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Sensory to motor transformation during innate and adaptive behavior in the cockroach

vorgelegt von

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"The study of roaches may lack the aesthetic values of bird-watching and the glamour of space flight, but nonetheless it would seem to be one of the more worthwhile of human activities."

- H.E. Evans, Life on a Little Known Planet

Contents

Abstract

Animal behavior is the result of processing and integrating various internal and external information. It can be highly flexible and vary between individuals. In insects, the mushroom body output region is an essential higher-order brain area in this process. Integration of various sensory and internal information takes place here as well as memory formation.

To investigate adaptive behavior, we established classical and operant conditioning paradigms with a focus on inter-individual differences: American cockroaches were trained harnessed as well as freely moving. To gain insight into the transformation from sensory input to motor output behind innate and adaptive behavior, we established an extracellular recording setup including different sensory stimulators: 1) We simultaneously recorded mushroom body output neurons (MBONs) and initial feeding behavior in single animals during odor stimulation and 2) we recorded MBON responses to different sensory modalities.

On the behavioral level, cockroaches were successful in memory formation across different paradigms and sensory modalities. Inter-individual differences regarding their cognitive abilities were discovered. Simultaneous neuronal and behavioral recordings revealed a correlation between MBON and feeding responses to food odors, which allowed for prediction of the behavior. Furthermore, neuronal recordings demonstrated that MBONs encode stimulus on- and off-responses, show adaptation during rapid successive stimulation and differ in response latencies to different sensory modalities.

Our results strengthen the idea that the mushroom body output region is not only important for memory formation. In addition, it is crucial for the integration as well as categorization of different sensory modalities. Moreover, it is involved in the sensory to motor transformation. Combining the successfully established behavioral and electrophysiological setups builds a solid base to investigate the role of MBONs in memory formation with high temporal resolution and with regard to inter-individual differences.

Zusammenfassung

Das Verhalten von Tieren ist das Ergebnis der Verarbeitung und Integration verschiedener interner und externer Informationen. Es kann sehr flexibel sein und von Individuum zu Individuum variieren. Bei Insekten ist die Ausgangsregion des Pilzkörpers, ein Gehirnareal höherer Ordnung, wesentlich in diesem Prozess. Hier finden die Integration verschiedener sensorischer und interner Informationen sowie die Gedächtnisbildung statt.

Um adaptives Verhalten zu untersuchen, haben wir klassische und operante Konditionierungsparadigmen eingeführt, wobei wir uns auf interindividuelle Unterschiede konzentriert haben: Amerikanische Schaben wurden sowohl in fixiertem als auch in freilaufendem Zustand trainiert. Um einen Einblick in die Umwandlung von sensorischen in motorische Informationen hinter angeborenem und adaptivem Verhalten zu erhalten, haben wir ein extrazelluläres Aufzeichnungssystem mit verschiedenen sensorischen Stimulatoren aufgebaut: 1) Wir zeichneten simultan die Ausgangsneurone der Pilzkörper (MBONs) und das initiale Fressverhalten in einzelnen Tieren während einer Geruchsstimulation auf und 2) wir zeichneten die Reaktionen der MBONs auf die Stimulation mit verschiedene sensorische Modalitäten auf.

Auf der Verhaltensebene waren die Schaben bei der Gedächtnisbildung über verschiedene Paradigmen und Sinnesmodalitäten hinweg erfolgreich. Zusätzlich wurden interindividuelle Unterschiede hinsichtlich ihrer kognitiven Fähigkeiten festgestellt. Simultan aufgenommene neuronale und verhaltensbezogene Daten zeigten eine Korrelation zwischen den Reaktionen der MBONs und den Fütterungsreaktionen auf Futtergerüche, was eine Vorhersage des Verhaltens ermöglichte. Darüber hinaus zeigten die neuronalen Aufzeichnungen, dass MBONs Beginn und Ende eines Stimulus kodieren, eine Anpassung bei schnell aufeinanderfolgenden Stimulationen zeigen und sich in den Reaktionslatenzen auf verschiedene sensorische Modalitäten unterscheiden.

Unsere Ergebnisse bestärken die Annahme, dass die Ausgangsregion der Pilzkörper nicht nur für die Gedächtnisbildung wichtig ist, sondern darüber hinaus entscheidend für die Integration sowie die Kategorisierung verschiedener sensorischer Modalitäten ist. Außerdem ist sie an der sensorischen zur motorischen Transformation beteiligt. Die Kombination der erfolgreich etablierten verhaltensbiologischen und elektrophysiologischen Versuchsanordnungen bildet eine solide Basis, um die Rolle der MBONs bei der Gedächtnisbildung mit hoher zeitlicher Auflösung und im Hinblick auf interindividuelle Unterschiede zu untersuchen.

General introduction

The role of the cockroach as model organism

Cockroaches as model organism for experiments investigating learning behavior are stated already in early 20th century (Szymanski, 1912; Turner, 1912). Even though other insects such as fruit flies (*Drosophila melanogaster*) and honey bees (*Apis melifera*) have been more extensively studied in neuroethological contexts, cockroaches offer some advantages over other insects. With their relatively large bodies and brains compared to other insects, American cockroaches (*Periplaneta americana*) are well-suited for behavioral and physiological studies. From an evolutionary perspective, cockroaches as basal insects are an interesting model organism to investigate, as it is contrary to model organisms such as fruit flies and honey bees that are evolutionary in a higher-order (Farris, 2005; Strausfeld et al., 2009; Bellen et al., 2010; Misof et al., 2014). Additionally, American cockroaches usually do not fly under regular laboratory conditions, which simplifies experiments with freely moving animals compared to fruit flies and honey bees. Their physiological robustness makes them eminently suitable for electrophysiological studies, which are difficult with more sensitive insects, especially in long-term recordings. Even recordings in freely moving cockroaches have been performed successfully (Mizunami et al., 1998a; Okada et al., 1999; Martin et al., 2015).

American cockroaches live in gregarious structures (Lihoreau et al., 2019), are domiciliary, are usually found in dark and moist places and avoid brightness (Bell, 1990), which fits their circadian rhythm with the active phase during the night (Harker, 1956). In these low-light conditions, olfactory information plays an important role in navigation, mating and food searching (Seelinger, 1990). Against the widely held view that cockroaches eat everything, they clearly avoid some food sources like citrus fruits and peppermint (Sakura and Mizunami, 2001; Yoon et al., 2009) and have preferred food sources, mostly carbohydrate rich foods (Laupraset et al., 2006). With their particularly sensitive antennae that detects slight changes in odor concentration they are able to detect their preferred food sources (Tichy et al., 2020a). Due to the characteristic length of their antennae, the variety of receptors on it (Schaller, 1978) and the segmentation of odor detection on the antennae (Paoli et al., 2020) cockroaches are able to scan wide areas. A specific class of olfactory receptor neurons is also responsive to mechanical stimuli (Waldow, 1975, 1977; Ernst et al., 1977) that provide relevant information for high-frequency navigation (Camhi and Johnson, 1999) and potential escape behavior to air puffs (Grandcolas, 1998). Their antennae are further equipped with cold, moist and dry receptors (Yokohari, 1978; Nishikawa et al., 1992). Visual inputs are detected by two light sensitive ocelli and two relatively large compound eyes. *P. americana*, which have two types of color receptors with their peaks of perception at 365 nm (narrowband UV-sensitive photoreceptor) and 507 nm (broadband green-sensitive photoreceptor) (Goldsmith and Ruck, 1958; Walther, 1958; Mote and Goldsmith, 1970). Detecting wavelengths of the green spectrum is particularly relevant for cockroaches to escape from dangerous situations with potential predators (Okada and Toh, 1998; Laurent Salazar et al., 2013). However, they lack the ability to detect red colors (Goldsmith and Ruck, 1958; Walther, 1958; Mote and Goldsmith, 1970), which is particularly useful for experimentalist to conduct experiments under red-light conditions, which is perceived as darkness by the cockroaches.

Equipped with the above described properties, *P. americana* is well suited as an invertebrate model organism and allows for supplemental investigations, additional to the established invertebrate model organisms.

Learning and memory in insects

Insects are appropriate model organisms for neuroethological investigations, because of their lower complexity than vertebrates and also because of their behavioral repertoire. Especially with their cognitive abilities they play an important role for investigations of learning and memory. Even though learning and memory has been researched in a broad range of insects such as ants (Piqueret et al., 2019; Czaczkes, 2022), crickets (Matsumoto and Mizunami, 2004; Matsumoto, 2022), locusts (Simões et al., 2016), mosquitos (Kaur et al., 2003), moths (Hartlieb et al., 1999; Daly and Smith, 2000) and parasitic and parasitoid wasps (Meiners et al., 2003; Hoedjes et al., 2011). Here the focus will be on cockroaches in comparison with the two most extensively studied model organisms, bees and flies (McGuire et al., 2005; Menzel, 2012, 2021; Giurfa, 2015; Modi et al., 2020).

Honey bees and bumble bees show complex forms of learning in a broad repertoire of olfactory and visual, classical and operant learning paradigms (Zhang et al., 2012; Avargues-

Weber and Giurfa, 2013; Chittka, 2017; Loukola et al., 2017). The proboscis extension response (PER) is extensively used as conditioned response for classical conditioning in honey bees. This paradigm is based on the classical conditioning experiment by Pavlov (1927). In his best-known experiment on dogs, he combined the appearance of food with the ringing of a bell. Before the conditioning phase, the dog started salivating only when food was present (unconditioned response), but after pairing of the food as unconditioned stimulus (US) with the ringing bell as conditioned stimulus (CS) the dog started salivating also when only the ringing bell was present (conditioned response; Fig. 1a). The PER is elicited similar to the salivation in Pavlov's experiments by food presentation, in the case of honey bees specifically sugar solution was used as food source (Fig. 1b).

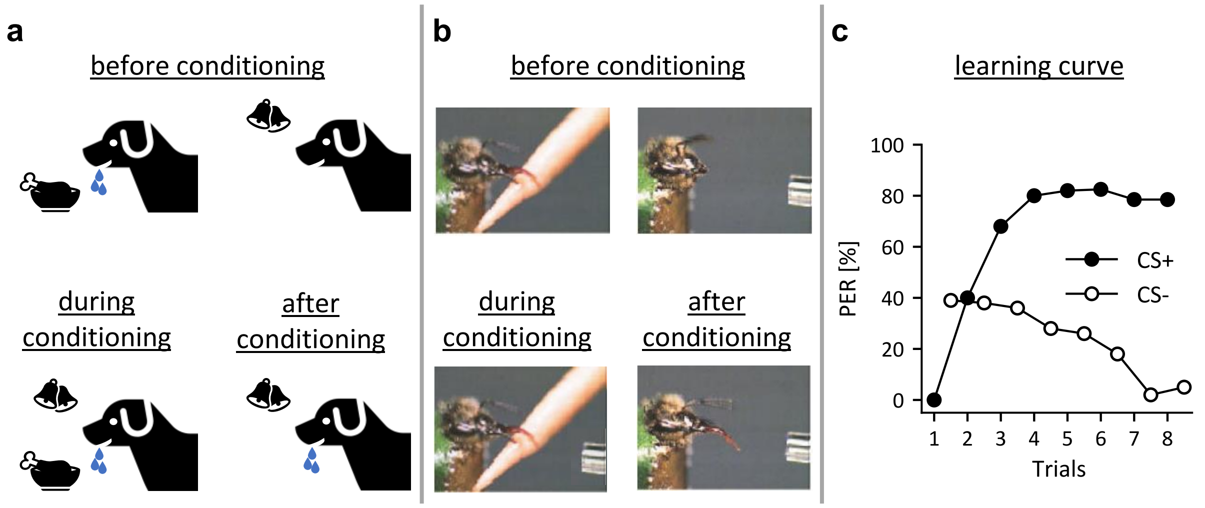

Figure 1 Classical conditioning in different species. **a** Schematic illustration of the classical conditioning paradigm by Pavlov (1927) **b** Classical conditioning paradigm with a honey bee that shows PER as conditioned response only to sugar solution before and during conditioning, but responds after conditioning to odor only as well (adapted from Menzel and Giurfa (2001)) **c** Exemplary learning curve during a differential conditioning task in honey bees (adapted from Menzel and Giurfa (2001)).

The strong intrinsic drive of bees for sugar consumption leads to a high motivation in training and a great learning success (Menzel and Giurfa, 2001; Fig. 1c). Initially odors were applied as CS (Takeda, 1961; Bitterman et al., 1983), further visual stimuli were used (Avarguès-Weber and Mota, 2016) and more recently even combinations of both were successfully used as CS (Becker et al., 2019; Riveros et al., 2020; Gil-Guevara et al., 2022). Other than that, bees are also capable of performing other tasks that involve operant visual (Avarguès-Weber et al., 2011; Kirkerud et al., 2017; Nouvian and Galizia, 2019) and spatial conditioning (Tsvetkov et al., 2019). It is remarkable that bees are also successful in more complex tasks (Zhang et al., 2012). They are able to discriminate between different patterns (Wehner, 1967; Giurfa et al., 1999) and can distinguish between concepts of 'sameness' and' difference' (Giurfa et al., 2001). Based on this, they can categorize face-like stimuli (Avarguès-Weber et al., 2010), discriminate between different human faces (Dyer et al., 2005) and differentiate between images of complex environments (Dyer et al., 2008). Further, they are able to generalize visual information based on numbers (Gross et al., 2009) and learn complex paths (Zhang et al., 2000).

Fruit flies are not capable of learning on such high level, compared to honey bees, but have been investigated successfully for more than 100 years in neuroscience and are a key molecular model system (Bellen et al., 2010). Their genetic accessibility as well as manipulability have allowed major steps forward in acquiring knowledge in this field (Davis, 1993; Keene and Waddell, 2007). Additionally, the relative small number of neurons make them accessible for connectome studies, providing researchers with the connectome responsible for learning and memory in the larva and adult fly (respectively: Eichler et al., 2017; Takemura et al., 2017). Typical learning paradigms in adult fruit flies are group assays in a T-maze apparatus developed by Tully and Quinn (1985). Commonly two odors are presented sequentially and one of the odors (CS) is paired with an electric shock as punishment (US), leading to avoidance of this odor. Drosophila larvae are mostly conditioned in groups on agarose filled Petri-dish either supplemented with sugar (US) or plain while an odor (CS) is present. Memory tests are usually conducted with two odors that were presented on opposite sides on an extra Petri-dish where successfully conditioned larvae stay closer to the rewarded odor (Michels et al., 2017). Both in adults and larvae more and more studies considering individuality in learning contexts and setups were established that allow observation of single animals (Lesar et al., 2021; Smith et al., 2022).

Cockroaches in comparison have not been investigated as much as honey bees or fruit flies, but have a number of relevant advantages as described above that can help to provide valuable insights. Similarly to the PER in bees, Hosono et al. (2016) established the maxillary-palpi extension response for classical conditioning in cockroaches elicited by pairing an odor (CS) with sugar solution (US). In various additional studies, it has been demonstrated that cockroaches learn successfully in classical and operant tasks (Balderrama, 1980; Barraco, 1981; Watanabe et al., 2003). Although cockroaches were investigated significantly less than other insects, they have been targeted in early operant conditioning experiments (Szymanski, 1912; Turner, 1912, 1913; Gates and Allee, 1933). More recent studies focused on olfactory conditioning where classical conditioning paradigms were used to train the cockroaches and testing took place in an open arena where they could show their odor preference (Watanabe et al., 2003; Sato et al., 2006). Further, it could be demonstrated that cockroach are able to learn under harnessed conditions (Watanabe et al., 2003) . In others, salivation (Watanabe and Mizunami, 2006, 2007) or maxillary-palpi extension response (Hosono et al., 2016) were used as conditioned response and memory up to 24 h could be indicated. Cockroaches were equally successful in operant olfactory conditioning paradigms (Sakura and Mizunami, 2001; Wada-Katsumata and Schal, 2021). Except for pure olfactory conditioning experiments, various visuo-spatial learning experiments were conducted with fixed cockroaches by pairing an appetitive odor (US) with a green light (CS) whereupon cockroaches responded by pointing with their antenna to the light source (Kwon et al., 2004; Lent and Kwon, 2004; Pintér et al., 2005; Pomaville and Lent, 2018). However, only few experiments were conducted with operant visual (Brown and Strausfeld, 2009) and spatial (Mizunami et al., 1998b) conditioning paradigms.

Inter-individual differences in learning

For a long time, inter- individual differences in invertebrates have mostly not been considered, but it is evident that it plays a relevant role. More and more studies pay attention to this issue (Kralj-Fišer and Schuett, 2014) and take into account that even clonal insects like aphids show different personality traits (Schuett et al., 2011). Even though group assays are a helpful method to get relatively fast large sample sizes, they are always limited to the group choice and show only the collective personality (Steymans et al., 2021). Decision making in a group of animals can be affected by many different factors like social modulation (van den Bos et al., 2013) or group composition (Planas-Sitjà et al., 2018; Cook et al., 2020; Nicolis et al., 2020). In

American cockroaches it has been stated that this can even lead to shifts in odor preference (Laurent Salazar et al., 2017; Calvo Martín et al., 2021; Günzel et al., 2021), which makes focusing on individual behavior, especially in the context of learning and memory, important.

Individuality can already occur on the level of sensory perception and processing (Honegger et al., 2020; Linneweber et al., 2020). However, regarding learning and memory in insects few studies have focused on the genetic variation (Dukas, 2008), others focused on the behavioral metrics (Borstel and Stevenson, 2021) and some focused on intra-specific differences, depending on different requirements for individuals in a colony (Muth, 2021). Lesar et al. (2021) used a Y-maze assay for individual Drosophila larvae and found that they perform switch-like learning behavior. This supports findings from Pamir et al. (2011, 2014) who argued that average learning rates in honey bees consist of individuals that show step-wise learning from one trial to the next. This led to the hypothesis of 'learners' that show the learned behavior consistently versus 'non-learners' that never show the learned behavior as two separated groups. Further evidence for this effect comes from elementary visual learning tasks in honey bees as well (Finke et al., 2021). Another issue raised on this topic is how individuals perform across different learning tasks and sensory modalities. Recent studies found a negative correlation between landmark and olfactory learning (Tait et al., 2019) and positive correlation between the performance in elementary and non-elementary visual tasks (Finke et al., 2021) in honey bees. Whereas no correlation between learning of different modalities (visual and olfactory) was found in individual bumble bees (Smith and Raine, 2014). However, it remains an open question, how cognitive abilities across different tasks and sensory modalities are related.

From peripheral sensory perception to the mushroom body

Olfactory and visual cues perceived by insects are processed in the brain via well researched sensory pathways. For insects it is known that both perceptions send projection neurons (PNs) to the mushroom body (MB) (Galizia and Rössler, 2010; Nishino et al., 2012; Vogt et al., 2014; Li et al., 2020b). Olfactory input from the antennae arrives in the antennal lobes (AL), which is equivalent to the olfactory bulb in vertebrates (Shepherd, 1972; Boeckh et al., 1987; Bargmann, 2006). In cockroaches and other insects, parallel pathways from functional units of the AL, called glomeruli, project with uni- and multiglomerular PNs to the MB calyces and the lateral horn (Galizia and Rössler, 2010; Watanabe et al., 2017; Fuscà and Kloppenburg, 2021; Fig. 2). PNs of the cockroach project to different zones of the MB calyx, depending on their source among the antennal sensilla (Watanabe et al., 2017). In addition to olfactory information, mechanosensory information are also detected by the antenna and are projected to the MB calyces (Strausfeld and Li, 1999a; Okada et al., 2003). The visual input in insects projects from the retina via different layers of the optic lobes (lamina, medulla, lobula) to the MB calyces (Reischig and Stengl, 2002; Borst, 2009; Dyer et al., 2011; Nishino et al., 2012; Yagi et al., 2016; Li et al., 2020; Fig. 2a). Input neurons responding to light stimulation in the cockroach are known to extend radially along the inner layer of the MB calyces (Nishikawa et al., 1998).

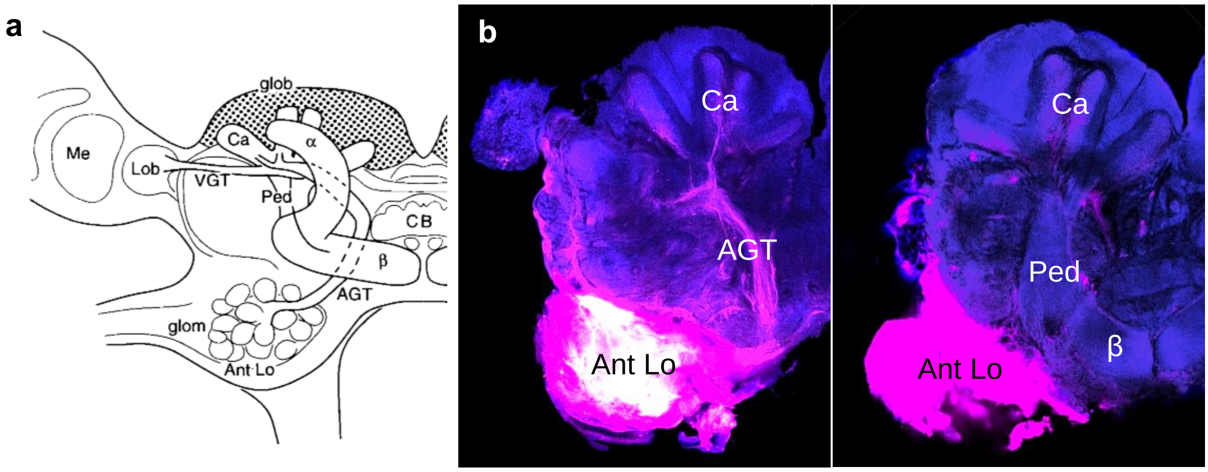

Figure 2 Left hemisphere of *P. americana*. **a** Schematic visualization of the cockroach central nervous system (adapted from Li and Strausfeld (1997)). Olfactory projection neurons lead from the glomeruli (glom) in the antennal lobe (Ant Lo) over the antennoglomerular tract (AGT) to two mushroom body calyces (Ca) per hemisphere. Visual projection neurons lead from the medulla (Me) over the lobula (Lob) and the visual glomerular tract (VGT) to the calyces. Coming from the calyces, two stalks merge to the pedunculus (Ped) and terminate in the α and β lobes. CB: central body; glob: globuli cells **b** Different layers of a whole brain scan showing the antennoglomerular tract projecting from the antennal lobe to the calyces (left) and the pedunculus deriving from the calyces leading to the β lobe (right).

The MB itself has changed evolutionary and is therefore quite diverse across insects (Farris, 2005; Strausfeld et al., 2009). The cockroach as an evolutionary basal insect has a prominent MB with two calyces that project rectilinear with Kenyon cells over the pedunculus into the

MB α and β lobe in each brain hemisphere (Weiss, 1974; Strausfeld and Li, 1999b; Fig. 2). Further, relatively specific in cockroaches four giant input neurons (calycal giants) project to the MB calyces with dendritic arborizations in the lateral horn and the neuropil anterior the MB α and β lobes (Nishino and Mizunami, 1998; Takahashi et al., 2017). Efferent and recurrent neurons located around the lobes integrate multimodal information (Homberg, 1984; Li and Strausfeld, 1999; Strausfeld and Li, 1999a) and are likely underlying memory formation for different sensory modalities (Vogt et al., 2014). It is stated that these encode visual (Homberg, 1984; Strausfeld and Li, 1999b; Strube-Bloss and Rössler, 2018; Schmalz et al., 2022), olfactory (Homberg, 1984; Li and Strausfeld, 1999; Strube-Bloss et al., 2012), tactile (Homberg, 1984; Li and Strausfeld, 1997; Strausfeld and Li, 1999a), gustatory (Homberg, 1984; Masek et al., 2015; Owald and Waddell, 2015), auditory (Li and Strausfeld, 1997; Strausfeld and Li, 1999a) and sensory-motor (Mizunami et al., 1998a; Okada et al., 1999) input in various insects.

Different roles of the mushroom body output

As various sensory modalities are represented in MBONs, the MB output acts as an integrational area, but is mostly known for memory formation (Strube-Bloss et al., 2011, 2016; Aso et al., 2014b; Hige and Turner, 2015; Owald and Waddell, 2015). Sensory information is projected via Kenyon cells to the MB lobes, where MBONs are located. This area gets additional input from dopaminergic neurons (Aso et al., 2014a). In vertebrates it has been shown that dopaminergic neurons play a crucial role in predicting the valence of sensory cues and enforcing neuronal plasticity (Schultz, 2015, 2016). Studies in insects stated that dopaminergic neurons play a similar role as in vertebrates by modulating the connection from Kenyon cells to MBONs (Riemensperger et al., 2005; Waddell, 2010; Aso et al., 2014a; Cohn et al., 2015; Felsenberg et al., 2017; McCurdy et al., 2021; Springer and Nawrot, 2021). In fruit flies two dopaminergic clusters are reported that are assumed to integrate parallel pathways of reward and punishment learning (Hige and Turner, 2015; Felsenberg et al., 2018; Yamazaki et al., 2018). In recent studies even specific connections between Kenyon cells and MBONs were reported that are relevant for aversive learning by monitoring the MBON postsynaptic site (Hancock et al., 2022). However, insect memory formation, particularly appetitive learning, does not only involve dopaminergic, but also octopaminergic signaling (Schwaerzel et al., 2003; Unoki et al., 2006; Burke et al., 2012; Mizunami and Matsumoto, 2017; Sabandal et al., 2020).

In honey bees the focus of investigation of MBONs was more on the population level. Electrophysiological approaches gave insights about temporal dynamics and activation patterns of MBONs (Strube-Bloss et al., 2011, 2016, 2021). The precise temporal resolution of neuronal activity during presentation of CS leads to the conclusion that MBONs predict the valence of stimuli and in turn cause the expression of the conditioned response (Strube-Bloss et al., 2011). However, it should be noted that plasticity in invertebrates was not only found in the MB output region but also in upstream regions like the antennal lobe and boutons in the MB calyx (Faber et al., 1999; Rath et al., 2011; Haenicke et al., 2018; Anton and Rössler, 2021; Franco and Yaksi, 2021; Marachlian et al., 2021). Nevertheless, in this work we focus on the MB output region.

The MB output integrates not only reward and punishment, but also internal states that are encoded in dopaminergic neurons and probably forward this information to Kenyon cells and MBONs (Cohn et al., 2015; Siju et al., 2020). Other studies also argued that valence is encoded in MBONs, which might guide behavioral output (Aso et al., 2014b; Modi et al., 2020; Siju et al., 2020). To this end, it has been stated that MBONs are required for decision making and distinct behaviors like food seeking and avoidance (Bräcker et al., 2013; Lewis et al., 2015; Tsao et al., 2018). Anatomical studies stated more and more that MBONs project to premotor areas (Li and Strausfeld, 1997, 1999; Okada et al., 2003; Aso et al., 2014a) and even directly to descending neurons (Hsu and Bhandawat, 2016; Emanuel et al., 2020; Li et al., 2020a). This indicates that the MB output region is involved in the process of integrating internal and external inputs and in transforming them into motor output.

Aim of this work

Here, we introduce cockroaches as model organism to study individual learning behavior and the underlying neuronal basis of stimulus processing in the MB that leads to specific behavior. With this work we build the basis to investigate the relevance of the MB output for sensory-motor transformation and memory formation in future studies. We established experimental learning protocols and built up an electrophysiological setup adapted from Strube-Bloss et al. (2011). We made use of the specific advantages of cockroaches as model organism and the high temporal resolution, which the electrophysiological setup allows.

(1) Thus, we got insights in the learning abilities of *P. americana* in classical and operant learning tasks with different stimulus modalities. Using the learning protocols that we developed and adapted for cockroaches, we emphasize the relevance of individual behavior and support the hypothesis of individual learning types in insects.

(2) By simultaneously recording MBON and behavioral responses during olfactory stimulation, we gained more insights about olfactory valence on the behavioral and neuronal level. Based on our results and machine learning approach, we argue that MBONs are involved in the decision making process in the sensory-motor pathway.

(3) With additional visual stimulations during extracellular recordings of MBONs, we expanded the knowledge about visual representation in respect to color identity, intensity, stimulus on- and offsets and adaptation. Additional, multimodality was investigated in the same MBON population by comparing response properties between olfactory, mechanical and visual stimuli.

This work builds a solid base to make use of the advantages of cockroaches, by combining the learning paradigms with the electrophysiological approach we established to gain further insights in plasticity of the MB output region.

Chapter I: Cockroaches show individuality in learning and memory during classical and operant conditioning

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Cockroaches Show Individuality in Learning and Memory During Classical and Operant Conditioning

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Animal personality and individuality are intensively researched in vertebrates and both concepts are increasingly applied to behavioral science in insects. However, only few studies have looked into individuality with respect to performance in learning and memory tasks. In vertebrates, individual learning capabilities vary considerably with respect to learning speed and learning rate. Likewise, honeybees express individual learning abilities in a wide range of classical conditioning protocols. Here, we study individuality in the learning and memory performance of cockroaches, both in classical and operant conditioning tasks. We implemented a novel classical (olfactory) conditioning paradigm where the conditioned response is established in the maxilla-labia response (MLR). Operant spatial learning was investigated in a forced two-choice task using a T-maze. Our results confirm individual learning abilities in classical conditioning of cockroaches that was reported for honeybees and vertebrates but contrast long-standing reports on stochastic learning behavior in fruit flies. In our experiments, most learners expressed a correct behavior after only a single learning trial showing a consistent high performance during training and test. We can further show that individual learning differences in insects are not limited to classical conditioning but equally appear in operant conditioning of the cockroach.

Keywords: classical conditioning, operant conditioning, insect cognition, learning and memory, cockroach, insect behavior, personality

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INTRODUCTION

A behavioral syndrome defines a consistent behavior of an individual that is correlated across time and contexts. Animal personality (Gosling and Vazire, 2002) is expressed in long-term differences among individuals across a variety of behavioral traits such as boldness-shyness, exploration-avoidance, activity level, sociability, or aggression (Sih et al., 2004a,b; Dingemanse and Wolf, 2010). While consistent behavioral traits have been heavily studied in vertebrates, literature on individuality and personality in invertebrates is still scarce (for review, see Kralj-Fišer and Schuett, 2014). The small amount of available data on invertebrate personality may be partly due to the traditional belief that invertebrates express stereotyped stimulus-response behaviors with little individual differences (e.g., Brembs, 2013). Invertebrate studies have primarily been conducted in the context of collective behavior in social contexts and mostly investigated how individual personalities influence the colony behavior (e.g., in cockroaches: Planas-Sitjà et al., 2018; Planas-Sitjà and Deneubourg, 2018, ants: Pinter-Wollman, 2012, spiders: Grinsted et al., 2013; Wright et al., 2014, pea aphids: Schuett et al., 2011; and crickets: Rose et al., 2017).

At the level of animal cognition, inter-individual performance differences may reflect variation in cognitive ability independent of animal personality. However, individual cognitive styles may also inflict personality (Carere and Locurto, 2011). Individuality has been intensively studied in learning and memory. Learning and memorizing the relevance of environmental cues is of major importance for the survival of virtually all animals. Individuals of a species can vary substantially in their learning performances as has been shown for both vertebrates (e.g., Gosling, 2001; Gallistel et al., 2004; Groothuis and Carere, 2005; Kolata et al., 2005; Schuett and Dall, 2009; Kotrschal and Taborsky, 2010; David et al., 2011) and invertebrates (for review, see Dukas, 2008).

In insects, studies have focused on bumblebees and honeybees. Bumblebees have been studied in a variety of tasks (Chittka et al., 2003). For example, individual bumblebees that learn only a single flower parameter (odor or color) were more efficient in several ways than those that had learned two: they made fewer errors, had shorter flower handling times, corrected errors faster, and transitions between flowers were initially more rapid (Chittka and Thomson, 1997). It has further been shown that individual bumblebees consistently differ in their ability to learn to discriminate stimuli from the visual and olfactory modality (Muller and Chittka, 2012). A systematic analysis of classical conditioning experiments in the honeybee found that the group-average learning behavior did not adequately represent the behavior of individual animals. This result was consistent across a large number of datasets including olfactory and tactile conditioning collected from more than 3,000 honeybees obtained during absolute and differential classical conditioning (Pamir et al., 2011, 2014). Gradually increasing learning curves reflected an artifact of group averaging and the behavioral performance of individuals was characterized by an abrupt and often step-like increase in the level of response (Pamir et al., 2011), a result that directly matches observation in vertebrates (Gallistel et al., 2004) but contradicts earlier findings in the fruit fly (*Drosophila melanogaster*) in which the group-average behavior has been described to represent the probabilistic expression of behavior in individuals (Quinn et al., 1974).

In the present work, we asked whether cockroaches show individuality in their learning performances, both in classical and operant conditioning tasks. Behavioral learning studies that used olfactory and visual cues demonstrated that cockroaches can be assayed for classical conditioning tasks while animals are immobilized (Watanabe et al., 2003; Kwon et al., 2004; Lent and Kwon, 2004; Watanabe and Mizunami, 2006) or able to move freely (Watanabe et al., 2003; Sato et al., 2006; Hosono et al., 2016). In some experiments, after classical olfactory conditioning, memory tests were performed in an open arena where cockroaches could freely choose to approach different odors (Watanabe et al., 2003; Sato et al., 2006). Open arenas and T-mazes have been used successfully for operant conditioning in cockroaches. Balderrama (1980) demonstrated for the first time that cockroaches could be trained to associate different odors with either sugar or salt solution in an open arena. Mizunami et al. (1998) studied place memory using a spatial heat maze with and without visual cues. More recent studies by Mizunami and colleagues (Sakura and Mizunami, 2001; Sakura et al., 2002) confirmed and extended operant conditioning of cockroaches in the open arena. Barraco et al. (1981) successfully trained cockroaches in a spatial discrimination task using an electric shock to punish either a left or right turn in a T-maze. Employing stimuli of different modalities, we show in the present study that cockroaches demonstrate individuality in their ability to learn and memorize stimuli employing both classical and operant conditioning tasks.

MATERIALS AND METHODS

Insects

All experiments were conducted with adult male *Periplaneta americana*. Animals were kept under a reverse light-dark cycle (12 h : 12 h) at 26°C in laboratory colonies at our rearing facilities at the University of Cologne. Cockroaches were allowed to drink water and fed on oat *ad libitum*. However, water was removed 4 days before training to increase motivation. All experiments were conducted in the active phase (scotophase) of the animals.

Experimental Setups

For classical conditioning, cockroaches were harnessed in custom-made fixation cylinders (**Figure 1A**) after anaesthetization at 4°C. After fixation, only the animals' head protruded allowing free movement of the antennae and mouthparts. After habituation in the experimental room for 16 h, cockroaches were placed in front of a 10 ml plastic syringe mounted in a holder and an exhaust system behind removing odor-loaded air (**Figure 1A**). Stimuli were diluted in mineral oil (Acros Organics™, Geel, Belgium) and odor concentrations were adjusted to match the vapor pressure of the odor with the lowest value (trans-cinnamaldehyde). Dilutions were as follows (in % v/v): isoamyl acetate (99+ %, pure, Acros Organics™, Geel, Belgium): 26.27%, butyric acid (> 99%, Aldrich, Darmstadt, Germany): 2.56%, trans-cinnamaldehyde (≥ 98%, Merck, Darmstadt, Germany) undiluted. Ten microliters of each odor were given on a piece of filter paper inserted in a 10 ml plastic syringe for olfactory stimulation. A filter paper without any odor nor the solvent was used as control stimulus testing for mechanical stimulation (Air). Isoamyl acetate and butyric acid were used as conditioned stimuli (CS+ or CS−), while trans-cinnamaldehyde served as control odor without any assigned contingency (reward or punishment). Odors were chosen based on choice behavior of cockroaches in preliminary tests in a T-maze, in which no preference was found between isoamyl acetate and butyric acid.

For operant conditioning, a custom-made flexible maze was used. Walls made from polyvinyl chloride allowed easy cleaning with alcohol between single trials. The maze was positioned on a ground plate and different tunnel pieces were combined to form a T-Maze (**Figure 1B**). Shutters allowed closing the start and target boxes (20 cm × 28 cm × 4 cm). All experiments with the T-maze were conducted under red light (**Figure 1B**).

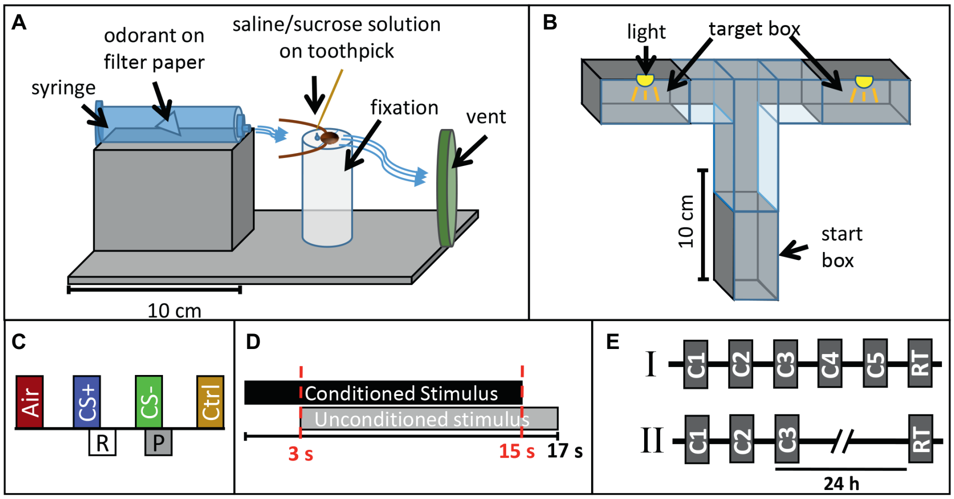

FIGURE 1 | Experimental design. Sketch of **(A)** the classical conditioning setup and **(B)** the operant conditioning setup. **(C)** Presentation of one training block in the classical conditioning paradigm. Each block contains an air puff (Air), the rewarded odor CS+ paired with sugar solution (R), the punished odor CS− paired with saline solution (P), and the control odor (Ctrl, cinnamaldehyde). **(D)** Time sequence of the conditioned stimulus (CS, odor) and unconditioned stimulus (US, sucrose or saline solution) in a single training trial during classical conditioning. **(E)** Operant conditioning protocols (I) with five consecutive training trials (C1–C5) and a retention test (RT) after 35 min and (II) with three consecutive training trials (C1–C3) and a retention test after 24 h.

Training and Test Procedures

First, we established a novel classical conditioning paradigm in the harnessed cockroach, training the animals to exhibit a specific movement of the maxilla-labia (mouthparts) as conditioned response behavior. We termed this response the maxilla-labia response (MLR). When touching the antennae and mouthparts with sucrose solution, cockroaches start to quickly move and extend their maxillae and labium, the most central mouthparts, to reach for and suck the solution. When saline solution is presented, animals touch and taste the solution without ingesting and show clear avoidance behavior (retraction of the mouthparts). In each single trial, the occurrence or non-occurrence of the MLR was recorded as a binary response (0/1). Only if the MLR was observed within the first 3 s of odor presentation (before US-onset, see **Figure 1C** and description below), it was counted as conditioned response. This novel paradigm for classical conditioning of the cockroach is similar to the proboscis extension response paradigm used in classical conditioning of honeybees, first established by Takeda (1961) and later standardized by Bitterman et al. (1983).

For classical conditioning, each block of training consisted of (1) one stimulation with a simple filter paper without an odorant to test for a mechanical response to the air puff (Air), (2) one CS+ presentation (reinforced conditioned stimulus) paired with 20% sucrose solution as positive reinforcer (unconditioned stimulus, US), (3) one CS− presentation (punished stimulus) paired with 20% saline solution as negative reinforcer, as well as (4) one stimulation with a control odor (cinnamaldehyde, Ctrl), which was not paired with a US (**Figure 1C**). In each CS+ or CS− presentation, the respective odor (CS) was presented for 15 s. Three seconds after odor onset, the US was delivered by touching the maxillary palps with sucrose or saline solution and animals were allowed to drink the respective solutions for 14 s (**Figure 1D**). In the case of the negative reinforcer, most animals did not drink the saline solution voluntarily but were "forced" to taste the salt in all trials by touching their mouthparts with the toothpick. We performed all experiments in two independent groups for which the identities of the CS+ odor and the CS− were reversed. For retention tests, the same pattern of odor presentation as in conditioning trials was used except that no US was presented.

Three differential classical conditioning experiments were conducted. In each block of training trials, the two control stimuli (air, cinnamaldehyde) were separated from the two CSs with an interstimulus interval (ISI) of 45 s, whereas the ISI between CS+ and CS− was 32 s. The first experiment was designed to investigate differential learning with an acquisition phase that consisted of five blocks of trials (each block contained one presentation of air, the CS+, the CS−, and a control stimulation, respectively) with an inter-block interval of 10 min. A retention test was conducted after 10 min. The second and third experiments were designed to investigate memory retention after differential learning at two different time intervals (1 h and 24 h). Due to the length of the experiment, only three training blocks with an inter-block interval of 10 min were used for these two experiments.

For operant conditioning, each cockroach was allowed to acclimate in the start box for 15 min before training. At the beginning of a training trial, the shutter was opened and the cockroaches were allowed to walk freely and enter the target boxes. When entering one of the target boxes for the first time, the shutter was closed and the animal was subjected to a 5 min light exposure (punishing stimulus, US). Whenever an animal entered the same target box again in a subsequent trial, it was again subjected to the light punishment. All animals which did not start moving within the first 3 min in two consecutive attempts were excluded from the experiment.

Two different operant conditioning paradigms were used. In the first one, animals were trained in five trials with an inter-trial interval (ITI) of 35 min and memory retention was tested after 35 min. In the second, animals were trained in three trials with an ITI of 35 min and a retention test was performed 24 h later (**Figure 1E**).

Statistics

The results were analyzed with Matlab R2019a (The MathWorks, Natick, Massachusetts, USA) and IBM SPSS Statistics Version 25.0 (IBM Corp., Armonk, New York, USA) and visualized with Matlab R2019a and Photoshop (Adobe Inc., San José, California, USA).

We analyzed spontaneous responses to different odors in two groups of animals. We pooled the behavioral response to odor presentations in the first training trial and before US presentation across all individuals that had been treated in parallel and under identical experimental conditions. Chi-squared tests were used to compare responses to different odors. Additionally, we calculated the Phi coefficient to analyze the correlation between odor responses across individuals.

For the statistical analysis of the classical conditioning experiments we applied three different statistical tests. First, one-way ANOVA was used to test the evolution of responses along training trails. Second, a two-way repeated measure ANOVA was used to compare the reinforcement type (CS+ and CS−) and the reinforcement type × trial interaction. Although ANOVA is usually not allowed in case of dichotomous data such as the MLR, Monte Carlo studies have shown that ANOVA can be used under certain conditions (Lunney, 1970), which all are met by the experiments reported here. Third, χ^2 tests were used for (1) comparison of responses to the CS+ and CS− in a given trial, (2) comparison of spontaneous responses and retention tests, (3) comparison of the last training trial and retention tests, and (4) comparison between different retention tests.

For further analysis of classical conditioning experiments, we pooled all animals with the same conditioning pattern regardless of the odor that was used as CS+ or CS−. To analyze the response to CS+, we excluded all animals showing spontaneous responses to the CS+ in the first trial. For analyzing responses to the CS−, we excluded all animals that did not respond to it in the first trial. This is a common procedure to exclude spontaneously responding animals and to visualize the learning curve (Pamir et al., 2011; Giurfa and Sandoz, 2012).

For all operant conditioning paradigms, decisions in the forced two-choice tasks were analyzed with a binomial test, since chance level of choosing one of two directions randomly was $p = 0.5$.

Analysis of Individuality

To analyze individuality of learning behavior, we followed the analyses in Pamir et al. (2011, 2014). For the analyses in the classical conditioning paradigm, we only considered animals that did not show a correct response to either the CS+ or the CS− in the very first trial and before the US was presented. Two subgroups were formed for training trials and test trial following the definition in Pamir et al. (2011). For any given trial, the first subgroup included animals that expressed the correct behavior in the present trial and in the previous trial (previous correct behavior, pC). The second subgroup included animals showing the correct behavior in the present trial but did not show it in the previous trial (previous incorrect behavior, pI). The same subgroup definitions were used for the retention test with regard to a correct or incorrect response during the final training trial. We compared the two subgroups in each trial and in the retention test with a χ^2 test at a significance level at $\alpha = 0.05$. The two subgroups are represented with upward and downward pointing triangles, respectively. Filled (open) symbols indicate that significance could (not) be established.

Following the analyses in Pamir et al. (2014), we formed separate subgroups of animals that showed a correct behavior for the first time in the second (or third) trial and tracked the subgroup behaviors across subsequent conditioning trials and the memory test. This allows to assess the robustness of the expression of a correct behavior across trials and the transfer of the behavioral expression during training to the short-term and long-term memory test situation.

Finally, in order to analyze the initiation of correct behavior, we computed for each trial the fraction of animals that responded correctly for the first time in this trial as well as the fraction of animals that never behaved correctly (non-learners, Pamir et al., 2014).

RESULTS

Spontaneous Response Toward Different Odors

We first analyzed the spontaneous and naive responses to each odor (isoamyl acetate, butyric acid, and cinnamaldehyde) during the very first conditioning trial before the reinforcing stimulus (US) was presented. **Figure 2** shows the group averaged responses to all three odors. In all experiments, approx. 60% of the animals showed a spontaneous MLR in presence of isoamyl acetate, which was significantly higher than for butyric acid and cinnamaldehyde for all experiments (χ^2: $p < 0.001$). The number of spontaneous responses to butyric acid and cinnamaldehyde was only significantly different in the first experiment (χ^2: $p = 0.01$) in which animals responded more often spontaneously to butyric acid. Isoamyl acetate is the main component of the banana blend and thus strongly associated with food and attractive for cockroaches (Lauprasert et al., 2006). This is most likely the reason for very high spontaneous response rates to isoamyl acetate. In addition, we found a significant positive correlation between responses to isoamyl acetate and butyric acid in both experiments [**Figure 2**: (1) $\Phi = 0.0258$, $p = 0.016$; (2) $\Phi = 0.143$, $p = 0.017$]. However, there was no significant correlation for other odor pairings, which might be due to the generally low spontaneous response rates to cinnamaldehyde and butyric acid.

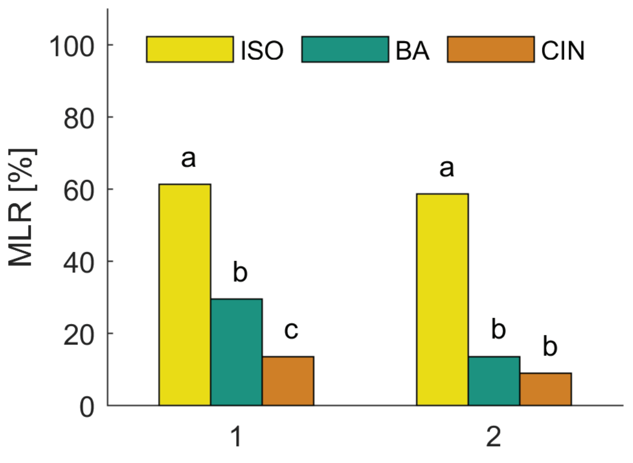

FIGURE 2 | Spontaneous responses to isoamyl acetate (ISO), butyric acid (BA), and cinnamaldehyde (CIN). Analysis of the MLR in the first trial of two classical conditioning experiments shows that ISO elicits significantly more spontaneous responses than BA and CIN (χ^2: $p < 0.001$). The spontaneous response to BA is only significantly higher compared to CIN in the first experiment (χ^2: $p = 0.01$).

A Novel Paradigm for Classical Olfactory Conditioning

We established a novel paradigm for classical conditioning in harnessed cockroaches (**Figure 1A**). The occurrence or absence of the maxilla-labia response (MLR, see section "Materials and Methods") was recorded as the conditioned response (CR) behavior. In this study, we used different protocols for differential olfactory conditioning (**Figures 1C,D**) to investigate the expression of the CR during learning and memory retention at two different time-points.

In a first protocol, we tested whether cockroaches are able to associate an odor with a reward or punishment during five consecutive training trials (inter-trial interval 10 min) followed by a retention test (after 10 min). We trained two groups of animals for which the odors isoamyl acetate and butyric acid were presented as CS+ and CS− with reversed contingencies (**Figures 3A,B**, respectively). The two odors did not elicit the same level of spontaneous responses (cf. section "Spontaneous Response Toward Different Odors"). Due to the high initial spontaneous response to isoamyl acetate, the average level of MLR was consistently high and did not significantly increase across the five trials when isoamyl acetate was used as CS+ (**Figure 3A**). However, responses to the punished odor (CS−, butyric acid) decreased significantly (one-way ANOVA: $F_{4,\ 260} = 4.23$; $p < 0.002$).

When butyric acid was used as CS+, responses showed a tendency to increase over the five training trials. In this case, responses to isoamyl acetate as CS− slightly decreased; however, this effect was not significant over the five trials. Animals still showed approximately 30% responses to the CS− in the fifth trial.

Responses to the control odor cinnamaldehyde only decreased significantly when isoamyl acetate was the CS+ (one-way ANOVA: $F_{4,\ 260} = 3.11$; $p = 0.016$), but did not change when butyric acid was used as CS+.

Overall, responses to the CS+ and CS− differed significantly when isoamyl acetate was rewarded (two-way repeated measures ANOVA: $F_{4,\ 49} = 3.095$; $p = 0.02$). When butyric acid was rewarded, CS+ and CS− did not differ significantly over trials (**Figure 3B**).

For further analyses, we pooled all animals according to CS+ and CS− and excluded those that did not behave correctly in the first trial, respectively. In both cases, correct behavior increased significantly across training trials [one-way ANOVA: **Figure 3C** (CS+): $F_{4,\ 230} = 8.808$; $p < 0.001$; **Figure 3D** (CS−): $F_{4,\ 190} = 15.544$; $p < 0.001$]. However, neither the behavior to CS− in **Figure 3C**, the CS+ in **Figure 3D**, nor the behavior to the control odor changed significantly over trials. Moreover, the interaction between trial and treatment was significant for CS+ (two-way repeated measures ANOVA: $F_{4,\ 43} = 12.156$, $p < 0.001$) and CS− (two-way repeated measures ANOVA: $F_{4,\ 35} = 17.591$, $p < 0.001$) and in both cases the behavior in retention tests were significantly different from each other (**Figures 3C,D**).

In addition, we excluded animals that did not behave correctly specifically either to the CS+, the CS- or the control. Accordingly, we could see that the effect of increasing correct behavior over trials was not only due to the exclusion of spontaneous responding or not responding animals (see **Supplementary Figure S1**).

Expression of Short-Term and Long-Term Memory After Classical Conditioning

To test memory retention after differential classical conditioning at a short- and long-term range we conducted a new experiment. A group of cockroaches were differentially trained during three consecutive trials. The group was then split in half and retention tests were performed either 1 h after the last training trial or 24 h after. In **Figure 4**, we show the training trials as unseparated groups, but the statistical analysis that include the training trials was conducted with splitted groups, which are shown in **Supplementary Figure S2**. The experiment was repeated with reversed contingencies of the odors. Responses to mechanical air stimulation (filter paper alone) did not vary and always stayed below 1.5%.

Overall, responses to the CS+ were significantly different from the CS− across three training trials in both groups (two-way repeated measures ANOVA: **Figure 4A**: $F_{2,\ 183} = 9.266$, $p < 0.001$; **Figure 4B**: $F_{2,\ 91} = 13.016$; $p < 0.001$). Responses to the CS− decreased significantly in both experiments (one-way ANOVA: **Figure 4A**: $F_{2,\ 552} = 7.181$; $p < 0.001$; **Figure 4B**: $F_{2,\ 276} = 3.291$; $p = 0.002$) while responses to the CS+ did not increase significantly. Responses to the control odor cinnamaldehyde decreased significantly only when butyric acid was used as CS+ (one-way ANOVA: $F_{2,\ 276} = 3.291$; $p = 0.039$).

During the 1 h test animals that received isoamyl acetate as CS+ maintained the elevated response level as group averaged performance, thus the retention test after 1 h was not significantly different to the response level in the last training trial (**Figure 4A**, χ^2: $p = 0.143$). Interestingly, the response level to isoamyl acetate was significantly higher in the 24 h retention test compared to the 1 h retention test (χ^2: $p < 0.001$), as well as in comparison

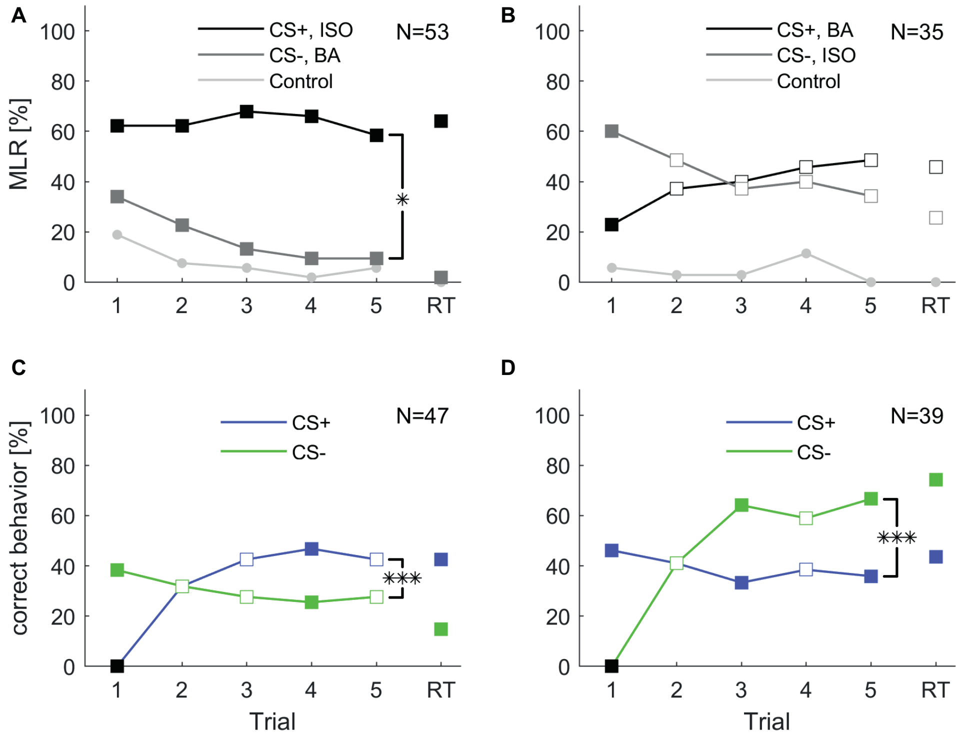

FIGURE 3 | Classical olfactory conditioning over five trials with a memory retention test after 10 min. Filled squares indicate trials in which a significant difference was found between CS+ and CS− (χ^2 test). Asterisks indicate that responses changed for the conditioned stimuli (CS+ and CS−) over trials (two-way ANOVA, repeated measures: treatment × trial interaction *: $p < 0.05$; ***: $p < 0.001$). **(A)** When isoamyl acetate (ISO) was the rewarded odor (CS+, black), the average group response did not increase during training. Responses to the punished odor BA (CS−, dark gray) decreased significantly over trials (one-way ANOVA: $p = 0.002$) and odor × trial interaction was significant (two-way repeated measures ANOVA: $p = 0.02$). **(B)** When BA was rewarded (CS+, black), responses increased considerably, however not significantly (one-way ANOVA: $p = 0.208$). Responses to ISO decreased, but again, not significantly (one-way ANOVA: $p = 0.189$). The odor × trial interaction was not significant (two-way repeated measures ANOVA: $p = 0.113$). **(C)** When excluding all spontaneous responding animals to the rewarded odor, response to CS+ increased significantly (one-way ANOVA: $p < 0.001$). Responses to the CS− decreased over trials, but not significantly (one-way ANOVA: $p = 0.686$). The treatment × trial interaction was significant (two-way repeated measures ANOVA: $p < 0.001$). **(D)** When excluding all not spontaneous responding animals to the punished odor the number of animals that behaved correctly to CS− over trials increased significantly (one-way ANOVA: $p < 0.001$), while response to CS+ did not change significantly over trials (one-way ANOVA: $p = 0.814$). The treatment × trial interaction was significant (two-way repeated measures ANOVA: $p < 0.001$). Black squares in **(C,D)** indicate the exclusion of animals that did not behave correctly in the first trial.

to the response level at the end of training (χ^2: $p < 0.001$) (**Supplementary Table S1**). When butyric acid was the rewarded odor (**Figure 6B**), response levels to the CS+ in both memory tests (after 1 and 24 h) were not different from each other (χ^2: $p = 0.475$), nor from the response level at the end of training (χ^2: 1 h: $p = 0.826$; 24 h: $p = 0.149$). The response level to the CS− resumed the initial high spontaneous response levels to isoamyl acetate during 1 and 24 h retention. All results are summarized in **Supplementary Table S2**.

For the next step of analysis, animals that did not behave correctly in the first trial were excluded and for both CS+ and CS− the percentage of correct behaving animals increased (**Figures 4C,D**, one-way ANOVA: CS+: $F_{2,537} = 33.537$; $p < 0.001$; CS−: $F_{2,306} = 43.027$; $p < 0.001$). The only other significant effect was the decrease of correct behavior to the CS− when all correct responding animals were excluded (one-way ANOVA: $F_{2,537} = 3.569$; $p = 0.029$). Moreover, the interaction between trial and treatment was significant in both cases (two-way repeated measures ANOVA: **Figure 4C**: $F_{2,178} = 46.719$; $p < 0.001$; **Figure 4D**: $F_{2,101} = 39.158$; $p < 0.001$).

When excluding all spontaneously responding animals, performance in the retention test stayed at the same level as at the end of training. However, the increase of the CS+ retention test after 24 h was significant (χ^2: $p < 0.001$). After exclusion of the nonspontaneous responders, performance in all retention tests stayed similar compared to the third trial of training. However, the percentage of correct behaving animals to the CS− decreased after 1 h (χ^2: $p < 0.001$) and 24 h (χ^2: $p < 0.001$).

Individual Learning Performance During Classical Conditioning

To test whether differences in learning performance exist among individual animals, we followed the analyses suggested in Pamir et al. (2011, 2014). Each of the two groups trained in the five trial classical conditioning experiment (**Figures 3C,D**) were divided into two subgroups (cf. section "Materials and Methods"): (1) individuals that behaved correctly in two consecutive trials (previous correct behavior, pC) and (2) individuals that did not behave correctly in the previous trial but started behaving

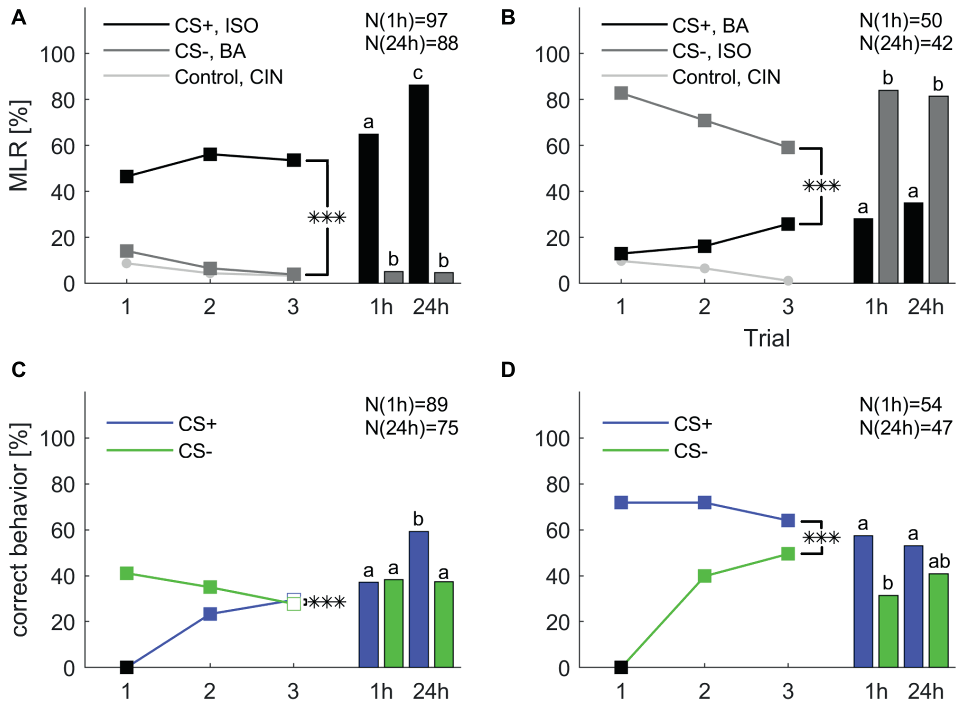

FIGURE 4 | Classical olfactory conditioning over three trials with memory retention tests after 1 and 24 h. Filled squares indicate trials in which a significant difference was found between CS+ and CS− (χ^2 test). Asterisks indicate a significant treatment × trial interaction (***: $p < 0.001$). Letters above the bars indicate significant differences between retention tests (χ^2). **(A)** When isoamyl acetate (ISO) was rewarded (CS+, black) and butyric acid (BA) punished (CS−, dark gray), responses significantly decreased for the CS− (one-way ANOVA: $p < 0.001$), but did not change for the CS+ (one-way ANOVA: $p = 0.155$). Overall, the odor × trial interaction was significant (two-way repeated measures ANOVA: $p < 0.001$). **(B)** When butyric acid was rewarded (CS+, black) and isoamyl acetate was punished (CS−, dark gray), responses to the CS+ increased, however, not significantly (one-way ANOVA: $p = 0.06$), but decreased significantly for the CS− (one-way ANOVA: $p = 0.002$). The odor × trial interaction was again significant (two-way repeated measures ANOVA: $p < 0.001$). **(C)** When excluding all spontaneous responding animals to the rewarded odor the response to CS+ increased significantly (one-way ANOVA: $p < 0.001$). The response to CS− decreased significantly over trials (one-way ANOVA: $p = 0.029$). The treatment × trial interaction was significant (two-way repeated measures ANOVA: $p < 0.001$). **(D)** When excluding all not spontaneous responding animals to the punished odor the number of animals that behaved correctly to CS− over trials increased significantly (one-way ANOVA: $p < 0.001$). The response to CS+ did not change significantly over trials (one-way ANOVA: $p = 0.38$). The treatment × trial interaction was significant (two-way repeated measures ANOVA: $p < 0.001$). Black square in **(C,D)** indicate the exclusion of animals that did not behave correctly in the first trial.

correctly in the present trial (previous incorrect behavior, pI). Previous correct behaving animals always showed a higher level of correct behavior than the average correct behavior across all animals (**Figure 5**, red upward triangles), while the previous incorrect behaving individuals always showed lower correct behavior (**Figure 5**, red downward triangles).

Next, we analyzed the across-trial behavior of those animals that showed their first correct behavior already in the second trial (i.e., after only a single pairing of CS and US) and find that this subgroup showed consistently high rates of correct responses across all trials and during retention with retention scores above 90%, both for CS+ and CS− (dark gray curves in **Figures 5A,C**). Individuals that started to respond correctly in the third trial (after two pairings of CS and US, light gray curves in **Figures 5A,C**) showed lower correct response levels than those animals that had started in the first trial but, still, these were comparably high considering the fact that the average response levels (blue and green curve in **Figures 5A,C**, respectively) included also the high performance group (dark gray curves). This indicates that fast learners are also good learners and parallels previous findings in the honeybee (Pamir et al., 2014).

In the histograms of **Figures 5B,D**, we counted for each trial separately how many animals responded correctly for the first time in that trial. From all animals that showed learning, most of them showed the correct response after a single conditioning trial. The second largest group behaved correctly for the first time after two conditioning trials. However, a substantial portion of animals never behaved correctly (black bars in **Figures 5B,D**) and this group is larger for a correct behavior toward the CS+.

Individuality in Operant Learning

We then tested learning, memory retention, and individual differences in an operant conditioning task. Cockroaches were trained to avoid a punishment and were tested for their memory for up to 24 h. For this, we designed a forced two-choice paradigm where an individual cockroach is placed in a T-maze during repeated training trials (**Figure 1B**, cf. Materials and Methods). In each trial, the cockroach was allowed to choose one of the arms and entered a target box. In the first trial and irrespective of the animal's choice, it experienced an aversive bright light stimulus. Whenever the animal chose the same side in subsequent trials, the same aversive stimulus was elicited.

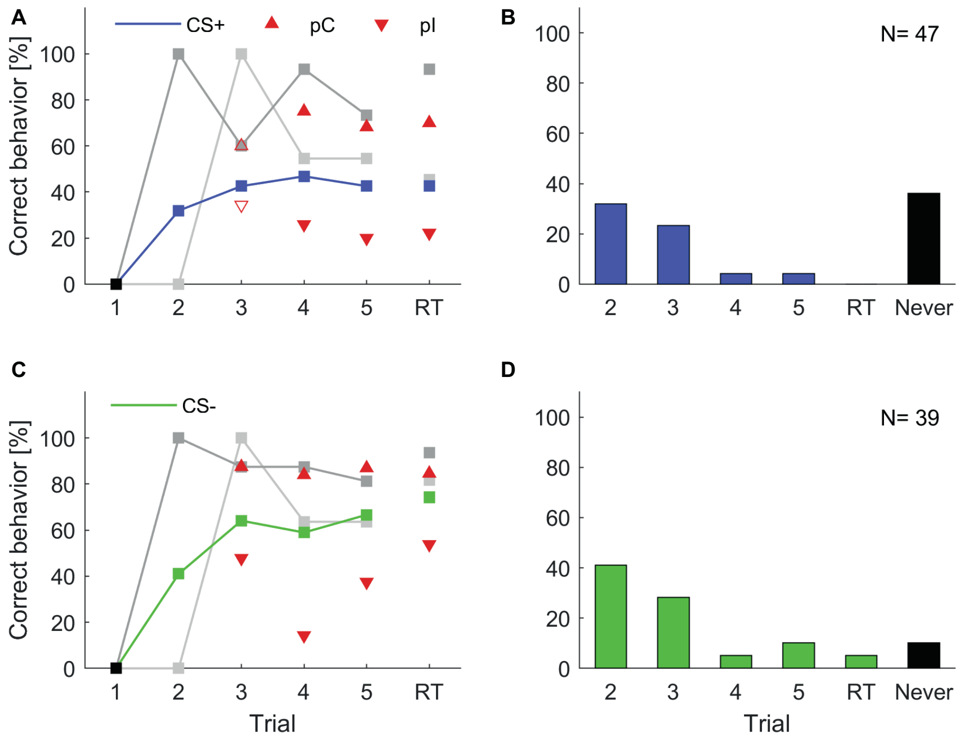

FIGURE 5 | Individual learning abilities in classical olfactory conditioning. Data for isoamyl acetate as CS+ and butyric acid as CS+ were pooled, likewise data for isoamyl acetate as CS– and butyric acid as CS– were pooled. Correct learning is represented by **(A)** increasing responses to the rewarded odor in the case of CS+ and **(C)** increasing response-suppression to the punished odor in the case of CS–. **(A,C)** Upward pointing triangles depict animals, which correctly behaved in the respective trial and already showed a correct behavior in the previous trial (pC). Triangles pointing downward depict animals that correctly behaved in the respective trial but did not do so in the previous trial (pI). Filled triangles indicate a significant difference of subgroups in the respective trials, empty triangles represent subgroups that were not significantly different (χ^2; $\alpha = 0.05$). Animals behaving correctly in trial two, i.e., after a single training trial (dark gray; CS+: $N = 15$; CS–: $N = 16$) showed consistently high probabilities of correct responses in subsequent trials (cf. Pamir et al., 2014). Similarly, high response scores were observed for animals responding correctly for the first time in trial three (light gray; CS+: $N = 11$; CS–: $N = 11$). Black squares indicate the exclusion of animals that did not behave correctly in the first trial. **(B,D)** Proportion of animals behaving correctly for the first time in the indicated trial. A high percentage of animals expressed a correct behavior already after a single training trial. A substantial proportion of animals never behaved correctly (black bars).

Learning was thus expressed in avoiding the side (left or right) that resulted in the punishment with the bright light stimulus.

In a first experiment, animals were trained for five consecutive trials and short-term memory retention was tested 35 min after the last trial. In a second experiment, animals were trained for three trials and a long-term retention test was performed 24 h later (**Figure 1E**). Animals in the first group significantly learned to avoid the punished side from the third trial onward (binomial tests: $p < 0.01$). Animals showed correct memory for the punished side in the retention test 35 min after (**Figure 6A**). Cockroaches in the second group did not significantly show learning after two training trials. However, memory for the correct side was expressed in the 24 h retention test (binomial test: $p = 0.014$, **Figure 6B**).

To study individual differences in these operant learning and memory tasks, animals were again attributed to two subgroups. In the short-term memory experiment, animals in the subgroup showing the correct behavior in two consecutive trials (pC) always performed better than the group average while animals in the subgroup pI consistently showed fewer correct choices in the present trial. Behavioral choices of previous correct deciding animals (pC) and previous incorrect deciding animals (pI) significantly differed in the third and fourth trials during training (χ^2: $p < 0.05$). This difference was not significant in the fifth learning trial, nor in the retention test after 35 min (χ^2: trial 3: $p = 0.161$; trial 4: $p = 0.186$, **Figure 6A**).

In the long-term memory experiment, the two subgroups (pC and pI) again differed significantly after two training trials. During the 24 h retention test, the subgroup of animals that had shown a correct decision in the last training trial significantly outperformed those animals that had made an incorrect decision in the last training trial (**Figure 6B**: χ^2: $p < 0.05$).

DISCUSSION

In the present work, we show that the adult American cockroach, *Periplaneta americana*, can solve classical olfactory and operant spatial conditioning tasks. In both cases, animals could learn to establish a conditioned response to the rewarded stimulus (CS+), and to diminish their responses to the punished odor (CS–) despite the fact that, in the classical conditioning task, the two odors were not equally important to the animals (high spontaneous

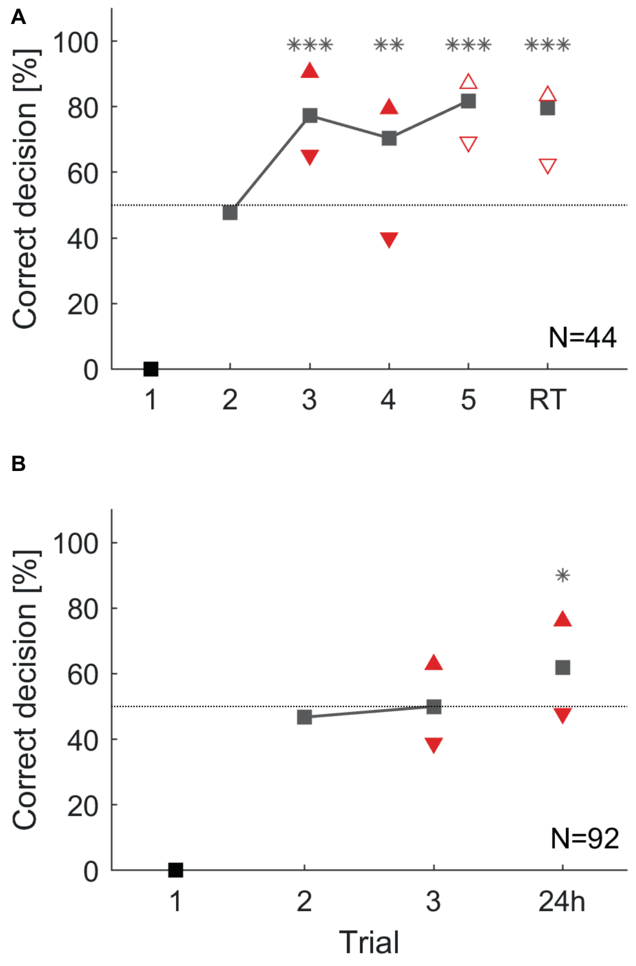

FIGURE 6 | Overall group performance and individual choices during an operant conditioning task in a T-maze **(A)** over five consecutive trials and a 35 min retention test (RT). From the third trial on cockroaches chose significantly more often the correct direction (**: $p < 0.01$; ***: $p < 0.001$). **(B)** Operant conditioning over three trials and a retention test after 24 h. However, this time cockroaches did not learn during training but chose significantly more the correct direction in the 24 h retention test (*: $p < 0.05$). Triangles that point upward depict animals that chose the correct direction in two consecutive trials (pC). Triangles that show downward depict animals that chose the correct direction in the present trial but did not choose it in the previous trial (pI). Filled triangles differ significantly from each other within a trial and empty ones do not (χ^2; $\alpha = 0.05$).

responses to isoamyl acetate). Overall, training resulted in the successful expression of short-term memory and long-term memory (after 24 h) in both conditioning tasks. We further show that cockroaches express individuality in their learning and memory performance in classical and operant conditioning.

Classical Olfactory Conditioning in the Cockroach

For the present study, we established a novel classical conditioning paradigm in harnessed cockroaches that allows to observe the expression (or non-expression) of a discrete conditioned response behavior, the **m**axilla-**l**abia **r**esponse (MLR) during learning and memory retention. The development of this paradigm was inspired by the highly successful proboscis extension reflex (PER) paradigm in the honeybee (e.g., Kuwabara, 1957; Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012; Menzel, 2012) in which the extension (or non-extension) of the proboscis is observed as a discrete conditioned response behavior.

A number of previous studies have investigated classical conditioning in the cockroach using training and test conditions that differ fundamentally from our MLR paradigm. In studies by Watanabe et al. (2003), Sato et al. (2006), and Liu and Sakuma (2013), in the German cockroach, unrestrained cockroaches were placed in a cylindrical chamber during repeated conditioning trials with one odor paired with sucrose reward (CS+) and a second odor paired with salt punishment (CS−). Sato et al. (2006) could prove that beyond simple olfactory discrimination learning, cockroaches exhibited excellent learning performance in an occasion setting paradigm in which a visual context defines the contingency between olfactory CSs (conditioning stimuli) and gustatory USs (unconditioned stimuli). Watanabe et al. (2003) extended their classical conditioning protocol in unrestrained cockroaches to harnessed cockroaches that were subsequently tested under freely moving conditions in a test arena where they could choose between the two previously conditioned odors. This paradigm, however, did not establish a clear conditioned response observable during training and thus expression of a conditioned response behavior is only accessible during memory retention and under conditions different from training. Classical conditioning leads to an increase in response of salivary neurons to an odor associated with sucrose reward in the cockroach (Watanabe and Mizunami, 2006). After differential conditioning, one odor paired with sucrose and another odor without reward, the sucrose-associated odor induced an increase in the level of salivation, but the odor presented alone did not, proving classical conditioning of salivation in cockroaches (Watanabe and Mizunami, 2007). Classical conditioning of salivation has first been shown a century ago by Pavlov in his famous dog experiments (Pavlov, 1927). Restrained cockroaches were further used to study spatial (e.g., Kwon et al., 2004) or visual-olfactory associative learning and memory (e.g., Lent and Kwon, 2004; Pintér et al., 2005; Lent et al., 2007) by quantifying the antennal projection response (APR) of animals that were tethered in the middle of an arena (Pomaville and Lent, 2018). The APR is based on the observation that antennal motor actions can be elicited by different modalities, including olfactory, tactile, and visual stimuli (e.g., Menzel et al., 1994; Erber et al., 1997). Conditioning the APR consists in quantifying directed antennal movements toward the direction of a rewarded visual stimulus and was inspired by operant conditioning of bees to extend their antennae toward a target in order to receive a reward (e.g., Menzel et al., 1994; Kisch and Erber, 1999). The advantage of training immobilized insects provides a powerful technique for studying the neuronal basis (by, e.g., employing neurophysiological and pharmacological techniques) of learning and memory in a simpler nervous system compared to vertebrates.

Initial Response Behavior During Classical Conditioning

Stimuli used for studying olfactory learning and memory in insects mostly employ odors that are relevant in the natural context, such as communication signals (i.e., pheromones) or food-related odors. Isoamyl acetate constitutes the most salient compound of the banana blend and is perceived as the smell of banana (Schubert et al., 2014). This odor is clearly food related and thus highly attractive for cockroaches (Lauprasert et al., 2006). This likely explains why, in our olfactory conditioning experiments, we observed a high level of initial responses to isoamyl acetate in the first trial (**Figure 2**). Consequently, it was difficult to observe learning (i.e., increasing conditioned response levels) when this odor was paired with a sucrose reward (CS+) since response levels were consistently high (> ~60%) from the first trial on and throughout training (**Figure 3A**). In the 24 h retention test, however, the MLR to isoamyl acetate was significantly increased compared to the response in the last training trial (**Figure 4A**), indicating that a long-term memory had been established. When isoamyl acetate was paired with salt punishment (CS−), animals learned to suppress their responses during training (**Figure 4B**). Initial responses to butyric acid were significantly lower (~ 30%) at the beginning of training in all cases (**Figure 2**). When associated to sugar, responses increased but never exceeded 50% even after five training trials. Spontaneous responses to butyric acid were completely abolished during training and memory retention when paired with punishment (**Figure 3B**). Concluding, the two odors employed in our study were not equally attractive to the animals.

Operant Spatial Conditioning in Cockroaches

A frequently used setup for operant conditioning is a Y- or T-maze, which is extensively used to study operant learning and decision-making in rodents. T- or Y-maze (dual choice) experiments in invertebrates have been used broadly to study visual or olfactory absolute and differential learning in free-flying bees (e.g., for review: Srinivasan et al., 1998; Giurfa et al., 1999, 2001; Avarguès-Weber et al., 2011; Nouvian and Galizia, 2019), in ants (e.g., Dupuy et al., 2006; Camlitepe and Aksoy, 2010), and in wasps (Hoedjes et al., 2012). In cockroaches, operant learning has repeatedly been studied in open arenas (e.g., Balderrama, 1980; Sakura and Mizunami, 2001; Sakura et al., 2002). The first work on operant conditioning in cockroaches was carried out by Balderrama (1980) who trained free-moving cockroaches individually in a simple training chamber to associate two artificial odors to sucrose and salt solutions, respectively, and testing discriminatory learning performance by measuring the odor preference before and after training. Spontaneous preference for one of the odors before training could be modified already with one trial and retention lasted up to 7 days. To date, there are only two studies that have challenged cockroaches in T-maze tasks, the first testing the influence of feces pheromones on directional orientation (Bell et al., 1973), while the second investigated effects of protein synthesis inhibiting drugs on learning and retention by training animals to avoid shock on one of the sides (Barraco et al., 1981). Our reason to perform an operant learning paradigm in the T-maze was to establish a forced binary choice that can be analyzed during acquisition and memory retention in a defined trial design. Electric shock as used for a punishing stimulus in the previous study by Barraco et al. (1981) seems a rather unnatural aversive stimulus that is unlikely to appear in nature. We decided to use bright light as negative reinforcer since cockroaches naturally avoid bright light and seek shelter in darkness (Turner, 1912).

Cockroaches started to avoid the side that was punished after a few trials. However, training results were variable across the two experiments. Previous studies concluded that cockroaches show unpredicted searching behavior (Balderrama, 1980). Similarly, we could observe different traits in behavior, which might partly underlie the variance in choice behavior. For example, some cockroaches show a high explorative behavior, possibly in search for an exit from the maze, and these did not seem to care much about the reinforcing stimulus while others stayed almost immobile throughout a trial and moved little. The punishing effect of light is limited because it has no harming consequence for the animal. They may thus habituate to the aversive light stimulus. The T-maze experiments in Barraco et al. (1981) using electric shock as negative reinforcer resulted in surprisingly high correct choice rates. However, a strong light seems to be repellent for most cockroaches since they normally try to hide in a dark place when, e.g., the light in a room is switched on. In future experiments, we want to explore whether a paradigm for appetitive operant conditioning can lead to higher levels of correct choice performance in cockroaches.

Individual Behavioral Expression of Learning and Memory

Our approach to study individuality in learning performance during classical conditioning was inspired by two previous studies by Pamir et al. (2011, 2014) that investigated a large number of datasets on classical appetitive conditioning in the honeybee. In these studies the authors were able to extract from an immense amount of data that honeybees express individual learning behavior and that a group of animals can be separated into at least two subgroups, learners and non-learners. Both studies by Pamir and colleagues have investigated behavioral learning and memory expression only toward the CS+. We have extended their analysis including behavioral learning and memory expression toward the CS− (**Figures 5C,D**).

After exclusion of individuals that did not respond correctly in the first trial, as for the honeybee (Pamir et al., 2014), a large fraction of animals (>35%) never showed the correct behavior to the CS+ odor in any of the learning trials or the retention test (**Figures 5B,D**). These animals may be considered non-learners. When taking into account only those animals that expressed the correct conditioned behavior at least once during the training session, we find that those animals expressed this behavior for the first time after average 1.7 conditioning trials toward the CS+ and after average 1.8 conditioning trials toward the CS−. Indeed, the largest fraction (50%) of responding animals showed a correct conditioned response behavior for the first time already after a single conditioning trial (single-trial learning), both toward the CS+ and the CS−. In effect, 86.6% of learners showed a first correct behavior to the CS+ or CS− already after the first or second conditioning trial, indicating rapid learning after a single or two trials. These numbers match closely those obtained in the honeybee where typically ~50% of individuals in a group of honeybees showed a conditioned response after a single training trial (Pamir et al., 2014). Moreover, the correct expression of learned behavior in fast learners is remarkably stable as can be seen when following the across-trial CR behavior of the subgroup of cockroaches that showed a correct behavior after a single conditioning trial (dark gray curve in **Figures 5A,C**). When looking at short-term memory retention in those animals (**Figures 5A,C**), 93.3 and 93.8% expressed the correct behavior during the test for CS+ and CS−, respectively. Conversely, of the fraction of animals that showed the correct behavior during short-term memory retention for CS+ and CS−, 95 and 82.8%, respectively, were fast learning individuals expressing the correct behavior after a single or two training trials. Similarly, Pamir et al. (2014) reported that honeybees that responded earlier showed a higher long-term memory retention than those responding later. Taken together, our results indicate that (1) individual cockroaches are able to learn efficiently during only one or two conditioning trials, and (2) fast learners are also good learners that robustly express the correct behavior throughout the training session and achieve very high retention scores.

Thus, in line with the results on honeybees reported by Pamir et al. (2011, 2014), we conclude from our results that the gradually increasing group-average learning curve does not adequately represent the behavior of individual animals. Rather, it confounds three attributes of individual learning: the ability or inability to learn a given task (learners vs. non-learners), the fast acquisition of a correct conditioned response behavior in learners, and a high robustness of the conditioned response expression during consecutive training and memory retention trials. Moreover, we could establish the same general result in an operant learning task in the cockroach. The latter result is in line with a study in bumblebees (Muller and Chittka, 2012) observing that some individuals were consistently better than others in associating different cues with reward or punishment in an operant learning task.

Interestingly, these congruent results in the honeybee and cockroach, two evolutionary far separated species, are in contrast to the long-standing notion on learning abilities in fruit flies. An early report on olfactory learning in *Drosophila melanogaster* by Quinn et al. (1974) using a meanwhile well-established and heavily used group assay for classical olfactory conditioning of flies concluded that the expression of behavior in the individual was probabilistic such that a group of flies can be treated as homogeneous with respect to the ability to acquire a correct CR behavior. This notion has been challenged by a more recent study (Chabaud et al., 2010), but awaits further conclusive investigation. We hypothesize that fruit flies exhibit individual learning performance that is very similar to those observed in the honeybee and established for the cockroach in this study.

Possible Causes for the Individual Expression of Learned Behavior

What could be the underlying causes for the observed individuality in behavioral learning performance? At the neuronal circuit level, learning-induced plasticity has been observed at different sites within the system. Two studies in honeybees found correlations between the behavioral performance in individuals and the expression of plasticity in the nervous system. Rath et al. (2011) performed calcium-imaging in the projection neurons of the antennal lobe. For their analysis, they formed two subgroups of learners and non-learners based on their conditioned response behavior and reported that, as a result of classical olfactory conditioning, odor response patterns in the projection neuron population became more distinct in learners but not in non-learners. Haenicke et al. (2018) performed Ca-imaging from the projection neuron boutons in the mushroom body calyx of the honeybee and found that the level of neuronal plasticity correlates significantly with the level of behavioral plasticity across individual animals in classical olfactory conditioning. Mushroom body output neurons have been shown to convey the valence of odors following classical conditioning in bees (Strube-Bloss et al., 2011, 2016) and flies (e.g., Aso et al., 2014; Hige et al., 2015). In bees, the level of observed plasticity in these neurons after classical conditioning again correlates with the behavioral performance during the retention test (Strube-Bloss, d'Albis, Menzel & Nawrot, unpublished data). Thus, individuality in the conditioned response performance during memory retention has been linked to the underlying plasticity in the neural circuitry.

In bees, a significant correlation between their sensitivity to sucrose concentration and learning performance during an olfactory task has been reported (Scheiner et al., 2004). Pamir et al. (2014) re-analyzed data from Scheiner et al. (2001) showing that sucrose responsiveness, interpreted as a proxy to the state of satiety, correlates with learning performance, both in olfactory and tactile classical conditioning.

In addition, a number of studies have linked variations in learning abilities with genetic variation across individuals. In the honeybee, for example, animals that performed well in olfactory/mechanosensory conditioning also performed well in visual learning (Brandes and Menzel, 1990). On the other hand, good and poor learners from strains selected for olfactory conditioning differed significantly in their visual learning values. Thus, genetic differences exist between different strains and such genetic variation can account for differences in learning in individuals (e.g., Brandes et al., 1988; Brandes and Menzel, 1990). Another study on honeybees considering individual differences in a latent inhibition learning task (learning that some stimuli are *not* signals of important events) also proved a genetic predisposition for learning this task (Chandra et al., 2000). Furthermore, a very recent study on honeybees showed that genetic determinism underlies the trade-off between appetitive and aversive learning (Junca et al., 2019). In a different study, fruit flies were trained to associate a chemical cue (quinine) with a particular substrate. It showed that individuals still avoided this substrate several hours after the cue had been removed, were expected to contribute more alleles to the next generation. From about generation 15 onward the experimental populations showed marked ability to avoid oviposition substrates that several hours earlier had contained the chemical cue (for review, see: Mery and Kawecki, 2002; Dukas, 2008). Indeed, genetic variation might underlie individuality in behavior in general and in learning behavior specifically. However, to our knowledge, genetic variation has not been studied in cockroaches in relation to behavioral traits. Unfortunately, maturation and reproduction cycles in cockroaches are rather long.

Outlook

In the present study, we investigated individual learning performance and learning speed in single learning tasks (classical olfactory conditioning or operant place learning). In future studies, we will extend our analyses of individuality in two directions. First, we will investigate whether the behavioral performance of individuals is consistent across different learning paradigms, i.e., whether good and fast learners in one classical conditioning paradigm will also perform above average in different classical or operant conditioning tasks. To our knowledge, there is only one invertebrate study where something comparable was published with honeybees (Tait et al., 2019). Second, we are interested in consistency across days or weeks investigating whether a high/low performance of one individual is equally high/low during a repetition of the same or similar task at a later point in time.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

AUTHOR CONTRIBUTIONS

CA, JB, and ND conducted the experiments. CA, ND, and MN designed the experiments and the experimental setups and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01539/full#supplementary-material

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Chapter II: The mushroom body output encodes behavioral decision during sensory-motor transformation

The mushroom body output encodes behavioral decision during sensory-motor transformation

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Abstract

Animal behavioral decisions are dynamically formed by evaluating momentary sensory evidence on the background of individual experience and the acute motivational state. In insects, the mushroom body (MB) has been implicated in forming associative memories and in assessing the appetitive or aversive valence of sensory stimuli to bias approach versus avoidance behavior. To study the MB involvement in innate feeding behavior we performed extracellular single-unit recordings from MB output neurons (MBONs) while simultaneously monitoring a defined feeding behavior in response to timed odor stimulation in naïve cockroaches. All animals expressed the feeding behavior almost exclusively in response to food odors. Likewise, MBON responses were invariably and strongly tuned to the same odors. Importantly, MBON responses were restricted to behaviorally responded trials, which allowed the accurate prediction of the occurrence versus non-occurrence of the feeding behavior in individual trials from the neuronal population activity. During responded trials the neuronal activity generally preceded the onset of the feeding behavior, indicating a causal relation. Our results contest the predominant view that MBONs encode stimulus valence. Rather, we conclude that the MB output dynamically encodes the behavioral decision to inform downstream motor networks.

Introduction

Animal behavioral decisions are based on the processing of momentary environmental conditions on the background of innate and experience dependent behavioral biases. In insects, and specifically in nocturnal species, olfactory cues play a major role in a variety of behavioral decisions involved e.g. in mating, oviposition, or navigation. During foraging, locating food sources and evaluating their quality is fundamental for survival and requires the accurate recognition of appropriate food odors to inform behavioral decisions.

Feeding behavior in insects is experimentally accessible through the registration of well-defined behaviors, e.g. in the proboscis extension response (PER) in bees (Bitterman et al., 1983) and flies (Yetman and Pollack, 1987; Shiraiwa and Carlson, 2007) and the maxilla-labia response (MLR) in cockroaches (Arican et al., 2020). The individual decision of an animal about executing a feeding behavior can be modulated by its internal and behavioral states (see Discussion). These influences may enter at different stages of sensory-motor processing including the mushroom body (MB) (Devineni and Scaplen, 2022).

While olfactory processing and learning in insects is being studied in great detail, we still lack understanding of how and at which stage of the recurrent sensory-motor pathway behavioral decisions are formed. Here we take advantage of the experimental accessibility in the cockroach that allows us to simultaneously monitor feeding behavior and record from central brain neurons in the individual animal with high temporal resolution. The MB is an evolutionary old and homologous central brain structure in insects (Strausfeld et al., 2009). The MB output integrates sensory input of different modalities (Li and Strausfeld, 1999; Yagi et al., 2016; Strube-Bloss and Rössler, 2018) through sensory projections with the internal state, the behavioral state and external sensory context (Cohn et al., 2015; Tsao et al., 2018; Siju et al., 2020; Aimon et al., 2022) through a large number of recurrent, mostly neuromodulatory input (see Discussion). While the MB function has predominantly been assigned to the formation and recall of short and long term associative memories (Heisenberg, 2003; Menzel, 2012; Hige et al., 2015; Owald et al., 2015), recent studies in untrained animals have demonstrated an important role of the MB in processing attractive and repulsive sensory stimuli in the context of innate behaviors (Bräcker et al., 2013; Lewis et al., 2015; Tsao et al., 2018; Siju et al., 2020) as well as its involvement in state-dependent sensory-motor transformation (Okada et al., 1999; Aimon et al., 2022).

Previous studies, mostly conducted in honey bees (Strube-Bloss et al., 2011, 2016) and fruit flies (Aso et al., 2014b; Owald and Waddell, 2015; Hancock et al., 2022), have provided accumulated evidence that distinct populations of MB output neurons (MBONs) establish a code for the valence of a sensory stimulus with respect to its behavioral relevance and specifically so as a consequence of associative learning (see Discussion). Here we demonstrate that a subpopulation of MBONs in the cockroach faithfully predicts the occurrence or non-occurrence of a defined feeding behavior in the cockroach on a single trial basis. Our results therefore contest the prevailing view that the output of the MB merely encodes the valence of sensory stimuli and we conclude instead that, at its output, the MB represents an integrated signal of internal state, momentary environmental conditions and experience-dependent memory to encode a behavioral decision.

Results

Feeding behavior is expressed in response to food odors

We start out with analyzing the odor specific feeding behavior in individual animals. We presented a set of 10 different odors and one additional control stimulus (clean air, Fig. 1) to each animal. Each stimulus is repeated 10 times (trials). The continuous recording of the animals' mouthparts allowed for the detection of a behavioral feeding response on a trial-by-trial basis. For each of the 10 trials per odor we thus obtained a binary data (no MLR vs. MLR) on the animal's feeding response.

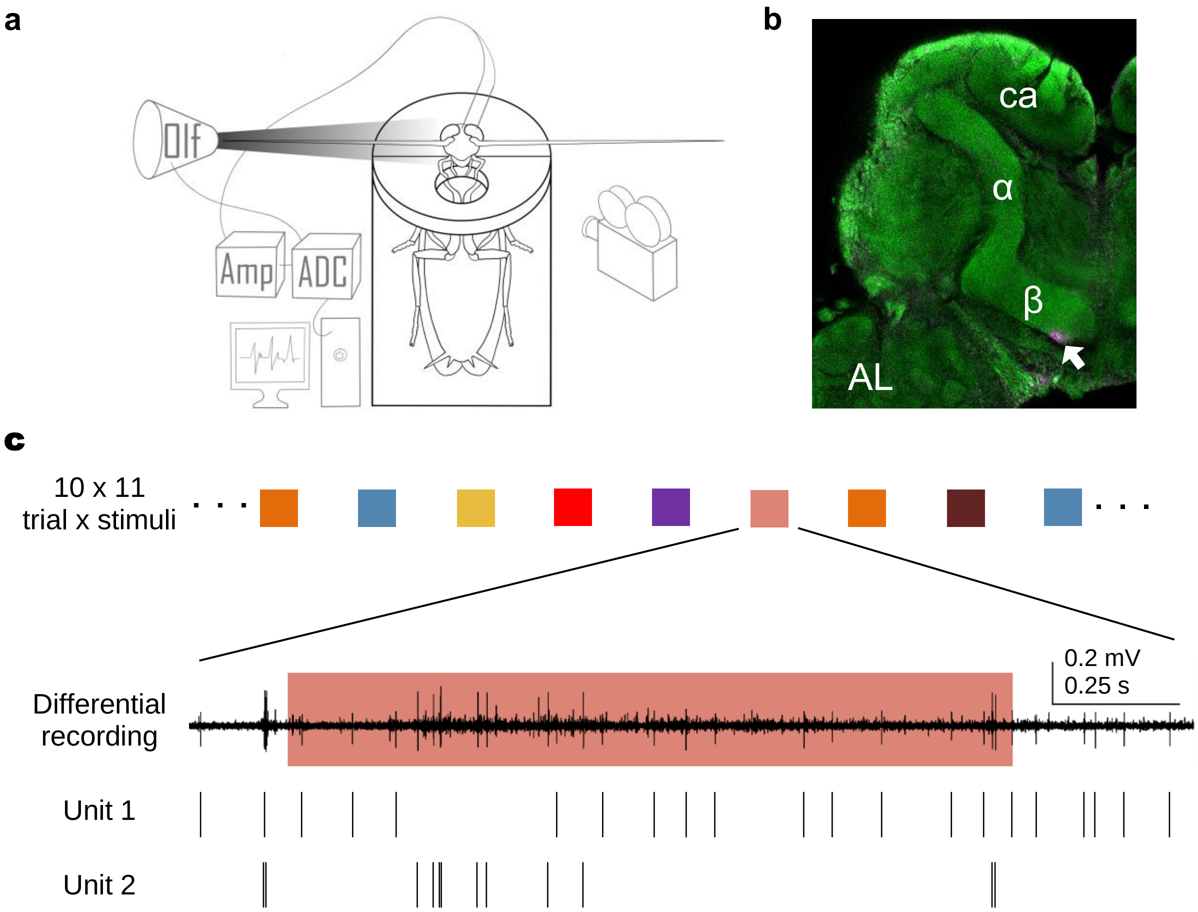

Figure 1: Extracellular single-unit recordings from MB output with simultaneous recording of feeding behavior. a Schematic illustration of a harnessed cockroach with inserted electrodes connected to an amplifier (Amp) and analog to digital converter (ADC). A computer-controlled odor supply system (Olf) provides the odor stimulus to the antenna from its tip. A camera records the mouthparts of the cockroach allowing for the detection of the maxilla-labia response (MLR). **b** Background staining with Lucifer Yellow of the right hemisphere of a cockroach brain. The MB α and β lobes are clearly visible. The position of the electrode tip, shown in magenta, (white arrow) is located at the border of the MB β lobe where the primary neurites of the MBONs leave the MB. AL: antennal lobe, ca: mushroom body calyx. **c** Stimulation pattern with odor and control stimulations in pseudo-randomized order. Extracellular differential recording during a single stimulation of 2 s duration (light red shading) with the odor cinnamaldehyde and the corresponding spiking activity of two single units.

We observed a clear overall behavioral response pattern across the set of tested odors in the group of animals (Fig. 2a). Only a small subset of four odors triggered repeated feeding behavior. In contrast, and consistently across all animals, almost no responses were evoked by the presentation of the remaining six odors. The few sparsely distributed responses to this subset of odors matches the low spontaneous response probability to the control stimulus (overall 2.2%). Importantly, the three responded odors isoamyl acetate (Iso), cinnamaldehyde (Cin) and benzaldehyde (Ben) are known food odors in the cockroach (Arican et al., 2020; Khoobdel et al., 2021).

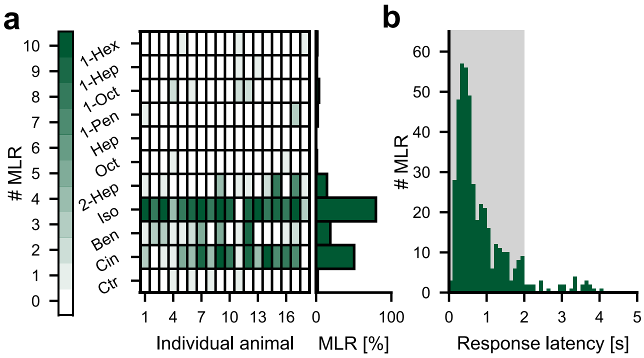

Figure 2: Behavioral feeding response exclusively occurs to food odors. a Number of observed feeding responses (MLR) over 10 trials per stimulus for the control and all odors in individual animals (matrix) and average across all animals (horizontal bar histogram) during the 2 s odor presentations. Stimuli: 1-hexanol (1-Hex), 1-heptanol (1-Hep), 1-octanol (1-Oct), 1-pentanol (1-Pen), heptanal (Hep), octanal (Oct), 2-heptanone (2-Hep), isoamyl acetate (Iso), benzaldehyde (Ben), trans-cinnamaldehyde (Cin), control (Ctr). **b** Distribution of single trial behavioral response latency during and after odor presentation (binwidth = 100 ms), gray shaded area depicts stimulus presentation. The mean (median) behavioral response time was 776 ms (632 ms).

Neuronal population response is dominated by food odors

To gain insight into the role of the MB output in sensory-motor transformation we recorded extracellular single unit activity from the output region of the MB β lobe throughout the experiment (Fig. 1). In a first analysis we quantified the single neuron and population activity in response to odor stimulation. Across the MBON population we observed clear and consistent responses only to the three food odors that also evoked consistent behavioral responses (Fig. 3d). In Fig. 3a we show, as an example, the single-trial spike trains of a single MBON and the corresponding trial-averaged firing rates in Fig. 3b. This neuron shows a strong response to Iso, a weak response to Cin and no response to Ben. Analysis of the trial-averaged neuronal population rate in Fig. 3c shows different population response profiles for the three odors. The fraction of responding neurons was largest for Iso and smallest for Ben, reflecting the behavioral odor response pattern in Fig. 2a. The control stimulus did not evoke a response in any neuron.

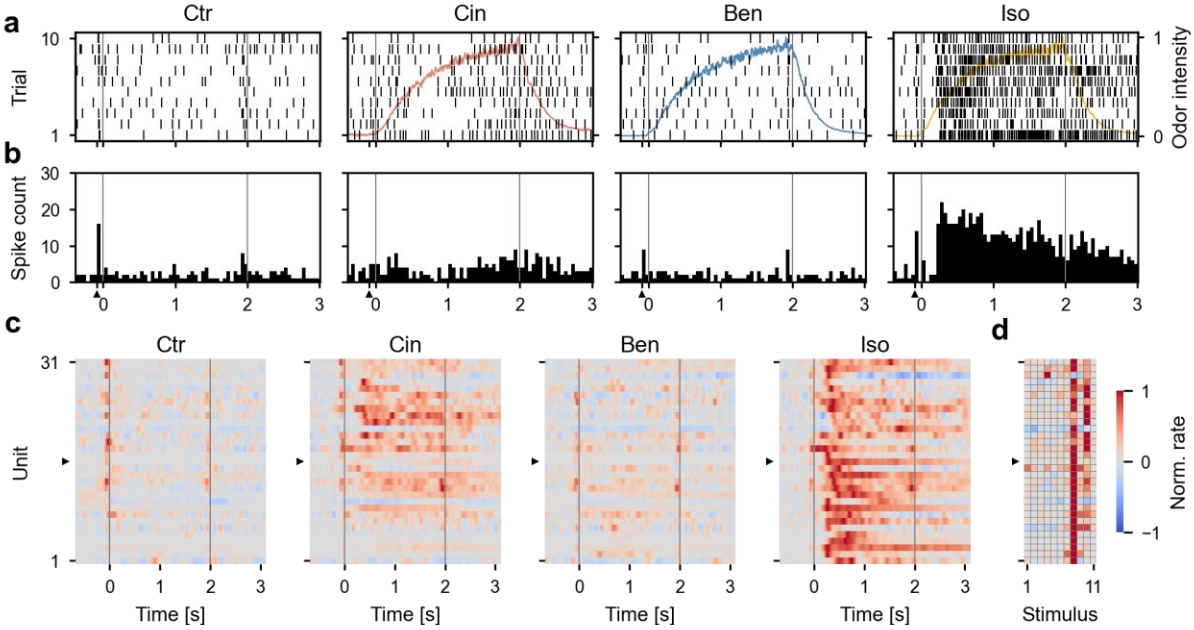

Figure 3: Neuronal population response during odor stimulation. a Exemplary spike raster plots of unit 16 (animal 10) during 10 repeated presentations of four different stimuli (Ctr = control, Cin = cinnamaldehyde, Ben = benzaldehyde, Iso = isoamyl acetate). The normalized trial-averaged odor concentration as measured with the PID is shown in the background (light color). **b** Peristimulus time histograms across all 10 stimulus trials (binwidth = 50 ms). Upward pointing triangle (▲) depicts the time point of valve switch at t = -90 ms before the odor arrives at the antenna tip (t = 0 ms). **c** Normalized trial-averaged firing rates across the population of 31 neurons. Color code indicates changes in the normalized firing rates relative to the baseline firing rate. **d** Matrix depicts normalized time-averaged firing rates across all units and all stimuli during stimulus onset (order of odors left to right as in Fig. 2a top to bottom). Odor stimulus was presented in the interval 0 – 2 s. Rightward pointing triangle (▶) in c & d indicates unit 16 shown in a & b.

MBON responses reflect behavioral feeding responses to food odors

We now consider the relation of single neuron responses and behavioral responses to the three food odors (Cin, Ben, Iso). To this end, and for each animal, we sorted all trials in two groups with respect to their behavioral response (MLR vs. no MLR). As a first result we find that the response spike count was consistently higher in behaviorally responded trials than in behaviorally unresponded trials for all recorded neurons (Fig. 4a).

Next, we analyzed the time-resolved response rate for the two behavioral conditions. The exemplary firing rate profile of a single MBON in Fig. 4b demonstrates a clear and strong trial-averaged response during trials that lead to a behavioral response, while no response occurred during behaviorally unresponded trials. Averaging the normalized firing rates across all MBONs confirms this picture where behaviorally responded trials show a prominent increase in firing rate in response to the olfactory stimuli whereas behaviorally unresponded trials did not evoke a discernible response (Fig. 4c), indicating a differential involvement of MBONs in the two behaviorally distinct categories.

To study in more detail the neuronal representation of odor and behavioral category we performed a principal component analysis (PCA) on the MBON population. Considering the 1st and 2nd principal components in Fig. 4d, we find that for each of the three odors, the respective trajectory during behaviorally responded trials (MLR) separates from the trajectory during behaviorally unresponded trials (no MLR), confirming our result on the averaged neuronal responses in Fig. 4c. In line with previous results in the honey bee (Strube-Bloss et al., 2011) we also observe a distinct representation of all three food odors in the principal component space.

Neuronal responses precede behavioral responses

What is the relative timing of behavioral and neuronal response? To answer this question, we estimated behavioral and neuronal response latencies at the single-trial level. In each behaviorally responded trial we determined the first video frame after odor onset ($t = 0$) that indicated a MLR. The overall distribution of behavioral response latencies shown in Fig. 2b is skewed towards the time of odor onset. In Fig. 4e-g we compare, per odor, the cumulative distributions of neuronal and behavioral single trial responses. While for Cin (Fig. 4e) and Ben (Fig. 4f) the neuronal responses are detected earlier than the behavioral responses, the situation is more difficult to assess for Iso (Fig. 4g) where neuronal and behavioral responses were overall fastest.

Note, that we applied a conservative strategy for estimating the single trial neuronal response onset, which minimizes the possibility of a premature response detection. At the same time and due to the stochastic nature of spike responses this approach tends to overestimate single trial latencies, specifically in weakly responded trials. To alleviate this estimation bias we used an additional approach where for each neuron we estimated its response latency from the trial averaged firing rate across all behavioral response trials for a given odor. This resulted in average single unit response latencies of 353 ms (range: 40-1,658 ms), 577 ms (67-1,821 ms) and 208 ms (27-1,575 ms) for the three odors Cin, Ben and Iso, respectively, indicating that individual MBONs express different response latencies.

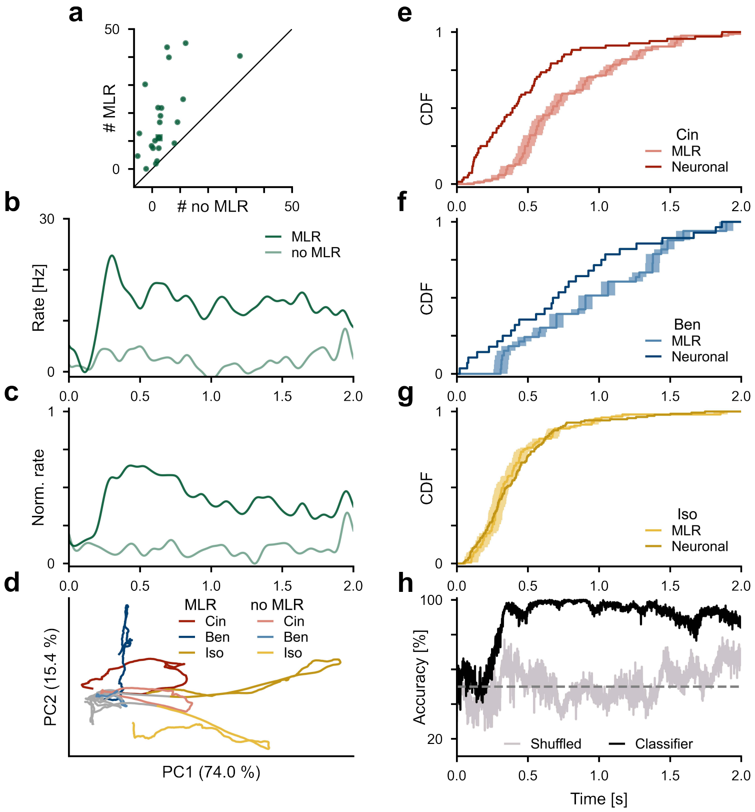

Figure 4: Mushroom body output reliably predicts behavioral response to food odors. a The median spike count during presentation of the food odors (Cin, Ben, Iso) computed across trials and per neuron is larger in behaviorally responded trials (MLR, ordinate) than in behaviorally unresponded trials (no MLR, abscissa) in all MBONs. **b** The time-resolved trial-averaged firing rate of a single MBON (unit 15, animal 10) shows a strong response to stimulation with food odors in behaviorally responded (MLR) but not in unresponded (no MLR) trials. **c** Across neuron average (N = 23) of normalized firing rate responses to food odors sorted by the animals' behavior indicate a clear neuronal response only to behaviorally responded trials but none to behaviorally unresponded trials. **d** First against the second principal component (PC1, PC2) of MBON population response separated by stimulus (Cin, Ben, Iso) and by behavioral response (MLR vs. no MLR) before (gray) and during (colored) stimulus presentation indicates separability of odors and behavioral state. **e-g** Across the single-trial MLR (weak line; shading refers to the 100 ms duration of the individual video frame) and single-trial neuronal response onset time (strong line) during presentation of Cin (e), Ben (f) and Iso (g). Only trials, which exhibit a behavioral response are considered (MLR). **h** Accuracy of single-trial prediction of behavioral response from neuronal population activity (black) sharply increases ~250 ms after stimulus onset and reaches almost perfect prediction. Averaged single trial prediction accuracy of behavioral response (MLR) based on a logistic regression classifier. The test data set (black) is compared to a control data set with randomly shuffled MLR labels (gray).

Neuronal population activity allows for the single-trial prediction of the behavioral response

Can we decode the neuronal response to predict feeding behavior in the single trial? To answer this question, we trained and tested a machine learning classifier in a time-resolved manner. To this end we first labeled the single trial neuronal responses according to their behavioral outcome (class label MLR vs. no MLR). We then separated the complete data set in a training and a test set. The former was used to train the classification algorithm in order to predict the class label based on the single-trial neuronal population activity. Classification performance was then evaluated on the test data set and quantified as accuracy. In a cross-validation approach we repeated the procedure on different splits of training and test data sets. The result in Fig. 4h (black curve) shows the average accuracy as a function of trial time. Initially, after stimulus onset, accuracy is at chance level (50%, horizontal dashed line) before it rises sharply to plateau at a high level of >90% accuracy. As a control we repeated the complete classification approach on the same data set, albeit with randomly shuffled behavioral class labels. As a result, accuracy did not significantly deviate from chance level.

Discussion

Over the past decade, a series of experimental studies on associative olfactory conditioning have concluded that the MB output encodes the valence of a sensory stimulus (see Introduction). The large majority of studies have been conducted in the fruit fly *Drosophila melanogaster*. With few exceptions (e.g. Sayin et al., 2019; Siju et al., 2020), these experiments evaluated learning induced plasticity at the level of MBONs and behavioral memory expression during a memory retention test in a behavioral group assay (Tully and Quinn, 1985) in separate groups of animals that underwent the same classical conditioning protocol (Aso et al., 2014b; Hancock et al., 2022). This approach did not allow to match neuronal and behavioral responses in the same individual and on a trial-to-trial basis.

By taking advantage of the experimental accessibility in the cockroach that allows us to simultaneously record neuronal spiking activity and feeding behavior with high temporal resolution in the individual animal, we were in the position to perform combined trial-based analyses of neuronal and behavioral responses. Our results demonstrate a tight link between the neuronal response at the MB output and the actual execution of a defined feeding behavior on a trial-to-trial basis where the occurrence or non-occurrence of behavior could be faithfully predicted with a single-trial classification approach. Our results contest the predominant view that the MB merely encodes sensory stimulus valence that provides a stimulus dependent behavioral preference. From our data we conclude that the MB output momentarily encodes a behavioral decision that is required for the execution of a behavior. Indeed, it has been shown that MBONs project to premotor areas (Li and Strausfeld, 1997, 1999; Okada et al., 2003; Aso et al., 2014a) and it has recently been shown that they can also make direct connections to descending neurons that innervate the ventral nerve cord (Hsu and Bhandawat, 2016; Emanuel et al., 2020; Li et al., 2020) in fruit flies and cockroaches.

Our conclusion is in line with earlier experimental observations in the cockroach (Mizunami et al., 1998; Okada et al., 1999) and recent experimental interpretations in the fruit fly (Hige et al., 2015; Lewis et al., 2015; Tsao et al., 2018; Siju et al., 2020; Aimon et al., 2022), which have suggested a tighter and acute involvement of the MB output in motor control. By investigating innate behavior in the fruit fly, Tsao et al. (2018) conclusively showed that MBON output is required for the expression of food-seeking behavior. This result implies that MBON activity should causally precede the behavioral execution. With respect to the precise relative timing of MBON activity and behavioral response, our analyses show that, on average, the onset of physiological spiking responses in the recorded MBON population indeed preceded the feeding behavior of the mouth parts. Stimulus response latencies differed across individual MBONs where several neurons showed fast stimulus-response times as short as 40 ms while others show considerably late response onsets after several hundred milliseconds and thus during the actual behavior. This feature of MBON specific response latencies matches earlier results in MBON recordings from naïve honey bees that reported a considerable fraction of fast responding MBONs that establish a rapid encoding of odor identity within ~70-80 ms (Strube-Bloss et al., 2012). Interestingly, we find that both, neuronal and behavioral response latencies are odor specific. Isoamyl acetate, the major single molecule component of banana blend (Schubert et al., 2014) and highly attractive both for flies and cockroaches (Schubert et al., 2014; Arican et al., 2020; Khoobdel et al., 2021), provoked the fastest and strongest neuronal as well as the fastest behavioral responses. We may further suggest that the later MBON responses occurring during behavior reflect on the behavioral state of the animal and rely on feedback signals (Mizunami et al., 1998; Okada et al., 1999). Indeed, in the fruit fly it has been shown that ongoing walking behavior through feedback via dopaminergic, octopaminergic and serotonergic neuromodulatory neurons strongly can dynamically influences MB activity (Cohn et al., 2015; Siju et al., 2020; Aimon et al., 2022).

In summary we hypothesize that the MB lobes are positioned at the center of the sensory-motor loop where it continuously integrates sensory evidence and monitors the animal’s metabolic and current behavioral state to form behavioral decision that is encoded in the MB output.

Materials and Methods

Animals

For all experiments adult male *Periplaneta americana* were used. Laboratory colonies were kept at 26 °C with a reversed light-dark cycle (12 h: 12 h) and fed with oat flakes and water *ad libitum*. All experiments were conducted during the scotophase, the natural active phase of *P. americana*.

Experimental setup

For data acquisition an extracellular recording setup was used (Fig. 1). The recording electrode (adapted from Okada et al., 1999; Strube-Bloss et al., 2011) consisted of three polyurethane coated copper wires (Ø 14 µm; Electrisola, Escholzmatt, Switzerland) glued together with hard sticky wax (Siladent, Goslar, Germany). A Teflon coated silver wire (Ø 125 µm, World Precision Instruments) was used as reference. Wires were fixed to a head stage that was connected to a preamplifier (PA 103, Electronics Workshop, University of Cologne, Germany). Main amplification using a 4-channel amplifier (MA 102 differential amplifier, Electronics Workshop, University of Cologne, Germany) was performed in differential mode from all three possible pair combinations of electrode wires. Amplified signals were bandpass filtered (300 Hz to 5 kHz), A/D converted with 16-bit amplitude resolution and a sampling rate of 25 kHz using a data acquisition unit (CED Micro 1401 mk II, Cambridge, UK) and stored on a PC.

An odor supply system (adapted from Strube-Bloss et al. (2011) was customized (by the workshop of the Department of Biology and an electrical engineer of the Institute of Zoology at the University of Cologne, Germany). The air stream (3.5 LPS charcoal filtered air) was split into two pathways. The first provided a permanent airstream (regulated with a restrictor, ø = 0.25 mm). The second passed through 12 magnetic valves (LFAA1200118H, Lee, Sulzbach, Germany). Each valve enables computer-controlled switching to pass the airstream either through an empty glass bottle (100 ml volume) or through a glass bottle (100 ml volume) filled with 5 ml of a pure odorant. The air outlet was placed at the tip of the right antenna aligned to its longitudinal axes such that the complete antenna was covered by the airstream (Fig. 1a).

A video camera (Logitech QuickCam Pro) was positioned in front of the animal's head to capture movement of its mouthparts, enabling the detection of a behavioral response (MLR, Arican et al., 2020).

All parts of the setup were placed in a Faraday cage covered in opaque fabric for shielding. Experiments were conducted in red light (643 nm), invisible to the animals (Goldsmith and Ruck, 1958; Walther, 1958; Mote and Goldsmith, 1970).

Animal preparation

Animals were anesthetized by cooling to 4 °C and harnessed in a custom-made holder (Fig. 1). The neck was fixed with hard sticky wax. Maxillary palps and the first basal segment of the antennae (scapus) were fixed with periphery wax (Sigma Dental Surgident, Systems, Handewitt, Germany) to avoid movement and contact with the electrodes. A small window was cut between the compound eyes and above the bases of the antennae. Trachea and head glands on top of the brain were removed and the animal was placed in the Faraday cage with a magnetic stand. Visible contours of the brain were used as landmarks to place the electrode above the β lobe in the right brain hemisphere, the silver wire was placed in the left compound eye. The recording electrode was slowly inserted axially with a micromanipulator (Luigs & Neumann SM-6, Ratingen, Germany) while monitoring the recording signal until the typical large amplitude extracellular action potentials of MBONs were detected. Then the head was covered with periphery wax to avoid dehydration of the brain and electrode movement.

Stimulus protocol

Ten odors we used for stimulation: 1-hexanol (Merck KGaA, Darmstadt, Germany), 1-heptanol (Merck KGaA, Darmstadt, Germany), 1-octanol (Thermo Fisher Scientific, Waltham, MA, USA), 1-pentanol (Merck KGaA, Darmstadt, Germany), heptanal (Thermo Fisher Scientific, Waltham, MA, USA), octanal (Merck KGaA, Darmstadt, Germany), 2-heptanone (Thermo Fisher Scientific, Waltham, MA, USA), isoamyl acetate (Thermo Fisher Scientific, Waltham, MA, USA), benzaldehyde (Merck KGaA, Darmstadt, Germany) & trans-cinnamaldehyde (Merck KGaA, Darmstadt, Germany). This set of odors is composed of single molecular odors that are perceivable by cockroaches (Sakura et al., 2002; Arican et al., 2020; Paoli et al., 2020; unpublished data). The food odors isoamyl acetate (Iso), benzaldehyde (Ben) and trans-cinnamaldehyde (Cin) were included because they are known to be able to elicit feeding behavior in cockroaches (Arican et al., 2020) and are clearly attributable to the specific food sources banana, almond, and cinnamon, respectively. The stimulation protocol comprised 10 trials per odor and additional 10 trials for the control stimulus (clean air). The total of 110 stimulations were presented in a different pseudorandomized order to each animal. The odors were presented for 2 s with an inter-trial interval (ITI) of 30 s (Fig. 1c).

To control for stimulus timing, we performed separate calibration measurements where odor concentration was measured at the antennal tip and base using a high temporal resolution photoionization detector (200B miniPID, Aurora Scientific, Aurora, ON, Canada). Odors consistently arrived at the antennal tip position with a delay of 90 ms with respect to the time of valve switching. We defined this time point as stimulus onset for subsequent analyses. In addition to the odor response some neurons showed a very brief spiking response that occured with high temporal precision immediately after valve switching and before the odor arrived at the antennal tip (Fig. 3b, ▲). We argue that switching a valve between the clean and the odorous air-stream caused a very brief interruption of the air stream detectable as a brief mechanical stimulus. We therefore interpret this initial brief and accurately timed neuronal response as a mechanosensory response. In addition, visual stimulation with either ultraviolet or bluish green light generated responses in a large subset of neurons (not shown) reflecting the MB role in multisensory integration.

Data processing and spike sorting

Preprocessing of extracellular recordings was performed with the software Spike 2 (v7.2, CED, Cambridge, UK). Semi-automated spike detection and a template-based spike sorting algorithm (Spike 2) was used. For spike detection we defined a threshold of minimum three times the signal's standard deviation as computed outside the stimulation intervals. Templates of the spike waveform were generated by the software and subsequently manually revised. Simultaneous video recordings (10 fps with constant frame rate) were made with the Spike 2 Video Recorder (v1.05, CED, Cambridge, UK) and analyzed manually in Spike 2 for MLR detection. In total, recordings of 21 animals were analyzed. From 19 animals 31 single units could be extracted and the MLR could be analyzed in 18 animals. In 15 animals neuronal signals and behavior were analyzed simultaneously.

Visualization of recording position

To visualize the recording tract, the tip of the electrode was dipped in Alexa Fluor 647 Hydrazide (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) before insertion into the brain. After recording, the brain was dissected in fresh cockroach Ringer's solution (185 mM NaCl, 4 mM KCL, 6 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Hepes, 35 mM Glucose, pH 7.2) and the whole brain was moved to 4% Formaldehyde in 0.1 M PBS overnight on a shaker at 4 °C. On the next day, the tissue was washed with 0.1 M PBS and 0.2% Triton X-100 in 0.1 M PBS with each solution three times for 10 min. Afterwards, the brain was incubated in 0.5% Lucifer Yellow CH dilithium salt (Merck KGaA, Darmstadt, Germany) diluted in 0.1 M PBS overnight on a shaker at 4 °C and washed again with 0.1 M PBS (5x 10 min) the next day. Finally, the brain was dehydrated in ethanol (30%, 50%, 70%, 90%, 95%, 2x 100%; 10 min each step), cleared in methylsalycylate (VWR Chemicals, Radnor, PA, USA) and mounted in it. The recording tract was visualized using a confocal laser scanning microscope (Leica TCS SP8, Wetzlar, Germany) and the scans were processed with ImageJ (FIJI based on ImageJ 1.53c, Wayne Rasband, NIH, USA).

Firing rate estimation

For analysis and visualization of neural and behavioral data custom written code in Python 3 was used. Firing rates were estimated by kernel convolution (Nawrot et al., 1999; Meier et al., 2008) with a time resolution of 1 ms. Trials were first aligned to stimulus onset (t = 0). Four different kernel functions were used: (1) a symmetric non-causal (i.e. centered) Gaussian kernel $k(t) = \frac{a}{\sqrt{2\pi\sigma}} exp(-t^2/(2\sigma^2))$ supported on $[-3\sigma, 3\sigma]$, (2) an asymmetric and strictly causal exponential kernel $k(t) = a \cdot exp(-t/\tau)$ supported on $[0, 5\tau]$, (3) an asymmetric non-causal alpha-shaped kernel (Krofczik et al., 2008) $k(t) = \frac{a \cdot t_0}{\tau^2} exp(-t_0/\tau)$ for $t_0 > 0$ and 0 otherwise aligned to its center of gravity ($t_0 = t + 1.6783 \cdot \tau$) and supported on $[-5\tau, 5\tau]$, (4) a strictly causal alpha-shaped kernel aligned at $t_0 = t$ supported on $[0, 6\tau]$. All kernels were normalized to unity such that $\int_{-\infty}^{\infty} k(t)\, dt = 1$.

For each neuron rates were estimated from single trials and from the pooled responses across trials.

To compute the baseline-corrected trial-averaged firing rate responses to odor stimulation per neurons we pooled spike trains across the respective set of trials and performed convolution with a Gaussian kernel (σ = 50 ms) and divided the result by the number of trials. We then subtracted the baseline firing rate as averaged across the baseline time window [-20 s, -0.5 s].

To obtain normalized firing rates, we averaged the firing rates per neuron and divided them by the maximum firing rate of the respective neuron over all odors.

Principal component analysis

For PCA we selected units from animals that showed a behavioral response (MLR) to food odors (Cin, Ben, Iso) during at least one trial and at most during nine trials per odor such allowing for comparison between behaviorally responded and unresponded trials. Six animals (seven neurons) fulfilled this condition. Single trial rate estimates (non-causal alpha kernel, $\tau = 200\, ms$) were used. The mean firing rate in the baseline window $[-20\, s, -0.5\, s]$ was subtracted in each single trial. Single trial rates were grouped by odor identity and behavioral outcome (MLR vs. no MLR) before trial-averaging. All vectors were used together for PCA.

Response latencies

To determine single trial neuronal response latencies, we used the baseline uncorrected data. Latencies were estimated from rate estimates with the causal exponential kernel ($\tau = 250\ ms$). For this, baseline activity was estimated in the time window $[-20\ s, -0.5\ s]$. For each trial the 97-percentile of the baseline firing rate in the time window $[-20\ s, -0.5\ s]$ was determined and used as threshold. If this value was smaller than $2.25 \cdot max(k(t))$ the threshold was set to $2.25 \cdot max(k(t))$. Response latency was determined as the time point of the first threshold crossing of the response rate from below within the time window $[\ 0\ s, 2\ s]$. This conservative procedure minimizes the chance to detect any premature response caused by spontaneous activity. As a consequence, there is a tendency to overestimate single trial response latencies when a response is detected only after integrating several true response spikes.

Based on the estimated firing rates of the single-trial analysis population onsets were analyzed. The single trials were grouped by their odor and unit identity. Baseline and stimulus activity was averaged across all trials in these groups. For each group the 97-percentile of the baseline firing rate was determined as response threshold. Neuronal onsets were detected in the group firing rate stimulus activity of the group, if a value was greater than the threshold as the border. If a neuronal onset was detected, the time point of the first crossing from below was considered as the time point of the neuronal onset. The mean across all units per odor was estimated. Units without a detected onset were ignored.

Prediction of behavioral outcome

For single trial prediction of a behavioral response from population activity during stimulation with food odors (Cin, Ben, Iso) we employed logistic regression classification with L2-norm (Python package Scikit-learn, Pedregosa et al., 2011) with regularization strength of 1.0. For training and testing the classifier we constructed neuronal pseudo populations (Rickert et al., 2009) as follows. We first selected those animals that expressed feeding behavior (MLR) in at least 10 and at most 20 out of total 30 stimulation trials resulting in a population of 17 units. We then estimated single trial firing rates using a causal alpha kernel ($\tau = 50\ ms$) and subtracted the mean firing rate in the baseline window $[-20\ s, -0.5\ s]$. All trials were grouped into two behavioral classes (MLR vs. no MLR). For each neuron and both classes, trials were then split into test (67%) and training set (33%). Training and testing were performed for each time point separately by randomly drawing (with replacement) 50 training and 20 test samples such that each sample consisted of a vector of 17 single trial firing rates. The mean and standard deviation of the training samples was estimated. Training and test data was centered by this mean and scaled by this standard deviation. Prediction accuracy was calculated as the percentage of correctly predicted single trial behavioral outcomes in the test set. The training and test procedure was repeated 24 times based on independent random splits into test and training sets. As a means of control, the complete process is repeated for a surrogate data set with randomly shuffled MLR labels, predicting a performance at chancel level (50%).

Data Availability

The data sets generated for this study are available on request to the corresponding authors.

Authors contributions

CA and MN designed the research. CA conducted the experiments. CA and FS analyzed the data. CA and MN wrote the manuscript. MS-B supported the adaptation of the recording setup. MS-B and WR revised the manuscript.

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.

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Chapter III: Visual processing and multimodality in the mushroom body output of *P. americana*

Abstract

The environment provides diverse sensory stimuli with a lot of variety, which implies the need to process this information appropriately. The responsible brain area to integrate and evaluate this information in insects is the mushroom body (MB) output region, that is mostly investigated in the context of learning and memory. Sensory, especially visual processing in the MB output of untrained animals was less intensively studied. To gain a better understanding of sensory encoding in MB output neurons (MBONs), in vivo extracellular recordings during sensory stimulation with focus on visual stimuli were conducted in cockroaches. We analyzed the responses to visual stimuli on different properties and compared the representation of visual, olfactory and mechanical stimuli within the same MBON population. We found differences between the representation of wavelengths and intensities and a fast adaptation to visual stimuli. About 35.7% of the recorded MBON population were unimodal and 60.8% were multimodal. Strikingly response latencies were explicitly different between the stimulus modalities. With this study we launched a systematical analysis of visual encoding and the aspect of multimodality in cockroach MBONs. In a next step, the gained knowledge and the established setup can be used to investigate the role of MBONs in sensorimotor processing of different sensory modalities or for learning and memory of these stimuli, on a single trial as well as on an individual animal level.

Introduction

Making use of sensory information provided by the environment is crucial for survival. Thus, animals need the ability to process them appropriately. In insects, higher order brain centers like the mushroom body (MB), the lateral horn and the central complex are relevant to process this information so that it is useful for the animal (Thiagarajan and Sachse, 2022). The MB and especially its output that is mostly investigated as learning and memory center in many species (Menzel and Manz, 2005; Strube-Bloss et al., 2011; Hige et al., 2015; Owald et al., 2015) plays a major role in processing environmental input, since it integrates distinct sensory information (Homberg, 1984; Schildberger, 1984; Rybak and Menzel, 1998; Li and Strausfeld, 1999; Strube-Bloss and Rössler, 2018).

The strong focus on olfaction and olfactory conditioning of studies investigating the role of the MB output (Strube-Bloss et al., 2011, 2012; Aso et al., 2014b; Hige et al., 2015; Owald et al., 2015; Dolan et al., 2018) led to a neglect of visual features in the MB output. Doubtless, olfactory cues play a major role in insects, especially in nocturnal cockroaches. However, visual capacities of nocturnal insects are similar to those of diurnal insects (Warrant and Dacke, 2011). Visual cues coming from optic lobes also land in the MB calyces (Nishikawa et al., 1998; Li et al., 2020b) and are further processed in MB output neurons (MBONs) (Schildberger, 1984; Li and Strausfeld, 1999; Vogt et al., 2014; Schmalz et al., 2022). Schmalz et al. (2022) found that some honey bee MBONs encode color identity and others encode intensity of colored lights. Especially intensity coding might be relevant for cockroaches whose photoreceptors are optimized to low light conditions (Heimonen et al., 2006). The primary processing site of vision in cockroaches was investigated by looking at sensitivity to different wavelengths and adaptation (Walther, 1958; Mote and Goldsmith, 1970; Butler, 1971; Ignatova and Frolov, 2022). Less is investigated on higher processing levels, although optical input is relevant for the behavior of cockroaches and influences, for example, escape behavior (Okada and Toh, 1998; Laurent Salazar et al., 2013). Further, the presence of different wavelengths and intensities affects the overall locomotion (Zhukovskaya et al., 2017). It was also shown that, similar to other insects like honey bees (Avarguès-Weber et al., 2011; Nouvian and Galizia, 2019) and fruit flies (Gerber et al., 2004; Liu et al., 2006; Vogt et al., 2014), cockroaches are able to learn visual cues (Szymanski, 1912; Turner, 1912; Lent and Kwon, 2004; Brown and Strausfeld, 2009; Pomaville and Lent, 2018).

It has often been stated that the MB output is involved in sensory-motor and decision making processes (Mizunami et al., 1998a; Okada et al., 1999; Hige et al., 2015; Lewis et al., 2015; Tsao et al., 2018), which makes it necessary to understand fundamental representation of different sensory stimuli in higher order brain areas. Various sensory input channels like those providing olfactory and visual information are integrated by insects, especially in food related contexts (McMeniman et al., 2014; Nicholas et al., 2018; Oh et al., 2021). An additional sensory information can even reverse behavior from avoidance to attraction (Cheng et al., 2019) and affect the learning processes and memory formation (Becker et al., 2019; Gil-Guevara et al., 2022). The process of integration and evaluation is continuous, allowing instantaneous decision making (Lewis et al., 2015). Therefore, it is relevant how different kinds of sensory information are represented over the MBON population.

Since MBONs are a relevant part of the sensory-motor pathway and only little is known about their role in visual contexts, we want to expand the knowledge on visual representation in MBONs. To get an idea of this process, we performed *in vivo* extracellular recordings at the output level of the MB β lobe, while stimulating with visual, olfactory and mechanical stimuli. We made use of the precise controllability of visual stimuli and focused on the neuronal response to stimulus on- and offsets and on neuronal adaptation for different wavelengths and intensities. Further, we examined MBON properties regarding multimodality.

Material and methods

This chapter is based on the same data and analyses as in Arican et al. (2022), with an additional visual stimulation protocol after the olfactory stimulation. Therefore, the repeating methods are kept short and only the additional parts are described in full detail.

Animals

Adult male *Periplaneta americana* were used for all experiments. The colonies were kept at 26 °C with a reversed light-dark cycle (12 h: 12 h) and the experiments were conducted during the scotophase. All animals were fed with oats and water *ad libitum*.

Experimental setup

The experiments were conducted in an extracellular recording setup with olfactory and visual stimulators (Fig. 1a). Three differential polyurethane coated copper wires (Ø 14 µm; Electrisola, Escholzmatt, Switzerland) that were connected to a preamplifier (PA 103, Electronics Workshop, University of Cologne, Germany) and an amplifier (MA 102 differential amplifier, Electronics Workshop, University of Cologne, Germany) were used as electrodes and a silver wire (Ø 125 µm, World Precision Instruments, Sarasota, FL, USA) was used as reference. For olfactory and mechanical stimulation, an odor supply system, as described in Arican et al. (2022), was used. The visual stimulation was performed with two light-emitting diodes (LEDs) with wavelengths of 365 nm (NCSU275T, Nichia, Tokushima, Japan) and 505 nm (NCSE119AT, Nichia, Tokushima, Japan). The LEDs were placed on top of the odor supply system to illuminate the animal from its right side. The amplifier and stimulation devices were connected to a computer-controlled analog to digital converter (CED Micro 1401 mk II, Cambridge, UK). A video camera (Logitech QuickCam Pro) was positioned in front of the animal's head to detect movement of the mouthparts during sensory stimulation (MLR, Arican et al., 2020). The recordings were only analyzed for the duration of olfactory stimulation (Arican et al., 2022), because the cockroaches did not respond initially to the light stimuli (see General discussion).

The experiments were conducted in a Faraday cage that was covered in opaque fabric under red light (643 nm).

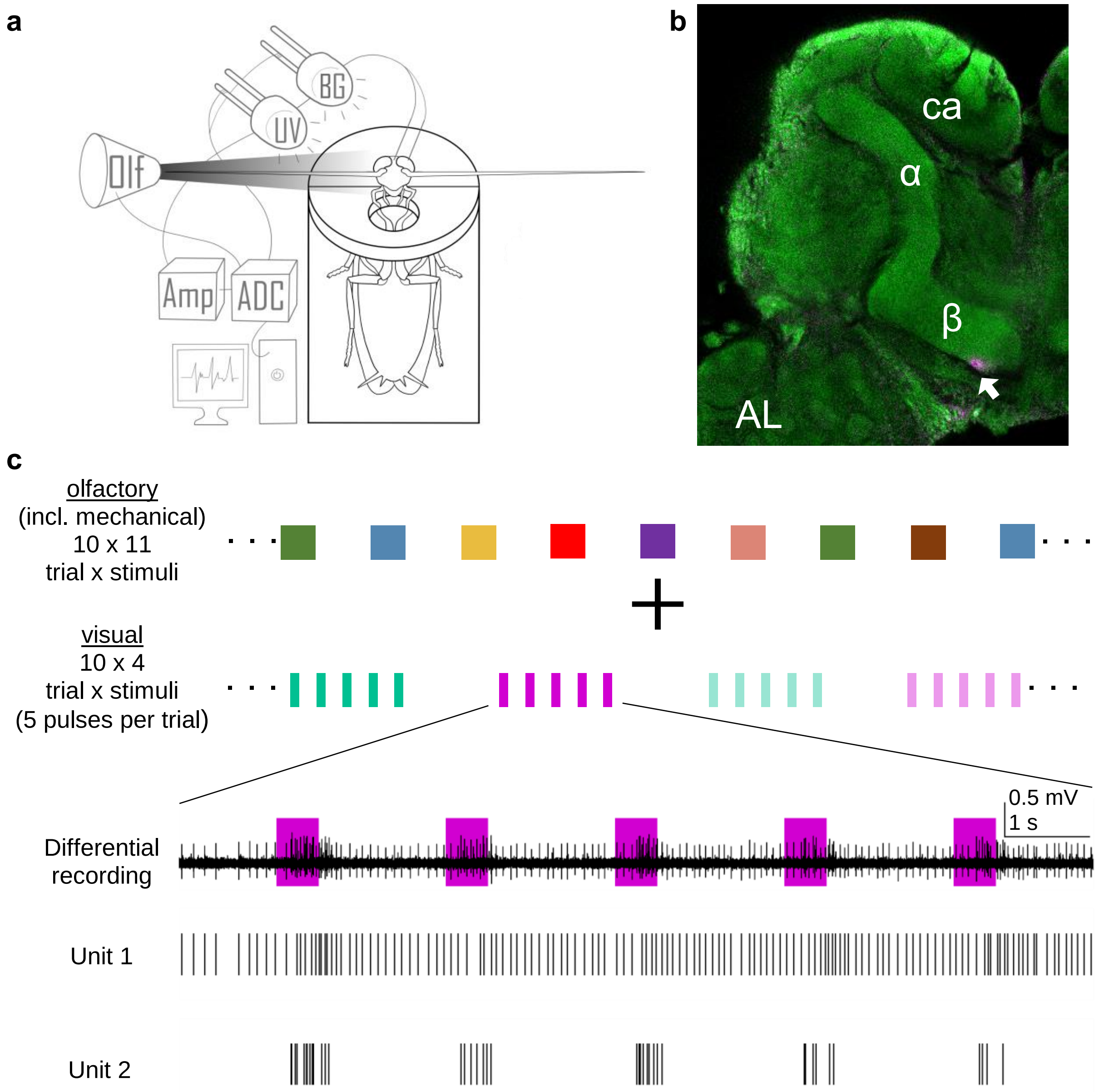

Figure 1 Extracellular single-unit recording during olfactory, mechanical and visual stimulation (adapted from Arican et al. (2022)). **a** Schematic illustration of a fixed cockroach in an extracellular recording setup with electrodes connected to an amplifier (Amp) and an analog to digital converter (ADC). Olfactory and mechanical stimuli were presented with an odor supply system (Olf), visual stimuli were presented with a bluish green (BG) and an ultraviolet (UV) LED. All stimulations were computer controlled. **b** Right hemisphere of a cockroach brain with prominent MB α and β lobes and the electrode position shown in magenta (white arrow) at the border of the β lobe. AL = antennal lobe; ca = MB calyx **c** Stimulation pattern showing parts of the olfactory, mechanical and the following visual stimulation protocol. For olfactory stimulation, 10 odors and a control stimulus (clean air) were presented (2 s) each 10 times in a pseudorandomized order. Each olfactory stimulus included a mechanical stimulus before the odor arrived at the antennal tip. For visual stimulation, two colors (BG, UV) were presented in two relative intensities (10%, 100%). Each of the four visual stimuli was presented in 10 trials consisting of five pulses (each pulse: 0.5 s; inter-pulse interval: 1.5 s). Exemplary differential recording during an UV light (100%) trial consisting of five pulses and two units that were sorted from the differential recording.

Animal preparation

Anesthetized animals were harnessed in a custom-made holder and the head was partly fixed with hard sticky wax (Siladent, Goslar, Germany), thus they were only able to move their maxillae and labium. Through a small window in the head capsule, the electrodes were inserted in the MB β lobe region (Fig. 1b). The silver wire was placed in the left compound eye. Once the electrodes perceived an accurate signal, the head was covered with periphery wax (Sigma Dental Surgident, Systems, Handewitt, Germany).

Stimulation

The stimulation protocol started with the odor stimulation and ended with visual stimulation. During odor stimulation, 10 odors (1-hexanol (1-Hex; Merck KGaA, Darmstadt, Germany), 1-heptanol (1-Hep; Merck KGaA, Darmstadt, Germany), 1-octanol (1-Oct; Thermo Fisher Scientific, Waltham, MA, USA), 1-pentanol (1-Pen; Merck KGaA, Darmstadt, Germany), heptanal (Hep; Thermo Fisher Scientific, Waltham, MA, USA), octanal (Oct; Merck KGaA, Darmstadt, Germany), 2-heptanone (2-Hep; Thermo Fisher Scientific, Waltham, MA, USA), isoamyl acetate (Iso; Thermo Fisher Scientific, Waltham, MA, USA), benzaldehyde (Ben; Merck KGaA, Darmstadt, Germany) & trans-cinnamaldehyde (Cin; Merck KGaA, Darmstadt, Germany)) and a control (clean air) were presented each 10 times for 2 s in a pseudorandomized order with an inter-trial interval of 30 s (Fig. 1c).

The odor arrived 90 ms after the valve switch at the antennal tip, but we detected neuronal responses before the arrival of the odor (see Arican et al. (2022)). Due to the switch of the magnetic valve between two bottles of the odor supply system, we argue that a very brief mechanical stimulus was present before every olfactory stimulation. Therefore, we analyzed the time window before the odor onset [0 s, 0.09 s] for mechanical stimulation and the olfactory onset was shifted by 90 ms, which let us separate the mechanical from the olfactory stimulus.

The visual stimulation protocol started 33 s after the last olfactory stimulus. For stimulation, two wavelengths (bluish green (BG): 505 nm; ultraviolet (UV): 365 nm) were presented each in two relative intensities (100%, 10%). The intensities were adjusted by the input current, which was 500 mA (100%) or 50 mA (10%). The wavelengths were chosen due to the peaks of the spectral sensitivity in the cockroach compound eye (Goldsmith and Ruck, 1958; Mote and

Goldsmith, 1970). Each trial consisted of five pulses (stimulus duration: 0.5 s, inter-pulse interval: 1.5 s). Four trials were always presented in the following order: 1. BG 100%, 2. UV 100%, 3. BG 10%, 4. UV 10%, which was repeated 10 times with an inter-trial interval of 10 s (Fig. 1c).

Data processing and spike sorting

Extracellular recordings were processed with semi-automated spike detection and spike sorting provided by the software Spike 2 (v7.2, CED, Cambridge, UK). For spike detection, the threshold was set to minimum three times the standard deviation of the baseline. Detected spikes were sorted in templates, automatically generated by the software and further manually revised. In total, 36 units from 21 animals were detected. Due to electrode drifts, not all units were usable for the data analysis of all stimulation protocols. Therefore, 33 units were used for analysis of the visual stimulation protocol, 31 units for the olfactory and mechanical stimulation protocol and 28 units for all protocols.

Visualization of recording position

To visualize the recording position retrospectively, the electrode was dipped in a fluorescent dye (Alexa Fluor 647 Hydrazide, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) before inserting it into the brain. After the recording, the whole brain was dissected and processed with the protocol for background staining with Lucifer Yellow CH dilithium salt (Merck KGaA, Darmstadt, Germany) as described in Arican et al. (2022). After mounting the brain in methylsalycylate (VWR Chemicals, Radnor, PA, USA), the electrode tract was visualized using a confocal laser scanning microscope (Leica TCS SP8, Wetzlar, Germany) and scans were further processed with ImageJ (FIJI based on ImageJ 1.53c, Wayne Rasband, NIH, USA).

Data analysis

All data were analyzed and illustrated with custom written code in Python 3.

Firing rate estimation

Firing rates were estimated by kernel convolution (Nawrot et al., 1999; Meier et al., 2008) using three different kernel functions with a time resolution of 1 ms: (1) A symmetric non-causal (centered) Gaussian kernel $k(t) = \frac{\alpha}{\sqrt{2\pi}\sigma}\exp(-\frac{t^2}{(2\sigma^2)})$ supported on [-3σ, 3σ] (σ = 50 ms), (2) an asymmetric and strictly causal exponential kernel $k(t) = a \cdot exp(-t/\tau)$ supported on [0, 5τ], and (3) an asymmetric non-causal alpha-shaped kernel (Krofczik et al., 2008) $k(t) = \frac{\alpha \cdot t_0}{\tau^2}\exp(-\frac{t_0}{\tau})$ for t_0>0 and otherwise aligned to its center of gravity ($t_0 = t + 1.6783 \cdot \tau$) and supported on [-5$\tau$, 5$\tau$]. For all analyses, except for the principal component analysis (PCA; described below), rates of each unit were estimated from pooled responses across trials.

For baseline corrected rates, the pooled rates across trials before stimulus onset were averaged across the baseline time window and subtracted from the rates of interest. Due to stimulus dependent inter-trial intervals, the baseline time window was adjusted to the respective interval (olfactory & mechanical: [-20 s, -0.5 s]; visual: [-2.5 s, -0.5 s]). To calculate normalized firing rates, the firing rates of a single unit were divided by the maximum trial-averaged firing rate of the respective unit over all stimuli.

Differences in the firing rates between subsequent pulses were calculated by averaging the firing rates during [0 s, 0.5 s] or immediately after [0.5 s, 1 s] each pulse for stimulus on- and offset responses. These firing rates were averaged over trials for pulses grouped by the stimulus. Then the second to fifth trial-averaged pulses were subtracted from the averaged firing rates of the respective previous pulse. For statistical analysis, Friedman tests were calculated (Python package scipy) with a following Nemenyi post-hoc test (Python package scikit_posthocs).

Neuronal onset detection

Neuronal onsets were detected for the three most responded olfactory stimuli (Cin, Ben, Iso), all visual stimuli and the mechanical stimulus. Therefore, thresholds were defined for each unit and stimulus type. The threshold was set to the 97-percentile of the respective trial-averaged baseline firing rate estimated with the causal exponential kernel (olfactory & visual: τ = 50 ms; mechanical: τ = 10 ms). If the threshold was lower than $2.25 \cdot \max(k(t))$ it was set to $2.25 \cdot \max(k(t))$. The threshold crossing of the trial-averaged firing rate was defined as response latency for each stimulus in each unit. For olfactory and mechanical stimuli, the response latency was calculated during stimulation (olfactory: [0 s, 2 s];

mechanical: [0 s, 0.09 s]) and for visual stimuli, latencies were calculated for stimulus onsets [0 s, 0.5 s] and offsets [0.5 s, 1 s] of the first pulse.

Principal component analysis

The PCA was performed for the three most responsive olfactory stimuli (Cin, Ben, Iso) and all visual stimuli. Due to their short time window, the mechanical stimulus was not included in the comparative analysis. For the analysis, multimodal units that responded at least to one olfactory and one visual stimulus were selected, which was fulfilled by eight units. The PCA was based on single trial rates estimated with the non-causal alpha kernel (τ = 150 ms). For each single trial, the mean firing rate of the respective baseline time window (olfactory: [-20 s, -0.5 s]; visual: [-2.5 s, -0.5 s]) was subtracted. The baseline corrected single trial rates were averaged for each stimulus and used for the PCA.

Results

The MB output population encodes on- and offset responses of visual stimuli

To gain insights into visual representation in the MB output, we stimulated *P. americana* with two different wavelengths (365 nm, 505 nm) in two relative intensities (100%, 10%) while recording extracellularly in the region of the MB β lobe (Fig. 1). Exemplary single unit raster plots and peri stimulus time histograms show a clear response to the visual stimuli (Fig. 2a,b). The example of unit 10 responded to all of the four stimuli. However, the population response pattern differs stronger between the colors than between the intensities (Fig. 2c). Distinctly more units responded to the BG light then to UV light. The difference in the response pattern between the intensities is more pronounced for BG and mostly the firing rate was higher to the lower intensity (e.g. unit 25: Fig. 2c (gray triangle), Suppl. Fig. 2). Additionally, we detected a distinct offset response, which was shorter than the onset response (Fig. 2d,e).

Comparing the response latencies to stimulus onsets, the range is larger for the higher intensities, but no difference was found between the colors (Fig. 2d). Grouping the latencies by stimulus on- and offset regardless of stimulus identity shows clearly that the offset latency is much shorter (Fig. 2e). The median (mean) of the neuronal response latency after the stimulus onset is 148 ms (202.46 ms) whereas it is 26 ms (39.91 ms) after the stimulus offset. Some of the offset responses are immediately after the stimulus offset and we can not preclude that these are residuals of the stimulus onset. However, excluding neuronal responses that started within the first 10 ms after the offset, does not have a significant effect. The median (mean) is with 43 ms (64 ms) still lower than the median and mean of the onset responses.

MBONs adapt from the second trial on

Neuronal on- and offset responses were detected for each unit. Response matrices confirm the impression that more units encoded on- and offsets of BG then of UV (Fig. 3a,d). To further investigate visual adaptation in MBONs, all five short pulses (0.5 s) per trial were considered in the following analysis. Firing rates were estimated for each stimulus averaged over the units responding to the respective stimulus on- or offset (depicted in the response matrices; Fig. 3a,d). This led to strong responses to the stimulus onset during the first pulse for all stimuli (Fig. 3b). The second peak in firing rate immediately after the first BG stimulus offset, let us assume that most of the units that responded to BG onset also seem to encode the stimulus offset, which was not the case for UV onset coding units. Looking at all pulses for stimulus onset coding units, the firing rates decreased at the second pulse, but did not decrease to baseline level. Comparing the difference in firing rates between the pulses shows that the average rate decreased by up to 5 Hz from the first to the second pulse for all stimuli and stayed at the same level for the following pulses (Fig. 3c).

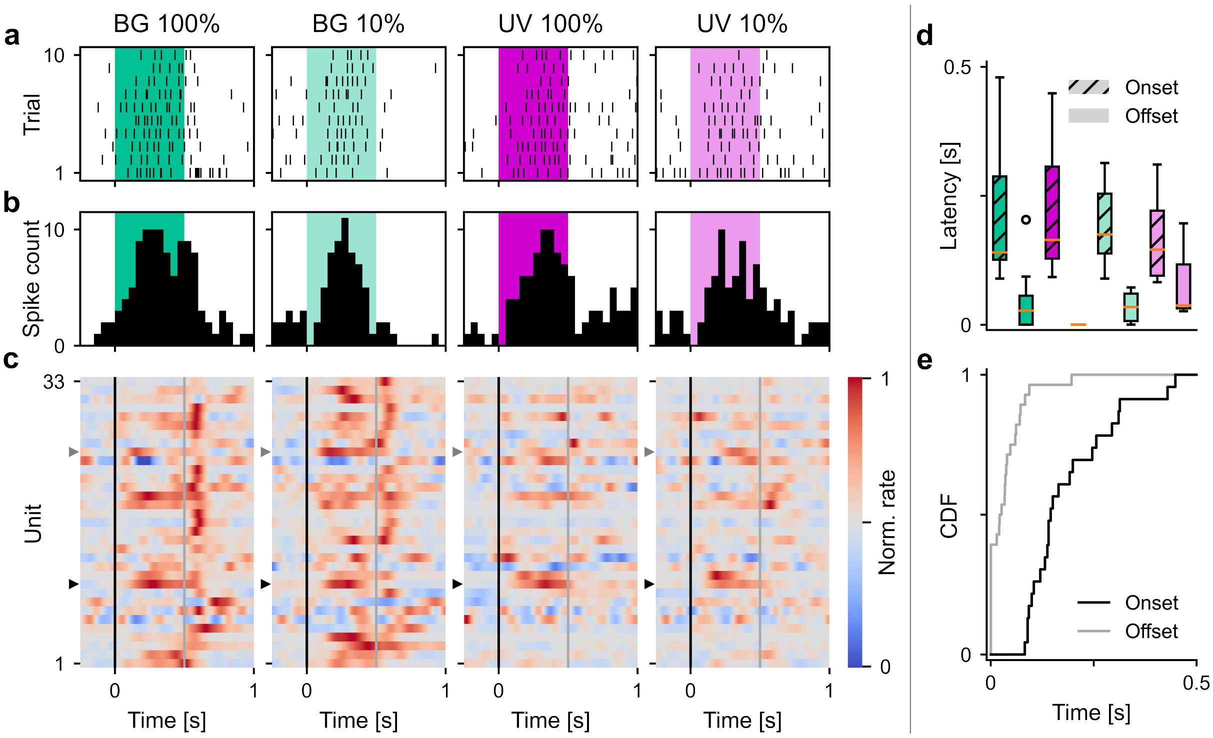

Figure 2 Single unit and population response to visual stimulation. **a** Spike raster plots for unit 10 during repeated presentation of visual stimuli. Color shaded areas depict time windows of stimulus presence (bluish green: 505 nm, 100%; light bluish green: 505 nm, 10%; purple: 365 nm, 100%; light purple: 365 nm, 10%). **b** Peristimulus time histograms across 10 trials (binwidth = 50 ms) shown for the respective stimuli. **c** Trial-averaged normalized firing rates estimated with a Gaussian kernel during presentation of BG and UV light in two intensities (onset: black line; offset: gray line). The color code represents changes in the firing rate over time. Black triangles (▶) indicate unit 10 that is depicted in a and b. Gray triangles (▶) indicate unit 25 (Suppl. Fig. 2). **d** Latency of neuronal response onsets to stimulus onsets (hatched) and to stimulus offsets (plane) of four different visual stimuli illustrated in boxplots. Red lines depict the median. **e** Cumulative distribution function (CDF) of neuronal response onsets to stimulus onsets (black line) and offsets (gray line) detected for trial-averaged firing rates during visual stimulation disregarding the stimulus identity.

More units responded to the stimulus offset than to the stimulus onset especially in the case of BG (Fig. 3a,d). Contrary to the behavior of onset coding units, the firing rate of units responding to the BG offset was actually highest during the stimulus offset (Fig. 3e). The units that encoded the offsets of both BG intensities and 10% UV, show the same adaptive behavior as the units that encoded stimulus onsets. Whereby the average change in firing rate was lower for 10% UV (ca. 2. Hz) than for the BG stimuli (ca. 5 Hz, Fig. 3f). Units that responded to the offset of 100% UV had higher firing rates after the stimulus onset (Fig. 3e).

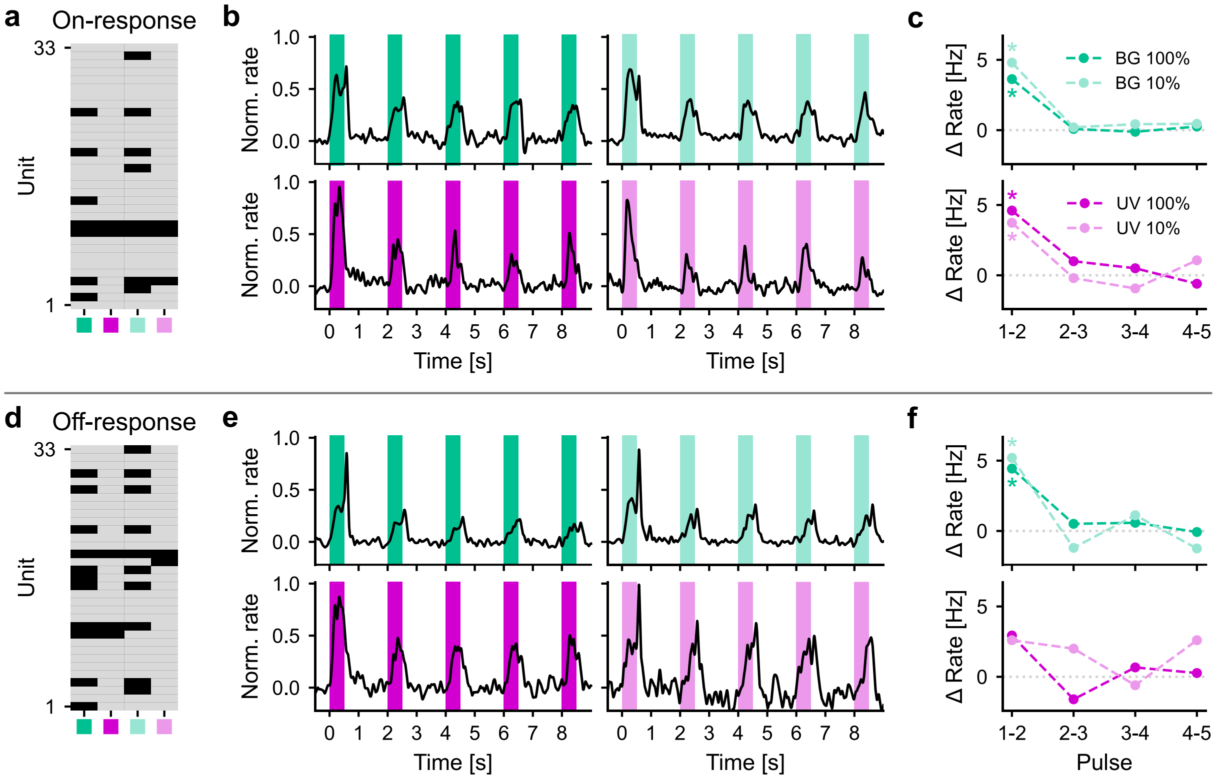

Figure 3 Behavior of responsive units to fast pulses of visual stimuli. **a,d** Response matrices indicate units that responded (black cells) during (a) and after (d) visual stimulation within 500 ms. **b,e** Normalized firing rates estimated with a Gaussian kernel of units that responded to the respective stimulus on- (b) or offset (e) as indicated in the response matrices. Firing rates were normalized for each color and intensity separately. **c,f** Changes in firing rates of the responding units during (500 ms, c) and after (500 ms, f) visual stimulation (BG: bluish green, UV: ultraviolet; intensities: 100% and 10%). The averaged firing rates of the first four pulses were each subtracted from the averaged firing rate of the respective following pulse. Asterisks indicate consecutive pulses that differed significantly in their firing rate (p-values < 0.005).

Multimodal representation in the mushroom body output

To get a better overview of how the MB output encodes different sensory modalities, neuronal responses were depicted for all stimuli and the ratio of units responding to the different modalities were analyzed (Fig. 4). As already described in Arican et al. (2022)) the three odors that had the highest neuronal responses are the food odors Ben, Cin and Iso. The number of units that responded to Iso was with approximately 82.1% by far the most compared to all stimuli (Fig. 4a). Most units that respond to any visual stimulus responded to both intensities of BG (10% intensity: 28.6%; 100% intensity: 21.4%) whereas UV elicited a response in few units (10% intensity: 10.7%; 100% intensity: 7.1%). Exactly 50% of the units responded to the mechanical stimulus. However, about 60.8% of all units encoded more than one stimulus modality and only 35.7% responded unimodally (Fig. 4b).

Further, a principal component analysis (PCA) for three odors with the highest neuronal response (Iso, Ben, Cin) and for four visual stimuli was performed with data of multimodal units regarding olfactory and visual stimuli. The trajectories of the olfactory and visual stimuli divide in different directions whereby the visual trajectories stay closer to each other than the olfactory trajectories (Fig. 4c). To gain insight into neuronal timing, the latencies were analyzed, grouped by either sensory modality (Fig. 4d) or the stimulus identity (Fig. 4e). The fastest responses were detected for the mechanical stimulus followed by visual and olfactory stimuli respectively (Fig. 4d). The median latency of visual stimuli (196 ms) is much faster than of olfactory stimuli (279 ms). This is also evidenced by the latencies for individual visual and olfactory stimuli (Fig. 4e) and seems not to be dependent on stimulus identity. Responses to the mechanical stimulus were usually fast and short (Suppl. Fig. 1), but it is important to note that the change in air pressure was very short and that only responses during the first 90 ms could be considered.

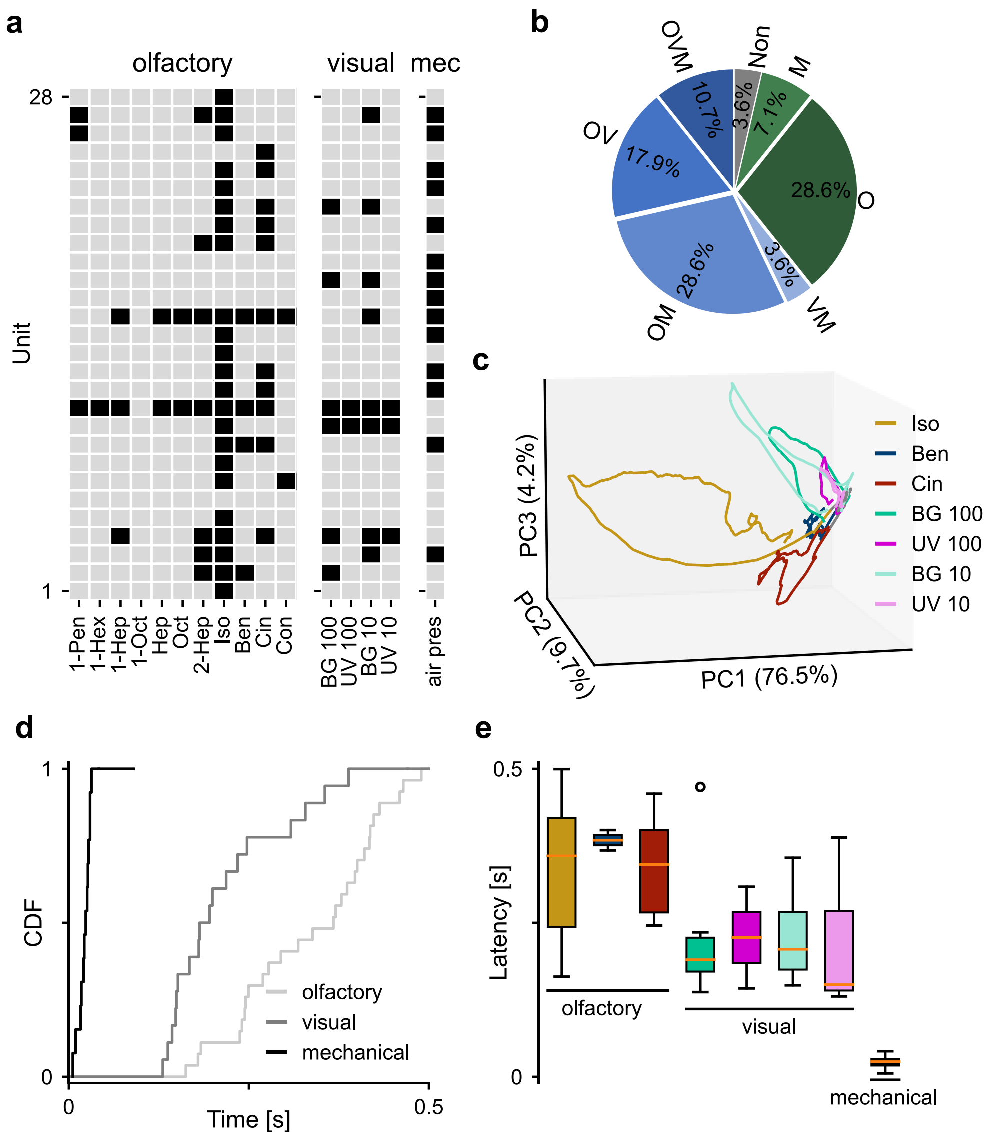

Figure 4 Representation of multimodality in the MB output. **a** Response matrix indicating units that responded (black cells) to particular olfactory, visual and mechanical (mec) stimuli. Olfactory stimulation included 10 odors (see Material and methods) and a control stimulus (clean air; Con), visual stimulation included two wavelengths (UV: 365 nm, BG: 505 nm) in two relative intensities (100%, 10%). The mechanical stimulus was a brief change in air pressure (air pres). **b** The pie chart depicts proportions of units responding to olfactory (O), visual (V), mechanical (M), no stimulus (Non) or any combination of different stimulus types. **c** Principal component analysis of the MB output population including only units that responded to at least one of the three odors (Iso, Ben, Cin) and to any of the visual stimuli. The first three principal components (PC1–PC3) depict the time window before (gray) and during (colored) stimulation. **d** Cumulative distribution function (CDF) of neuronal onsets grouped by olfactory (including only Iso, Ben and Cin), visual (all stimuli) and the mechanical stimulus within the first 90 ms (mechanical) or 500 ms (olfactory and visual) after stimulus onset. **e** Latency of neuronal onsets to stimulus onset for three olfactory stimuli, four visual stimuli and one mechanical stimulus depicted in boxplots. Red lines depict the median.

Discussion

With this study we contributed to build a basis for deeper investigations on sensory representation in the higher order brain area the MB output, with a focus on visual stimuli. Most studies on the MB output focused on learning and memory (Okada et al., 2007; Strube-Bloss et al., 2011, 2016; Aso et al., 2014b; Owald et al., 2015) and especially visual stimuli were almost exclusively investigated in the context of memory formation in fruit flies (Aso et al., 2014b; Vogt et al., 2014). Although in several other untrained insects visual representation in MBONs was reported (Schildberger, 1984; Rybak and Menzel, 1998; Li and Strausfeld, 1999), only few systematical studies exist on visual and multimodal processing in MBONs (Strube-Bloss and Rössler, 2018; Schmalz et al., 2022).

To analyze the representation of visual information in MBONs in our approach, we made sure that cockroaches were able to perceive the visual stimuli that we presented, by choosing the wavelengths dependent on the relative peak sensitivity of their UV and green receptors (Goldsmith and Ruck, 1958; Mote and Goldsmith, 1970). Both wavelengths elicited responses in the MBONs with different response patterns on the population level (Fig. 2c). Distinctly more units responded to BG (505 nm) then to UV (365 nm), corresponding to the peripheral level of the visual pathway, which has a ratio of 3 (UV): 5 (green) photoreceptors (Butler, 1971). On a behavioral level, cockroaches avoid higher wavelengths since they imply danger, leading to fleeing behavior (Okada and Toh, 1998; Laurent Salazar et al., 2013) and increase locomotion in general (Zhukovskaya et al., 2017). On the other hand, UV light that is encoded only by few units leads to more immobility (Zhukovskaya et al., 2017) and freezing behavior (Novikova et al., 2021). Therefore, we hypothesize that BG might be more valuable for the cockroach which is in line with the broader sensitivity in the color spectrum of the green receptors compared to the UV receptors (Mote and Goldsmith, 1970).

In contrast to our findings, honey bee MBONs responded strongest to UV compared to blue and green light (Schmalz et al., 2022). Honey bees are attracted strongest to UV light, which also increases their mobility (von Helversen, 1972; Labhart, 1974) and modulates learning performance in cross modal conditioning tasks (Becker et al., 2019). However, they have no sensitivity differences at the photoreceptor level (Becker et al., 2019; supplements). Also, behaviorally they follow different life strategies compared to the cockroach. In a subset of honey bee MBONs, Schmalz et al. (2022) found a positive correlation between stimulus intensity and firing rate. Interestingly, we found that most neurons responded with higher firing rates to the lower intensity (e.g. unit 25: Fig. 2c (gray triangle), Suppl. Fig. 2), which is in line with the optimization to low light conditions on the receptor level (Heimonen et al., 2006; Frolov and Ignatova, 2020), but needs to be further investigated with more intensity steps.

Neuronal responses to stimulus offsets in MBONs as we found them in the cockroach (Fig. 2,3) were also reported in honey bees (Schmalz et al., 2022) and fruit flies (Vrontou et al., 2021). This cue might imply safety for cockroaches, like when they reach a shelter (Okada and Toh, 1998), which is a valuable information for the survival of a cockroach. Some neurons seem to encode stimulus on- and offset, but the response latency to the offset is mostly shorter than to onset (Fig. 2d,e), which might be relevant for decoding it.

Cockroach retinal sensitivity is affected after adaptation to green and UV light (Walther, 1958). In the olfactory pathway, adaptation has also already been found in early stages (Bhandawat et al., 2007; Krofczik et al., 2008; Nagel and Wilson, 2011). Further, Kenyon cells that project olfactory and visual information from the MB calyx to MBONs show spike frequency adaptation mediated by current injection (Demmer and Kloppenburg, 2009). We first investigated adaptation to visual stimuli, because of the better controllability of the stimulus. The adaptation we found seems to be independent of the color identity and was already detectable in the second stimulus pulse. However, the adaptation remained at the same level from the second pulse on and did not decrease to baseline activity. Only in UV offsets we had no significant decrease in firing rate between the first and second pulse (Fig. 3f). Especially in the case of 100% relative intensity the detected responses are probably residue of the stimulus onset response. To gain better insights about stimulus offset representation in MBONs, experiments are required with longer intervals between stimulus on- and offsets. Further, more recordings of UV sensitive units are needed to compare responses to UV with responses to BG more reliably.

Natural environments never consist of single sensory modalities. Cross-modal information are beneficial for more precise perception and behavioral output of sensory information and can lead to a reduction of response latency (Chatterjee et al., 2022; Martorell and Medan, 2022). This brings about the need for an integrational center, which is the MB in insects. The MB perceives olfactory, visual, auditory, mechanical, tactile, gustatory, thermo-sensory and hygro-sensory information from different brain regions (Strausfeld and Li, 1999a; Nishino et al., 2003; Galizia and Rössler, 2010; Frank et al., 2015; Masek and Keene, 2016; Marin et al., 2020; Thiagarajan and Sachse, 2022) which are further processed in MBONs (Schildberger, 1984; Li and Strausfeld, 1999; Vogt et al., 2014; Strube-Bloss and Rössler, 2018).

Former MBON recordings during stimulation with different stimuli already revealed that the representation of stimulus modalities varies between MBONs (Li and Strausfeld, 1999; Strube-Bloss and Rössler, 2018). In cockroaches, up to four different modalities were tested in recordings of single MBONs (auditory, mechanical, olfactory, visual), some of which responded to all modalities (Li and Strausfeld, 1999). In this study, only 10.7% of the units responded to all modalities (olfactory, mechanical, visual) (Fig. 4b). From 35.7% unimodal units none responded only to visual stimuli, which might indicate that visual information can always be modulated by other stimuli; however, this requires further examination of the interplay of different modalities presented simultaneously.

Apart from binary responsiveness, features of multimodal MBONs are clearly separated due to the stimulus modality in honey bees (Strube-Bloss and Rössler, 2018). This is comparable to our findings, whereas it seems that the multimodal cockroach MBONs differ stronger within modalities. Especially the odors in the PCA (Iso, Ben, Cin) separate more from each other (Fig. 4c) than shown in honey bees, which is potentially due to the difference in value of the odors for cockroaches while the two floral odors, tested in honey bees, might not differ in the value for them.

Since MBONs are argued to be part of the sensory-motor pathway that is involved in the decision making process (Mizunami et al., 1998a; Okada et al., 1999; Hige et al., 2015; Lewis et al., 2015; Tsao et al., 2018), their responses need to be fast and accurate. Mechanical stimuli elicited by far fastest neuronal responses that decreased fast (Fig. 4d,e, Suppl. Fig. 1). This might be an important quick and short response to elicit fleeing from predators, that can be triggered by air puffs (Grandcolas, 1998). Interestingly, the latencies of the same modality are roughly in the same range (Fig. 4d,e) which might mirror the need of rapidity in certain sensory modalities. In particular, mechanosensory information are the relevant information in low-light situations, where visual information might be insufficient.

With this study we could contribute to the overall understanding of the representation of different sensory modalities in the MB output with a special focus on visual stimuli and visual adaptation. Future investigations on the interplay of these sensory modalities (visual, olfactory, mechanical) are needed in order to understand how this shapes behavior in complex and changing environment and how they affect each other in the context of learning and memory.

General discussion

Behavioral learning experiments as foundation to investigate underlying neuronal mechanisms

Over the past decades, many behavioral learning paradigms have been established and reinvented for insects, mostly for fruit flies and honey bees (McGuire et al., 2005; Giurfa, 2015; Menzel, 2021). These were essential to shed light on the underlying neuronal mechanisms of learning and memory (Davis, 1993; Menzel and Giurfa, 2001; Menzel, 2012; Modi et al., 2020). In our studies, cockroaches were used to build a supplementary unit to investigate this topic. Classical olfactory and operant spatial paradigms partly inspired by and adapted from earlier studies in different insects (Barraco, 1981; Menzel and Giurfa, 2001; Watanabe et al., 2003) were established successfully (Arican et al., 2020).

When designing behavioral experiments for later use in electrophysiological setups, we opted for more straightforward experiments that lead to reliable learning rates. The classical conditioning paradigm with harnessed cockroaches that we established contributes an ideal starting point for experiments with simultaneous *in vivo* MB recordings with the opportunity to analyze single training trials (Arican et al., 2020, Fig. 1a). Various opportunities also exist in the selection of appropriate stimuli as CS and US for the learning paradigms. Olfactory stimuli are of major relevance for cockroaches e.g. in mating, navigation and foraging and earlier studies showed that cockroaches are capable of olfactory learning (Balderrama, 1980; Watanabe et al., 2003; Hosono et al., 2016), which made them a valid choice for classical conditioning experiments. However, working with odors can be challenging regarding the controllability of the experimental settings. Delivering the stimuli with clear on- and offsets, tuning the concentration, unraveling mechanical from the olfactory stimulus and generally fast temporal stimulation in single odors or mixtures is tough. For better control of odors, more complex apertures are needed (Raiser et al., 2017; Tichy et al., 2020b). To monitor the odors, we recorded exemplary plumes with a photoionization detector (200B miniPID, Aurora Scientific, Aurora, ON, Canada), which was not possible during the experiments, because of its disruptive sound. Another issue in using olfactory stimuli is the high initial response to some odors (Arican et al., 2020, Fig. 2), which brings noise in the data.

Some exploratory experiments with repeated presentation of odors of different chemical classes with different functional groups gave us insight about the initial response of untrained cockroaches (Fig. 1a). While some odors that are strongly associated with one specific food source (e.g. isoamyl acetate with banana; Schubert et al., 2014) elicited high MLR rates (Arican et al., 2020, Fig. 2; Arican et al., 2022, Fig. 2), others like 2-heptanol and 2-octanol that are not so easily assignable did as well. Due to their initial high response, these might be potential candidates for future studies on aversive conditioning. These eight odors already elicited a variety of response patterns, indicating the value of the odors for cockroaches, which makes the comparability of these odors difficult.

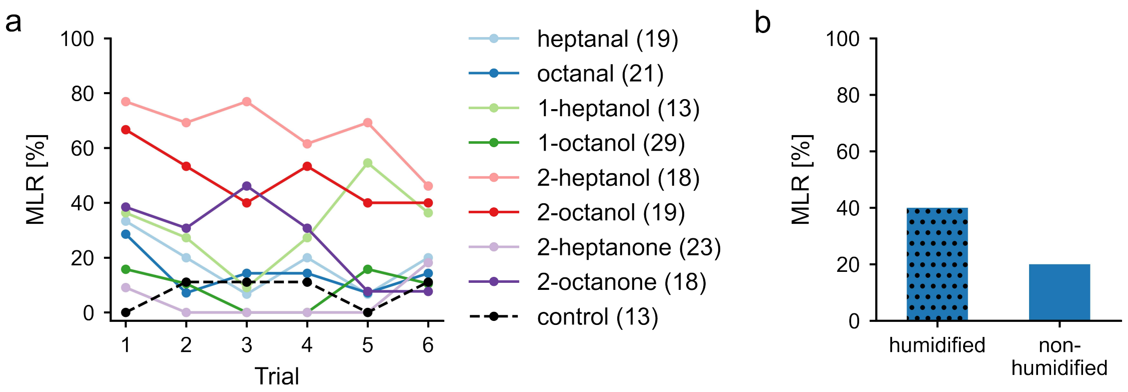

Figure 1 Initial feeding behavior (MLR) of untrained cockroaches. **a** Averaged MLR to repeated presentation of eight different odors and a clean air puff (control). Each stimulus (2 s) was presented six times with an inter-trial interval of 10 min. The odor presentation was performed manually with a syringe filled with an odorant-soaked or dry filter paper. Number in brackets indicate the animals tested with each odor. The used setup was the same as described in Arican et al. (2020). **b** Averaged MLR to a single presentation of octanal (2 s) per animal. Instead of manual presentation (as in a), the odor was presented with an automated odor supply system (described in Arican et al. (2022)) that was equipped with syringes filled with odorant-soaked filter papers. The airstream was either humidified (dotted bar, N = 20) or non-humidified (plane bar, N = 10).

It is well known that initial behavior is dependent on internal states like satiety (Inagaki et al., 2014; Grunwald Kadow, 2019; Vogt et al., 2021; Devineni and Scaplen, 2022). Lin et al. (2014) showed that water deprived fruit flies change from avoiding humidity to being attracted by it. In a small sample of untrained and water deprived (seven days) cockroaches octanal was presented with a humidified or non-humidified airstream. The initial response to the humidified airstream was twice as strong as to the non-humidified airstream (Fig. 1b), which might be dependent on the thirstiness of the animal.

Subsequent appetitive conditioning with non-humidified octanal that had a relatively low initial response rate, showed a clear learning success even without excluding animals that responded before the first US presentation (Fig. 2a). The learning success becomes even more evident after excluding the spontaneous responders (Fig. 2b). Other than that, experiments with permanent airstreams to reduce mechanical stimulation during odor presentation revealed the effect and necessity of the mechanical stimulus for learning (Fig. 2). Neither the olfactory nor the mechanical stimuli alone could cause a significant learning performance. We assume that learning success depends on the relevance of the US to an animal as it has been shown, for example, in mosquitos (Wolff et al., 2019). Cockroaches mainly use the mechanical stimulation of an air plume for orientation and adding a pheromone to the plume leads up to 100% of the male cockroaches walking towards the odor source (Willis and Avondet, 2005). The relevance of the mechanical stimulus and especially of the combination with olfactory information might be related to the learning rates we observed (Fig. 2). This goes along with the generally high learning rates in worker honey bees in olfactory learning tasks (General introduction, Fig. 1c), since odors are a relevant information to be successful in their main task of collecting pollen from flowers.

In our operant approach, a highly aversive light stimulus that elicits either fleeing (Okada and Toh, 1998; Laurent Salazar et al., 2013) or freezing (Novikova et al., 2021) behavior was used as punishment (Arican et al., 2020, Fig. 1b). The experiments were conducted in a custom-built T-maze that could be a candidate for future experiments with simultaneous extracellular recordings in freely moving cockroaches. However, a major issue in experiments with the maze was the motivation of the animals to start moving and decide for one direction. This might be solved by adding an airstream to the maze, because bimodal sensory input increased the rate of initial behavior in fixed and freely moving cockroaches (Willis and Avondet, 2005; Fig. 2). Adding an airstream would also allow better control of the odor distribution in the maze.

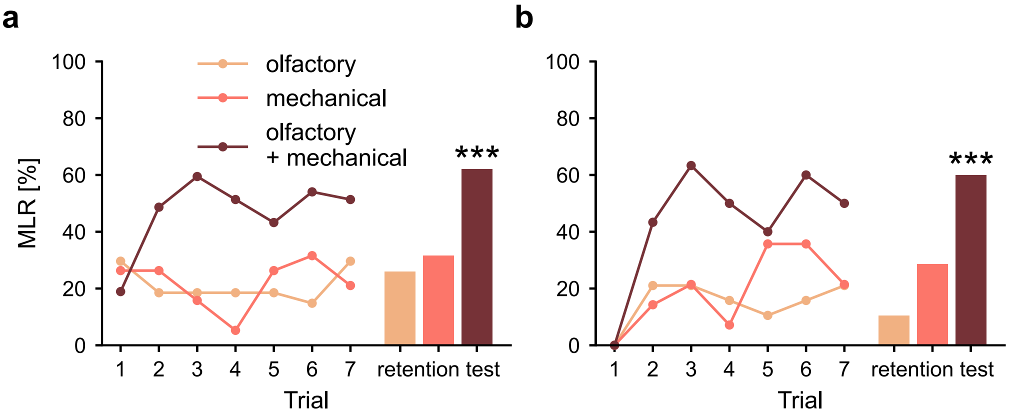

Figure 2 Appetitive classical conditioning paradigm over seven training trials with a retention test after 1 h and three different CS. The conditioning protocol is based on the protocol of Arican et al. (2020). Instead of manual presentation, the CS was presented with an automated odor supply system (described in Arican et al. (2022)). It was equipped with an adjustable permanent airstream and syringes filled with odorant-soaked filter paper instead of glass bottles. Octanal and a clean air puff (mechanical) were used as CS. Octanal was provided in a permanent clean airstream without mechanical stimulus (olfactory) or without a permanent airstream in an odor puff (olfactory + mechanical). Asterisks indicate significant differences between the first response before training and the respective retention test determined with χ^2 tests (***: $p < 0.001$). **a** All animals that were trained and tested are depicted. (olfactory: N = 27; mechanical: N = 19; olfactory + mechanical: N = 37) **b** Animals that responded to the first CS presentation before providing the US were excluded. (olfactory: N = 19; mechanical: N = 14; olfactory + mechanical: N = 20).

Despite the aversion to light, cockroaches are able to get valuable information from it and learn for example that a specific light source can give insight into food localization (Kwon et al., 2004; Lent and Kwon, 2004; Pomaville and Lent, 2018). In exploratory experiments, we could show that only about 1.28% of the cockroaches (N = 78) responded spontaneously with MLR to bluish green and ultraviolet light presented with the LEDs described in Chapter III (data not shown). Due to better controllability of light sources and the low spontaneous MLR rate, which are great advantages over olfactory CS, we conducted additional exploratory experiments to find out if visual stimuli can elicit MLR. Rewarding with sugar solution worked equally well for both wavelengths and led to a learning rate of about 40% that does not contain notable spontaneous responses (Fig. 3). It took three training trials to increase the MLR rate which might be due to the strong initial avoidance to light.

Since the cockroaches were relatively successful in simple learning paradigms, it would be interesting to investigate the limits of cockroach cognition in future experiments. From an evolutionary perspective, cockroaches as more basal insects with large MBs would be good comparable organisms to bees that have high cognitive abilities (Zhang et al., 2012; Avarguès-Weber and Giurfa, 2013; Chittka, 2017; Loukola et al., 2017).

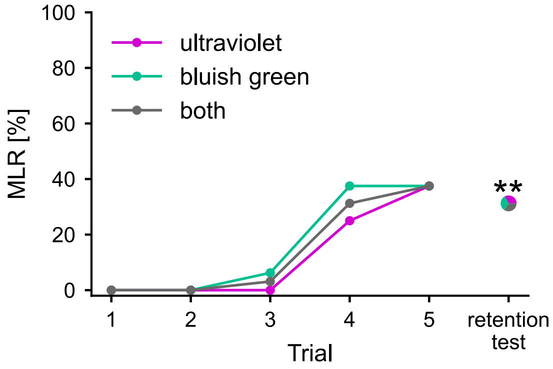

Figure 3 Visual appetitive classical conditioning paradigm over five training trials with a retention test after 10 min. The conditioning protocol is based on the classical conditioning paradigm in Arican et al. (2020). Instead of olfactory stimuli, visual stimuli were presented as CS. Used CS were bluish green (505 nm) and ultraviolet (365 nm) light stimulation with a relative intensity of 10% as described in Chapter III. Learning rates were depicted for each wavelength individually (ultraviolet, bluish green) and the average of both wavelengths (gray). Asterisks above the marker of the retention tests indicate significant differences between the first response before training and the respective retention test determined with χ^2 tests (**: $p < 0.005$). bluish green: N = 16; ultraviolet: N = 16.

Inter-individual differences in learning

Inter-individual difference in behavior is something one would expect in insects, because differences can occur on many levels from sensory perception to behavior (Honegger et al., 2020; Jafari and Alenius, 2021). Many variable natural parameters exist within the same species like sex, age and ancestors (Mery and Kawecki, 2002; Brown and Strausfeld, 2009; Münch et al., 2010; Jiao et al., 2022) and many internal and external conditions can affect each individual (Devineni and Scaplen, 2022). Individuality in cognitive abilities in insects was already reported in 1977 by McGuire and Hirsch, but was not considered in many studies. In Arican et al. (2020), we stated inter-individual differences in cognitive abilities in cockroaches. We found individuality in the appetitive and the aversive conditioned response. With that we strengthened the hypothesis of Pamir et al. (2011, 2014) that insect populations can be divided into subgroups of animals that learn reliably (‘learners’) and those that never learn (‘non-learners’) a specific task. Other studies that focused on individual differences in insect cognition investigated whether these abilities are the same across different sensory modalities and if they are task dependent (Smith and Raine, 2014; Tait et al., 2019; Finke et al., 2021).

To get a better idea of generalizability of cognitive capacities in individual cockroaches we used the data from Arican et al. (2020) and compared the performance to the CS+ and CS- during a differential conditioning task (Fig. 4). The animals that showed at least one correct response in the respective trial were separated depending on whether they responded correctly only to the CS+ (blue), only to the CS- (green) or to both (gray). With increasing trial count, the number of animals that behaved correctly to both CS is strikingly low, whereas the number of animals that behaved at least to one of the two CS correctly increased over trials. Also, the animals that never responded correctly over all trials after the first training trial to at least one of the stimuli were grouped (Fig. 4, hatched bar). They were separated depending on whether they never behaved correctly only to the CS+, only to the CS- or to both. Only one animal never responded correctly to both stimuli. This indicates differences in the ability to learn appetitive or aversive tasks within an animal and does not speak for general low or high cognitive capacities in individual cockroaches. Further, it precludes that missing odor perception or similar inabilities are the reason why some animals did not learn. Regarding the underlying neuronal mechanism, it is in line with the parallel pathways that are responsible for appetitive and aversive memory formation stated in fruit flies (Hige and Turner, 2015; Yamazaki et al., 2018).

Approaches like ours that consider only binary results might overlook some aspects, which could be improved by employing multi-factorial analyses of a conditioned response (Borstel and Stevenson, 2021) or at least by using a gradual metric. For example, one could record from the M15 or M17 muscle that is responsible for the MLR in cockroaches (Schmitt et al., 2014), as it has been done with the honey bee M17 muscle that is responsible for the PER (Smith and Menzel, 1989b, 1989a).

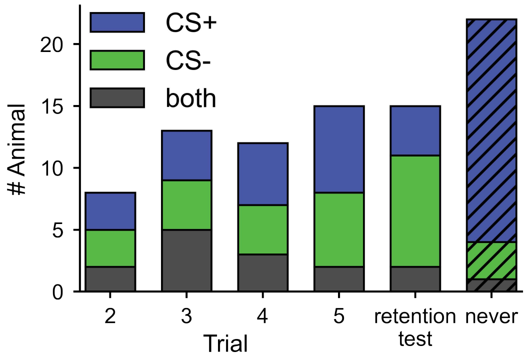

Figure 4 Behavioral responses of cockroaches during classical differential conditioning (data from short-term memory paradigm in Arican et al. (2020)). Plane bars indicate the number of animals that responded correctly either to the CS+ (MLR), the CS- (no MLR) or to both in the indicated trials and the retention test. Hatched bar indicates the number of animals that never responded correctly either to the CS+, the CS- or to both in all training trials and the retention test, excluding the initial response before the first training trial.

Inter-individual differences within a population generate robustness to environmental changes (Jandt et al., 2014). This applies also to cognitive abilities, from which for example trade-offs between appetitive and aversive learning abilities were reported (Junca et al., 2019). The underlying neuronal mechanisms that lead to behavioral flexibility and to individual differences have been investigated on different levels (Honegger et al., 2020; Jafari and Alenius, 2021; Devineni and Scaplen, 2022), but the mechanisms on higher order brain areas regarding inter-individual differences in cognitive abilities are still unclear. Thus, future experiments with physiological approaches investigating memory formation on individual level, should consider analyzing inter-individual differences as well.

Different roles of the mushroom body output

The MB as a higher order brain center and especially the MB output covers different functions. Anatomically, they clearly integrates various sensory pathways (Strausfeld and Li, 1999a; Nishino et al., 2003; Galizia and Rössler, 2010; Miroschnikow et al., 2018; Marin et al., 2020; Thiagarajan and Sachse, 2022). Also, MBON responses to diverse sensory stimuli were stated many times in various species (Schildberger, 1984; Li and Strausfeld, 1999; Nishino et al., 2003; Strube-Bloss and Rössler, 2018). With our studies, we added knowledge about the processing of olfactory, visual and mechanical stimuli in cockroach MBONs. We could show a diverse response pattern over the recorded MBON population (Chapter III, Fig. 4), leading to the hypothesis that sensory stimuli can be processed independently or in interdependency. The ability to process different stimuli independently is important under conditions where some sensory information is missing and insects need reliable information (e.g. in darkness). Whereas the ability to process stimuli interdependent is important to generate a preferably realistic picture of the environment. Generally, various sensory inputs need to be processed and whether sensory inputs are considered can be dependent on their intensity (Gil-Guevara et al., 2022). Integrating sensory inputs has been shown to affect different behaviors like motion, locomotion, foraging, feeding, decision making and conditioned responses in insects (Kulahci et al., 2008; McMeniman et al., 2014; Gepner et al., 2015; Becker et al., 2019; Cheng et al., 2019; Riveros et al., 2020; Oh et al., 2021; Chatterjee et al., 2022; Gilad et al., 2022). On the behavioral level, we found an increase of learning performance in a bimodal paradigm compared to a unimodal paradigm (Fig. 2). Thus, a relevant next step to understand the integration on a neuronal level better, would be to record MBONs during parallel presentation of different stimuli. To further understand the effects on memory formation, the training effects at the MB output level need to be compared between uni- and multimodal trained animals.

Anatomical functional structures for sensory processing in cockroaches have been already reported from the antenna over the antennal lobe to the MB calyces and for the Kenyon cells that project through the pedunculus and the MB lobes (Nishikawa et al., 1998; Strausfeld and Li, 1999b; Nishino et al., 2012; Watanabe et al., 2017; Paoli et al., 2020). Intracellular recordings with simultaneous stainings of MBONs and the cockroach specific calyceal giant feedback neurons indicate structural and functional separation at the MB output level (Takahashi et al., 2019). Few exemplary interdependencies between sensory inputs like the effect of vinegar on CO2 avoidance that is encoded in fruit fly MBONs are stated (Lewis et al., 2015), but the full picture of sensory integration on the MB output level remains an open question.

In this work, we mainly focused on the representation of visual and olfactory stimuli in the MB output (Arican et al., 2022; Chapter III). We found differences in the representation between the modalities, which seem not to encode stimulus identity. During olfactory stimulation we could detect feeding behavior on a single trial level, that correlated strongly with the neuronal responses (Arican et al., 2022; Fig. 4a-c). This indicates that MBONs encoded information that is related to the feeding behavior. To investigate the initial feeding response to the odors, we used untrained animals. Only few other studies used naïve insects to investigate the functions of the MB output, but found various information that seems to be encoded there. In honey bee MBONs, responses dependent on intensity and general on-/off-status of visual stimuli were reported (Schmalz et al., 2022). In fruit flies, it is argued that MBONs encode odor valence (Aso et al., 2014b). Another discussed area that is involved in internal valence coding in insects is the lateral horn that also interacts with MBONs (Sachse and Beshel, 2016; Dolan et al., 2018, 2019; Das Chakraborty et al., 2022).

Behavioral outputs are highly affected by external and internal states (Cohn et al., 2015; Devineni and Scaplen, 2022). The underlying networks are often located at the MB output level (Bräcker et al., 2013; Lewis et al., 2015; Hattori et al., 2017; Tsao et al., 2018; Sayin et al., 2019; Siju et al., 2020). A more frequent investigated topic in MBONs is learning and memory (Strube-Bloss et al., 2011, 2016; Hige and Turner, 2015; Owald and Waddell, 2015). In this work, we established the behavioral foundation for further investigation of the underlying neuronal mechanism of learning and memory. Using the advantages of our methods and of cockroaches as model organism, the following aspects could be examined on a neuronal level in the context of memory formation: (1) integration of multimodal sensory inputs leading to changes in learning rates (Fig. 2), (2) inter-individual differences between learning and not learning animals (Arican et al., 2020, Fig. 5) and (3) the differences between learned and not learned tasks within an individual (Fig. 4).

All aspects that were discussed in this section are attributed to the MB and lead to behavioral output. Anatomical connections between MBONs and premotor areas (Li and Strausfeld, 1997, 1999; Okada et al., 2003; Aso et al., 2014a) and even direct connections to descending neurons (Hsu and Bhandawat, 2016; Emanuel et al., 2020; Li et al., 2020a) were stated in several insect species. Thus, the driving question is whether and how MBONs are involved in the sensory-motor process. We were able to predict the feeding response with high accuracy in a machine learning approach, trained and tested with behavioral and electrophysiological data during olfactory stimulation (Arican et al., 2022, Fig. 4h). Others also found correlations between MB output activity and motor responses (Mizunami et al., 1998a; Okada et al., 1999; Aimon et al., 2022), but with the high temporal resolution of our setup, we were able to add temporal aspects to our analyses. We found a wide range of neuronal response latencies, but interestingly also very fast responses. Fast responses, as also seen in honey bees, are necessary for rapid categorization of the input, leading to fast behavioral responses (Strube-Bloss et al., 2012, 2021). Usually, neuronal responses were faster than the behavioral responses (Arican et al., 2022, Fig. 4e-g), which strengthens the hypothesis that MBONs play a relevant role in the sensory-motor transformation process.

It remains a challenge to understand the whole mechanistic background of the integration processes between all the external sensory inputs and the various internal states and how this leads to behavior that is plastic at the same time. There is a lot of evidence that at least a subpopulation of MBONs plays a major role in these processes and we contributed to these topics with our established methods.

The cockroach as complementary model organism for the study of the mushroom body

In this study, we decided to work with cockroaches not only to have an additional comparable insect model organism, but also to use the advantages that cockroaches have over other species. Their robustness guarantees a higher success rate for extracellular recordings and makes long-term recordings from the same animal possible. The method of extracellular recordings was chosen to enable recordings of MBON activity simultaneously with the behavior. Insect studies that simultaneously record neuronal and behavioral responses were rarely done and if so usually calcium imaging was used (Seelig et al., 2010; Aimon et al., 2022). Calcium imaging has the huge disadvantage of low temporal resolution, whereas extracellular recordings have a high temporal resolution, which is necessary to align the recordings with fast responses like the MLR in cockroaches (Arican et al., 2022, Fig. 2b, 4e-g).

In the late 1990s the differential extracellular recording method was used to record from freely moving cockroaches (Mizunami et al., 1998a; Okada et al., 1999). In these studies, they made use of the robustness of the cockroaches and the fact that they do not attempt to fly under standard laboratory conditions. For the recording, they used long copper wires and the cockroaches could move freely in an X-shaped arena. This could be an easily feasible next step without the need to set up things like treadmills or other workarounds for physiological recordings from freely moving animals. However, the maze we used in purely behavioral experiments could be improved to have a better readout than we had with the binary choice in the T-maze (Arican et al., 2020). For example, the maze could be improved by adding more target chambers (Laupraset et al., 2006; Khoobdel et al., 2021) or an airstream as discussed above.

In a first step, we decided to work with fixed cockroaches, because previous studies already showed that it is possible to train harnessed cockroaches (Watanabe et al., 2003; Kwon et al., 2004; Lent and Kwon, 2004). We decided to work mainly with olfactory stimuli, because cockroach olfactory receptor neurons are precise in their respondence (Tichy et al., 2020a), which should lead to a precise odor representation in the animals. Since cockroaches are capable of perceiving visual stimuli (Goldsmith and Ruck, 1958; Mote and Goldsmith, 1970) we could use it as a comparable modality for further investigations. Visual stimuli have the advantage that they are easier to control and can have a sharp on- and offset easily compared to olfactory stimuli. During the experiments, we realized the high sensitivity of cockroaches to mechanical stimuli while we tried to minimize the mechanical stimulus during the odor presentation (Arican et al., 2022; Chapter III). During behavioral experiments, in which we compared uni- and bimodal conditioning (Fig. 2), we observed the relevance of the mechanical stimulus. The sensitivity might be due to their relatively long, thin and flexible antennae and signal danger, which might elicit fleeing behavior like when they are exposed to light (Okada and Toh, 1998; Laurent Salazar et al., 2013). More experiments are needed to understand the interdependency between the olfactory and the mechanical stimuli and the general processing of mechanical stimuli. Further, we aimed the region of the MB β lobe as recording site because of the easier accessibility than the MB α lobe. The β lobes are positioned more central, whereas the α lobes are closer to the ocelli and the compound eyes (General introduction, Fig. 2).

The next step will be to train harnessed cockroaches with classical conditioning paradigms that we established for olfactory (Arican et al., 2020) and visual (Fig. 3) stimuli, during extracellular recordings of MBONs. For the recordings in this study, we automated everything from sensory stimulation through electrophysiological and behavioral recordings. We developed a computer-controlled odor supply system (Arican et al., 2020) and a computer-controlled visual stimulation device (Arican et al., 2022) with the help of the workshop of the Department of Biology and an electrical engineer of the Institute of Zoology at the University of Cologne. The stimulation devices and the camera to record the behavior were all controlled by the same software (Spike 2, CED, Cambridge, UK), to guarantee error-free alignment of the neuronal and behavioral recordings to the stimulation. To better exploit the already high temporal resolution of the neuronal recordings, the camera is already updated for future experiments with higher framerates (90 fps, Basler ac2040-90um, Ahrensburg, Germany). Another future project will be to establish a tool for automated MLR detection, potentially we would use DeepLabCut (Mathis et al., 2018; Nath et al., 2019) for this approach. This would save time and could provide more specific data about the strength of the movement. To do noise-free extracellular recordings during conditioning and to avoid possible error sources by manual conditioning in the electrophysiological setup, we developed a computer-controlled pump to provide liquids as US. The pump can be controlled with Spike 2, to have aligned timing with the physiological and behavioral recordings. Therefore, a micropump (mp6-liq, Bartels, Dortmund, Germany) that can release little amounts of liquids was used. With that pump single drops of sugar solution can be provided to the cockroaches, to have a closed loop conditioning and recording setup.

Conclusion

In this work, we established behavioral and electrophysiological setups for cockroaches to make use of the advantages of these animals. We investigated the behavior of trained animals with focus on inter-individual differences and investigated sensory processing of the MB output leading to innate behavior in individual animals and compared different sensory modalities.

This work emphasizes the ability of cockroaches to learn in different paradigms with different kinds of sensory modalities. Further, we confirm the importance of inter-individual differences in insects and stress the existence of this difference in task dependent cognitive abilities (Arican et al., 2020).

Behavioral and neuronal recordings during olfactory stimulation showed valence dependent responses on both levels. Further analysis revealed the predictability of the behavior based on the neuronal data, indicating the relevance of MB output in the sensory to motor transformation (Arican et al., 2022).

Visual stimulations demonstrated that some MBONs respond to on- and offsets, some behave dependent on the stimulus intensity and some adapt to fast repetitions of visual stimuli. Comparing the representation of different sensory modalities in MBONs, we found stronger differences between the modalities than within the stimuli (Chapter III).

With this we contributed to the overall understanding of sensory processing and the formation of innate and adaptive behavior.

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Appendix

Contribution statement

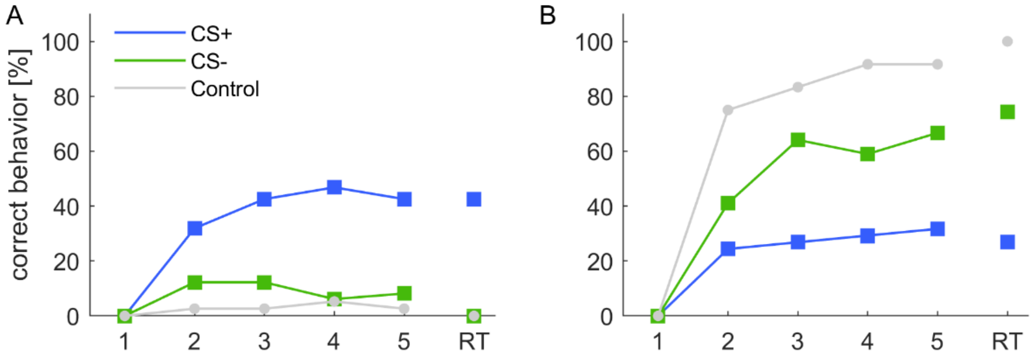
All research ideas were generated in cooperation with Prof. Dr. Martin P. Nawrot and me. The data were mostly collected by me. Part of the data in Chapter I was collected by students supervised by Prof. Dr. Martin P. Nawrot and me. Most of the data analyses were made by me. Felix Schmitt contributed to the data analysis of Chapter II. Figures were all generated by me. The written text in Chapter I was generated with Prof. Dr. Martin P. Nawrot, Dr. Nina Deisig and me. The written text of Chapter II was generated by Prof. Dr. Martin P. Nawrot, Felix Schmitt and me. The written text of Chapter III was generated only by me.

Data accessibility statement

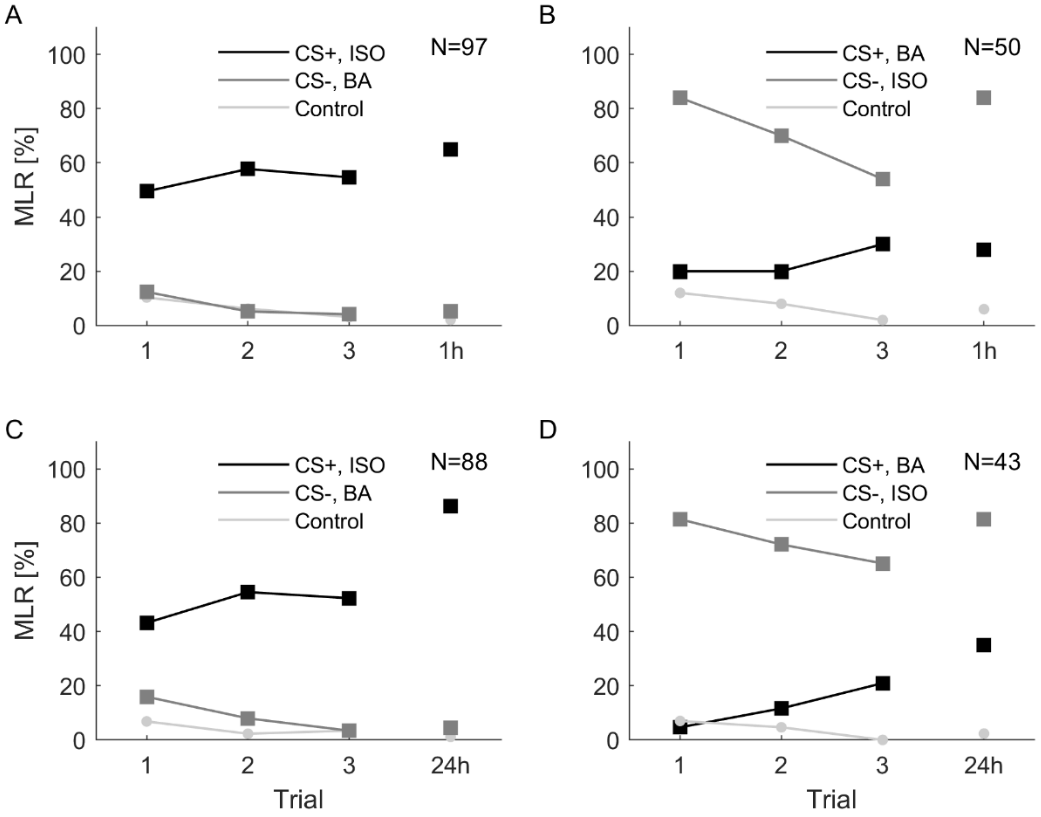
All data are stored on lab intern data servers. The datasets generated for the published studies are available on request to the corresponding author.

Supplemental material - Chapter I

Supplementary Material



Supplementary Figure 1 Classical olfactory conditioning over five trials with a memory retention test (RT) after 10 minutes. A) Animals that did respond spontaneously in the first trial to the respective stimulus were excluded. The correct behavior to CS+ (blue) increased significantly over trials (one-way ANOVA: $p < 0.001$, N = 47) while the behavior towards the CS- (green) and control (gray) odors did not change over trials (CS-: p = 0.133, N = 49; control: p = 0.394, N = 76). B) Animals that did not respond spontaneously in the first trial to the respective stimulus were excluded. The correct behavior to CS- increased significantly over trials (one-way ANOVA: $p < 0.001$, N = 39). Unexpectedly, the behavioral expression also increased (the expression of the MLR decreased) towards the CS+ (one-way ANOVA: p = 0.003; N = 41) albeit to a lesser extent and mostly from trial one (spontaneous response) to a rather constant level represented by a small fraction of all animals. This might be explained to some extent with spontaneously responding non-learners that expressed spontaneous responses towards the CS+ odor with a low probability or with the satiety state of the animals. The observed decrease of the MLR towards the control odor is expected, as this odor was not rewarded. In many protocols for differential conditioning the CS+ odor is rewarded and the CS- odor is not rewarded which results in a decrease of the conditioned response to the non-rewarded odor (e.g. Pamir et al., 2011). Since, the sample size is too low we could not test for an increase in correct responses to the control odor (N = 12). In our experiments this corresponds with the reduction of the MLR (increase of correct behavior) towards the control odor.

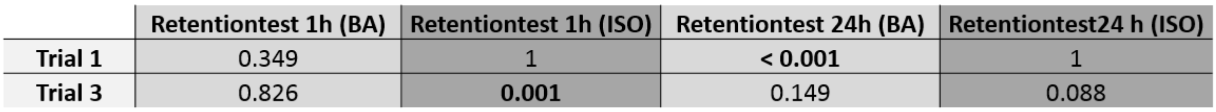


Supplementary Figure 2 Classical olfactory conditioning over three trials with memory retention tests after 1 h and 24 h. A) Isoamyl acetate (ISO, black) was used as CS+, butyric acid (BA, dark gray) was used as CS- and cinnamaldehyde (light gray) was used as control. The retention test was after 1 h. B) BA (black) was used as CS+, ISO (dark gray) was used as CS- and cinnamaldehyde (light gray) was used as control odor. The retention test was after 1h. C) ISO (black) was used as CS+, BA (dark gray) was used as CS- and cinnamaldehyde (light gray) was used as control odor. The retention test was after 24 h. D) BA (black) was used as CS+, ISO (dark gray) was used as CS- and cinnamaldehyde (light gray) was used as control odor. The retention test was after 24 h**.**

Supplementary Table 1 Comparison between training trials and retention tests. Isoamyl acetate (ISO) was used as CS+ and butyric acid (BA) was used as CS-. Data is depicted in Supplementary Figure 1 A&C. Chi² test was used to analyze differences in the number of conditioned responses between retention tests and the respective trial 1 or 3 and p-values are listed. Numbers in bold are < 0.05 and indicate significant difference between the training trial and retention test.

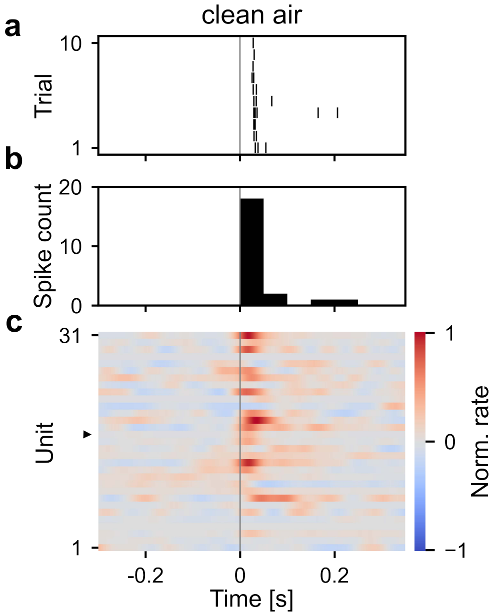
	Retentiontest 1h (ISO)	**Retentiontest 1h (BA)**	**Retentiontest24 h (ISO)**	**Retentiontest 24h (BA)**
Trial 1	**0.03**	0.076	**< 0.001**	**0.0129**
Trial 3	0.143	0.733	**<0.001**	0.7

Supplementary Table 2 Comparison between training trials and retention tests. Butyric acid (BA) was used as CS+ and isoamyl acetate (ISO) was used as CS-. Data is depicted in Supplementary Figure 1 B&D. Chi² test was used to analyze differences in the number of conditioned responses between retention tests and the respective trial 1 or 3 and p-values are listed. Numbers in bold are < 0.05 and indicate significant difference between the training trial and retention test.

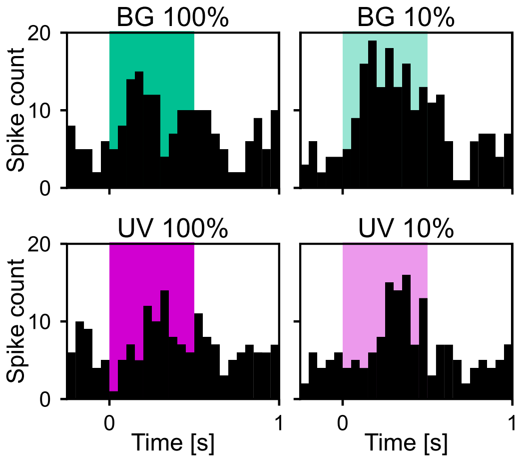


	Retentiontest 1h (BA)	Retentiontest 1h (ISO)	Retentiontest 24h (BA)	Retentiontest24 h (ISO)
Trial 1	0.349	1	**< 0.001**	1
Trial 3	0.826	**0.001**	0.149	0.088

Supplemental material – Chapter III



Supplementary Figure 1 Single unit and population response to a mechanical stimulus. **a** Spike raster plot of an exemplary unit during repeated presentation of the mechanical stimulus in the control (clean air) trial of the olfactory stimulation protocol. **b** Peristimulus time histogram across 10 trials (shown in a, binwidth = 50 ms). **c** Trial-averaged normalized firing rates estimated with a Gaussian kernel during mechanical stimulation. The color code represents changes in the firing rate over time. Triangle (▶) indicate unit 17 that is depicted in a and b. The gray line in all subplots indicates the onset of the mechanical stimulus.



Supplementary Figure 2 Exemplary peristimulus time histogram (binwidth = 50 ms) of unit 25 (related to Fig. 2c) across 10 trials during stimulation. Color shaded areas depict time windows of stimulus presence with bluish green (BG) and ultraviolet (UV) light in two relative intensities (10%, 100%).

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Erklärung zur Dissertation

gemäß der Promotionsordnung vom 12. März 2020

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Teilpublikationen:

Arican, C., Bulk, J., Deisig, N., and Nawrot, M. P. (2020). Cockroaches show individuality in learning and memory during classical and operant conditioning. *Front. Physiol.* 10, 1–14. doi:10.3389/fphys.2019.01539.

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