 0.001
			block 3	0.9899	0.0393	10615	25.158	< 0.001
			light 2	-0.0207	0.0331	28	-0.624	0.54
			light 3	0.0177	0.0331	28	0.535	0.60
			time:block 2	-0.0006	0.0009	10615	-0.735	0.46
			time:block 3	-0.0038	0.0009	10615	-4.448	< 0.001

Table S 3 cont.

group	light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
procaine	L(0)	angle	Intercept	1.2990	0.0473	14155	27.490	< 0.001
			time	0.0068	0.0002	14155	37.981	< 0.001
			block 2	0.1399	0.0117	14155	11.922	< 0.001
			block 3	0.1174	0.0117	14155	10.004	< 0.001
			light 2	-0.0226	0.0565	38	-0.400	0.69
			light 3	-0.1433	0.0565	38	-2.534	0.02
			time:block 2	-0.0012	0.0003	14155	-4.901	< 0.001
			time:block 3	-0.0002	0.0003	14155	-0.597	0.55
	L(-1)		Intercept	1.8169	0.0394	14155	46.079	< 0.001
			time	-0.0034	0.0002	14155	-21.716	< 0.001
			block 2	-0.0754	0.0103	14155	-7.359	< 0.001
			block 3	-0.0386	0.0103	14155	-3.766	< 0.001
			light 2	-0.0829	0.0549	38	-1.511	0.14
			light 3	0.0354	0.0549	38	0.644	0.52
			time:block 2	0.0008	0.0002	14155	3.515	< 0.001
			time:block 3	0.0005	0.0002	14155	2.454	0.01
	L(+1)		Intercept)	1.6409	0.0377	14155	43.507	< 0.001
			time	-0.0029	0.0002	14155	-18.757	< 0.001
			block 2	-0.0831	0.0100	14155	-8.349	< 0.001
			block 3	-0.1175	0.0100	14155	-11.811	< 0.001
			light 2	0.1220	0.0436	38	2.800	0.01
			light 3	0.0612	0.0436	38	1.404	0.17
			time:block 2	0.0014	0.0002	14155	6.435	< 0.001
			time:block 3	0.0013	0.0002	14155	6.115	< 0.001

Table S 3 cont.

group	light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
procaine	L(on)	angle	Intercept	1.6409	0.0377	14155	43.507	< 0.001
			time	-0.0029	0.0002	14155	-18.757	< 0.001
			block 2	-0.0831	0.0100	14155	-8.349	< 0.001
			block 3	-0.1175	0.0100	14155	-11.811	< 0.001
			light 2	0.1220	0.0436	38	2.800	0.01
			light 3	0.0612	0.0436	38	1.404	0.17
			time:block 2	0.0014	0.0002	14155	6.435	< 0.001
			time:block 3	0.0013	0.0002	14155	6.115	< 0.001
		angular speed	Intercept)	1.6409	0.0377	14155	43.507	< 0.001
			time	-0.0029	0.0002	14155	-18.757	< 0.001
			block 2	-0.0831	0.0100	14155	-8.349	< 0.001
			block 3	-0.1175	0.0100	14155	-11.811	< 0.001
			light 2	0.1220	0.0436	38	2.800	0.01
			light 3	0.0612	0.0436	38	1.404	0.17
			time:block 2	0.0014	0.0002	14155	6.435	< 0.001
			time:block 3	0.0013	0.0002	14155	6.115	< 0.001

Table S 4: Summary of Linear Mixed Model results for angle difference and angular speed difference in a round arena after injections. Effects of treatment, dark period or light period (trial), light identity and interaction of treatment and trial were analyzed on angle difference and angular speed difference. Sham-treated animals were compared with vehicle-injected or procaine-injected animals after injections into the CX/SMP region. Degrees of Freedom (DF)

light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
L(0)	angle difference	Intercept	0.1750	0.0519	14871	3.375	< 0.01
		dark period	0.0014	0.0002	14871	7.723	< 0.001
		treatment - procaine	0.1785	0.0804	64	2.221	0.03
		treatment - vehicle	0.0646	0.0882	64	0.733	0.47
		light 2	-0.0169	0.0250	132	-0.677	0.50
		light 3	0.0775	0.0250	132	3.100	< 0.01
		dark period:treatment - procaine	-0.0013	0.0003	14871	-4.333	< 0.001
		dark period:treatment - vehicle	0.0000	0.0003	14871	0.084	0.93
L(-1)		Intercept	0.0556	0.0336	14871	-1.656	0.10
		dark period	-0.0005	0.0002	14871	-2.984	< 0.01
		treatment - procaine	-0.0535	0.0495	64	-1.080	0.28
		treatment - vehicle	0.0087	0.0544	64	0.161	0.87
		light 2	-0.0116	0.0236	132	-0.490	0.63
		light 3	-0.2135	0.0237	132	-9.029	< 0.001
		dark period:treatment - procaine	0.0005	0.0003	14871	2.052	0.04
		dark period:treatment - vehicle	-0.0001	0.0003	14871	-0.484	0.63
L(+1)		Intercept	0.0236	0.0312	14871	0.756	0.45
		dark period	-0.0004	0.0002	14871	-2.608	0.01
		treatment - procaine	-0.1327	0.0450	64	-2.950	< 0.01
		treatment - vehicle	-0.0345	0.0494	64	-0.699	0.49
		light 2	-0.1737	0.0243	132	-7.149	< 0.001
		light 3	-0.0401	0.0243	132	-1.651	0.10
		dark period:treatment - procaine	0.0010	0.0003	14871	3.589	< 0.001
		dark period:treatment - vehicle	-0.0001	0.0003	14871	-0.416	0.68

Table S 4 cont.

light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
Empty	angle difference	Intercept	-0.3294	0.0347	14871	-9.488	< 0.001
		dark period	-0.0002	0.0002	14871	-0.871	0.38
		treatment - procaine	0.0063	0.0458	64	0.138	0.89
		treatment - vehicle	-0.0325	0.0503	64	-0.646	0.52
		light 2	0.4806	0.0347	132	13.861	< 0.001
		light 3	0.4425	0.0347	132	12.764	< 0.001
		dark period:treatment - procaine	-0.0002	0.0003	14871	-0.654	0.51
		dark period:treatment - vehicle	0.0000	0.0003	14871	-0.062	0.95
L(on)		Intercept	-0.1848	0.0396	14871	-4.664	< 0.001
		light period	-0.0007	0.0002	14871	-4.330	< 0.001
		treatment - procaine	-0.1085	0.0610	64	-1.780	0.08
		treatment - vehicle	0.0105	0.0669	64	0.157	0.88
		light 2	-0.0876	0.0207	132	-4.229	< 0.001
		light 3	-0.0220	0.0207	132	-1.063	0.29
		dark period:treatment - procaine	0.0007	0.0003	14871	2.553	0.01
		dark period:treatment - vehicle	-0.0002	0.0003	14871	-0.648	0.52
	angular speed difference	Intercept	-0.2231	0.0784	14871	-2.847	< 0.01
		dark period	-0.0011	0.0004	14871	-2.661	0.01
		treatment - procaine	0.1882	0.1185	64	1.588	0.12
		treatment - vehicle	0.1172	0.1301	64	0.901	0.37
		light 2	0.0967	0.0477	132	2.029	0.04
		light 3	0.1939	0.0477	132	4.069	< 0.001
		dark period:treatment - procaine	-0.0009	0.0006	14871	-1.359	0.17
		dark period:treatment - vehicle	-0.0005	0.0007	14871	-0.755	0.45

Table S 5: Summary of Linear Mixed Model results for distance difference and speed difference in a round arena after injections. Effects of treatment, dark period or light period (trial), light identity and interaction of treatment and trial were analyzed on distance difference and speed difference. Sham-treated animals were compared with vehicle-injected or procaine-injected animals after injections into the CX/SMP region. Degrees of Freedom (DF)

light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
L(0)	distance difference	Intercept	3.0605	1.2070	14871	2.536	0.01
		dark period	0.0182	0.0040	14871	4.587	< 0.001
		treatment - procaine	-0.7036	1.8798	64	-0.374	0.71
		treatment - vehicle	1.5052	2.0636	64	0.729	0.47
		light 2	-0.2089	0.5461	132	-0.382	0.70
		light 3	-1.4436	0.5461	132	-2.643	< 0.01
		dark period:treatment - procaine	0.0097	0.0064	14871	1.526	0.13
		dark period:treatment - vehicle	-0.0083	0.0070	14871	-1.187	0.24
L(-1)		Intercept	-0.4900	0.9115	14871	-0.538	0.59
		dark period	-0.0006	0.0036	14871	-0.165	0.87
		treatment - procaine	1.2382	1.4044	64	0.882	0.38
		treatment - vehicle	-1.8478	1.5418	64	-1.199	0.24
		light 2	-0.3907	0.4695	132	-0.832	0.41
		light 3	-1.9136	0.4695	132	-4.076	< 0.001
		dark period:treatment - procaine	-0.0061	0.0058	14871	-1.053	0.29
		dark period:treatment - vehicle	0.0160	0.0063	14871	2.527	0.01
L(+1)		Intercept	-2.0894	0.6680	14871	-3.128	< 0.01
		dark period	-0.0075	0.0036	14871	-2.076	0.04
		treatment - procaine	0.2076	0.9658	64	0.215	0.83
		treatment - vehicle	0.5420	1.0602	64	0.511	0.61
		light 2	0.1996	0.5165	132	0.386	0.70
		light 3	1.8902	0.5165	132	3.660	< 0.001
		dark period:treatment - procaine	-0.0081	0.0058	14871	-1.392	0.16
		dark period:treatment - vehicle	-0.0071	0.0064	14871	-1.114	0.27

Table S 5 cont.

light position	dependent variable	fixed variable	Estimate	standard error	DF	t	p
Empty	distance difference	Intercept	-4.4865	0.7990	14871	-5.615	< 0.001
		dark period	-0.0084	0.0037	14871	-2.249	0.025
		treatment - procaine	-0.3465	1.0381	64	-0.334	0.74
		treatment - vehicle	-0.2702	1.1396	64	-0.237	0.81
		light 2	6.1357	0.8222	132	7.462	< 0.001
		light 3	6.6833	0.8223	132	8.128	< 0.001
		dark period:treatment - procaine	0.0029	0.0060	14871	0.483	0.63
		dark period:treatment - vehicle	0.0063	0.0066	14871	0.947	0.34
L(on)		Intercept	-8.8488	2.1121	14871	-4.190	< 0.001
		light period	-0.0414	0.0051	14871	-8.055	< 0.001
		treatment - procaine	0.7869	3.3321	64	0.236	0.81
		treatment - vehicle	0.4644	3.6580	64	0.127	0.90
		light 2	-5.7401	0.7620	132	-7.533	< 0.001
		light 3	0.0533	0.7620	132	0.070	0.94
		dark period:treatment - procaine	-0.0099	0.0083	14871	-1.189	0.23
		dark period:treatment - vehicle	-0.0060	0.0091	14871	-0.656	0.51
	speed difference	Intercept	0.0799	0.1033	14871	0.773	0.44
		dark period	0.0017	0.0004	14871	3.961	< 0.001
		treatment - procaine	-0.0581	0.1571	64	-0.370	0.71
		treatment - vehicle	0.0825	0.1724	64	0.478	0.63
		light 2	0.0437	0.0601	132	0.728	0.47
		light 3	0.0809	0.0601	132	1.346	0.18
		dark period:treatment - procaine	0.0005	0.0007	14871	0.690	0.49
		dark period:treatment - vehicle	-0.0023	0.0008	14871	-2.995	< 0.01

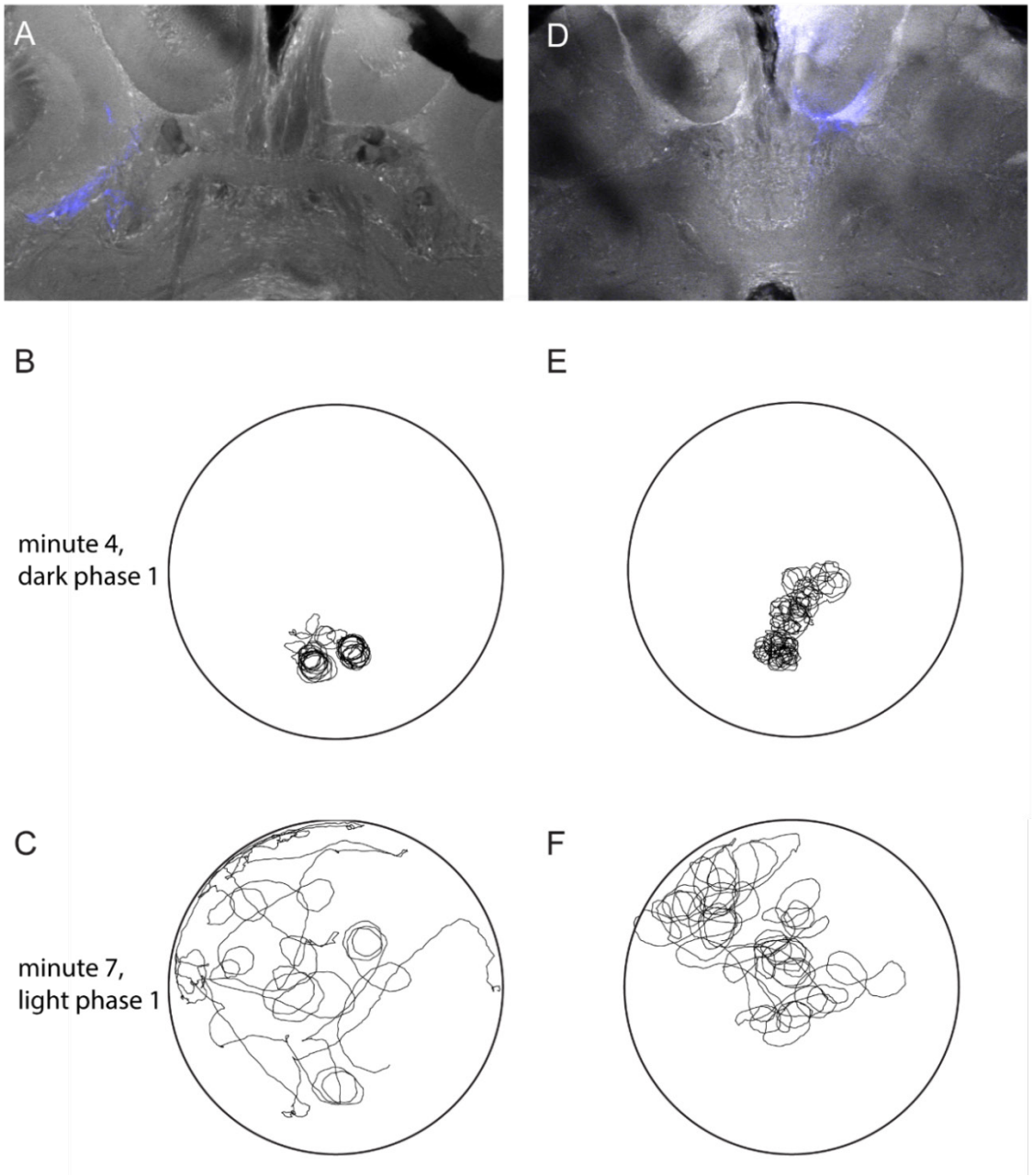


Figure S 1. Circling behavior in two bees injected into lateral sites in the CX/SMP. Bees were injected with procaine solution with Alexa dye (blue) into the CX/SMP in the left hemisphere **(A)** and in the right hemisphere **(B)**. Dye went from the main injection site to the PB and CBU. To identify the neuropils DAPI staining and auto-fluorescence of the tissue (grey) was used. Bees circled into the contra-lateral direction of the injection site when it was dark: Circling clockwise **(B)** and circling anti-clockwise **(E)**. When light were switched on in a sequence, the two bees partially disrupted the circling behavior and targeted the light **(C,F)**.

Figure S 2: With experience, sham-treated bees increased orientation towards the L(-1) or L(+1). Means of angle difference **(A-E)** or means of angular speed difference **(F)** (grey dots) with added regression line (dashed line) (GLMMs, Table S 4, $p < 0.05$). In panels **A-E** positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position. **(A)** The sham group increasingly turned towards L(on) over light phases (Table S 4). Bees increasingly turned and away from L(0) **(B)**, and towards L(-1) **(C)** and/or L(+1) **(D)** (Table S 4). No significant effect of dark period n mean angle difference for the empty side was found **(E)** (Table S 4). **(F)** Over dark periods, the sham group showed an increasing drop of angular speeds (negative angular speed difference) (Table S 4).

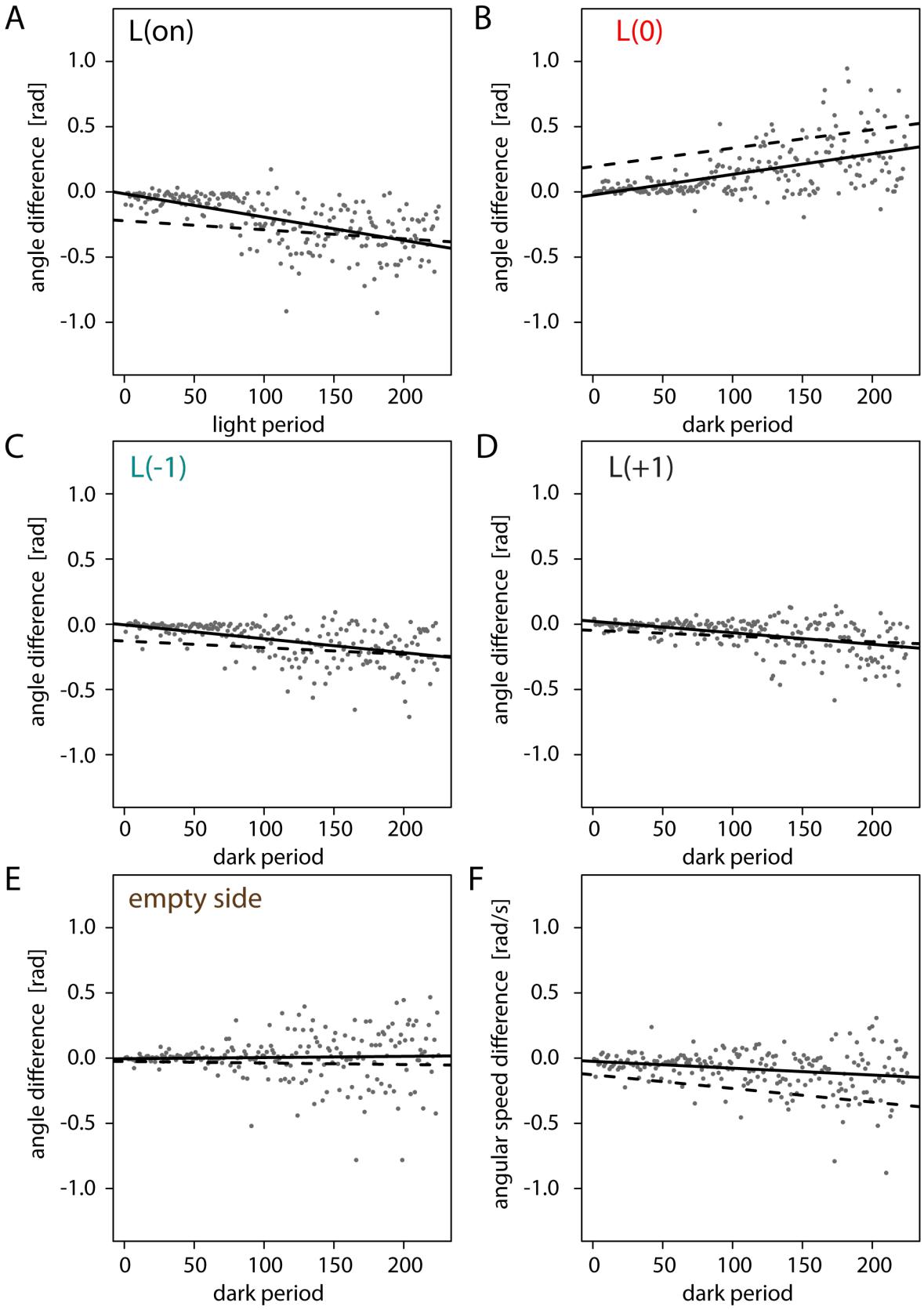

A
L(on)
angle difference [rad]
light period
B
L(0)
angle difference [rad]
dark period
C
L(-1)
angle difference [rad]
dark period
D
L(+1)
angle difference [rad]
dark period
E
empty side
angle difference [rad]
dark period
F
angular speed difference [rad/s]
dark period

Figure S 3: With experience, vehicle-treated bees increased orientation towards the L(-1) or L(+1). Means of angle difference **(A-E)** or means of angular speed difference **(F)** (blue dots) with added regression line (dashed line) (GLMMs, Table S 4, $p < 0.05$). In panels **A-E** positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position. **(A)** The sham group increasingly turned towards L(on) over light phases (Table S 4). Bees increasingly turned and away from L(0) **(B)**, and towards L(-1) **(C)** and/or L(+1) **(D)** (Table S 4). No significant effect of dark period n mean angle difference for the empty side was found **(E)** (Table S 4). **(F)** Over dark periods, the sham group showed an increasing drop of angular speeds (negative angular speed difference) (Table S 4).

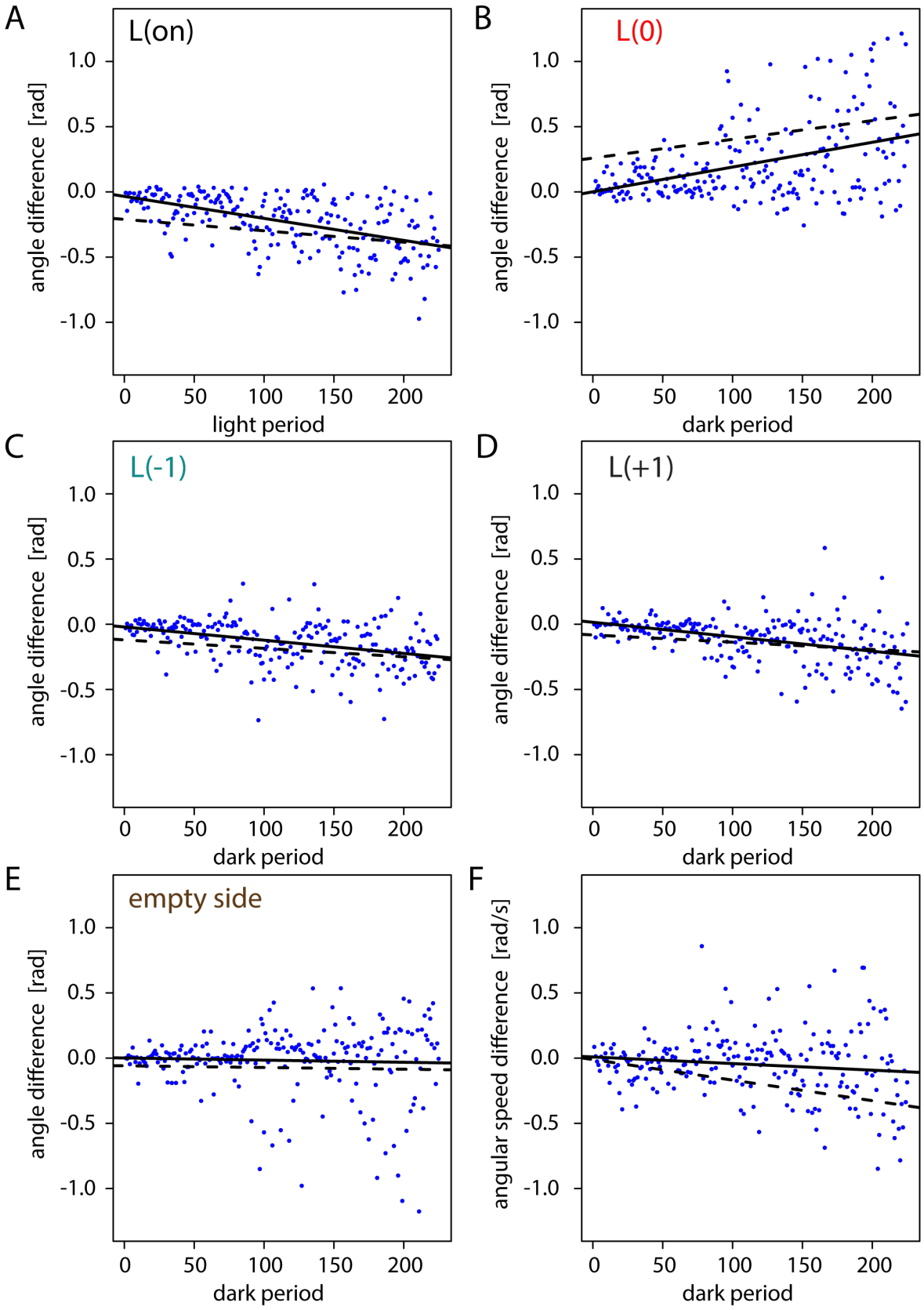

A
L(on)
angle difference [rad]
light period
B
L(0)
angle difference [rad]
dark period
C
L(-1)
angle difference [rad]
dark period
D
L(+1)
angle difference [rad]
dark period
E
empty side
angle difference [rad]
dark period
F
angular speed difference [rad/s]
dark period

Figure S 4: Bees injected with procaine did not orient towards L(+1). Means of angle difference **(A-E)** or means of angular speed difference **(F)** (magenta dots) with added regression line (dashed line) (GLMMs, Table S 4). In panels **A-E** positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position. The procaine group did not increase turns towards L(on) **(A)** over light phases (Table S 4). Bees did not turn and away from L(0) **(B)**, and towards L(-1) **(C)** and/or L(+1) more frequently over dark periods. **(D)** (Table S 4). Bees did not change orientation to the empty side over dark periods (Table S 4) **(E)**. **(F)** Over dark periods, the procaine group showed an increasing drop of angular speeds (negative angular speed difference) (Table S 4).

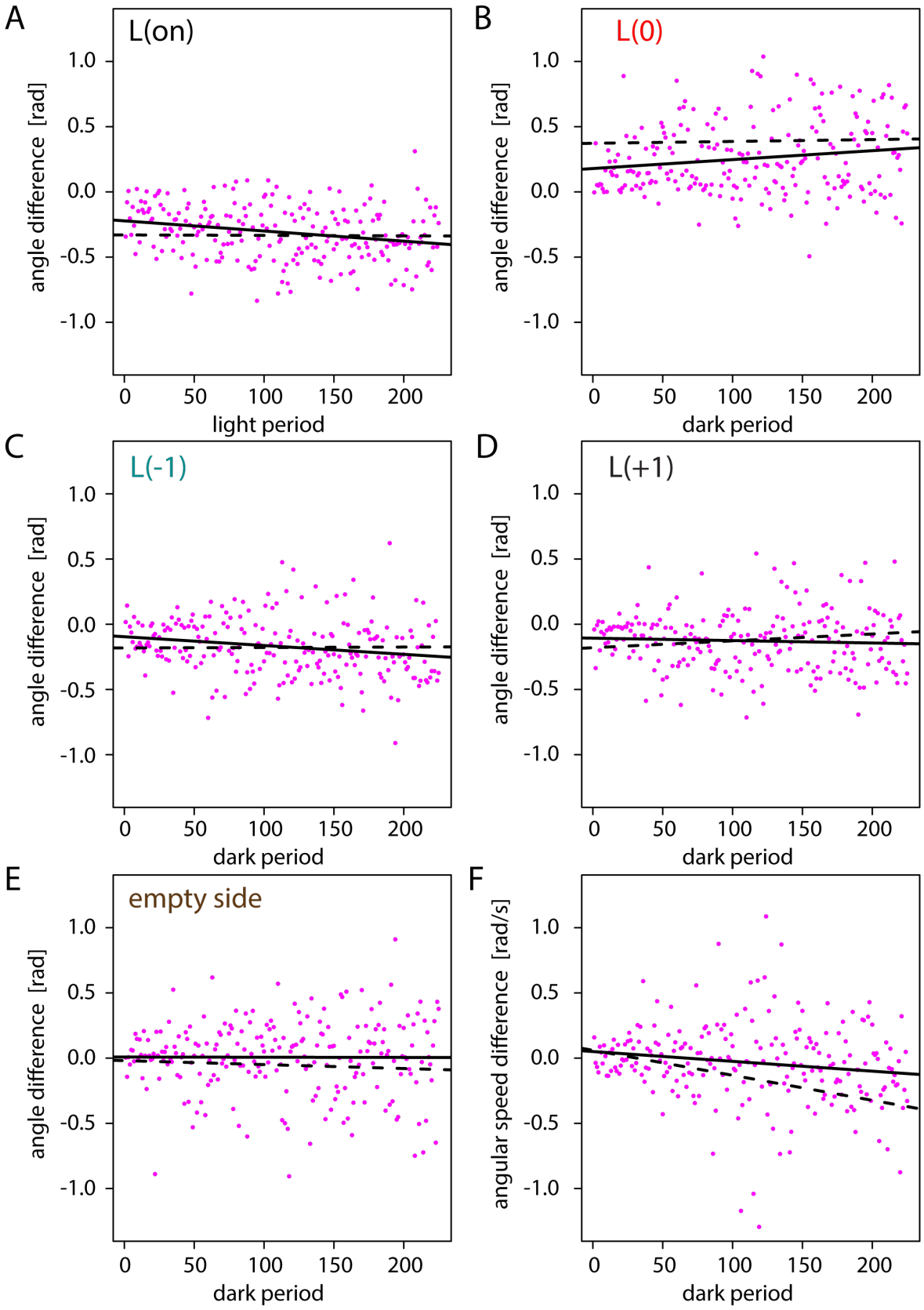

A
L(on)
angle difference [rad]
light period
B
L(0)
angle difference [rad]
dark period
C
L(-1)
angle difference [rad]
dark period
D
L(+1)
angle difference [rad]
dark period
E
empty side
angle difference [rad]
dark period
F
angular speed difference [rad/s]
dark period

Figure S 5: Individual plots for angle differences calculated for L(0) in the dark period after sham injections. Positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position.

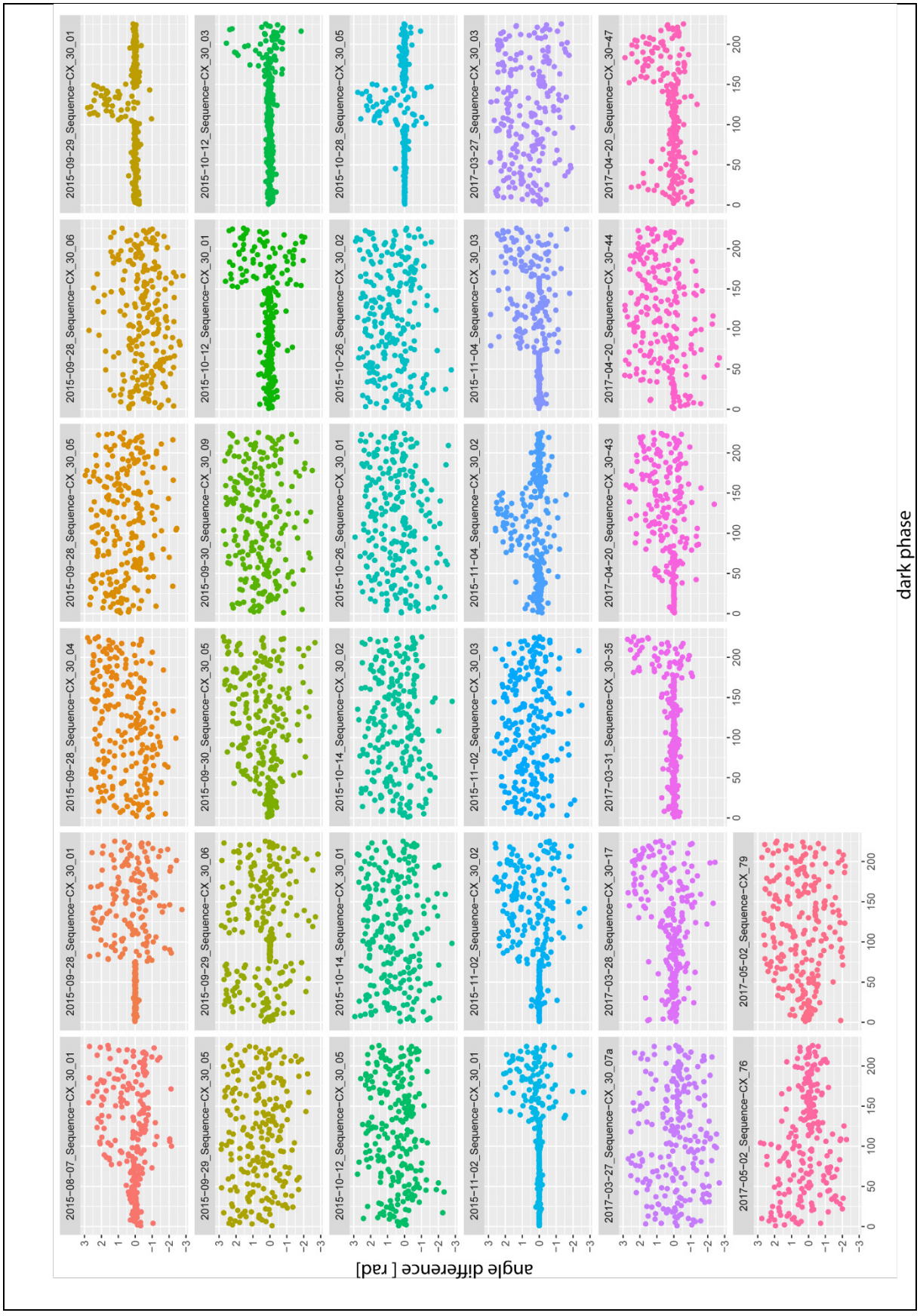

2015-08-07_Sequence-CX_30_01
2015-09-28_Sequence-CX_30_01
2015-09-28_Sequence-CX_30_04
2015-09-28_Sequence-CX_30_05
2015-09-28_Sequence-CX_30_06
2015-09-29_Sequence-CX_30_01
2015-09-29_Sequence-CX_30_05
2015-09-29_Sequence-CX_30_06
2015-09-30_Sequence-CX_30_05
2015-09-30_Sequence-CX_30_09
2015-10-12_Sequence-CX_30_01
2015-10-12_Sequence-CX_30_03
2015-10-12_Sequence-CX_30_05
2015-10-14_Sequence-CX_30_01
2015-10-14_Sequence-CX_30_02
2015-10-26_Sequence-CX_30_01
2015-10-26_Sequence-CX_30_02
2015-10-28_Sequence-CX_30_05
2015-11-02_Sequence-CX_30_01
2015-11-02_Sequence-CX_30_02
2015-11-02_Sequence-CX_30_03
2015-11-04_Sequence-CX_30_02
2015-11-04_Sequence-CX_30_03
2017-03-27_Sequence-CX_30_03
2017-03-27_Sequence-CX_30_07a
2017-03-28_Sequence-CX_30_17
2017-03-31_Sequence-CX_30_35
2017-04-20_Sequence-CX_30_43
2017-04-20_Sequence-CX_30_44
2017-04-20_Sequence-CX_30_47
2017-05-02_Sequence-CX_76
2017-05-02_Sequence-CX_79
angle difference [rad]
dark phase

Figure S 6: Individual plots for angle differences calculated for L(-1) in the dark period after sham injections. Positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position.

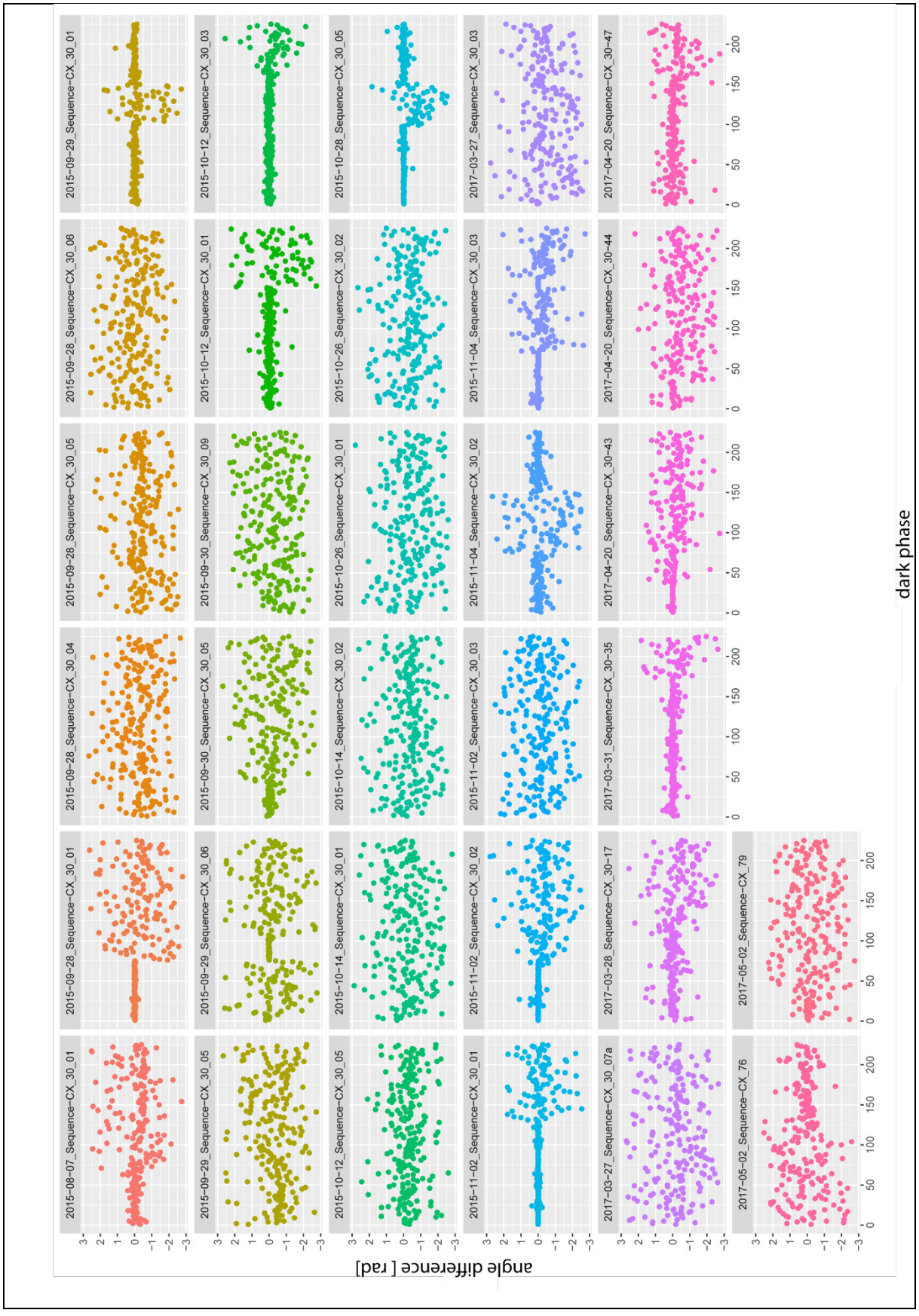

2015-08-07_Sequence-CX_30_01
2015-09-28_Sequence-CX_30_01
2015-09-28_Sequence-CX_30_04
2015-09-28_Sequence-CX_30_05
2015-09-28_Sequence-CX_30_06
2015-09-29_Sequence-CX_30_01
2015-09-29_Sequence-CX_30_05
2015-09-29_Sequence-CX_30_06
2015-09-30_Sequence-CX_30_05
2015-09-30_Sequence-CX_30_09
2015-10-12_Sequence-CX_30_01
2015-10-12_Sequence-CX_30_03
2015-10-12_Sequence-CX_30_05
2015-10-14_Sequence-CX_30_01
2015-10-14_Sequence-CX_30_02
2015-10-26_Sequence-CX_30_01
2015-10-26_Sequence-CX_30_02
2015-10-28_Sequence-CX_30_05
2015-11-02_Sequence-CX_30_01
2015-11-02_Sequence-CX_30_02
2015-11-02_Sequence-CX_30_03
2015-11-04_Sequence-CX_30_02
2015-11-04_Sequence-CX_30_03
2017-03-27_Sequence-CX_30_03
2017-03-27_Sequence-CX_30_07a
2017-03-28_Sequence-CX_30-17
2017-03-31_Sequence-CX_30-35
2017-04-20_Sequence-CX_30-43
2017-04-20_Sequence-CX_30-44
2017-04-20_Sequence-CX_30-47
2017-05-02_Sequence-CX_76
2017-05-02_Sequence-CX_79
angle difference [rad]
dark phase

Figure S 7: Individual plots for angle differences calculated for L(+1) in the dark period after sham injections. Positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position.

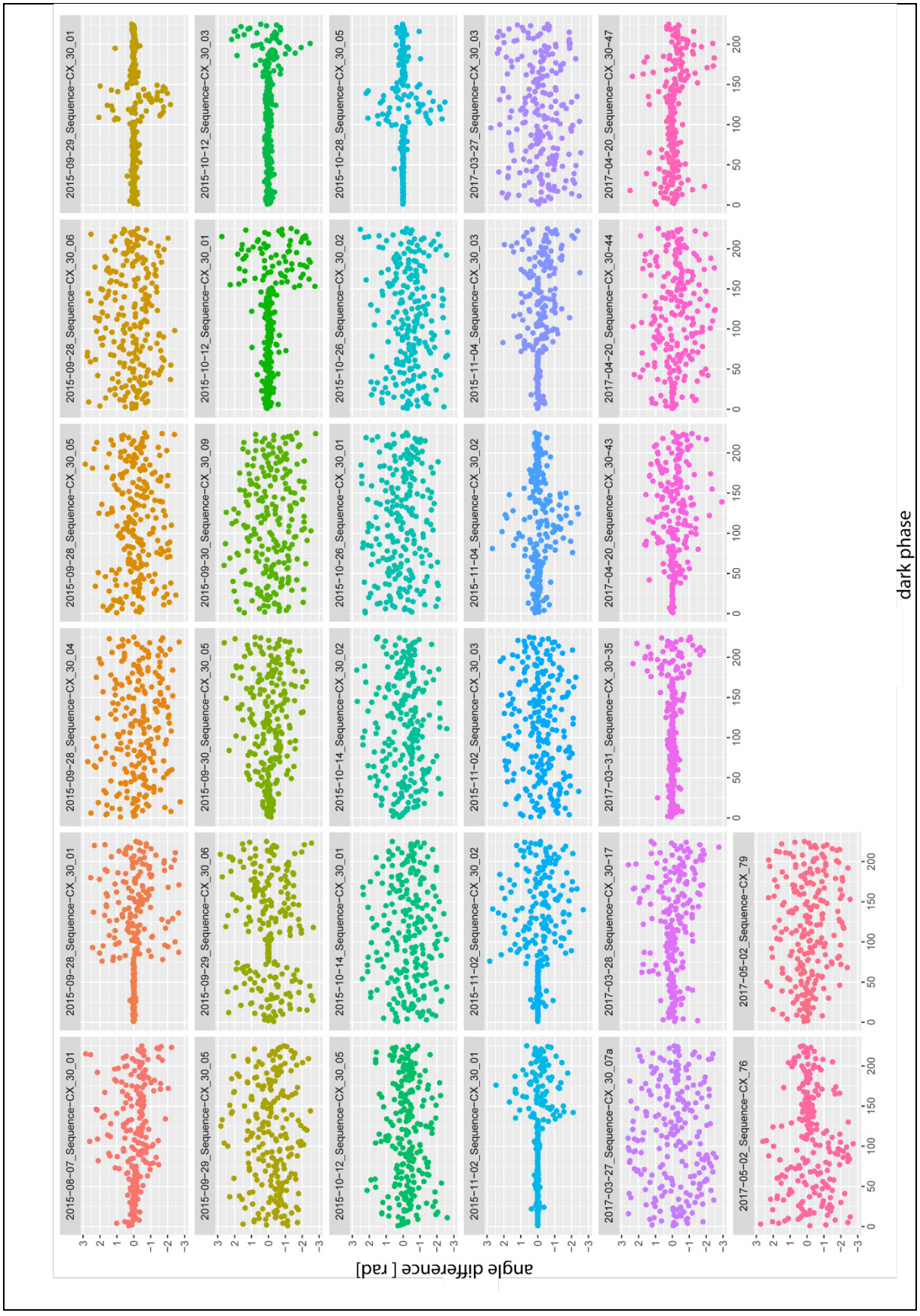

2015-08-07_Sequence-CX_30_01
2015-09-28_Sequence-CX_30_01
2015-09-28_Sequence-CX_30_04
2015-09-28_Sequence-CX_30_05
2015-09-28_Sequence-CX_30_06
2015-09-29_Sequence-CX_30_01
2015-09-29_Sequence-CX_30_05
2015-09-29_Sequence-CX_30_06
2015-09-30_Sequence-CX_30_05
2015-09-30_Sequence-CX_30_09
2015-10-12_Sequence-CX_30_01
2015-10-12_Sequence-CX_30_03
2015-10-12_Sequence-CX_30_05
2015-10-14_Sequence-CX_30_01
2015-10-14_Sequence-CX_30_02
2015-10-26_Sequence-CX_30_01
2015-10-26_Sequence-CX_30_02
2015-10-28_Sequence-CX_30_05
2015-11-02_Sequence-CX_30_01
2015-11-02_Sequence-CX_30_02
2015-11-02_Sequence-CX_30_03
2015-11-04_Sequence-CX_30_02
2015-11-04_Sequence-CX_30_03
2017-03-27_Sequence-CX_30_03
2017-03-27_Sequence-CX_30_07a
2017-03-28_Sequence-CX_30_17
2017-03-31_Sequence-CX_30_35
2017-04-20_Sequence-CX_30_43
2017-04-20_Sequence-CX_30_44
2017-04-20_Sequence-CX_30_47
2017-05-02_Sequence-CX_76
2017-05-02_Sequence-CX_79
angle difference [rad]
dark phase

Figure S 8: Individual plots for angle differences calculated for L(0) in the dark period after vehicle injections. Positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position.

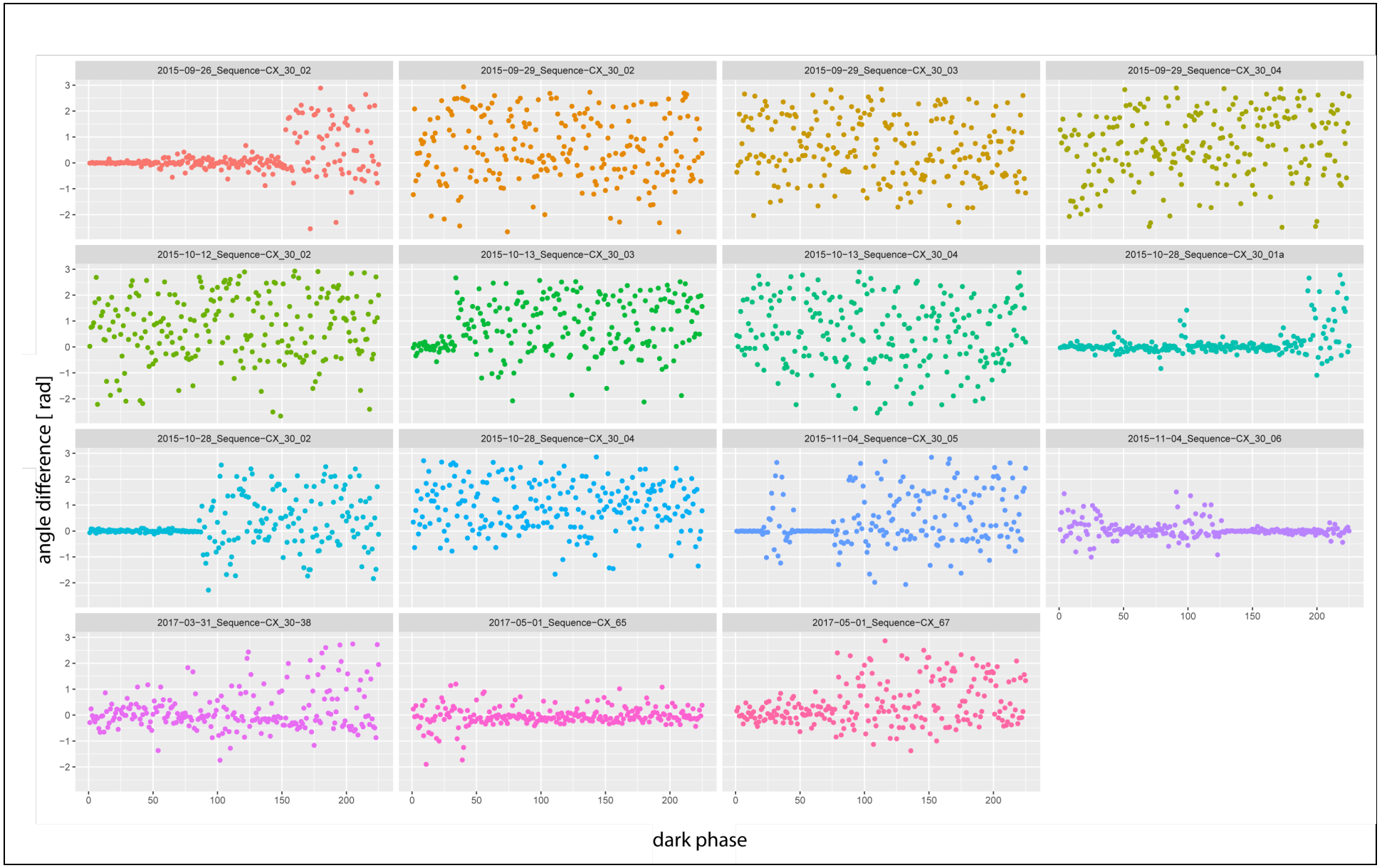

2015-09-26_Sequence-CX_30_02
2015-09-29_Sequence-CX_30_02
2015-09-29_Sequence-CX_30_03
2015-09-29_Sequence-CX_30_04
2015-10-12_Sequence-CX_30_02
2015-10-13_Sequence-CX_30_03
2015-10-13_Sequence-CX_30_04
2015-10-28_Sequence-CX_30_01a
2015-10-28_Sequence-CX_30_02
2015-10-28_Sequence-CX_30_04
2015-11-04_Sequence-CX_30_05
2015-11-04_Sequence-CX_30_06
2017-03-31_Sequence-CX_30-38
2017-05-01_Sequence-CX_65
2017-05-01_Sequence-CX_67
angle difference [rad]
dark phase

Figure S 9: Individual plots for angle differences calculated for L(-1) in the dark period after vehicle injections. Positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position.

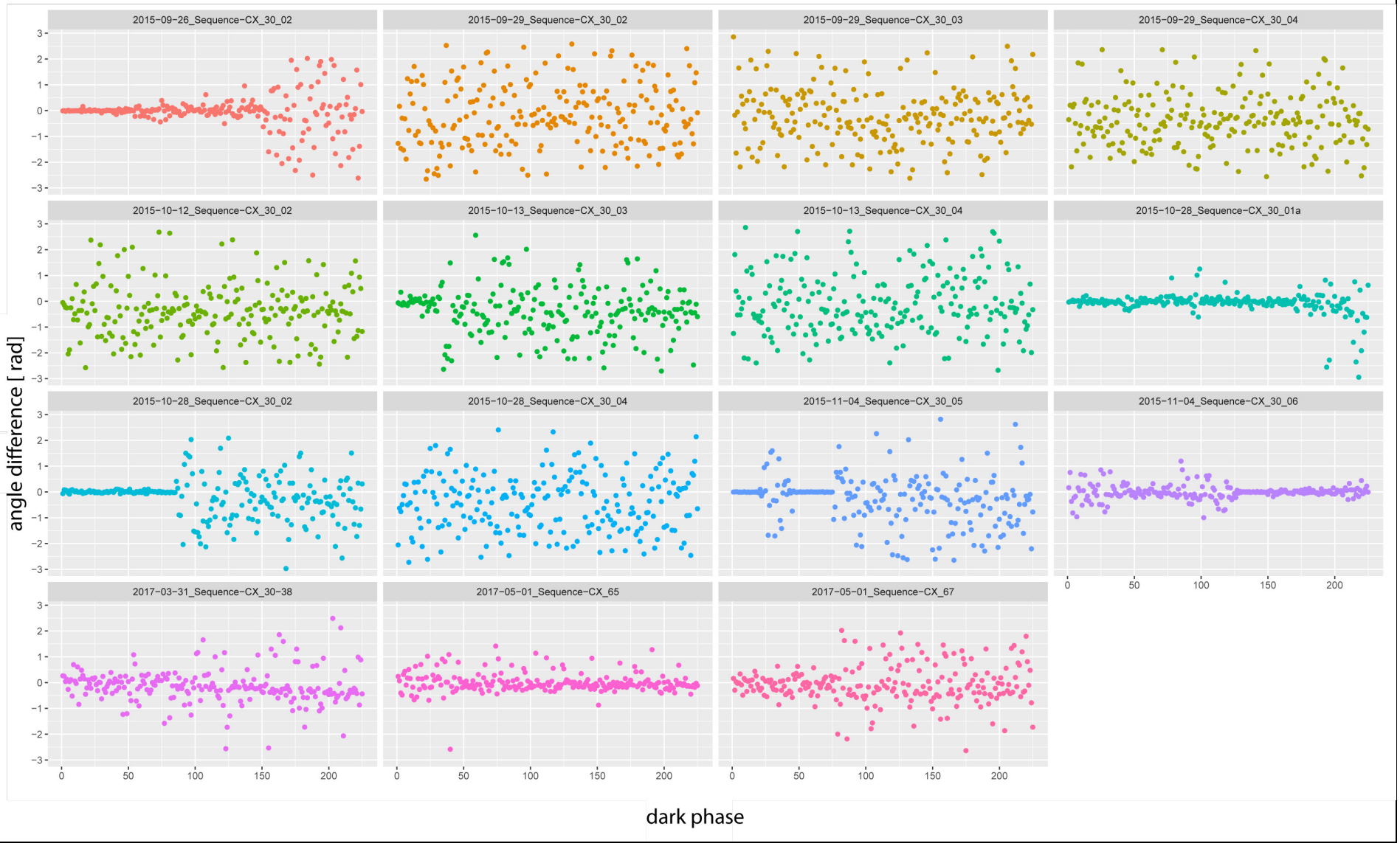

2015-09-26_Sequence-CX_30_02
2015-09-29_Sequence-CX_30_02
2015-09-29_Sequence-CX_30_03
2015-09-29_Sequence-CX_30_04
2015-10-12_Sequence-CX_30_02
2015-10-13_Sequence-CX_30_03
2015-10-13_Sequence-CX_30_04
2015-10-28_Sequence-CX_30_01a
2015-10-28_Sequence-CX_30_02
2015-10-28_Sequence-CX_30_04
2015-11-04_Sequence-CX_30_05
2015-11-04_Sequence-CX_30_06
2017-03-31_Sequence-CX_30-38
2017-05-01_Sequence-CX_65
2017-05-01_Sequence-CX_67
angle difference [rad]
dark phase

Figure S 10: Individual plots for angle differences calculated for L(+1) in the dark period after vehicle injections. Positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position.

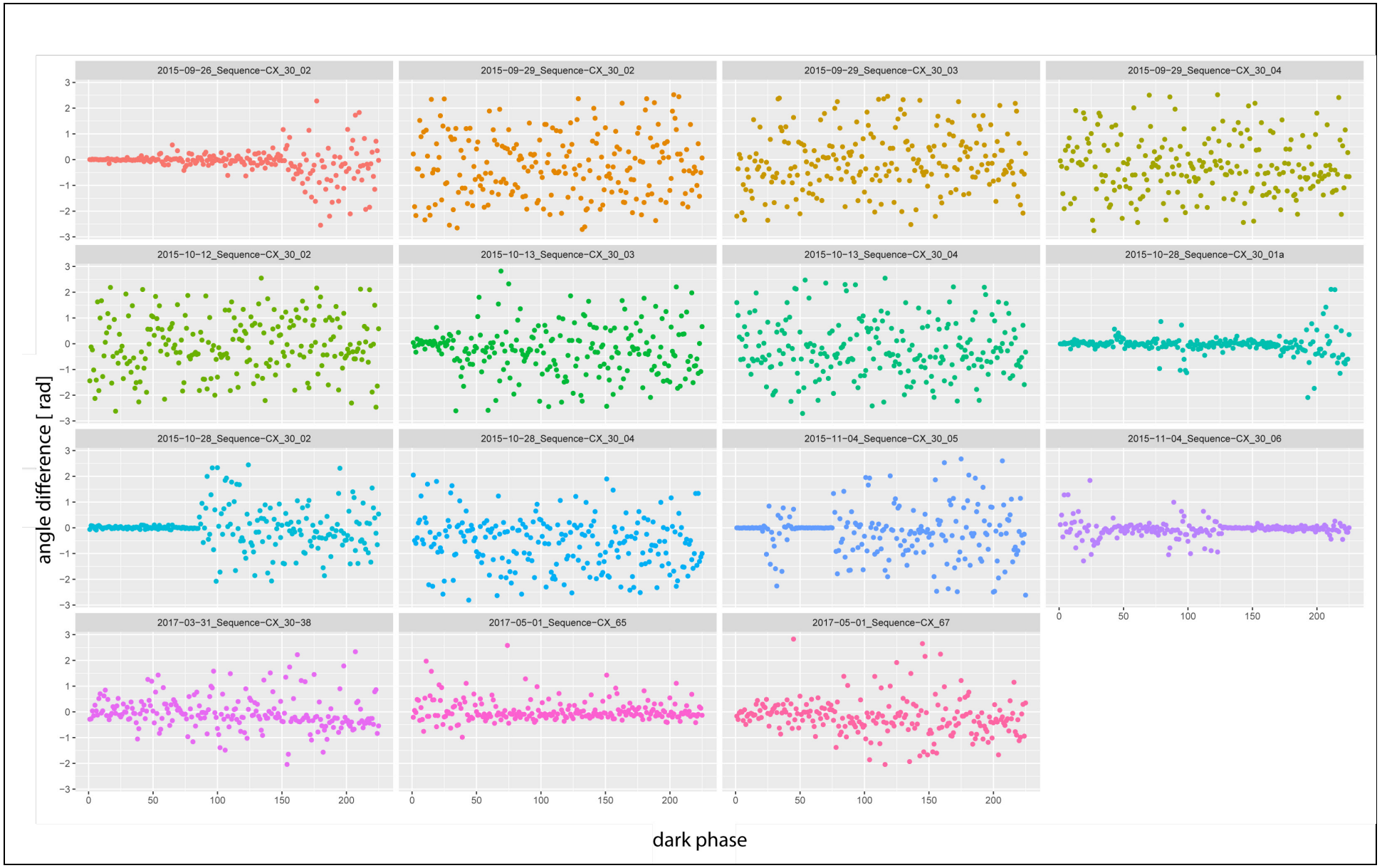

2015-09-26_Sequence-CX_30_02
2015-09-29_Sequence-CX_30_02
2015-09-29_Sequence-CX_30_03
2015-09-29_Sequence-CX_30_04
2015-10-12_Sequence-CX_30_02
2015-10-13_Sequence-CX_30_03
2015-10-13_Sequence-CX_30_04
2015-10-28_Sequence-CX_30_01a
2015-10-28_Sequence-CX_30_02
2015-10-28_Sequence-CX_30_04
2015-11-04_Sequence-CX_30_05
2015-11-04_Sequence-CX_30_06
2017-03-31_Sequence-CX_30-38
2017-05-01_Sequence-CX_65
2017-05-01_Sequence-CX_67
angle difference [rad]
dark phase

Figure S 11: Individual plots for angle differences calculated for L(0) in the dark period after procaine injections. Positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position.

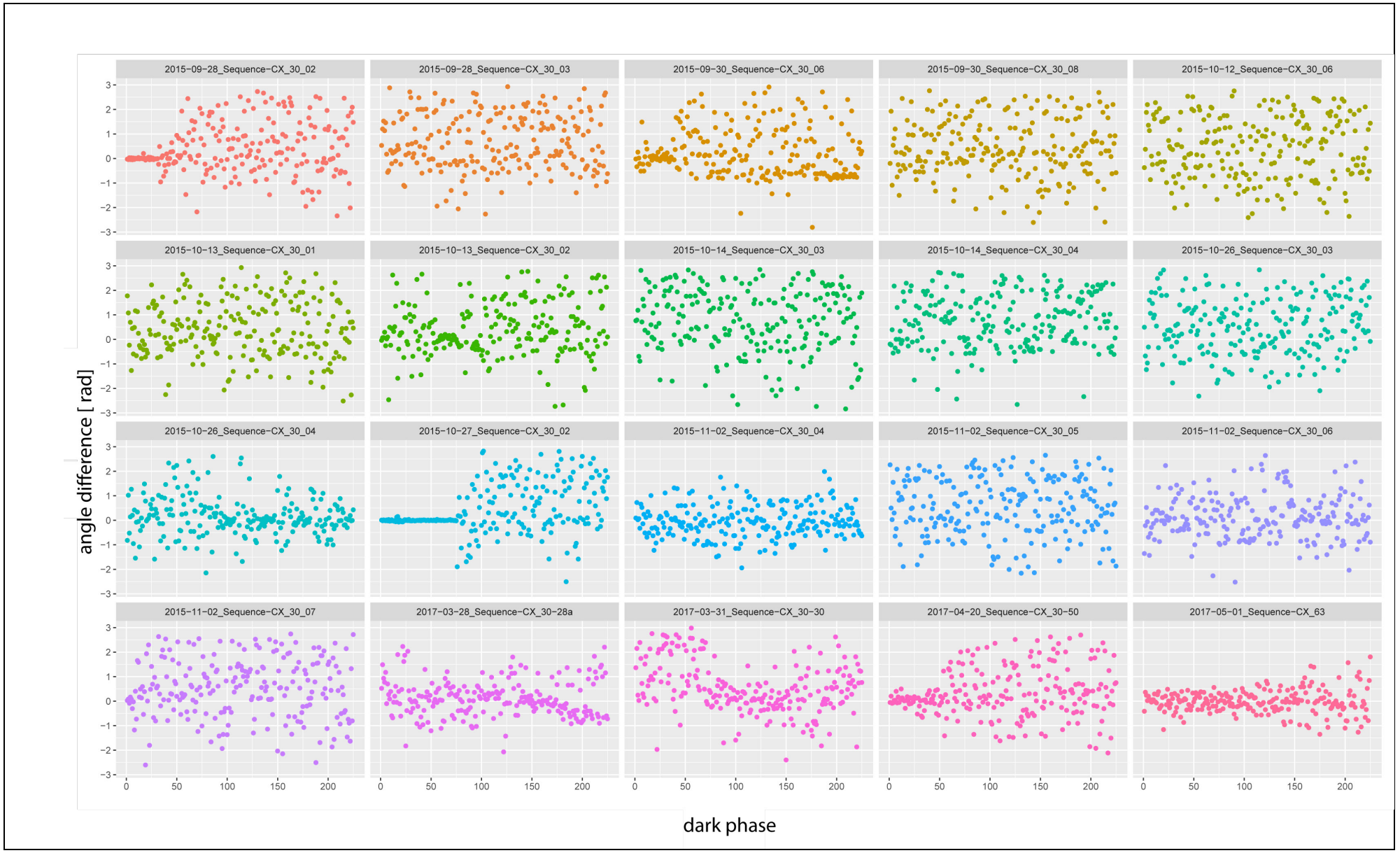

2015-09-28_Sequence-CX_30_02
2015-09-28_Sequence-CX_30_03
2015-09-30_Sequence-CX_30_06
2015-09-30_Sequence-CX_30_08
2015-10-12_Sequence-CX_30_06
2015-10-13_Sequence-CX_30_01
2015-10-13_Sequence-CX_30_02
2015-10-14_Sequence-CX_30_03
2015-10-14_Sequence-CX_30_04
2015-10-26_Sequence-CX_30_03
2015-10-26_Sequence-CX_30_04
2015-10-27_Sequence-CX_30_02
2015-11-02_Sequence-CX_30_04
2015-11-02_Sequence-CX_30_05
2015-11-02_Sequence-CX_30_06
2015-11-02_Sequence-CX_30_07
2017-03-28_Sequence-CX_30-28a
2017-03-31_Sequence-CX_30-30
2017-04-20_Sequence-CX_30-50
2017-05-01_Sequence-CX_63
angle difference [rad]
dark phase

Figure S 12: Individual plots for angle differences calculated for L(-1) in the dark period after procaine injections. Positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position.


2015-09-28_Sequence-CX_30_02
2015-09-28_Sequence-CX_30_03
2015-09-30_Sequence-CX_30_06
2015-09-30_Sequence-CX_30_08
2015-10-12_Sequence-CX_30_06
2015-10-13_Sequence-CX_30_01
2015-10-13_Sequence-CX_30_02
2015-10-14_Sequence-CX_30_03
2015-10-14_Sequence-CX_30_04
2015-10-26_Sequence-CX_30_03
2015-10-26_Sequence-CX_30_04
2015-10-27_Sequence-CX_30_02
2015-11-02_Sequence-CX_30_04
2015-11-02_Sequence-CX_30_05
2015-11-02_Sequence-CX_30_06
2015-11-02_Sequence-CX_30_07
2017-03-28_Sequence-CX_30-28a
2017-03-31_Sequence-CX_30-30
2017-04-20_Sequence-CX_30-50
2017-05-01_Sequence-CX_63
angle difference [rad]
2
0
-2
0
50
100
150
200

Figure S 13: Individual plots for angle differences calculated for L(+1) in the dark period after procaine injections. Positive values indicate orientation away from the respective light's position, and negative values indicate orientation towards respective light's position.

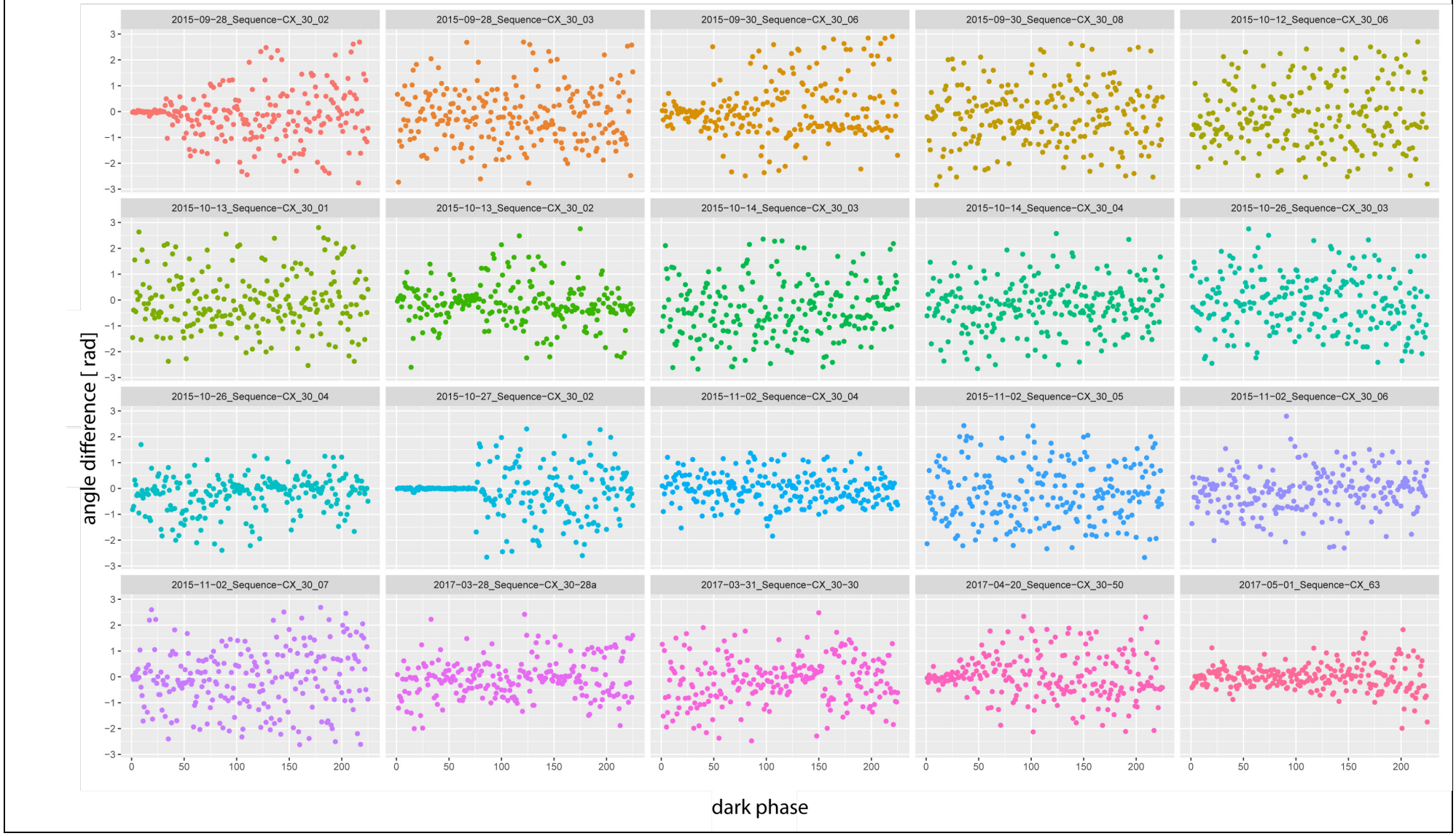

2015-09-28_Sequence-CX_30_02
2015-09-28_Sequence-CX_30_03
2015-09-30_Sequence-CX_30_06
2015-09-30_Sequence-CX_30_08
2015-10-12_Sequence-CX_30_06
2015-10-13_Sequence-CX_30_01
2015-10-13_Sequence-CX_30_02
2015-10-14_Sequence-CX_30_03
2015-10-14_Sequence-CX_30_04
2015-10-26_Sequence-CX_30_03
2015-10-26_Sequence-CX_30_04
2015-10-27_Sequence-CX_30_02
2015-11-02_Sequence-CX_30_04
2015-11-02_Sequence-CX_30_05
2015-11-02_Sequence-CX_30_06
2015-11-02_Sequence-CX_30_07
2017-03-28_Sequence-CX_30-28a
2017-03-31_Sequence-CX_30-30
2017-04-20_Sequence-CX_30-50
2017-05-01_Sequence-CX_63
angle difference [rad]
dark phase

Figure S 14: With experience, sham-treated approached L(+1). Means of distance difference **(A-E)** or means of speed difference **(F)** (grey dots) with added regression line (dashed line) (GLMMs, Table S 5). In panels **A-E**, positive values indicate an increase in distance from the respective light's position, and negative values indicate an approach to respective light's position. The sham group increasingly approached towards L(on) **(A)** over light phases. Bees increasingly walked away from L(0) **(B)**, and towards L(+1) **(D)** or the empty side **(E)** but not towards L(-1) **(C)**. Over dark periods, the sham group showed larger increases in speed (positive speed difference) **(F)**.

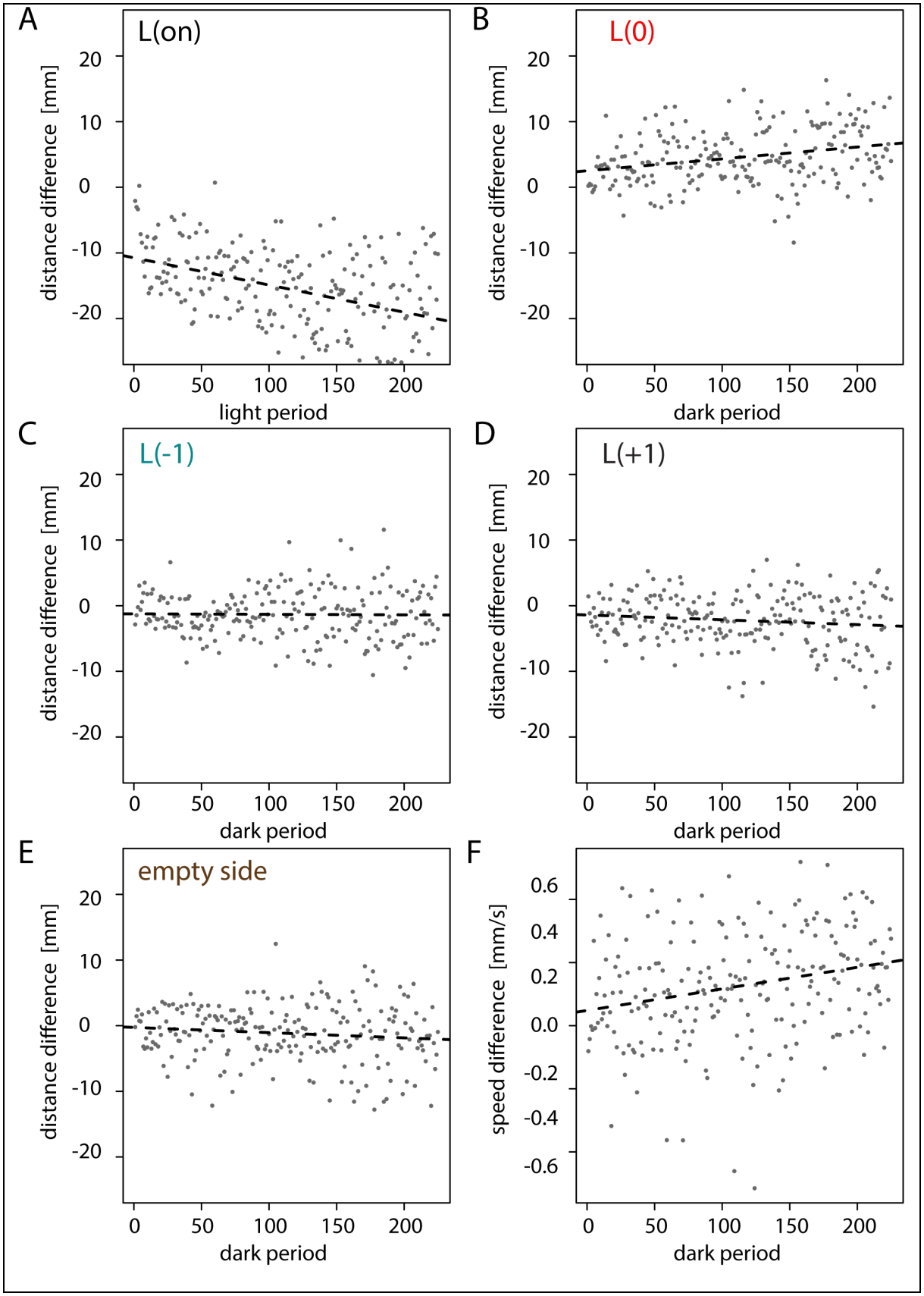

A
L(on)
distance difference [mm]
light period
B
L(0)
distance difference [mm]
dark period
C
L(-1)
distance difference [mm]
dark period
D
L(+1)
distance difference [mm]
dark period
E
empty side
distance difference [mm]
dark period
F
speed difference [mm/s]
dark period

Figure S 15: With experience, vehicle-treated approached L(+1). Means of distance difference **(A-E)** or means of speed difference **(F)** (blue dots) with added regression line (dashed line) (GLMMs, Table S 5). In panels **A-E**, positive values indicate an increase in distance from the respective light's position, and negative values indicate an approach to respective light's position. The sham group increasingly approached towards L(on) **(A)** over light phases. Bees increasingly walked away from L(0) **(B)**, and towards L(+1) **(D)** or the empty side **(E)** but not towards L(-1) **(C)**. Over dark periods, the sham group showed smaller increases in speed (positive speed difference) **(F)**.

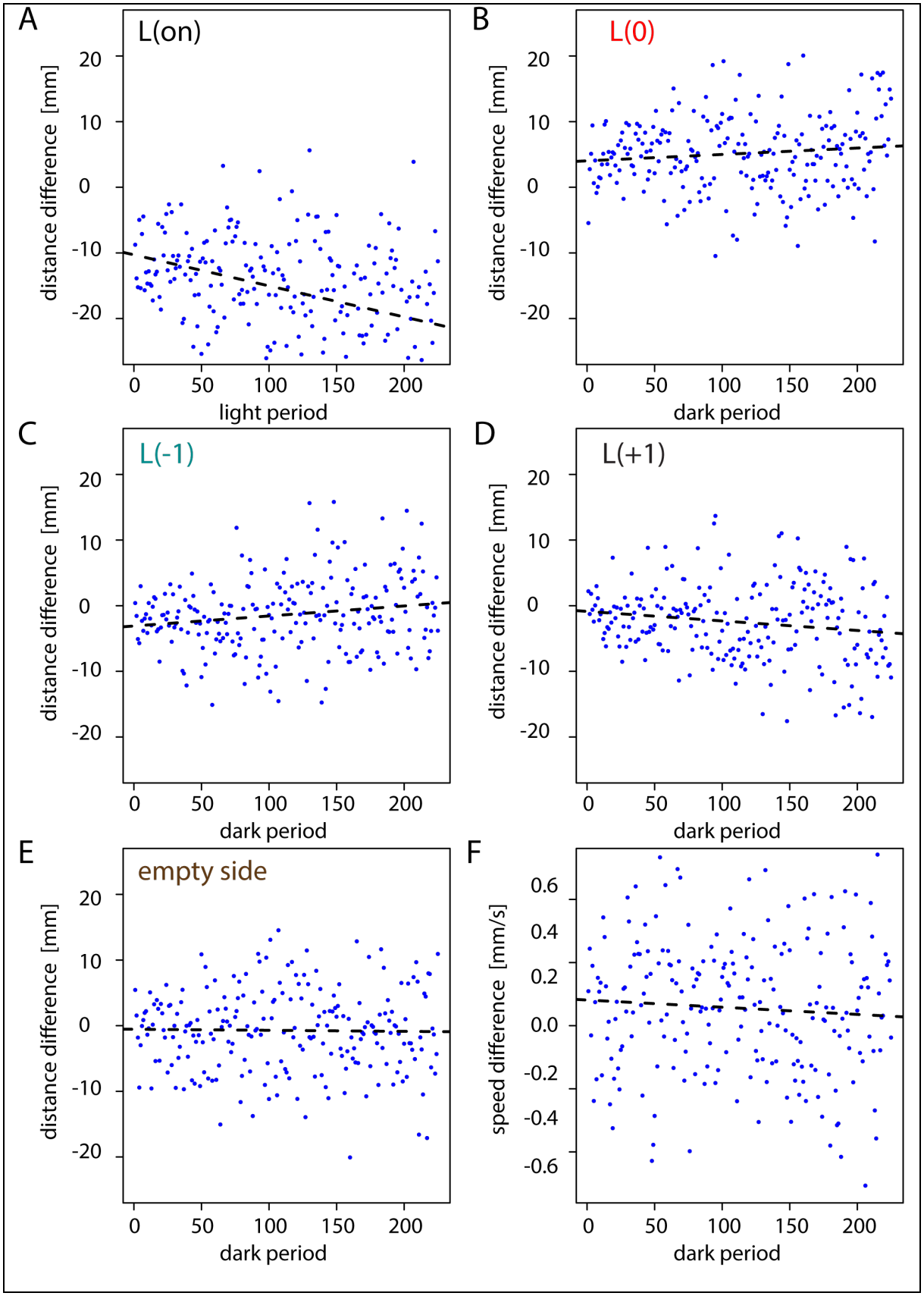
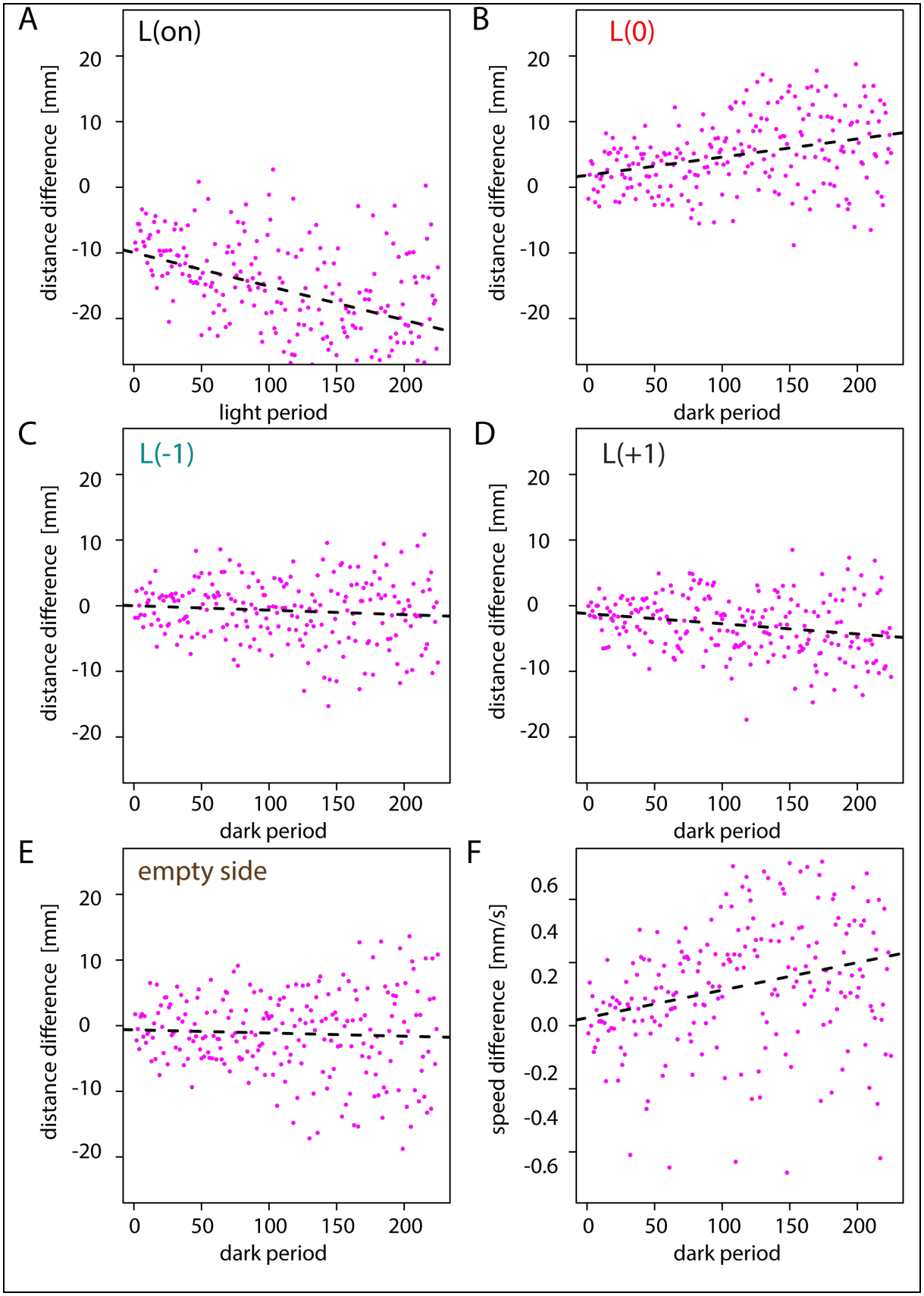

A
L(on)
distance difference [mm]
light period
B
L(0)
distance difference [mm]
dark period
C
L(-1)
distance difference [mm]
dark period
D
L(+1)
distance difference [mm]
dark period
E
empty side
distance difference [mm]
dark period
F
speed difference [mm/s]
dark period

Figure S 16: With experience, procaine-treated approached L(+1). Means of distance difference **(A-E)** or means of speed difference **(F)** (magenta dots) with added regression line (dashed line) (GLMMs, Table S 5). In panels **A-E**, positive values indicate an increase in distance from the respective light's position, and negative values indicate an approach to respective light's position. The sham group increasingly approached towards L(on) **(A)** over light phases. Bees increasingly walked away from L(0) **(B)**, and towards L(+1) **(D)** or the empty side **(E)** but not towards L(-1) **(C)**. Over dark periods, the sham group showed larger increases in speed (positive speed difference) **(F)**.


A
L(on)
distance difference [mm]
light period
B
L(0)
distance difference [mm]
dark period
C
L(-1)
distance difference [mm]
dark period
D
L(+1)
distance difference [mm]
dark period
E
empty side
distance difference [mm]
dark period
F
speed difference [mm/s]
dark period

Chapter V

The evolution of honey bee dance communication: a mechanistic perspective

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Abstract

Honey bee dance has been intensively studied as a communication system, and yet we still know very little about the neurobiological mechanisms supporting how dances are produced and interpreted. Here, we discuss how new information on the functions of the central complex (CX) of the insect brain might shed some light on possible neural mechanisms of dance behavior. We summarize the features of dance communication across the species of the genus *Apis*. We then propose that neural mechanisms of orientation and spatial processing found to be supported by the CX may function in dance communication also, and that this mechanistic link could explain some specific features of the dance form. This is purely a hypothesis, but in proposing this hypothesis, and how it might be investigated, we hope to stimulate new mechanistic analyses of dance communication.

Introduction

Honey bee (*Apis*) dance communication is arguably the most lauded of all forms of animal signaling. Bees dance to signal the location of valuable resources to their nestmates, and dances are effective in recruiting additional foragers to those resources (Seeley, 1995). It has been described as the only known form of 'symbolic communication' in the invertebrates (von Frisch, 1967). All bees in the genus *Apis* dance, but outside of *Apis* there is nothing quite like it. Thanks to visionary work by Martin Lindauer (1956b; 1961), we have had a possible phylogenetic scenario for the evolution of dance for some time. It is still not clear, however, how a bee might convert a foraging trip to a functionally referential signal (sensu Blumstein, 1999), or how a recruit might interpret a dance to identify a foraging location. Here, we discuss how new findings from insect neurobiology may shed some light on this problem. We begin by briefly summarizing the features of dance communication across the genus *Apis*. We then review new research into how orientation and spatial relationships are processed by the central complex [CX, an unpaired cluster of neuropils (see Glossary) in the center of the insect brain]. We argue that neural mechanisms of spatial processing may have been exapted for new functions in dance communication. Exaptation (sensu Deacon, 2012) refers to a new adaptive function evolving by a shift or expansion of existing functions. We propose that pre-existing mechanisms for spatial processing, involving mostly the CX, adopted new functions in the evolution of dance performance and dance

following, and that this mechanistic relationship explains some of the specific features of honey bee dance communication. Having set out this hypothesis, we conclude by suggesting how it could be explored experimentally.

The structure of dance communication in *Apis mellifera*

In European honey bees (*Apis mellifera*), dances are performed in the contexts of foraging and nest site selection. On returning to the hive, successful *A. mellifera* foragers sometimes perform highly stereotyped dance movements (**Figure 1**). For resources more than a few hundred meters away from the nest, the dance can be described as a repeating figure-of-eight movement performed on the vertical surface of the comb hanging inside the hive (**Figure 1**).

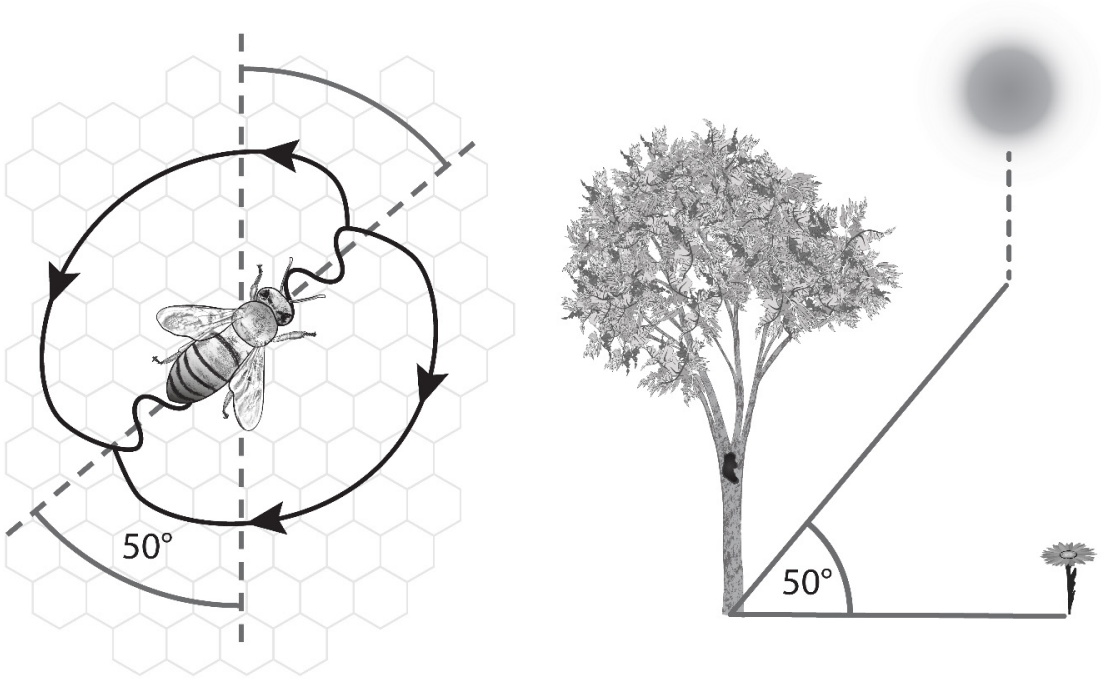


Figure 1: Schematic of the waggle dance of *Apis mellifera*. Dances advertise resources found by foragers. The dance is usually executed on vertical combs inside the hive (left). The angle of the waggle phase of the dance relative to vertical on the comb corresponds to the direction to the advertised resources on departure from the hive relative to the solar azimuth (right). The duration of the waggle phase correlates with the amount of optic flow experienced during the flight to the resources. At the end of the waggle phase the forager loops back to the beginning and repeats the movement.

At the junction between the two loops of the figure of eight, the bee takes a stride and leans forward, vibrating her wings and waggling her abdomen rapidly from side to side in the famous and distinctive 'waggle run' of the dance (Dyer, 2002; Tautz et al., 1996). The wing vibrations produce both acoustic signals and jets of air directed behind the dancing bees (Michelsen, 2012). Features of the waggle run correlate with the distance and direction of the resources found by the forager. Since these dances appear to represent quantitative information about the position of foraging sources in a new (and apparently arbitrary) form compared to the original information, they have been described as 'symbolic communication' (Couvillon, 2012; Dyer, 2002; Preece and Beekman, 2014; von Frisch, 1967). For foraging resources located close to the hive (typically less than a hundred meters), the duration of the waggle phase is extremely short; consequently the figure-of-eight form deforms into a sickle or round shape, but the very brief waggle phases of these dances still contain some directional information (Gardner et al., 2008; Griffin et al., 2012; Preece and Beekman, 2014; Sen Sarma et al., 2004).

Apis mellifera: what is communicated when dancing?

A. mellifera most typically dance on vertical wax frames within the dark nest cavity. The orientation of the waggle phase relative to vertical on the comb correlates with the direction of the resource relative to the solar azimuth on departure from the hive (**Figure 1**); hence the angle of the waggle run relative to vertical is considered a signal of direction for *A. mellifera* (von Frisch, 1967). There is flexibility to directional signaling in this species. European honey bees will sometimes dance on the horizontal board at the hive entrance in the sun, in which case their dances point directly towards the resource (Esch, 2012; von Frisch, 1967). If the image of the sun is reflected in a mirror such that it is visible at the bottom of the frames inside the dark hive then the bees orient their waggle phases to signal the direction of the food relative to this image of the sun (Esch, 2012; von Frisch, 1967).

The duration of the waggle phase in time correlates with the distance of the resource from the hive (Gardner et al., 2008; Schürch et al., 2013). More precisely, the duration of the waggle phase correlates with the amount of retinal image flow (i.e. optic flow; see Glossary) experienced by the bee during her flight (Esch et al., 2001; Srinivasan et al., 2000). The amount of optic flow is usually highly correlated with distance travelled (Barron et al., 2005;

Tautz et al., 2004), and the relationship between waggle duration and the distance to the resource is best described by a linear function, albeit with significant variation around a linear fit (Gardner et al., 2008; Schürch et al., 2013). The speed and number of dance circuits performed correlates with the relative value of the gathered resources (Barron et al., 2007; Tautz, 2008).

Recruits attend to the movements of the dancer, often following close behind her. Recruits must follow more than one waggle phase in order to gain information on the location of the indicated resource (Tanner and Visscher, 2008), but how they 'read' the dance is still unclear. Multiple stimuli could signal the position and movements of the dancer, including physical contact with her body by the antennae of the followers (Rohrseitz and Tautz, 1999; von Frisch, 1967), substrate-borne vibrations generated by the dancer (Tautz, 1996) and acoustic signals (Kirchner et al., 1991; Kirchner et al., 1988; Michelsen, 2003), as well as air flows and narrow directional jets of air generated by the vibrating wings of the dancer (Michelsen, 2003; Michelsen, 2012). Any or all of these might be used by followers to track a dancer's movements. In *A. mellifera*, there is considerable variation in waggle runs both within and between dances for the same location (Couvillon et al., 2012; Schurch and Couvillon, 2013; Schürch et al., 2013). Authors disagree over whether recruits must follow behind the dancer to gain information from the dance (Judd, 1995; Michelsen, 2012; Rohrseitz and Tautz, 1999), or whether recruits can read a dance from side-on (Tanner and Visscher, 2009). Either way, following more waggle runs increases the chance of a recruit successfully locating a foraging source, and presumably recruits have the capacity to improve their estimate of resource location by combining (and perhaps averaging) information obtained from successive waggle runs (Tanner and Visscher, 2009; Tanner and Visscher, 2008).

Odors detected by recruits during the waggle dance also provide important information, but the contribution of odors to dance communication in *A. mellifera* has been controversial (Couvillon, 2012; Esch, 2012; Wenner and Wells, 1990). Dancers produce a specific pheromonal bouquet, which attracts recruits to them, but this does not provide any spatial information (Esch, 2012; Thom et al., 2007). The specific floral odor of the resource collected by the dancer is also a source of information used by recruits to help them locate the indicated resources (Couvillon, 2012; Esch, 2012; Grüter et al., 2008; Grüter and Farina,

2009).

Variation in dance across *Apis* and beyond: insights for a model of dance evolution

Dance is unique to the genus *Apis*, but social recruitment of foragers is certainly not. Opinions still differ as to whether the sister group to tribe Apini are the Bombini (bumblebees) or Meliponini (stingless bees) (Oldroyd and Wongsiri, 2006; Thompson and Oldroyd, 2004), but both of these tribes feature social recruitment of foragers to food sources. Bumblebees do not signal the location of found resources, but they do advertise that they have found something by an energetic zig-zagging run within the hive to alert nestmates (Dornhaus and Chittka, 1999; Dornhaus and Chittka, 2001). The floral odor clinging to the returning forager provides information to recruits about the discovery (Dornhaus and Chittka, 1999; Dornhaus and Chittka, 2001). The stingless bees are by far the most diverse group of these three tribes (with 36 genera within the tribe; (Michener, 2000), and their social recruitment mechanisms vary. The most commonly reported are non-directed alerting runs rather similar to those of bumblebees, but some species have directional social recruitment systems (Lindauer, 1961; Lindauer and Kerr, 1958; Nieh, 2004). It seems reasonable to propose that social recruitment of foragers is ancestral to the Apini (l'Anson Price and Grüter, 2015).

Apis is the only extant genus of Apini. Mapping the differences in the dances between the extant species of the genus to the consensus *Apis* phylogeny suggests a plausible scenario for how dance may have evolved (**Figure 2**). This model for dance evolution was first proposed by Lindauer (1956a) and has been updated by Oldroyd and Wongsiri (2006), Couvillon (2012) and l'Anson Price and Grüter (2015).

The dwarf honey bees (*A. andreniformis* and *A. florea*) are basal to the genus (**Figure 2**). These nests form a single sheet of comb hanging from a tree limb. Dances are performed on a horizontal surface at the top of the comb, and dancers point their waggle runs directly to the resource using celestial cues and/or landmarks to orient the dance (Dyer, 2002). It has been proposed (Couvillon, 2012; von Frisch, 1967) that ancestral honey bees communicated first by excitatory runs (perhaps similar to bumblebees) that involved shaking of the body and were aligned toward the foraging site.

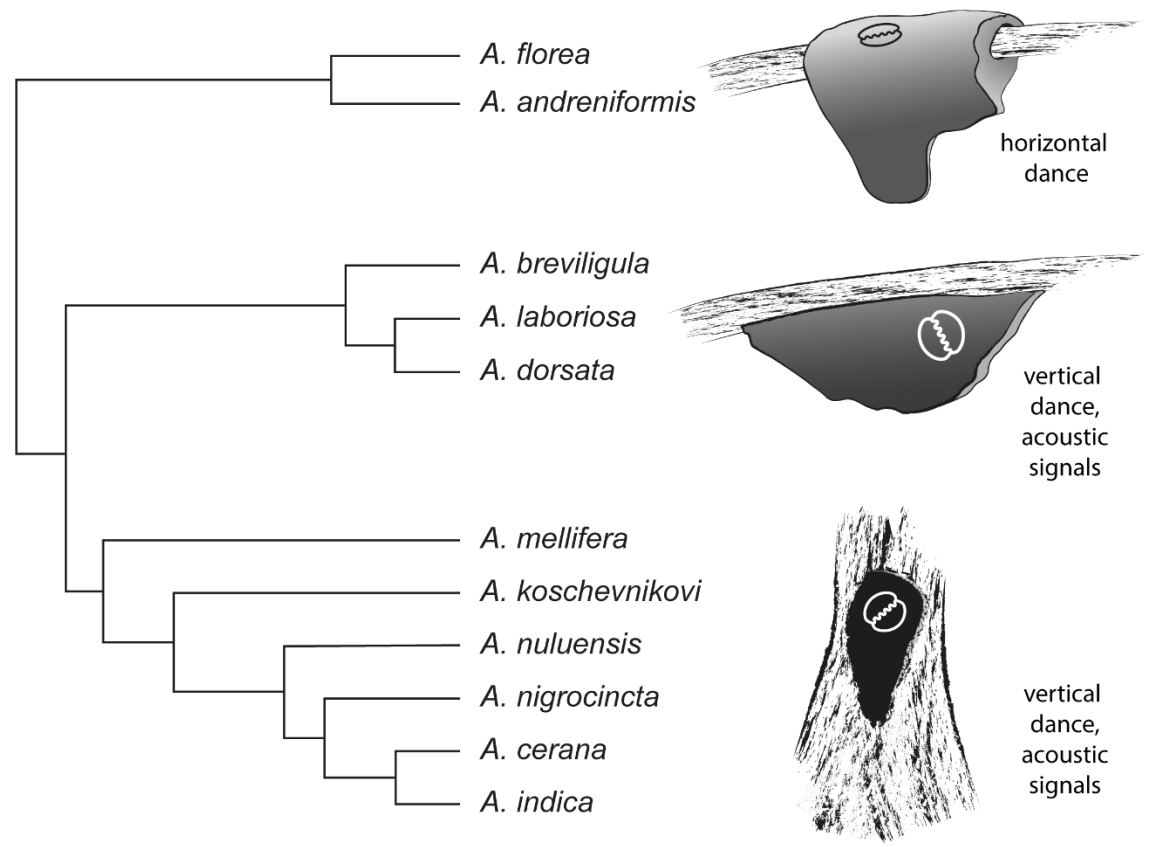


Figure 2: Diversity of dance and nest forms within the genus *Apis*. Dwarf honey bees *A. andreniformis* and *A. florea* dance on the horizontal surface (black dance trace) at the top of their nests suspended from tree branches, and orient their waggle phases to point toward the advertised resources. Giant honey bees (the *A. dorsata* clade) dance on the vertical surface (white dance traces) of their comb and orient their waggle phases with respect to gravity, similar to the dances of the cavity-nesting species (the clade including *A. mellifera*). Phylogeny adapted from Lo (2010)

The simple waggle dance could be thought of as re-enacting the departure direction of the forager bee from the hive. Over time it is assumed that the dance evolved to became more stereotyped to resemble the neat figure-of-eight waggle dances of the extant dwarf bees. Orienting dances relative to gravity on a vertical comb, and adding acoustic signals to the waggle phase are considered to be derived dance features. Vertical dances evolved in species that build combs in cavities (*A. mellifera* and *A. cerana*) or under ledges (*A. dorsata* and *A. laboriosa*), where there is no horizontal dance floor. Sound pulses and air jets are interpreted as adaptations to make waggle dances more apparent in low-light environments such as a cavity, or underneath a sheet of close-packed bees (as in the giant honey bees like *A. dorsata*). In the migratory *A. dorsata*, dances also occur in the context of the migration of

the colony. Dances precede the departure of the colony from either its original nest site (Dyer and Seeley, 1994) or from bivouac sites along the colony migration route (Robinson, 2012). In this special case dances appear to indicate the direction for the swarm to move on departure, but it is not clear if they indicate any specific distance (Dyer and Seeley, 1994; Robinson, 2012). The cavity-nesting species *A. mellifera* and *A. cerana* have dances that are so similar it is possible for them to function across species (Su et al., 2008; Tan et al., 2008).

A phylogenetic analysis of dance evolution suggests therefore that the original dances can be thought of as "a symbolic enactment of the foraging flight" (Couvillon, 2012; von Frisch, 1967; Wilson, 1971), since the waggle run points directly in the direction to be flown. If this interpretation is correct, then the evolutionary innovation that may have led to the dance motor pattern could have initially been as simple as an outbound forager delaying her departure from the hive and performing part of her departing flight vector (including beating her wings) while still clinging to the comb. The neat figure-eight looping behavior that is so characteristic of dancing may have evolved later as a mechanism to enable the dancer to hold a position on the comb for multiple circuits while being followed.

The waggle dance of *A. mellifera* still indicates the vector from the hive to the food source, but uses a gravitational reference to substitute for a celestial reference. From this perspective, the mechanisms supporting dance communication must therefore involve the mechanisms of orientation of flight. Below, we summarize new findings on the neural mechanisms of orientation and path integration (see Glossary) in bees and other insects, and propose how they might function in dance communication.

The central complex and its role in orientation and path integration in walking and flying insects

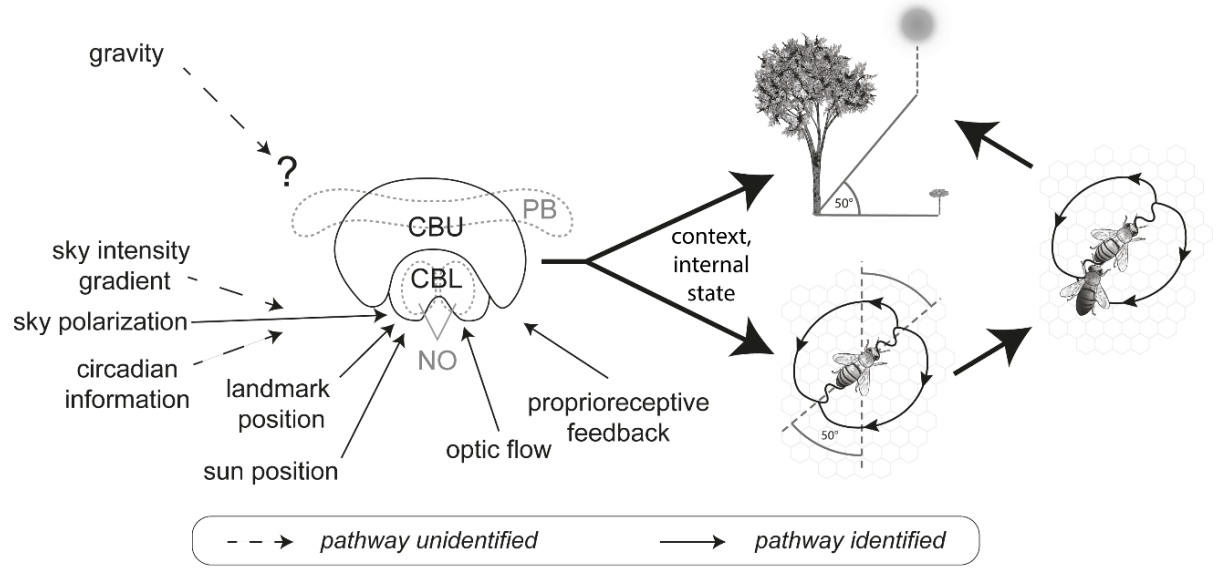
Recently a series of studies of the CX has transformed our understanding of how insects process their position in space (**Figure 3**) (Pfeiffer and Homberg, 2014; Plath and Barron, 2015; Turner-Evans and Jayaraman, 2016; Varga et al., 2017). The CX consists of the protocerebral bridge (PB), the central body (CB) and the noduli (NO) in pterygote insects (**Figure 3A**). The CB is divided into an upper division [CBU, termed fan-shape body (FB) in the fruit fly] and a lower division [CBL, termed ellipsoid body (EB) in the fruit fly]. The CX receives sensory information via tangential neurons from the surrounding protocerebrum;

the majority of this sensory input is visual information indirectly relayed from the optic neuropils. The tangential neurons have ramifications outside of the CX and connect to the PB (TB neurons), the CBU (TU neurons), the CBL (TL neurons) and the NO (TN neurons). Information is transmitted between the PB, CBU and CBL via columnar neurons, which create a columnar organization (vertical slices) in all three structures. In the NO, two distinct layers are found which are interconnected exclusively with the CBU or with the CBL. Important input and output regions for the CX are the adjacent lateral accessory lobes (LAL) in either hemisphere. For more detail on CX architecture and connectivity see (Heinze et al., 2013; Heinze and Homberg, 2008; Lin et al., 2013; Wolff et al., 2015).

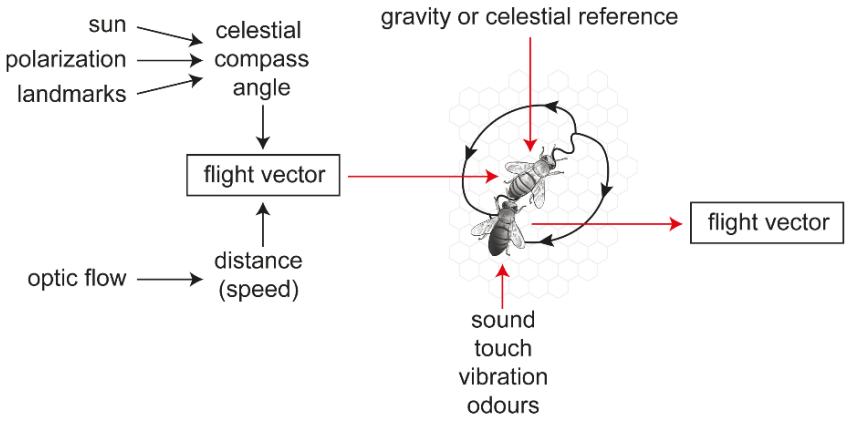
For a foraging flight, it is essential that a bee is able to identify the directions of the home hive, the foraging site, and its current heading relative to some common reference. It has long been known that the pattern of polarized light in the sky is a vital reference by which bees orient. More recent research in several insects has shown that neurons in the CX form a map-like organization of *E*-vector orientations (el Jundi et al., 2014; Heinze and Homberg, 2007; Pfeiffer and Homberg, 2014) which can provide celestial compass information that will help an insect to identify its orientation relative to celestial cues. The compass neuron network comprises groups of excitatory and inhibitory tangential and columnar neurons with activity maxima elicited by different azimuths of the celestial body or different *E*-vector angles (see Glossary). Polarotopy in the network is stabilized by antagonistic integration across neurons that are active at *E*-vector angles shifted by 90° (Bockhorst and Homberg, 2015). The celestial compass pathway has been reconstructed in the honey bee (Brockmann and Robinson, 2007; Held et al., 2016; Mota et al., 2011; Zeller et al., 2015), and is very similar to the described pathways found in other bees (Pfeiffer and Kinoshita, 2012), locusts (Homberg et al., 2003; Homberg and Paech, 2002).

Figure 3: Inputs to the central complex involved in orientation, and proposed to also be involved in dance. (A) The CX is composed of the central body upper unit (CBU), the central body lower unit (CBL), the protocerebral bridge (PB) and the noduli (NO). The figure shows a summary of inputs that have been identified in various insect species entering the CX (solid arrows) and potential inputs to the CX with as-yet-unidentified pathways (dashed arrows). The relevant references are included in the text. We propose that these inputs, which carry different forms of spatial information, along with processing within the CX support both the calculation of the vector displayed in the dance (upper right) and the execution of the dance movement (lower right). Which behavior is performed (dance or flight) depends on the context and state of the bee. **(B)** Information flow between dancer and recruit. For the dancer, celestial information and optic flow information gathered during flight are integrated into a single flight vector reflecting the shortest path between the hive and the resources. The flight vector information is transformed to specific dance movements: dance orientation and waggle duration, oriented relative to either gravity or celestial references depending on the bee species. Odors attract recruits to dancers, and recruits sense the dance movement through sound, touch and vibration. Recruits then transform information gathered from the dance to a flight vector. Red arrows indicate which parts of this hypothesis present the greatest challenges for a neurobiological interpretation.

A


gravity
?
PB
CBU
CBL
NO
sky intensity gradient
sky polarization
circadian information
landmark position
sun position
optic flow
proprioreceptive feedback
context, internal state
50°
50°
pathway unidentified
pathway identified

B


sun
polarization
landmarks
celestial compass angle
flight vector
optic flow
distance (speed)
gravity or celestial reference
flight vector
sound
touch
vibration
odours

It seems that processing within the CBL helps a moving insect maintain a heading relative to celestial or other visual cues, or to execute a turn to a new heading. Activity in one column of the EB corresponds to the orientation of the animal in relation to either visual stimuli (allothetic cue; see Glossary) or to the proprioceptive signals provided by walking (idiothetic cue; see Glossary) (Seelig and Jayaraman, 2015). Interestingly, the EB activity is maintained beyond the presentation of either visual stimuli or the animal moving – thus providing a possible mechanism for spatial working memory in flies (Seelig and Jayaraman, 2015). These findings were based on calcium imaging, capturing activity profiles created by all neurons in the EB at once. A recent study based on extracellular recordings in the cockroach CBL region discovered neurons that responded variously to allothetic and idiothetic cues alone or in combination (Varga and Ritzmann, 2016). This would support orientation relying on external and self-motion cues.

The CBL is also important for organizing an insect's change in orientation relative to the external landmarks or cues; most of the relevant studies have been done with walking or tethered insects. For example, initiation of locomotion and turning behavior in cockroaches and crickets is preceded by a change in firing rate in CBL neurons (Guo and Ritzmann, 2013; Kai and Okada, 2013; Martin et al., 2015). Further, stimulation of neurons with predictive firing patterns elicited the same walking and turning responses observed when recording from these cells (Martin et al., 2015). We note here that these studies are based on extracellular recordings; it is therefore possible that some neural responses were recorded from neurons bypassing rather than entering the CBL.

The CX is core to mechanisms underlying orientation and movement in the environment. However, it remains to be investigated how the insect brain integrates the spatial information gathered on a foraging flight to be able to navigate a direct route (a single vector) from a nest to a food source and back. Ethological studies suggest that some insects (especially walking ants and flying bees) use path integration to find the shortest route, and even use novel short-cuts from food sources to the nest (Collett and Collett, 2000b; Wehner and Srinivasan, 2003). As we have described, it is now well established that the CX supports the capacity to both represent and control orientation relative to either idothetic and allothetic cues. Heinze (pers comm) has reported tantalizing new findings that the TN neurons projecting to the NO in the tropical nocturnal sweat bee *Megalopta genalis* might

have the capacity to code speed. If this finding proves to be correct, it shows that the CX system processes within it all the information needed for a path integration calculation. We propose it is most parsimonious to imagine a homebound vector calculated by the CX system could be used to determine a dance movement as well as to determine a flight vector.

How orientation mechanisms and the CX might be involved in generating the dance

The hypothesis that the CX is involved in dance signaling was first suggested by Brockmann & Robinson (2007). As discussed above, for *A. mellifera* the directional information obtained relative to the sun during flight must be translated into directional information relative to gravity when dancing on the vertical comb. How could this be done? A possible inference from current studies of the CX is that it is able to use any spatial reference to generate an orientation signal, and that its output is not bound to any specific form of spatial reference (Seelig and Jayaraman, 2015; Varga et al., 2017). Thus, there may not need to be a specific mechanism for switching of a reference frame for orientation in the CX, but information on gravity must be available to the CX system if gravity is to be used as an orientational reference frame by dancers.

One candidate site for sensing orientation relative to gravity (i.e. geosensing) is the neck, since any inclination of the thorax in comparison to the head due to gravity would lead to a different pressure of the head onto the thorax. Manipulations of mechanosensory hairs located at the neck leads to disorientated geotactic behavior and a disorientated dance (Lindauer and Nedel, 1959; von Frisch, 1967). Projections from these hairs to the subesophageal ganglion have been found in the honey bee (Brockmann and Robinson, 2007). Other possible sites for geosensing include the joint between the thorax and the abdomen, and the leg joints (Srinivasan, 2011). However, these have not been investigated in relation to dance behavior to our knowledge, and how geosensing might be integrated into the CX network still needs to be explored.

Dancers can also update their directional estimate of the food source over the course of the day as the sun moves across the sky, demonstrating a time-compensation aspect to their celestial compass (for discussion see Srinivasan, 2011). When bees are stopped from foraging for a time after learning a food source and then receive some nectar from that food source,

some bees start to dance the direction of the food source indicating the correct position of the sun at that time despite having not left the hive to update their information on solar position (Lindauer, 1960). Zeller et al (2015) identified a possible circuit for interaction between neurons carrying polarization information and neurons sensitive to circadian information suggesting a possible locus for time compensation of the celestial compass. This system could also be involved in the generation of time-compensated dance output.

How distance information is transformed from flight to dance is currently challenging to understand. The amount of optic flow experienced in flight en route to the food source determines duration of the waggle phase (Esch, 2001). For dance, however, the distance aspect of the vector output of the CX network must be transformed to a waggle phase of a certain duration rather than a flight of a certain amount of optic flow. How this might be done is not clear.

How might the dance be interpreted by recruits?

The key information that dance followers gain from the dance is that a profitable food source exists, along with information on its direction, an estimate of distance, and its odor. As discussed above, recruits mostly track the position of the dancer using their antennae (Dyer, 2002; Esch, 2012; Michelsen, 2012). Open-nesting honey bees can also see the dancer. Recruits must transform a vector indicated by the sensed dance movement back into a flight vector. In open-nesting species, the direction component of the dance is usually oriented with respect to celestial cues, therefore the direction estimated from the dance is in the same reference frame as that of a flight. For cavity-nesting species, however, the direction estimated from the dance can be oriented with respect to gravity. In flies and cockroaches the CX heading estimation is not bound to any specific reference frame (Seelig and Jayaraman, 2015; Varga et al., 2017). If the same is true for bees, then the CX system could enable direction estimation to operate with respect to any reference frame. No 'switching mechanism' would be needed within the CX since the context of the currently executed movement would provide the reference. To translate a dance vector to a flight vector, however, it must be the case that flight headings orient to visual and/or celestial cues and do not use gravity, whereas dance and dance-following headings orient to gravity.

How distance information signaled by the duration of a waggle phase might be translated

by a recruit back to the amount of optic flow perceived during flight is another tough question. As discussed above, evidence suggests quite a high level of imprecision in both the execution of the waggle movement and the vectors flown by recruits. Perhaps this imprecision reflects sensorimotor constraints in the translation of vector information stored by the CX into dance movements, and back again (Beekman et al., 2005).

Recruits are also able to pick up odor cues from the collected resource from the dancer, and it is common for the dancer to donate nectar to recruits via trophallaxis (Farina, 2000; Farina et al., 2005). In this case, the well-studied mechanisms of olfactory learning in the bee antennal lobe and mushroom body (Galizia, 2014) would enable the recruit to associate the odor of the dancer with nectar reward, which would establish the odor of the nectar source as a rewarding goal (Reinhard et al., 2004a; Reinhard et al., 2004b).

Investigating the neural basis of the waggle dance

By emphasizing the relationships between aspects of dance behavior and aspects of orientation and foraging behavior, and by considering the properties of neural systems now known to be involved in orientation and foraging, we have proposed that neural systems might have been exapted during evolution to new functions in dance. This is, of course, a hypothesis that needs to be tested. Dance is, by its nature, a movement and a social interaction, which means that it cannot be studied using harnessed bees; this, in turn, rules out using electrophysiological approaches with current technology. However, the anatomical and electrophysiological exploration of neuronal pathways involved in spatial orientation and navigation in other insect systems now has great momentum, and will certainly provide insights that will help us understand orientated behaviors, including dance.

The challenge that we face is to relate the responses of specific groups of neurons to what the animal is doing in its natural environment. el Jundi et al. (2014) artfully demonstrated a roadmap for how that might be done by first manipulating the natural stimuli to carefully observe the change in behavior, then demonstrating the same changes in behavior occur in response to carefully selected artificial stimuli applied in a lab setting, and finally by recording neuronal responses to these artificial stimuli using electrophysiology. Can we apply these principles to similarly dissect the dance to its mechanism?

If we can find out which cues trigger a switch between using path integration information to execute a flight vector or to execute a dance vector, we would be one step closer, at least, to understanding the dance behavior. We also urgently need a better understanding of dance as a pattern of motor activation. The behavior is well described (Tautz et al., 1996), but what muscles are involved?

Neuropharmacological methods have been used to explore dance (Barron et al., 2009; Barron et al., 2007) and to uncover how dance changes due to ingestion of pesticide (Schricker and Stephen, 1970). To make further progress, targeted neuropharmacology by microinjection of specific agonists and antagonists into specific brain regions (Søvik et al., 2016) could provide a method for testing the role of the CX in dance. Substances known to alter dance behavior and navigation after systemic treatments could be injected into different brain regions to determine whether bees would still be able to find food sources which had been visited before. A challenge is whether the bee could recover well enough from such an invasive procedure to participate in dances or follow them before the pharmacological agent has worn off. Perhaps injection of microcapsules into the head capsule could be a method of delivering a slow-release drug to areas of the bee brain. The ultimate goal would be to combine long-term acting drugs such as irreversible antagonists with long-term observation of treated bees in the hive to uncover changes in behavior. RFID tagging of bees (Perry et al., 2015) is a useful tool for this kind of research.

Very ingenious neurogenomic analyses have identified some candidate genomic pathways that are potentially involved (Sen Sarma et al., 2010; Sen Sarma et al., 2009). Other techniques that would be transformative could be genetic transformation of bees with piggyBAC and CRISPR/Cas9 (see Glossary) or similar technologies (Kohno et al., 2016; Schulte et al., 2014), or bee-scaled microcanulae or microelectrode backpacks (similar to those now used in free-ranging small mammals; (Fan et al., 2011). We acknowledge that highly insightful recordings have been made from free-moving insects (Kai and Okada, 2013; Martin et al., 2015), but currently it will be technically challenging to record from a bee that is interacting with other bees in a hive such that she may undertake or follow a dance.

Conclusions

In this Commentary, we have discussed the hypothesis that processing in the CX could contribute to both the production and interpretation of the honey bee waggle dance. We acknowledge that the core of this hypothesis was first proposed by visionary neuroethologist Harald Esch who wrote:

"The role of image motion during foraging and dancing can help to investigate the "nature" of bee dances. The waggle dance might be a "symbolic replay" of a foraging flight… We suspect that the whole waggle dance is an act of conditioning: A recruit "learns" the location of a feeding site during attendance of a symbolic replay of a foraging flight inside the hive. A food sample delivered by the dancer through trophallaxis serves as a reward. We know that bees can perform most of the behaviors that are required for this task" (Esch, 2012).

Although there has been enormous success in dissecting the phenomenon of dance behavior, thus far there has been little progress in studying the neural mechanisms involved. This is because it is an extremely hard task. Bees only dance in a hive, and no one has yet persuaded any bees to execute dances in a laboratory setting, making the dance a very difficult phenotype to investigate experimentally. Learning more about the neurobiology of the bee brain is allowing us to flesh out this hypothesis. If indeed dance evolved by exaptation of orientation and learning systems, then while dance can still be described as a functionally referential signal, the form of the dance is far from arbitrary and reflects a hive-bound replay of a foraging flight.

Acknowledgments

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Supplemental Material

Glossary

Allothetic: a navigational reference external to the subject

E-vector: electric vector – the component of light that interacts with matter (Horváth and Varjú, 2004), functionally also the angle of polarisation.

Idiothetic: a navigational reference internal to the subject

Neuropil: a region of dense nerve tracts, connectivity and synaptic contacts in the insect brain (Strausfeld, 2012)

Optic flow: the progression of objects in a visual scene across the eye as an animal moves through the scene (Zeil et al., 2009)

Path integration: the integration of all distances travelled and all angles steered, which results in the shortest return path (home vector) (Collett and Collett, 2000a)

PiggyBAC: a transposon system that has proved effective for the stable introduction of gene sequences into the genomes of various insect species (Schulte et al., 2014)

Polarization: the scattering of light by the Earth's atmosphere. In a theoretical world, the degree of polarization is 100% if the incident angle between light and molecules in the atmosphere is 90° (Wehner, 2001)

Polarotopic map: a map of e-vectors (polarization angles)

Rotational optic flow: the lateral progression of objects in a visual scene across the eye as an animal turns (Zeil et al., 2009)

Translational optic flow: the progression of objects in a visual scene across the eye around the axis of movement through the environment, caused by the animal's movement (Zeil et al., 2009)

Conclusions and Outlook

The mushroom bodies and the central complex have both been implicated in visual processing, locomotor control and spatial learning. This thesis was motivated by one overarching research question: what are the particular functional roles of the MBs and the CX in locomotion, visual orientation and learning tasks? I will here consolidate my main findings and present implications and prospects.

Multisensory integration by the MBs and the CX – functionally antagonistic, divided or sequential?

In this thesis, I have demonstrated the importance of studying the role of different brain regions with the same behavioral assay. The microinjection technique described in **Chapter II** and used in **Chapters III - V** has limitations, the most important one being that no injection will be completely reproducible. The major advantage, however, is that the drug effect is still fairly localized if small volumes are used and provides currently the best method for selective manipulation of neural activity in specific brain region of honey bees. Microinjections further allow investigations of behaviors in insects with a different ecologies to the current insect genetic model systems (e.g. *Drosophila melanogaster*) which can contribute to understanding the neural basis of behavior (Webb and Wystrach, 2016). I have used the strengths of this method to create several comprehensive data sets, which contribute to the ongoing identification of the roles of the different insect brain regions in visually oriented behavior.

The CX has been established as the site where a representation of the animal's orientation in relation to external landmarks is created (Varga et al., 2017). The MBs, on the other hand, are clearly involved in associative learning (Heisenberg, 1998; Zars, 2000). While past research has mainly focused on olfactory learning (Giurfa, 2007), my findings presented in **Chapter III** suggest an important role of the MBs in color learning also. After training the response to the learned color stimulus was to run away. I showed, that this response was impaired when neural activity in the CX was reduced. It would be interesting to see, whether a similar effect could be achieved in appetitive learning. This would also clarify if only learning is affected or if procaine might also have an effect on sensation of shocks. Since the bees escaped the shock-paired light field in the initial and the last trials, it is

unlikely, however, that the effect was purely due to an attenuated sensation to shocks. It might proof useful to increase the sample size even further, since we found some effects that could be due to a bias – for example the preference for green in the initial preference test observed in the group injected into the MBC with vehicle solution. This could be verified or rejected with a higher sample size and additional counter-balanced color learning assays. Additionally, it would be worthwhile to vary the assay using lights of the same wavelength but with different intensities, as intensity was shown to have an effect on learning in this particular assay (Kirkerud et al., 2017). Furthermore, it would be interesting to see if the impairment in running away from the shock-paired blue side found in the procaine group after CX injections, can be reproduced when the behavioral response to a learned visual stimulus is not running but a different motor response, e.g. extending the proboscis. If a similar effect can be found, this would support our hypothesis that information of the learned stimulus might be passed on indirectly from the MBs to the CX to regulate the motor response, i.e. to run away.

Early research in flies and cockroaches proposed an antagonistic role of the MBs and the CX in locomotor control: inhibiting or ablating the MBs increases walking activity (Heisenberg, 1998; Zars, 2000) and inhibiting or ablating the CX decreases walking activity (Strauss, 2002). As reviewed in **Chapter I**, recent research has shown that the role of the CX in motor control is far more complex. The CX is involved in turning, reaching across gaps and climbing (e.g., Varga et al., 2017). Furthermore, a different study showed that the initial walking activity was reduced after inhibiting or ablating the MBs in fruit flies when compared to controls (Serway et al., 2009). In **Chapter IV**, I present similar findings for honey bees: After anesthetizing the MBCs, walking activity was reduced within the first 15 minutes after treatment. However, in the light of other findings, it seems that, while control animals exhibit higher walking activity compared to animals with disturbed MBs, walking activity in control animals declines faster compared to animals with disturbed MBs. Thus, MBs seem to be involved in structuring activity bouts and rest periods relative to the external environment.

The SMP was suggested as a candidate region where an indirect connection from the MBs to the CX might be found (Strausfeld and Hirth, 2013). The CX receives visuospatial information (**Chapter I**) which is integrated to a head direction signal. In **Chapter IV**, I

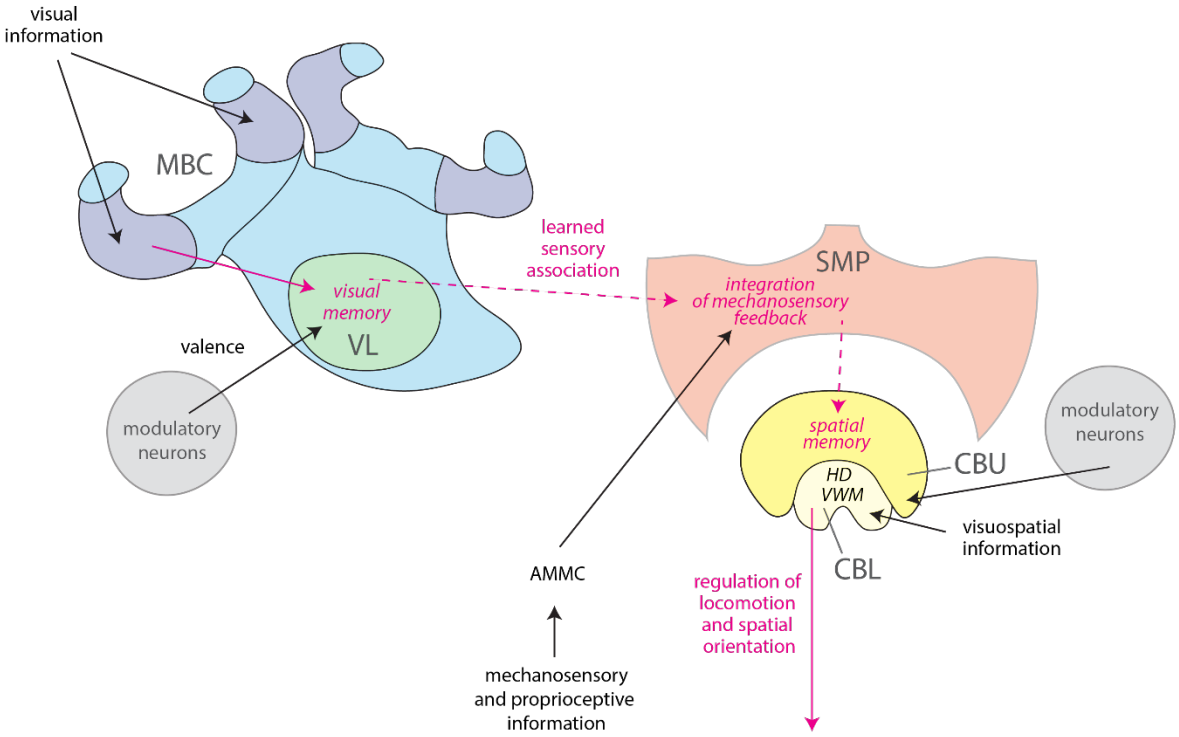
showed that the SMP with its connections to the CX was important for turning while walking in dark, low light conditions and conditions with distinct visual cues. In the dark, the animal had to rely on mechanosensory cues received by the antennae and the feet. Since the SMP receives mechanosensory and proprioceptive inputs in other insects (Ignell et al., 2005; Strausfeld, 1976), I concluded that the SMP might process idiothetic information arising from motion and proprioceptive cues to be passed on to the CX for further processing.

Potential differences in duration of behavioral effects caused by procaine-injections, could be due to differences in diffusion and elimination of the drug, which in turn could be due to distribution and activity differences of acethylcholinesterase (AChE) in the brain. AChE seems to be the only cholinesterase in honey bees (Shapira et al., 2001). AChE might therefore be the enzyme which hydrolyzes procaine in the insect brain. In Chapter III, the procaine effect on exhibiting a conditioned response to a learned stimulus after injections into the VLs seem to have lasted for most if not the entire conditioning phase (15 - 24 minutes). This was similar to results found for olfactory learning (Muller et al., 2003). However, after injections into the CX with procaine the effect on running away from the conditioned light field seemed to have worn off after about half of the conditioning trials (~ 20 minutes). Walking activity was affected for ~ 10 minutes after injections into the MBCs and turning behavior was affected for ~ 5 minutes after injections into the CX/SMP (Chapter IV). However, in circling animals the effect duration was longer (~ 15 - 20 minutes). This was comparable to results found for procaine effects on walking activity (Kaiser and Libersat, 2015) or optomotor responses (Kathman et al., 2014) in cockroaches. This suggests, that the location of injection affected the duration. This could be due to different levels of AChE in the different brain regions and could be investigated by matching AChE levels identified by with the duration of the behavioral effect.

Taken together, the MBs and the CX have a clear functional division: the MBs form multisensory associations, which are weighted according to different behavioral states; the CX provides orientation information in relation to external information and internal feedback. However, the findings presented here support also a functional sequence: learned and weighted information could be passed on indirectly from the MBs to the CX in order

to produce a goal-directed and oriented motor response in relation to the environment (Figure 1). This does not mean, however, that all motor responses due to sensory integration in the MBs would be need to be regulated by the CX. Other motor responses such as extending the proboscis in response to a positively associated odor could still be initiated by descending pathways directly connecting to MB extrinsic neurons (Heisenberg, 1998; Strausfeld et al., 1984) I further found that the SMP and CX are further involved in spatial learning in honey bees. This supports findings in flies (Ofstad et al., 2011) and furthermore showed that honey bees are able to learn complex spatial relationships of visual stimuli.

Figure 1: Information flow model for visually oriented behaviors. Supporting evidence for processes from this thesis is marked in magenta. Dashed arrows indicate hypothetical processes. Evidence from other studies in different insects is marked in black. The MBs (blue) receive visual information, e.g. color information, in the collar regions of the MBCs (dark blue). Processed visual information is passed on to the VLs of the MB (green). The VLs are a likely site for association of visual information with positive or negative reinforcement (valence) to produce visual memories. Outputs from the VLs project into the SMP (orange), which also receives mechanosensory and proprioceptive feedback information from the antennal mechanosensory and motor center (AMMC) in the brain. Integrated information from the SMP might be forwarded to the CX (yellow). The CBL (light yellow) receives visuospatial information, e.g. visual patterns leading to the formation of visual working memory (VWM). Integrated with information about body angle, a representation of the orientation in relation to external landmarks is created (head direction: HD). Integration of VWM and HD could lead to a longer-lasting spatial memory, possibly located in the CBU (dark yellow). The CX could now contribute to the regulation of locomotion and orientation in space according to valence coded visual information from the MBs ('is something good or bad'), spatial memories ('where was the animal's goal located before'), VWM ('where are the external references located at this moment') and HD ('how is the animal's body oriented in relation to external landmarks'). Both, the CX and the MBs receive modulatory input. CX: central complex, CBU: upper division of the central body, CBL: lower division of the central body, MBs: mushroom bodies, VLs: ventral lobes, SMP: superior medial protocerebrum, AMMC: antennal mechanosensory and motor center.


visual
information
MBC
visual
memory
VL
valence
modulatory
neurons
learned
sensory
association
SMP
integration
of mechanosensory
feedback
spatial
memory
HD
VWM
CBU
CBL
modulatory
neurons
visuospatial
information
AMMC
regulation of
locomotion
and spatial
orientation
mechanosensory
and proprioceptive
information

Outlook

The studies presented in this thesis corroborate the importance of investigating functions of different brain regions with the same bioassay. A future challenge will be to investigate functions of the different brain regions in the same animal. A combination of pharmacological and electrophysiological techniques could be a promising approach. The work presented here is only part of what could be done with such a data set – further studies are planned in collaboration with groups using advanced algorithms to analyze movement patterns. This would provide further detailed microanalyses of individual as well as treatment effects on locomotion, orientation and visual learning in the presented assays.

Findings from Chapter III led to the hypothesis that weighted and associated sensory information could be passed on from the MBs to the CX via the SMP. I propose a combination of several established techniques to test this hypothesis: Microinjections into the VLs and subsequent electrophysiological recordings from CX unit in walking insects. Many units in the CX precede or correlate with locomotor changes (Varga et al., 2017). It would be quite insightful to investigate if activity patterns in these units change if a previously conditioned visual stimulus versus a non-conditioned visual stimulus were presented to the insect. If information about the learned sensory information was passed on from the MB to the CX, I would expect a change in the activity pattern in the recorded CX units. Another worthwhile topic to explore is, which has only been used in suggested flow models so far, is the modulatory input to both, the MBs and the CX. Further studies in mapping the modulatory neurons might help to address questions, such as, the question, if MBs and CX share modulatory input from similar regions. One candidate region could be the PPL1 or PPM3 region (in fruit flies) which provides dopaminergic input to both, the CX and the MBs (Strausfeld and Hirth, 2013).

With all the benefits of a controlled laboratory experiment in mind, it is equally important to relate findings back to the natural environment and ecology of the studied animal (Webb and Wystrach, 2016). High conservation between the brain structures and pathways between different insects, allows us to generalize findings across different orders to a certain degree. However, it is important to remember that the same structures or neural processes have been exapted to produce different behaviors in different insects (e.g., Fahrbach, 2006;

Farris, 2015; Webb and Wystrach, 2016). In Chapter V, I revisited the hypothesis that the CX is also involved in the waggle dance in honey bees in the light of recent CX research: If representation of the insect's orientation is independent of a particular reference system, the same processes enabling orientation and navigation during a foraging flight could be used to produce the waggle dance.

To investigate the neural basis of complex behaviors such as navigation, orientation during flight or the waggle dance will be a challenge in the future. I demonstrated that microinjected honey bees can be released from their harnesses and be studied while freely moving in arenas. In future experiments, we could tackle some of the big questions in insect orientation and navigation, for example how insects path-integrate. Insects trained to fly through tunnels to a food source could be tested for their ability to use optic flow to measure distance after injections into the CX. To go a step further, homing abilities could be investigated in tagged or marked insects which would be released at a feeding site after injections into the CX or other brain regions. To uncover neural mechanisms underlying dance is already proving to be one of the more challenging questions. It might be challenging but very interesting to microinject returning foragers with a longer lasting drug, such as tetrodotoxin, and observe if the dance rate in those animals decreased. Likewise, honey bee recruits could be injected after having followed a dance to investigate if they are still able to find the advertised foraging site.

Ultimately, studying the neural basis underlying complex behaviors in insects could uncover fundamental mechanisms and processes generalizable across the animal kingdom. Neuroethological research using insects provides great insights into how small brains solve complex tasks. Findings can not only guide us to uncovering similar mechanisms in vertebrates but can also serve as inspiration for applied fields such as robotics or artificial intelligence.

Research on limb movement and dynamics of walking and flying insects has been applied to build micro-aircrafts (e.g., Yanghai et al., 2017), swimming robots (e.g., Kwak and Bae, 2017) and robots walking across complex environments to perform dangerous tasks (e.g., Chou et al., 2015; Go et al., 2006; Zhong et al., 2017; Zhong et al., 2016). For example, flying robots have been modelled after dragonflies using information about wing shape, body

balance and aerodynamics (de Croon et al., 2009). Water beetles use swimming hairs on their legs to which move across water by drag-powered swimming. This has inspired the design and functionality of a new fast-swimming robot (Kwak and Bae, 2017). Light-driven soft robots to perform tasks in confined spaces were created by implementing movement and shape characteristics of caterpillars (Rogóż et al., 2016).

Besides design and movement of robot appendages, sensory processing and higher order integration has served as model for neuro-computational control. Neurons responding to looming stimuli in locusts have been mirrored in a computational model for a vehicle collision detection system (Manfred, 2017). Some aerial robots steer and land with the help of an insect-inspired visual based guidance algorithm (Srinivasan, 2011). Implementation of a MB-like network has led to successful reward-based motor learning in a climbing robot (Arena et al., 2017). These are just a few examples of insect-inspired applications in robotics, but they already provide an impression of the numerous ways to use neuroethological research to build more successful and advanced artificial agents.

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Appendix I

Current progress in understanding the functions of the insect central complex

Original Publication

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Current Opinion in Insect Science

Current progress in understanding the functions of the insect central complex

Jenny Aino Plath[a,b] and Andrew B Barron[a]

The central complex is a group of neuropils in the center of the insect brain which performs higher sensory integration. This region is involved in diverse vital behavioral processes including visual processing, motor coordination, orientation and navigation. Little is known of the circuit organization and properties within this region, and we here review recent progress toward a functional understanding of the central complex. Since central complex research is increasingly limited to just a few model systems, we argue that studies of the central complex in species with broad behavioral repertoires and strong navigational capabilities such as bees and ants will aid in determining the functions of this region.

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Introduction

The central complex (CX) spans across the midline connecting both hemispheres of the insect brain, and is highly interconnected with the surrounding protocerebrum [1]. Exciting new studies include analyses of CX network structures and properties and explore the involvement of the CX in processing of polarized light, motion processing, spatial memory and motor control [2••]. In this review we focus on these behavioral functions.

Research is presently dominated by a few insect species: the discoid cockroach *Blaberus discoidalis*, the monarch butterfly *Danaus plexippus*, the fruit fly *Drosophila melanogaster* and the desert locust *Schistocerca gregaria*. The CX is conserved across insects and other closely related arthropod groups [1,3,4]; hence many functions are most likely to be quite generalizable across other insects as well. We discuss future directions for CX research.

Brief anatomy and connections of the central complex

All parts of the CX are interconnected. The CX neuropils are the protocerebral bridge (PB), the central body (CB) and two noduli (Figure 1a). The CB is divided into the upper unit (CBU) and the lower unit (CBL); also termed fan-shaped body (FB) and ellipsoid body (EB) respectively in the fruit fly CX literature [5•]. The PB and the CB are structured in columns created by the distinct arborization pattern of the columnar neurons [2••] (Figure 1b,c).

Three large fiber tracts lead from and to the CX: the anterior bundles, the isthmus tracts and fibers connecting to the PB [2••]. The CX does not seem to have direct connections to the mushroom bodies (MB) [2••,6], except for a recently discovered neuron in the butterfly brain [7•].

The CX mainly receives indirect visual input [2••], and probably indirect mechanosensory and olfactory input [8–10]. Two parallel visual pathways have been identified in locusts, bees and butterflies [7•,11–14]. The anterior pathway originates in the visual neuropils and does not directly enter the CBL, but enters indirectly via the anterior lobe of the lobula, the anterior optic tubercle and the median and lateral bulb (Figure 2b). In the locust polarized light input is conveyed to the CX via this pathway, and this is assumed to be the case for bees and butterflies as well [7•,12,13].

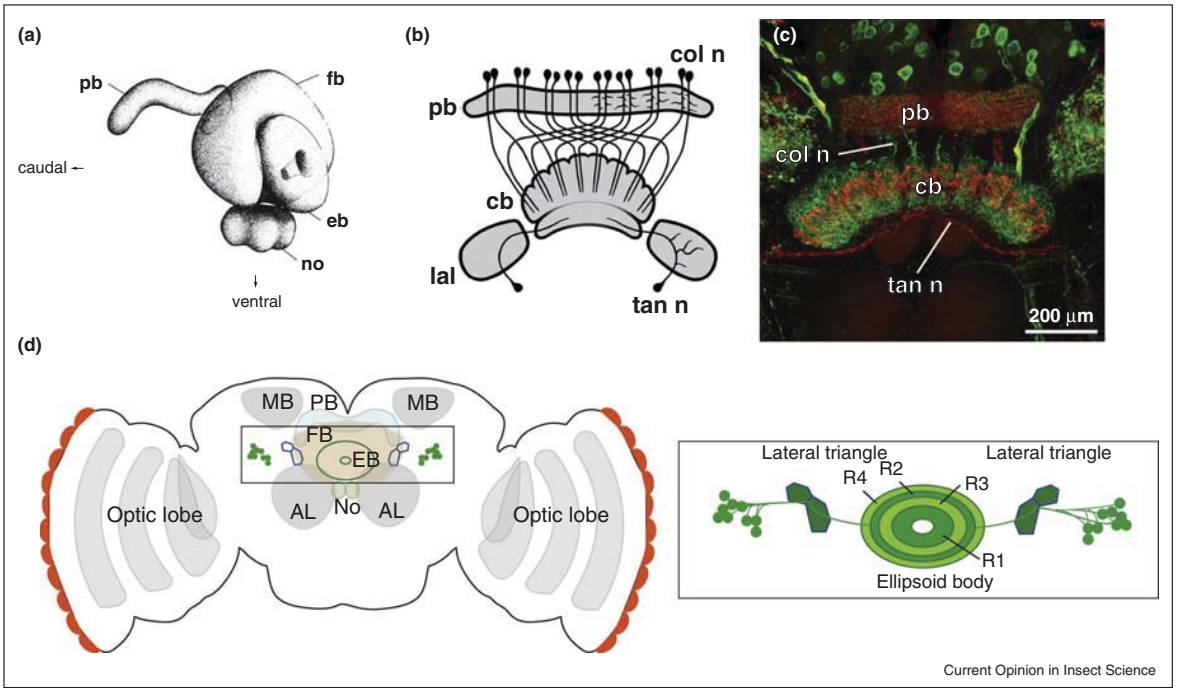
Functions of the central complex

During their daily foraging activities insects have to find their way to food sources and back to their nests or hiding places in known and unknown terrain. To be successful the animal needs navigation and orientation skills, spatial memory and a quickly updated visual working memory. The animal needs to select and initiate the most appropriate motor outputs to affect locomotion and foraging. The CX is involved in all these processes (Table 1) and recent progress has been made to determine how.

Processing of polarized light

Many insects navigate with the help of celestial cues including the position of the sun, the pattern of polarized light and the chromatic gradient of the sky, for example [7•,15].

Figure 1



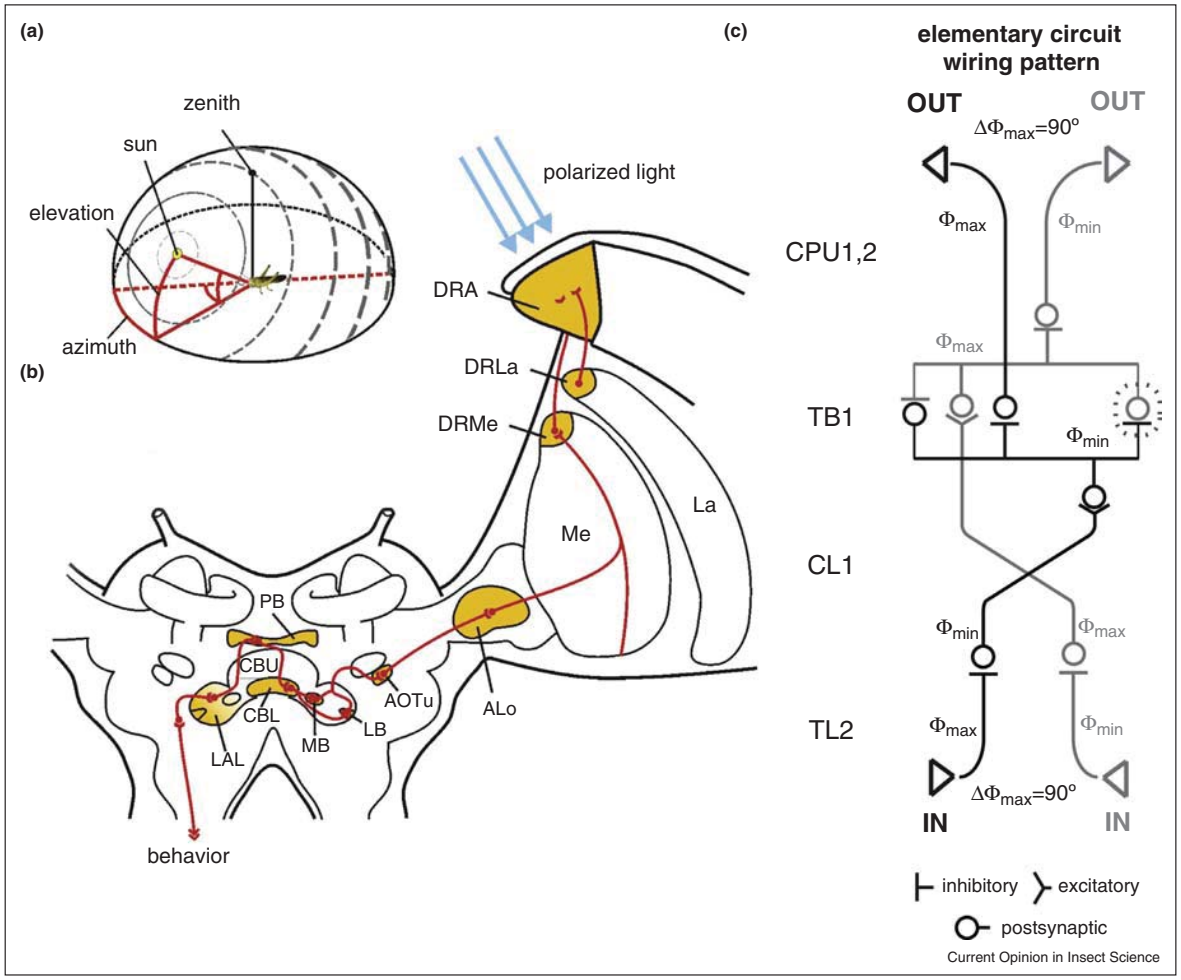
Central complex structure and architecture. **(a)** Central complex neuropils in the fruit fly: protocerebral bridge (pb), fan-shaped body (fb) and ellipsoid body (eb), which combined are termed central body, and the noduli (no). **(b)** Schematic drawing of connections between the central complex neuropils in the cockroach. Columnar neurons (col n) connect between the protocerebral bridge (pb) and the central body (cb). Tangential neurons (tan n) provide input and output connections to the adjacent lateral accessory lobe (lal). **(c)** Original staining of the central complex neuropils and neuronal connections in the cockroach *Periplaneta americana* (red: allatostatin-like immunoreactivity, green: tachykinin-like immunoreactivity). **(d)** Architecture of the central brain of the fruit fly showing the mushroom bodies (MB), the antennal lobes (AL), the noduli (NO), the ellipsoid body (EB) the fan-shaped body (FB) and the protocerebral bridge (PB). Marked in green are the ring neuron groups R1–R4 and in blue the lateral triangle. Originals: (a) modified from [55] with permission of Springer-Verlag; (b) by Carsten Heuer from [56]; (c) from [56] as modified from [3] with the permission of Elsevier; (d) reprinted from [24] by permission from Macmillian Publishers Ltd: *Nature*, copyright (2013).

Scattering in the atmosphere results in a linear polarization of sun light (Rayleigh scattering, [16,17]). A property of polarized light is the electric field vector (*E*-vector), which indicates the orientation of polarization. Different *E*-vectors are arranged in a concentric pattern around the sun's position (Figure 2a). This is used by many insects to orientate and navigate, even when the sun is blocked by clouds.

In locusts, polarized light information enters the CX via the anterior visual pathway (Figure 2b). Neurons in the PB columns are specific in their response to *E*-vector orientation and differ in their peak activity from one column to the next, spanning over 180° across the entire PB [2••,18]. Thus, the PB network provides a central polarotopic representation of the sky polarization pattern. It has therefore been suggested that the CX is the main neuropil to process celestial compass information [2••,15,19]; but how does this processing work?

The representation of a specific *E*-vector angle in the individual columns in the PB likely arises from an antagonistic integration of different input paths 20••]. As illustrated in Figure 2c, information about the preferred *E*-vector enters the CX via tangential neurons (TL2). There is a strong indication that the information is passed on inverted via an inhibitory synapse to columnar neurons (CL1). This reduces the activity in the CL1 neurons and the downstream tangential neurons in the PB (TB1). The model suggests that a pair of TB1 neurons integrates information coming from two TL2-CL1-TB1 networks: one TB1 neuron being inhibited and one TB1 neuron being disinhibited by the same *E*-vector angle. The preferred *E*-vector angles of the paired TB1 neurons are 90° apart so that when one TB1 neuron is excited it inhibits its paired partner. Each TB1 neuron displays robust antagonistic responses to the preferred and to the antipreferred angle (perpendicular to preferred angle) as a result (Figure 2c).

Figure 2



Processing of polarization in the central complex. **(a)** *E*-vectors of polarized light are arranged in concentric circles around the sun and can be used by insects to navigate. The sun's position is determined by the azimuth and the elevation. **(b)** The anterior polarization pathway originates in the dorsal rim areas of the compound eye (DRA: dorsal rim area) and the visual neuropils; (DRLa, DRMe: dorsal rim areas of lamina and medulla). Information enters the central body lower unit (CBL) via the anterior lobe of the lobula (ALo), the anterior optic tubercle (AOTu), the lateral bulb (LB) and the medial bulb (MB). In the central complex the information is relayed via the central body upper unit (CBU) and the protocerebral bridge (PB) to be processed and generate behavioral output via the lateral accessory lobe (LAL). **(c)** Proposed circuit for processing of polarized light. Information about the preferred *E*-vector angle (Φ_{max}) enters the central body by TL2 neurons and is passed inverted on via an inhibitory synapse to CL1 neurons and further to the TB1 neurons in the PB. Two TB1 neurons integrate information coming from two such networks which are tuned antagonistically to the same *E*-vector. The information subsequently leaves the PB via the CPU1 and CPU2 neurons. Originals: (a) from [21] as modified from [57] with permission of John Wiley and Sons and Elsevier, (b) from [21] with permission of Elsevier; (c) modified from [20••] with permission of the American Physiological Society.

Furthermore, some tangential neurons have two activity peaks at different solar azimuths for certain solar elevations [21], one being at the solar and one being at the antisolar position. Since the activity maxima at different solar azimuths differ between units, the locust can identify the correct position of the sun at certain elevations solely based on *E*-vector information without requiring other celestial compass information.

Additionally, animals seem to use polarized light information to stay on course [20••]: neurons downstream of the TL2 neurons exhibited adapting responses when stationary polarization input was given and non-adapting responses when rotating polarization input was given, providing a possible simple neural mechanism for maintaining a constant heading relative to the *E*-vector.

Table 1

Functions of the central complex in different insects.

Insect species investigated	Function of the central complex	Part of central complex involved	Method used	Ref.
Schistocerca gregaria, possibly *Drosophila melanogaster* and *Danaus plexippus*	Processing of polarized light by *E*-vector tuning of tangential neurons and antagonistic coding in tangential-columnar neuron networks	PB, CBL	Intracellular recordings	[7•,20••,25•]
Schistocerca gregaria	Determination of sun position by different activity peaks at solar and antisolar position for different solar elevations	CBL	Intracellular recordings	[21]
Schistocerca gregaria	Processing of looming stimuli	PB, CBU, CBL	Intracellular recordings	[22]
Schistocerca gregaria, Drosophila melanogaster	Processing of translational movement	PB, CBU (FB), CBL	Intracellular recordings, calcium imaging	[22,23]
Drosophila melanogaster	Activity modulation by behavioral state	CBU (FB), CBL (EB)	Intracellular recordings, calcium imaging	[23,24]
Drosophila melanogaster	Visual working memory for spatial orientation depends on different molecular mechanisms of the ring neurons in the EB	CBL (EB)	Calcium imaging, histology and behavioral analysis using an LED arena	[28,29]
Drosophila melanogaster	Visual place memory relying on visual patterns	CBL	Behavioral analysis using an LED arena	[30]
Blaberus discoidalis, *Gryllus bimaculatus*	Neural activity correlated with walking activity and turning	CBL, CBU or not specified	Extracellular multichannel recording in tethered or free-walking animals, Procaine injections	[32–35]
Drosophila melanogaster	Groups of dendrites from columnar neurons encode the fly's position in relation to a visual landmark, which continues when no visual cues are present during walking and when the animal has stopped	CBL (EB)	Calcium imaging in tethered animals on a track ball	[24,31••]

Motion and spatial information processing

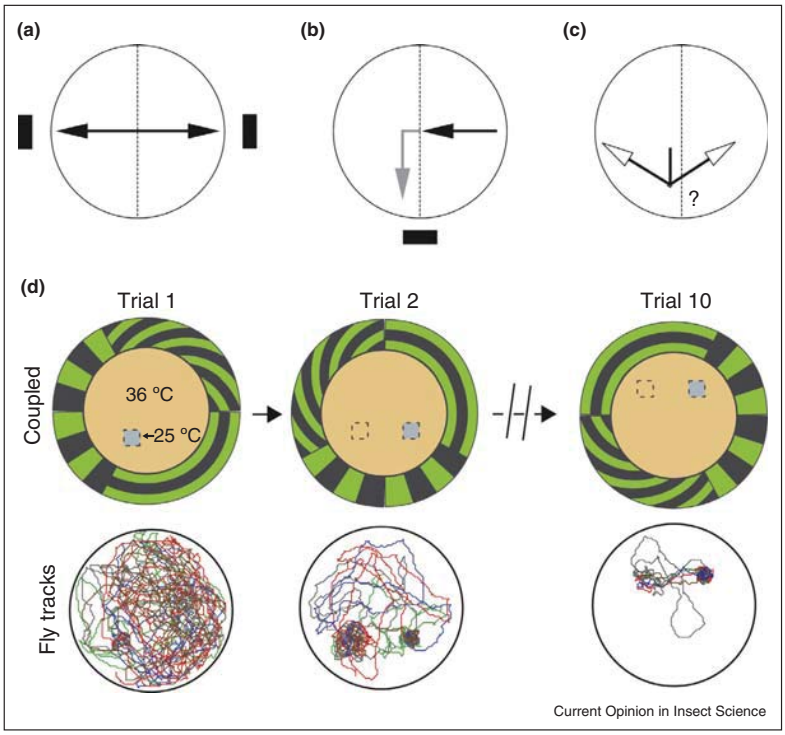
During flight it is imperative for the animal to react to approaching objects such as obstacles or predators. Antagonistic responses to opposite stimuli in the CX also seems to play a role here:

In the locust many CX units showed excitation to a looming stimulus displayed to one eye and inhibition when displayed to the other eye [22]. Similar response patterns were found in the fruit fly when forward motion versus backward motion was perceived. [23]. Interestingly, the animal's state influenced motion processing: neuronal responses to visual stimuli were measured during flight but not during rest [23]. Seelig *et al.* [24] focused on responses of the dendritic arborization of EB neurons (ring neurons) in the fruit fly [24] (Figure 1d). The dendrites form condensations (microglomeruli) in the lateral triangle (lateral bulb, Figures 1d and 2b) and receive visual input. Here, responses to visual stimuli were diminished during flight but not during walking. Thus, it is argued that responses of the ring neuron dendrites relate to a modulation of motor output and providing behaviorally relevant visual information than to direct motor control [24]. Furthermore, the response patterns indicated that the microglomeruli in this region were arranged as a spatial map relating to the visual field of the fly [24]. Lin *et al.* [25•] suggest that several such topographical maps may occur in the CX. Whether these topographical arrangements are organized by similar networks as those that have been found for the polarization pathways in the PB remains to be investigated.

Spatial memory

An important aspect of orientation and navigation is a quickly updated visual working memory (VWM) as well as visual and spatial memory. The detour paradigm has been developed as a lab assay to test VWM in fruit flies [26]. This assay makes use of the Buridan's paradigm in which the fly walks between two opposing black stripes [27] (Figure 3a). In the detour paradigm the stripes disappear and a new stripe appears perpendicularly (Figure 3b). After a successful orientation toward the new stripe it is removed so that the fly is left without visual cues (Figure 3c). In 80% of the cases the fly will turn toward its original heading using idiothetic (use of internal cues when navigating) information of the initial path. Several recent studies have shown that different

Figure 3



Spatial learning paradigms used to test CX function in fruit flies. (a–c) Detour paradigm: **(a)** when black bars are displayed on the walls of the arena the fly walks between them. **(b)** When the fly has successfully oriented toward one of the black bars, the two black bars disappear and one black bar appears perpendicularly. **(c)** When the fly has successfully oriented toward the black bar, the bar disappears and the fly is left without visual cues and memory of the previously visible bars can be tested. **(d)** Visual place memory: the fly has to find the cooled tile in a heated arena. Time to find the cool tile in relation to the visual pattern displayed on the arena walls decreases with number of trials. Originals: (a–c) reprinted from [26] by permission from Macmillian Publishers Ltd: *Nature*, copyright (2008); (d) reprinted from [30] by permission from Macmillian Publishers Ltd: *Nature*, copyright (2011).

sets of ring neurons in the EB are needed for intact VWM function in the detour paradigm [26,28,29].

The EB is also involved in visual place learning [30] in the fruit fly (Figure 3d). Ofstad *et al.* [30] presented an assay in which the fly had to find a cool tile in a heated arena in relation to a visual pattern projected on the walls. The time the flies needed to find the tile decreased over successive learning trials, but when EB neurons were silenced learning was impaired [30].

Seelig and Jayaraman [31••] investigated neuronal activity during landmark orientation in tethered but walking fruit flies. Groups of columnar neurons originating in the EB encode for the fruit fly's orientation in relation to a landmark. Intriguingly, the EB activity profile was maintained in the absence of visual cues when the fly was walking as well as when the fly stopped, indicating a formation of visual working memory or short-term memory [31••]. Hence, this network provides a possible basis for navigation relying on path integration by maintaining a representation of the animal's position even when visual landmarks are no longer available.

Sensory information processing for motor control

Whether the CX directly initiates and controls motor output remains an open question. Newly developed techniques that allow recording of neural activity while a cockroach or cricket is walking are great advances toward clarifying this matter [32,33].

In cockroaches various activity patterns in CB units were found in response to wide-field visual motion stimuli which elicit visually guided behavior [34]. Walking was diminished when parts of the CX were anesthetized, thus showing that CX activity is necessary for initiation of locomotion. Furthermore, in both crickets and cockroaches activity changes of different CX units were correlated with specific directions of turns, while other units were attuned to walking activity regardless of turning direction, or were attuned to turning

activity in general [33,35]. The majority of recorded activity changes preceded locomotion. This evidence favors a more direct role of the CX in initiation of locomotion. Some of the neurons, however, changed their firing rate after the locomotion onset or change. This might indicate a feedback pathway to the CX. Kai and Okada [33] suggest that the ongoing activity during walking could arise from reafferent control for mechanosensory inputs, and from exteroceptive and proprioceptive inputs due to movement of body parts.

Interestingly, in cockroaches only a few neurons responded to antennal stimulation when it occurred after an active movement of the antenna to a rod [35]. In contrast, many neurons responded to imposed antenna stimulation. The authors suggest that the neurons responding to self-generated antennal contact might also be arranged in a map-like representation similar to the *E*-vector representation in the PB.

Toward an understanding of the central complex functions

Recent studies of the CX have begun analyzing the neuronal architecture of the CX neuropils [7•,25•,36]. They confirm that the CX comprises a network for complex information processing and integration. Features of CX neural systems include neurons with symmetrical morphologies and connections on both brain sides, converging and diverging pathways and numerous parallel pathways. Network architectures include tiling of neurons, which is the spreading of neighboring arborization without overlap to increase innervation surface and to minimize functional redundancy in the innervated area [37].

A model for horizontal and vertical signal propagation in the CX used the new network information from the fruit fly [38]. In horizontal propagation the signal passes from an input node to many output nodes. In vertical propagation the signal is passed from an input node to an output node. Remarkably, the pattern of the CX network indicated a high efficiency in horizontal as well as vertical signal propagation, which was mainly related to the inclusion of hubs in the network — these being highly interconnected clusters of neurons.

Interestingly, Liu *et al.* identify two loops in the CX network, which could be related to a reverberation function [25•]. Reverberation is defined as the persistence of neural activity in a circuit network beyond the stimulus [39], and is associated with consolidating memories during sleep in mammals [40], or to working memory [41]. It remains to be investigated if reverberation in the CX is connected to similar processes. However the CX has been implicated in both reverberation and sleep in insects: Donlea and colleagues showed that sleep could be induced by activation of neurons connecting to the FB, which were also shown to be crucial for sleep homeostasis [42]. Further, when sleep was induced after massed training (short interval between training trials) long-term memory was formed [43], while massed training alone did not lead to long-term memory formation.

Conclusions and future prospects

The studies reviewed here show great progress toward uncovering the functional roles of the CX (Table 1). New techniques such as recording from free-walking insects and advanced neuronal tracing technologies will help to further map the numerous functions the CX is associated with.

However, neuroethological studies on the CX are increasingly involving only a few insect species. The current functions localized to the CX are all important for movement and navigation. It is therefore unfortunate that the CX has been barely explored in central-place foraging ants and bees for which navigation is so important and well developed. In recent years, new techniques to investigate navigation, spatial orientation and visually guided behavior in bees and ants have been developed or improved [44–50]. They include tracking in the field with harmonic radar [49] and radio frequency identification tags [48], and 3D reconstruction of an insect's environment [50]. This makes it possible to study navigational and visual orientation in great detail and in large numbers. Further, the honey bee has been established as a powerful model system for learning and memory using free-flying bees as well as harnessed honey bees [51–53]. We propose that ant and bee species could be ideal for further study of the role of the CX in orientation and navigation and would help to complete a comparative analysis of the CX functions.

It is still a mystery how insects with a much smaller brain compared to vertebrates can solve similar complex navigational tasks [54]. Representation of body orientation in reference to a visual landmark in the fruit fly EB [31••] is an exciting finding which provides a vital starting point from which to further uncover the underlying mechanisms.

Analysis of the CX is gaining momentum rapidly, and more knowledge of this region will fill a critical gap in our comprehension of the insect's brain.

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Appendix II

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

Original Publication

Pages a12-a22 of this thesis have been removed as they contain published material under copyright. Removed contents published as:

Søvik, E., Plath, J. A., Devaud, J. M., Barron, A. B. (2016) Neuropharmacological Manipulation of Restrained and Free-flying Honey Bees, Apis mellifera. *Journal of Visualized Experiments*, vol. 117, e54695, doi.org/10.3791/54695.

Appendix III

Different Roles for Honey Bee Mushroom Bodies and Central Complex in Visual Learning of Colored Lights in an Aversive Conditioning Assay

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Different Roles for Honey Bee Mushroom Bodies and Central Complex in Visual Learning of Colored Lights in an Aversive Conditioning Assay

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The honey bee is an excellent visual learner, but we know little about how and why it performs so well, or how visual information is learned by the bee brain. Here we examined the different roles of two key integrative regions of the brain in visual learning: the mushroom bodies and the central complex. We tested bees' learning performance in a new assay of color learning that used electric shock as punishment. In this assay a light field was paired with electric shock. The other half of the conditioning chamber was illuminated with light of a different wavelength and not paired with shocks. The unrestrained bee could run away from the light stimulus and thereby associate one wavelength with punishment, and the other with safety. We compared learning performance of bees in which either the central complex or mushroom bodies had been transiently inactivated by microinjection of the reversible anesthetic procaine. Control bees learned to escape the shock-paired light field and to spend more time in the safe light field after a few trials. When ventral lobe neurons of the mushroom bodies were silenced, bees were no longer able to associate one light field with shock. By contrast, silencing of one collar region of the mushroom body calyx did not alter behavior in the learning assay in comparison to control treatment. Bees with silenced central complex neurons did not leave the shock-paired light field in the middle trials of training, even after a few seconds of being shocked. We discussed how mushroom bodies and the central complex both contribute to aversive visual learning with an operant component.

Keywords: visual learning, operant learning, mushroom bodies, central complex, honey bees, procaine

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INTRODUCTION

Learning of a predictive relationship between a stimulus or an action and a certain outcome is essential for an animal's survival. Honey bees are excellent learners, quickly forming association between stimuli of different sensory modalities and meaningful appetitive and aversive stimuli (Giurfa, 2007). Over the past decades, research has been dedicated to uncover the neural

mechanisms and processes underlying learning in bees, and honey bees have been established as a powerful model to investigate learning and memory (Menzel, 1999, 2001, 2012; Giurfa, 2003, 2007). Learning assays are typically performed with free-flying bees as well as harnessed bees (Menzel, 1999, 2001; Giurfa, 2003, 2007; Menzel, 2012). Free-flying bees readily learn olfactory as well as visual stimuli. Appetitive learning and memory dynamics have been studied extensively using odors and colors or shapes paired with sucrose rewards.

Harnessed bees have been used in the proboscis extension response (PER) assay, in which the conditioned stimulus (CS) is paired with a sucrose reward (unconditioned stimulus: US) which leads to an extension of the proboscis (Bitterman et al., 1983; Felsenberg et al., 2011; Giurfa and Sandoz, 2012). Olfactory conditioning is easily studied with this assay since 50–60% of the trained bees already respond to an odor after one CS-US pairing (Bitterman et al., 1983; Felsenberg et al., 2011). It has proven difficult, however, to achieve successful conditioning of color stimuli with rewards or punishment in harnessed honey bees. Differential conditioning with a reward-paired color stimulus and a non-rewarded color stimulus resulted in moderate learning rates when the antennae were ablated (Kuwabara, 1957; Hori et al., 2006, 2007; Niggebrugge et al., 2009), when the bee was able to turn her head easily (Dobrin and Fahrbach, 2012) or when the color stimulus was combined with movement (Balamurali et al., 2015). Colored light, however, has been used successfully as a context for olfactory learning in PER when presented as an occasion-setter (Mota et al., 2011) or in a reinstatement paradigm (Plath et al., 2012). The difficulty in establishing robust visual learning in the PER assay has inhibited functional analyses of roles of different brain regions in visual learning in bees.

Here we used a recently developed aversive visual conditioning assay: the Automated Performance Index System (APIS) (Kirkerud et al., 2017) to analyze the roles of central processing regions of the bee brain in visual learning. This system was an adapted version of the one used for aversive olfactory conditioning (Kirkerud et al., 2013; Schott et al., 2015; Wehmann et al., 2015). In the APIS assay bees are able to move freely in a conditioning chamber, which is equipped with LEDs to provide visual stimuli of different wavelengths and intensities. Visual stimuli can be paired with low voltage electric shocks. Tracking of the animal's position is fully automated thanks to infrared sensors in the chamber. The chamber can be used to investigate differential learning presenting light in half of the chamber and light with different properties in the other half. One light field is paired with electric shock, so that the bee needs to cross over to the other half of the chamber to avoid being shocked. The assay has been extensively tested with different light stimuli including light of different wavelengths and intensities (Kirkerud et al., 2017). Bees easily learn to associate 465 nm light (blue for humans) and 590 nm light (yellow for humans) but not 525 nm light (green for humans; in the following, we use the human colors instead of the wavelengths for simplicity) with the aversive shock stimulus. In this study, we paired blue light with shocks in one half of the chamber and illuminated the "safe" part of the chamber with green light. Bees can be treated pharmacologically and then their behavior can be assessed in the APIS chamber. Here, we investigated the role the mushroom bodies (MBs) and the central complex (CX) in visual learning.

MBs and the CX are considered the main integrative centers in the insect brain, and both regions could be involved in learning an appropriate behavioral response to a visual stimulus. We investigated the behavioral consequence of silencing of the input region of the MBs, the collar region in the mushroom body calyces (MBC), and the vertical lobes (VL) as the output region of the MBs. The collar region receives direct visual input from the lobula and medulla in honey bees (Ehmer and Gronenberg, 2002; Gronenberg and Lopez-Riquelme, 2004). A recent study has found two types of Kenyon cells in the fruit fly MBC that respond to either light intensity or wavelength (color) information relayed from the optic neuropils (Vogt et al., 2016). Interestingly, in flies both types of neurons are required for learning and memory in an aversive differential conditioning, either testing different intensities or different wavelengths. The output of the collar region in the mushroom bodies terminates in an inner layer of the vertical lobes in honey bees (Strausfeld, 2002). It has been repeatedly shown that the vertical lobes play a crucial role for different forms of olfactory learning and memory formation in honey bees (Menzel, 1999, 2012) and fruit flies (Heisenberg, 2003; Keene and Waddell, 2007; Busto et al., 2010; Davis, 2011), but visual learning has only been investigated sparsely so far.

The CX comprises a group of unpaired neuropils in the center of the insect brain. One important role of the CX is generation of motor outputs according to processed internal and external stimuli (Pfeiffer and Homberg, 2014; Plath and Barron, 2015). The CX is essential for the initiation and termination of walking, turning and climbing behavior in fruit flies (Strauss and Heisenberg, 1993; Martin et al., 1999; Strauss, 2002; Poeck et al., 2008; Triphan et al., 2010), cockroaches (Guo and Ritzmann, 2013; Guo et al., 2014; Martin et al., 2015) and crickets (Kai and Okada, 2013) and is considered as site for action selection and goal-directed behavior (Libersat and Gal, 2013; Strausfeld and Hirth, 2013; Barron et al., 2015; Fiore et al., 2015; Barron and Klein, 2016). A role of the CX in visual learning of patterns and spatial features has been shown in various behavioral assays using fruit flies (Liu et al., 2006; Neuser et al., 2008; Wang et al., 2008; Pan et al., 2009; Hou et al., 2011; Ofstad et al., 2011; Kuntz et al., 2012, 2017).

In this study, we used the transient and local anesthetic procaine to selectively silence neural activity in these three brain regions. Procaine is a reversible blocker of voltage-gated Na^+- and other voltage-gated channels to a lesser degree and has been established as a means to study olfactory learning and memory in honey bees (Muller et al., 2003; Devaud et al., 2007, 2015). Procaine has also been utilized to show that silencing the central body reduces spontaneous walking and optomotor responses (Kathman et al., 2014; Kaiser and Libersat, 2015). Our expectation was that mushroom bodies are needed for this form of visual conditioning with a strong operant component. This allowed the bee to learn from consequences of her behavior and not only from a stimulus-stimulus pairing. Interrupting processing in the collar region and blocking the further processing in the output regions of the mushroom bodies could lead to an impairment in performance in aversive

visual learning which can be measured in the APIS assay. We hypothesized further that learning of the stimulus-shock pairing would remain intact when the central complex was anesthetized but the reaction of running away from the stimulus would be impaired. We discuss how our results will contribute to uncovering mechanisms underlying visual learning in insects.

MATERIALS AND METHODS

Animals and Surgical Procedure

For all experiments, honey bees were collected from two established queen-right colonies at Macquarie University in Sydney, Australia. Foragers were collected at the hive entrance while leaving for a foraging bout. Bees were immobilized on ice and harnessed in PER tubes (Bitterman et al., 1983; Felsenberg et al., 2011). To prepare the animals for injections, the bee's neck was filled with soft dental wax to prevent movement of the head. A stripe of wax was positioned loosely over the antennae to prevent their movement during the operation.

For MBC injections, we entered through the ocellar tract. The lens of the median ocellus was carefully pushed outwards with the tip of a micro-scalpel and a small incision was made into the neurilemma sheath covering the brain to ease entering of the micropipette.

To access the brain for intracerebral injections (VL and CX), a window was cut into the head capsule with three cuts: One above the antennal stems (dorsal), one below the median ocellus (ventral), and one at the border of the right eye (Devaud et al., 2007). The created flap was opened and held in place with soft dental wax. The glands and trachea above the brain were carefully moved aside and a small incision was made into the neurilemma above the target structure to enable a smooth entry of the micropipette during injections. After injections the flap was carefully released to close the window and sealed with a drop of eicosane (Sigma-Aldrich Australia) melted at ∼35°C). For detailed demonstration of the procedure please refer to Søvik et al. (2016).

Injections

In the following study four different treatment groups were compared: procaine-injected animals (procaine/proc), saline-injected animals (vehicle/veh), animals that underwent the operation and injection procedure without having any solution injected into the brain (sham), and non-treated animals (NT), which were directly transferred to the chamber after catching.

To locally and temporarily inhibit neural activity, the drug procaine was used. In the honey bee procaine reduces Na^+- and K^+-currents and spiking activity in mushroom body neurons (Devaud et al., 2007). Procaine HCl (Sigma-Aldrich Australia) was dissolved in physiological saline (7.54 g/L NaCl, 0.448 g/L KCl, 0.872 g/L $MgCl_2 \times 6\ H_20$, 0.735 g/L $CaCl_2 \times 2H_20$, 54.72 g/L Sucrose, 4.95 g/L D-glucose, and 2.38 g/L HEPES, pH = 6.7, 500 mOsm, Sigma-Aldrich Australia, see Burger et al., 2013) as a stock solution of 40% (w/v). On the day of the experiment, the solution was diluted with additional saline to create a 20% (w/v) procaine solution. Physiological saline was also used as a control solution. To identify the injection site afterwards, both solutions contained 0.5 mg/ml dextran Alexa fluor 546 or dextran Alexa fluor 568 (10.000 MW, Molecular probes by Life technologies, Carlsbad, CA, USA). Microinjections were performed with a microinjector (Eppendorf, Hamburg, Germany) and an electronic micromanipulator (Luigs & Neumann Feinmechanik und Elektrotechnik, Ratingen, Germany). Micropipettes were pulled from glass capillaries (World Precisions Instruments, Sarasota, FL, USA) using an electrode puller (Scientific & Research Instruments, Karnataka, India). The tips were broken to an outer diameter of 10–15 μm. The injection volume was adjusted and rechecked both before and after every animal by measuring a droplet injected into mineral oil.

Injections into the MBC occurred via the ocellar tract of the median ocellus. The micropipette was brought to the opening of the removed lens and then finely adjusted until the micropipette was just above the incision made earlier. The micropipette was then inserted to a maximum injection depth of ∼215 μm and a volume of ∼2 nL was injected. The micropipette was removed and the bee was quickly transferred into the conditioning chamber (**Figure 1A**).

To target the center of the VL, ∼1 nL of solution was injected into each lobe at a depth of ∼60 μm and at an angle of 68–70° relative to the brain surface. A stereomicroscope fluorescent adapter was then used to visualize the injection site (Green- Light and Filter Set; NIGHTSEA, Lexington, MA, USA). Successful injections were identified by spreading of the fluorescent dye throughout the VL. To target the CX, ∼0.5 nL of solution was injected at a depth of ∼330 μm and at an angle of 68–75° relative to the brain surface; entering at the midline between the VLs. Successful injections were identified using laser scanning confocal microscopy (see below).

Behavioral Assay

Honey bees were conditioned in the APIS chamber, designed and manufactured at the University of Konstanz, Germany with an aversive visual conditioning paradigm established in (Kirkerud et al., 2017). Tracking of the bee and delivery of stimuli in APIS are fully automated which eliminates human error or bias. Due to the design of the chamber, bees can only move in almost straight lines, either toward or away from a stimulus, and any turn made by the animal is tracked as a complete reversal by the sensors. Shock and light stimuli were controlled with a script loaded into the system software. The program utilizes sensor feedback to determine the bee's location and initiates stimuli at specified time points. The operation of the chamber and the assay used are similar to methods used earlier in flies (Zars et al., 2000; Claridge-Chang et al., 2009).

Following injection, the bee was quickly placed into the chamber and allowed to acclimate for 15 min while freely moving around in the dark. The conditioning protocol consisted of one unreinforced preference test followed by nine reinforced training trials (**Figure 1B**), and ending with four unreinforced test trials (**Figure 1C**). In each trial, a blue light field ($\lambda^B = 465$ nm, Luminous intensity: 105 mcd) was switched on in the half of the chamber where the bee was situated and a green light field ($\lambda^G = 525$ nm, Luminous intensity: 119 mcd) illuminated the

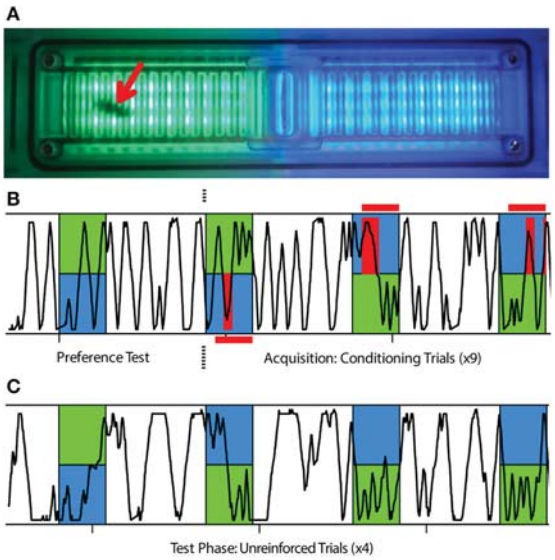

FIGURE 1 | APIS learning assay used in this study. (A) The APIS chamber can be illuminated with two different light fields of varying wavelengths and intensities; in this case light appearing green to humans and light appearing blue to humans. The chamber is equipped with an electrifiable grid to deliver 10 V shocks to the bee's feet and with infrared sensors to automatically track the bee's movement. A bee in the chamber (red arrow) could only move in a straight line, either toward or away from a stimulus, and turns were scored as a reversal of direction as detected by the infrared sensors. **(B,C)** Typical running trace of a bee in the chamber. Blue and green indicate illumination wavelength and red indicates when shocks were available (red horizontal bars) or delivered (red vertical bars) to the bee. Blue light was always illuminating the half of the chamber in which the bee was located at light-onset. **(B)** After an acclimatization period of 15 min post-injection, the bee was exposed to 14 s of both green and blue illumination as a preference test. The bee was then subjected to nine conditioning trials in which, after 3 s of illumination, the bee experienced shocks on the blue side for another 11 s, but not on the green side. **(C)** Subsequently, the bee was tested four times with 14 s of illumination without shocks to determine the post-training response to blue and green light fields.

opposite half. All trials lasted 14 s and were presented at regular intervals of 44 s (from onset to onset). For the training trials, electric shock pulses (10 V, 4 Hz, 100 ms) were activated 3 s after light onset. These shock pulses were delivered to the feet of the bee through the metal grid as long as movement sensors on the blue side were triggered. This meant that the bee could either escape the shocks by crossing from the shock-predicting blue side to the safe green side or potentially avoid them completely by escaping within 3 s and remain on the green side until the end of the trial. Since bees were always located on the blue half at trial onset (Figure S3), there was an inherent bias in the calculated preference toward this side. Once the behavioral assay was complete, the bee was quickly placed onto ice and anesthetized for dissection.

Histology and Imaging

Once anesthetized, the bee's head capsule was opened and the brain was removed in 0.1 M PBS (Sigma-Aldrich Australia) using forceps and a fresh breaker-blade piece. Whole brains were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hattfield, PA, USA) in 0.1 M PBS overnight in a chilled room (16°C). Brains were then washed in 0.1 M PBS (3 × 10 min) at room temperature (22°C) and stored in the fridge (4°C). Samples were either washed daily with fresh 0.1 M PBS or they were processed immediately for histology.

Whole brains were incubated in 250 µL DAPI (2 µg/ml, Sigma-Aldrich Australia) in 0.1 M PBS and 0.2% Triton-X 100 (Sigma-Aldrich Australia) overnight. Brains were then washed in 0.1 M PBS (3 × 10 min) followed by an ethanol dehydration series (i.e., 50, 70, 90, 98, 100, 100% 10–30 min each step) and cleared in methylsalicylate (Sigma-Aldrich Australia).

Brains were then mounted on previously prepared slides with a cavity well. Wells were created with glass cover slips (Marienfeld-Superior, Lauda-Koeningshofen, Germany) and custom made aluminum slides (manufactured at the University of Konstanz, Germany) secured together using DPX mounting medium (Sigma-Aldrich Australia). Cleared brains were mounted in the well using DPX mounting medium and sealed with another cover slip.

Samples were imaged (4.77 µm slice) using an Olympus Fluoview inverted confocal microscope (FV-1000 IX81) located at Macquarie University in Sydney, Australia. DAPI staining and auto-fluorescence of the tissue was used to identify the neuropils and determine the location of the injection site marked by the Alexa dye (**Figure 2**).

All injections in the CX group were located in the central body (**Figures 2E,F**). One injection in the vehicle group (**Figure 2E**, red dot with black border), and one injection in the procaine group (**Figure 2F**, red dot with black border), was located at the border of the lower division of the central body and some dye was also found in the noduli; indicating that those areas were possibly affected as well. Since the performance in APIS was very similar for both injection sites, results were presented for all combined CX injections.

Data Analysis

The data was analyzed and graphed using R 3.3.2 (R Core Team, Vienna, Austria) and RStudio 1.0.136 (RStudio Inc., Boston, MA, USA) with a custom written script. As a measurement for learning, the Performance Index (PI) was calculated: difference between time spent on the green side of the chamber and time spent on the blue (shocked) side of the chamber divided by the total trial time:

$$PI = \frac{t\,(green) - t\,(blue)}{t\,(green) + t\,(blue)}$$

This resulted in a variable ranging from −1 to 1, where positive values indicate that the bee spent more time on the safe side than on the shocked side, negative values the opposite. A bee that had learnt to associate the blue light with shock would run away from the blue side shortly after light-onset and avoid returning to the blue side. As a consequence, the relative time spent on the green side increased leading to higher PI-values (**Figure 3A**). A bee that had not learnt, spent equal amounts of time on each side

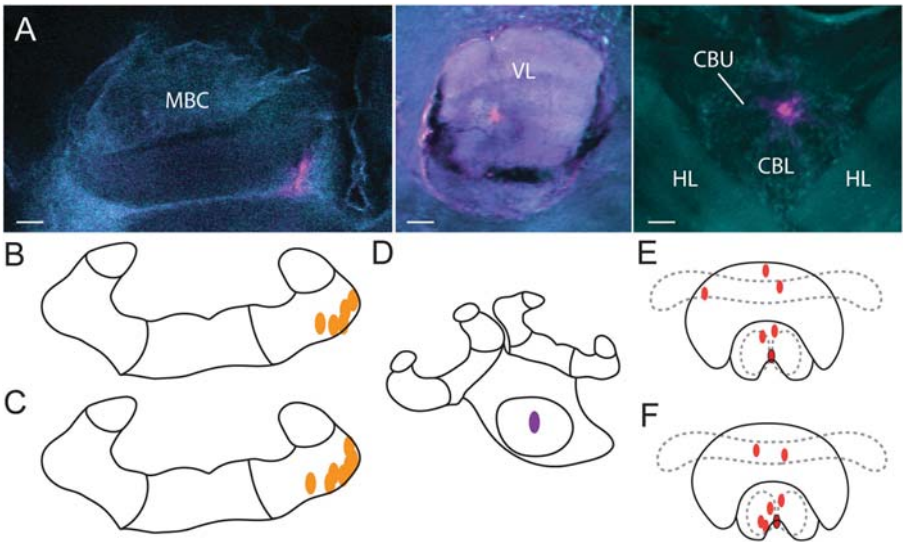

FIGURE 2 | Injection sites. (A) Alexa dye injections are shown in magenta (false color) in the MBC (left), VL (middle) and the CX (right). A DAPI-counterstain and auto-fluorescence of the brain tissue (false colored in cyan) allowed us to identify brain neuropils. Orientation of all three scans was aligned with rostral (neuraxis) facing upwards. Injections of vehicle **(B)** and of procaine solution **(C)** into the MBC as identified by the CLSM scans. Injections into the VL **(D)** were identified visually with fluorescent light and were all located in the center. Injections of vehicle **(E)** and of procaine solution **(F)** into the central body (red dots) and injections located at the border of the lower division of the central body with spread into the noduli (red dots with black border). MBC, mushroom body calyces; VL, ventral lobes; HL, horizontal lobes; CBU, upper division of the central body; CBL lower division of the central body; Scale bar = 30 μm.

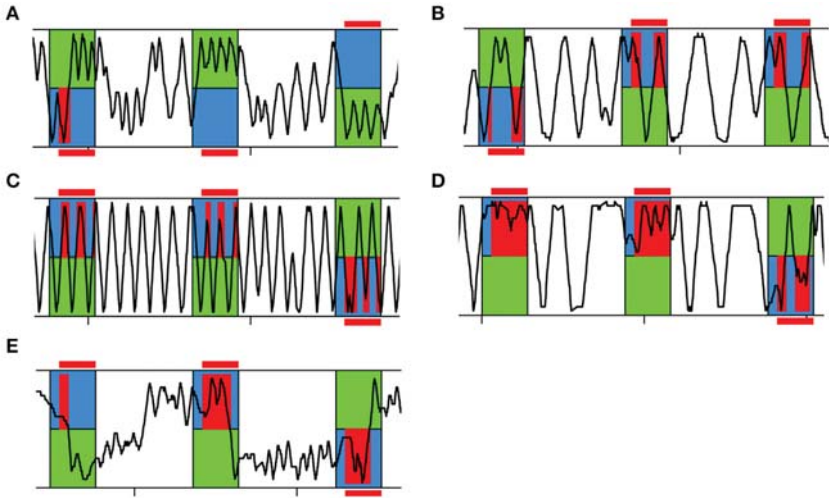

FIGURE 3 | Representative running traces of individual bees in APIS. Three training trials are shown. The bee was exposed to 14 s of blue and green light fields. After a 3 s delay the bee experienced shock when located on the blue side (red). **(A)** Typical running trace of a bee spending more time on the green side than on the blue side, thus achieving high Performance Indices (PIs). **(B)** Typical running trace of a bee spending more time on the blue side than on the green side, thus achieving low PIs. **(C)** Typical running trace of a bee with an equal number of reversals on the green and blue side, thus achieving a Reversing Difference close to zero. **(D)** Representative running trace of a bee reversing more often on the blue side than on the green side, thus achieving a negative Reversing Difference. **(E)** Typical running trace of a slowly responding bee taking a long time to cross over to the green side at the beginning of each trial and after light-onset, thus achieving a high Crossing Latency.

or more time on the blue side. A bee that had not learnt, would be expected to have lower PI-values (**Figure 3B**).

To investigate the movement pattern of the bee in more detail we further analyzed how many reversals of direction were performed in the chamber. We analyzed the total number of reversals per trial and the Reversing Difference: number of reversals performed on the blue side subtracted from the number of reversals performed on the green side of the chamber divided by the total number of reversals:

$$Reversing\ Difference = \frac{reversals\,(green) - reversals\,(blue)}{reversals\,(green) + reversals\,(blue)}$$

A bee that had learnt to avoid returning to the blue side typically ran back and forth on the green side (**Figure 3A**). If a bee had not learnt to avoid the blue side, we found two patterns: either she was running back and forth in the whole chamber (**Figure 3C**) or she was running back and forth on the blue side (**Figure 3D**). In the former case, the number of reversals performed would be equal for both sides (Reversing Difference close to zero). In the latter case, the number of reversals performed was higher on the blue side than on the green side (negative Reversing Difference).

As another parameter for learning performance as well as to evaluate the reaction to the shock-paired light, we analyzed how fast an animal would cross over to the green side after light-onset (Crossing Latency). If the bee managed to cross over under 3 s, she could completely avoid being shocked due to the delay of the shock-onset after light-onset, assuming she would not then return to the blue side (**Figure 3A**, second and third trial shown). If Crossing Latency was higher than 3 s she would experience shocks on the blue side (**Figure 3E**).

For statistical analysis of PI, Speed, Reversing Difference, Crossing Latency and Position in Chamber (at light-onset), the calculated data were fitted to linear mixed models with trial and treatment (procaine, vehicle, sham, NT) as fixed effects and bee identity as a random effect to correct for repeated measurements in the training, as well as the test phase (lme function in the R nlme package, Pinheiro et al., 2016). For statistical analysis of Reverses per Trial the calculated data were fitted to generalized linear mixed models (Poisson distribution) with trial and treatment (procaine, vehicle, sham, NT) as fixed effects and bee identity as a random effect to correct for repeated measurements in the training, as well as the test phase (glmer function in the R lme4 package, Bates et al., 2015). Statistical differences were determined *post-hoc* with the Tukey's range test using the R multcomp package (Hothorn et al., 2008). Since bees with lower speeds could not perform well in this assay in which performance is based on movement, animals with lower speeds than 2.1 cm/s were excluded from the analysis (Figure S1).

RESULTS

Control Animals Learned to Remain on the Green Side

In this study, we investigated color learning and how the animal's behavior in response to a learned stimulus changed. We first studied the behavior of the non-treated (NT) and sham-treated control groups. NT and sham-treated bees both developed a preference for the safe green side after few trials of color-shock conditioning (**Figure 4**). For both control groups PIs increased over the course of training (**Figure 4A**). PIs corresponded to around 39% of the first trial spent on the green side which increased to 61% (NT) and 72% (sham) in last trial. Increase of PIs from the first to the last trial was significant for both, NT animals (paired t-test, df = 25, $t = -2.682$, $p = 0.013$) and for sham-treated animals (paired t-test, df = 39, $t = -5.4861$, $p < 0.001$). In the test phase both groups continued to spend more time on the green side (**Figure 4A**).

We further explored how running and reversing in the chamber changed in response to the first light-shock pairing. Sham-treated animals were slower than NT-animals in the training but not in the test phase (**Figure 4B**). After five conditioning trials both groups performed on average three to five more reversals on the green side (**Figure 4C**). The total number of reversals performed in the chamber remained constant in that period (Figure S2A). Both groups crossed over to the green side after 2 to 4 s into the trial (**Figure 4D**). In the last training trial 20 out of 26 NT-animals and 21 out of 40 sham-treated animals crossed over under 3 s (data not shown). Taken together, after learning to associate blue light with shock the control bees ran away from the blue side before or shortly after shock-onset and thereafter ran back and forth on the green side.

Procaine Injections into the MBC Did Not Impair Performance in the Visual Learning Paradigm

We then examined how silencing of neurons of a collar region in the MBC with procaine injections changed the bees' behavior in the APIS assay (**Figure 5**). Procaine- and vehicle-injected animals were compared to sham-treated animals which were operated on in the same way. Overall, we observed no impairment of the bees' performance in the learning assay due to the injections. All bees were able to avoid the blue side after a few trials and moved normally. Curiously, we found a difference between PIs for all three groups in the preference test (**Figure 5A**). However, this did not seem to have an effect on the training where all groups performed similarly. Neither speed (**Figure 5B**), Reversing Differences (**Figure 5C**), Reversals per Trial (Figure S2B) or Crossing Latencies (**Figure 5D**) after the second trial were affected by injections (Table S1).

Procaine and Vehicle Injections into the VLs Impaired Performance in the Visual Learning Assay

Next, we investigated which role the VL as part of the MB output played in visual learning (**Figure 6**). Surprisingly, injections into the VL with either, procaine or vehicle solution resulted in impairment of color learning. Both groups achieved mean PI-values around zero, indicating that they spent equal amount of

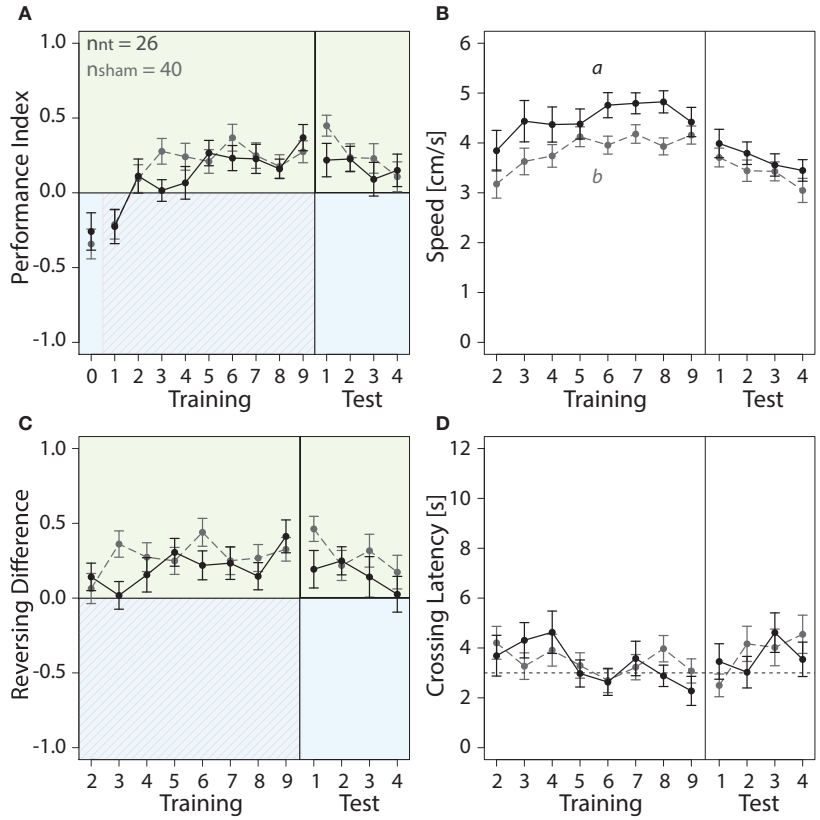

FIGURE 4 | With training, bees of sham and NT control groups learned to spend more time on the safe green side than the shocked blue side. Means ± SEM are plotted for all variables. Non-treated animals (NT) are shown in black, sham-treated animals (sham) in gray. No effect of the different injection methods used for the different regions on any of the four variables shown was found (ANOVA, $p > 0.05$). Sham-treated animals were therefore pooled into one group to compare with NT animals. Significant treatment effects determined with an LMM ($p < 0.05$, Table S1) are indicated with letters a and b. Bees were subjected to one preference test (0) nine training trials and four test trials. Control animals spent more time on the green side and avoided the shock-paired blue side (shocked period indicated by red diagonal lines) after a few trials. **(A)** No effect of treatment on Performance Index was found in training or in the test phase (Table S1). **(B)** An LMM indicated a significant effect of treatment on speed (Table S1). After one conditioning trial, speed was lower in sham-treated animals than in NT-animals in the training (*post-hoc* Tukey HSD, $z = -2.188$, $p = 0.03$), but no significant effect of treatment on speed was found in the test phase (Table S1) **(C)** Number of reversals on the green side was higher after one conditioning trial. No significant effect of treatment was found in training or in the test phase (Table S1). **(D)** Crossing Latency approached the 3-s threshold (horizontal dashed line) over the course of training, which corresponds to the delay between light-onset and shock-onset. **(A)** No significant effect of treatment on Crossing Latency was found for training or in the test phase (Table S1).

time on both sides (**Figure 6A**). This was not the case in sham-treated animals, which preferred the safe green side after two trials. Thus injection of the vehicle (with or without procaine), but not the insertion of the micropipette itself impaired learning of the light-shock pairing. Lower PIs in vehicle and procaine groups were not the result of impaired locomotion, since speed (**Figure 6B**) was not affected by treatment (Table S1). Furthermore, vehicle and procaine groups with injections into the VLs showed equal number of turns on the green side as on the blue side (**Figure 6C**), while Reversals per Trial (Figure S2C) remained unaffected. This indicated that the bees were either running back and forth from one side of the chamber to the other or were spending equal amounts of time running back and forth on each side. However, Crossing Latencies (**Figure 6D**) were found not to be significantly different (Table S1). Thus, vehicle- and procaine-treated bees ran away from the shocks after a similar delay as sham-treated bees in most trials.

Procaine Injections into the CX Changed Behavioral Responses in the Visual Learning Paradigm

Lastly, we explored how an animal's performance in the APIS-chamber was changed by silencing neural activity in the CX with procaine (**Figure 7**). Procaine-treated animals did not show a preference for the green side in the middle trials of the training. Rather, they remained on the shock-paired blue side longer than vehicle- and sham-treated animals. PIs were lower in procaine-treated animals in the training (**Figure 7A**). In fact, these bees

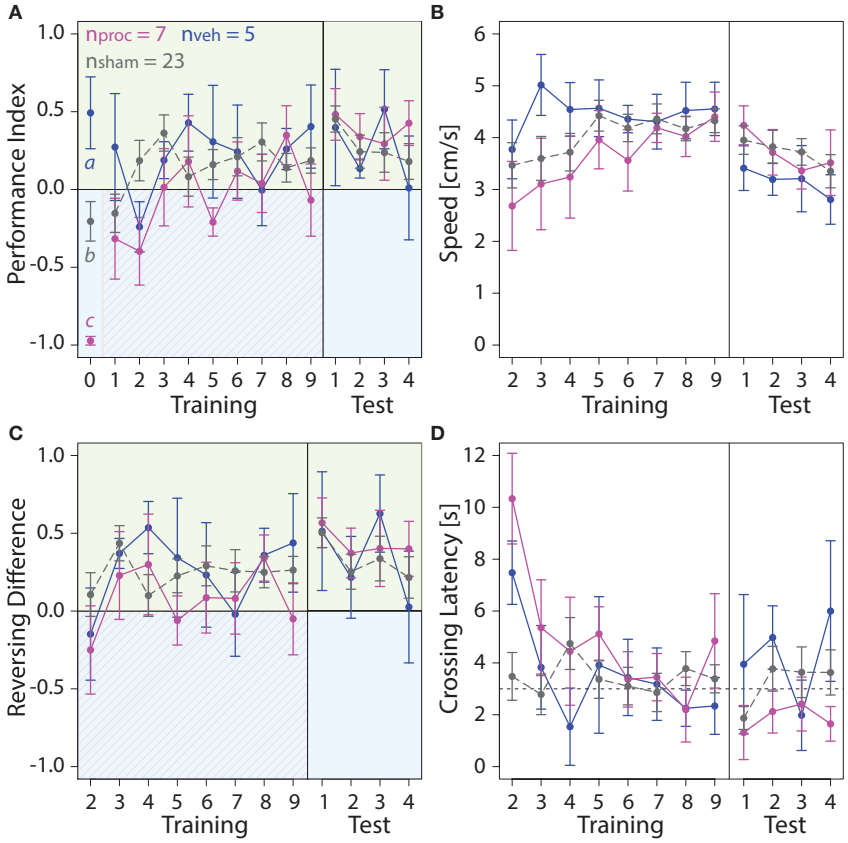

FIGURE 5 | Comparison of behavior in the APIS assay for bees injected with the vehicle (blue) or procaine solution (magenta) into the MBC, or sham-treated bees (gray). All groups learned to spend more time on the green side. Means ± SEM are plotted for all variables. Significant treatment effects determined with an LMM ($p < 0.05$, Table S1) are indicated with letters a, b, and c. Bees were subjected to one preference test (0) nine training trials and four test trials. **(A)** An LMM indicated an effect of treatment on Performance Index (PI) in the preference test (Table S1). Treatment comparison with a Tukey HSD *post-hoc* test revealed differences in PIs of vehicle and sham groups ($z = 2.631$, $p = 0.02$), PIs of procaine and sham groups ($z = -3.310$, $p = 0.003$) and PIs of procaine and vehicle groups ($z = -4.657$, $p < 0.001$). An LMM indicated a significant difference between PIs of procaine and sham groups in training (Table S1), but a Tukey *post-hoc* test, which corrects for multiple testing indicated no difference between PIs of these groups ($z = 2.080$, $p = 0.09$). No effect of treatment on PIs was found for the test phase (LMM, Table S1). All bees spent more time on the green side and avoided the shock-paired blue side (shocks indicated by diagonal lines) after a few trials. **(B)** Speed did not differ between experimental groups (LMM, Table S1). **(C)** Number of reversals on the green side was higher after one conditioning trial. No effect of treatment on Reversing Differences was found in training or in the test phase (Table S1). **(D)** Crossing Latency approached the 3-s threshold (horizontal dashed line) over the course of training, which corresponds to the delay between light-onset and shock-onset. No significant effect of treatment on Crossing Latency was found for training or in the test phase (Table S1).

spent 60–70% of the trial duration on the blue side in the middle of the training. Hence, the animals either did not leave the blue side or returned to the blue side more often. This behavior was not due to an impairment in locomotion since we found no differences in speed (**Figure 7B**) in the training (Table S1). However, toward the end of the training and in the test phase procaine-treated bees preferred the green side and PIs were similar to those found for vehicle- or sham-treated bees. We further explored if the ability to reverse in the chamber might have been affected. Procaine-treated bees did not reverse in the chamber less often than vehicle- or sham-treated bees (Figure S2D) (Table S1). But they performed on average three to four more reversals on the blue side than on the green side in the middle trials of training (**Figure 7C**). In contrast, vehicle- and sham-treated bees performed on average three to five more reversals on the green side in the same trials. Additionally, Crossing Latency was found to be on average 6 to 8 s in the middle trials for procaine-treated bees (**Figure 7D**). This was about twice as long as Crossing Latencies found for vehicle-treated and sham-treated bees and around 40–60% of the trial duration. Thus, procaine-treated bees did not leave the blue side even when the shocks were delivered for more than 3 s. Differences in Crossing Latencies were not due to different starting positions at light-onset in the training (Figure S3D) (Table S1).

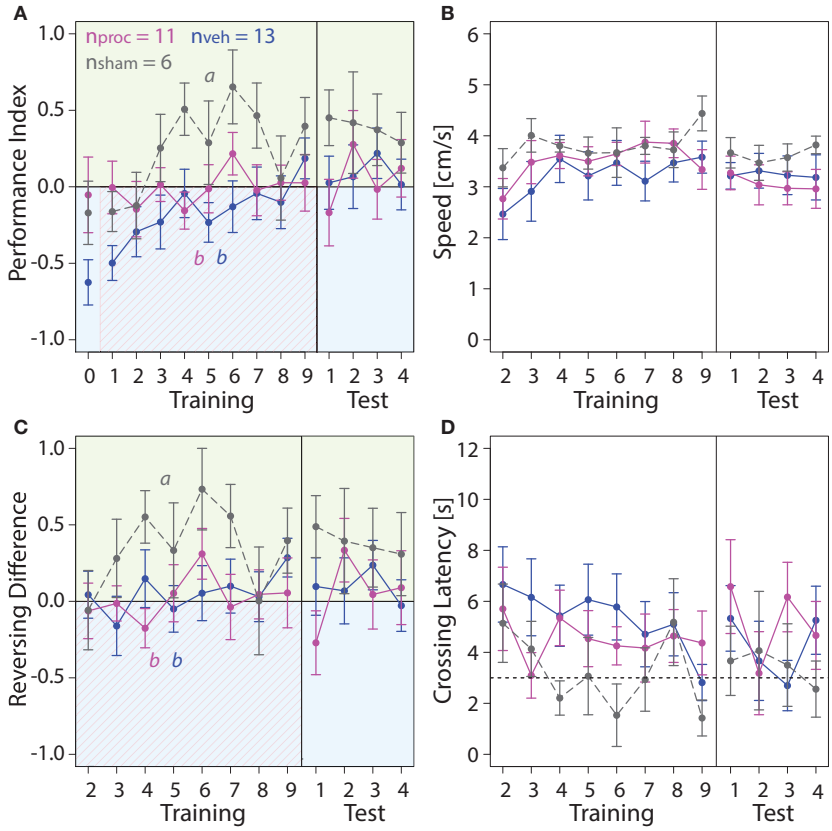

FIGURE 6 | Comparison of behavior in APIS for bees injected with vehicle (blue) or procaine solution (magenta) into the VLs, or sham-treated bees (gray). Learning to differentiate the shock-paired blue side and the safe green side was impaired in procaine and vehicle groups. Means ± SEM are plotted for all variables. Significant treatment effects determined with an LMM ($p < 0.05$) are indicated with letters a and b. Bees were subjected to one preference test (0) nine training trials and four test trials. **(A)** An LMM indicated an effect of treatment on Performance Index (PI) in the training but not in the test phase (Table S1). Treatment comparison with a Tukey HSD *post-hoc* test showed differences in PIs of vehicle and sham groups ($z = -4.217$, $p < 0.001$) and PIs of procaine and sham groups ($z = -2.638$, $p = 0.02$). **(B)** Speed did not differ between experimental groups (LMM, Table S1). **(C)** Reversing Differences were affected by treatment in the training but not in the test phase (LMM, Table S1). Treatment comparison with a Tukey HSD *post-hoc* test showed differences in Reversing Difference of vehicle and sham groups ($z = -3.107$, $p = 0.005$) and Revering Differences of procaine and sham groups ($z = -3.567$, $p = 0.001$). **(D)** Crossing Latency approached the 3-s threshold (horizontal dashed line) over the course of training, which corresponds to the delay between light-onset and shock-onset. No significant effect of treatment on Crossing Latency was found for training or in the test phase (Table S1).

DISCUSSION

About a decade ago the MBs were believed to process mainly olfactory information to generate meaningful associations to other stimuli. The CX was believed to primarily process visual and spatial information. Amongst other recent studies this study has shown this division might not necessarily be so clear. Our data indicate that the VLs as part of the MB output as well as the CX are involved in differential visual learning in the APIS assay.

Mushroom Body Function Was Required for Visual Learning with a Choice Component

Control bees escaped the shock-paired light field and avoided returning to it after only a few conditioning trials (**Figure 4**). These results were congruent with data obtained from untreated forager bees conditioned in the same assay in Konstanz, Germany (Kirkerud et al., 2017), and confirms the robustness of the paradigm across continents. While the operation and injection is an invasive procedure, we found that sham-treated animals recovered well and showed no deficits in learning performance compared to NT animals. In contrast, bees with silenced VLs escaped the shock-paired light field but failed to remain in the safe light field (**Figure 6**). Instead, they ran back and forth in the chamber resulting in lower PIs. This behavior indicated that they most likely failed to associate one light field with danger and the other light field with safety. We found a similar behavior in bees injected with the vehicle only. A similar phenomenon was found when injections of PBS into the MB lobes led to a reduced performance in olfactory reversal learning in comparison to injections into the calyces (Boitard et al., 2015). However, no effect of the vehicle was found when observing neural activity

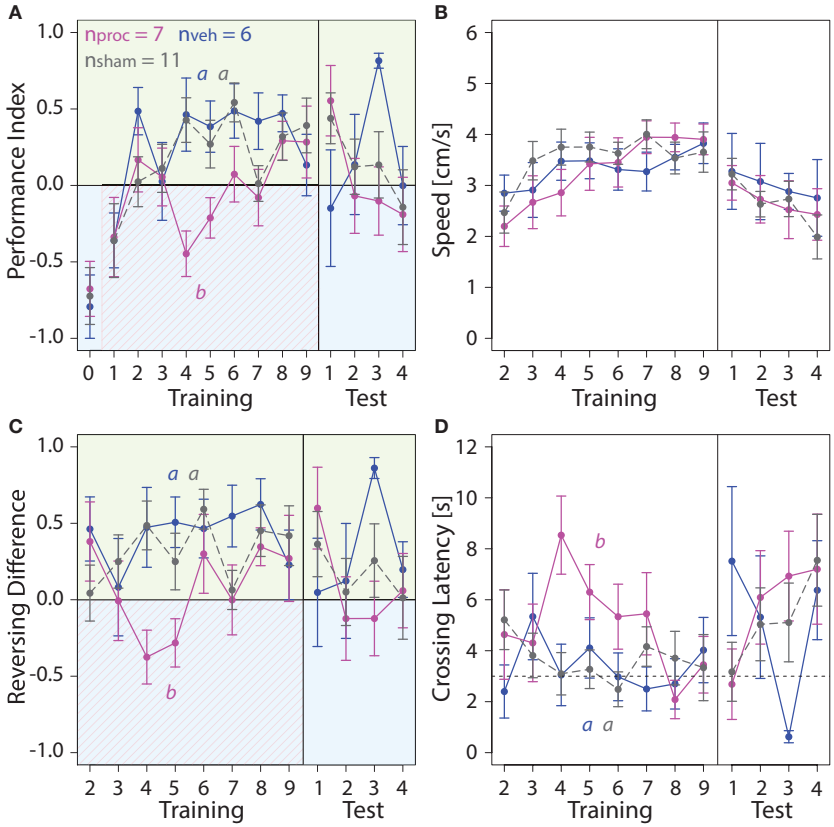

FIGURE 7 | Comparison of behavior in APIS for bees injected with vehicle (blue) or procaine solution (magenta) into the CX, or sham-treated bees (gray). Bees injected with procaine into the CX did not run away from the shock-paired blue side. Means ± SEM are plotted for all variables. Significant treatment effects determined with an LMM ($p < 0.05$, Table S1) are indicated with letters a and b. Bees were subjected to one preference test (0) nine training trials and four test trials. **(A)** Performance Indices (PIs) were affected by treatment in the training but not in the test phase (LMM, Table S1). Treatment comparison with a Tukey HSD *post-hoc* test showed differences in PIs of procaine and sham groups ($z = -2.512$, $p = 0.03$) and PIs of procaine and vehicle groups ($z = -3.052$, $p = 0.006$). **(B)** Speed did not differ between experimental groups (LMM, Table S1). **(C)** An LMM indicated an effect of treatment on Reversing Differences in the training but not in the test phase (Table S1). Treatment comparison with a Tukey HSD *post-hoc* test revealed differences in Reversing Difference of procaine and sham groups ($z = -2.629$, $p = 0.02$) and Reversing Differences of procaine and vehicle groups ($z = -2.995$, $p = 0.008$). **(D)** In vehicle and sham groups Crossing Latency approached the 3-s threshold (horizontal dashed line) over the course of training, which corresponds to the delay between light-onset and shock-onset. An LMM revealed an effect of treatment on Crossing Latency in the training but not in the test phase (Table S1). Treatment comparison with a Tukey HSD *post-hoc* test showed differences in Crossing Latencies of procaine and sham groups ($z = 2.467$, $p = 0.04$) and Crossing Latencies of procaine and vehicle groups ($z = 2.532$, $p = 0.03$).

changes due to injections using calcium imaging (Girardin et al., 2013).

When targeting one collar region of the MBC with procaine we found no deficits in performance (**Figure 5**). But since the honey bee collar region receives color input (Ehmer and Gronenberg, 2002; Gronenberg and Lopez-Riquelme, 2004) and the VLs were clearly involved in visual learning in APIS, it is possible that silencing neurons in only one of the eight collar regions in all MBCs might not have been sufficient to impair performance in the APIS assay. Further studies impacting all collar regions are necessary to clarify, but technically this would be extremely tricky to do.

In freely moving fruit flies, MB function was required for a visual paradigm with color stimuli and aversive reinforcement (Vogt et al., 2014, 2016). Similar to the paradigm presented here, blue and green light fields were presented simultaneously rather than sequentially. These findings stand in contrast to other studies implicating no involvement of the MBs in visual learning. Mutant flies (*Drosophila melanogaster*) with severely underdeveloped MBs and interrupted MB input were either conditioned by being shaken while illuminated with one color (Heisenberg et al., 1985) or trained with heat stimuli in a differential visual assay while being tethered in a flight simulator (Wolf et al., 1998). In both cases, mutant flies showed no learning deficits. In the latter case the fly was able to terminate the heat stimulus by turning left or right until the adjacent 90°-quadrant of the arena was faced and the arena was then illuminated with light of a different color. This suggests that the MBs are involved

in color learning which includes a choice situation rather than learning of sequentially presented color stimuli in a differential paradigm. Indeed, it has been shown that MBs are required to make a choice of responding to conflicting information of color and shape or color and position based on saliency (Tang and Guo, 2001; Zhang et al., 2007).

In both, bees and flies the dominant input to the MBs is olfactory, but it appears that MBs are also crucial for learning of visual information in bees in a binary-choice assay. Strausfeld (2012) and Farris (2015) argue that processing of visual information in the MB in insects is largely driven by the ecological relevance in the animal's life and the nature of visual input received. Large MBs with developed calyces are therefore not limited to species which rely predominately on olfactory information to navigate in their environment. They can also be found in aquatic beetle species which navigate mainly by vision (Lin and Strausfeld, 2012). It remains to be investigated if the MBs play a role in visual learning in other insect orders as well.

Silencing Neurons in the Central Complex Affected the Behavioral Response

We also found that silencing of neurons in the CX led to a change in behavior (**Figure 7**). Procaine-treated bees spent more time in the safe light field than on the shock-paired light field in the second and third trials and in the end of the training. This indicates, that learning of the light-shock pairing might still have been present. In the middle of the training period, however, procaine-treated bees remained on the shock-paired side of the chamber even after several seconds of shocks being delivered. This was not a result of an impaired ability to initiate reversals or an inability to walk in a straight line (Figure S2D). Nor was it caused by a major deficit in locomotion since speed was not found to be affected by procaine-injections, and rather bees appeared unable to execute an avoidance of the shocked light field.

But why was the effect not visible in the first learning trials? It seems very unlikely that procaine was only active in the middle trials of the training. Cockroaches with central bodies silenced by procaine showed deficits in locomotion and optomotor responses immediately after injections (Kathman et al., 2014; Kaiser and Libersat, 2015). Another explanation is that the response in the first trials might have mainly been driven by a direct reaction to the shocks, resulting in a short-lasting reflex-like escape maneuver. Initial responses to the shock could have been initiated by more direct and faster-processing "escape-pathways" generating a quick behavioral response to an obnoxious stimulus without involving the CX. Various escape reactions in insects have been proposed that bypass the higher processing centers of the brain (Horridge, 1962; Card, 2012). Is it possible that silencing of the CX only interfered with coordinating a motor response to a learned visual stimulus, but not an escape response from an aversive stimulus? In this case, a learned response to the blue light field would have been impaired but not the response to the shock itself. Toward the end of the training the procaine-effect seemed to have worn off, since the bees rapidly increased the proportion of time spent on the safe green side.

The CX has been implicated as the site to generate goal-directed behavior and to modulate movement in insects (Strausfeld and Hirth, 2013; Barron et al., 2015; Plath and Barron, 2015). Various studies have shown that the CX is crucial for spatial orientation memory (Neuser et al., 2008; Kuntz et al., 2012, 2017), visual pattern memory (Liu et al., 2006; Hou et al., 2011) and visual place learning (Ofstad et al., 2011) in fruit flies. A recent study has shown that a group of neurons in the ellipsoid body (part of the CX in the fruit fly) represents the orientation of the animals in relation to a visual stimulus (Seelig and Jayaraman, 2015). Taken together, the CX clearly has a role in visual learning and memory involving spatial orientation of the cues in fruit flies and possibly in other insects. We propose that the CX might also initiate the appropriate responses to learned stimuli which are processed by the MBs such as color stimuli.

Information about a Learned Stimulus Might Be Conveyed Indirectly to the Central Complex

Taken together, we showed that both, the MBs and the CX contributed to the behavioral response to a learned light stimulus. We propose the MBs integrated the coinciding shock and light information and the CX initiated the escape from the light field. We summarized the information flow between the different

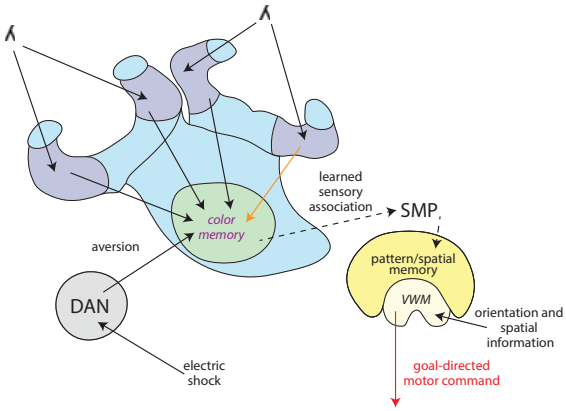

FIGURE 8 | Information flow model for differential color learning in a binary choice assay. Information about the light wavelength (λ) enters the collar region (dark blue) of the MBC from the optic neuropils. Visual information is passed on from the collar region to the VL (light green) via Kenyon cells. This process was partially disrupted by a procaine injection into one collar region (orange arrow). Electric shock information is passed on from the ventral nerve cord to dopaminergic neurons (DAN, gray) which modulate MB output. In the VL wavelength information is associated with aversion and most likely color memories are formed here. This process was disrupted by procaine-injections into the VL (marked in purple). Information about the learned sensory association might be passed on indirectly to the CX (yellow) via the superior medial protocerebrum (SMP). The CX receives orientation and spatial information and processes how the animal is orientated in relation to its environment using visual working memory (VWM). The CX initiates a goal-directed motor response, possibly modified in regards to the learned sensory association. This process was disrupted by procaine-injections into the CX (red arrow).

brain regions with the addition of other findings from different insect orders (**Figure 8**). To integrate coinciding shock and light information, both stimuli need to be received by the MBs. In the fruit fly γ lobe (part of the VL), a descending Kenyon cell carrying olfactory information forms synapses along the axon with a set of MB output neurons. Dopaminergic neurons modulate these individual compartments in relation to the internal state of the animal (Cohn et al., 2015). In flies, a group of these dopaminergic neurons (PPL1 cluster) carry information of aversive stimuli such as electric shocks (Waddell, 2013; Kaun and Rothenfluh, 2017). It needs to be studied, however, if this process is also found in other insect orders. In fruit flies, olfactory short-term memory is formed in the γ lobes which transitions into long-term memory to α and β lobes via the α' and β' lobes. Kenyon cells which convey wavelength and intensity information to the collar (Vogt et al., 2016) descend into the γ lobes in fruit flies. It remains to be investigated where exactly visual memories relating to color information are formed and where they transition from short-term to long-term memories.

A great question remains, whether there is a connection between the MBs and the CX. A direct connection between the MBs and the CX has not been found so far, with the exception of a single neuron recently discovered in the monarch butterfly (Heinze et al., 2013). An indirect connection could be found in the superior medial protocerebrum (Strausfeld and Hirth, 2013), which comprises outputs from the MBs carrying visual information in fruit flies (Ito et al., 1998) as a well as inputs to the upper division of the central body found in different insects (Strausfeld and Hirth, 2013; Pfeiffer and Homberg, 2014). It is therefore possible that information about the learned sensory association generated by the MBs is passed on indirectly to the CX in order to produce the conditioned response. Evidence for a connection between the MBs and CX manifesting in behavior was found when a sensory preconditioning paradigm involving cross-modal stimuli was investigated (Zhang et al., 2013). Here, an olfactory stimulus and a visual stimulus based on elevation were pre-conditioned. Then one stimulus was paired with reinforcement. A subsequent test of the other stimulus produced a response, even though it was never reinforced. Tested individually, blocking part of the MBs abolished olfactory memory and blocking part of the ellipsoid body (part of the CX in the fruit fly) abolished visual elevation memory. Remarkably, when the olfactory stimulus was reinforced after pre-conditioning and MBs were blocked, animals responded to the visual elevation stimulus. Thus, an association of the two CSs must have occurred in the pre-conditioning.

To explore the connection between the MBs and the CX will be a challenge in the future. The vast knowledge gained about learning and memory in the honey bee field in combination with pharmacological techniques (Felsenberg et al., 2011; Søvik et al., 2016) and assays such as APIS could provide a powerful tool to uncover how the different brain regions interact.

AUTHOR CONTRIBUTIONS

All authors (JP, BE, NK, US, CG, and AB) contributed substantially to the design and conception of the experiments, analysis and interpretation of the data and revision of the manuscript. JP, BE, and US contributed to acquisition and analysis of the data. JP and NK contributed to R analysis and statistical analysis of the data. JP and BE drafted the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnbeh.2017.00098/full#supplementary-material

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