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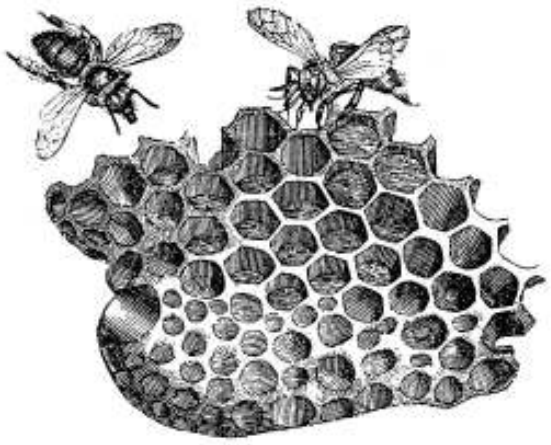


INTRODUCTION

1. Learning and Memory: Basic Definitions

In the course of life, animals acquire experiences, which are an indispensable part of their survival. These experiences increase an animal's knowledge about its environment and allow producing adaptive behavior upon future encounters with the events that generated such experiences. Learning and memory are the two basic capacities that articulate this scenario: ***Learning*** is any relatively permanent change in response that occurs as a result of experience acquired individually (Bitterman et al., 1979). Such acquisition may lead, depending on factors such as the number of experiences gathered, their spacing in time, etc, to the formation of ***memory***, which is defined as the capacity to encode, store and retrieve the acquired information in the nervous system (Ebbinghaus, 1913). Different types of memories are established by variations in experimental procedures (e.g. repetition during training, massed vs. spaced trials, etc.), which are distinguishable in terms of their duration (i.e. stability), contents and resistance to extinction (Ebbinghaus, 1885). In this sense, a basic distinction acknowledges that memory is organized in at least two forms: a transient and unstable ***short-term memory (STM)***, and a robust, long-lasting ***long-term memory (LTM)***. Both memory types exhibit distinct temporal courses and underlying molecular processes (Kandel, 2001).

Associative learning is defined as the capacity to learn the predictive links existing between related events in an animal's environment. It allows to extract the logical structure of the world and thus to reduce uncertainties of future events (Pearce, 1987; Rescorla, 1988). Two main paradigms of associative learning are usually distinguished. One is the paradigm of ***classical (Pavlovian) conditioning*** (Pavlov, 1927) and the other is the paradigm of ***operant or instrumental conditioning*** (Skinner, 1938). While the former relies on learning stimulus – stimulus associations (Pavlov, 1927), the latter relies on acquiring associations linking actions and specific outcomes (reinforcements) of these actions (Skinner, 1938). In classical conditioning the action of the animal is irrelevant for the contingency between stimuli to be acquired; in operant conditioning, on the contrary, it is the action of the animal that determines the occurrence of reinforcement. A considerable amount of literature has been produced on these two learning forms, which have been studied in invertebrates and

vertebrates, including humans. In the following, I will focus exclusively on classical conditioning as it constituted the main experimental framework for most of my works.

1.1. Classical Conditioning: Principles and Definitions

This conditioning form was first discovered by Ivan Pavlov. Pavlov was a Russian physiologist, who at the beginning was not interested by learning but by the physiology of digestion, which he studied using dogs as experimental animals. During his studies, he discovered by chance that dogs began salivating when his assistant came into their sight, and before they received their food. Since the salivary response is an innate response, Pavlov suggested that the salivation in the presence of the assistant represented an acquired

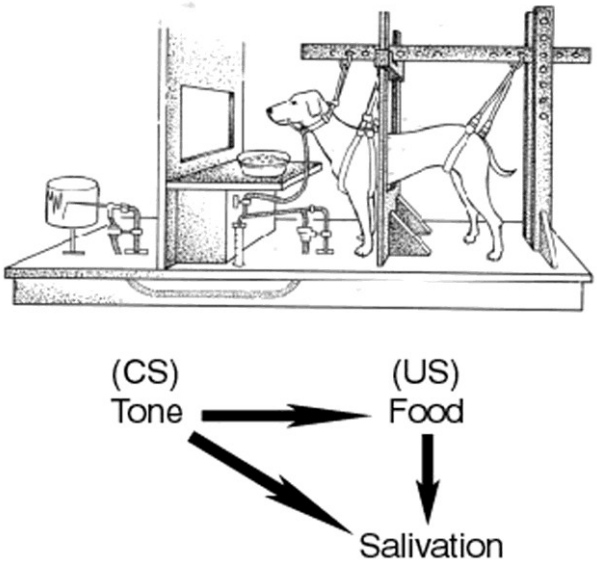


Figure 1: Illustration of the classical conditioning experiment conducted by Pavlov. The upper part shows the harnessing setup with the experimental subject. The lower part shows the scheme of associations underlying Pavlovian conditioning and leading either to innate salivation (US -> Salivation) or to acquired (conditioned) salivation (CS -> Salivation).

expectation of the food. To test this hypothesis, Pavlov made his subject, a harnessed dog (Fig. 1), to associate the food, evoking the reflex of salivation, with a neutral stimulus unable to evoke it, which was the sound of a metronome. The saliva was collected through a tube and the experimenter could observe and record the salivary response from behind the screen.

Pavlov paired the presentation of both stimuli, sound and food, in such a way that the sound of the metronome always anticipated the food (forward pairing) in order to create the food expectation. After experiencing the sound-food pairings several times, the dogs learned to salivate to the sound of the metronome itself, thus showing the acquisition of a novel response as a consequence of the training. Pavlov then termed the different components of his protocol as: 1) Unconditioned Stimulus (US), the biologically relevant stimulus eliciting an

innate, reflexive response (here the food); 2) Unconditioned Response (UR), the reflexive response elicited by the US (here salivation to the food); 3) Conditioned Stimulus (CS), the stimulus that is originally neutral but that acquires the capacity to evoke a learned response (here the sound of the metronome); 4) Conditioned Response (CR), the learned response to the CS (here salivation to the metronome sound).

Thus in his work on classical conditioning, Pavlov concluded that the basis of classical, Pavlovian conditioning resides in the fact that subjects learn to associate an originally neutral stimulus (conditioned stimulus – CS) with a biologically significant stimulus (unconditioned stimulus – US) that elicits a reflexive response (unconditioned response) (Pavlov, 1927). Originally, the CS does not elicit a behavioural response but once an association between the US and the CS is established, the response to the CS will resemble the response to the US.

Different variants of the basic Pavlovian scheme can be conceived. In its basic form (see above), the animal learns a simple link between a CS and a US (CS+, with + indicating the presence of the US), a situation termed *absolute Pavlovian conditioning.* In *differential Pavlovian conditioning,* the animal learns that one CS (CS_1) is reinforced, while another CS (CS_2) is non-reinforced (CS_1+ vs. CS_2-). In the former case, an animal has to learn to respond to CS+ alone, which is unambiguously associated with reinforcement; in the latter, it has to learn to respond to CS_1+ and not to CS_2- because both are unambiguously associated with reinforcement and with the absence of it, respectively.

1.2. Classical Conditioning in Invertebrates

Decades of research have established invertebrates as standard models for the study of classical conditioning. This is because invertebrates learn simple associations and have a relatively simple nervous system that allows associative phenomena to be traced to the cellular and molecular levels in different kinds of laboratory preparations (Giurfa, 2013).

Aversive classical conditioning, for example, has been studied in the mollusc *Aplysia.* In this sea slug, skin sensory neurons make direct synapses onto motor neurons that control the defensive gill withdrawal reflex; upon a light touch to the naïve animal's siphon, these

sensorimotor synapses fail to transmit and the gills are not withdrawn. If, however, the touch is repeatedly paired with a noxious stimulus, such as an electric shock, that does elicit gill withdrawal, the light touch alone eventually comes to elicit a gill withdrawal, whereas, before such training, it did not (Kandel et al. 1979; Walters et al. 1979; Carew et al. 1981; Hawkins et al. 1983; Abrams and Kandel 1988; Hawkins et al. 1989; Byrne et al. 1990; Hawkins et al. 1998).

Classical conditioning has also been intensively studied in the fruit fly *Drosophila melanogaster*. Flies can easily be trained to associate an odor (the CS) with an aversive electric shock (the US). The typical procedure consists in training groups of flies alternatively presented with two different odours, one paired with an electric shock (CS_1+), and another non-paired with the shock (CS_2-) (Tully and Quinn, 1985). Retention is measured in a T-maze where conditioned flies must choose between the CS_1+ and the CS_2-. Note, however, that despite its recurrent description as a Pavlovian protocol, the procedure employed involves operant components as the flies freely move within the maze and their actions determine therefore whether a shock will be experienced or not. Although flies certainly associate the odours as CS with the shock or absence of shock as US, the protocol does not exclude the occurrence of operant learning.

On the contrary, true Pavlovian learning can be studied in another preparation conceived for the honeybee *Apis mellifera*, an insect that has emerged as a powerful model for the study of learning and memory (Giurfa, 2003, 2007; Hammer, 1993, 1997; Menzel, 1999, 2001). Similarly to the reflexive salivation of dogs, an appetitive reflex, the **proboscis extension response** (henceforth PER), was known to occur in the bee (Frings, 1944; Frings and Frings, 1949), and other insects (Minnich, 1921, 1926) upon stimulation of gustatory organs, e.g. the antennae, tarsi or mouth parts, with sugar solution. In the fifties, a Japanese researcher, Matsutaro Kuwabara, realized that this appetitive response could be conditioned in harnessed bees using visual stimuli as CS and sucrose solution delivered to the tarsi as US (Kuwabara, 1957). However, his work did not reach broad impact probably due to the fact that a critical procedural aspect to follow for this protocol to work was the necessity to cut the antennae of the bees prior to conditioning. Indeed, Kuwabara mentioned that bees in his protocol started extending the proboscis to the sucrose solution before it reached the antennae or mouth parts. This was an undesirable effect as the US was supposed to elicit PER only upon contact. He speculated that this effect was due to the presence of hygroreceptors on the

antennae, which sensed the approaching aqueous mass of sugar solution delivered on a small spoon. Therefore, he decided to cut the bees' antennae to avoid this problem, and to elicit the response by stimulating the tarsi with sucrose solution. Depriving the bees of their antennae is not necessarily an ideal handling of the experimental subjects. A damaged animal will probably be less responsive than an intact animal. The low acquisition rates observed in antennae-deprived bees despite long conditioning procedures (Hori et al., 2006; Hori et al., 2007) may be related to this fact. Later, as described below, an olfactory version of this protocol was developed, which allowed to study Pavlovian olfactory learning to an unprecedented level of detail (Bitterman et al., 1983; Takeda, 1961) (see below). This achievement contributed, in part, to the success of the honeybee as one of the most popular models in the neurosciences of learning and memory.

2. The Honey Bee as a Model for the Study of Learning and Memory

The study on honey bee behaviour was pioneered by Karl von Frisch (1886 – 1982). He became famous for the discovery of the honey bee dance, a communication behaviour where a successful forager transmits information to other foragers within the hive about the distance and direction of a profitable food source (von Frisch, 1967). In a natural context and despite their small size, honey bees exhibit an extremely rich behavioural repertoire. At the social level, honey bees exhibit reproductive division of labour (with sterile and reproductive castes), generational overlap and cooperative brood care (Wilson, 1971). During their lifetime, exhibit 'caste polyethism' a term used to indicate that individuals go through different caste stages and perform different tasks at different ages (Robinson and Page, 1989; Wilson, 1971). A newly emerged bee perform the most basic task which is cell cleaning. It progresses with being a nurse where the individual takes care of the queen and feeds larvae. Afterwards the individual starts being a guard gathering first experiences with the outer world. Usually around the 2nd week of life, the bee becomes a forager. During this phase, the bee will start experiencing outer environment and actively uses its cognitive abilities to navigate between the hive and the food sources and to learn and memorise various food-related stimuli. To this end, it exhibits of a rich sensory perception and developed motor performances.

Indeed, bees see the world in colour (Menzel and Backhaus, 1991), perceive shapes and patterns (Giurfa and Lehrer, 2001; Srinivasan, 1994) and resolve movements with a high

temporal resolution (Srinivasan et al., 1999). Their olfactory sense is able to distinguish a large range of odours (Guerrieri et al., 2005) and the mechanosensory perception is also extremely rich due to thousands of hair cells all around the body and proprioreceptors inside the body (Dacher et al., 2005; Erber et al., 1998; Scheiner et al., 2005).

More importantly, the learning capacities of honey bees, which set the basis for their floral constancy, i.e. the fact that bees remain truthful to the same flower species as long as it offer profitable nectar or pollen reward, are amenable to the laboratory for detailed study. After almost a century of honey bee research, different protocols have been established to assess the bees' learning and memory capabilities (Giurfa, 2007). Here we will focus on one of these protocols, the olfactory conditioning of the PER (Bitterman et al., 1983; Takeda, 1961).

Yet, before presenting the current knowledge on honey bee learning and memory gained through the olfactory conditioning of PER, I will focus on innate US responsiveness and show how the PER preparation allowed characterizing in detail different aspects of how bees respond to the sucrose reward, and how this analysis provided important insights into the organization of labor within the colony and the individual learning itself.

2.1. Sucrose Responsiveness in Honey Bees

Long before research on PER conditioning started, it was well known that PER could be elicited by stimulating gustatory organs like the antennae, tarsi or mouth parts with sugar solution. The PER had thus been detected in bees (Frings, 1944; Frings and Frings, 1949), flies (Minnich, 1926) and butterflies (Minnich, 1921) among others. Much later, the spontaneous PER upon sucrose stimulation was used to quantify the appetitive responsiveness of bees (i.e. their tendency to respond to sucrose of different qualities) without involving any learned component (Page et al., 1998). This is done by presenting restrained bees with increasing sucrose concentrations delivered to the antennae and determining either the lowest concentration from which the bee starts responding, discriminating it from water (sucrose threshold), or the number of sucrose concentrations to which it responds (sucrose score).

This quantification allowed differentiating nectar- and pollen foragers in terms of their different responsiveness to sucrose (Page and Erber, 2002; Pankiw and Page, 1999; Scheiner

et al., 2004). Nectar foragers exhibit higher thresholds (i.e. lower responsiveness) than pollen foragers, which exhibit lower thresholds and thus higher responsiveness (Page et al., 1998). Although this difference may appear counterintuitive at first sight, the currently accepted explanation is that nectar foragers are more selective when collecting nectar, and thus only respond to the highest sucrose concentrations, which provide the highest energy gain to the individual and the colony. Sucrose responsiveness thresholds have been shown to vary with multiple factors such as age, caste, sex, (Amdam et al., 2006; Page et al., 1998; Pankiw and Page, 1999; Scheiner et al., 2001a, b), foraging experience, genotype, feeding status (Pankiw and Page, 2001), and season (Scheiner et al., 2003), among others.

Later works showed that pollen foragers, being more responsive to sucrose, perform better than nectar foragers during the appetitive PER conditioning (Pankiw and Page, 1999; Scheiner et al., 1999; Scheiner et al., 2001a). This result reflects the fact that the subjective value of sucrose reward, which can be estimated via PER responsiveness, may vary between bees. Thus, the same sucrose concentration may be efficient to support conditioning in some bees while it may not be rewarding enough for other bees which will learn less efficiently when provided with this concentration as US (Scheiner et al., 2005). *Summa summarum*, bees showing higher sucrose responsiveness perform better during the associative appetitive PER conditioning. In other words, sucrose responsiveness allows us to dissect the effects of genotype and division of labour on associative appetitive learning.

As an important neuronal signalling component, and in facilitating or depressing behavioural responsiveness, three biogenic amines have been examined for their role in the modulation of sucrose responsiveness. These are octopamine, tyramine and dopamine (Scheiner et al., 2002). Modulation was tested by injecting these substances into the bees and determining if and how sucrose responsiveness changed. Octopamine and tyramine increased sucrose responsiveness whereas dopamine decreased it. The decrease induced by dopamine was also induced by a dopamine receptor agonist ADTN (Scheiner et al., 2002).

2.2. The Appetitive Olfactory Conditioning of PER in the Honey Bee

As mentioned above, besides providing insights into sucrose responsiveness PER was used as reflex to be conditioned in a Pavlovian protocol first established by Takeda (1961) who was inspired by the early work of Kuwabara (1957; see above). Kuwabara had reported that PER

can be conditioned using colours as CS. Takeda decided to replace colours by odours and set the basis for the olfactory conditioning of PER.

In this protocol, each bee is restrained in an individual harness so that it can only freely move its antennae and mouth-parts (mandibles and proboscis) (Fig. 2).

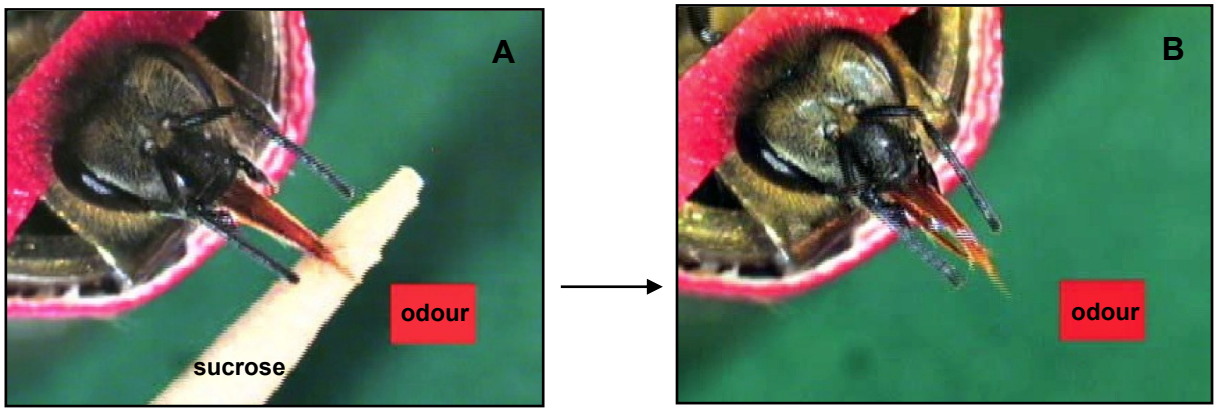


Figure 3: Appetitive olfactory conditioning of proboscis extension reflex (SER). **A)** A harnessed bee is trained with paired presentations of an odorant (CS) blown to the antennae and the sugar reward (US) delivered on a toothpick, following an absolute – conditioning design (a single odorant reinforced). **B)** After a successful conditioning, the bee extends its proboscis to the odour CS alone, which has been learned as predictor of the sucrose US.

The antennae are the bees' main chemosensory organs. When the antennae of a hungry bee are touched with sucrose solution, the animal reflexively extends its proboscis to reach out to and suck the sucrose (PER). Thus, the sucrose solution inducing the PER is the appetitive US. In the case of classical conditioning, a bee learns to associate the US with a neutral odorant which is blown to the antennae. The odorant does not release such a reflex in naive animals and serves as the CS. The coupling of the CS and the US in a forward-pairing manner results in acquisition of the conditioned odorant (Fig. 2).

The protocol has emerged through the years as a unique tool to access the neural and molecular bases of Pavlovian learning and memory in honey bees as it allowed dissecting the neural circuits underlying CS (odour) and US (sucrose) processing (Giurfa and Sandoz, 2012). Before describing these circuits it is important to provide a general overview of the honey bee brain.

2.3. The Architecture of the Honey Bee Brain

The brain of a honey bee (Fig. 3) has a volume of approximately 1 mm^3 and contains around 960.000 neurons (Witthöft, 1967). In general, the honey bee brain is divided into the

subesophageal ganglion (SEZ) and the supraesophageal zone (SPZ) (Ito et al., 2014). The SPZ comprises several main regions or neuropiles, such as the antennal lobes (AL), the optic lobes (OL), the mushroom bodies (MB), a vast region surrounding the MB generally termed as the protocerebral lobes (PL), a small lateral protrusion of the PL, known as the lateral horn (LH), and the central complex (CX). ALs are the primary olfactory neuropiles and receive direct inputs mainly from chemoreceptors located on the antennae. The OLs are visual processing neuropiles receiving information from the photoreceptors. The MBs occupy approximately 30% of the brain and are higher-order integration centres exhibiting segregated multimodal input and integrated multimodal output; these structures have been historically associated with the presence of memory traces, in particular long-term ones (Menzel, 1999; Menzel and Giurfa, 2001). The PL exhibits also a multimodal organization, with visual and olfactory afferences arriving at different regions of this structure. The CX seems to be involved in different forms of visual processing, from patter recognition (Liu et al., 2006), to polarized light processing (Heinze et al., 2009; Heinze and Homberg, 2007; Homberg et al., 2011) and visual-course setting (Neuser et al., 2008; Strauss, 2002), among others.

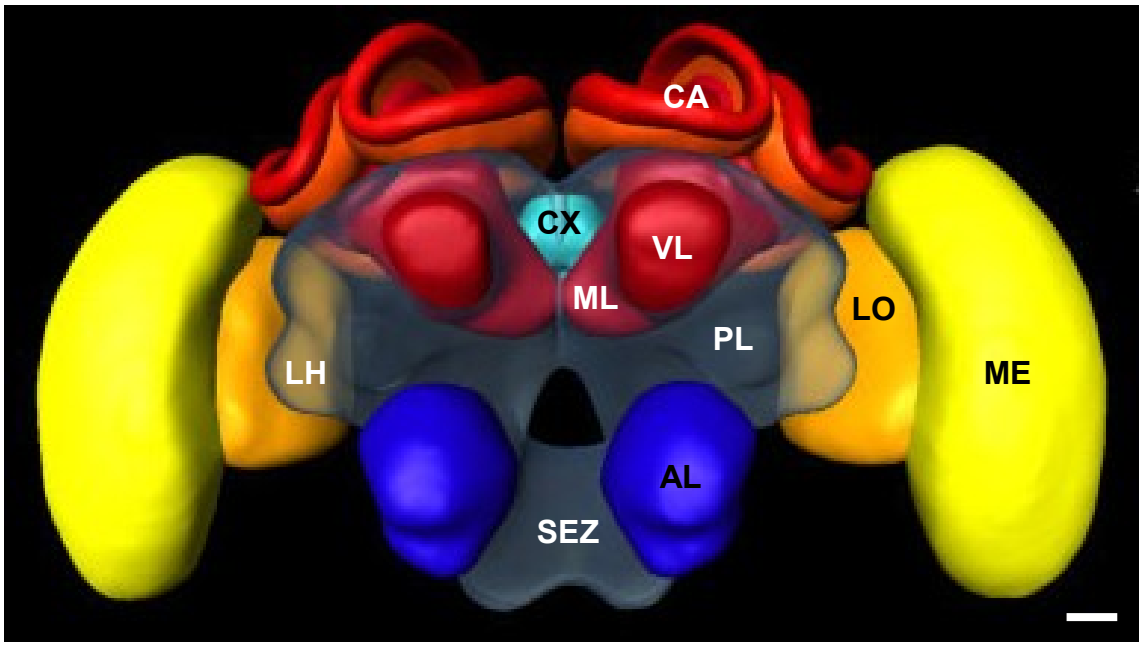


Figure 3: 3D-reconstruction of a honey bee brain. This frontal view depicts a honey bee brain with its neuropiles. AL: antennal lobe; CA: calyx of the mushroom body (MB); VL: vertical lobe; ML: medial lobe; CX: central complex; LO: lobula of the optic lobe (OL); ME: medulla; PL: protocerebral lobe; LH: lateral horn; SEZ: subesophageal zone. Scale = 100 µm; adapted from Brandt et al. 2005.

The AL receives input from 60,000 chemosensory receptor neurons located in specialized cuticular structures (sensilla) on the antennae (Esslen and Kaissling, 1976; Menzel and Muller, 1996). The AL consists of approximately 160 globular structures termed glomeruli, whose number corresponds to that of the olfactory receptor genes recently indentified on the honey bee genome (Robertson and Wanner, 2006). Neurons with the same receptor class project to the same glomerulus. Each glomerulus is a localized site of synaptic interactions between different cell populations: the afferences from olfactory receptors, the lateral connections between glomeruli provided by local inhibitory (GABAergic, histaminergic) interneurons (LIN) and the efferent projection neurons (PN), which connect the AL with the MB, several regions in the PL and the LH. PNs run in three distinct main fibre tracks, namely the antenna-cerebral tracts, currently termed as medial, lateral and mediolateral antennal lobe tract (m-ALT, l-ALT, and ml-ALT) (Abel et al., 2001; Kirschner et al., 2006).

The OL is formed by three succeeding neuropiles: the lamina (LA), the medulla (ME) and the lobula (LO) (Kien and Menzel, 1977b). These are the main centres of visual processing in the bee brain receiving and processing information from the photoreceptors located within ommatidia in the compound eyes.

The lamina is the first visual neuropile, in which the axons of the photoreceptors connect to first order processing interneurons, the lamina monopolar cells (LMC). The LA is made of thousands of optical cartridges, each receiving an axon bundle (containing the nine photoreceptor cell axons) from the overlying ommatidium, as well as the axons of four different types of monopolar cells. Additionally, tangential, centrifugal and horizontal fibers can be found within each cartridge. The spatial arrangement of photoreceptor axons and LMCs within a cartridge remains constant throughout the lamina, thus retaining the retinotopic organization.

The outer chiasm (Ribi, 1974) forms the connection between the LA and the second visual neuropile, the ME. Fibres coming from the anterior part of the LA project to the posterior ME while posterior fibres from the LA project to the anterior ME. The retinotopic organization is retained but it is reversed in the medulla. ***The medulla*** is also built of distinct cartridges, receiving the axons of long visual fibres (UV receptors) and from the four LMC of the corresponding cartridge in the LA. Processing of spectral information in the distal part of

the medulla is not understood yet, but it seems that neurons already respond with spectral opponency (Kien and Menzel, 1977a), which sets the basis for their colour vision capacities.

The direct connection between the medulla and the third optic ganglion, ***the lobula (LO)***, is formed by monopolar cells from the ME, which constitute ***the inner chiasm***. In much the same way as the outer chiasm, neuronal fibres cross the horizontal plane, so that fibres coming from the posterior part of the medulla project to the anterior part of the LO, and vice versa. Therefore, the LO presents the same retinotopic arrangement of visual cartridges as the LA. Neurons responding to directional movement, but also double spectral-opponency neurons, seem to be present in the lobula (Hertel and Maronde, 1987). These neurons project either to the MBs on both brain sides (lobula tract), to the Anterior Optic Tuberculum (AOTU), or build the great commissure (GC) connecting both eyes. Recently, our group has been able to achieve the first optophysiological recordings of neural activity at the neuronal-ensemble level in the visual circuits of the honeybee (Mota et al., 2013; Mota et al., 2011b). It was shown that in the Anterior Optic Tuberculum retinotopy is ventrally-dorsally reversed and, that color processing is compartmentalized in this structure in a way that may refer to a chromatic compass for sky-light-based navigation (Mota et al., 2013).

Different tracts leave the optic lobes to reach ***the MBs***. The most important tract connecting the two optic lobes to the MBs is the anterior superior optic tract (a.s.o.t.) which contains about 300 neurons from the dorso-medial part of the lamina and projecting to the collar and the basal ring of the calices on both brain sides. The anterior inferior optic tract (a.i.o.t.) coming from the ventral part of the medulla joins the a.s.o.t. and projects to the collar and basal rings on both sides. Lastly, the lobula tract (lot) connects each LO with the same regions in the MBs. Interestingly, in both collar and basal ring of the calyces, a strict segregation of information coming from the ME and the LO is observed (Ehmer and Gronenberg, 2002). Moreover, within the collar, projections from the dorsal and ventral parts of the medulla are also segregated. Furthermore, extrinsic lobula neurons also connect with the optic lobe of the contralateral side (Hertel et al., 1987).

The honeybee MB is a higher order multisensory processing/integration centre, which consists of ~160,000 densely packed neurons, the ***Kenyon cells*** (KC) (Mobbs, 1982, 1984). Their dendrites form the input sites, ***calyces (CA),*** which receive afferences from different sensory pathways (olfactory, visual, gustatory, mechanosensory) and brain regions (AL, OL, SEG) (Strausfeld, 2002) and which project to the output regions of the MBs, the ***α- and β-***

lobes (Mobbs, 1982), currently identified as vertical (VL) and medial lobes (ML) (Strausfeld, 2002). Furthermore, the KCs are morphologically differentiated into subpopulations: ***inner compact cells (ICC), non-compact cells (NCC) and outer compact cells (OCC)*** (Farris et al., 1999). The cell bodies of the ICC are around 4–5 μm and tightly packed into a cone-shaped region at the centre of each calyx. NCC have larger cell bodies (6–7 μm) and fill up the remaining space within each calyx up to the dorsal border. Dendrites of NCC are detected in the lip and collar regions of the calyces. The OCC are around 6-7 μm as well and their appearance is similar to that of the ICC. They are located lining the outsides of the calyces. Dendrites of OCC can be found in the collar region (Mobbs, 1982). The calyx is segregated into several subregions. Each subregion receives afferents from sensory organs: the lip region receives olfactory afferents; the collar region (col) visual afferents; and the basal ring (br) mixed afferents, in which its inner half is innervated by olfactory afferents and outer half by visual afferents (Ehmer and Gronenberg, 2002; Gronenberg, 2001; Kirschner et al., 2006). The segregation among these subregions continues within the VL layers (Strausfeld, 2002). From both VL and ML, the information is further relayed to other brain regions via the so-called MB extrinsic neurons (MBEN) (Rybak and Menzel, 1993).

Recurrent inhibitory feedback to the MBs is provided by the ***A3-v cluster of the protocerebral calycal tract (PCT)***, which connects the major output regions of the MB, the α- and β-lobes and pedunculus, with its major sensory input site, the calyces (Rybak and Menzel, 1993). The ***second PCT cluster (A3-d)*** provides local feedback presumably onto MBENs to premotor neurons (Okada et al., 2007). Inhibitory modulation by these GABAergic feedback neurons can be crucial to solve higher-order learning problems (Devaud et al., 2007).

The PL represents a large mass between the AL and the OL (Brandt et al., 2005). Not much is known about the morphological and functional segregations of the PL in honey bee. A prominent structure has been however thoroughly studied. It is an anterior part of the PL, located dorsal to the AL, ventrolateral to the α-lobe of the MB and known as the anterior optic tubercle (AOTU). It is a spherical neuropile that receives input from OL (Mota et al., 2011a). That PL receives information from the visual sensory organ, appears to be conserved across insect models. Prior works in *Drosophila*, the blowfly and the bumble bee show that this structure is segregated into smaller distinctive neuropiles. These are characterised by the

innervations of the visual projection neurons arriving from the OL (Otsuna and Ito, 2006; Paulk et al., 2008; Strausfeld and Okamura, 2007) and are termed 'optic glomeruli'.

The LH is a prominent neuropile that is a small lateral protrusion of the protocerebral lobe (Rybak, 1994), compromising at least 4 sub-compartments (Kirschner et al., 2006). It is innervated by various projection neurons (PNs) leaving the AL via different antenno-cerebral tracts (ALTs) (Abel et al., 2001; Kirschner et al., 2006; Muller et al., 2002). Through these various ACTs the LH is connected to larger parts of protocerebral areas and the MBs (Müller et al. 2002; Kirschner et al. 2006). A recent work in the LH of honey bee revealed the principles of olfactory coding occurring at the input of the LH which share similarities with those occurring in the AL (Roussel et al., 2014). This work shows that the LH exhibits a specific coding pattern in response to odorants, thus supporting previous evidences in the locust suggesting that the LH may be involved in simple coding of olfactory stimulus, bilateral olfactory integration and multimodal integration (Gupta and Stopfer, 2012).

The CX is another higher order structure formed by a group of interconnected neuropiles located centrally in the protocerebrum (Homberg, 1985; Strauss, 2002). In the honey bee, it comprises the central body (CB), segregated as an upper (CBU) and a lower (CBL) division, the protocerebral bridge (PB), an arched neuropile located dorsally to the CBU, and by two globular structures located posterior to the PB called noduli (NO). Relatively little is known about the neuroanatomy and physiology of the CX in the honey bee except that its interneurons respond to visual stimuli (Homberg, 1985; Milde, 1988). In other insects, the CX participates in polarization vision, visual information processing and memory storage, spatial orientation and locomotion control (for review see Boyan and Reichert, 2011; Pfeiffer and Homberg, 2014) so that similar roles could be ascribed to the CX of the honey bee.

The SEZ is a fusion of the mandibular, maxillary, and labial neuromeres (Rehder, 1988). This region processes the gustatory, olfactory and mechanosensory input from the proboscis. It seems to be particularly important for gustatory coding (Rehder, 1988; Sanchez et al., 2007). From there, projections are sent to motor neurons controlling the muscles of the mouthparts, thereby mediating the proboscis extension reflex (PER). This region is important for associative appetitive learning as it contains the cell body of an important modulatory neuron involved in olfactory appetitive conditioning, the VUMmx1, which substitutes for sucrose in appetitive olfactory conditioning (Hammer, 1993) (see below).

2.4. The Neural Circuits Underlying Appetitive Olfactory Learning in the Honey Bee

Having described the main structures of the bee brain and their known functionalities, it is now possible to go back to the appetitive olfactory conditioning of PER (see above and Fig. 2) and trace the US (sucrose) and CS (olfactory) components to the neural level.

2.4.1. Appetitive US (sucrose) Pathway Underlying Olfactory PER Conditioning

In the honey bee, the US (sucrose) processing pathway starts at the level of the gustatory receptors which are localized within gustatory sensilla localized on the antennae, tarsi and mouth parts (de Brito Sanchez, 2011; de Brito Sanchez et al., 2007). Sucrose receptor neurons from the mouthparts converge to the SEZ and somehow (the synaptic regions are unknown) connect with a neuron called VUMmx1 (abbreviation for "ventral unpaired median neuron of the maxillary neuromere 1") (Fig. 4). It responds with long-lasting spike activity to sucrose solution delivered both at the antennae and the proboscis (Hammer, 1993). The dendrites of VUMmx1 arborise symmetrically in the brain and converge with the olfactory pathway at three sites: the ALs, the calyces of the MBs, and the LH, which are key processing stages of olfactory information (CS pathway, see above) in the bee brain.

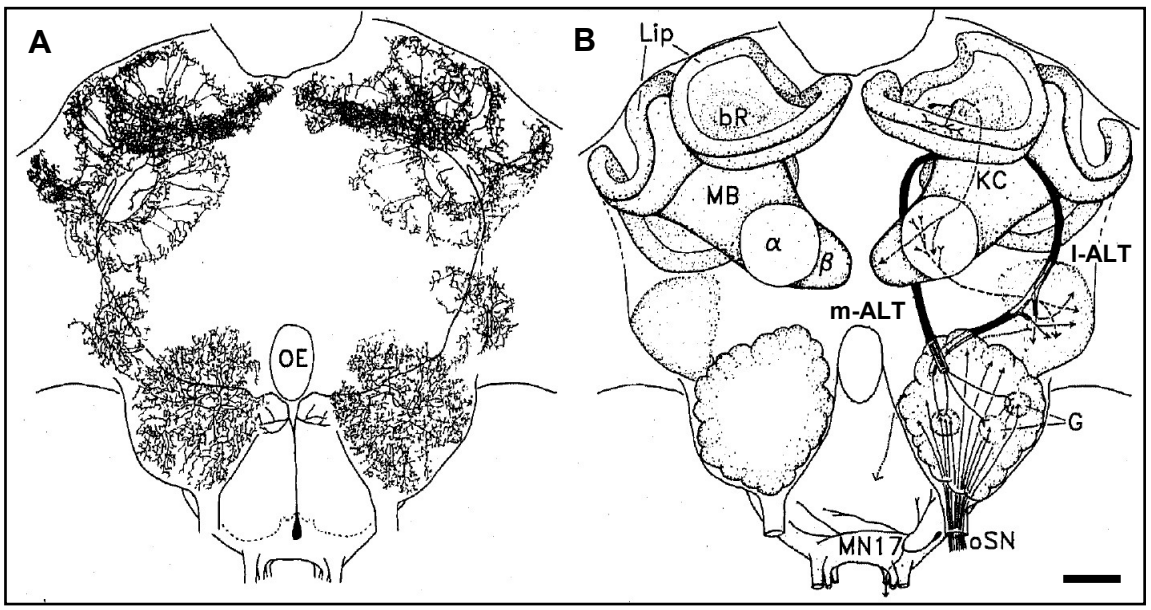


Figure 4 (previous page): Morphology of the VUMmx1 (ventral unpaired medial maxillar 1) neuron; adapted from Hammer 1993. A: VUMmx1 neuron sends its projection to the antennal lobes (AL), lateral horns (LH) and calyces (lip and basal ring, bR) of the mushroom bodies (MBs) and conveys the appetitive reinforcement signal. B: Olfactory pathway in honey bee showing the afferences from olfactory sensory neurons (oSN) to the glomeruli (G) of the AL. Olfactory information processed in the AL is then relayed by ALTs (l- and m-ALT are depicted) to the MBs which consist of Kenyon cells (KC). The output region of the MBs, the α- and β- lobes is also shown. Scale = 100 µm.

Behavioural learning of an olfactory stimulus can be induced by substituting the sucrose reward in PER conditioning by an artificial depolarisation of VUM_{mx1} immediately after olfactory stimulation (forward pairing) (Hammer, 1993). If depolarization precedes olfactory stimulation (backward pairing), no learning is observed. The same forward-backward effect is seen when sucrose is used as reward under similar experimental conditions. In all cases the bees' response was quantified in terms of the number of spikes of M17 (Rehder, 1987), a muscle controlling the movement of the proboscis. The results thus show that VUM_{mx1} constitutes the neural correlate of the US in associative olfactory learning.

Classical conditioning relies on the fact that a CS acquires the capacity of replacing the US as it becomes a reliable predictor of reinforcement. This was evident in recordings of VUMmx1 activity after olfactory conditioning (Hammer, 1993). After training a bee to discriminate a rewarded (CS_1+) from a non-rewarded odorant (CS_2-), it was found that VUMmx1 fired to the CS_1+ and not to the CS_2- (Hammer, 1993). Thus, CS_1+, the odorant that reliably predicted the US, acquired the capacity of activating VUMmx1. At the same time, VUMmx1 continued to respond to the US when it was presented unexpectedly, i.e. not preceded by a predictive odorant, but it diminishes its responses to predictable sucrose (Menzel and Giurfa, 2001). Thus, the VUMmx1 neuron has the characteristic properties of a system that provides information on reinforcement-prediction error that is critical to associative learning (Schultz and Dickinson, 2000).

A fundamental characteristic of the VUMmx1 neuron is that it belongs to a group of octopamine-immunoreactive neurons (Kreissl et al., 1994). Activity of VUMmx1 corresponds therefore to the release of the biogenic amine octopamine (OA) by this neuron on its target structures. Thus, in appetitive PER conditioning, octopamine mediates the reinforcing properties of sucrose reward in the bee brain (Hammer, 1993; Hammer and Menzel, 1998; Farooqui et al., 2003). OA was shown to be necessary and sufficient to substitute for the sucrose reward (Hammer and Menzel, 1998) by pairing an odorant with injections of OA as a substitute for sucrose into the MBs or ALs (but not the LH) lobe. This experiment produced a lasting, learning-dependent enhancement of proboscis extension (Hammer and Menzel, 1998).

Several octopamine receptors have been characterised in honey bees (Balfanz et al., 2014; Grohmann et al., 2003; Hauser et al., 2006). However, silencing the expression of only one octopamine receptor, AmOA1, in the honey bee antennal lobe using double-stranded RNA was sufficient to impair appetitive olfactory learning (Farooqui et al., 2003). In a recent pharmacological study (Behrends and Scheiner, 2012), it was shown that activation of this receptor by administration of octopamine leads to a modulation of sucrose sensitivity, in this case a sensitivity increase in newly emerged bees, which are in principle insensitive.

It is notable that reinforcement signalling in the bee brain does not seem to follow the same logic as in the fruit fly brain. Recently, an interconnection between octopaminergic and dopaminergic pathways was discovered in *Drosophila*, which plays a crucial role in appetitive olfactory conditioning (Burke et al., 2012; Liu et al., 2012). Specifically, a subset of dopaminergic neurons was found, which possess octopaminergic receptors allowing them to receive signals from peripheral octopaminergic neurons signalling the presence of sucrose. These dopaminergic neurons convey the sucrose-reward signal to the mushroom bodies. Their afferences are spatially segregated from those of other subsets of dopaminergic neurons which convey punishment signals to the mushroom bodies (Burke et al., 2012; Liu et al., 2012).

2.4.2. Olfactory CS Pathway Underlying Olfactory PER Conditioning

The olfactory CS processing starts with the olfactory detection of odour molecules. It starts at the level of the antennae, where olfactory receptor neurons are located within olfactory sensilla (sensilla placodea; Esslen and Kaissling, 1976). Sensory neurons endowed with molecular olfactory receptors convey information about odorants to the antennal lobe. Information is processed further in the AL before being conveyed through the PNs to higher order centres such as the LH and the MB (see above and Giurfa and Sandoz, 2012). In the MB, the olfactory input areas are the calyces, specifically the lip and the basal ring subregions. The convergence of these different regions of the olfactory pathway with VUMmx1 arborisations representing the US pathway is particularly remarkable as it provides the structural basis for CS–US associations.

Neural activity at the different stages of the CS processing pathway has been measured using various recording techniques including electrophysiological and opto-physiological means (Abel et al., 2001; Denker et al., 2010; Joerges et al., 1997; Kirschner et

al., 2006; Mauelshagen, 1993; Szyszka et al., 2005; Yamagata et al., 2009). At the level of neuropiles, olfactory processing has been intensively studied using the calcium imaging technique. These studies have established that odorants are encoded as odor-specific spatiotemporal patterns of glomerular activity at the level of the AL (Carcaud et al., 2012; Joerges et al., 1997; Sachse et al., 1999). By performing calcium imaging experiments shortly (i.e. ca. 15 min) after PER conditioning, it was found that olfactory differential conditioning (CS_1+ vs. CS_2-) induces an increase in the intensity of the glomerular activation pattern for the rewarded odorant CS_1. No change was recorded in the pattern of the non-rewarded odorant CS_2 (Faber et al., 1999). In addition, a decorrelation of the patterns of odors CS_1 and CS_2 was found, suggesting that their discriminability was improved (Faber et al., 1999). This conclusion was recently confirmed and extended by Rath and co-workers (Rath et al., 2011) who also employed calcium imaging to measure antennal lobe activity two to five hours after differential conditioning. They found that the response patterns to CS_1 and CS_2 became more different in bees that learned to discriminate between the two odorants, but not in bees that did not successfully discriminate between them.

Calcium imaging has been also applied in the case of studies performed at the level of the MBs. Recordings of Kenyon cells showed that the combinatorial olfactory code at this level is sparse and temporally sharpened as a consequence of pre- and postsynaptic processing within the mushroom body microcircuits (Szyszka et al., 2005), and due to the probable action of inhibitory recurrent neurons A3-v mentioned above. These responses can be modified by associative learning as shown by PER conditioning studies coupled to calcium imaging recordings. While repeated stimulation with an odour leads to a non-associative decrease in the response strength of Kenyon cells, the pairing of an odour with sucrose induces an associative prolongation of Kenyon-cell responses. After conditioning, Kenyon-cell responses to a rewarded odour (CS_1+) recover from the decrease induced by repetition, while the responses to a non-rewarded odour (CS_2−) decrease further. The spatiotemporal pattern of activated Kenyon cells changes for both odours when compared with the response before conditioning but the change is stronger for the CS_2− (Szyszka et al., 2008).

Finally, at the level of the LH, calcium-imaging recording discovered an odour- and pheromone-specific coding. Odour-similarity relationships are mostly conserved between the AL and the LH (Roussel et al., 2014). Since this discovery is very recent, no study has

analyzed until now whether the olfactory code existing at the level of the AL is subjected to modifications that are induced by associative olfactory learning.

Taken together, these studies provide an overview about odour processing at several stages along the CS pathway and about the modifications of neural activity induced by olfactory learning in these stages.

2.5. Appetitive Memories Induced by Olfactory PER Conditioning

Appetitive olfactory memories are acquired through PER conditioning and can be retrieved minutes, hours or days later, depending on different experimental factors (Menzel, 2001; Menzel et al., 2001). Among these factors, one can cite the kind of CS, the intensity of the US (i.e. the amount and/or quality of sucrose solution received during conditioning), the number of conditioning trials and the intertrial interval (Menzel et al., 2001).

Different memory phases (short-term, early and late; medium-term, early and late and long-term, early and late) have been characterized accurately in terms of their dynamics and molecular substrates (Menzel, 1999) (Fig. 5). One pairing of an odorant with sucrose (i.e. one conditioning trial) leads to short-term (STM: in the range of sec to min), mid-term (MTM: in the range of hours) an early long-term memory (e-LTM) that can be retrieved 24–48h after conditioning. This e-LTM depends on translation but not on gene transcription and is not,

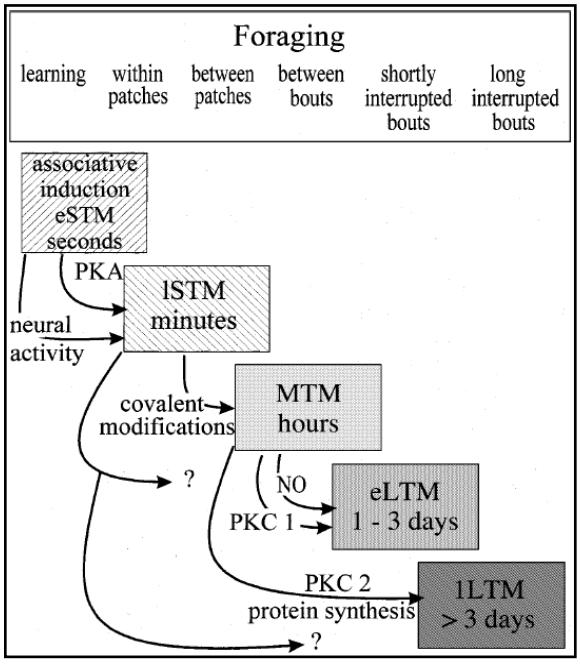


Figure 5: Summary diagram of the memory phases of the honey bee and their known molecular substrates. A relationship between the temporal organization of these memories and the natural foraging cycles is also shown; adapted from Menzel 1999.

therefore, affected by transcription inhibitors such as actinomycin D. Three conditioning trials, however, induce not only STM, MTM and e-LTM but also a stable late long-term memory (l-LTM) that can be retrieved 72h or more after conditioning (Fig. 6). Unlike e-LTM, l-LTM requires gene transcription and can therefore be inhibited by actinomycin D (Eisenhardt, 2006; Giurfa and Sandoz, 2012; Menzel, 1999, 2001; Schwärzel and Müller, 2006).

Trial spacing is a critical factor for the induction of different types of memory. Generally, massed trials (i.e. trials succeeding each other in a fast sequence) lead to less stable memories compared to spaced trials (i.e. trials separated in time) which lead to stabilized memories. Longer intertrial intervals lead to better acquisition and retention (Menzel et al., 2001).

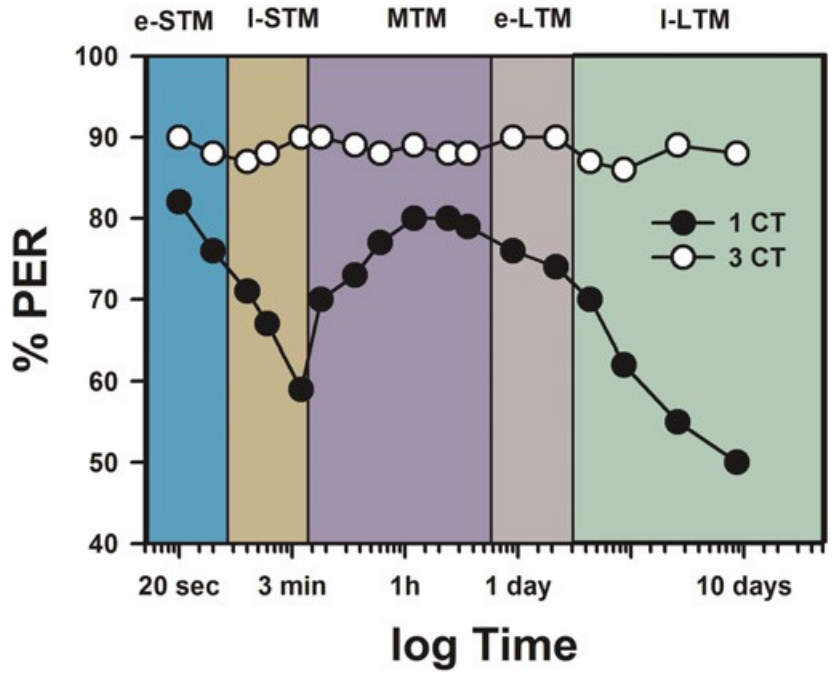


Figure 6: Olfactory memory phases in honeybees. Retention performance (measured as PER percentage: % PER) depending on trial number and the respective underlying memory phases (after Menzel 2001). The memory retention of a single CS-US association (1 CT; black dots) is compared with three (or more) conditioning trials (3 CT; white dots) with 10-min inter-trial intervals. 1 CT allows good retention performance for up until 1 d. 3 CT induce high performance for several days with two forms of long-term memory, one that depends on translation of already-present mRNA (early-LTM: e-LTM), the other critically depending on de novo gene transcription (late-LTM: l-LTM, starting at 3 d); adapted from Giurfa & Sandoz 2012.

The formation of stable l-LTM induces structural modifications in the honey bee brain in accordance with the fact that memories may reside in novel, stabilized connections within olfactory networks that require protein synthesis. These modifications have been found in the AL and in the calyces of MBs (Hourcade et al., 2010; Hourcade et al., 2009).

In the AL, learning-dependent structural modifications concern the volume of the AL glomeruli. An association with a volume increase in a subset of glomeruli is observable three days after an successful appetitive olfactory conditioning (Hourcade et al., 2009). This increase is odour-specific but does not reflect enhanced activity in the glomeruli encoding the learned odorant as a different pattern of glomerular variation is seen in any case. Rather it may reflect the modulatory effect of VUMmx1 on the inhibitory network of the AL.

In the calyces of the MBs, the neuronal modifications are detectable at the level of the microglomeruli that are present in the calyces. These microglomeruli constitute the interaction site between presynaptic afferent neurons (e.g. PNS coming from the ALs in the case of olfactory information) and the postsynaptic Kenyon cells which make the MBs. Hourcade et al. have reported that the density of olfactory but not visual microglomeruli increases after a successful olfactory l-LTM formation and that this increase is abolished by a protein synthesis inhibitor (Hourcade et al., 2010).

3. The Advent of a New Conditioning Protocol for Harnessed Bees: the Aversive Olfactory Conditioning of the Sting Extension Reflex

The previous sections highlighted the fact that for approximately a century, research on honey bee learning and memory has focused almost exclusively on appetitive learning, exploiting the fact that bees can learn about a variety of sensory stimuli or to perform certain behaviours if these are rewarded with sucrose solution, the equivalent of nectar collected in flowers. We have seen that this natural foraging scenario is amenable to the laboratory through the olfactory conditioning of PER and how, during the last 50 years, immense progresses have been made in deciphering the neural and molecular bases of ***appetitive*** learning and memory using bees as a model.

On the contrary, not much was known about the capacity of honey bees to learn aversive events in their environment. In the fruit fly *Drosophila melanogaster*, the other insect that has emerged as a powerful model for the study of learning and memory, aversive learning has been the dominant framework (Busto et al., 2010; Davis, 2005; Heisenberg, 2003; Keene and Waddell, 2007; Margulies et al., 2005). In the fruit fly, olfactory aversive conditioning consists in training groups of flies in a T-maze which allows alternated

presentation of two different odours, one (CS_1+) paired with the US of an electric shock, and another (CS_2-) non-paired with the shock (Tully and Quinn, 1985). Retention is tested afterwards in a dual-choice situation as flies have to choose between the CS+ and the CS- without aversive reinforcement. Successful learning and retention result in CS+ avoidance. This behavioural protocol has allowed the dissection of aversive learning at the cellular and molecular level and identifying the cellular location of different aversive memory traces (Busto et al., 2010; Davis, 2005; Heisenberg, 2003; Keene and Waddell, 2007; Margulies et al., 2005).

Due to obvious differences in behavioural and motivational contexts, in addition to the impossibility to equate US nature and strength, it has been difficult to compare appetitive and aversive learning in bees and flies, respectively, despite their fundamental contribution to understanding learning and memory at multiple levels. As a consequence, the question of whether the mechanisms underlying learning and memory in these two insect models are general or rather specific has remained elusive. Yet, in the last five years, a new conditioning protocol has been established in honey bees, which was conceived to fill this gap (Vergoz et al., 2007a). This protocol is the aversive conditioning of the sting extension response (SER) which is a defensive response to potentially noxious stimuli (Breed et al., 2004). This unconditioned response can be elicited by means of electric-shock delivery to a harnessed bee (Núñez et al., 1997). As no appetitive responses are involved in this experimental context, true punishment (aversive) learning could be studied for the first time in harnessed honey bees.

Inspired by the work of Núñez and coworkers, who used the SER to study the presence of an opioid-like system in honey bees (Núñez et al., 1997), and by the well-established protocol of olfactory PER conditioning (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Takeda, 1961), the protocol of olfactory conditioning of SER was successfully established by Vergoz et al. (Vergoz et al., 2007a). In this protocol, forward-pairing of an odour with an electric shock results in bees learning this contingency and therefore extending their sting in response to the previously punished odour (Vergoz et al., 2007a)(Fig. 7).

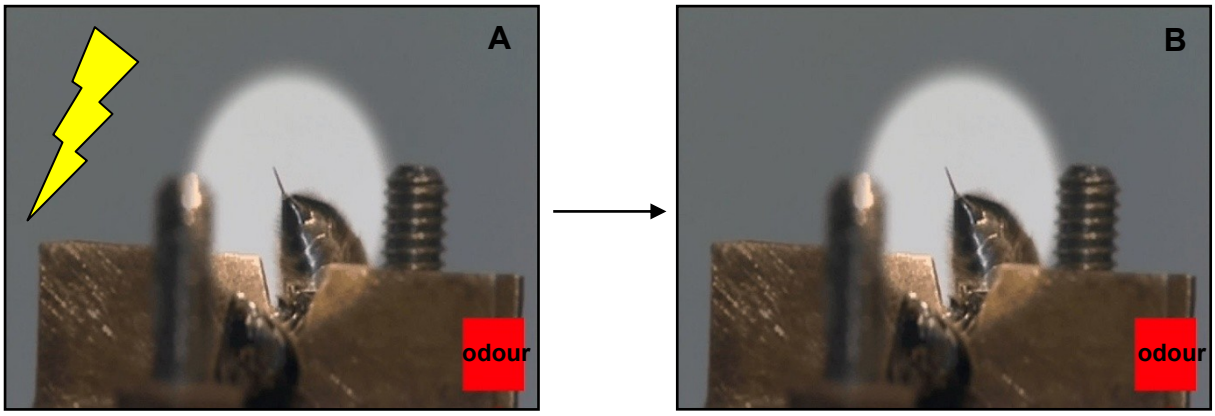


Figure 7: Aversive olfactory conditioning of sting extension reflex (SER). A: A harnessed bee is trained explicitly paired presentations of an odorant (CS) and the electric shock (US) following an absolute – conditioning design (a single odorant reinforced). B: After a successful conditioning the bee would extend its sting in the presence of the CS alone.

To this end, bees are fixed individually on a metallic holder so that they build a bridge between two brass plates through which a 2 sec mild electric shock (7.5 V) is delivered by a stimulator (60 Hz - AC current) (Fig. 7A). Bees treated in this way extend their sting reflexively in response to the electric shock (Burrell and Smith, 1994; Núñez et al., 1997) (Fig. 7B). Bees of a 'paired group' are trained with explicitly paired presentations of an odour (the CS) and the electric shock (the US) following an absolute-conditioning design (a single odorant reinforced). As a control for this kind of conditioning, an 'explicitly unpaired group' of bees is presented with unpaired presentations of odour and shock. Figure 8A shows that bees from the paired group learn the odour – shock association and increase conditioned SER to the punished odour during trials. In contrast, bees in the explicitly unpaired group show no significant change in responsiveness to the odour during trials. Thus, the increase of SER observed in the paired group is due to associative learning and not to the simple experience with the odour and the shock. One hour after conditioning, bees of the paired group still remember the conditioned odour while bees of the unpaired do not respond to the odour (Fig. 8A, black and white bars). Therefore, an aversive memory retrievable 1 h after learning is established in the paired but not in the explicitly unpaired group (Vergoz et al., 2007a).

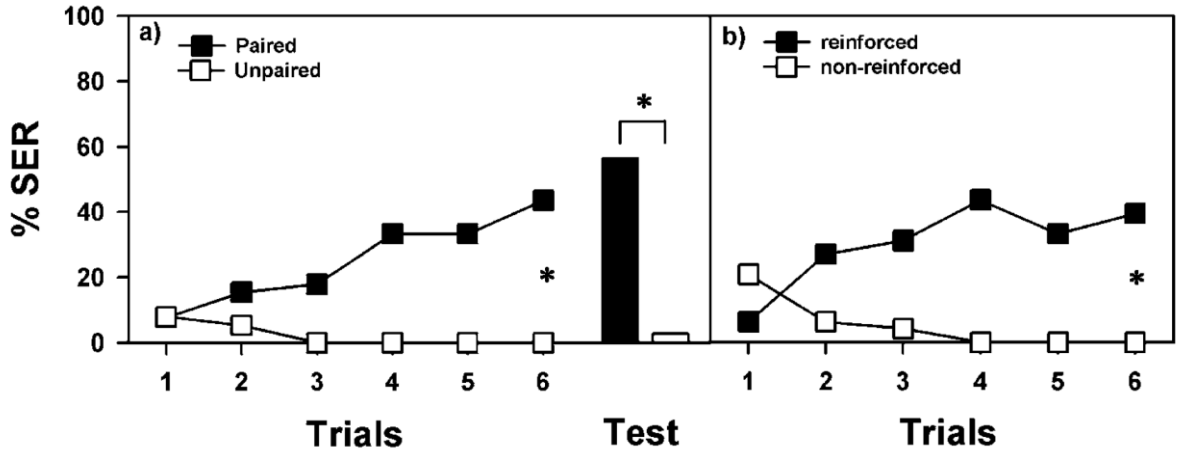


Figure 8: Associative olfactory conditioning of the sting extension reflex (SER) in honeybees. A: Responses (SER) of bees trained with an odorant explicitly paired with an electric shock (black squares) and with odorant and unpaired electric shock (white squares) during 6 trials. Only the bees in the paired group learned the association and extended their sting as a response to the odorant. One hour after conditioning an olfactory aversive memory was present in the paired (black bar), but not in the unpaired, group (white bar). B: Responses (SER) of bees (n = 48) trained to discriminate an odorant reinforced with an electric shock (black squares) and a non-reinforced odorant (white squares) during 12 trials (6 reinforced and 6 non-reinforced). Bees learned to discriminate between odorants as a result of conditioning; adapted from Vergoz *et al.* 2007

Moreover, in a differential-conditioning design (Fig. 8B) in which each bee acts as its own control, bees learn to extend their sting to an odour paired with an electric shock and not to respond to another non-reinforced odour. Bees are conditioned during 6 reinforced and 6 non-reinforced trials, presented in a pseudo-random sequence. The resulting learning curves (Fig. 1d) show that bees learn to discriminate between odours as a result of conditioning. Thus, olfactory conditioning of SER is truly associative and does not rely on the simple exposure to the training stimuli, independently of their outcome (Vergoz et al., 2007a).

The use of the term 'aversive' is fully appropriate in the case of this protocol because it was shown that after aversive differential SER conditioning using one odour punished with shock (CS_1+) and the other not (CS_2-), bees released individually in a mini Y-maze under red light (i.e. in the dark for bees), which presented the two odours used previously, CS_1 and CS_2, avoided significantly the odorant which was coupled with the shock (CS_1+). The aversive nature of SER conditioning in honey bees is clearly emphasised by this result (Carcaud et al., 2009).

3.1. Aversive US Pathway Underlying Olfactory SER Conditioning

As mentioned above, in appetitive PER conditioning, octopamine mediates the reinforcing properties of sucrose reward in the bee brain (Farooqui et al., 2003; Hammer, 1993; Hammer and Menzel, 1998). In the fruit fly, dopamine was found to mediate the aversive properties of the electric-shock reinforcement used in olfactory conditioning (Aso et al., 2012; Aso et al., 2010; Claridge-Chang et al., 2009; Schwaerzel et al., 2003). In order to establish whether dopaminergic signalling is also crucial for aversive US signalling in bees, neuropharmacological experiments were first performed in order to block this signalling and determine whether olfactory SER conditioning was possible (Vergoz et al., 2007a). Separate groups of bees were injected into the brain through the medium ocellus 30 min before differential conditioning with Ringer solution (control), mianserine or epinastine (octopaminergic blockers) or fluphenazine or flupentixol (dopaminergic blockers).

Ringer-injected bees learned to discriminate the punished from the non-punished odor (Fig. 9A). One hour later, they remembered the aversive association and extended their sting in response to the previously punished odorant. Octopaminergic antagonists (mianserine or epinastine) did not affect performance at any of the concentrations used in these experiments. Figure 9B shows that mianserine-injected bees learned to discriminate the two odorants and responded with SER only to the odorant paired with the electric shock. Retention tests also showed significant discrimination. Thus, octopaminergic antagonists did not impair aversive olfactory learning in honey bees. Dopaminergic antagonists (fluphenazine and flupentixol) had, on the contrary, a dramatic effect on aversive olfactory learning. Flupentixol-injected bees did not learn to discriminate between odorants. Consequently, they did not show discrimination in the tests performed one hour later (Fig. 9C). Fluphenazine had a similar effect although with less effectiveness. These results showed therefore that dopamine-, but not octopamine signalling, is necessary for aversive olfactory learning in honey bees (Vergoz et al., 2007a).

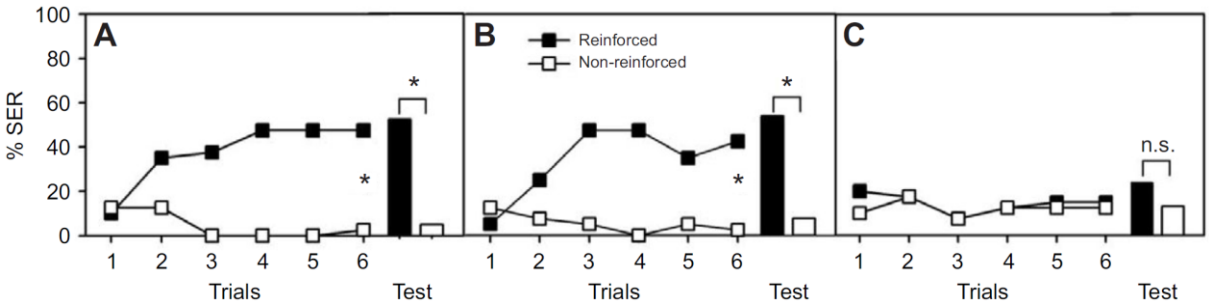


Figure 9: The effect of octopamine and dopamine receptor antagonist on aversive olfactory conditioning in honey bee. Responses (SER) of bees trained to discriminate an odorant reinforced with an electric shock and a non-reinforced odorant during 12 acquisition trials (six reinforced and six non-reinforced). A retention test was conducted 1h after the last acquisition trial. SER responses are shown for (A) control bees injected with Ringer solution into the brain, (B) bees injected with the octopaminergic antagonist mianserine into the brain and (C) bees injected with the dopaminergic antagonist flupentixol into the brain. Ringer solution- and mianserine-injected bees learned to discriminate the reinforced from the non-reinforced odorant and remembered the difference 1h later. Flupentixol-injected bees did not learn to discriminate the reinforced from the non-reinforced odorant, nor did they respond appropriately in the retention tests. These results show that dopamine but not octopamine receptors are required for aversive olfactory learning in honey bees; adapted from Tedjakumala and Giurfa, 2013

These results prompt a precise neuroanatomical characterization of dopaminergic neurons in the honey bee brain. This characterization is necessary because immunocytochemistry studies using an antiserum against dopamine were performed 25 years ago (Schäfer and Rehder, 1989) but the technique used to stain candidate dopaminergic neurons does not allow to differentiate whether labelled neurons were neurons producing dopamine (true dopaminergic neurons) or neurons incorporating dopamine.

Dopamine-like immunoreactive neurons were identified in most parts of the brain and in the suboesophageal ganglion (Schäfer and Rehder, 1989). Only the optic lobes were devoid of staining. Approximately 330 dopamine-immunoreactive cell bodies were found in each brain hemisphere plus the corresponding suboesophageal hemi ganglion. Most of the stained cell bodies were situated within three clusters: two (C1 & C2) below the α-lobe of the mushroom body, in the inferior medial protocerebrum, and one rather below the lateral calyx (C3). Other stained cell bodies lied dispersed or in small groups around the protocerebral bridge, below the optic tubercles, proximal to the inferior rim of the lobula, and in the lateral and inferior somatal rind of the suboesophageal ganglion (SEG). Due to limitations of the staining technique, not all of the dendritic arborizations and axons of these neurons could be visualized so that where and how dopaminergic circuits contact the olfactory pathway remains to be determined (Schäfer and Rehder, 1989). This information is crucial to study where the association between the odor CS and the electric shock US takes place.

Besides, a dissection of the contribution of the three dopaminergic receptors identified in the honey bee, AmDOP1 (Blenau et al., 1998; Mustard et al., 2003), AmDOP2 (Humphries et al., 2003; Mustard et al., 2003) and AmDOP3 (Beggs et al., 2005), to US signalling in aversive learning is necessary. AmDOP1 and AmDOP3 have been related to the vertebrate D1-like (up-regulates c-AMP) and D2-like family of dopamine receptors (down-regulates c-AMP), respectively (Beggs et al., 2005; Blenau et al., 1998) while AmDOP2 appears to be

related to invertebrate octopamine receptors and constitutes a distinct 'invertebrate type' dopamine receptor (Humphries et al., 2003). From a functional point of view, it can be referred to as a 'D1-like receptor' because it also up-regulates cAMP. The lack of specific pharmacologic blockers of these receptors has precluded until now straightforward analyses of their implication in aversive learning. Impairment of aversive learning yields conflicting evidence with respect to this topic: while pharmacological blocking with vertebrate antagonists indicated that AmDOP2 receptors are necessary for aversive learning (Vergoz et al., 2007a), analyses of transcript levels of dopaminergic receptor genes suggested, on the contrary, that impairment of aversive learning is associated with an *increase* of AmDOP2 receptors (Geddes et al., 2013). More experiments are necessary to elucidate if and how these different receptors contribute to aversive learning.

An interesting twist to the study of aversive learning and dopaminergic signalling is the discovery that 20-hydroxyecdisone (20-E), a metabolite of the steroid hormone ecdysone, which intervenes in insect development and reproduction (Riddiford et al., 2000), impairs aversive but not appetitive conditioning in bees (Geddes et al., 2013). This impairment seems to be achieved in part via the dopamine/ecdysone receptor gene AmGPCR19, which is the honeybee orthologue of the dopamine/ecdysone receptor gene 48 (DmDopEcR) identified in Drosophila (Srivastava et al., 2005). Thus, exogenous 20-E injection determines both a reduction in AmGPCR19 levels and a decrease in aversive learning performances, therefore indicating that aversive learning in honey bees can be modulated by ecdysteroids (Geddes et al., 2013).

3.2. Olfactory CS Pathway Underlying Olfactory SER Conditioning

Having described the olfactory circuit in the bee brain in the case of the appetitive olfactory PER conditioning (see above), we will summarize here the information on odor processing upon olfactory SER conditioning that is available up to now. So far, only one study has been achieved, which recorded olfactory activity in the antennal lobe during differential SER conditioning using calcium imaging. The study addressed the question of whether punishment learning induces changes in the neural representation of the learned odorants (Roussel et al., 2010).

No differences were found between glomerular responses to the punished odor (CS_1+) and to the non-punished odour (CS_2-) in bees that learned the discrimination in spite of the fact that in appetitive olfactory PER conditioning, changes in neural activity have been found after differential conditioning (see above: Faber et al., 1999; Rath et al., 2011) but see (Peele et al., 2006).

A possible explanation for this lack of difference between the neural responses to the CS_1+ and the CS_2- could be that the aversive olfactory memory traces are located downstream to the antennal lobe, for instance, in the mushroom bodies (Gerber et al., 2004). Another possibility relates to the timing of the neural activity recording. In this case, recordings were obtained in parallel to conditioning (i.e. during conditioning trials) taking advantage of the fact that SER conditioning enables simultaneous recording of behavioral output (sting extension) and calcium variation at the neural level. Note that such simultaneity is in principle not possible in PER conditioning because proboscis extension induces muscular activity that interferes with stable calcium-signal recordings in the brain. In the case of SER conditioning, changes in neural activity in response to the CS_1+ and the CS_2-, if any, could be only detectable some time after conditioning, upon later memory formation. Further experiments are required in which antennal lobe activity should be measured at different time intervals following conditioning. Similarly, focusing on higher-order structures such as the mushroom bodies would be crucial.

3.3. Aversive Memories Induced by Olfactory SER Conditioning

In the first works performed on olfactory SER conditioning, bees showed the presence of aversive memories in retention tests performed 1h after conditioning (Carcaud et al., 2009; Vergoz et al., 2007a). This period corresponds, in appetitive PER conditioning, to mid-term memory, which is independent of protein synthesis and thus relatively labile (see above: Menzel, 1999). In a further work, the question of whether olfactory SER conditioning also leads to the formation of long-term memories was explicitly addressed (Giurfa et al. 2009).

As in the appetitive conditioning, several crucial parameters of the conditioning protocol affect memory retention following olfactory SER conditioning. Variation in parameters such as the interstimulus interval (ISI) and the intertrial interval (ITI) determined different memories (Giurfa et al., 2009). In the case of ISI, bees subjected to aversive

conditioning were able to associate an odorant to the electric shock as long as the stimuli were forward paired (odour onset followed by shock onset), regardless of the duration of the ISI. This was not the case if the pairing between CS and US was backward, i.e. the odorant is introduced after the electric shock.

A more critical factor for memory formation was ITI. As in the appetitive olfactory conditioning, spaced trials induced better retention and more robust memories. Bees were conditioned in a differential conditioning protocol with spaced trials (intertrial interval of 10 min), and subjected to retention tests 1, 24, 48 and 72h after training. An independent group of bees was used for each retention time. All groups learned to discriminate the CS_1+ from the CS_2- and reached comparable levels of discrimination at the end of training. After conditioning, bees responded more to the CS+ than to the CS- in all retention intervals assayed (Fig. 11A). These results show that spaced trials lead to a robust long-term memory that is retrievable even three days after training (Giurfa et al., 2009).

Such a long-term memory was studied with respect of its molecular basis. Specifically, the possible dependency of 3-day long-term memory on *de novo* protein synthesis was analyzed. In the two hours following conditioning, bees were injected in the brain through the ocellar tract either with PBS (control group), anisomycin (a translation inhibitor) or actinomycin D (a transcription inhibitor). Seventy-two hours after conditioning, retention performances varied depending on treatment (Fig. 10). Retention performance was significant in control bees injected with PBS but not in bees injected either with anisomycin or with actinomycin D, thus showing that both translation and transcription are essential events for long-term memory formation of the odor-shock association (Fig. 10)(Giurfa et al., 2009).

These results show that aversive learning can induce a robust and stable l-LTM that relies on protein synthesis as it depends both on translation and transcription. Bees have the capacity to remember aversive experiences long after they took place. The biological contexts in which such capacity could be applied are multiple. On the one hand, foragers could avoid in this way returning to food places in which negative experiences, or eventually unfulfilled expectations, occurred, thereby enhancing foraging efficiency. On the other hand, it may be adaptive to memorize and remember during long periods the smell of predators in order to exhibit appropriate defensive responses to them.

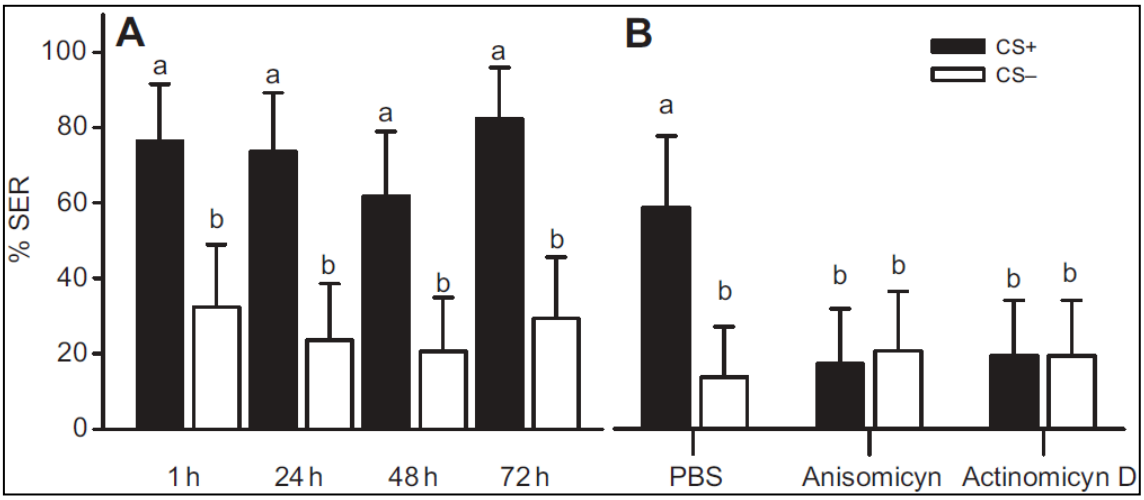


Figure 10: Memory retrieval in aversive conditioning. **A:** Memory retention after SER differential conditioning. Four groups of bees were trained in parallel (acquisition) and tested afterwards after different retention intervals (1, 24, 48 and 72h post-conditioning). Different letters indicate significant differences. All groups remembered the discrimination learned during training. **B:** Dependency of late long-term memory (l-LTM; 72h retention) on translation and transcription. Three groups of bees were trained in parallel (acquisition) and tested 72h after the last acquisition trial and after injection of PBS, anisomycin or actinomycin D. Different letters indicate significant differences. Only the group injected with PBS (control) remembered the discrimination learned during training; inhibition of transcription (actinomycin D) or translation (anisomycin) resulted in an absence of l-LTM; adapted from Tedjakumala and Giurfa, 2013.

3.4. Aversive Shock Responsiveness and Colony Organization

As for the appetitive PER to sucrose solution, studies were performed to determine whether bees vary in their shock responsiveness and how such variation may influence task organization within the hive. Firstly, Roussel et al. (2009) determined whether or not sucrose responsiveness (see above) in forager bees correlates with responsiveness to electric shocks of varying voltage. Like PER for sucrose, SER allows direct quantification of response thresholds to a stimulus that, in this case, is fully independent of a foraging context. Proboscis extension responses to a logarithmic series of sucrose solutions of increasing concentration were measured in a first phase, and sting extension responses to a series of shocks of increasing voltage were measured in a second phase. In another group of bees the reversed sequence (first shock, then sucrose) was employed. Neither the responses to the electric shocks nor the responses to the sucrose solutions differed significantly between these two groups, thus showing that the order of stimulation was irrelevant.

As expected, bees significantly increased PER to sucrose solutions of increasing concentration (Fig. 11a) and, similarly, bees significantly increased SER to electric shocks of increasing voltage (Fig. 11b). The increase in PER and SER responses with sucrose concentration and voltage value does not, however, answer the question of whether the bees responding more to concentrated sucrose are also those responding more to the highest voltages. To answer this question, both a sucrose-responsiveness score and a shock-responsiveness score were determined for each bee (Roussel et al., 2009). Scores were quantified as the sum of all responses made along the whole sequence of tested stimulations. Scores varied, therefore, from 0 (no response to any stimulus tested in the series) to 6 (responses to all six stimuli of the series) (Fig. 11c).

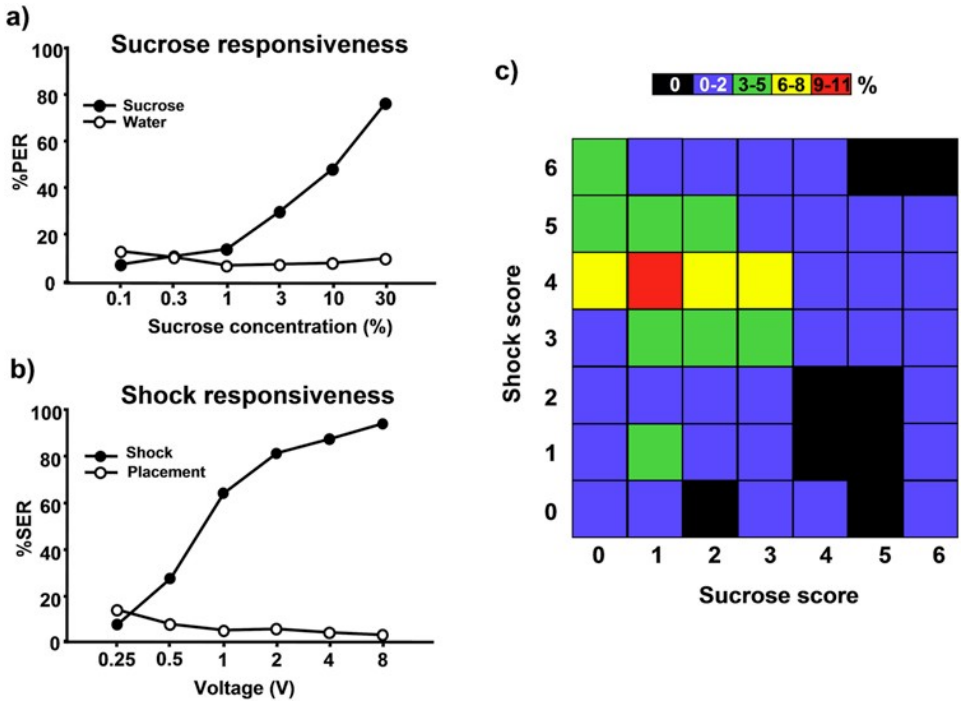


Figure 11 (previous page): Relationship between sucrose and shock responsiveness in honey bees. a) Sucrose responsiveness curves showing % of PER to a series of sucrose solutions of increasing concentration (black circles) and % of PER of the same bees to the presentation of water (control – white circles). Bees increased their response to sucrose solution of increasing concentrations. b) Shock responsiveness of the same bees showing % of SER to a series of shocks of increasing voltage (black circles) and % of SER of the same bees to placements in the same setup without shock delivery (control – white circles). Bees increased their response to shocks of increasing voltage. c) A 7x7 matrix of correlation between sucrose and shock responsiveness scores in the same bees. Scores varied from 0 (no response to any stimulus tested in the series) to 6 (responses to all six stimuli of the series). Colors assigned to each box represent the percentage of bees exhibiting a particular combination of sucrose and shock responsiveness scores. No significant correlation exists between sucrose and shock responsiveness scores (R =20.03; t (N-2) =20.42; NS); adapted from Tedjakumala and Giurfa, 2013.

Results of this analysis were represented as a 7x7 matrix in which one axis is defined by sucrose-responsiveness scores and the other axis by shock-responsiveness scores (Fig. 11c). Colours assigned to each box represent the percentages of bees exhibiting a particular combination of sucrose and shock responsiveness scores. Figure 11c shows no clear relationship between appetitive and aversive responsiveness, i.e. the correlation analysis performed on the two scores was non-significant. In other words, there was no correlation between responsiveness to two stimuli of opposed hedonic value such as sucrose and electric shock (Roussel et al., 2009).

In studies in which PER was used, correlated responsiveness was found for stimuli that are related to the appetitive search for food in which bees engage during foraging activity (Erber et al., 2006; Humphries et al., 2005; Scheiner et al., 2004). It seems coherent that responsiveness to odours (which are characteristic of food sources) and to light (which elicits foraging flight), as well as motor activity, are correlated in the same bees (Erber et al., 2006; Humphries et al., 2005; Scheiner et al., 2004). This variety of related sensitivities can be grouped in a "*foraging behaviour syndrome*" (Pankiw, 2005), defined as a set of correlated behaviours reflecting between-individual consistency in behaviour across multiple foraging situations (Sih et al., 2004). Yet, this syndrome may just constitute a part of the complex behavioural tuning within a hive.

Several behavioural syndromes may coexist in an insect society. A "*defensive behavior syndrome*" could be conceived, in which a correlated set of defensive traits could be linked to sensitivity to electric shock. For instance, responsiveness to shock could correlate with defensive responsiveness to alarm pheromone components such as isopentyl Acetate (IPA), the main component of the sting pheromone (Boch et al., 1962), and 2-Heptanone, an alarm substance released by mandibular glands (Shearer and Boch, 1965). Foraging and defensive syndromes would constitute independent insulated modules coexisting within the same individual and defining its tendency to act as a forager or as a defender (Roussel et al., 2009).

3.5. Shock Sensitivity and Olfactory SER Conditioning

In Pavlovian learning, in which an animal learns that a conditioned stimulus (CS) acts as a predictor of the unconditioned stimulus (US), sensitivity to the US, which directly determines its salience for the animal, plays a critical role for learning efficiency and rate (Rescorla and Wagner, 1972). Higher sensitivity to an US results in better learning performances as shown by studies relating sucrose sensitivity and appetitive PER conditioning; bees which are highly sensitive to sucrose show better appetitive learning performances (Scheiner et al., 2003; Scheiner et al., 1999; Scheiner et al., 2005; Scheiner et al., 2001a, b). Does shock responsiveness affect in a similar way olfactory aversive learning in bees?

To answer this question, shock-responsiveness scores were determined in a group of honey bee foragers (see above), which were then divided into two subgroups according to their scores: bees exhibiting highest response selectivity and responding only to the highest shock voltages (scores 1 to 3; 'low responsiveness group') and bees exhibiting generalized, non-selective responses to 4 to 6 of the voltages tested including lower ones ('high-responsiveness group'). On the next day, bees were trained in a differential conditioning procedure to discriminate an odour paired with shock (CS+) from an odour not paired with shock (CS-). Both groups of bees learned to discriminate the CS+ from the CS- and remembered this information one hour later. Yet, the high-responsiveness group showed a higher percentage of conditioned responses to the CS+ than the low-responsiveness group. Responses to the CS- did not differ between groups. In the retention tests, bees of the high-responsiveness group also responded more to the CS+ than bees of the low-responsiveness group while no differences were found for the CS-.

These results show that the more responsive a bee is to electric shocks, the better it learns to associate an odor with this noxious stimulus. Similarly, In the case of sucrose reinforcement, the more responsive a bee is to sucrose, the better it learns and memorizes CS-US associations in appetitive olfactory and tactile learning protocols (Scheiner et al., 2003; Scheiner et al., 1999; Scheiner et al., 2005; Scheiner et al., 2001a, b). Taken together these results underline the crucial role of US sensitivity for learning and retention performances as underlined by models of classical conditioning, where US salience directly affects learning rate (Rescorla and Wagner, 1972).

3.6. Shock Sensitivity, Olfactory SER Conditioning and Caste Specialization within the Hive

We have seen so far that honey bee foragers exhibit a shock responsiveness that does not necessarily correlate with sucrose responsiveness, and that their US sensitivity directly determines their learning success in olfactory SER conditioning. Do these principles apply to other honeybee castes and do castes differ from each other in terms of these variables?

To answer this question, a first study focused on a comparison between guards and foragers in terms of shock responsiveness and aversive learning. Foragers were collected upon arrival at a feeder containing sucrose solution to which they were previously trained, thus ensuring that they were real nectar foragers. Guards were collected at the hive entrance after eliciting attack by means of a mechanical disturbance. One day after determining shock responsiveness scores of these two groups of bees, they were subjected to differential conditioning. Retention tests were again performed 1h after the last conditioning trial.

Shock responsiveness differed significantly between guards and nectar foragers, the responses of foragers to shocks being generally higher than those of guards, especially for lower voltages. Thus, guards are less sensitive to electric shocks than nectar foragers. Figure 7b shows that both guards and nectar foragers learned to discriminate between the CS+ and the CS- and remembered the aversive association one hour later. Yet, although both groups responded similarly to the CS- during conditioning, at the end of training, nectar foragers responded significantly more to the CS+ than guards. The same difference was found in the retention tests as nectar foragers remembered significantly better the CS+ than guards (which reflected their better acquisition) but did not differ in their response to the CS-.

Thus, the more responsive, and presumably more sensitive, foragers are the ones learning and remembering better aversive associations. Although this result appears surprising, it may be adaptive for guards to be less sensitive, and presumably more tolerant, to noxious stimuli. Accordingly, they would assign low values to an aversive reinforcement, thus determining lower acquisition and retention performances. Such a low sensitivity of guards to noxious stimuli may indeed be adaptive for honeybees, as defensive responses are costly for the colony (especially when recruitment takes place), and defensive responses should not be triggered by any kind of aggression, but rather by situations that are potentially dangerous for the colony.

Neural-based explanations could account for the difference found between guards and foragers in shock responsiveness and aversive conditioning. Dopamine levels in the bee brain depend on age (Schulz and Robinson, 1999; Taylor et al., 1992) so that older bees have more dopamine in their brains. Foragers, which are generally older than guards, are more sensitive to shock and thus more prone to learn aversive associations than guards (Roussel et al., 2009). Nurse bees are the youngest adult members of the colony and stay in close contact with the queen. Dopamine levels are even lower in nurses compared to guards and foragers (Schulz and Robinson, 1999; Taylor et al., 1992) and as a consequence, shock sensitivity should be lower and olfactory SER conditioning less successful in these bees. In addition, nurses are exposed to queen mandibular pheromone (QMP) via their close contacts with the queen. QMP is a chemical blend that has priming and acute effects on social control within the colony (Sandoz et al., 2007). Among these effects, QMP induces young workers to feed and groom the queen and primes bees to perform colony-related tasks (Keeling et al., 2003; Slessor et al., 1988).

Olfactory SER conditioning of nurse bees has been studied in relation to the presence of the queen and QMP (Vergoz et al., 2007b). One of the key components of QMP, homovanillyl alcohol (HVA), bears a striking structural resemblance to dopamine. The presence of this compound within the pheromone blend suggested that dopamine function in the brain of recipient young bees might be affected by exposure to QMP (Beggs et al., 2007). Indeed, exposure to QMP, and more precisely to HVA, affected dopamine levels, levels of dopamine receptor gene expression, and cellular responses to this amine in young worker bees. These results showed that dopamine levels in the bee brain not only depend on age but also on the contact with QMP (Beggs et al., 2007).

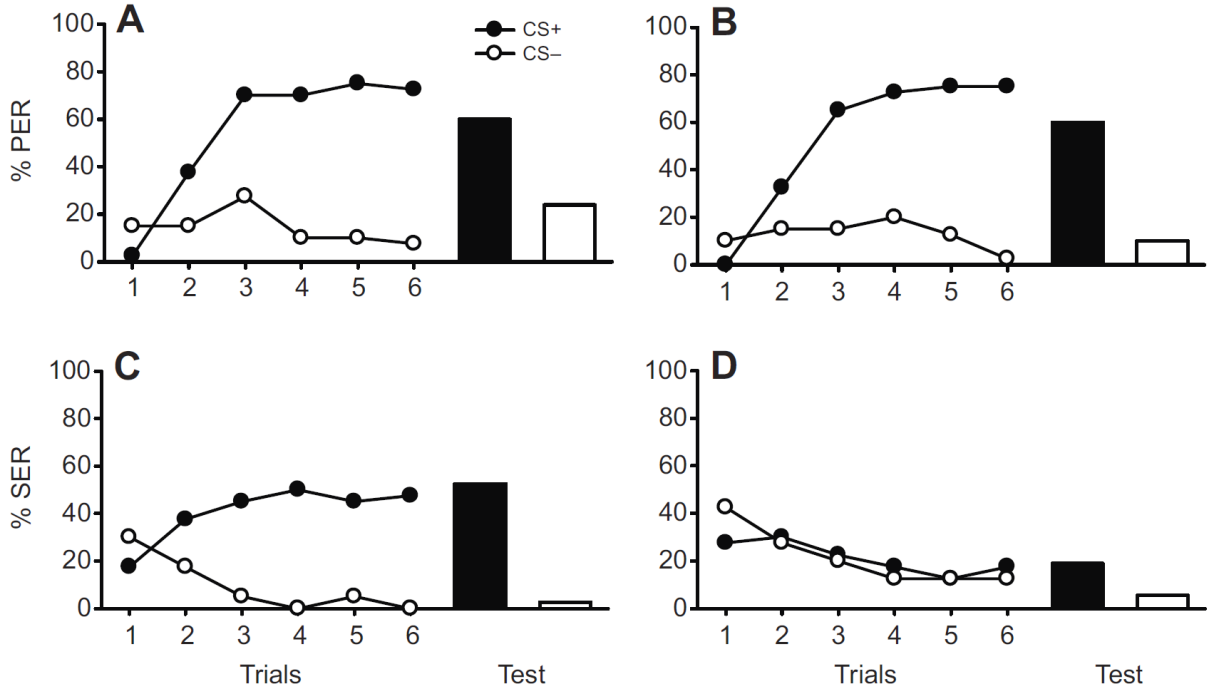


Figure 12: Effects of queen mandibular pheromone (QMP) on appetitive learning (A,B) or aversive learning (C,D) in 6-day-old workers. (A,B) Associative olfactory conditioning of the PER in control (non-exposed) bees and bees exposed to QMP, respectively. Bees were trained to discriminate between an odorant paired with sucrose (CS+) and a non-reinforced odorant (CS–). After 12 conditioning trials (six CS+ and six CS–), control bees clearly learned to discriminate between the two odorants and remembered this 1h after the last conditioning trial (A). QMP-exposed bees also learned to discriminate between the two odorants and remembered this 1h after the last conditioning trial (B). (C,D) Associative olfactory conditioning of the SER in control (non-exposed) bees and bees exposed to QMP, respectively. After 12 conditioning trials, control bees learned to discriminate between the two odorants and remembered this 1h later (C). QMP-exposed bees did not learn to discriminate between the two odorants; 1h after the last conditioning trial, the percentage of bees responding to the two odorants was similar (D); adapted from Tedjakumala and Giurfa, 2013.

How does this inhibition of dopaminergic signaling affect aversive olfactory learning in young bees? To answer this question, Vergoz et al (2007b) examined QMP's impact on associative olfactory learning in young bees (6-day old) exposed to QMP from the time of adult emergence. Bees of the same age maintained under identical conditions but without exposure to QMP were used as controls. These two groups were in turn subdivided into two groups, one trained following appetitive PER conditioning to discriminate an odour reinforced with sucrose from a non-reinforced odour (Fig. 12A, B), and another trained following aversive SER conditioning to discriminate an odour reinforced with shock from a non-reinforced odour (Fig. 12C, D) (Vergoz et al., 2007b). Both exposed and non-exposed bees learned the appetitive discrimination and showed retention one hour later (Fig. 12A, B). Interestingly, while non-exposed young bees (Fig. 12C) learned the aversive discrimination and remembered it one hour later, bees of the same age exposed to QMP failed to show

aversive learning and retention (Fig. 12D). Thus, QMP suppresses aversive olfactory learning in young bees but leaves their appetitive learning intact (Vergoz et al., 2007b). A possible interpretation of these results is that the inhibition exerted by QMP on aversive learning increases the probability that young nurses remain in close contact with their queen by impeding aversive experiences around her (Vergoz et al., 2007b).

The effect of QMP on associative learning of young bees reminds that of ecdysteroid hormones like 20-E when injected into adult bees (see above). Indeed, 20-E impairs aversive nut not appetitive learning. Moreover, higher impairment of aversive learning when bees are 2-day old correlates with higher levels of endogenous ecdysone (Hartfelder et al., 2002). Recent results have shown in addition that like 20-E (see above), QMP impairs aversive learning inducing a concomitant reduction of the AmGPCR19 receptor (Geddes et al., 2013). The ecdysone/dopamine signalling pathway would be therefore implied in aversive US signalling as well as in social regulation.

Taken together these results show how aversive olfactory SER conditioning has helped uncovering unsuspected aspects of social organization and division of labour within the hive. These articulate on specific and variable stimulus sensitivities, which in turn reflect complex regulation of biogenic-amine levels and neural signalling, which determine not only distinct aversive learning performances but mainly different behavioural roles and syndromes (i.e. sets of correlated behaviours across situations; see (Sih et al., 2004)) within the hive.

Objectives

The aim of this thesis was to characterise the dopaminergic neuronal network in the brain of honey bee which mediates the noxious/aversive signalling. Until the establishment of the aversive SER conditioning protocol (Vergoz et al., 2007a), most studies on honey bee learning and memory have used the appetitive context. SER conditioning offered therefore the opportunity to study for the first time the principles and mechanisms underlying aversive learning in bees.

Yet, the review of the knowledge gained in these last seven years based on SER conditioning allows identifying gaps which still have to be filled and questions that need to be answered. A precise knowledge about aversive US signalling in the honey bee brain is still missing so that we aimed at providing a multi-level characterization of dopaminergic signalling in the bee brain. Using tools from behavior, pharmacology, neuroanatomy and molecular biology we studied different aspects of dopaminergic signaling in order to understand its role in olfactory SER conditioning.

Specifically, we asked **(1)** how dopaminergic as well as other forms of aminergic signaling, mediate US responsiveness (i.e. responsiveness to electric shocks) in individual bees; **(2)** which dopaminergic circuits can be identified in the honey bee brain and how do these circuits relate to other stimulus-processing circuits to which they could assign an aversive value upon conditioning, and **(3)** what is the molecular impact of aversive conditioning on dopaminergic receptor expression in the mushroom bodies of the bee brain and how this potential impact affects behavioral responses to electric shocks.

The ***first part of our work*** consisted of a pharmacological study of the modulation of aversive US signalling in the brain. We used a combination of behavioural quantification of shock responsiveness to a series of increasing voltages, with injections of neurotransmitter antagonists in the bee brain. We aimed at answering if and how responsiveness to electric shocks is mediated by dopaminergic signalling in the brain. Additionally, we also studied the possible modulation of shock responsiveness by other existing neurotransmitters.

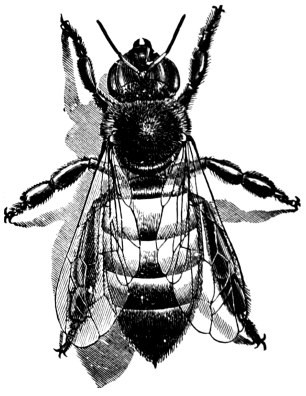
The ***second part of our work*** consisted of a neuroanatomical description of the dopaminergic neuronal circuits existing in the brain. Our aim was to provide an accurate neuroanatomical description of the circuits which could be involved in aversive US signalling. We used immunocytochemistry to visualise dopaminergic innervations in various

neuropiles of the bee brain and achieved multiple reconstructions of dopaminergic neurons using state-of-the-art neuroanatomical techniques. This study was performed to provide insights into the CS-US connectivity available in the bee brain, which could mediate different forms of aversive learning.

Finally, ***the third part of our work***, focused on long-term variations in the expression of specific dopaminergic receptors in the bee brain following aversive conditioning. We aimed at determining whether aversive learning induces long-term molecular changes in the Kenyon cells of the mushroom bodies, which have been associated with long-term memory. We asked whether the expression of various receptor types, including dopaminergic ones, varies three days after aversive conditioning and determined the consequences of this variation for shock responsiveness.

1

Pharmacological modulation of aversive responsiveness in honey bees



1st PART

Pharmacological modulation of aversive responsiveness in honey bees

Stevanus Rio Tedjakumala, Margaux Aimable and Martin Giurfa (2014)

Front. Behav. Neurosci., 07 January 2014

In the first part of our work, we studied the neural bases of innate aversive responsiveness in honey bees. We quantified noxious-stimulus responsiveness in harnessed bees by measuring their sting extension response (SER) to a series of electric shocks of increasing voltage. We studied the neuropharmacological bases of such responsiveness by using a combination of behavioral quantifications and injection of antagonists of target neurotransmitters inthe bee brain. In this way, we determined the effect of blocking these different forms of neurotransmission on shock responsiveness of harnessed bees.

We focused on the biogenic amines octopamine, dopamine and serotonin, and on the ecdysteroid 20-hydroxyecdisone. We found that both octopamine and 20-hydroxyecdisone are dispensable for shock responsiveness while dopamine and serotonin act as down-regulators of sting responsiveness. As a consequence, antagonists of these two biogenic amines induce an increase in shock responsiveness to shocks of intermediate voltage; serotonin, can also increase non-specific responsiveness.

We thus provided the first evidence of the involvement of biogenic amines in the central control of sting responsiveness to noxious stimuli and discussed the implications of our results. We suggested that different classes of dopaminergic neurons exist in the bee brain

and we defined at least two categories: an instructive class mediating aversive labeling of conditioned stimuli in associative learning, and a global gain-control class which down-regulates responsiveness upon perception of noxious stimuli. Serotonergic signaling together with down-regulating dopaminergic signaling may play an essential role in attentional processes by suppressing responses to irrelevant, non-predictive stimuli, thereby allowing efficient behavioral performances.

Pharmacological modulation of aversive responsiveness in honey bees

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Abstract

Within a honey bee colony, individuals performing different tasks exhibit different sensitivities to noxious stimuli. Noxious-stimulus sensitivity can be quantified in harnessed bees by measuring the sting extension response (SER) to a series of increasing voltages. Biogenic amines play a crucial role in the control of insect responsiveness. Whether or not these neurotransmitters affect the central control of aversive responsiveness, and more specifically of electric-shock responsiveness, remains unknown. Here we studied the involvement of the biogenic amines octopamine, dopamine and serotonin, and of the ecdysteroid 20-hydroxyecdisone in the central control of sting responsiveness to electric shocks. We injected pharmacological antagonists of these signaling pathways into the brain of harnessed bees and determined the effect of blocking these different forms of neurotransmission on shock responsiveness. We found that both octopamine and 20-hydroxyecdisone are dispensable for shock responsiveness while dopamine and serotonin act as down-regulators of sting responsiveness. As a consequence, antagonists of these two biogenic amines induce an increase in shock responsiveness to shocks of intermediate voltage; serotonin, can also increase non-specific responsiveness. We suggest that different classes of dopaminergic neurons exist in the bee brain and we define at least two categories: an instructive class mediating aversive labeling of conditioned stimuli in associative learning, and a global gain-control class which down-regulates responsiveness upon perception of noxious stimuli. Serotonergic signaling together with down-regulating dopaminergic signaling may play an essential role in attentional processes by suppressing responses to irrelevant, non-predictive stimuli, thereby allowing efficient behavioral performances.

Keywords: neuromodulation, honeybee, stingextensionresponse (SER), aversiveresponsiveness, octopamine, dopamine, serotonin, 20-hydroxyecdisone

Introduction

Honey bees are a well-established model for the study of learning and memory (Giurfa, 2007; Menzel, 1999). In the laboratory, associative olfactory learning is studied using harnessed bees subjected to Pavlovian protocols such as the appetitive conditioning of the proboscis extension reflex (PER) (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Takeda, 1961) and the aversive conditioning of the sting extension reflex (SER) (Carcaud et al., 2009; Giurfa et al., 2009; Vergoz et al., 2007). In the former, bees learn to associate an odorant as conditioned stimulus (CS) with sucrose solution as unconditioned stimulus (US). In the latter, bees learn the association between an odorant as CS and an electric shock as US. Although much is known about PER conditioning in terms of underlying circuitries, neural structures and neurotransmitters (Giurfa, 2007; Giurfa and Sandoz, 2012; Menzel, 1999), less is known about SER conditioning given its recent establishment (Tedjakumala and Giurfa, 2013; Vergoz et al., 2007).

A proper characterization of the SER protocol implies a thorough analysis of the unconditioned response, the sting extension response. This response is elicited by noxious stimuli (Breed et al., 2004) and can be systematically triggered in harnessed bees by the delivery of a mild electric shock (Lenoir et al., 2006; Núñez et al., 1997; Núñez et al., 1983; Vergoz et al., 2007). Sting responsiveness to shocks varies among bees within a colony (Lenoir et al., 2006; Roussel et al., 2009). For instance, foragers exhibit higher sting extension responsiveness than guards when stimulated with a series of increasing voltages. Based on this different sensitivity, they also learn better odor-shock associations (Roussel et al., 2009). These results demonstrate the crucial role of US sensitivity for learning and retention performances as underlined by models of classical conditioning, where US salience directly affects learning rate (Rescorla and Wagner, 1972). They also show that sensitivity to noxious stimulations may determine behavioral biases and specializations within the hive, thus contributing to the social organization of the colony (Roussel et al., 2009; Tedjakumala and Giurfa, 2013).

Biogenic amines play a crucial role in the control of insect responsiveness. Unconditioned appetitive responsiveness, measured through PER to a series of increase concentrations of sucrose solution (Pankiw and Page, 2003, 1999, 2000; Scheiner et al., 2004), is modulated by octopamine (OA) and dopamine (DA) signaling (Scheiner et al.,

2002). For instance, feeding or injection of both OA and tyramine, an OA precursor, significantly increase PER to sucrose stimulation (Scheiner et al., 2002); on the contrary, DA decreases sucrose responsiveness when injected into the thorax but has no effect if fed. Consistently, injection or feeding of the DA receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN) reduces sucrose responsiveness significantly (Scheiner et al., 2002). Whether or not these biogenic amines affect unconditioned *aversive* responsiveness remains unknown. In particular, the implication of these neurotransmitters in the control of the *unconditioned SER* (i.e. in electric-shock responsiveness) has not been studied until now. In isolated abdominal preparations, OA potentiates reflexive sting extension responses and this potentiation persists for at least 3 h (Burrell and Smith, 1995). Yet this analysis does not reveal how aminergic signaling at the brain level drives sting responsiveness and the perception of noxious stimulations.

Studies on olfactory SER conditioning have shown that DA and the ecdysteroid 20-hydroxyecdisone (20E) are differently involved in this form of aversive learning (Geddes et al., 2013; Vergoz et al., 2007). DA is thought to mediate the aversive properties of electric shock as blocking of DA signaling impairs aversive learning and retention (Vergoz et al., 2007). 20E increases the expression of the DA receptor gene, Amdop2, and reduces the expression of the putative dopamine/ecdysone receptor gene, Amgpcr19, that tends to be highly expressed in the brains of foragers exhibiting strong aversive learning (Geddes et al., 2013); as a consequence, higher levels of 20E correlate with deficient aversive learning performances (Geddes et al., 2013). Serotonin (5-HT) has been repeatedly related to aggressiveness in invertebrates (Kravitz and Huber, 2003) and SER is a component of aggressive/defensive behaviors (Breed et al., 2004); however, the potential role of 5-HT in aversive responsiveness has not been addressed until now. Here we studied the involvement of the biogenic amines OA, DA and 5-HT, and of the ecdysteroid 20E in sting responsiveness to electric shocks of increasing voltage. We used pharmacological blocking procedures to determine if and how these different neural signaling pathways affect shock responsiveness in honey bees. As variations in shock responsiveness correlate with diverse behavioral specializations within the colony (Roussel et al., 2009; Tedjakumala and Giurfa, 2013), our experiments allow discussing the functions of biogenic amines for the social organization of the hive.

Materials and Methods

Insects

Honey bees, *Apis mellifera*, were obtained from outdoor colonies. Nectar foragers were collected twice a day, between experimental series, from an artificial feeder to which they were previously trained. The feeder was located at 20 m from the hive and contained sucrose solution 40% (weight/weight). Nectar foragers were used because of their higher shock responsiveness (Roussel et al., 2009) and to reduce the high variability in biogenic amine titers that exist between different castes within a colony (Wagener-Hulme et al., 1999). We aimed, in this way, at ensuring that pharmacological treatments act on comparable levels.

Once captured, the bees were brought to the laboratory and chilled on ice for 5 minutes until they stopped moving. They were then harnessed on individual holders (**Figure 1**) designed for aversive stimulation via delivery of an electric shock (Carcaud et al., 2009; Giurfa et al., 2009; Vergoz et al., 2007). Holders consisted of two brass plates fixed to a Plexiglas plate. Brass plates were connected to the output of the stimulator (60 Hz – AC current). The resistance measured between the two plates in the presence of the bee was 200-300 KΩ. Conductance gel was applied below the thorax to ensure efficient shock delivery. Low melting-point wax was used to immobilize the head and facilitate drug injection. Once fixed, each bee was fed with a droplet (5 µl) of sucrose solution 30% and kept resting for 1.5h.

Measuring Shock Responsiveness

Responsiveness to electric shock was measured using the SER. We stimulated bees with increasing voltages and recorded whether the bee extended its sting. The following voltages were applied in ascending order: 0.25, 0.5, 1, 2, 4 and 8 V. By alternating between a non-shocked (placement) and a shocked phase every 5 minutes, each individual was given, in this way, a 10-minute shock interval. Each shock trial lasted 20 s; it consisted of 10 s of familiarization in the setup, followed by 2 s of electric shock; afterwards, the bee stayed for another 8 s before being replaced by the next test subject. Placement trials, in which the bee was placed in the setup during 20 s without shock delivery, were interspersed between shock trials to avoid sensitization. The inter-stimulus interval was approximately 1 min.

As shock responsiveness can vary from day to day depending on weather and/or intracolonial conditions, the response of experimental groups was always measured in parallel with that of their corresponding control groups.

Pharmacological Drugs and Injections

A tiny hole was pricked into the cornea of the median ocellus to allow the insertion of a 10 µl-syringe (World Precision Instrument), which was used to inject 200 nl of each drug solution. Drugs were injected into the brain of immobilized bees along the median ocellar nerve (**Figure 1**). The ocellar nerve consists of a thick fiber bundle, approximately 40 µm in diameter, which runs medially and caudally from the dorsal margin of the head capsule into a depth of 300 µm into the protocerebrum. Previous works have shown that drugs migrate through the ocellar tract into the bee brain and that drug distribution is fast (less than 5 min) and homogenous within the brain (Menzel et al., 1999). After use, syringes were cleaned in PBS, ethanol and distilled water, completing three full wash cycles in each case.

The following substances, were injected 30 min before the experiment: *epinastine hydrochloride* (OA receptor antagonist (Roeder et al., 1998)), *cis-(Z)-flupentixol dihydrochloride* (DA receptor antagonist (Blenau et al., 1998)), *methiothepin mesylate* (5-HT antagonist (Blenau and Thamm, 2011)), *cyproheptadine hydrochloride sesquihydrate* (5-HT antagonist; (Howarth et al., 2002)), *ketanserin* (5-HT antagonist; (Howarth et al., 2002; Wedemeyer et al., 1992)), *20E* (ecdysteroid; (Geddes et al., 2013)), and PBS (control). Three different drugs were thus used to study the role of 5-HT in aversive responsiveness, which had never been addressed until now.

All substances, except for 20E and PBS, were obtained from Sigma-Aldrich France. 20E was kindly provided by Dr. Rodrigo Velarde (Wake Forest University, Winston-Salem, USA); PBS was obtained from EUROMEDEX (Strasbourg, France). Injection time was chosen based on previous experiments which have shown that the effects of aminergic blockers reach a stable level approximately 30 min after drug application (Blenau and Erber, 1998; Mercer and Erber, 1983; Scheiner et al., 2002; Vergoz et al., 2007).

20E was first dissolved in 1 ml isopropanol 100% to prevent crystallization of the steroid, resulting in a stock solution of 10 mg/ml, which was then diluted down to 1 mg/ml in

PBS. For all other substances, 1 mg was diluted in 1 ml PBS. Final concentrations obtained were 3.5 mM of epinastine, 1.97 mM of flupentixol, 2.21 mM of methiothepin, 2.85 mM of cyproheptadine, 1.83 mM for ketanserin and 2.08 mM of 20E. To test for dose-response effects, we prepared for each drug, except for cyproheptadine, two additional dilution series of 1:100 and 1:10000; for cyproheptadine only the additional dilution of 1:100 was used. In all case, aliquots were made and kept in -20 °C until use. Each aliquot was used for one whole week and kept during this time in 4 °C.

Data Analysis

The occurrence of SER was recorded during the 2 s of electric stimulation in shock trials, and during the corresponding 2 s without stimulation in placement trials. An observable sting extension was given a score of 1; incomplete sting movements were scored as 0. Sting responsiveness (% of bees responding to a given voltage) was then calculated. Two-way ANOVA (Statistica, StatSoft) was used to compare each treatment against its PBS control and for inter-treatment comparisons. ANOVA procedures are applicable in the case of binary response variables despite their lack of normality if comparisons imply equal cell frequencies and at least 40 degrees of freedom of the error term (Lunney, 1970; Matsumoto et al., 2012), conditions which were fulfilled by our experiments. Under these conditions, the use of repeated-measurement ANOVA allowed, not only within-group analysis, but also between-group comparisons. An alpha level of 0.05 was used throughout.

Results

Effects of OA Blocking on Aversive Responsiveness

PBS-injected bees (n = 41) showed a typical increase in responsiveness with increasing voltages (Roussel et al., 2009), which reached 100% at 8 V. Injection of the OA blocker epinastine did not have a significant effect on shock responsiveness (**Figure 2A**). All three epinastine concentrations assayed (3.5 mM: n = 40; $3.5x10^{-2}$ mM: n = 41; $3.5x10^{-4}$ mM: n =

41) induced the same responsiveness as the PBS control (Two-way ANOVA: $F_{3,159} = 1.48$, $p = 0.22$).

Responses to placements in the setup (**Figure 2B**) interspersed between shock trials remained low along the experiment and were unaffected by epinastine ($F_{3,159} = 0.48$, $p = 0.70$). Thus, neither were the bees sensitized nor did epinastine change their basal responsiveness.

Effects of DA Blocking on Aversive Responsiveness

Injection of the DA blocker flupentixol into the bee brain induced a significant increase in shock responsiveness compared with PBS-injected bees (**Figure 3A**; $F_{3,160} = 5.46$, $p < 0.01$). There were no significant differences between flupentixol-injected bees ($F_{2,120} = 1.08$, $p = 0.34$), thus showing that all three concentrations of this drug had the same enhancing effect. Each of the three flupentixol concentrations assayed increased responsiveness to intermediate voltages with respect of the responsiveness exhibited by PBS-injected bees (1.97 mM: n = 41 $F_{1,80} = 7.28$, $p < 0.01$; 1.97×10^{-2} mM: n = 41 $F_{1,80} = 13.02$, $p < 0.001$, and 1.97×10^{-4} mM; n = 41: $F_{1,80} = 7.07$, $p < 0.01$). Tukey post hoc tests showed that increases with respect to PBS-injected bees were significant for 1 V in all three concentrations ($p < 0.001$ for all three comparisons) and for 0.5 V in the intermediate concentration (1.97×10^{-2} mM: $p < 0.05$) but not for the other voltages despite barely non-significant results in the intermediate voltage of 2 V. Thus, by blocking the DA system, responsiveness to electric shocks of intermediate voltage was significantly increased.

Responsiveness during placement trials remained low and constant both for PBS- and flupentixol-injected bees (**Figure 3B**; $F_{3,160} = 0.41$, $p = 0.74$) so that the neither the injection procedure nor the placement trials per se affected basal responsiveness.

The fact that the blocking of DA signaling increased shock responsiveness was unexpected as it had been previously found that this signaling mediates the reinforcing properties of the electric shock (Vergoz et al., 2007). Its suppression was expected to *lower* shock responsiveness. We thus decided to verify this finding. We performed a replicate of this experiment to verify the enhancing effect of flupentixol using the highest concentration previously used (1.97 mM: n = 69) and a PBS group as a control (n = 68). The results of this

replicate (**Figure 3C**) confirmed that blocking the DA system via flupentixol injection increases shock responsiveness ($F_{1,135}$ = 4.07, p < 0.05). Responses in placement trials remained low and unaffected by flupentixol (not shown) so that there were no differences between flupentixol- and PBS-injected bees in these trials ($F_{1,135}$ = 0.01, p = 0.97). Finally, we performed a further replicate aimed at testing again the lowest flupentixol concentration previously used ($1.97x10^{-4}$ mM). This was necessary as in the first replicate (**Figure 3A**) a significant effect was already visible at this lowest concentration, thus raising the question of whether this effect really reflected a flupentixol blockade of dopaminergic receptors or was rather a non-specific effect due to the use of an immoderate drug concentration. We thus measured shock responsiveness in flupentixol-injected bees using the same concentration ($1.97x10^{-4}$ mM; n = 49) and in PBS-injected bees (n = 46). Results from this replicate (**Figure 3D**) showed that contrarily to the highest flupentixol concentration, which consistently enhanced responsiveness in different replicates (**Figures 3A,C**), the lowest concentration did not induce a significant increase of responsiveness with respect to PBS-injected bees ($F_{1,93}$ = 0.03, p = 0.87). Responses in placement trials did not differ between flupentixol- and PBS-injected bees ($F_{1, 93}$ = 0.008, p = 0.93).Thus, the flupentixol concentration of $1.97x10^{-4}$ mM was probably on the verge of significance and certainly not excessive. This ensures that our flupentixol concentrations were moderate and targeted specifically dopaminergic receptors.

Effects of 20E on Aversive Responsiveness

We then tested the effect of 20E on sting responsiveness to aversive stimulations. It has been reported that sting responsiveness is unaffected by injection of 20E (Geddes et al., 2013); yet, a single concentration was used in this study (0.312 mM) so that caution is required before generalizing this conclusion. Because 20E levels correlate inversely with aversive learning success (Geddes et al., 2013), injection of a higher concentration of 20E could impair sting responsiveness.

Injection of 20E did not affect sting responsiveness to electric shocks (**Figure 4A**) compared to PBS controls ($F_{3,158}$ = 0.93, p = 0.43). For all three 20E concentrations assayed (2.08 mM: n = 41, $2.08x10^{-2}$ mM: n = 40, $2.08x10^{-4}$ mM: n = 40) responses to increasing voltages augmented in a similar way as that of control bees (n = 41). Responses during placement trials (**Figure 4B**) interspersed between shock trials remained low along the

experiment and were unaffected by 20E ($F_{3,158}$ = 0.72, p = 0.54). A second replicate of this experiment (**Suppl. Figure 1**) yielded the same results: for all three concentrations tested the response of 20E-injected bees was the same as that of control bees both in shock (**Suppl. Figure 1A:** $F_{3,143}$ = 1.90, p = 0.13) and in placement trials (**Suppl. Figure 1B:** $F_{3,143}$ = 0.69, p = 0.55).

Thus, irrespective of the concentration of 20E used, the proportion of bees responding reflexively with sting extension to electric shocks increased in a similar way as in control bees, thus showing that 20E did not induce variations in shock sensitivity.

Effects of 5-HT Blocking on Aversive Responsiveness

The role of 5-HT in aversive responsiveness was studied in more detail given the lack of prior reports on the role of this biogenic amine in aversion and aggression in honey bees. Three different blockers of 5-HT signaling were used: while cyproheptadine shows potent non-competitive inhibition (Howarth et al., 2002), ketanserin and methiothepin show potent competitive inhibition in the presence of 5-HT (Howarth et al., 2002; Vleugels et al., 2013). Methiothepin acts as a non-specific antagonist of all known 5-HT receptors (Am5-HT_{1A}, Am5-$HT_{2\alpha}$ and Am5-HT_{7}) with the exception of Am5-$HT_{2\beta}$ (Schlenstedt et al., 2006; Thamm et al., 2010; Thamm et al., 2013). Ketanserin is an antagonist of Am5-HT_{2} receptor (Thamm et al., 2013); its effect on other Am5-HT receptors is unknown. Finally, cyproheptadine antagonizes both the Am5-$HT_{2\alpha}$ and the Am5-$HT_{2\beta}$ receptors (Thamm et al., 2013).

Effects of 5-HT Blocking by Methiothepin

Injection of the 5-HT blocker methiothepin into the brain induced an increase in shock responsiveness with respect of PBS-injected bees (n = 41) which was close to significance (**Figure 5A**; $F_{3,160}$ = 2.62, p= 0.052). There were no significant differences between methiothepin-injected bees ($F_{2,120}$ = 0.03, p = 0.96), thus showing that all three concentrations of this drug had the same enhancing effect. Nevertheless, pairwise comparisons between the PBS control and each of the methiothepin concentrations yielded a significant result in each case (2.2 mM: n = 41, $F_{1,80}$ = 4.62, p < 0.05; $2.2x10^{-2}$ mM: n = 41, $F_{1,80}$ = 4.32, p < 0.05; $2.2x10^{-4}$ mM: n = 41, $F_{1,80}$ = 5.75, p < 0.05). Thus, when analyzed separately, all three

concentrations increased significantly sting responsiveness to electric shock. Tukey tests showed that increases with respect to PBS-injected bees were significant for 1 V in all methiothepin concentrations (2.2 mM and $2.2x10^{-4}$ mM: $p < 0.01$, $2.2x10^{-2}$ mM: $p < 0.001$). Thus, methiothepin injections increased significantly the responsiveness to an electric shock of intermediate voltage. On the contrary, they did not affect the basal responsiveness in placement trials as SER remained low and similar to that of PBS controls (**Figure 5B**; $F_{3,160} = 1.58$, $p = 0.20$).

A replicate of this experiment was performed in order to include a methiothepin concentration lower than those used above. This was necessary because in pharmacological experiments, the effect of a drug should be tested at a concentration as low as possible to avoid possible side effects such as a lack of specificity due to excessive drug concentration. Given that in the previous replicate the effect of methiothepin already saturated at the lowest concentration ($2.2x10^{-4}$ mM), we now tested the effect of methiothepin $2.2x10^{-8}$ mM (n = 50) to demonstrate that in our previous experiment this drug was used at moderate concentrations. In parallel, the effect of the highest concentration (2.2 mM) was again tested (n = 50), together with the corresponding PBS control (n = 49). **Figure 5C** shows that, as in the previous replicate, the highest concentration of methiothepin induced a significant increase of responsiveness during shock trials when compared to the control (2.2 mM: $F_{1,97} = 4.75$, $p < 0.05$). On the contrary, the lowest concentration did not (2. $2x10^{-8}$ mM: $F_{1,97} = 0.48$, $p = 0.49$). Neither the high nor the low methiothepin concentration affected basal responsiveness in placement trials (**Figure 5D**) in which SER remained low and similar to that of PBS controls ($F_{2,146} = 6.00$, $p = 0.55$). The differential effect of these two methiothepin concentrations on shock responsiveness shows that our experiments were done at reasonably moderate drugs concentrations, so that the enhancing effect induced by higher methiothepin concentrations was indeed through blockade of 5-HT receptors.

A further replicate of this experiment was performed using the highest concentration of methiothepin (2.2 mM; n = 67) and a corresponding PBS group (n = 65) to verify the enhancing effect of methiothepin on shock responsiveness (**Suppl. Figure 2**). The response of methiothepin-injected bees showed again an increase of responsiveness to electric shocks which, in this case, was close to significance (**Suppl. Figure 2A**: $F_{1,130} = 3.34$, $p = 0.07$). Placement trials also showed an increased in responsiveness in methiothepin-injected bees with respect of PBS-injected bees (**Suppl. Figure 2B**: $F_{1,130} = 8.30$, $p < 0.01$), thus showing

that methiothepin induced in this case a general, non-specific increase in responsiveness. Interestingly, this increase occurred at the begin of the experiment and vanished along placement trials ($F_{5,650} = 28.34$, $p < 0.001$), thus showing a potential habituating effect.

Taken together these findings indicate that the 5-HT system plays a significant role in sting responsiveness to electric shocks and that it can even underlie general arousal and non-specific responsiveness.

Effects of 5-HT Blocking by Ketanserin

Injection of the 5-HT blocker ketanserin induced a significant increase in shock responsiveness to electric shocks with respect of the PBS control (**Figure 6A**: $F_{3,162} = 2.92$, $p < 0.05$). There were significant differences between the three groups of bees injected with ketanserin ($F_{2,120} = 3.89$, $p < 0.05$), as the increase in shock responsiveness was more evident for the higher ketanserin concentration (1.83 mM). This conclusion was confirmed by the pairwise comparisons between PBS controls (n = 43) and the three ketanserin groups: only the highest concentration increased significantly shock responsiveness (1.83 mM: n = 40, $F_{1,81} = 6.25$, $p < 0.05$) while the other two concentrations did not (1.83×10^{-2} mM: n = 41, $F_{1,82} = 0.18$, $p = 0.67$; 1.83×10^{-4} mM: n = 42, $F_{1,83} = 0.54$, $p = 0.46$). Tukey tests used to compare PBS responses and responses at the highest ketanserin concentration revealed significant differences at 1 V ($p < 0.05$).

In placement trials (**Figure 6B**) there was no difference between ketanserin- and PBS-injected bees ($F_{3,162} = 0.95$, $p = 0.41$). Yet, in both cases, an increase in general responsiveness was observed at the beginning of the placement trials, which vanished afterwards in successive placement trials ($F_{5,600} = 27.20$, $p < 0.001$).

Thus, sting responsiveness to electric shocks was increased by the injection of ketanserin in its highest concentration. As for one of the methiothepin replicates (**Suppl. Figure 2B**), we observed an increase of placement responsiveness, which returned afterwards to basal levels along trials.

Effects of 5-HT Blocking by Cyproheptadine

Injections of cyproheptadine induced the clearest increase in shock sensitivity (**Figure 7A**: $F_{2,87} = 7.81$, $p < 0.001$), and thus in sting responsiveness, with respect to PBS controls (n = 30) and previous 5-HT antagonists (see above). There were no significant differences between the two groups of cyproheptadine-injected bees ($F_{1,58} = 0.87$, $p = 0.35$), thus showing that both concentrations (2.85 mM: n = 30; 2.85×10^{-2} mM: n = 30) had the same enhancing effect. Indeed, each cyproheptadine concentration taken separately increased significantly sting responsiveness with respect of PBS controls (2.85 mM: $F_{1,58} = 13.80$, $p < 0.001$; 2.85×10^{-2} mM: $F_{1,58} = 8.12$, $p < 0.01$). Tukey tests comparing PBS responses and responses of the two cyproheptadine concentrations revealed significant differences at 1 and 2 V in both cases ($p < 0.001$).

Responses during placement trials (**Figure 7B**) interspersed between shock trials remained low along the experiment and were unaffected by cyproheptadine ($F_{2,87} = 0.50$, $p = 0.61$). Thus, blocking 5-HT signaling by cyproheptadine determined a significant increase in shock but not in placement responsiveness.

A replicate of this experiment was performed in order to include a cyproheptadine concentration lower than those used above. Given that in the previous replicate the effect of cyproheptadine already saturated at the lowest concentration tested (2.85×10^{-2} mM), we now tested the effect of cyproheptadine 2.85×10^{-8} mM (n = 49) to demonstrate that in our previous experiment this drug was used at moderate concentrations. In parallel, the effect of the highest concentration (2.85 mM) was again tested (n = 50), together with the corresponding PBS control (n = 49). **Figure 7C** shows that, as in the previous replicate, the highest concentration of cyproheptadine induced a significant increase of responsiveness during shock trials when compared to the control (2.85 mM: $F_{1,97} = 4.79$, $p < 0.05$) while the lowest concentration did not ($F_{1,75} = 2.65$, $p = 0.11$). Neither the high nor the low cyproheptadine concentration affected basal responsiveness in placement trials (**Figure 7D**) in which SER remained low and similar to that of PBS controls ($F_{2,145} = 0.0005$, $p = 0.99$). The differential effect of these two cyproheptadine concentrations on shock responsiveness shows that our experiments were done at reasonably moderate drugs concentrations, so that the enhancing effect induced by higher methiothepin concentrations was indeed through blockade of 5-HT receptors.

Discussion

Our study provides the first neuropharmacological dissection of the neurotransmitter systems underlying the central control of sting responsiveness to noxious stimuli in honey bees. By injecting pharmacological antagonists into the bee brain, we determined the effect of blocking different forms of neurotransmission on shock responsiveness. We found that both OA and 20E are dispensable for shock responsiveness while DA and 5-HT act as repressors of sting responsiveness; antagonists of these two biogenic amines induce an increase in shock responsiveness to shocks of intermediate voltage.

Octopamine and Aversive Responsiveness

OA blocking through epinastine did not affect sting responsiveness to electric shock. Injection of three different concentrations of the OA antagonist epinastine into the bee brain did not induce any change in SER thus indicating that this biogenic amine is not involved in the central control of this reflexive response. Epinastine was chosen due to its high specificity and affinity to OA receptors (Roeder et al., 1998). Mianserin, another drug previously used as OA antagonist in the bee, was avoided due to its side-effects on the serotonergic system (Fernández et al., 2012). Epinastine blocks specifically AmOA1, the only OA receptor identified so far in the honey bee (Farooqui et al., 2004; Grohmann et al., 2003). This OA receptor is thus dispensable for shock responsiveness.

Isolated abdominal preparation have been used to show that OA reduces the level of rhythmic neuromuscular activity during stimulated stinging response trials, but does not alter the activity in pre-stimulation baseline trials or post-stimulation recovery trials. Local applications of OA at the level of the abdominal preparations showed that OA also potentiates SER and this potentiation persisted for at least 3h (Burrell and Smith, 1995). Yet, the same isolated abdominal preparations showed no differences between castes in sting responsiveness (Burrell and Smith, 1994) although it is clear that such differences exist (Lenoir et al., 2006; Roussel et al., 2009). It was thus concluded that "*any effect of caste must arise in more anterior ganglia and/or in the brain*" (Burrell and Smith, 1994). In our case, we conclude that the lack of effect of epinastine in our *in toto* preparation shows that the abdominal effects of OA are under central control.

The lack of effect of OA antagonism on shock responsiveness is in agreement with the notion of modularity of appetitive vs. aversive behaviors (Roussel et al., 2009; Tedjakumala and Giurfa, 2013). In this scheme, OA is predominantly associated with appetitive behavior: OA is crucial for appetitive responsiveness as feeding or injection of both OA and tyramine, an OA precursor, significantly increase PER to sucrose stimulation (Scheiner et al., 2002). Also, in appetitive olfactory PER conditioning, OA is said to mediate the reinforcing properties of sucrose reward (Farooqui et al., 2003; Hammer, 1993; Hammer and Menzel, 1998). Therefore, pairing an odor with injections of OA in the bee brain leads to olfactory learning in harnessed bees, which exhibit afterwards PER to this odor (Hammer and Menzel, 1998). In cricket visual and olfactory learning, pharmacological blocking of OA receptors impairs the acquisition of appetitive but not aversive learning (Unoki et al., 2005, 2006). In *Drosophila* mutants that have the biosynthetic pathway to OA blocked, learning of an odor-sucrose association is also impaired (Schwaerzel et al., 2003). These mutants can, however, learn an aversive olfactory discrimination, in which they have to avoid an odorant previously paired with an electric shock (Schwaerzel et al., 2003). Recently, the exclusiveness of OA neurotransmission for appetitive reinforcement signaling has been revised in the fruit fly where a group of dopamine neurons was found to signal sugar reward to the mushroom bodies, the site where appetitive memory traces are formed (Liu et al., 2012b). These DA neurons are selectively required for the reinforcing property of, but not a reflexive response to, the sugar stimulus, which is mediated by OA. Thus, OA-dependent memory formation requires signaling through DA neurons (Burke et al., 2012). These experiments indicates that sweet taste engages a distributed OA signal that reinforces memory through discrete subsets of mushroom-body-targeted DA neurons (Burke et al., 2012). Furthermore, OA signaling also intervenes in the consolidation of aversive, intermediate-term memory in Drosophila (Wu et al., 2013). Following odor-shock learning, the anterior paired lateral (APL) neurons release OA to the α' and β ' Kenyon cells of the mushroom bodies and this signal is necessary for the consolidation of anesthesia resistant memory, a component of intermediate term memory retrievable 3 h after conditioning.screte subsets of mushroom-body-targeted DA neurons.

Dopamine and Aversive Responsiveness

Flupentixol was chosen as antagonist of the dopaminergic system due to its high binding affinity to D1- as well as D2-like receptors (Kokay and Mercer, 1996). Among various

dopaminergic antagonists assayed in olfactory SER conditioning, it proved to be highly effective to impair olfactory acquisition and mid-term retention (Vergoz et al., 2007), thus indicating that dopaminergic signaling underlies the aversive reinforcement properties of the electric shock. Other dopaminergic antagonists such as fluphenazine have been assayed on aversive olfactory conditioning and had less effect on behavioral performances (Vergoz et al., 2007).

In the present work, dopaminergic blocking through flupentixol induced an increase of shock responsiveness for low/intermediate voltages (0.5–1 V), thus reflecting an enhancement in shock sensitivity. At higher concentrations no differences between flupentixol and PBS-injected bees was found, probably because of a ceiling effect. The increase of shock sensitivity at lower voltages was observed for different concentrations of flupentixol and in different replicates of this experiment, thus showing that the effect was robust and repeatable. The result thus indicates that DA acts as a depressor of sting responsiveness to electric shocks so that when its effect is antagonized, responsiveness increases.

This results is consistent with those of studies in which the effect of DA on sucrose responsiveness was analyzed (Scheiner et al., 2002). DA *decreases* sucrose responsiveness when injected into the thorax. Also, injection or feeding of the DA receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN) reduces sucrose responsiveness significantly (Scheiner et al., 2002). Although we did not test 6,7-ADTN, it can be predicted that injection of this DA receptor agonist should also decrease shock responsiveness. In olfactory PER conditioning, injection of DA into the antennal lobes reduces significantly olfactory retention both after one and three conditioning trials (Macmillan and Mercer, 1987). DA seems, therefore, to play a depressing role in a series of appetitive and aversive responses.

Yet, a different role for DA was suggested based on protocols of aversive conditioning in bees, crickets and flies. Besides the above-mentioned fact that a subset of DA neurons convey an appetitive reinforcement signal to MBs, the role of the dopaminergic system in insect learning has been related to aversive-reinforcement signaling in the insect brain. In crickets, pharmacological blocking leads to an impairment of visual and olfactory aversive learning (Unoki et al., 2005, 2006). In adult fruit flies, blockade of DA neurons impairs olfactory aversive learning (Schwaerzel et al., 2003); activation of a specific subset of DA neurons (distinct from that conveying appetitive signals, see above) in mutant flies substitutes for shock reinforcement in aversive olfactory conditioning (Aso et al., 2012; Aso et al., 2010;

Claridge-Chang et al., 2009); similar results were obtained in *Drosophila* larvae where activation of DA neurons contingent to odor presentation results in odor avoidance (Schroll et al., 2006), thus showing that a specific subset of DA neurons substitute for aversive reinforcement in aversive learning. As mentioned before, in the honey bee, a similar conclusion was originally reached in aversive olfactory SER conditioning (Vergoz et al., 2007): in this case, injection of the DA antagonists flupentixol into the bee brain suppresses the capacity to learn and retrieve odor-shock associations (Vergoz et al., 2007), thus suggesting that, in this case too, DA mediates aversive-reinforcement signaling necessary for aversive learning. Importantly, specific controls showed in some (but not all) of these studies that DA blockade or activation did neither affect motor responses nor sensory perception, so that in the framework of aversive conditioning DA does not down-regulate behavior in a non-specific way; instead, it acts specifically as an aversive reinforcement signal.

How is it then possible to reconcile these two functions? If DA blockade facilitates behavior owing to the general depressor effect of this biogenic amine, why were motor and sensory functions unaffected in the conditioning protocols discussed above despite DA blockade? If DA signaling mediates the aversive reinforcing properties of the electric shock and its blockade impairs aversive learning, why does its blockade in shock responsiveness experiments (this work) *enhance* shock sensitivity? This result was unexpected as we assumed, based on the previous work on olfactory SER conditioning (Vergoz et al., 2007), that blocking the dopaminergic system would diminish the aversive reinforcement properties of the electric shock, thus decreasing shock responsiveness. Importantly, in our work as in that on olfactory SER conditioning (Vergoz et al., 2007) the same antagonist (flupentixol), the same injection site (ocellar tract) and dose (1.97 mM) were used, so that functional differences are not due to these experimental variables.

A possible explanation for this dual function is to assume the existence of, at least, two different classes of dopaminergic neurons mediating different functions: one acting as a *general gain control system*, with the specific role of down-regulating responsiveness and another acting as *instructive* neurons in aversive associative learning which mediates aversive US signaling. Owing to these different functions, their brain targets could be different. While the first class would exhibit extensive and broad branching within the entire brain in order to be able to modulate different motivational components (appetitive, aversive) and sensory modalities (olfactory, visual gustatory, etc), the second class would exhibit a specific

connectivity with respect to CS processing circuits (olfactory, visual) in order to facilitate CS-US associations and provide instructive (i.e. valence) information to the targeted CS circuit (Giurfa, 2006). These two classes may also differ in terms of the dopaminergic receptors they express.

In vertebrates, dopaminergic receptors are generally classified in two main families, the D1-like and D2-like receptors (Jaber et al., 1996; Neve et al., 2004). Activation of the D1-like family is coupled to increases in cAMP concentration and is typically excitatory, while D2-like activation reduces cAMP and is typically inhibitory. In the honey bee, three different DA receptors have been identified: AmDOP1 (Blenau et al., 1998), AmDOP2 (Humphries et al., 2003) and AmDOP3 (Beggs et al., 2005). AmDOP1 and AmDOP3 have been related to the vertebrate D1-like and D2-like family of dopamine receptors, respectively (Beggs et al., 2005; Blenau et al., 1998). AmDOP2 appears to be more closely related to invertebrate OA receptors but it has been referred to as a 'D1-like receptor' because it up-regulates cAMP (Humphries et al., 2003). In the case of olfactory SER conditioning, DA blockade by means of vertebrate D1-like and D2-like receptor blockers SCH23390 and spiperone, respectively, yielded different results: while SCH23390 did not impair olfactory SER conditioning, spiperone significantly impaired acquisition and retention, thus suggesting that D1-like and D2-like DA receptors contribute differently to the signaling of US reinforcement by the instructive DA neurons (Vergoz et al., 2007). In addition, the fact that 20E (see below) impairs olfactory SER conditioning, thus acting on the instructive DA neurons, but leaves intact shock responsiveness to electric shock (Geddes et al., 2013) reaffirms the heterogeneity of the DA signaling mechanisms in the bee brain.

We suggest that the first class of DA neurons, acting as general gain control system, could mediate responding adaptively to appropriate stimuli in the insect's environment. It may therefore mediate attentional processes in which perception is focused on one stimulus (or group of related stimuli), while filtering out other simultaneous stimuli that are less relevant at any moment (Posner et al., 1980). Attentional processes, similar to those described in vertebrates, can also be identified in insects (Dyer and Chittka, 2004; Giurfa, 2004; Miller et al., 2011; van Swinderen, 2011; Van Swinderen and Andretic, 2011b) and, in the case of *Drosophila*, a neural correlate of such processes is a transient increase in a 20-30 Hz local field-potential recorded in a region of the brain called the medial protocerebrum (van Swinderen and Greenspan, 2003). Current views relate DA levels in the insect brain with

arousal levels (Van Swinderen and Andretic, 2011a). Transient attenuation of DA release in fly mutants attenuates the 20-30 Hz responsiveness to the object to be attended and oral delivery of methamphetamine, which increases DA release, rescues this responsiveness (Andretic et al., 2005). Thus, gain-control DA neurons may modulate selective attention in the insect brain, acting on a series of nervous circuits underlying different forms of sensory-motor performances.

Different classes of dopaminergic neurons have been identified in the fruit fly which mediate appetitive (Burke et al., 2012; Liu et al., 2012a) and aversive (Aso et al., 2012) reinforcing functions. Yet, suppressing DA signaling in mutants does not affect sensitivity to electric shocks with respect to wild-type flies (Riemensperger et al., 2011). This result does not invalidate our findings as in the fruit fly experiments, flies were tested in groups and not individually, and were subjected to a single voltage (60 V) during one minute so that no sensitivity curves were established. Appropriate behavioral measurements should show whether suppression of DA signaling does indeed leave shock sensitivity unaffected in fruit flies as claimed (Riemensperger et al., 2011), or whether it increases sensitivity to voltages lower than the one tested, consistently with our findings. In any case, we posit that besides the instructive category of DA neurons available in bees and flies, a different class of dopaminergic neurons exist which provide a down-regulating control of responsiveness upon perception of potentially noxious stimulation.

20E and Aversive Responsiveness

20-hydroxyecdisone (20E) is a metabolite of the steroid hormone ecdysone, which intervenes in insect development and reproduction (Riddiford et al., 2000). This ecdysteroid impairs aversive but not appetitive conditioning in bees (Geddes et al., 2013). Two-day old bees are deficient in olfactory SER learning (Geddes et al., 2013) in agreement with higher titers of ecdysteroids occurring at this age (Hartfelder et al., 2002). This impairment seems to be achieved in part via the dopamine/ecdysone receptor gene *Amgpcr19*. Exogenous 20E injection determines both a reduction in AmGPCR19 levels 3 h after injection and a decrease in aversive learning performances of adult (6-day old) bees (Geddes et al., 2013). The same 20E injection does not modify the levels of the three dopaminergic receptors known in the bee, AmDOP1, AmDOP2, and AmDOP3, 3 h after injection (McQuillan, 2013). Taken

together these results indicate that at this delay the decrement of aversive learning induced by 20E occurs via AmGPCR19 and not via the AmDOP receptors.

The fact that 20E injection did not affect shock responsiveness indicates that the decrement in olfactory SER conditioning induced by this ecdysteroid is not due to a loss of US sensitivity. 20E could then exert a negative effect on the other components of this associative learning: it may reduce olfactory perception and/or impair the associability of CS and US pathways. Following our suggestion concerning the existence of at least two classes of dopaminergic neurons (see above), we suggest that the negative effect of 20E on aversive learning is mediated by the instructive neurons specifically involved in aversive associative learning, but not by the gain-control dopaminergic neurons. Accordingly, flupentixol impairs aversive olfactory learning (Vergoz et al., 2007) and modifies shock responsiveness [this work], whereas 20E triggers a different side-effect, impairing the learning, but not the perception to shock stimuli (Geddes et al., 2013). This suggests that flupentixol and 20E may bind/block different DA receptors and even trigger different signal cascades.

5-HT and Aversive Responsiveness

Three different blockers of 5-HT signaling were used in our work. The clearest effects on SER were obtained with cyproheptadine, which shows potent non-competitive inhibition in the presence of 5-HT (Howarth et al., 2002; Vleugels et al., 2013) and antagonizes both the Am5-$HT_{2\square\square}$ and the Am5-$HT_{2\square}$ receptors (Thamm et al., 2013). In this case, the two higher cyproheptadine concentrations assayed (2.85 mM and $2.85x10^{-2}$ mM) increased significantly shock sensitivity at intermediate voltages but not placement responsiveness.

Methiothepin acts as a competitive inhibitor in the presence of 5-HT, and antagonizes in a non-specific way all known 5-HT receptors (Am5-HT_{1A}, Am5-$HT_{2\square}$ and Am5-HT_{7}) with the exception of Am5-$HT_{2\beta}$ (Schlenstedt et al., 2006; Thamm et al., 2010; Thamm et al., 2013). Injections of this drug also increased significantly the responsiveness to electric shocks of intermediate voltage but to a lower extent than cyproheptadine. Global comparisons between responses to the methiothepin concentrations and PBS responses in all three replicates yielded barely non-significant results ($p = 0.052$, $p = 0.07$ and lastly $p = 0.06$). Yet, pairwise comparisons between single-dose responses and PBS responses were significant in

three out of four cases. In one of the four cases, methiothepin induced a general, non-specific increase in responsiveness visible at the begin of the placement trials. SER returned to basal levels along consecutive placement trials thus showing that the increased excitability following 5-HT blockade was reduced probably via habituation processes.

Finally, ketanserin is a competitive antagonist of Am5-$HT_{2\beta}$ receptor (Thamm et al., 2013). Only the highest concentration of this drug increased significantly shock responsiveness with respect to PBS controls. In this case, increase of responsiveness in the first placement trial was present both in ketanserin groups and in PBS controls so that it cannot be attributed to 5-HT inhibition.

Taken together, our results provide the first analysis of the role of 5-HT in aversive responsiveness in honey bees. Injection of three different 5-HT antagonists increased to different extents shock responsiveness to intermediate voltages and in some cases, to placement trials. These results indicate that the serotonergic system acts as a depressor of aversive responsiveness and probably of a broader spectrum of behaviors. Since clearer increases in shock sensitivity were observed with cyproheptadine, it may be suggested that inhibition of general responsiveness by 5-HT requires both Am5-$HT_{2\alpha}$ and Am5-$HT_{2\beta}$ receptors. When only one of these receptors is targeted, as seems to be the case for methiothepin (Am5-$HT_{2\alpha}$) and ketanserin (Am5-$HT_{2\beta}$) increases in responsiveness are still visible but to a lower extent, thus suggesting an additive effect of 5-HT neurotransmission via these two receptors.

The notion of 5-HT mediating a general inhibitory system is supported by results obtained in appetitive olfactory PER conditioning. In this framework, injection of 5-HT impairs the acquisition and retrieval of olfactory memories (Bicker and Menzel, 1989; Menzel et al., 1999; Mercer and Menzel, 1982). An inhibitory role of 5-HT signaling was also found in a variant of PER conditioning used to study latent inhibition, a decrement in learning performance which results from the nonreinforced preexposure of the odor to be conditioned (Fernández et al., 2012). In this case, blockade of 5-HT by injection of ketanserine and methysergide suppresses latent inhibition and rescues learning of the pre-exposed odor. It was thus suggested that latent inhibition could be the consequence of increased levels of 5-HT, resulting from repeated unrewarded CS exposure (Fernández et al., 2012). Higher levels of 5-HT would determine an inhibitory state (or a state of reduced excitability) and would thus impair CS-US associations.

5-HT neurotransmission would thus intervene in the modulation of a broad spectrum of behaviors, acting as a general gain-control system facilitating behavioral inhibition. Serotonergic neurons in the optic ganglia can modulate visual responses such as the motion-sensitive visual antennal reflex, a typical direction specific antennal response to a stripe pattern moving up- and downward (Erber and Kloppenburg, 1995; Kloppenburg and Erber, 1995). Consistently with our hypothesis, 5-HT application into the ipsilateral lamina, lobula, and medulla, the main visual areas of the insect brain, leads to an immediate and long lasting (at least 30 min) decrease of the reflex when the ipsilateral compound eye is stimulated. In some cases, the response to stimulation of the contralateral eye is also reduced (Erber and Kloppenburg, 1995). Accordingly, 5-HT reduces background activity as well as responses to moving stripe patterns by motion-sensitive lobula neurons. The amplitudes of lobula field potentials evoked by moving stripe patterns are also reduced by application of 5-HT. Phototactic responsiveness is also strongly reduced by 5-HT but can be rescued by feeding bees a mixture of 5-HT and the Am5-HT_{1A} receptor antagonist prazosin over a 2-day period (Thamm et al., 2010).

All in all, the picture emerging from our and other studies is one in which 5-HT may allow responding adaptively to relevant stimuli of different valence (appetitive, aversive) and sensory modalities (visual, olfactory) by suppressing responses to irrelevant, non-predictive stimuli. Together with DA (see above), 5-HT may thus play an essential role in attentional processes, allowing an insect to cope efficiently with its environment.

Biogenic Amines and Task Division in the Hive

Task division in a social insect colony is a fundamental factor for sociality (Wilson, 1971). The *response-threshold model* has been proposed to explain the division of labor in social insects. It posits that differences in sensitivity to external stimuli exist between individuals and that individuals highly sensitive to a given stimulus are prospective candidates for becoming specialized in tasks involving such a stimulus (Page and Erber, 2002). Related sensitivities to stimuli usually encountered in an appetitive context can be grouped in a "foraging behavior (or appetitive) syndrome" (Page et al., 2006), defined as a set of correlated behaviors reflecting between-individual consistency in behavior across multiple foraging situations (Sih et al., 2004). Similarly, sensitivities to stimuli usually encountered in an

aversive/defensive context can define an "aversive syndrome" (Roussel et al., 2009; Tedjakumala and Giurfa, 2013). Biogenic amines may play an essential role for such specializations, modulating an individual's responsiveness to specific stimuli (Scheiner et al., 2004). For instance, OA and tyramine facilitate appetitive responses while DA inhibits appetitive aversive responses so that behavioral syndromes may be defined at the individual level through the fine balance between amines mediating appetitive and aversive responses.

In this scenario we propose that it is worth distinguishing between two different involvements of biogenic amines: on one hand some of them (OA, DA) may act as instructive signals in associative circuits attributing specific valences to stimuli to be learned (OA: appetitive; DA: aversive), and, on the other hand, they may provide global gain control systems facilitating behavioral responses through a decrease of responsiveness thresholds (OA) or, on the contrary, inhibiting such responses (DA and 5-HT) thereby determining more focused and appropriate stimulus responses. In such scenario, attentional control may be particularly relevant for the division of labor. Further studies should determine if and how response-threshold models need to incorporate such control mechanisms and whether biogenic amines such as 5-HT play a relevant role for task specialization.

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Figures

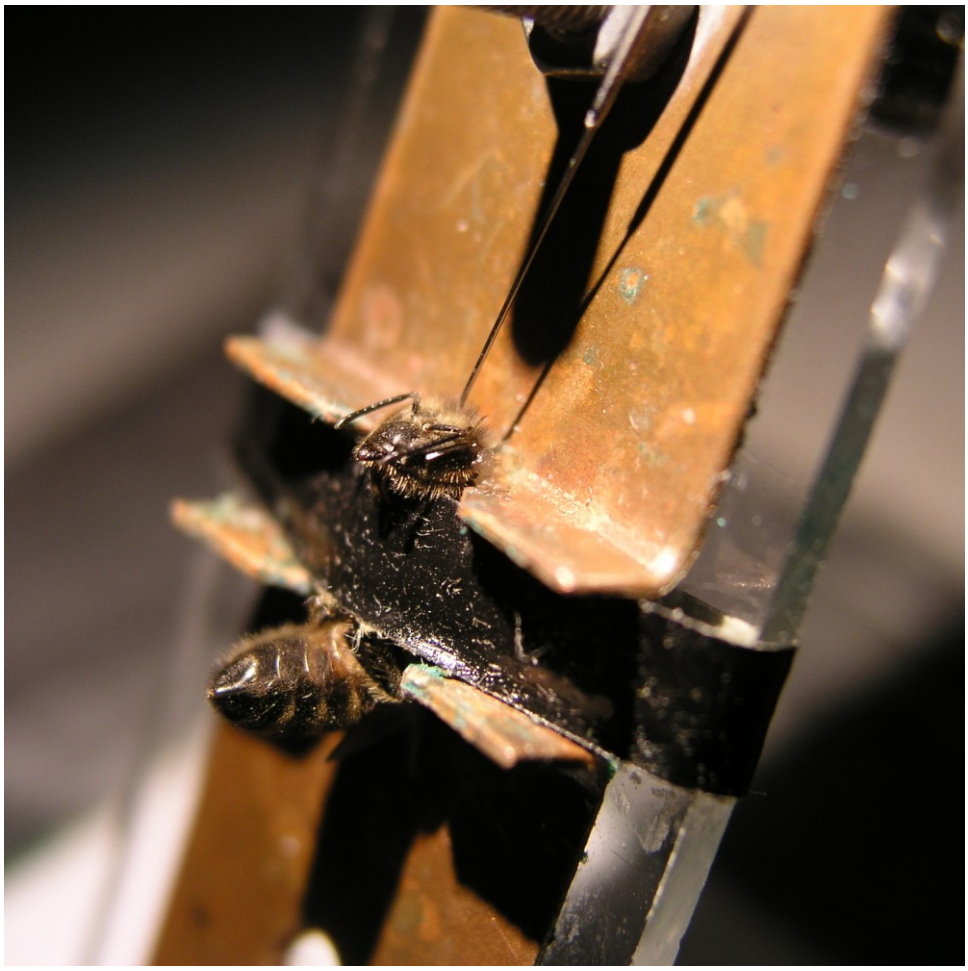


Figure 1: Brain injection via the ocellar tract in a honey bee harnessed on a shock delivery setup. A tiny hole was pricked into the cornea of the median ocellus to allow the insertion of a Hamilton syringe **(a)** located above the bee. The syringe allows delivery of the drug to be tested in the median ocellar tract **(b)**, which runs medially and caudally from the dorsal margin of the head capsule into the protocerebrum. The head of the bee is fixed to the metallic plate by means of a low-temperature melting wax **(c)** to reduce movements during injection. A girdle **(d)** is used to clamp the thorax to restrain mobility during the experiment. The bee acts as a bridge between the two metallic plates **(e)** fixed on a Plexiglas plate **(f)**. EEG cream was smeared on the two notches of the metallic plates to ensure good contact between the plates and the bee. The bee closes a circuit and receives a 2-s mild electric shock which induces the sting extension reflex (SER).

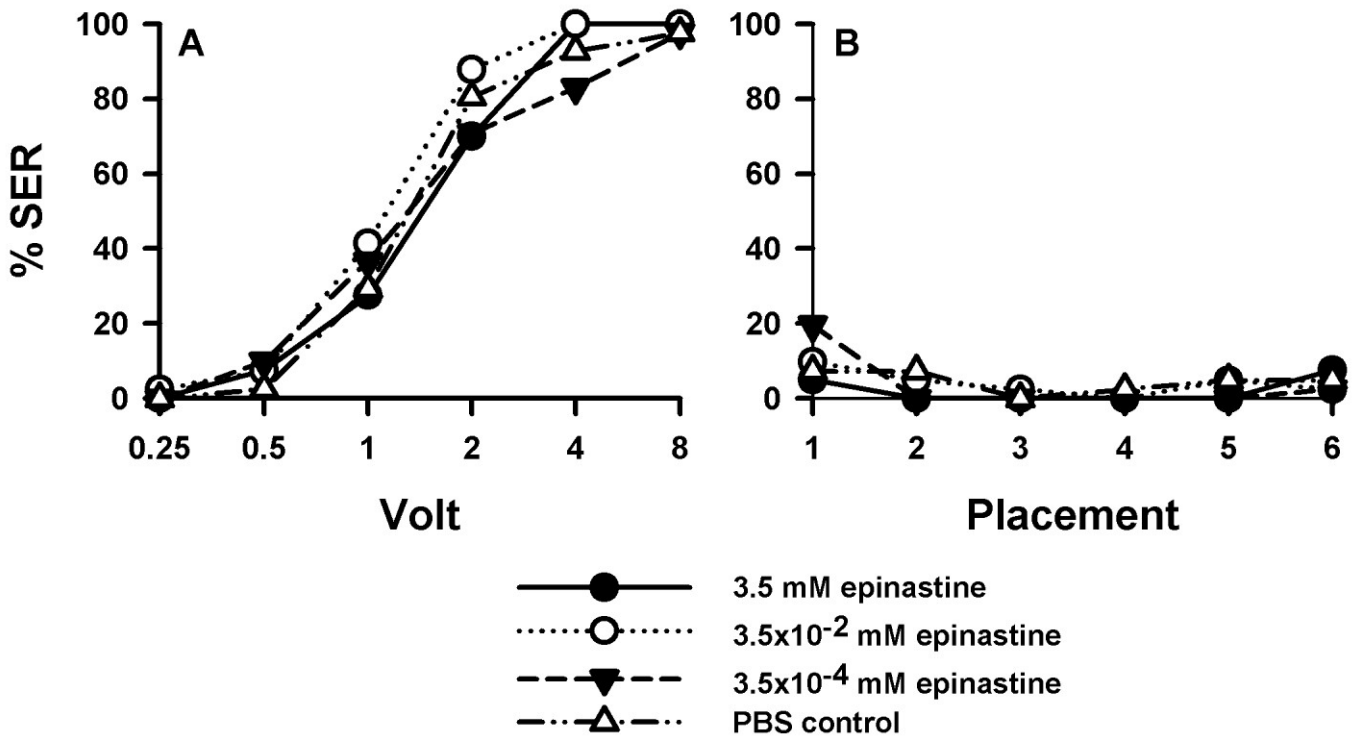


Figure 2: Effects of OA blocking on aversive responsiveness. Three different groups of bees were injected with three different concentrations of the OA antagonist epinastine (3.5 mM: n = 40; 3.5x10-2 mM: n = 41; 3.5x10-4 mM: n = 41). A fourth group was injected with PBS as a control (n = 41). Sting responsiveness was measured in response to increasing voltages during shock trials **(A)** and during placement trials in which the bees were placed in the setup without stimulation **(B)**. All three epinastine concentrations induced the same responsiveness as the PBS control both in the shock and in the placement trials, thus showing that OA does not play a significant role in sting responsiveness to a noxious stimulus.

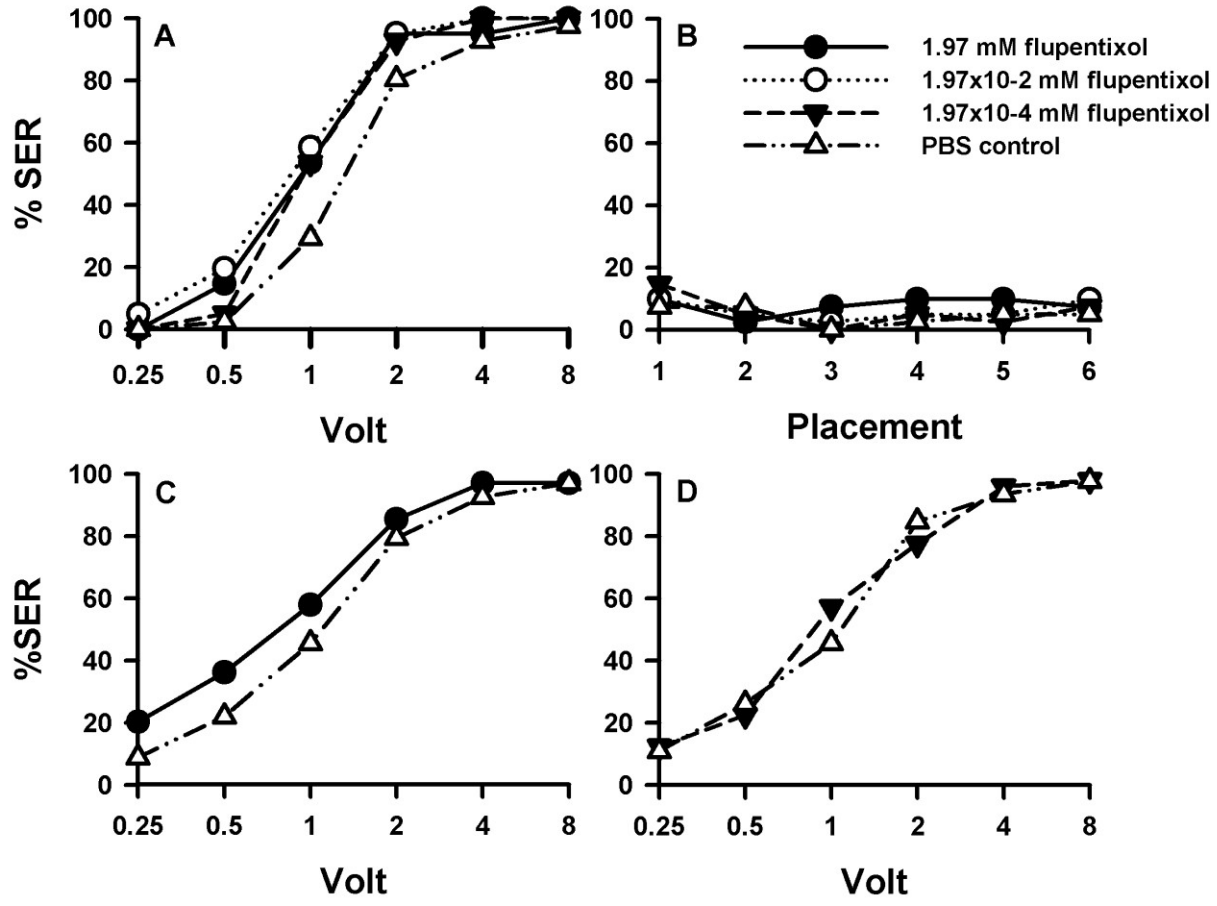


Figure 3: Effects of DA blocking on aversive responsiveness. Three different groups of bees were injected with three different concentrations of the DA antagonist flupentixol (1.97 mM: n = 41; $1.97x10^{-2}$ mM: n = 41; $1.97x10^{-4}$ mM: n = 41). A fourth group was injected with PBS as a control (n = 41). Sting responsiveness was measured in response to increasing voltages during shock trials **(A)** and during placement trials **(B)**. All three flupentixol concentrations induced an increase of responsiveness to electric shocks compared to PBS controls ($F_{3,160} = 5.46$, $p < 0.01$). No differences were found between flupentixol-injected and PBS-injected bees in the placement trials. **(C)** In a replicate of this experiment, another group of bees was injected with the highest flupentixol concentration (1.97 mM: n = 69) and their response to increasing voltages was measured. An increase in responsiveness to shocks with respect of the PBS control was verified (n = 68) ($F_{1,135} = 4.07$, $p < 0.05$) . Thus, DA signaling plays a significant inhibiting role in sting responsiveness to noxious stimuli as its blockade increased shock sensitivity. **(D)** In a further replicate the lowest flupentixol concentration ($1.97x10^{-4}$ mM; n = 49) was again tested with its corresponding PBS control (n = 46). In this case, flupentixol did not induce an increase of responsiveness with respect to PBS-injected bees ($F_{1,93} = 0.03$, $p = 0.87$). Thus, the flupentixol concentration of $1.97x10^{-4}$ mM was certainly not excessive and the effects of higher concentrations targeted specifically dopaminergic receptors.

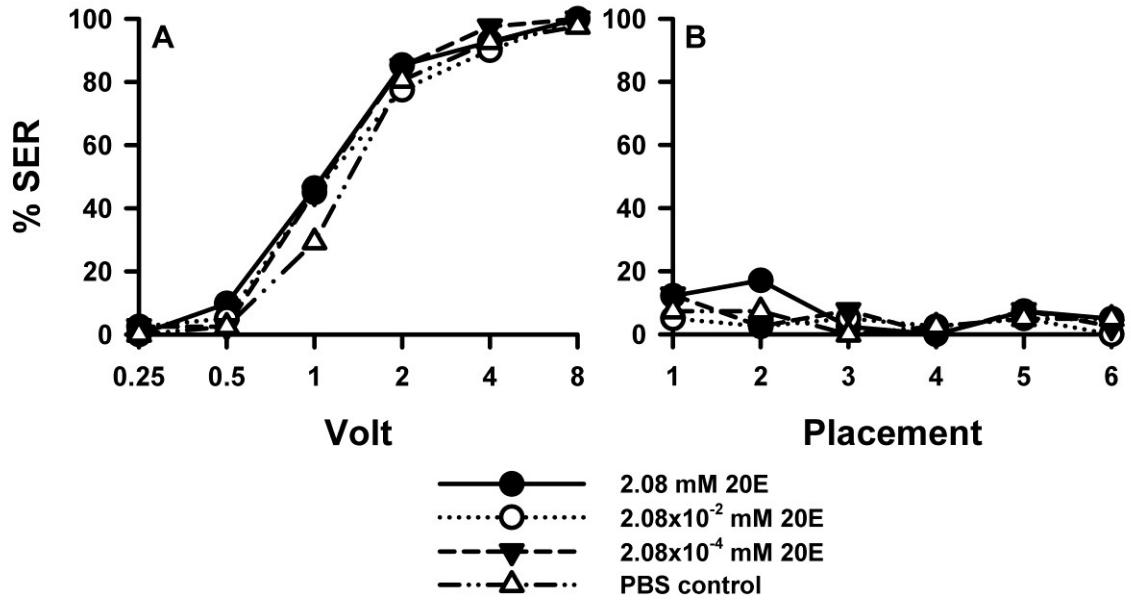


Figure 4: : Effects of 20E injection on aversive responsiveness. Three different groups of bees were injected with three different concentrations of 20E (2.08 mM: n = 41; $2.08x10^{-2}$ mM: n = 40; $2.08x10^{-4}$ mM: n = 40). A fourth group was injected with PBS as a control (n = 41). Sting responsiveness was measured in response to increasing voltages during shock trials **(A)** and during placement trials **(B)**. All three 20E concentrations induced the same responsiveness as the PBS control in the shock and in the placement trials, thus showing that 20E does not play a significant role in sting responsiveness to a noxious stimulus.

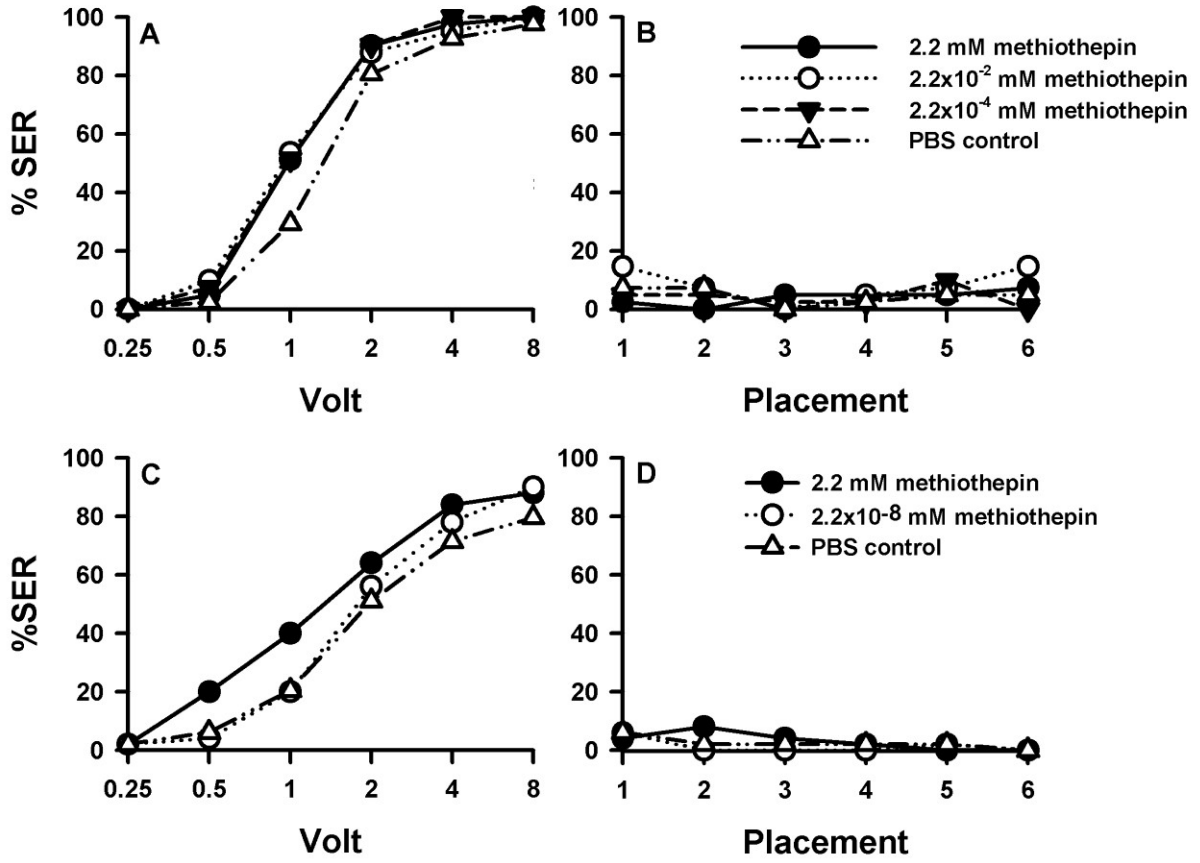


Figure 5: Effects of 5-HT blocking on aversive responsiveness. (A, B) Three different groups of bees were injected with three different concentrations of the 5-HT antagonist *methiothepin* (2.2 mM: n = 41; $2.2x10^{-2}$ mM: n = 41; $2.2x10^{-4}$ mM: n = 41). A fourth group was injected with PBS as a control (n = 41). Sting responsiveness was measured in response to increasing voltages during shock trials **(A)** and during placement trials **(B)**. Taken globally, the three methiothepin concentrations assayed induced an almost significant increase of shock responsiveness when compared to the PBS control ($F_{3,160} = 2.62$, p= 0.052). Yet, pairwise comparisons showed that each methiothepin concentration induced a significant increase of shock responsiveness with respect to PBS control ($p < 0.05$ for all three comparisons). There were no differences between methiothepin-injected and PBS-injected bees in the placement trials. **(C, D)** A further replicate using a lower concentration of methiothepin. Two different groups were injected with two different concentrations of methiothepin, the highest one used in the previous replicate (2.2 mM: n = 50) and a lower one ($2.2x10^{-8}$ mM: n = 50). A third group was injected with PBS as a control (n = 49). Sting responsiveness was measured in response to increasing voltages during shock trials **(C)** and during placement trials in which the bees were placed in the setup without stimulation **(D)**. The highest methiothepin concentration induced a significant increase of responsiveness during shock trials ($F_{1,97} = 4.75$, p = 0.032) while the lowest concentration did not ($F_{1,97} = 0.48$, p = 0.49). No differences were detected in the placement trials.

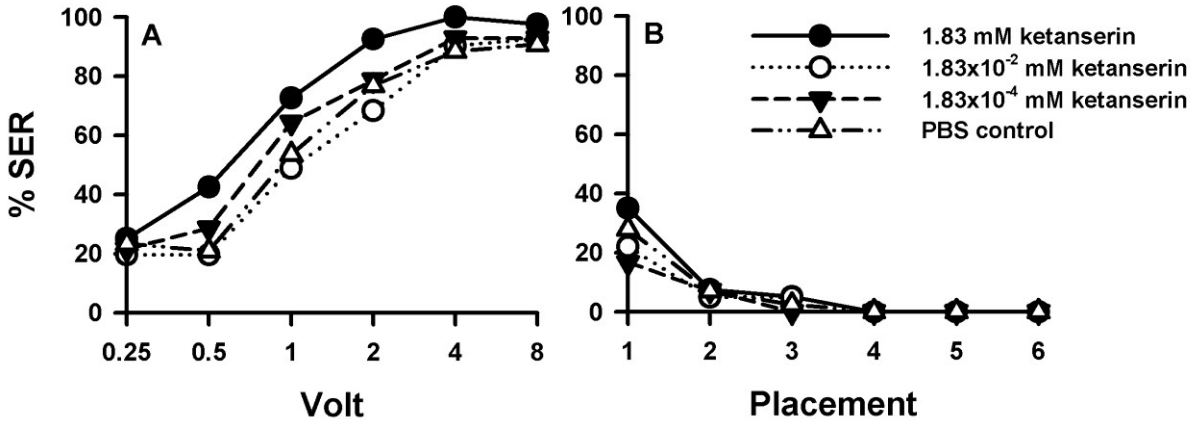


Figure 6: Effects of 5-HT blocking on aversive responsiveness. (A, B) Three different groups of bees were injected with three different concentrations of the 5-HT antagonist *ketanserin* (1.83 mM: n = 40; $1.83x10^{-2}$ mM: n = 41; $1.83x10^{-4}$ mM: n = 42). A fourth group was injected with PBS as a control (n = 43). Sting responsiveness was measured in response to a series of increasing voltages during shock trials **(A)** and during placement trials **(B)**. Only the highest ketanserin concentration increased significantly shock responsiveness with respect to the control (1.83 mM: $p < 0.05$) while the other two concentrations did not ($1.83x10^{-2}$ mM and $1.83x10^{-4}$ mM: NS in both cases). No differences were detected in the placement trials.

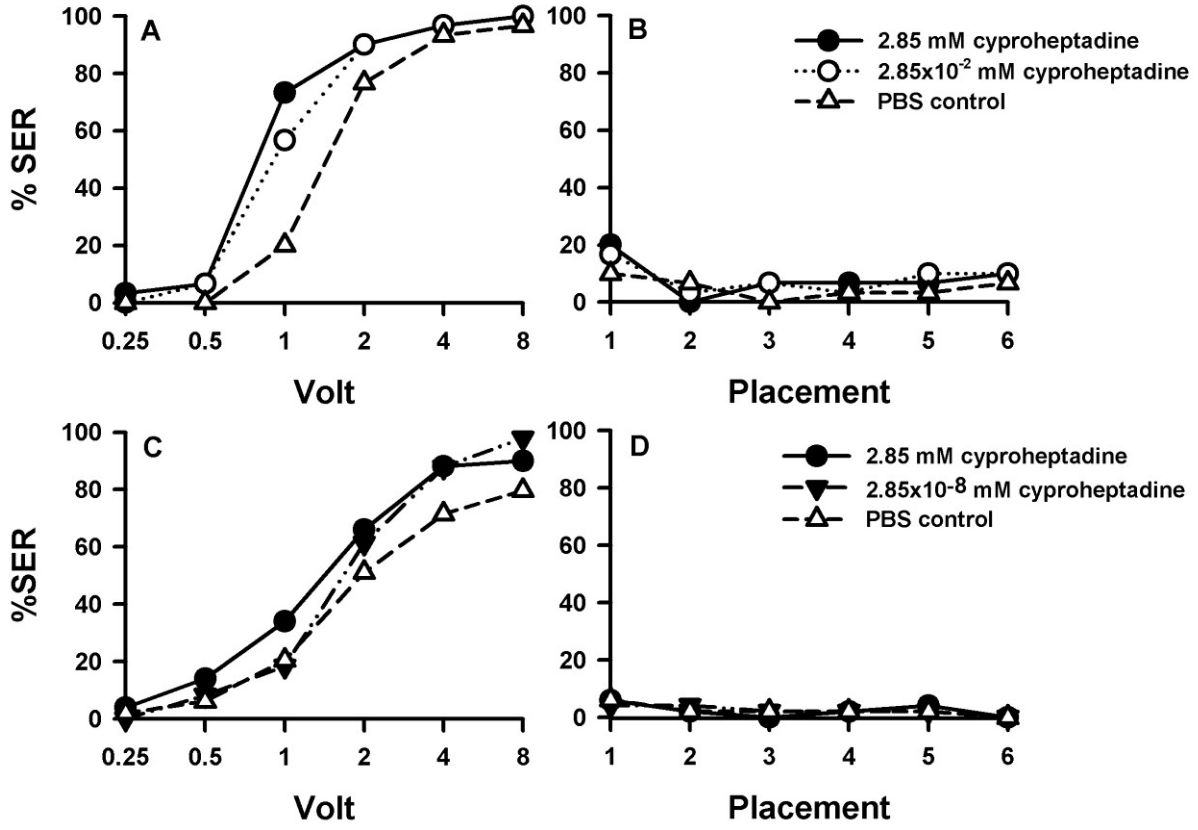
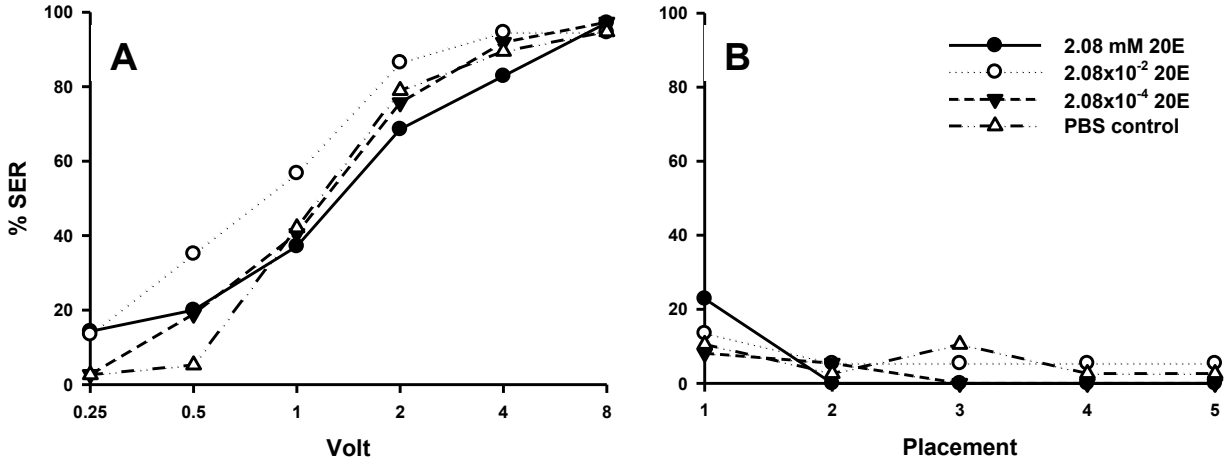
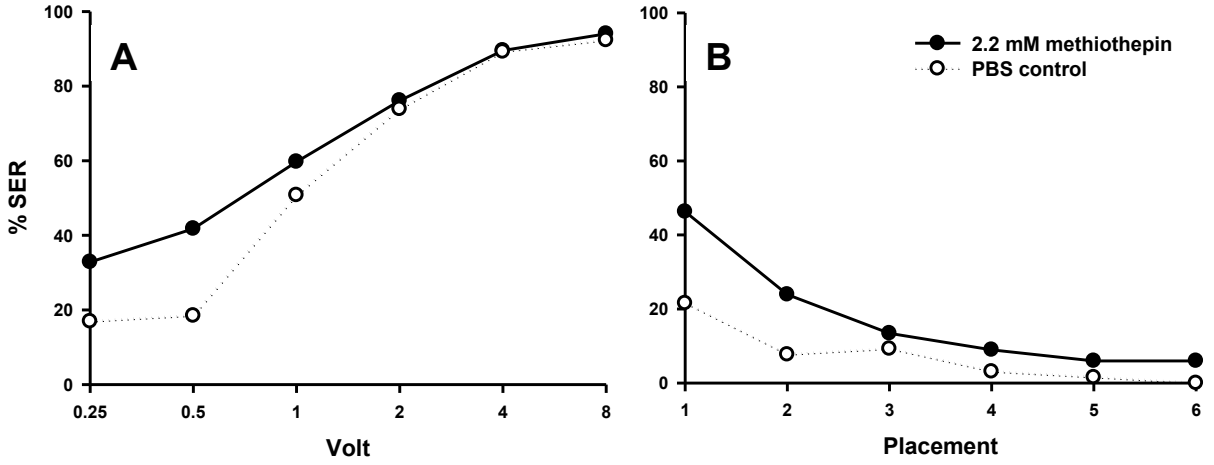


Figure 7: Effects of 5-HT blocking on aversive responsiveness. **(A, B)** Two different groups of bees were injected with two different concentrations of the 5-HT antagonist *cyproheptadine* (2.85 mM: n = 30; $2.85x10^{-2}$ mM: n = 30). A third group was injected with PBS as a control (n = 30). Sting responsiveness was measured in response to a series of increasing voltages during shock trials **(A)** and during placement trials **(B)**. Both cyproheptadine concentrations induced a significant increase of shock responsiveness when compared to PBS controls ($F_{2,87} = 7.81$, $p < 0.001$) but not change of responsiveness during the placement trials. **(C,D)** A further replicate using a lower concentration of cyproheptadine. Two different groups of bees were injected with two different concentrations of cyproheptadine, the highest one used in the previous replicate (2.85 mM: n = 50) and a lower one ($2.85x10^{-8}$ mM: n = 49). A third group was injected with PBS as a control (n = 49). Sting responsiveness was measured in response to increasing voltages during shock trials **(C)** and during placement trials in which the bees were placed in the setup without stimulation **(D)**. The highest concentration did induce a significant increase of responsiveness during shock trials ($F_{1,97} = 4.79$, $p = 0.031$). The lowest concentration did not ($F_{1,75} = 2.65$, $p = 0.11$). No differences were detected in the placement trials.

Supplementaries



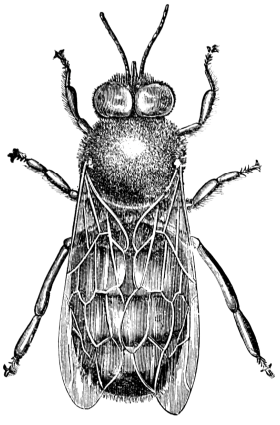
Supplementary Figure 1: Effects of blocking by 20E on aversive responsiveness. Three different groups of bees were injected with three different concentrations of 20E (2.08 mM: n = 35; $2.08x10^{-2}$ mM: n = 37; $2.08x10^{-4}$ mM: n = 37). A fourth group was injected with PBS as a control (n = 38). Sting responsiveness was measured in response to increasing voltages during shock trials (A) and during placement trials in which the bees were placed in the setup without stimulation (B). All three 20E concentrations assayed induced the same responsiveness as the PBS control in the shock and in the placement trials, showing that 20E does not play a significant role in sting responsiveness to a noxious stimulus.



Supplementary Figure 2: Effects of 5-HT blocking on aversive responsiveness. A group of bees was injected with the highest different concentrations of the 5-HT antagonist methiothepin (2.2 mM: n = 67). Another group was injected with PBS as a control (n = 65). Sting responsiveness was measured in response to a series of increasing voltages during shock trials (A) and during placement trials in which the bees were placed in the setup without stimulation (B). The highest methiothepin concentration induced an almost significant increase of responsiveness to the electric shocks compared to the PBS controls ($F_{1,130} = 3.34$, $p = 0.07$) and a significant increase of non-specific responsiveness in placement trials ($F_{1,130} = 8.30$, $p < 0.01$). These results show that 5-HT inhibits sting responsiveness to a noxious stimulus and non-specific responsiveness as its blockade through methiothepin increases both forms of responsiveness.

2

A tyrosine-hydroxylase characterization of dopaminergic neurons in the honey bee brain



2nd PART

A tyrosine-hydroxylase characterization of dopaminergic neurons in the honey bee brain

Stevanus Rio Tedjakumala, Jacques Rouquette, Marie-Laure Boizeau, Karen Mesce and Martin Giurfa
(*in prep.*)

In the second part of this work, we aimed at characterizing the neuroanatomy of dopaminergic (DA) neurons in the bee brain given the importance of dopaminergic signaling for aversive responsiveness and learning. Previous studies attempted a reconstruction of dopaminergic neurons in this insect but the methods employed to this end did not guarantee an efficient detection of DA neurons. Our study was conceived as a mean to characterize the basic connectivity of these neurons in different regions of the brain into which they could exert their modulatory/instructive role. Information about this connectivity could thus provide a tool to interpret the aversive behaviour of the honey bee. We performed immunocytochemical studies in which we applied an antibody raised against tyrosine hydroxylase (TH), a precursor of DA, and combined it with fluorescence imaging techniques using confocal microscopy to obtain a 3D reconstruction of different types of DA neurons.

Our results confirmed prior reports showing that a higher-order brain structure, the mushroom body (MB), is heavily innervated by relatively big clusters of DA neurons surrounding it. These clusters known as C1-, C2- and C3-clusters, comprise 75, 75 and 140 cell bodies, respectively. These values are lower for the C1 and C2-cluster, but higher for the C3-cluster than those previously reported. DA processes were also found in the protocerebral lobes (PL) and antennal lobes (AL); their somata were located at lateral rim of the deuterocerebrum. Together these DA neurons contact the olfactory circuit at different

processing stages (AL, PL, MB) and provide the neural basis for DA labeling of different forms of olfactory signaling.

DA neurons also innervate a central region of the brain termed the central complex (CX), which has been related to navigation and other forms of visual processing. This region is innervated by a part of the C3-cluster and by a set of neurons, renamed as C3b due to their morphological property of sharing innervations with the C3-cluster in the same neuropile. Besides, we provided the first evidence of DA processes in the optic lobe of the honey bee brain and identified a novel DA cluster therein, which we termed C4. 80 cell bodies can be quantified in this cluster and they are located at the dorsomedial border of the lobula. These neurons can thus provide instructive aversive signals for visual cues processed at this level.

All in all, our work provides an integrative, neuroanatomical study of DA neurons which allows appreciating the rich and extensive innervation pattern of DA neurons in the bee brain. We discuss putative functional aspects of the different DA clusters and compare them to those of the fruit fly, *Drosophila melanogaster* to yield a comparative perspective to our analyses of dopaminergic signaling and aversive learning in the bee.

A tyrosine-hydroxylase characterization of dopaminergic neurons in the honey bee brain

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Abstract

Dopamine (DA) plays a fundamental role as a modulator of insect behavior and as a value system in associative learning where it mediates reinforcing properties of unconditioned stimuli. Here we aimed at characterizing the dopaminergic neurons in the central nervous system of the honey bee, an insect that constitutes a model for the study of learning and memory. We used tyrosine hydroxylase (TH) immunoreactivity (ir) to ensure that the neurons detected synthesize DA endogenously. Our results were consistent with previous reports of DA-ir as we found three previously known main dopaminergic clusters, C1 to C3; the C1-cluster is located in a small region adjacent to the esophagus and the antennal lobe; the C2-cluster is situated above the C1-cluster, between the antennal lobe and the vertical lobe of the mushroom body; the C3-cluster is located below the calyces of the mushroom body. In addition, we found a novel fourth cluster, C4, located above the dorsomedial border of the lobula, which innervates the visual neuropils of the bee brain. Further smaller processes and clusters are described. The profuse DA innervation in the entire bee brain, and the specific connectivity of DA neurons with visual, olfactory and gustatory circuits provide the basis for DA modulation of these sensory modules and for value-based labeling of sensory cues of these modalities in associative learning.

Keywords: honey bee, *Apis mellifera*, brain, dopamine, dopaminergic neurons, tyrosine hydroxylase

Introduction

Honeybees are a well-established model for the study of learning and memory (Giurfa, 2007; Giurfa and Sandoz, 2012; Menzel, 1999, 2001). Several protocols are used in the laboratory to access the behavioral, neural and molecular bases of these capacities (Giurfa, 2007). Among these protocols, the olfactory conditioning of the sting extension response (SER) allows studying aversive learning and memory under controlled experimental conditions (Tedjakumala and Giurfa, 2013; Vergoz et al., 2007a). SER is a defensive response elicited in bees by potentially noxious stimuli (Breed et al., 2004). In the laboratory, it can be triggered by electric-shock delivery to a harnessed bee (Burrell and Smith, 1994; Núñez et al., 1997). Bees learn to associate an odorant as conditioned stimulus (CS) with an electric shock as the aversive unconditioned stimulus (US). Dopamine (DA) signaling is indispensable for aversive SER conditioning and memory formation as pharmacological blocking by different dopaminergic antagonists suppresses the capacity of bees to learn an odor-shock association through an inhibition of the aversive US pathway (Vergoz et al., 2007a).

In the fruit fly *Drosophila melanogaster*, DA signaling also mediates the reinforcing properties of aversive US (Aso et al., 2012; Aso et al., 2010; Claridge-Chang et al., 2009; Kim et al., 2007; Riemensperger et al., 2005; Schwaerzel et al., 2003a). Based on a protocol in which flies learn to avoid an odorant paired with an electric shock and choose a different odorant that was never paired with shock, neurogenetic experiments first showed that inhibiting DA signaling disrupts this form of learning (Schwaerzel et al., 2003b). Later, a subset of 12 DA neurons was identified in the so-called PPL1 cluster whose optogenetic activation paired with the presentation of an odorant was necessary and sufficient to support the formation of an aversive memory for that odorant (Claridge-Chang et al., 2009). Similar studies using thermogenetic mutants were able to pair odorant stimulation with the activation/inhibition of other subsets of DA neurons in the PAM and the PPL1 clusters thereby inducing/suppressing olfactory memories, respectively (Aso et al., 2012; Aso et al., 2010). Thus, a subset of DA neurons in the PPL1 and PAM clusters of fruit fly brain provide negative valence if engaged during odorant presentation. In other insects, DA signaling also mediates aversive US signaling. For instance, in the cricket, pharmacological blockade of DA neurons impedes the learning of aversive olfactory and visual associations (Unoki et al., 2005, 2006).

Recent evidence obtained in *Drosophila* has shown that the role of DA in reinforcement signaling is more complex than previously thought as anatomically distinct DA neurons were found, which can also provide an instructive signal for appetitive learning and memory (Burke et al., 2012; Liu et al., 2012b). Moreover, DA signaling in *Drosophila* does not exclusively refer to aversive or appetitive learning and memory as it is not limited to the neural representation of the aversive and appetitive reinforcements used in these conditioning forms. A growing body of behavioral and electrophysiological studies suggests that DA is involved in a broad spectrum of fruit fly behaviors such as sleep, arousal, visual attention, wakefulness and aggression modulation (Alekseyenko et al., 2013; Liu et al., 2012c; Ueno et al., 2012; Van Swinderen and Andretic, 2011).

In the honey bee, the role of DA seems also to be more complex than the original notion of a neurotransmitter mediating aversive reinforcement (Vergoz et al., 2007a). Recent pharmacological experiments revealed that the dopaminergic system down-regulates the unconditioned responsiveness to electric shocks (Tedjakumala et al., 2014). This responsiveness can be quantified by subjecting harnessed bees to a series of increasing voltages which increase their tendency to respond with a SER. Pharmacological blockade of the dopaminergic system results in an *increase* of the responsiveness to the aversive US. It was thus suggested that the dopaminergic system is heterogeneous in its functionality as besides mediating the reinforcing properties of an aversive US in the framework of associative aversive learning, it may act as a general inhibitor of aversive responsiveness, i.e. as a "gain-control system" (Tedjakumala et al., 2014). These results suggest the existence of at least two putatively functional classes of DA neurons, one controlling global aversive responsiveness through an inhibitory action and the other mediating aversive US in the case of aversive learning (Tedjakumala et al., 2014).

In the light of this heterogeneity, an accurate neuroanatomical characterization of DA neurons in the bee brain is necessary. This characterization should allow the identification of structures and neural modules of the bee brain that are targeted by DA neurons, thus providing the anatomical basis for associations stimulus-reinforcement and for modulations of behavioral responsiveness. Prior works reported the presence of putative dopaminergic neurons in the bee brain by means of immunocytochemistry (Schäfer and Rehder, 1989; Schurmann et al., 1989). These works used antibodies raised against DA, which may have some disadvantages. For instance, it has been shown that DA may diffuse differently to

adjacent neurons during signaling processes based on the density of its uptake sites (Stamford et al., 1988), thus leading to accounts including several false DA neurons. Also, not much is known about the metabolic rate of DA in the insect brain; DA may be present for longer periods, but it cannot be ruled out that it is synthesized shortly before release so that immunocytochemistry may miss several DA neurons if its timing is not appropriate. Finally, DA can be auto-oxidized (Sulzer and Zecca, 1999), which may pose a problem during brain extraction.

Here we used a different approach to characterize the dopaminergic neurons in the central nervous system of the honey bee as we targeted tyrosine hydroxylase (TH), an enzyme which is a precursor to DA (Fon and Edwards, 2001). TH allows to synthesize Dihydroxylphenylalanine (L-DOPA) from Tyrosine and L-DOPA is then converted into DA. Thus, by targeting TH, we make sure that the neurons analyzed in our work do indeed synthesize DA endogenously. Our neuroanatomical data were gathered by means of a combination of immunocytochemistry using fluorescence-coupled antibodies and 3D-imaging using image stacks captured from a single whole brain by a confocal microscopy. In this way, it was possible to reconstruct complete DA circuitries in the bee brain without eventual loss of tissues. For such a reconstruction, the dopaminergic circuits of the fruit fly were used as a reference to understand previously unknown DA processes in the bee brain. A complete characterization of DA neurons in the protocerebrum of *Drosophila* at a single cell resolution has been achieved using TH *GAL4*-transgene and TH antibody (Mao and Davis, 2009).

Materials and Methods

Insects

Honeybees *Apis mellifera* were obtained from outdoor colonies located in the apiary of the University Paul Sabatier. Only foragers were used for this study as it was reported that they have significantly higher dopamine levels than nurses or guards (Taylor et al., 1992). To this end, a feeder filled with 30% (weight/weight) sucrose solution was set at the apiary and bees were collected upon feeding.

Bees were brought to the laboratory and chilled on ice for approximately 5 minutes. Afterwards, they were individually harnessed in metal holders from which only the head capsule protruded. The bees were left for at least 1 h in resting conditions before dissection. In this way, potential alterations of dopamine levels due to stress and or the chilling anesthesia were reduced (Chen et al., 2008).

Dissection and Fixation

The head capsule was opened by cutting a frontal window between the compound eyes and the ocelli. The mandibles and the antennae were also cut following the side cuts, exposing the whole brain. The glands were removed to allow the fixative to access the brain optimally. This whole process took no longer than 30 seconds. Afterwards, the bee was immediately decapitated and the whole head capsule was fixed in 4% PFA (in bee ringer) at 4°C. During dissection, the compactness of the hypopharyngeal glands was controlled to make sure that the bees were old enough to be considered foragers (Maleszka et al., 2009).

The next day, the head capsule was immerged in phosphate-buffered saline (PBS) and the brain was removed from it. The trachea covering the brain was removed carefully. The brain was rinsed three times (20 minutes each) in PBS to remove the rest of PFA. The samples were immediately embedded in 5% low melting agarose (in phosphate buffered saline - PBS). A vibratome (Leica VT1000S) was used to section the samples at 80 - 160 µm. The sections were immediately placed in PBS for further processing. The whole-mount samples provided by one of us (Prof. Karen Mesce; University of Minnesota) were fixed following the procedure described in Mesce et al. (2001).

Immunocytochemistry

Brain slices were permeabilized and blocked in PBS solution containing 0.3% Triton X-100 and 5% normal goat serum (ngs) for 1 hour. The primary antibodies were α-tyrosine hydroxylase (TH) raised in rabbit and α-SynORF1 raised in mouse against the *Drosophila* synapsin gene (Klagges 1996), which is used to label synaptic structures (courtesy of Prof. Erich Buchner, Würzburg).

After blocking, we incubated the samples with both antibodies (□□TH 1:50 and □□Syn 1:50) during 48 hours. We then rinsed the primary antibodies multiple times (10 - 20 - 30 - 2 x 60 minutes) in 0.3% Triton X-100. The secondary antibodies were Alexa Fluor® 488 a-rabbit (Invitrogen) and DyLight 649 anti-mouse (Jackson ImmunoResearch) raised in goat. They were applied 1:100 for 24 hours.

After exposure to the secondary antibodies, brain slices were subsequently rinsed multiple times (10 - 20 - 30 - 2 x 60 minutes) in 0.3% Triton X-100 and mounted between coverslips in VECTASHIELD® Mounting Medium (Vector Labs). Whole mount samples were treated as described in Mesce et al. (2001).

Confocal Microscopy

Samples were imaged using a confocal laser scanning microscope (Leica TCS SP5 MP and LSM510 NLO - Carl Zeiss, Jena, Germany), with either a 25x oil objective (LCI Plan-Neofluar 25x/0.8) or a 20x water objective (PL APO 20x/0,5 on the Leica microscope and W Plan Apo 20x/1.0 on the Zeiss microscope). Ar-Kr and HeNe laser were used to excite Alexa Fluor® 488, Texas Red® DyLight 649 at 458, 543 and 633 nm, respectively. The emission was detected with a 500-530, 560-615 and 650-680 nm bandpass filter, respectively.

The images were collected as Z stacks with a Z step size between 0.410 and 0.709 µm. A visual field of view was registered with a pixel resolution of either 512 x 512 or 1024 x 1024 pixels. Each region of interest was captured by moving the visual field of view over the entire region, resulting in a huge and detailed mosaic image. The stacks were rendered for 3D reconstruction with Imaris. At least 5 samples were compared for each neuronal cluster considered to confirm the neuroanatomical processes reported.

Nomenclature

The different spatial axes of orientation used in this study follow the body axes of the honey bee. The nomenclature used for characterizing brain structures and pathways follows that proposed recently by the Insect Brain Name Working Group (Ito et al., 2014).

Results

Dopaminergic Cell Clusters

Prior work had shown that dopamine-like immunoreactive (DA-ir) neurons are present in most parts of the bee brain (Schäfer and Rehder, 1989). Approximately 330 dopamine immunoreactive soma were reported in each brain hemisphere and three clusters of dopaminergic neurons were described: one below the lateral calyx (C3 cluster) and two in the anterior-ventral protocerebrum (C1 & C2 clusters) (Fig. 1). The optic lobes were said to be devoid of dopaminergic label (Schäfer and Rehder, 1989).

Our results, based on TH-immunoreactivity (TH-ir), were consistent with the reported DA-ir (Schäfer and Rehder, 1989; Schurmann et al., 1989) as we were able to identify the three previously known main dopaminergic clusters, C1 to C3 (Schäfer and Rehder, 1989; Schurmann et al., 1989) (Fig. 1). Yet, in addition, we discovered a novel fourth cluster which we termed C4, which had been overlooked by prior works, and which is located above the dorsomedial border of the lobula (Figs. 1 and 7). This new discovery was possible, probably because we benefited from better reconstruction methods based on confocal microscopy and because of the TH antibody itself. TH-ir was detectable throughout the whole brain (supraesophageal zone - SPZ and subesophageal zone - SEZ).

Three-dimensional reconstructions of the C1-, C2- (Fig. 2A) and C3-clusters (Fig. 3-5) are shown for a better overview. The C1-cluster (Fig. 2B) is located in a small region adjacent to the esophagus (ES) and the antennal lobe (AL) at a depth of ca. 120 µm. The C2-cluster is more eccentric (Figs. 1 and 2C) and situated above C1; it is located between the AL and the vertical lobe (VL) at a depth of ca. 60 µm. The C3-cluster is located below the calyces (CA) of the mushroom body (MB) from the ventral to the dorsal part of the brain (Fig. 3B). As mentioned above, the C4 cluster is found on the dorsolateral border of the lobula (Figs. 1 and 8B) spanning from the anterior part of the brain down to a depth of ca. 120 µm. Other smaller cell clusters were also detected: two independent clusters with around 8 (Fig. 6B) and 16-20 somas (Fig. 7B) in each hemisphere around the protocerebral bridge (PB) dorsal to the central complex (CX), one below each optic tubercle with 2 to 3 somas (Fig. 9B), 6 - 8 somas at the border between the lobula and the deutocerebrum (Fig. 10B) and 4-6 somas in the rind between the AL and the SEZ (Fig. 11B).

Dopaminergic Cell Numbers

Our counting of dopaminergic neurons (Fig. 1) in the C1- and C2-clusters registered around 75 somas per cluster, which is less than the 100 somas (Schäfer and Rehder, 1989) and more than the 40 somas (Schurmann et al., 1989) previously reported. Each soma had a diameter of ca. 10 μm, coincident with that measured in the prior works. In the C3-cluster, we identified ca. 140 somas, which is more than the 80-90 somas (Schäfer and Rehder, 1989) and the 50 somas (Schurmann et al., 1989) previously reported. The somas of this cluster had diameters varying between 7 and 12 μm, in accordance with the previous reports. In the C4-cluster, which had not been previously detected, ca. 80 somas were identified. Their diameter varied between 8 and 10 μm.

Taking into account several other small cell clusters, we counted between 400 and 450 somas altogether per brain hemisphere, an estimation that surpasses the 350 and 120 somas reported by Schäfer and Rehder (1989) and Schurmann et al. (1989), respectively. TH-ir clusters and their processes innervate in a symmetrical manner both brain hemispheres. Signal intensity showed notable fluctuations for the newly discovered C4 cluster. It varied between samples and provided relatively weaker signals compared to those of the other clusters, a fact that may explain why it was overlooked by previous analyses of DA-ir.

Dopaminergic Innervation of Brain Regions

In the following sections we provide a detailed description of TH-immunoreactive neurons innervating different regions of the supraesophageal zone (SEZ).

Mushroom bodies (MBs)

MBs are prominent higher-order integration centers, which receive input from olfactory, visual, gustatory and mechanosensory afferences and from the lateral protocerebrum (Strausfeld, 2002). Each MB is made of approximately 170 000 Kenyon cells (Witthöft, 1967) and consists of a lateral and a medial calyx which are joined by short necks to a common pedunculus, which then divides into a medial and vertical lobe. The vertical lobe extends

forward to the front surface of the brain where its truncated end lies approximately 150 µm above the antennal lobe. The soma of the Kenyon cells (class I Kenyon cells) are located mainly in the bowl of each calyx and above its rim. Their dendrites ramify within the cup-shaped neuropil of the calyces whilst their projection fibers pass through the pedunculus, branch at its base, and send one process into the medial lobe and one into the vertical lobe. An additional 14,000 Kenyon cell bodies lie outside each calyx (class II Kenyon cells) and form a layer around the outer calyx wall. Their neurites penetrate the outer wall to project directly, towards the lower part of the vertical lobe which has been identified as the gamma lobe (Strausfeld, 2002).

The TH-ir in the MBs was coincident with the DA-ir reported in previous works (Schäfer and Rehder, 1989; Schurmann et al., 1989). The MB is innervated by the three main clusters C1, C2 and C3, both in the vertical and the medial lobe, the pedunculus and the calyces (Fig. 2-5). Neither the Kenyon cells nor any MB intrinsic neurons were labeled, since all the TH-ir arborizations innervating this structure leave it at some point. For the sake of precision we divided our description of MB innervation according to the three main regions of this structure: the lobes, the pedunculus and the calyces.

Medial and vertical lobes. The neurites of the C1- and C2-clusters meet at a point posterior to each cluster (Fig. 2D). Before meeting, they arborize laterally with intense staining around the outer medial and inferior border of the vertical lobe (VL), innervating various regions of the lateral protocerebrum (LP) (Fig. 2E). The TH-ir in the MB seems to come from the same bundle of neurites and can be detected in both the vertical (VL) and the medial lobe (ML). In the vertical lobe, it is possible to observe innervations of various layers, with a higher intensity found in the inferior part, which corresponds in part to the gamma lobe (Fig. 2E). The innervations cannot be distinctly traced as they appear as thin fiber-like arborizations. In the medial lobe, faint sporadic signals can be detected, indicating the presence of very fine dopaminergic branches. Neurons of the C1- and C2-clusters exhibit morphological properties similar to those of the A1- and A2-neurons reported in a study of MB extrinsic neurons of the honey bee (Rybak and Menzel, 1993b).

The C3-cluster sends one of its neurite bundles in direction of the midline of the brain. It splits into three main branches (Figs. 3A and 3C; branches termed 2a, 2b and 2c). One bundle runs anteriorly and splits medially and laterally at the border of the VL. It projects further ventrally and appears to envelop the VL at its outer dorsal border (Fig. 3A and 3C;

branch 2a); another branch continues medioventrally to the midline sending projections to the contralateral hemisphere (Figs. 3A and 3C; branch 2b), whilst the last branch projects posterior along the medial surface of the vertical lobe and turns dorsolaterally behind the pedunculus (PED) of the medial calyx (CA) (Fig. 3A and 5A; branch 2c). This thick branch terminates between the medial and lateral calyces (Fig. 5B). Its arborizations in the calyces will be described below. Furthermore, the lateral projection of the bundle coming from the C3 cluster runs anteriorly where it branches to innervate protocerebral lobe and to run further ventrally along the border of the vertical lobe. All these branches show strong TH-staining.

Pedunculus (PED). TH-ir originating from the layers of the vertical lobe continues further posterior and dorsally as fine fibers into the PED where they disperse into columns built by Kenyon cell axons (Fig. 2F arrows). An intensely stained bundle runs along the ventral and lateral border of the VL. It innervates the PED as a net of varicosities at the level where the PED starts to diffuse into the VL. Its origin can be located at the ventral border of the VL where it diverges from a group of several large processes. Due to the intertangled processes at this point, the neurites could not be further traced. The pedunculus neck (PEDN) is innervated by processes terminating as fine varicosities between the lateral and medial calyces (Fig. 2F).

Calyces (CAs). In the CAs, TH-ir exhibits an heterogeneous distribution (Fig. 5C). The lip and the collar, which receive olfactory and visual input, respectively (Ehmer and Gronenberg, 2002; Gronenberg, 1999; Strausfeld, 2002) present indistinct varicose arborizations whilst the basal ring, which receives olfactory and visual input (Gronenberg, 2001), provides comparatively weaker signals. Unfortunately, without single cell resolution it was impossible to determine the somas connected to these arborizations.

Central complex (CX)

The CX is a central structure in the bee brain which is made of four interconnected, midline spanning neuropils: the upper (UD) and lower (LD) divisions of the central body and, more posteriorly, the protocerebral bridge (PB) and a pair of ventral noduli (NO) (Jonescu, 1909; Kenyon, 1896). The CX seems to be involved in different functions such as sensory integration, motor control, and spatial learning (Pfeiffer and Homberg, 2014).

Similarly as in the MB, the TH-ir in the CX was consistent with the DA-ir characterized in prior works (Schäfer and Rehder, 1989; Schurmann et al., 1989). Dopaminergic processes in this neuropil can be traced to at least two origins. The first one contributes to the projections in the anterior part of the upper division of central body (CB). It derives from the big neurite bundles coming from the C3-cluster (Fig. 3A and 3C; bundle 1). One of these bundles contacts anteriorly a small region in the PL located superior to the vertical lobe and anterior to the PED. There it gives origin to numerous varicose processes. Afterwards it goes ventromedially to the midline where it innervates the upper division of the central body in the form of densely packed column-like arborizations, invading it compartment-wise (Fig. 4; bundle 1). The second one contributes to the projections in the posterior part of the upper division, the lower division of the central body, and the noduli (NO). It can be traced down to a set of around 8 labeled somas in each hemisphere which are disposed in a row posterior-laterally to the protocerebral bridge (PB) and dorsolaterally to the lateral calyx (lCA) (Fig. 6B arrow). We propose to call this cluster C3b (Fig. 6), because its somas also arborize into the central complex, similarly to neurons of the C3-cluster. To depict the complete morphology of the neurons from this cluster, a 3D reconstruction is included (Fig. 6A). Their neurites run ventrally and project anteriorly towards the ventral border of the ML (Fig. 6C). At this point, the neurites send one branch medially toward the ventral border of the central body from which the stained fibers enter the lower, upper divisions and the noduli (Fig. 6A and 6C arrow). The posterior part of the upper division is also innervated from its posterior surface by a number of thin fibers. The protocerebral bridge shows only a weak staining. In general, the intensity of the TH-ir in the posterior part of the central complex is relatively stronger than in the anterior part (Fig. 4C and 6D-E).

Optic lobes (OLs)

The OLs are responsible for processing visual information acquired via the photoreceptors located within the ommatidia of the compound eyes (Avargues-Weber et al., 2012). They comprise three main regions, the lamina, the medulla and the lobula. Dopaminergic processes in this neuropil were only detectable through TH-ir as previous works using DA-ir did not report dopaminergic innervation in this region of the bee brain (Schäfer and Rehder, 1989; Schurmann et al., 1989). These processes derive from a single cluster, which we termed C4 (Fig. 1 and 7A, B) and which is located at the dorsomedial border of the lobula. Its neurites

send processes to the optic lobe (Fig. 7B, asterisk) and the protocebral lobe (PL) at the level of the PEDN (pedunculus neck) where the terminals could not be detected (Fig. 7B, double asterisk).

Two TH-antibodies yielded different staining results for the processes in the optic lobe. The staining with the *antibody which was raised in rabbit* exhibited two subtypes of projections. One subtype consists of two projections which bypass the lobula (LO) and rise dorso- and ventroanteriorly. Each of them runs along the dorso- and ventroanterior border of the OL before innervating the medulla. Each bundle comprises large neurites that are intensely stained and that innervate the outer layer of the medulla. They seem to share the same projection tract running along the dorsal and ventral border of the optic lobe, the anterior superior optic tract (asot) and the anterior inferior optic tract (aiot) (Ehmer and Gronenberg, 2002). The projections of the second subtype enter exclusively into the lobula. A fan-shaped bundle of relatively large neurites runs ventrally and enters the lobula laterally. They innervate the lobula homogenously at different depths. Intense staining can be detected in the medulla's serpentine layer (Fig. 7C) and in column-like processes of the medulla (Fig. 7D). Moving towards the outer layer of the medulla, the neurites of the C3-cluster appear in the form of column-like thin fibers.

The staining with the *antibody which was raised in mouse* yielded only one type of projections, which is the second subtype provided by the staining with the rabbit anti-TH, which enters exclusively into the lobula (see above). Additionally, both antibodies stained the retina. We therefore reconstructed only the second subtype as its somas were traceable and could be verified by the two antibodies. Our 3D reconstruction was able to trace a process which projects uninterruptedly to the LO (Fig. 7A).

Other neuropils in the protocerebral lobe

Our TH staining revealed another projection, which can be traced back to the C3-cluster, that was not detected in prior works (Schäfer and Rehder, 1989; Schurmann et al., 1989). This projection reaches a small, non-described, yet defined neuropil, which is located posterior to the anterior dorsal protocerebral commisure (adpc), flanking dorsally the upper division of the

central body (Fig. 3D, 4B and 4C, asterisks). The innervations are strong and bleb-like in comparison with the size of its neurites. There are two other faint, observable projections in this neuropil. The first one (Fig. 3D double arrows) reaches the contralateral hemisphere. The second one (Fig. 3D arrow) leaves posteriorly and turns dorsoposteriorly around the PEDN (pedunculus neck), where it intertwines with other processes, for instance those originating from the C4-cluster, which renders its terminals in the protocerebral lobe untraceable.

Besides this novel projection, the TH-ir processes we obtained showed also coincidences with other DA-ir processes described before (Schäfer and Rehder, 1989; Schurmann et al., 1989). For instance, posterior to neuropil mentioned above (see Fig. 3D), dorsal to the medial calyx and anterior to the protocerebral bridge (PB), there are few somas (Fig. 8B arrow), which project ventrally crossing the neurite of the C3b-cluster. This cluster has been known as S_P-cluster. The projections continue to a neuropil dorsal to the great commisure (GC) where they send very thin side processes into the neuropil at the lateral border of the PB (Fig. 8). A 3D reconstruction allows an outlook of this cluster and its processes (Fig. 8A).

The anterior optic tubercles (AOTU) are small neuropils located in each hemisphere of the insect brain which are connected by two inter-tubercle tracts (Mota et al., 2011b). They are a major target of visual interneurons from the optic lobe, in particular from the lobula and the medulla (Mota et al., 2011b). They respond to chromatic information in a spatially and temporally segregated manner and intervene probably in navigation (Mota et al., 2013). We found that the AOTUs are innervated by dopaminergic varicose processes which, at least in part, can be traced back to a set of about five labeled fibers that run in the inter-tubercle tracts. Due to its location adjacent to the anterior optic tubercle (AOTU), this cluster is called AOTU-cluster. The 3D reconstruction of this cluster and its neurite bundle provides a better outlook (Fig. 9A). Their somas could not be determined (Fig. 9B, asterisk).

Below each AOTU, 2-3 somas (Fig. 9B) with a diameter of 20-25 µm project posteriorly to the ventrolateral border of the vertical lobe (VL) of the mushroom body, ascend and make widespread arborizations in the neuropil lateral to the PED (pedunculus) (Fig. 9C star). A few of their processes project towards the lobula (LO) (Fig. 9C arrow), but do not enter the optic lobe (OL).

At the ventroposterior border of the lobula, a cluster is located in the proximity of the LO, named S_L. The S_L-cluster and its neurite bundle can be observed better as the 3D reconstruction (Fig. 10A). It consists of 5-8 somas (Fig. 10B arrow) gives rise to a thin bundle of neurites which projects straight dorsally and makes a medial turn before reaching the lateral calyx of the MB. Some of the processes stay in the vicinity of the calyces, while some others project behind the central body across the midline of the brain. The terminals of these fibers could not be detected.

Antennal and dorsal lobes

The antennal and dorsal lobes are prominent neuropils in the bee brain. The antennal lobe (AL) is the primary olfactory neuropil and is constituted by ca. 160 globular subunits termed glomeruli. Glomeruli are interaction sites between the afferent projections of olfactory receptors on the antenna, local interneurons connecting glomeruli laterally and efferent projection neurons conveying the olfactory message reshaped by the AL to higher-order centers such as the lateral horn and the MB. The dorsal lobes (DL) receive mechanosensory input from the antennae (Pareto, 1972; Suzuki, 1975) and house the antennal motoneurons

In the AL, the TH-ir staining produced similar results as those of previous works having used DA-ir (Schäfer and Rehder, 1989; Schurmann et al., 1989). TH-ir could be detected in two small clusters of neurons termed S1 (Fig. 11B arrow) and S2 (Fig. 11B double arrows), which are located in subesophageal zone (SEZ). Each cluster comprises two somas with a diameter of around 10-20 μm. Both are located in the immediate region posterior to the AL, at the lateral border of the DL, with S1 being more ventral and anterior than S2. Their projections share a similar morphological pattern, rendering difficult their tracing to a single-cell resolution level despite their relatively large sizes (Fig. 11A). The neurites project medially to a neuropil in the DL where they arborize delicately (Fig 11B, asterisk) before entering the antennal lobe. The branches distribute themselves as fine processes all over the AL, making contacts both with the neurites and the glomeruli of the AL (Fig 11C arrows).

Discussion

We aimed at characterizing the dopaminergic neurons in the central nervous system of the honey bee. To this end, we used tyrosine hydroxylase (TH) immunoreactivity as this staining ensures that the neurons detected do indeed synthesize DA endogenously. Two differently raised commercial antibodies from two independent sources were used for the immunocytochemistry. One is a polyclonal TH antibody raised in rabbit and the other is a monoclonal antibody raised in mouse. Apart from the expression pattern in the optic lobes, both antibodies yielded similar results, which were consistent with the previous reports of DA-ir (Schäfer and Rehder, 1989; Schurmann et al., 1989) as we found three previously known main dopaminergic clusters, C1 to C3 (Schäfer and Rehder, 1989; Schurmann et al., 1989) (Fig. 1). Yet, in addition, we found a novel fourth cluster, C4, located above the dorsomedial border of the lobula, which innervated the visual neuropils of the bee brain (Figs. 1 and 7). The profuse DA innervation in the entire bee brain, and the specific connectivity of DA neurons with visual, olfactory and gustatory circuits provide the basis for DA modulation of these sensory modules and for value-based labeling of sensory cues of these modalities in associative learning.

The C1-, C2- and C3-clusters

The location and general connectivity of these three clusters are consistent with those reported in prior studies based on DA-ir (Schäfer and Rehder, 1989; Schurmann et al., 1989). There are, however, slight differences between these results and those of our work such as the number of somas or an unreported projection in the small neuropil flanking dorsally the central complex. It is possible that these differences were due to the following reasons: *Firstly, the thickness of the sections*. A disadvantage of sectioned samples is that they might lead to information loss. In prior studies, the slices had a thickness of around 10-12 µm, while ours were 80-160 µm thick. Additionally, whole mount samples were also used in our study. Thus, it cannot be ruled out that in the previous studies, some parts of the tissues were damaged or even lost during the cutting process, leading to a smaller number of somas detected in the C3-cluster. This might also allow explaining why the particular projection to the neuropil dorsal to the central complex was not previously reported. Yet, this argument

does not explain why in our study the number of somas in the C1- and C2-clusters was lower than those reported in earlier studies.

Secondly, the antibodies used. In prior studies, it was not clear against which DA antigen the antibody had been raised. As already mentioned, we used antibodies against TH to make sure that our putative DA neurons synthesize DA endogenously. The morphology of the neurons of the C1- and C2-clusters resembles that of the A1 and A2 mushroom-body extrinsic neurons (MBEN); while some neurons of the C3-cluster resemble the A6 MBENs as reported by Rybak and Menzel (1993a). In the honey bee brain, the A1 and A2 MBENs are located anteriorly and in the same depth as the C1- and C2-clusters and project in the same manner into the vertical lobe of the MB. Their branches envelop the vertical lobe and project to the protocerebral lobe unilaterally. Fine varicose fibers are detected both in the vertical and the medial lobe. Rybak and Menzel (1993a) counted an average of 50-60 stained neurons of the A1 and A2 type. This number is close to the 75 somas counted both in the C1- and C2-clusters, thus indicating that these clusters may consist of these two types of MBEN. Thus the A1 and A2 neurons described by Rybak and Menzel as MBEN may in fact be dopaminergic neurons of the C1- and C2-clusters. In Drosophila, the homologue of the C1- and C2-clusters may be the PAM cluster with respect of soma location and innervations. The neurons of the PAM cluster terminate in the horizontal lobes of the mushroom body and in neuropils adjacent to them (Mao and Davis, 2009).

The A6 MBEN of the bee is so far the only neuron which can be proposed as a model neuron for the C3-cluster. Its soma is located ventrally to the lateral calyx of the MB and it projects both to the vertical lobe and to neuropils in the contralateral hemisphere, in addition to the ipsilateral lateral horn. Even though the neurons of the C3-cluster could not be traced o this extent, it is possible to sort out a part of their massive projections in this way. Looking at the number of reported A6 neurons which ranges between 60 and 80, we may conclude that they are part of the 140 cells constituting the C3-cluster. The homologue of this cluster in Drosophila might be the PPL1- and the PPL2ab-clusters together. Firstly, the location of the somas of the PPL1-cluster is relatively close to the calyx and this cluster has terminals in the dorsal part for the fan-shaped body as one of the terminals of the C3-cluster (Liu et al., 2012c; Mao and Davis, 2009). Secondly, the terminals of the PPL2ab-cluster are detectable in the calyx of the mushroom body (Mao and Davis, 2009) similarly to the C3-cluster. It is notable

that C3-cluster is a big cluster consisting of different types of neurons, or possibly, sub-clusters.

The C4-cluster

Our TH-staining uncovered the presence of a previously unknown dopaminergic cluster, the C4-cluster. For the first time, and contrarily to what was previously concluded (Schäfer and Rehder, 1989; Schurmann et al., 1989), we showed the presence of putative DA neurons innervating the optic lobe of the honey bee. In accordance to the nomenclature of previous reports, the name adopted for this cluster follows the sequential order of the main dopaminergic clusters previously described in the honey bee brain.

The fact that the C4-cluster remained undetected in two parallel neuroanatomical characterizations of dopaminergic neurons (Schäfer and Rehder, 1989; Schurmann et al., 1989) is quite surprising due to the fact that Mercer et al. (1983) had reported faint dopamine expression in the optic lobes. Subsequent studies on DA expression verified this finding and showed that DA is, in fact, detectable in the optic lobes despite variable expression levels (Sasaki and Nagao, 2001; Taylor et al., 1992). In worker bees, and regardless of their age, DA expression levels in the optic lobes is relatively low compared to that in the protocerebrum (Mercer et al., 1983; Sasaki and Nagao, 2001). An age/caste effect exists nevertheless as DA levels are higher in bee foragers (Taylor et al., 1992). This might be due to the fact that foragers are less exposed to the queen and to the inhibitory effects of the queen mandibular pheromone (QMP) on worker dopaminergic signaling (Beggs et al., 2007; Vergoz et al., 2009; Vergoz et al., 2007b). One cannot rule out, therefore, that in prior studies the samples used for detect dopaminergic signaling in the optic lobes were obtained from relatively young bees rather than foragers, so that dopamine levels were not in a detectable range.

The morphology of the C4 neurons resembles in part to that of the MBENs connecting the calyces and the optic lobes (Ehmer and Gronenberg, 2002). The C4-cluster could comprise at least three different cell types. The first one shares the track with the asot along the dorsal border of the optic lobe. The second one shares the track with the aiot along the ventral border. The third one projects directly into the lobula and then innervates the serpentine layer of the medulla. These three types of neurons share only one single projection into the protocerebrum. It runs to the pedunculus neck where the neurites could not be

distinguished from other putative dopaminergic processes. Even though these neurons share several properties of the MBENs mentioned in prior studies, it is intriguing to see that the cluster is located exclusively at the dorsomedial border of the lobula. Neurons projecting to the asot and aiot have their somas in the dorsomedial edge of the medulla and the base of the lateral calyx adjacent to the ventral edge of the medulla and lobula, respectively (Ehmer and Gronenberg, 2002). C4-cluster somas cannot be located in these regions, suggesting that they might possess different morphological properties.

The first two cell types are stained only by the rabbit polyclonal antibody. The third cell type, however, is only stained by antibodies. Except this discrepancy, both antibodies yielded the same staining pattern. This pattern is indicative of a dopaminergic modulation of visual circuits and thus of visual processing at different levels, from the lamina to the lobula. The consequences of this modulation are still unknown. It is worth noting that dopaminergic signaling is crucial for aversive forms of learning as it mediates aversive reinforcement signaling (e.g. electric shock) and that in the bee visual forms of aversive learning exist which may depend on such signaling as they consist in associating visual chromatic/achromatic stimuli with electric shock (Mota et al., 2011a). Although the critical coincidence of the visual stimulus and the shock pathways necessary to support visual aversive learning and memory may be only at the level of the mushroom bodies, it is interesting to realize that such coincidence could occur at multiple levels upstream the mushroom bodies, thus providing multiple substrates for different forms of visual plasticity.

TH-immunoreactivity in the mushroom body

The mushroom body is a higher order processing center, which integrates various types of sensory information conveyed by visual, olfactory, mechanosensory and gustatory afferences arriving to segregated regions of the calyces and information conveyed by afferences of the lateral protocerebrum arriving at the gamma lobe, the lower portion of the vertical lobe (Strausfeld, 2002). Dopaminergic processes innervate both the calyces, the vertical and medial lobes and the pedunculus. TH-ir in the calyces is obvious in the lips and the collar regions, known as the input regions from olfactory and visual afferences, respectively. The vertical and medial lobes, sites associated with memory retrieval (Cano-Lozano et al., 2001) are variably innervated.

In the fruit fly *Drosophila melanogaster* studies on olfactory aversive and appetitive learning have shown that several classes of dopaminergic neurons provide distinct forms of reinforcement signals (appetitive, aversive; short term, long term) thus resulting in multiple forms of memories (Aso et al., 2012; Aso et al., 2010; Burke et al., 2012; Claridge-Chang et al., 2009; Liu et al., 2012a) . Two clusters have their dense terminals in the lobes, each with its unique zonal organization. The PAM cluster, whose resemblance with the C1- and C2-clusters has been underlined above, mediates the aversive reinforcement properties of the electric shock used as unconditioned stimulus in olfactory aversive conditioning so that its inhibition impairs aversive learning while its activation is sufficient and necessary to mediate aversive learning (Aso et al., 2012; Aso et al., 2010; Claridge-Chang et al., 2009).

Despite the relatively small number of neurons constituting it (12; see Mao and Davis, 2009), the *Drosophila* PPL1 cluster, a cluster which resembles the C3-cluster of the honey bee, provides aversive reinforcement signaling and regulates ARM levels and gating to LTM (Aso et al., 2010; Claridge-Chang et al., 2009; Placais et al., 2012). Moreover, the interplay between neurons in the PAM and PPL1 clusters results in a various robustness in aversive memory formation (Aso et al., 2012). At least three essential dopamine pathways to the MB can together induce aversive (shock-induced) memory in the fly. They arborize in different MB subdomains that are defined by specific combinations of intrinsic and extrinsic neurons (Aso et al., 2012). Each dopamine pathway for memory induction intersects the specific axonal compartment of Kenyon cells and the memories induced by the distinct dopamine neurons interact to tune the stability of the shock memory

Remarkably, dopaminergic signaling does not solely mediate aversive-reinforcement signaling to the mushroom bodies of the fruit fly. It has been recently shown that appetitive sucrose reinforcement is mediated by a hierarchical network in which peripheral signaling is mediated by octopaminergic neurons which further convey their signal to dopaminergic neurons of the PAM cluster impinging onto the mushroom bodies. Thus, a different form of dopaminergic neurons indicates the presence of reward to the fruit fly mushroom bodies and contributes to appetitive memory formation (Burke et al., 2012; Liu et al., 2012a).

Taken together the results obtained in the fruit fly have shown the fundamental importance of different subsets of dopaminergic neurons of the PAM and PPL1 clusters as neural correlates of reinforcement signaling in appetitive and aversive olfactory conditioning. In the bee, appetitive reinforcement seems to be independent of dopaminergic signaling as it

is mediated by a single octopaminergic neuron, the VUMmx1 neuron, which arborizes in the antennal lobes, lateral horn and mushroom bodies and whose activity substitutes for sucrose reward in appetitive olfactory conditioning (Hammer, 1993). Yet, the dependency of aversive olfactory SER conditioning on dopaminergic signaling has been demonstrated using pharmacological blockade (see Introduction)(Vergoz et al., 2007a). The specific neurons mediating the shock signaling in this aversive conditioning could, thus, be found in the C1-, C2 and/or C3-clusters given the apparent homologies with the PAM and PPL1 clusters of the fruit fly.

TH-immunoreactivity (TH-ir) in the central complex (CX)

The central complex is a group of four interconnected, midline spanning neuropils in the center of the bee brain. It consists of the upper and lower divisions of the central body and, more posteriorly, the protocerebral bridge and a pair of ventral noduli. Different lines of research have proposed a role of the central complex in sensory integration, motor control, and spatial learning and, in particular, in the processing of visual information (Homberg, 1985; Milde, 1988). Our work showed that TH-ir innervations in the central complex can be attributed to at least two clusters, the C3-cluster innervating the anterior upper division of the central body and the C3b-cluster innervating the posterior upper division, the lower division of the central body and the noduli.

The somas of the C3b-cluster can be located in the posterior region anterior to the protocerebral bridge. Interestingly, the PPM3 cluster in Drosophila can be located in a relatively similar region and possesses as many somas (8; see Mao and Davis, 2009) as the C3b-cluster. In the central complex, their innervations can be traced in the lower half of the fan-shaped body, the noduli (Alekseyenko et al., 2013; Mao and Davis, 2009) and the ellipsoid body (Liu et al., 2012c).

The presence of dopaminergic neurons in the central complex of *Drosophila* has been associated with sleep, arousal, wakefulness and aggression modulation (Alekseyenko et al., 2013; Ueno et al., 2012). So far, dopaminergic processes in this neuropil have not been associated with reinforcement-signaling functions for appetitive and/or aversive associative learning and memory, but this may be due to the fact that conditioning protocols in which dopaminergic function has been studied in the fly are mostly olfactory. Note, however, that

visual conditioning protocols exist in which a tethered fly is suspended in a visual circular arena and where it flies in order to avoid visually displayed patterns associated with the aversive reinforcement of a heat beam on the thorax (Wolf et al., 1992). For this conditioning form, the central complex plays a fundamental role, as two groups of horizontal neurons in one of its substructures, the fan-shaped body, are required for *Drosophila* visual pattern memory (Liu et al., 2006). Besides, a small set of neurons in the ellipsoid body, which is another substructure of the central complex and connected to the fan-shaped body, is also required for visual pattern memory. Both groups of neurons constitute, therefore, a complex neural circuit in the central complex for *Drosophila* visual pattern memory (Pan et al., 2009), which may benefit from a possible association with dopaminergic circuits conveying aversive reinforcement signaling.

Dopaminergic neurons as modulators of behavior

Besides their role as a circuitry for reinforcement signaling, dopaminergic neurons act as a more general modulatory system, generally depressing several behavioral components. For instance, dopamine decreases sucrose responsiveness (i.e. PER to increasing sucrose concentrations) when injected into the thorax. Also, injection or feeding of the DA receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN) reduces sucrose responsiveness significantly (Scheiner et al., 2002). In olfactory PER conditioning, injection of DA into the antennal lobes reduces significantly olfactory retention both after one and three conditioning trials (Macmillan and Mercer, 1987). In the case of aversive responsiveness (i.e. SER to increasing shock voltages), we showed that dopaminergic blocking induces an *increase* of shock responsiveness, thus reflecting an enhancement in shock sensitivity (Tedjakumala et al., 2014; see previous chapter). This result thus indicates that in its default mode, and besides its reinforcement-signaling role, dopaminergic signaling acts as a depressor of sting responsiveness to electric shocks so that when its effect is antagonized, responsiveness increases.

We suggested that a possible explanation for this dual function is to assume the existence of different classes of dopaminergic neurons mediating different functions: one acting as a general gain control system, with the specific role of down-regulating responsiveness and another acting as instructive neurons in aversive associative learning

which mediates aversive US signaling. Owing to these different functions, their brain targets could be different. While the first class would exhibit extensive and broad branching within the entire brain in order to be able to modulate different motivational components (appetitive, aversive) and sensory modalities (olfactory, visual gustatory, etc), the second class would exhibit a specific connectivity with respect to CS processing circuits (olfactory, visual) in order to facilitate CS-US associations and provide instructive (i.e. valence) information to the targeted CS circuit (Giurfa, 2006). In principle, the neural architecture of the dopaminergic circuits described in the present work provides the basis for these different functions. Further functional studies should address the possible heterogeneity of the different dopaminergic clusters in the bee brain. We hope that our analyses will constitute an important anatomical reference guiding such functional hypotheses in the future.

Acknowledgements

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Figures

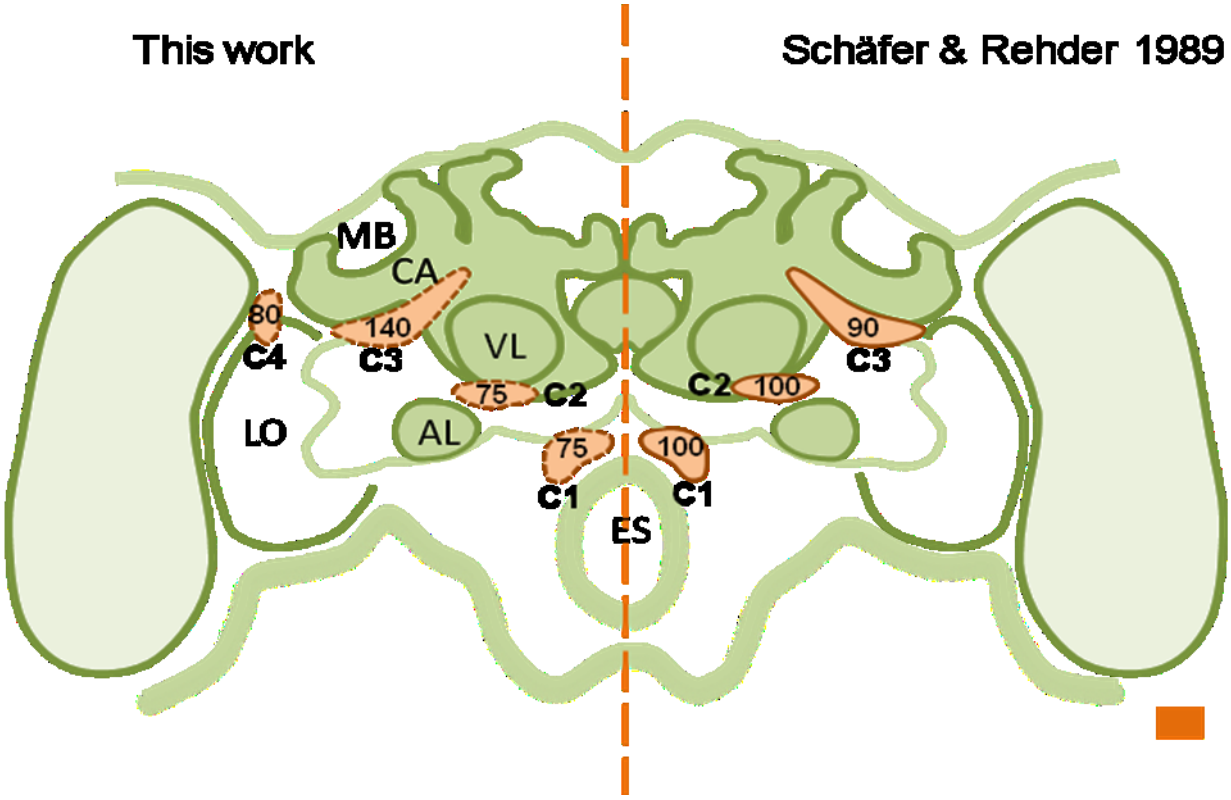

Figure 1. Overview of the honey bee brain showing the main dopaminergic cell clusters, C1 to C4, found in the supraesophageal zone (SPZ); ***left:*** results of this study obtained using the technique of TH staining; ***right:*** results of Schäfer and Rehder (1989) obtained using dopamine-like immunoreactivity (DA-ir). The numbers indicate the numbers of neurons per cell cluster. The main difference resides in the identification of the C4 cluster whose existence was unknown up to now. ES: esophagus; AL: antennal lobe, LO: lobula; MB: mushroom body; CA: calyces; VL: vertical lobe. Scale bar: 100 µm

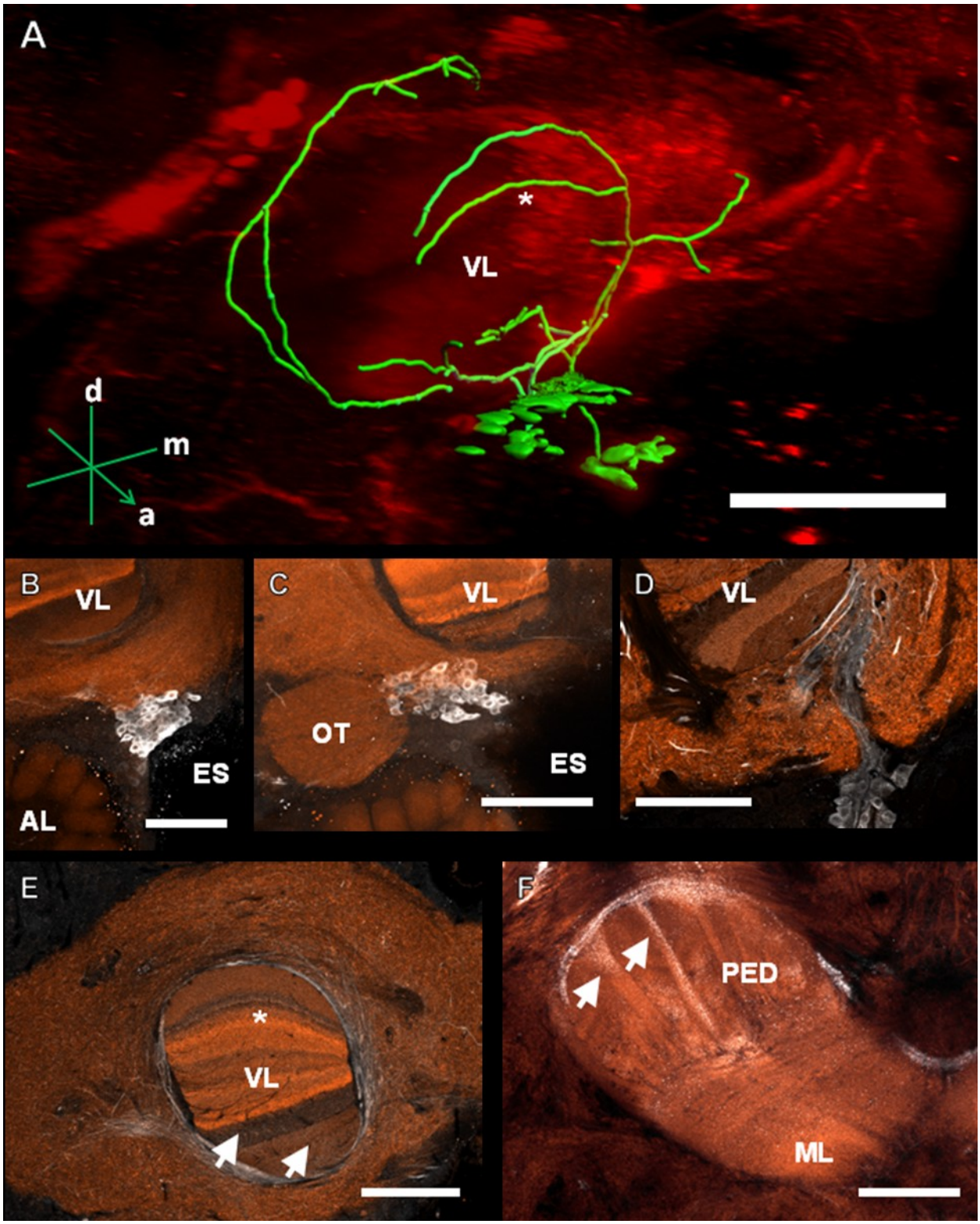
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Figure 2. The C1- and C2-clusters and their processes in the bee brain. **A.** 3D reconstruction of the C1- and C2-clusters. Confocal images of the C1- and C2-clusters were stacked and presented in an oblique angle along with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The reconstructed neurons wrap the vertical lobe (VL). The asterisk is highlighting an innervation into the VL as described in fig. 2E. **B.** The C1-cluster is shown; it is located adjacent to the esophagus (ES) and between the vertical lobe (VL) of the mushroom body and the antennal lobe (AL). **C.** The C2-cluster is shown; it is located below the ventral part of the VL, above the AL and medial to the optic tuberculum (OT). **D.** The neurite bundles of the C1- and C2-clusters meet at the ventromedial margin of the VL and then envelop and enter the VL. The lowers layers of the VL can be clearly seen. **E.** Fine fiber-like arborizations in the VL can be clearly observed in various layers of the VL (arrows). The neurite bundles envelop the VL dorsomedially and –laterally. Parts of the dorsomedial bundle enter the VL at one of its most dorsal layers (asterisk – see fig. 2A for the reconstruction), which presents Kenyon cell axons from the basal ring. Neurites arborize anteriorly to and out of the VL to make ramifications into the neuropils lateral and medial to the VL. **F.** The peduncle (PED) of the mushroom body is innervated by column-like varicosities (arrows). The main bundle can be traced back to processes running along the lateral border of the VL. Scale bar: 100 μm.

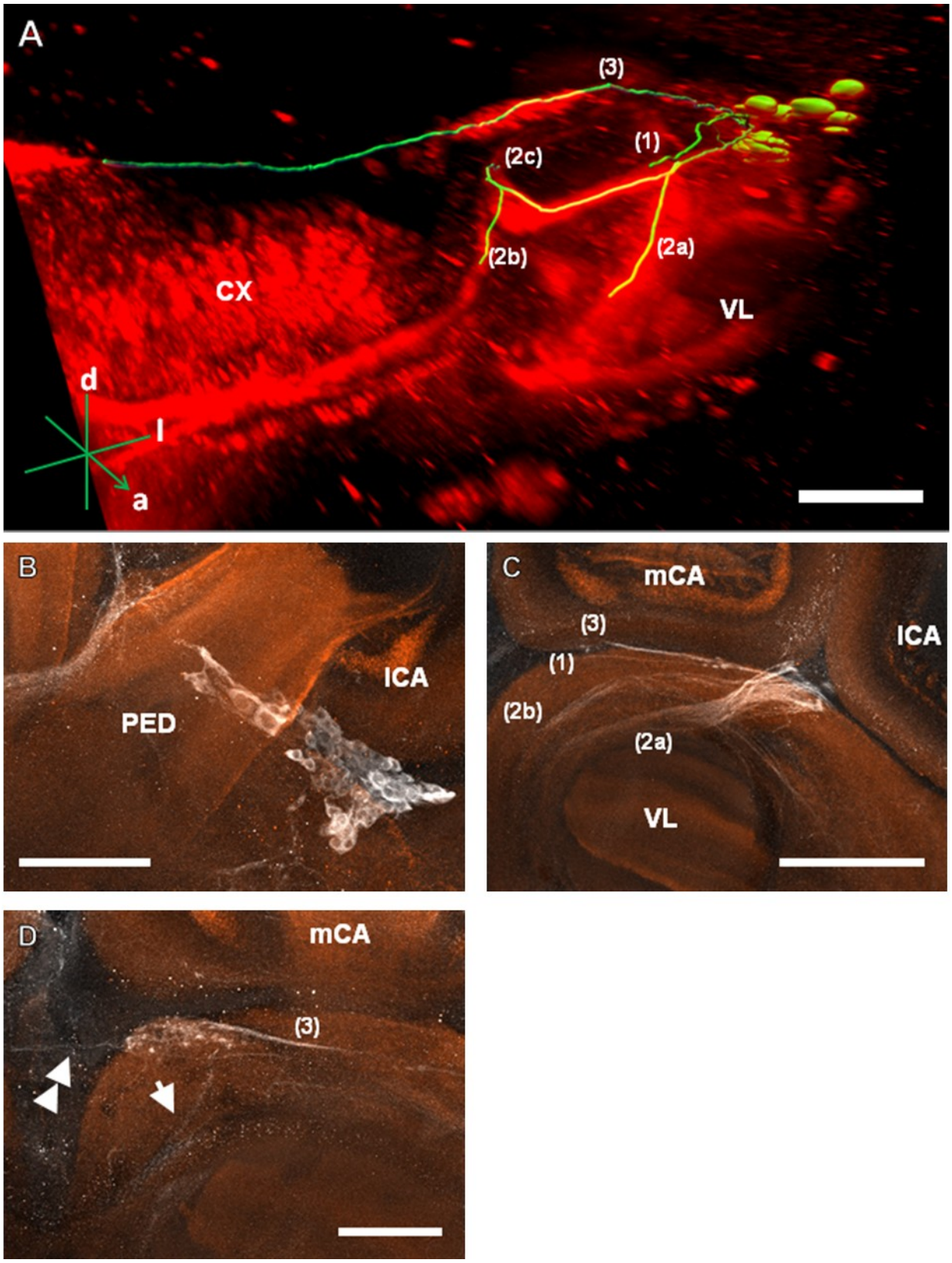
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Figure 3. The C3-cluster and its main processes into the bee brain **A.** 3D reconstruction of the C3-cluster and its main bundles (1; 2a, 2b, 2c; 3); confocal images of this cluster were stacked and presented in an oblique angle along with reference axes indicating the directions of the anterior (a), dorsal (d) and lateral (l) side of the stacks. The vertical lobe (VL) and the central complex (CX) are shown. **B.** The somas of the C3-cluster are located below the lCA of the mushroom body and adjacent to the pedunculus (PED). Various diameters can be observed among the somas, indicating their heterogeneity. **C.** Three main bundles can be traced from the C3-cluster. **(1)** the bundle projects in the upper division (UD) of the CX through the anterior part of the UD. **(2)** the bundle splits and sends one branch into the neuropil ventromedial to the vertical lobe **(2a)** and one branch to the contralateral side **(2b)**; one branch **(2c)** projects posterior, along the dorsal rim of the VL, making a loop behind the PED (see **Fig. 5** for **2c**). From between the lateral (lCA) and the medial calyx (mCA), the fibers invade the PED and the calyx neuropil (see **Fig. 5**). **(3)** a bundle projects to an unidentified neuropil, flanking dorsally the upper division of the central body and interconnects to the same region in the other brain hemisphere **D.** The innervations in the unidentified region ventral to the mCA. From this neuropil, one neurite bundle projects to the contralateral side (double arrows) and another one projects posteriorly (arrow). Scale bar: 100 µm.

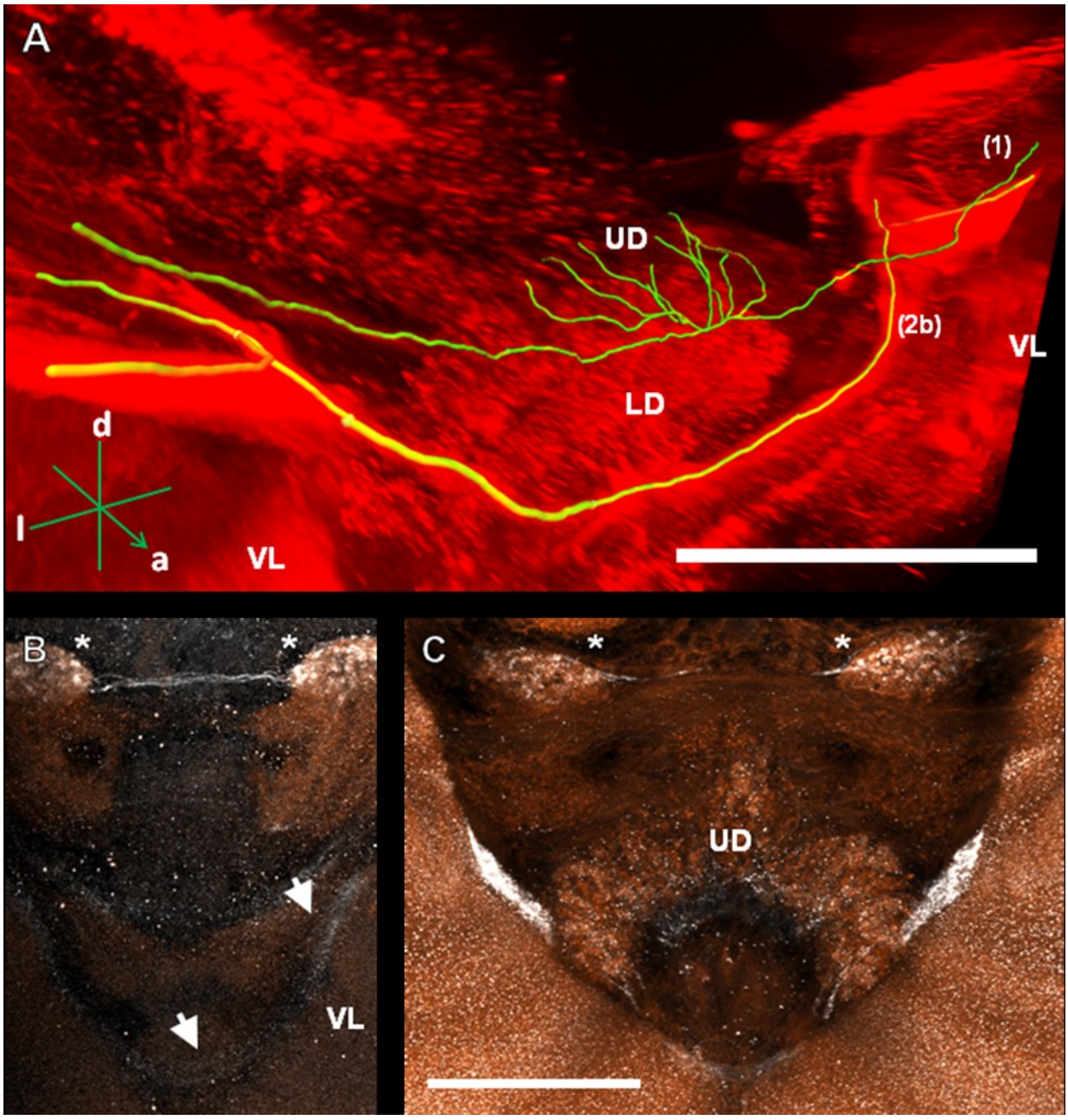

Figure 4. Two main tracks of the C3-cluster interconnecting both brain hemispheres. **A.** 3D reconstruction of these tracks; confocal images were stacked and presented in an oblique along with reference axes indicating the directions of the anterior (a), dorsal (d) and lateral (l) side of the stacks. Two main bundles from the C3-cluster interconnect both hemispheres. Bundle 1 projects into the upper division (UD) of the central complex (CX). Both vertical lobes (VL) are indicated for reference. **B.** A neurite bundle projects along the medial border of the vertical lobe (VL) of the mushroom body to the anterior part of the brain, bypassing the central body (CB) and projecting further to the other brain hemisphere (arrows). The unidentified regions (asterisks) flanking the CB dorsally are interconnected via another bundle. **C.** Another interconnecting neurite bundle projects to the anterior part of the UD of the CX before finally projecting further to the other brain hemisphere. Scale bar: 100 µm.

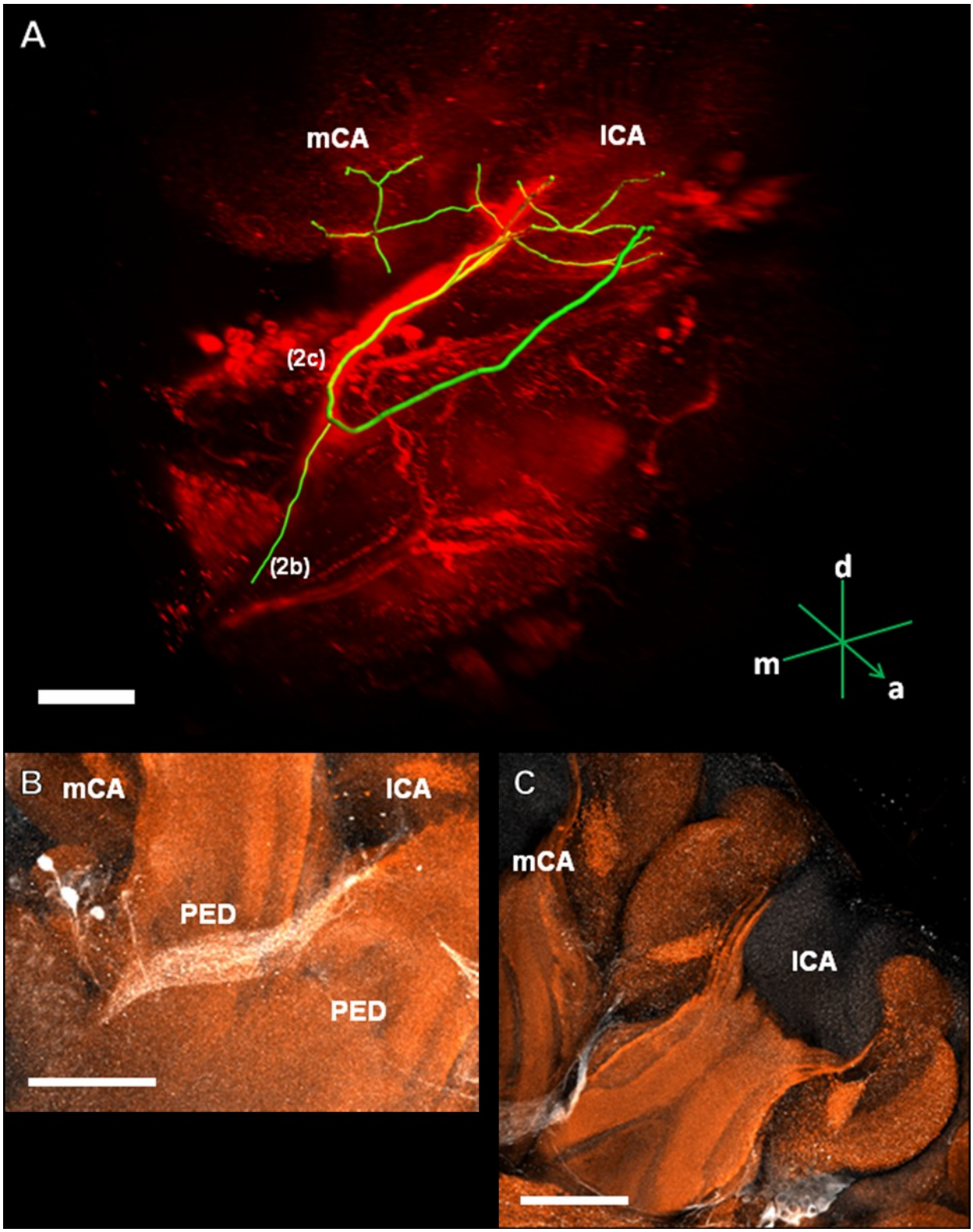
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Figure 5. The main track of the C3-cluster innervating the calyces (CA) of the mushroom body. **A.** 3D reconstruction of this track; confocal images were stacked and presented in an oblique angle along with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The medial (mCA) and the lateral (lCA) calyces are shown. One neurite bundle originating from the C3-cluster projects to the contralateral hemisphere **(2b)** and another one forms a loop **(2c)** before terminating in the calyces of the mushroom body. **B.** The loop envelops the posterior part of the PED. It goes towards the space between the two peduncles (PED) and projects into both calyces of the mushroom body. **C.** TH-ir in the calyces of the MB. Somas of the C3-cluster can be observed below lCA. Scale bar: 100 μm.

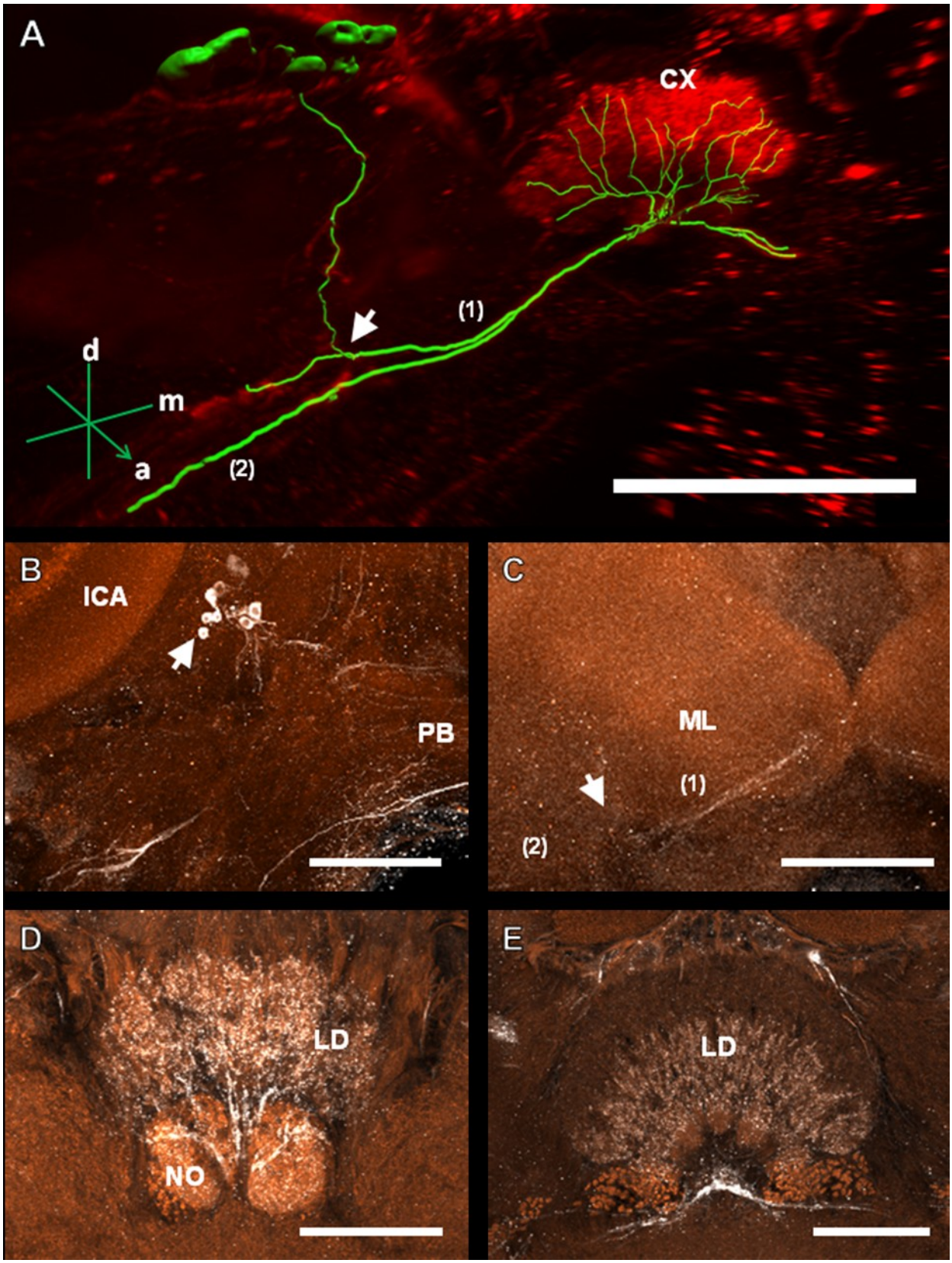
A
CX
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Figure 6. The C3b-cluster and its processes in the central complex (CX). **A.** 3D reconstruction of this cluster; confocal images of the C3b-cluster were stacked and presented in an oblique angle along with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The innervation of the CX is shown. One branch (1) projects medially toward the ventral border of the central body and enters the lower division (LD) and the noduli. Another branch (2) goes to the ventral border of the medial lobe (ML). **B.** The cell cluster (arrow) consists of around 8 somata located posterior to the pedunculus (PED) and medioventrally to the lateral calyx (lCA). The protocerebral bridge (PB) of the CX is shown. **C.** From the somata, the neurites project ventrally (arrow; see also Fig. 6A arrow) where they divide into two branches, innervating **(1)** the central body, its noduli and LD of the CX - and **(2)** the ventral border of ML. **D.** TH-ir in the NO and the posterior part of the LD. **E.** TH-ir in the LD of the CX. The projections enter the CX ventrally and originate symmetrically from both C3b-clusters. Scale bar: 100 µm.

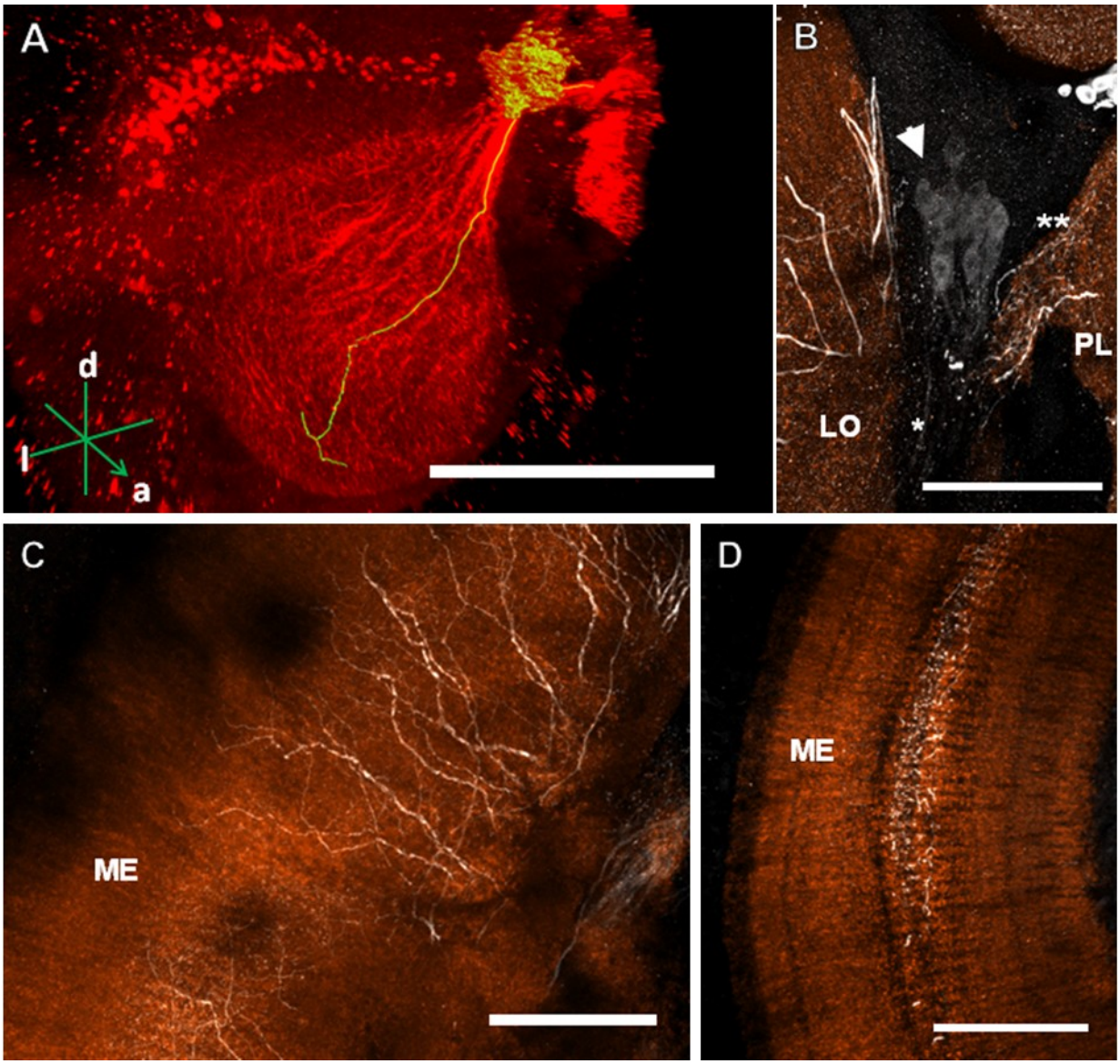

Figure 7. The C4-cluster and a traceable projection up to the lobula (LO). **A.** 3D reconstruction of the C4-cluster and of a single traceable neurite projecting to the LO; confocal images were stacked and presented in an oblique angle along with reference axes indicating the directions of the anterior (a), dorsal (d) and lateral (l) side of the stacks. **B.** Around 80 somas (arrow) are found in the C4-cluster. The neurite bundle (asterisk) projects toward the LO and the protocerebral lobe (PL) (double asterisk). **C.** Leaving the LO, the neurites arborize in the medulla (ME) in a column-like pattern. **D.** TH-ir in the serpentine layer of the ME. The neurites arborize laterally within the serpentine layer. Scale bar: 100 µm.

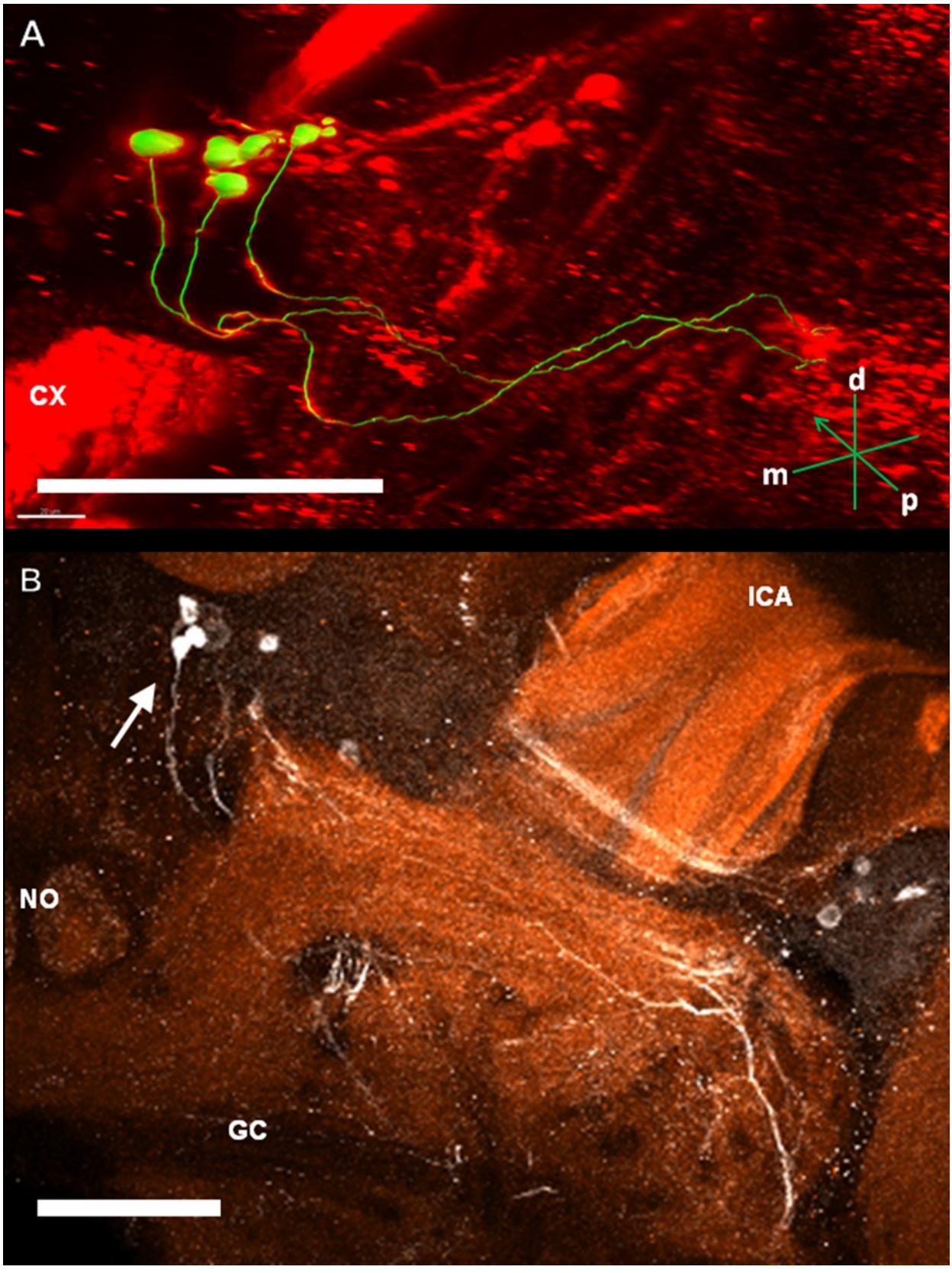
A
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Figure 8. The S_P cluster and its neurite bundle. **A.** 3D reconstruction of this cluster; confocal images of S_P-cluster are stacked and presented in an oblique angle along with reference axes indicating the directions of the posterior (p), dorsal (d) and medial (m) side of the stacks. The central complex (CX) is shown. **B.** Between 15-20 somata are found in this cluster (arrow). The neurite bundles can be distinguished into two main tracks projecting to neuropils dorsal to the great commisure (GC). The lateral calyx (lCA) and a nodulus (NO) are shown. Scale bar: 100 μm.

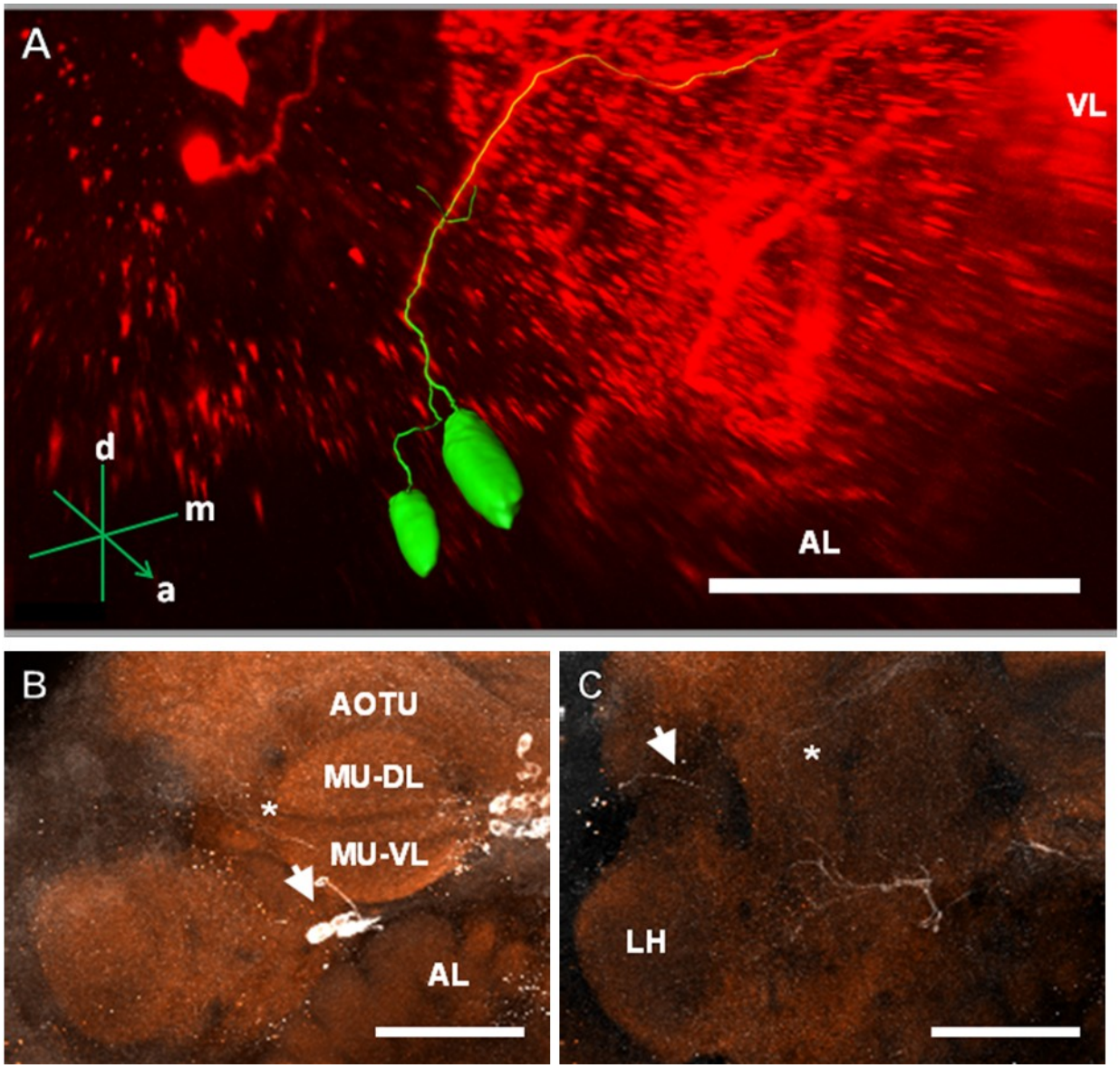


Figure 9. The anterior optic tubercle (AOTU) cluster and its neurite bundle. **A.** 3D reconstruction of the AOTU cluster; confocal images of this-cluster are stacked and presented in an oblique angle along with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The location of the vertical lobe (VL) and the antennal lobe (AL) is indicated. **B.** 2-3 somas (arrow) are located between the AOTU and the antennal lobe (AL). The two major units (MU) of AOTU, MU-dorsal lobe (MU-DL) and -ventral lobe (MU-VL), can be observed. Processes leaving the AOTU can be observed, which are unrelated to the AOTU-cluster (asterisk). **C.** The neurite bundle projects posteriorly below the AOTU along the ventrolateral border of the vertical lobe, making a turn dorsomedially (asterisk) and finally projecting into neuropils posterior to the medial lobe. Some processes toward the lateral border of the protocerebrum can be observed (arrow), superior to the lateral horn (LH). Their terminals could not be determined. Scale bar: 100 µm.

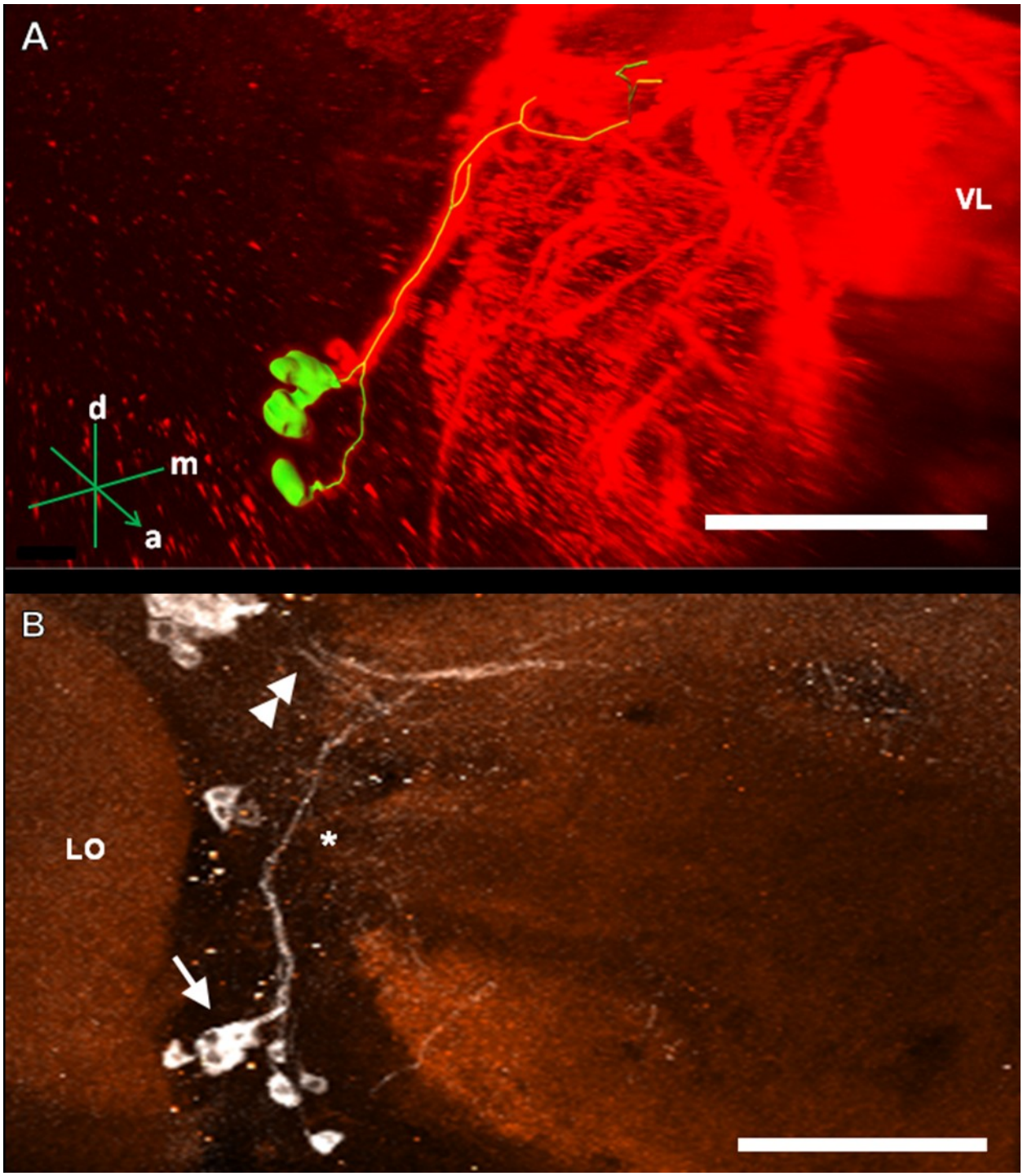


Figure 10. The S_L-cluster and its neurite bundle. **A.** 3D reconstruction of the S_L-cluster; confocal images of this cluster are stacked and presented in an oblique angle along with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The location of the vertical lobe (VL) is indicated. **B.** The S_L-cluster with its 5-8 somas (arrow) is located at the ventromedial border of the lobula (LO). Its neurite bundle projects dorsally and turns medially (asterisk), where it collate with processes coming from the C4-cluster (double arrow). Their terminals could not be determined. Scale bar: 100 µm.

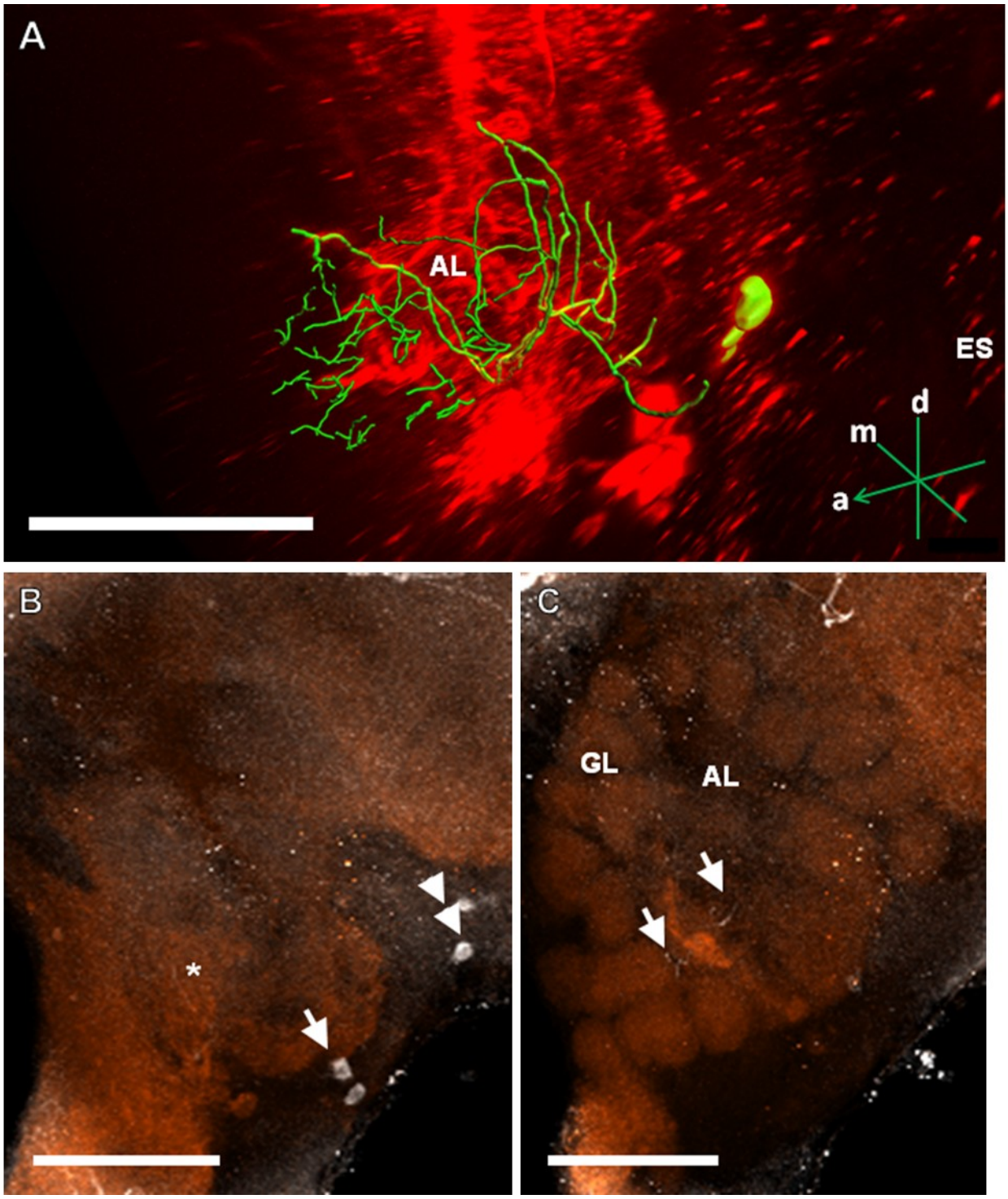
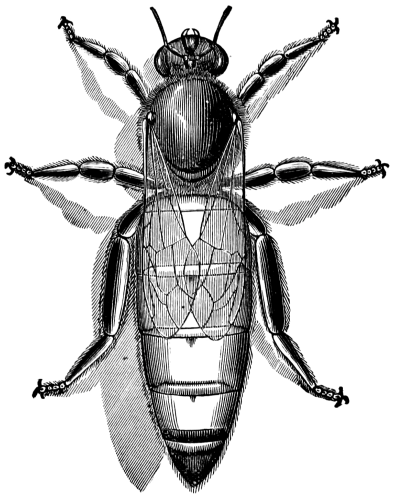


Figure 11. The S1- and S2-clusters and their processes in the antennal lobe (AL). **A.** 3D reconstruction of these clusters; confocal images were stacked and presented in an oblique angle angle along with reference axes indicating the directions of the anterior (a), dorsal (d) and medial (m) side of the stacks. The location of esophagus (ES) is indicated. **B.** The somas of the S1- (arrow) and S2-clusters (double arrow) can be observed at the lateral border of the deutocerebrum. **C.** The neurite bundles form delicate arborizations innervating the AL in the centre (arrows) and spread into the peripherally arranged glomeruli (GL). Scale bar: 100 µm.

3

Aversive learning increases the expression of dopamine-receptor genes in specific cell populations of the honey bee brain



3rd PART

Aversive learning increases the expression of dopamine-receptor genes in specific cell populations of the honey bee brain

Stevanus Rio Tedjakumala*, Henry James McQuillan*, Elodie Despouy, Elodie Urlacher, Alison Mercer§ and Martin Giurfa§ (*in prep.*)

The last part of this work studied the molecular mechanisms underlying aversive responsiveness and associative learning in the honey bee. Specifically, we analyzed whether aversive learning induces variations in the expression of specific receptor genes, thereby changing the aversive sensitivity to punishment. As in the 1st experimental chapter, we measured aversive responsiveness in bees by quantifying sting extension responses (SER) to electric shocks but we performed in addition a subsequent aversive conditioning in which bees had to learn to differentiate an odorant paired with shock from a different odorant not paired with shock. Three days after conditioning w performed a memory test and quantified again aversive responsiveness to electric shocks.

We found that aversive olfactory learning induces a long-term decrease in shock responsiveness three days after conditioning. We focused on the constitutive cells of the mushroom bodies, the Kenyon cells, and used laser-capture micro dissection followed by RT-qPCR to determine whether learning and/or memory retrieval induced long-term changes in receptor-gene expression in specific populations of Kenyon cells. We found that aversive learning, but not retrieval, promotes a specific long-term increase in the expression of the dopaminergic receptor genes Amdop2 and partially of Amdop1. No changes were detected for Amdop3, the ecdysteroid/dopamine receptor gene Amgpcr19

and the octopaminergic receptor gene Oa1, which mediates appetitive-reinforcement signaling.

Thus, learning induces an increase of specific receptor genes, which may mediate an equally specific long-term decrease of responsiveness to the shock punishment. These results indicate that specific forms of dopaminergic signaling act as down-regulators of behavioral responsiveness to reinforcing stimuli and that such down-regulation may reflect the acquisition of predictable expectations about these stimuli.

Aversive learning increases the expression of dopamine-receptor genes in specific cell populations of the honey bee brain

Stevanus Rio Tedjakumala[1,2,*], Henry James McQuillan[3,*], Elodie Despouy[1,2], Elodie Urlacher[3], Alison Mercer[3,§] and Martin Giurfa[1,2,§]

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Summary

Associative learning allows extracting the predictive relationships in an individual's environment, a capacity that is crucial for survival as it allows us to use past events to predict the future and to adjust our behavior accordingly (Wasserman and Miller, 1997). Multiple short-, medium-, and long-term mechanisms determine plastic changes in neural-circuit connectivity underlying learning and memory formation (Chen and Tonegawa, 1997; Kolb and Whishaw, 1998). At a single neuron level, molecular cascades underlying these capacities have been extensively investigated (Izquierdo et al., 2006; Kandel, 2001). However, the possibility that learning induces variations in the expression of specific receptor genes, thereby changing the sensitivity to environmental signals, has been less explored. Here we studied aversive responsiveness and learning in the honey bee, an insect that has yielded important insights into the cellular and molecular bases of learning and memory (Giurfa, 2007; Menzel, 1999). We measured aversive responsiveness in bees by quantifying sting extension responses (SER) to electric shocks (Roussel et al., 2009; Tedjakumala et al., 2014) and found that subsequent aversive olfactory learning (Giurfa et al., 2009; Tedjakumala and Giurfa, 2013; Vergoz et al., 2007) induces a long-term decrease in shock responsiveness three days after conditioning. To uncover the mechanisms mediating this decrease in sensitivity, we focused on Kenyon cells, the neurons that constitute the mushroom bodies (MBs), a structure of the insect brain that has been associated with learning and memory (Davis, 2011; Giurfa and Sandoz, 2012; Heisenberg, 2003; Menzel, 1999). We used laser-capture micro dissection followed by RT-qPCR to determine whether learning and/or memory retrieval induce long-term changes in receptor-gene expression in specific populations of Kenyon cells. We found that aversive learning, but not retrieval, promotes a long-term increase in the expression of the dopaminergic receptor genes *Amdop2* (Humphries et al., 2003; Mustard et al., 2003) and partially of *Amdop1* (Blenau et al., 1998; Mustard et al., 2003). No changes were detected for *Amdop3* (Beggs et al., 2005), the ecdysteroid/dopamine receptor gene *Amgpcr19* (Geddes et al., 2013) and the octopaminergic receptor gene *Oa1* (Grohmann et al., 2003), which mediates appetitive-reinforcement signaling (Farooqui et al., 2003). Thus, learning induces an increase of specific receptor genes, which may mediate a long-term decrease of responsiveness to the shock punishment. These results indicate that specific forms of dopaminergic signaling act as down-regulators of behavioral responsiveness to

reinforcing stimuli and that such down-regulation may reflect the acquisition of predictable expectations about these stimuli.

Results

Aversive Learning Induces a Specific Decrease of Shock Sensitivity

Stimulus sensitivity can be evaluated by measuring individual responses to a series of increasing values of a given stimulus modality and determining from which value an animal starts responding or the number of stimulations of the series to which it responds. In honeybees, differences between nectar and pollen foragers are mediated by different sensitivities to sucrose stimulation as shown by their proboscis extension reflex (PER) to increasing sucrose concentrations (Page et al., 2006; Scheiner et al., 2004). Nectar foragers respond only to higher sucrose concentrations while pollen foragers respond to a broader range of diluted concentrations (Scheiner et al., 2004). Aversive sensitivity in bees can be also estimated through responsiveness to a series of increasing voltages (Roussel et al., 2009; Tedjakumala et al., 2014). The response provided by harnessed bees subjected to these electric stimulations is the sting extension reflex (SER) (Núñez et al., 1983). Guards exhibit a lower responsiveness to a series of voltages compared to foragers, owing to their higher tolerance to potentially noxious stimulations (Roussel et al., 2009).

Stimulus sensitivity directly affects learning and memory as animals learn better about stimuli for which they are highly sensitivity. In appetitive conditioning protocols in which harnessed bees learn to associate an olfactory or a tactile stimulus as the conditioned stimulus (CS) with sucrose reward as the unconditioned stimulus (US), bees that are more responsive to sucrose learn faster and show higher performance than less responsive bees (Scheiner et al., 2003; Scheiner et al., 1999; Scheiner et al., 2005; Scheiner et al., 2001a; Scheiner et al., 2001b), and consequently remember better the learned appetitive associations (Scheiner et al., 2005; Scheiner et al., 2004). In aversive learning, in which honey bees learn to associate an odor (CS) with a mild electric shock (US) and exhibit afterwards SER to the odor that was learned as a predictor of the shock (Giurfa et al., 2009; Tedjakumala and Giurfa, 2013; Vergoz et al., 2007), foragers that are more responsive to the shock learn and memorize better the aversive associations (Roussel et al., 2009).

Although the direct influence of stimulus sensitivity on learning and memory performances has been well documented, if and how learning and memory retrieval affect subsequent stimulus sensitivity is less well understood. Here we asked whether learning odor-shock associations induces long-lasting changes in shock sensitivity. The repeated experience with the shock may render bees either more sensitive to it, or, on the contrary, less sensitive because of a decrease in the surprising effect of the shock underlying learning (Rescorla and Wagner, 1972). To answer this question we quantified shock responsiveness to a series of increasing voltages and then trained bees using a differential conditioning protocol, in which they had to learn to associate an odor (CS+) with a shock and a different odor (CS-) with the absence of shock (Vergoz et al., 2007). To determine whether this learning affects long-term shock sensitivity, responsiveness to a series of increasing voltages was quantified again, three days after conditioning.

To quantify shock responsiveness, we stimulated harnessed bees with the following voltages, applied in ascending order: 0.25, 0.5, 1, 2, 4 and 8 V (Roussel et al., 2009; Tedjakumala et al., 2014). Figure 1A shows a typical increase in responsiveness with increasing voltages (Roussel et al., 2009; Tedjakumala et al., 2014) (ANOVA for repeated measurements, $F_{5,195} = 50.99$, $p < 0.001$), which reached 100% at 8 V. Four hours after quantifying shock responsiveness, bees were subjected to differential SER conditioning. Bees were trained to discriminate 1-nonanol (CS+), which was paired with a 7.5 V electric shock as US, from 1-hexanal which was not paired with the shock (CS-). Each bee acted as its own control as it had to learn to respond to the CS+ and not to the CS-. Four CS+ and four CS- trials were presented in pseudo-random order. Individuals showing more responses to the CS+ than to the CS- during the training were considered as 'learners' while those showing more response to the CS- were considered as 'non-learners'(Roussel et al., 2010). Memory retrieval was assessed three days after conditioning, a post-training delay which corresponds to the presence of a long-term memory dependent on protein-synthesis (Giurfa et al., 2009). To this end, bees were presented with the CS+ and the CS- in a sequence that varied randomly from bee to bee, and without shock. While learners discriminated the CS+ from the CS- at the end of conditioning (Figure 1B; ANOVA for repeated measurements, $F_{1,19} = 54.37$, $p < 0.001$), non-learners did not master this discrimination despite having been submitted to the same conditioning protocol (Figure 1C; ANOVA for repeated measurements, $F_{1,19} = 0.19$, $p = 0.67$ NS). Memory retrieval was significant in the case of learners, which responded more to the CS+ than to the CS- in the absence of reinforcement (McNemar's test, $\chi^2 = 6.75$, $p < 0.01$). Their performance in the

test reached the same level as in the last conditioning trial performed three days before (Figure 1C; ANOVA for repeated measurements, $F_{1,19} = 2.92$, $p = 0.1$ NS). In the case of non-learners, no retrieval was observed ($\chi^2 = 48$, $p = 0.5$ NS).

Shock responsiveness of both learners (Figure 1D) and non-learners (Figure 1E) was measured immediately after the retrieval test. Learners exhibited a long-term significant decrease in shock sensitivity with respect to their original responsiveness (Figure 1D; ANOVA for repeated measurements, $F_{1,19} = 9.81$, $p < 0.01$). Non-learners, on the contrary, did not show any change in shock responsiveness (Figure 1E; ANOVA for repeated measurements, $F_{1,19} = 0.96$, $p = 0.34$ NS) despite having experienced the same conditioning protocol, and thus the same sensory stimulations. This result thus shows that the acquisition and/or retrieval of odor-shock associations induced a significant decrease of shock responsiveness.

Changes in responsiveness may reflect circadian variations in stimulus sensitivity. It may thus be possible that the variation found in learners was due to the fact that the original shock responsiveness was measured in the morning while the post-conditioning shock responsiveness was measured during the evening of the 3rd day after training. We thus conducted a control experiment in which the first two phases (measure of original shock responsiveness and differential aversive conditioning) were identical to those of the previous experiment but the third phase (measure of post-conditioning shock responsiveness) did not occur immediately after the retention test but 18 h after it, i.e. during the morning of the 4th day after training. In this way, shock responsiveness before and after conditioning was measured exactly during the same temporal window.

As in the previous experiment, bees showed an increase in responsiveness with increasing voltages (Figure S1A; ANOVA for repeated measurements, $F_{5,195} = 61.14$, $p < 0.001$). Learners were able to discriminate the CS+ from the CS- at the end of conditioning (Figure S1B; ANOVA for repeated measurements, $F_{1,19} = 77.07$, $p < 0.001$) while non-learners did not (Figure S1C; ANOVA for repeated measurements, $F_{1,19} = 0.001$, $p = 0.97$ NS). Three days after conditioning, memory retrieval was significant for learners (McNemar's test, $\chi^2 = 11.08$, $p < 0.001$) but not for non-learners (McNemar's test, $\chi^2 = 0.5$, $p = 0.48$ NS). When shock responsiveness was re-measured in the morning of the 4th day, we found again that learners exhibited a long-term significant decrease in shock sensitivity (Figure S1D; ANOVA for repeated measurements, $F_{1,19} = 19.94$, $p < 0.001$), whilst non-learners did not exhibit any change in sensitivity (Figure S1E; ANOVA for

repeated measurements, $F_{1,19} = 1$, $p = 0.33$ NS). This result thus confirms that aversive learning induces a long-term, stable decrease in shock sensitivity that was not subjected to circadian variations.

We then asked whether the modification of aversive responsiveness induced by aversive conditioning is specific for aversive stimulation and SER or is non-specific and translates to appetitive responsiveness and PER. To answer this question, we first quantified sucrose responsiveness (PER) by stimulating harnessed bees with a series of increasing sucrose concentrations (0.1%, 0.3%, 1%, 3%, 10% and 30% (Pankiw and Page, 1999)). Four hours later, we subjected the bees to differential SER conditioning and three days later we performed an aversive-memory retrieval test as in the previous experiments. Finally, immediately after the retrieval test, we measured sucrose responsiveness again to determine whether aversive conditioning induced a long-term decrease in sucrose sensitivity as in the case of shock sensitivity.

Figure S2A shows that naïve bees exhibited a typical increase of appetitive responsiveness with increasing sucrose concentration ($F_{5,195} = 51.5$, $p < 0.001$. During aversive conditioning, learners mastered the discrimination between the conditioned odorants (Figure S2B: $F_{1,19} = 61.69$, $p < 0.001$) while non-learners did not discriminate them (Figure S2C: $F_{1,19} = 2.92$, $p = 0.1$ NS). Three days later, memory retrieval was significant in the case of learners ($\chi^2 = 6.75$, $p < 0.01$) but not in the case of non-learners ($\chi^2 = 0$, $p = 1$, NS). Contrarily to what happened in the case of shock responsiveness, sucrose responsiveness did not change in learners with respect to their original responsiveness (Figure S2D: $F_{1,19} = 0.6$, $p = 0.45$ NS). Non-learners did also not modify their sucrose responsiveness (Figure S2E: $F_{1,19} = 0.02$, $p = 0.88$ NS). These results thus show that the acquisition and/or retrieval of aversive odor-shock associations did not induce any change in appetitive responsiveness. The decrease of aversive responsiveness induced by aversive conditioning is thus specific to the aversive modality and does not translate to other forms of unrelated responsiveness.

Aversive learning induces an Increase of Receptor-Gene Expression in Specific Kenyon Cell Populations of the Mushroom Bodies

We then searched for molecular mechanisms underlying the learning-dependent decrease in shock responsiveness. Dopaminergic signaling is particularly relevant in this context as

pharmacological experiments have shown that it down regulates shock responsiveness (Tedjakumala et al., 2014). Three dopaminergic receptors have been identified in the honey bee: AmDOP1 (Blenau et al., 1998; Mustard et al., 2003), AmDOP2 (Humphries et al., 2003; Mustard et al., 2003) and AmDOP3 (Beggs et al., 2005). We aimed at determining whether aversive learning and retrieval modify the expression levels of these dopaminergic receptor genes in the Kenyon cells, the constitutive neurons of the mushroom bodies. Mushroom bodies have proved to play a fundamental role for aversive learning and memory in the fruit fly *Drosophila melanogaster* (Busto et al., 2010; Davis, 2005, 2011; Heisenberg, 2003; Keene and Waddell, 2007). Furthermore, as dopamine can also activate a dopamine/ecdysteroid receptor in the fruit fly (Srivastava et al., 2005), and a similar receptor exists in the bee (AmGPCR19), which modulates aversive learning (Geddes et al., 2013), we also focused on *Amgpcr19* expression. Finally, we quantified the expression of the octopaminergic receptor gene *Amoa1* (Grohmann et al., 2003) knowing that octopaminergic signaling is dispensable both for aversive learning (Vergoz et al., 2007) and for the unconditioned sting extension response to electric shock (Tedjakumala et al., 2014).

We trained bees following the same differential conditioning protocol used in the previous section. As before, we distinguished between learners and non-learners, and we measured retrieval three days after conditioning. Figure S3 shows the learning and retention performances of learners and non-learners. While learners discriminated the CS+ from the CS- at the end of conditioning (Figure S3A; $F_{1,16} = 58.18$, $p < 0.001$), non-learners did not master this discrimination despite having been submitted to the same conditioning protocol (Figure S3B; $F_{1,17} = 3.4$, $p = 0.08$ NS). Memory retrieval was significant in the case of learners ($\chi^2 = 4.17$; $p < 0.05$). A significant decrease of responses was nevertheless observed between the last CS+ training trial and the CS+ test ($F_{1,19} = 3.4$, $p < 0.05$) showing that retrieval was not possible in all learners. In the case of non-learners, retrieval was not significant despite a tendency to respond more to the CS+ than to the CS- in some bees ($\chi^2 = 3.2$; $p = 0.07$).

Immediately after the retention test, at the time at which changes in shock responsiveness are detectable in learners (see Fig. 1D), brains were extracted and placed in Tissue Tek (Sakura) and frozen on dry ice. Ten learner brains were collected for further analyses. Five of these brains belonged to bees that showed perfect retrieval and thus responded to the CS+ and not to the CS-; the other five belonged to bees that did neither respond to the CS+ nor to the CS-. Ten non-learners were collected in the same way.

We used laser-capture microdissection to sample the inner (ICC) and non-compact Kenyon cells (NCC) of the mushroom bodies, whose somata are located within the calyx (Farris et al., 2004), the cup-shaped structure of the mushroom bodies. Serial sections (12 mm) were taken from areas of the brain in which the two populations of Kenyon cells were most clearly apparent (McQuillan et al., 2012). RNA was isolated from the cells and real-time quantitative PCR (RT-qPCR) was used to determine levels of expression of the receptor genes of interest in the two subpopulations of Kenyon cells as depending on learning/retention.

We determined the efficiency of several housekeeping genes (HKGs) as references for potential changes in receptor gene expression. Their stability was evaluated by Best-Keeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004). Normfinder suggested various HKGs combinations providing a robust internal control. We chose the combination of ef1a and Rps8 as it ensured the most stable reference for the various factors of analyses. We then compared expression levels of the receptor genes of interest, *Amdop1*, *Amdop2*, *Amdop3*, *Amoa1* and *Amgpcr19,* both in IC and NC Kenyon cells of learners and non-learners (Figure 2). Within each of these two categories of bees, we pooled the expression data irrespectively of retention performances in order to access the impact of learning itself.

Amdop2 was significantly more expressed in learners both in IC and in NC Kenyon cells (Figure 2B; $F_{1,18} = 10.23$, $p < 0.01$). On the contrary, no significant difference in *Amdop2* expression was found between cell types ($F_{1,18} = 0.51$, $p = 0.48$ NS). The interaction between learning performance and Kenyon cell subpopulation was also non significant ($F_{1,18} = 0.05$, $p = 0.83$ NS), thus showing that the increase of *Amdop2* was generalized and similar for both cell populations. Post-hoc analyses showed that within each cell type, the expression of *Amdop2* increased significantly in learners but not in non-learners (ICC and NCC: Fisher's LSD, $p < 0.05$ for both comparisons). In contrast, no significant variation was found for *Amdop3* whose levels were unaffected by learning (Figure 2C; $F_{1,18} = 0.67$, $p = 0.42$ NS). Differences in the expression of *Amdop3* were evident between IC and NC Kenyon cells ($F_{1,18} = 19.46$, $p < 0.001$) but these were independent of learning. Also no significant interaction between learning performance and Kenyon cell subpopulation was found ($F_{1,18} = 0.12$, $p = 0.74$ NS). Finally, *Amdop1* yielded an intermediate pattern of results when compared to *Amdop2* and *Amdop3* (Figure 2A); although the levels of *Amdop1* increased in learners with respect to non-learners, this variation was non-significant ($F_{1,18} = 3.54$, $p = 0.08$ NS). Specifically, no differences in

gene expression were found in NC Kenyon cells depending on learning performance whilst a non-significant increase was found in IC Kenyon cells. Furthermore, neither a significant difference in *Amdop1* expression between cell types ($F_{1,18}$ = 2.14, p = 0.16 NS) nor an interaction between learning performance and cell type was detectable ($F_{1,18}$ = 0.08, p = 0.78 NS).

In the case of *Amgpcr19* (Figure 2D) and *Amoa1* (Figure 2E), no significant differences between learners and non-learners were found (*Amgpcr19*: $F_{1,18}$ = 1.66, p = 0.21 NS; *Amoa1*: $F_{1,18}$ = 2.4, p = 0.14 NS). Variations in gene expression between IC and NC Kenyon cells existed for both genes (*Amgpcr19*: $F_{1,18}$ = 5.84, p < 0.05; *Amoa1*: $F_{1,18}$ = 90.14, p < 0.001) but these differences were independent of learning success. The interaction between learning performance and Kenyon cell subpopulation was non-significant both for *Amgpcr19* ($F_{1,18}$ = 0.24, p = 0.63 NS) and *Amoa1* ($F_{1,18}$ = 2.49, p = 0.13 NS).

We then determined whether the expression levels of the receptor genes *Amdop1*, *Amdop2*, *Amdop3*, *Amoa1* and *Amgpcr19* in IC and NC Kenyon cells was related with the retrieval performance of bees. To this end, we distinguished between bees that exhibited significant retrieval three days after conditioning and bees without retrieval. For all receptor genes considered, no significant variation in expression was found as related to retrieval success (Fig. 3; *Amdop1*: $F_{1,18}$ = 0.94, p = 0.34 NS; *Amdop2*: $F_{1,18}$ = 1.53, p = 0.23 NS; *Amdop3*: $F_{1,18}$ = 0.03, p = 0.87 NS; *Amgpcr19*: $F_{1,18}$ = 0.006, p = 0.94 NS; *Amoa1*: $F_{1,18}$ = 0.5, p = 0.49 NS). Similarly, no significant interaction between cell-type and retrieval performance was detected (*Amdop1*: $F_{1,18}$ = 1.19, p = 0.29 NS; *Amdop2*: $F_{1,18}$ = 0.28, p = 0.6 NS; *Amdop3*: $F_{1,18}$ = 0.24, p = 0.63 NS; *Amgpcr19*: $F_{1,18}$ = 0.3, p = 0.59 NS; *Amoa1*: $F_{1,18}$ = 0.72, p = 0.41 NS). Only a significant variation was found for the factor cell-type in three receptor genes (*Amdop3*: $F_{1,18}$ = 19.59, p < 0.001; *Amgpcr19*: $F_{1,18}$ = 5.86, p < 0.05; *Amoa1*: $F_{1,18}$ = 82.33, p < 0.001) but not in the other two (*Amdop1*: $F_{1,18}$ = 2.28, p = 0.15 NS; *Amdop2*: $F_{1,18}$ = 0.51, p = 0.48 NS). This difference reveals that IC and NC Kenyon cells are heterogeneous with respect to receptor-gene expression but this difference was independent of retrieval success.

Taken together, our results show that learning promoted a long-term, specific increase in the expression of some dopaminergic receptor genes (*Amdop2*, and partially *Amdop1*) but that this increase was not related to retrieval success itself.

Discussion

We quantified aversive responsiveness of honey bees before and after aversive olfactory learning and showed for the first time that learning induces a long-term modification of aversive responsiveness as bees become less sensitive to the shock after conditioning. We show that a possible molecular mechanism underlying this variation in aversive responsiveness is a long-lasting learning-dependent increase of the dopaminergic receptor gene *Amdop2* and partially of another dopaminergic receptor *Amdop1* in Kenyon cell populations of the mushroom bodies. Our results thus provide the first account on changes in receptor genes in the mushroom bodies following associative learning in insects and relate these changes with a learning-induced variation in reinforcement responsiveness.

Aversive learning induces long-term changes in shock responsiveness

Innate responsiveness to biologically relevant stimuli such as sucrose reward has been shown to vary in honey bees as depending on a broad spectrum of factors such as caste, patriline, age, season, nutrition and foraging experience (see Scheiner et al., 2004 for review). Here we add associative learning to the factors that may induce a significant change in reinforcement responsiveness. Aversive olfactory learning determined a long-term decrease in shock responsiveness observable three day after conditioning. This decrease was not due to sensory adaptation or fatigue as it was observed three days after conditioning with no shock experience in between. It was also not due to circadian variations of responsiveness as testing learners in different temporal windows yielded the same result, i.e. a long-term and stable decrease in shock responsiveness. Moreover it was specific of the aversive modality as aversive conditioning had no impact on appetitive sucrose responsiveness measured through PER to increasing sucrose concentrations.

The decrease of shock responsiveness was explicitly determined by the learning of odorant-shock associations as bees which had the same sensory experience with odorants and electric shocks but which did not learn the discrimination did not exhibit a decrease of shock responsiveness. The decrease was particularly visible for low and intermediate voltages and may thus reflect the acquisition of predictable expectations concerning the higher voltage used during conditioning (7.5 V). As a consequence, responses to lower voltages are decreased as these stimuli become less relevant and thus less effective for eliciting SER. In other words, the consequence of learning would be to render the bees more selective in their responses to voltages. Future work should determine whether

protocols of appetitive conditioning induce a comparable long-term decrease in appetitive (but not aversive) responsiveness to food rewards whose intensity is lower than that used for conditioning.

Aversive learning induces long-term changes dopaminergic-receptor expression in the mushroom bodies of the honey bee brain

Pharmacological blockade of dopaminergic signaling in the bee brain via the dopaminergic antagonist flupenthixol induces a short-term (30 min after drug injection) increase in shock responsiveness (Tedjakumala et al., 2014). It was therefore suggested that the dopaminergic system acts as a "general gain control system" mediating attention and adaptive responding to appropriate stimuli in the insect's environment and the filtering out of stimuli that are less relevant (Tedjakumala et al., 2014). Attentional processes, similar to those described in vertebrates, can also be identified in insects (Dyer and Chittka, 2004; Giurfa, 2004; Miller et al., 2011; van Swinderen, 2011; Van Swinderen and Andretic, 2011a). Current views relate dopamine levels in the insect brain with arousal levels (Van Swinderen and Andretic, 2011b). Thus, dopaminergic neurons may modulate selective attention in the insect brain, acting on a series of nervous circuits underlying different forms of sensory-motor performances.

We focused on dopaminergic receptor-gene expression in the mushroom bodies as these structures have been related to long-term memory formation, storage and retrieval (Busto et al., 2010; Davis, 2005, 2011; Giurfa, 2003; Heisenberg, 2003; Keene and Waddell, 2007; Menzel, 1999, 2001) and to attentional processes mediated by dopaminergic signaling (Andretic et al., 2005; Miller et al., 2011; van Swinderen and Greenspan, 2003). We performed a cell-population specific analysis differentiating between inner compact (ICC) and non-compact Kenyon cells (NCC), whose cell bodies are located within the mushroom-body calyces (Farris et al., 2004) and which show heterogeneous aminergic receptor-gene expression during the lifetime of the bee (McQuillan et al., 2012).

We found that aversive learning, but not long-term memory retrieval, induced significant variations of receptor-gene expression in Kenyon cells. As not all learners retrieved the aversive memory and some non-learners answered during the retrieval test as if they had formed such memory, no significant relationship was found between receptor-

gene expression and retrieval success. On the contrary, the relationship between receptor-gene expression and learning success was highly significant for *Amdop2,* and close to significance for *Amdop1*. Thus, two of the three dopaminergic receptor genes known in the honey bee exhibited a long-term increase in expression following aversive conditioning, which was concomitant of the significant long-term decrease in shock responsiveness induced by such conditioning. Neither *Amdop3* nor *Amgpcr* could be related with the decrease of shock sensitivity induced by aversive learning, thus indicating a potential different role for their corresponding receptors in the bee brain. *Oam1,* which has been related to appetitive sucrose signaling (Farooqui et al., 2003), was also unaffected as expected from the use of an aversive protocol for which octopaminergic signaling is dispensable (Vergoz et al., 2007). This absence of variation in *Oam1* expression may explain the fact that appetitive responsiveness to sucrose solution was unaffected by aversive conditioning.

Aversive learning thus triggers processes leading to a stable increase in dopamine receptor-gene expression which serves as the potential basis for the specific modulation of aversive responsiveness. Whether appetitive learning induces in turn a stable increase in the expression of the octopaminergic receptor gene *Oam1* remains to be determined.

The three receptor genes for which no changes in expression were found, *Amdop3, Amgpcr19 and Amoa1*, exhibited nevertheless significant variations in expression with respect to the two cell populations considered, ICC and NCC. While *Amgpcr19* and *Amoa1* were significantly more expressed in ICC than in NCC, *Amdop3* showed the reversed trend, thus suggesting a different pattern of action at the level of the mushroom bodies. These results confirm the heterogeneity of Kenyon cell populations with respect to receptor expression and signaling (McQuillan et al., 2012) and also indicate that the two dopamine-related receptor genes *Amdop3* and *Amgpcr19* play a signaling role different from that of *Amdop2* and eventually *Amdop1*. Firstly, activation of AmDOP3 receptors results in down regulation of intracellular levels of cAMP, a property characteristic of D2-like dopamine receptors, while activation of AmDOP1 and AmDOP2 receptors up-regulates intracellular levels of cAMP, thus rendering them more similar to D1 dopamine receptors (Beggs et al., 2005). Thus, the two receptor genes that increased their expression levels after aversive conditioning were the D1-like receptor genes, which both activate the cAMP signaling pathway, and potentially lead to protein synthesis necessary for stabilizing long-term changes in the bee brain (Menzel, 1999). Secondly, *Amdop3* is widely expressed in the brain but shows a pattern of expression that differs from that of either *Amdop1* or *Amdop2*.

While the latter intervene in the modulation of aversive responsiveness, consistently with the suggestion of a dopaminergic system acting as gain control system (Tedjakumala et al., 2014), *Amdop3* could participate in instructive signaling and thus mediate the reinforcing properties of the electric shock during conditioning.

Mechanisms of dopaminergic receptor-gene up-regulation

In vertebrates, learning and long-term memory are associated with temporally defined changes in gene expression that lead to the strengthening of synaptic connections in selected brain regions. In rats trained in a spatial discrimination-learning protocol, 19 genes that showed statistically significant changes in expression were found in specific cell populations of the hippocampus when comparing trained and naive animals (Robles et al., 2003). Also spatial learning in the Morris water maze determines distinct temporal gene expression profiles in the hippocampus associated with learning and memory (Cavallaro et al., 2002).

Up-regulation of dopaminergic receptor genes may be linked to molecular plasticity events triggered by learning. In this scenario increased activity in dopaminergic circuits involved in associative aversive learning may result in an increased number and weight of synaptic connections in these circuits, and thus in an increased number of the dopaminergic receptors and receptor genes participating in these circuits.

Conclusion

Our results thus show that aversive learning induces a specific increase of dopaminergic gene expression, giving rise to specific alterations in the sensitivity to environmental noxious stimulations. Bees became less reactive to lower voltages, based on their predictive experience with a shock of a higher voltage. Learning has thus profound effects on gene expression in the brain and on how reinforcing stimuli are subsequently evaluated.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and two figures.

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Figures

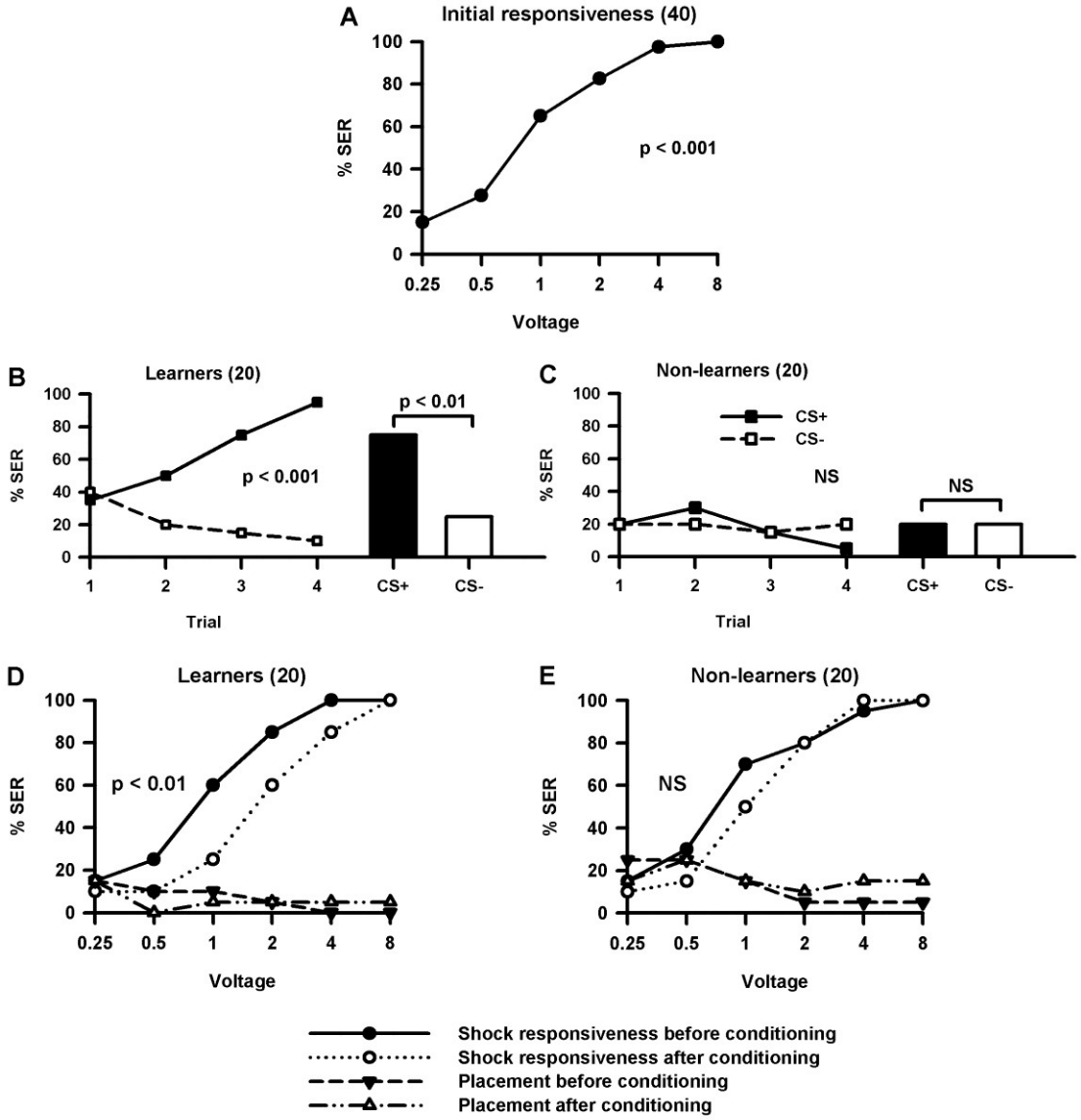


Figure 1. Learning-related modulation of shock responsiveness through aversive conditioning

(A) Shock responsiveness curve showing a typical SER response to increasing voltages ($F_{5,195} = 50.99$, $p < 0.001$). **(B)** During olfactory aversive conditioning, learners were able to discriminate the CS+ from the CS- ($F_{1,19} = 54.37$, $p < 0.001$). Three days after conditioning, learners responded significantly more to the CS+ during the retrieval test ($\chi^2 = 6.75$, $p < 0.01$). **(C)** During olfactory aversive conditioning, non-learners were not able to discriminate the CS+ from the CS- ($F_{1,19} = 2.92$, $p = 0.1$ NS). Three days after conditioning, non-learners did not respond to the conditioned odors during the retrieval test ($\chi^2 = 48$, $p = 0.5$ NS). **(D)** A significant decrease of shock responsiveness was evident in learners ($F_{1,19} = 9.81$, $p < 0.01$). **(E)** No significant variation of shock responsiveness was found in the case of non-learners ($F_{1,19} = 0.96$, $p = 0.34$ NS). Sample sizes are presented in parentheses.

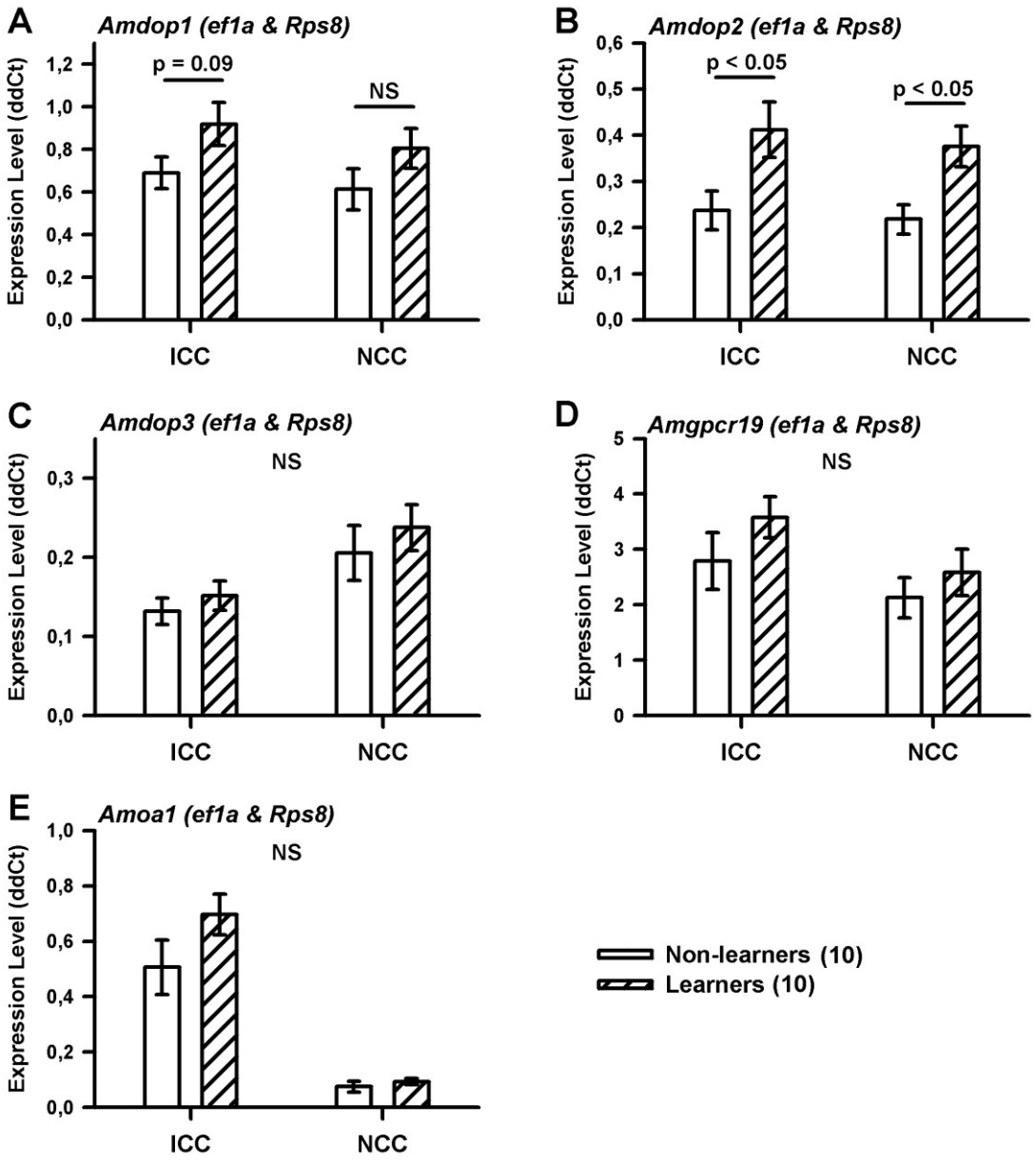

A
Amdop1 (ef1a & Rps8)
p = 0.09
NS
Expression Level (ddCt)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
ICC
NCC
B
Amdop2 (ef1a & Rps8)
p < 0.05
p < 0.05
Expression Level (ddCt)
0,6
0,5
0,4
0,3
0,2
0,1
0,0
ICC
NCC
C
Amdop3 (ef1a & Rps8)
NS
Expression Level (ddCt)
0,3
0,2
0,1
0,0
ICC
NCC
D
Amgpcr19 (ef1a & Rps8)
NS
Expression Level (ddCt)
5
4
3
2
1
0
ICC
NCC
E
Amoa1 (ef1a & Rps8)
NS
Expression Level (ddCt)
1,0
0,8
0,6
0,4
0,2
0,0
ICC
NCC
Non-learners (10)
Learners (10)

Figure 2. Normalized expression levels of receptor genes in ICC and NCC Kenyon cells of learners ad non-learners

(A) *Amdop1* shows a marginally non-significant increase in learners with respect to non-learners ($F_{1,18} = 3.55$, $p = 0.08$ NS); no difference was found between cell types ($F = 2.14$ $p = 0.16$ NS) and no significant interaction was observable ($F_{1,18} = 0.08$, $p = 0.78$ NS). **(B)** *Amdop2* shows a significant increase in learners with respect to non-learners ($F_{1,18} = 10.23$, $p < 0.01$). No difference was found between cell types ($F = 0.51$ $p = 0.48$ NS). No significant interaction was observable ($F_{1,18} = 0.05$, $p = 0.82$ NS). *Post hoc* analyses detected a significant increase of *Amdop2* expression both ICC and NCC ($p < 0.05$). **(C)** *Amdop3* expression did not vary significantly between learners and non-learners ($F_{1,18} = 0.67$, $p = 0.42$ NS). A significant difference was found between cell types ($F = 19.46$ $p < 0.001$) while no significant interaction was detected ($F_{1,18} = 0.12$, $p = 0.74$ NS). **(D)** *Amgpcr19* expression did not vary significantly between learners and non-learners ($F_{1,18} = 1.66$, $p = 0.21$ NS). A significant difference was found between cell types ($F = 5.84$ $p < 0.05$) while no significant interaction was detected ($F_{1,18} = 0.24$, $p = 0.63$ NS). **(E)** *Amoa1* expression did not vary significantly between learners and non-learners ($F_{1,18} = 2.4$, $p = 0.14$ NS). A significant difference was found between cell types ($F = 90.14$ $p < 0.001$) while no significant interaction was found ($F_{1,18} = 2.49$, $p = 0.13$). Bars show means ± S.E.; sample sizes are shown in parentheses.

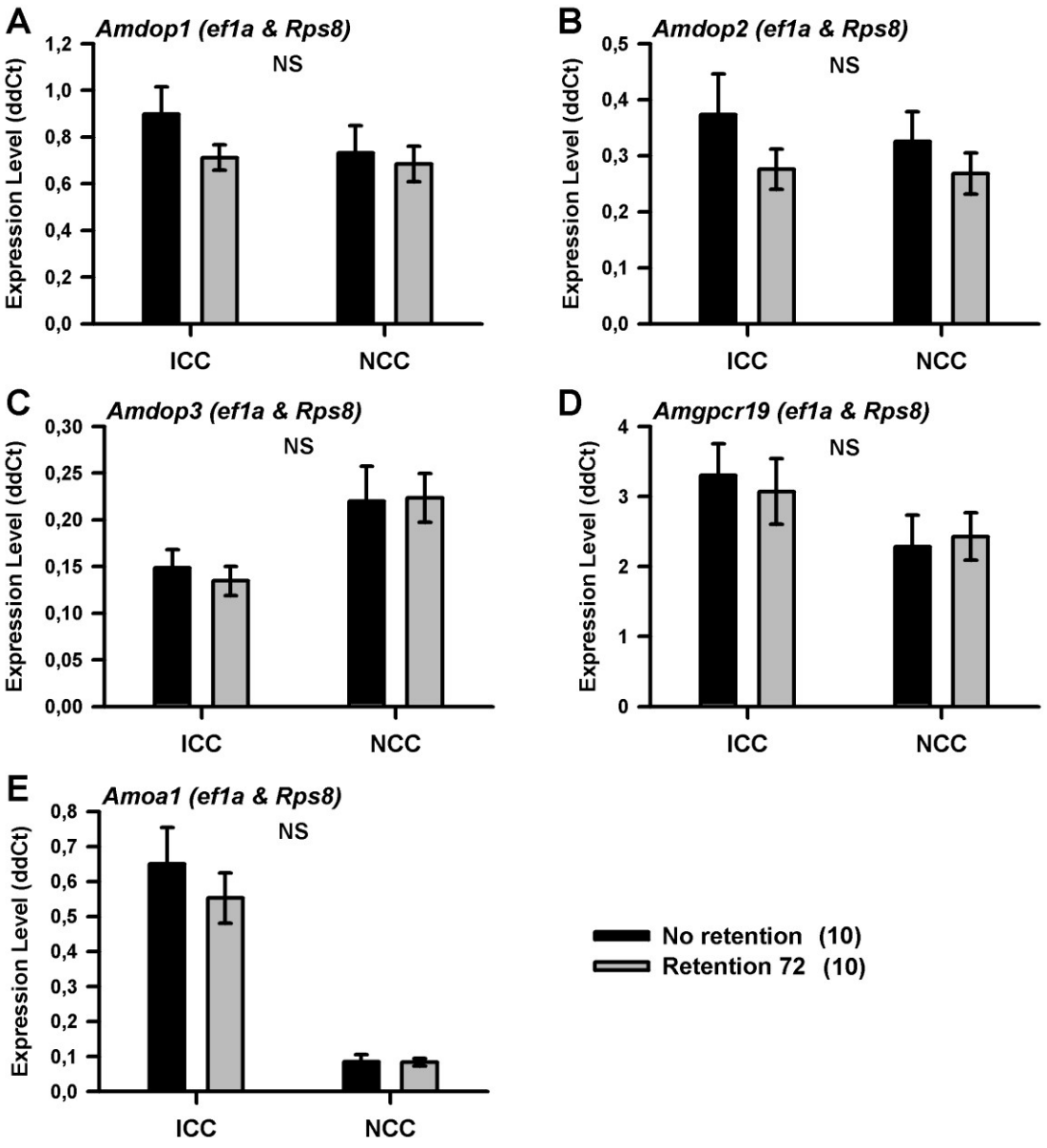

A
Amdop1 (ef1a & Rps8)
NS
Expression Level (ddCt)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
ICC
NCC
B
Amdop2 (ef1a & Rps8)
NS
Expression Level (ddCt)
0,5
0,4
0,3
0,2
0,1
0,0
ICC
NCC
C
Amdop3 (ef1a & Rps8)
NS
Expression Level (ddCt)
0,30
0,25
0,20
0,15
0,10
0,05
0,00
ICC
NCC
D
Amgpcr19 (ef1a & Rps8)
NS
Expression Level (ddCt)
4
3
2
1
0
ICC
NCC
E
Amoa1 (ef1a & Rps8)
NS
Expression Level (ddCt)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
ICC
NCC
No retention (10)
Retention 72 (10)

Figure 3. Normalized expression levels of receptor genes in ICC and NCC Kenyon cells of bees showing successful and non-successful retrieval

(A) *Amdop1* expression did not vary significantly between bees with successful and bees with non-successful retrieval ($F_{1,18}$ = 0.94, p = 0.34 NS). No significant difference was found between cell types ($F_{1,18}$ = 2.28, p = 0.15 NS). No significant interaction was found ($F_{1,18}$ = 1.19, p = 0.29 NS). **(B)** *Amdop2* expression did not vary significantly between bees with successful and bees with non-successful retrieval ($F_{1,18}$ = 1.53, p = 0.23 NS). No significant difference between cell types was detected ($F_{1,18}$ = 0.51, p = 0.48 NS). No significant interaction was found ($F_{1,18}$ = 0.28, p = 0.6 NS). **(C)** *Amdop3* expression did not vary significantly between bees with successful and bees with non-successful retrieval ($F_{1,18}$ = 0.03, p = 0.87 NS). A significant difference in expression was found between cell types ($F_{1,18}$ = 19.59, p < 0.001) while no significant interaction was found ($F_{1,18}$ = 0.24, p = 0.63 NS). **(D)** *Amgpcr19* expression did not vary significantly between bees with successful and bees with non-successful retrieval ($F_{1,18}$ = 0.006, p = 0.94 NS). A significant difference in expression was found between cell types ($F_{1,18}$ = 5.86, p < 0.05) while no significant interaction was found ($F_{1,18}$ = 0.3, p = 0.59 NS). **(E)** *Amoa1* expression did not vary significantly between bees with successful and bees with non-successful retrieval ($F_{1,18}$ = 0.5, p = 0.49 NS). A significant difference in expression was found between cell types ($F_{1,18}$ = 82.33, p < 0.001) while no significant interaction was observable ($F_{1,18}$ = 0.72, p = 0.41 NS). Bars show means ± S.E. Sample sizes are shown in parentheses.

Supplemental Information

Supplemental Experimental Procedures

Insects

Honey bees, *Apis mellifera*, were obtained from outdoor colonies. Nectar foragers were collected from an artificial feeder to which they were previously trained. Nectar foragers were used because of their higher shock responsiveness (Roussel et al., 2009).

Once captured, the bees were chilled on ice for 5 minutes until they stopped moving. They were then harnessed on individual holders designed for aversive stimulation via delivery of an electric shock (Carcaud et al., 2009; Giurfa et al., 2009; Vergoz et al., 2007). Holders consisted of two brass plates fixed to a Plexiglas plate. Brass plates were connected to the output of the stimulator (60 Hz – AC current). The resistance measured between the two plates in the presence of the bee was 200-300 KΩ. Conductance gel was applied below the thorax to ensure efficient shock delivery. Low melting-point wax was used to immobilize the head and facilitate drug injection. Once fixed, each bee was fed with a droplet (5 µl) of sucrose solution 30% and kept resting until aversive responsiveness measurements.

Shock responsiveness

Bees were stimulated with increasing voltages and the occurrence of a SER was recorded upon each stimulation. The following voltages were applied in ascending order: 0.25, 0.5, 1, 2, 4 and 8 V. By alternating between a non-shocked (placement) and a shocked phase every 5 minutes, each individual was given, in this way, a 10-minute shock interval. Each shock trial lasted 20 s; it consisted of 10 s of familiarization in the setup, followed by 2 s of electric shock; afterwards, the bee stayed for another 8 s before being replaced by the next test subject. Placement trials, in which the bee was placed in the setup during 20 s without shock, were interspersed between shock trials to avoid sensitization. The inter-stimulus interval was approximately 1 min.

Aversive olfactory conditioning

Bees were trained in a differential conditioning procedure to discriminate 1-nonanol (CS+) paired with a 7.5 V electric shock from 1-hexanal which was not paired with the shock (CS-). Odorants were obtained from Sigma Aldrich (Deisenhofen, Germany). Five µl of pure odorant were applied onto 1 cm^2 filter paper placed into a 20 ml syringe, thus allowing odorant delivery to the antennae. Each odorant was delivered for 5 s. An air extractor placed behind the bee prevented odorant accumulation, as well as possible contamination by pheromone release. The voltage was delivered during 2 s. The bees experienced four CS+ and four CS- trials in a pseudo-random sequence. Each conditioning trial lasted 1 min. The bee was placed in the stimulation site in front of the air extractor and left for 20 sec before being exposed to the odorant paired with the electric shock. The electric shock started 3 sec after odorant onset and finished with the odorant. The bee was then left in the setup for 35 sec and then removed. The intertrial interval (ITI) was always 10 min. Retrieval tests were performed 3 days after the last conditioning trial and consisted of presenting in a random order the CS+ and the CS- without reinforcement.

Both during the conditioning trials and the retrieval tests, SER was quantified during the presentation of the odorants (conditioned responses) and during the shock (unconditioned responses). Bees not responding to the shock were not used for the analyses. An observable sting extension was given a score of 1; incomplete sting movements were scored as 0. Sting responsiveness (% of bees responding to a given voltage) was then calculated.

Brain Preparation

After the retention test the bees were immediately sacrificed. The brain was taken out and embedded in Tissue-Tek (Sakura) to be frozen on dry ice and put into storage at -80 C until further processing. Later on, 12-µm brain sections were prepared in a cryostat (Leica). They were transferred onto 2-µm polyethylene naphthalate membrane glass slides (Leica) to be immediately frozen again for storage at -80C and ready for the following laser-capture microdissection (within the next 48h at latest).

Laser-capture microdissection

Laser capture microdissection (Leica) was applied to isolate specifically samples of Kenyon cells' subpopulation, inner compact and non-compact cells. The sections were briefly thawed to be quickly fixed in 75% ethanol (30 sec), rinsed in RNase free H2O, followed by washing in PBS (10 sec) and stained in 0.5% cresyl violet acetate in 0.1M NaAcetate buffer (5-10 sec). Afterwards, they were rinsed in RNase free H2O, dehydrated in increasing alcohol series as the following: 75% ethanol (30 sec) – 95% ethanol (30 sec) – 100% ethanol (1 min) and followed by xylene (5 min). The slides were drained and air dried for 10-15 min before proceeding directly to LCM. During all these processes RNase free materials were used.

Each sample included cells captured from between at least 10 sections of the lateral and medial mushroom body calyces of one bee. Samples of ICC and NCC were isolated from 10 bees per experimental group (balanced by selecting 5 showing 72h-memory and 5 not showing). Isolated cells were immediately frozen on dry ice, and RNA was isolated from the cells as soon as possible using a PureLink® miRNA Isolation Kit (invitrogen). RNA quality and quantity were assessed using the 2100 expert bioanalyzer, a pico assay chip, and reagents (Agilent) according to the manufacturer's protocols.

Real-time quantitative PCR

qPCR analysis was performed as previously described (Vergoz et al., 2009). Briefly, 1 ng of RNA was reverse-transcribed using VILO Supercript (Invitrogen). Gene-specific amplification products were generated using ExpressSYBR GreenER qPCR SuperMix (Invitrogen) and gene specific primer pairs (Amdop2, Amdop3, Amoa1 and Amgpcr19). Assay efficiencies were derived from standard curves generated using cDNA reverse-transcribed from a random whole-brain sample.

Receptor transcript abundances were determined using the ddCt method with assay efficiencies incorporated in the following formula: Normalized = (1 + Efficiency target,-dCt target)/(1 + Efficiency reference,-dCt reference). Using the software packages Best-Keeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004), a set reference genes were computed to look for the most stable combination. Duplicates were run of each sample.

Statistics

During the behavioral experiments, we recorded the SER to the electric-shock stimulation (aversive responsiveness experiments) or to the conditioned odorants (aversive conditioning experiments), i.e. whether bees extended their sting after the onset of the corresponding stimulation. Multiple responses during odorant presentation were counted as a single SER. The percentage of SER recorded was used to plot responsiveness/acquisition curves. Similar procedure was followed for PER in the case of appetitive responsiveness quantification. To analyze the variation of performance during trials, we used analyses of variance (ANOVAs) for repeated measurements both for between-group and for within-group comparisons. Monte Carlo studies have shown that it is permissible to use ANOVA on dichotomous data only under controlled conditions (Lunney, 1970), which are met by our experiments (equal cell frequencies and at least 40 degrees of freedom of the error term). Performances within a retention test were analyzed by means of a McNemar test.

Conditioning-related changes in receptor gene expression (ddCt values) were examined by one-way ANOVA. The analysis (Statistica, StatSoft) was conducted to compare target gene receptors' expression levels of learners against non-learners. An alpha level of 0.05 was used throughout.

Supplementary Figures

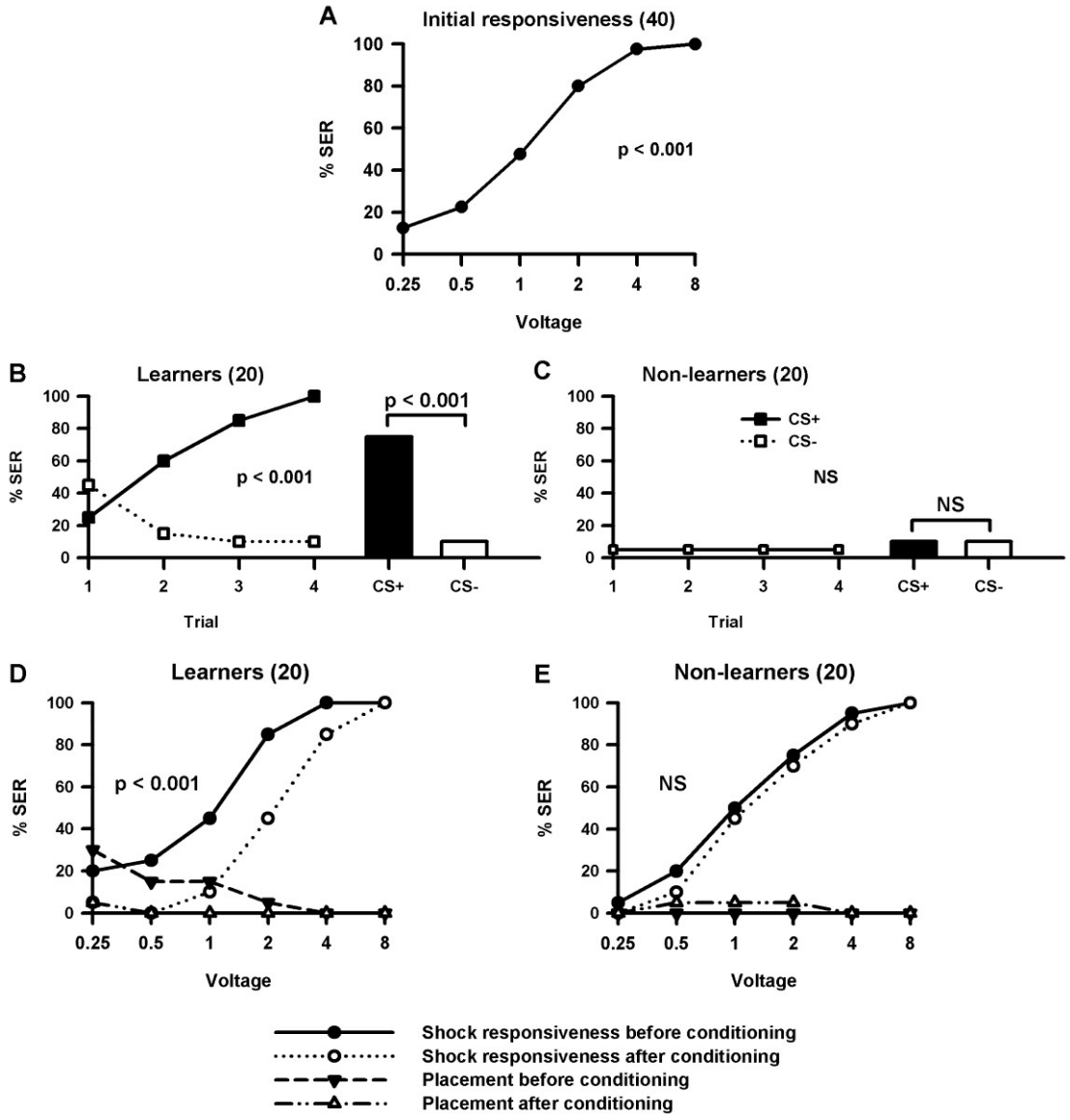


Figure S1. Learning-related modulation of shock responsiveness through aversive conditioning measured 18h after the retrieval.

(A) Shock responsiveness curve showing a typical SER response to increasing voltages ($F_{5,195} = 61.14$, $p < 0.001$). **(B)** During olfactory aversive conditioning, learners were able to discriminate the CS+ from the CS- ($F_{1,19} = 77.07$, $p < 0.001$). Three days after conditioning, learners responded significantly more to the CS+ during the retrieval test ($\chi^2 = 11.08$, $p < 0.001$). **(C)** During olfactory aversive conditioning, non-learners were not able to discriminate the CS+ from the CS- ($F_{1,19} = 0.001$, $p = 0.97$ NS). Three days after conditioning, non-learners did not respond to the conditioned odors during the retrieval test ($\chi^2 = 0.5$, $p = 0.48$ NS). **(D)** A significant decrease of shock responsiveness was evident in learners ($F_{1,19} = 19.94$, $p < 0.001$). **(E)** No significant variation of shock responsiveness was found in the case of non-learners ($F_{1,19} = 1$, $p = 0.33$ NS). Sample sizes are presented in parentheses.

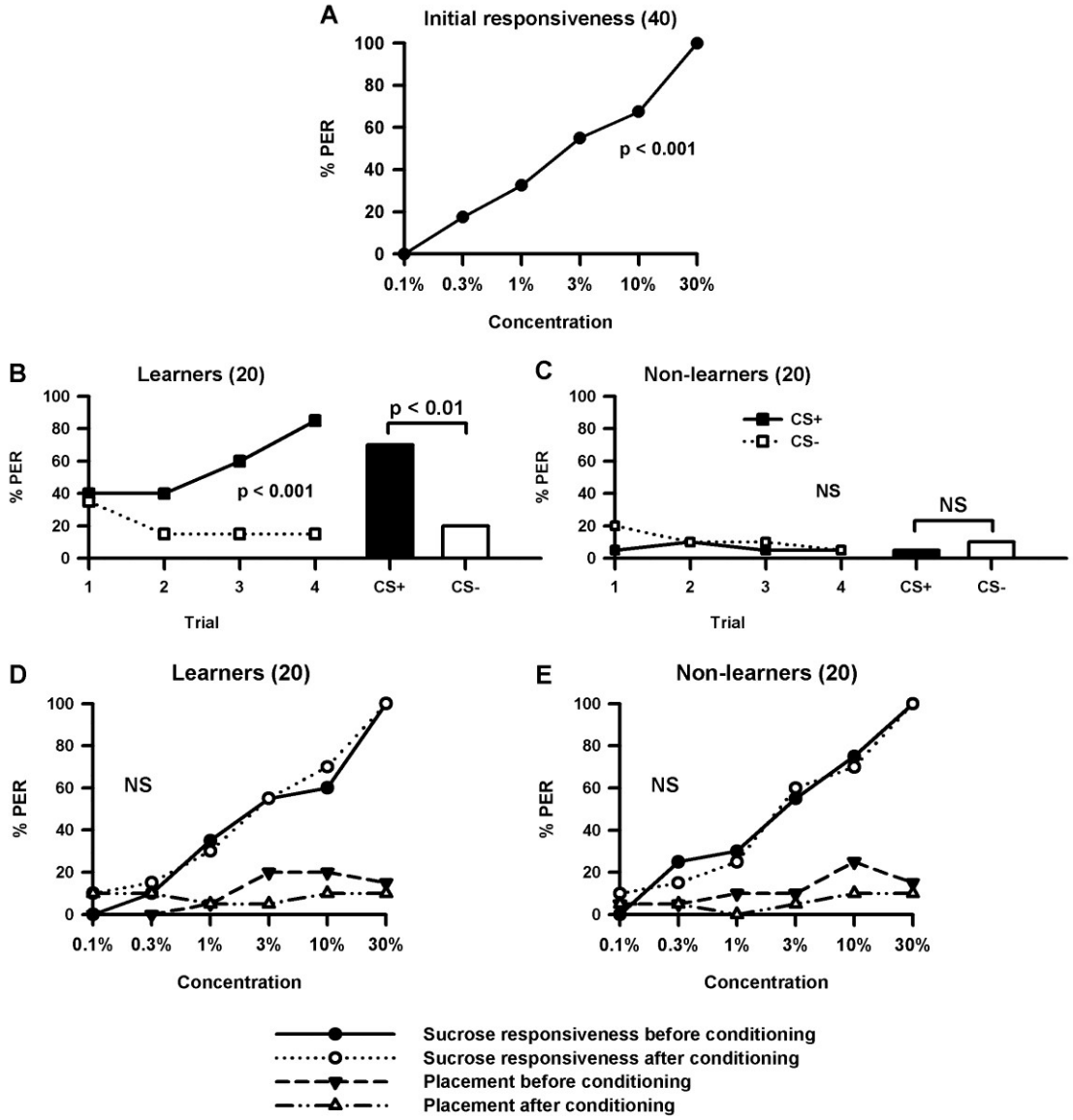


Figure S2. Absence of a learning-related modulation of sucrose responsiveness through aversive conditioning

(A) Sucrose responsiveness curve showing a typical PER increase to increasing sucrose concentrations ($F_{5,195}$ = 51.5, $p < 0.001$). **(B)** During olfactory aversive conditioning, learners were able to discriminate the CS+ from the CS- ($F_{1,19}$ = 61.69, $p < 0.001$). Three days after conditioning, learners responded significantly more to the CS+ during the retrieval test (χ^2 = 6.75, $p < 0.01$). **(C)** During olfactory aversive conditioning, non-learners were not able to discriminate the CS+ from the CS- ($F_{1,19}$ = 2.92, p = 0.1 NS). Three days after conditioning, non-learners did not respond to the conditioned odors during the retrieval test (χ^2 = 0, p = 1 NS). **(D)** No significant variation of sucrose responsiveness was found in the case of learners ($F_{1,19}$ = 0.6, $p < 0.45$). **(E)** Similarly, no significant variation of sucrose responsiveness was found in the case of non-learners ($F_{1,19}$ = 0.02, p = 0.88 NS). Sample sizes are presented in parentheses.

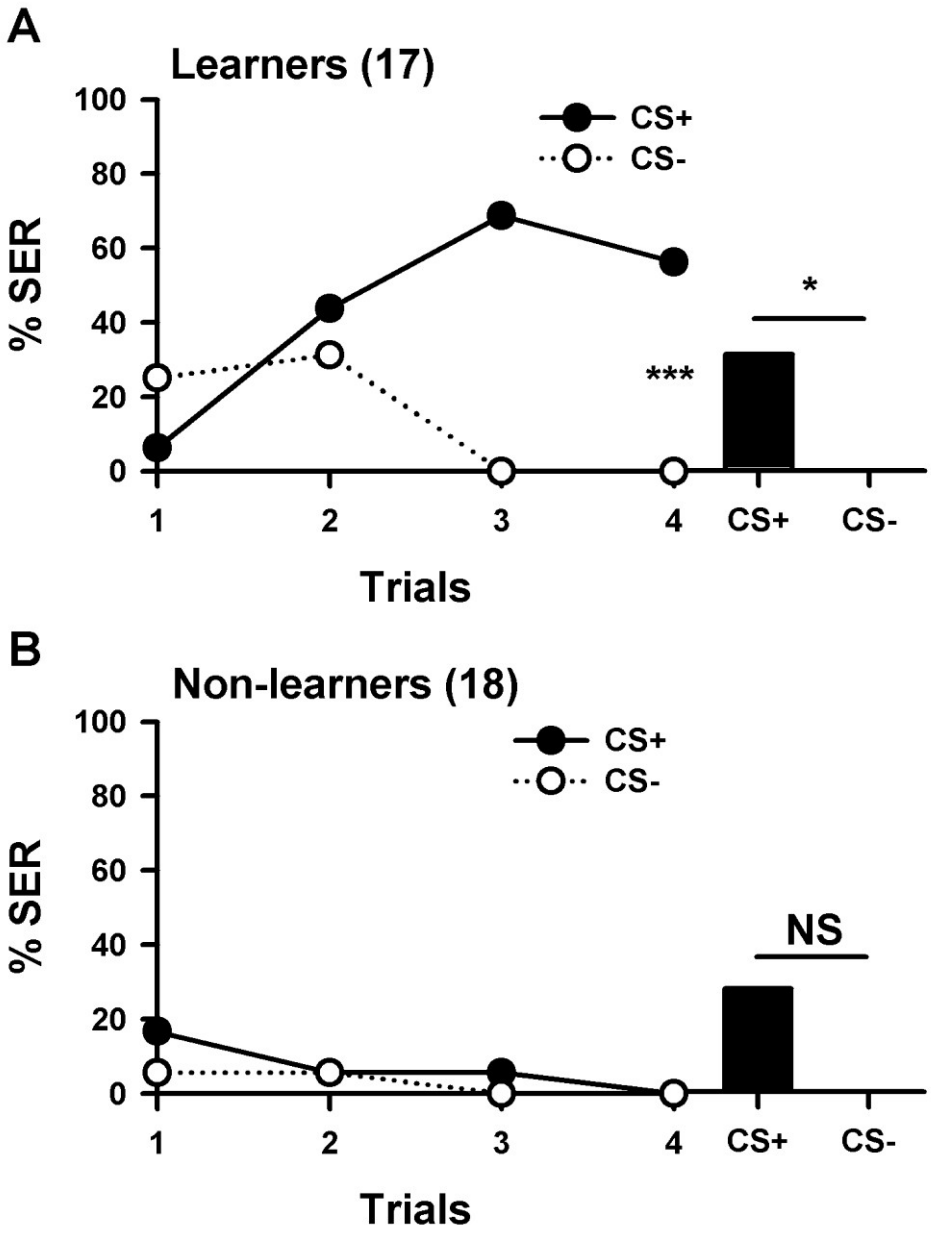
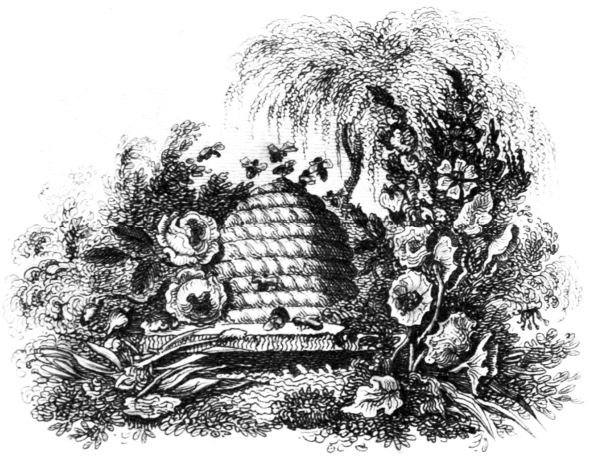


Figure S3. Acquisition curves of learners and non-learners during the aversive olfactory conditioning and the 72h-retrieval tests.

(A) Learners were able to discriminate the CS+ (black dots) from the CS- (white dots) at the end of conditioning ($F_{1,16} = 58.18$, $p < 0.001$). Three days after conditioning learners responded significantly more to the CS+ than to the CS- in the absence of reinforcement ($\chi^2 = 4.17$; $p < 0.05$) during the retrieval test. Sample size is shown in parentheses. **(B)** Non-learners were not able to learn the discrimination between the CS+ (black dots) and the CS- (white dots) ($F_{1,17} = 3.4$, $p = 0.08$). Three days after conditioning non-learners did not respond significantly more to the CS+ than to the CS- in the absence of reinforcement (McNemar test $\chi^2 = 3.2$; $p = 0.07$) during the retrieval test. Sample size is shown in parentheses.

DISCUSSION



DISCUSSION

The main goal of this thesis was to understand the behavioural, neural and molecular bases of aversive stimulus representation in the brain of an insect model, the honey bee, *Apis mellifera*. To this end, we focused on electric shock as an aversive stimulus that has been repeatedly used as unconditioned stimulus in various variants of aversive Pavlovian SER conditioning and characterised the role and circuitry of dopaminergic neurons in the bee brain as well as the role of dopaminergic receptor genes in noxious/aversive signalling. We performed, therefore, an integrative study combining various approaches, including behavioural, pharmacological, neuroanatomical and molecular analyses, with the goal of understanding the involvement of dopaminergic signalling in aversive US representation.

We firstly concentrated on understanding the role of diverse neurotransmitters in electric-shock signalling. We chose a neuropharmacological approach and injected pharmacological blockers of these neurotransmitters into the bee brain to determine whether such blockade affected SER to an increasing series of shock voltages. Our results confirmed that dopaminergic signalling underlies shock responsiveness, yet in a rather unexpected way. We thus postulated that different classes of dopaminergic neurons exist in the bee brain and that they mediate different functions with respect to electric shock signalling. Then, adopted a neuroanatomical approach and characterised the dopaminergic circuits in the bee brain using immunocytochemistry and 3D confocal reconstruction. We verified prior finding reporting the existence of three main dopaminergic clusters in the bee brain and identified a novel cluster in the visual region. Finally, we studied the molecular mechanisms underlying aversive responsiveness and associative learning to analyze whether aversive learning induces variations in the expression of specific receptor genes, thereby changing the aversive responsiveness to punishment.

In the following sections we will discuss main questions derived from our work. Note that as each result chapter constitutes an independent article, specific discussions can also be found therein.

1. Different Functionality of Dopaminergic Neurons (neuropharmacological study – responsiveness to electric shock, an aversive stimulus)

The first part of this thesis aimed at uncovering the neural bases of innate aversive responsiveness. To determine the neurotransmitters that modulate innate responsiveness to electric shocks, we injected antagonists of various neurotransmitters and quantified the sting extension reflex (SER) to a series of increasing voltages. We found significant modulation of SER upon blockade of two biogenic amines, dopamine (DA) and serotonin (5-HT). In both cases, an *increase* in shock responsiveness to shocks of intermediate voltage was found. These results thus show that in their default mode, these two biogenic amines act as repressors of sting responsiveness. In other words, their role is to inhibit unnecessary responsiveness to less intense noxious stimuli. This result was unexpected in the case of dopaminergic inhibition as the injection of DA antagonists inhibits (i.e. to *decrease*) aversive SER conditioning, a result that was explained through the inhibition of shock signalling during aversive learning. If this were the unique function of dopaminergic neurons, the blockade of DA circuits in our innate responsiveness experiment should also have decreased shock responsiveness, which was not the case. On the contrary, shock responsiveness decreased. Discussing how to reconcile these results is therefore mandatory.

1.1. Dopaminergic Modulation of Aversive Learning and Memory

In several insect models, the role of the dopaminergic system in insect learning and memory has been established and strongly related to aversive-reinforcement signalling in the insect brain. In crickets for example, pharmacological blocking leads also to an impairment of visual and olfactory aversive learning (Unoki et al., 2005, 2006). Also, in the fruit fly, *Drosophila melanogaster* neurogenetic blockade of DA neurons via specific mutants impairs olfactory aversive learning (Schwaerzel et al., 2003) while activation of a specific subset of DA neurons in other mutant flies substitutes for shock reinforcement in aversive olfactory conditioning (Aso et al., 2012; Aso et al., 2010; Claridge-Chang et al., 2009). Similar results were obtained in *Drosophila* larvae where activation of DA neurons contingent to odour presentation results in odour avoidance (Schroll et al., 2006), thus showing that a specific subset of DA neurons substitutes for aversive reinforcement in aversive learning.

Furthermore, the aversive learning and memory in the fly rely on the D1-like receptors in the MB, thus suggesting the necessity of cAMP-activating receptor type (Kim et al., 2007).

In the honey bee, the involvement of dopaminergic signalling in shock signalling in the brain was also suggested after combining aversive olfactory SER conditioning with DA blockade by pharmacological antagonists (Vergoz et al., 2007): flupentixol and spiperone suppressed the capacity to learn odour-shock associations (Vergoz et al., 2007), thus suggesting that, in this case too, DA mediates aversive-reinforcement signalling necessary for aversive learning.

Moreover, dopaminergic signalling is not solely modulated by the bioamine itself. 20-hydroxyecdisone (20E), which is a metabolite of the steroid hormone ecdysone and intervenes in insect development and reproduction (Riddiford et al., 2000), is known to affect the learning acquisition. It impairs aversive but not appetitive conditioning in bees (Geddes et al., 2013). Two-day old bees are deficient in olfactory SER learning (Geddes et al., 2013) in agreement with higher titres of ecdysteroids occurring at this age (Hartfelder et al., 2002). This impairment seems to be achieved in part via the dopamine/ecdysone receptor gene *Amgpcr19*. Thus, exogenous 20E injection determines both a reduction in AmGPCR19 levels and a decrease in aversive learning performances (Geddes et al., 2013), an effect similar to what has been achieved through blocking all dopamine receptors indiscriminately (Vergoz et al., 2007).

1.2. Dopaminergic Modulation of Aversive Responsiveness

We showed that dopaminergic blocking by injection of flupentixol, a general DA antagonist, resulted in a significant increase of shock responsiveness. This indicates that DA acts as a depressor of sting responsiveness to electric shocks so that when its effect is antagonized, the responsiveness increases. Because flupentixol is a generic blocker of DA receptors, this results raises the question of which of these receptors actually regulate shock responsiveness. In the case of olfactory SER conditioning, DA blockade by means of vertebrate D1-like and D2-like receptor blockers SCH23390 and spiperone, respectively, yielded different results: while SCH23390 did not impair olfactory SER conditioning, spiperone significantly impaired acquisition and retention, thus suggesting that D1-like and D2-like DA receptors contribute differently to the signalling of US reinforcement by the instructive DA neurons (Vergoz et al.,

2007). Taken together, these facts suggest the heterogeneity of the DA signalling mechanisms in the bee brain.

Injection of 20E also impairs olfactory SER conditioning but leaves intact shock responsiveness to electric shock (Geddes et al., 2013). Because 20-E injection determines a reduction of the levels of the dopamine/ecdysone receptor gene AmGPCR19, which is the honeybee orthologue of the dopamine/ecdysone receptor gene 48 (DmDopEcR) identified in *Drosophila* (Srivastava et al., 2005), it can be proposed that aversive learning in the honey bee depends on this dopamine/ecdysone receptor gene. At the same time, as 20E injection does not affect unconditioned shock responsiveness, it may be concluded that dopaminergic signalling exerting an inhibitory action on this responsiveness does not involve AmGPCR19. Furthermore, as the decrement in olfactory SER conditioning induced by 20-E is not due to a loss of US sensitivity, the inhibitory effect of this ecdysteroid could be exerted on the other components of the odour-shock learning: it might reduce olfactory perception and/or impair the associability of CS and US pathways.

1.3. Different Classes of Dopaminergic Neurons

Our results revealed at least two different functions of DA neurons. On the one hand, DA receptor blocking appears to facilitate behaviour owing to the general depressor effect of this biogenic amine without affecting motor and sensory functions in the conditioning protocols discussed above. On the other hand, DA signalling appears to mediate the aversive reinforcing properties of the electric shock, as its blockade impairs aversive associative learning and enhances its suppressor function in shock responsiveness experiments (the first part of this work) shock sensitivity. This dual function assume the existence of, at least, two different classes of dopaminergic neurons mediating different functions: one acting as a *general gain control system*, with the specific role of down-regulating responsiveness to noxious stimuli and another acting as *instructive* neurons in aversive associative learning which mediates aversive US signalling. Owing to these different functions, their brain targets could be different. While the first class would exhibit extensive and broad branching within the entire brain in order to be able to modulate different motivational components (appetitive, aversive) and sensory modalities (olfactory, visual gustatory, etc), the second class would exhibit a specific connectivity with respect to CS processing circuits (olfactory, visual) in order to facilitate CS-US associations and provide instructive (i.e. valence) information to the targeted

CS circuit (Giurfa, 2006). These two classes may also differ in terms of the dopaminergic receptors expression level. As mentioned above, the instructive class may be closely related to the AmGPCR19 receptor. With respect to Amdop receptors, uneven distributions of the D1- and D2-like dopamine receptors in the bee brain have been observed (Kokay et al., 1998; Kokay et al., 1999). Depending on the occurrence, classes of dopaminergic neurons may modulate certain populations of dopaminergic receptor and thus activate specific neuropils. As proven in the case of specific blocking of the dopamine/ecdysteroid receptor, activating distinct sets of receptor might lead to various behavioural outcomes.

Dopaminergic neurons acting as down regulators of behavioural responsiveness to noxious stimuli could mediate responding adaptively to appropriate aversive stimuli in the insect's environment. They may mediate attentional processes in which perception is focused on one stimulus (or group of related stimuli), while filtering out other simultaneous stimuli that are less relevant at any moment (Posner et al., 1980). Attentional processes, similar to those described in vertebrates, can also be identified in insects (Dyer and Chittka, 2004; Giurfa, 2004; Miller et al., 2011; van Swinderen, 2011; Van Swinderen and Andretic, 2011) and, in the case of *Drosophila*, a neural correlate of such processes is a transient increase in a 20-30 Hz local field-potential recorded in a region of the brain called the medial protocerebrum (van Swinderen and Greenspan, 2003). Current views relate DA levels in the insect brain with arousal levels (Van Swinderen and Andretic, 2011). Transient attenuation of DA release in fly mutants attenuates the 20-30 Hz responsiveness to the object to be attended and oral delivery of methamphetamine, which increases DA release, rescues this responsiveness (Andretic et al., 2005). Thus, gain-control DA neurons may modulate selective attention in the insect brain, acting on a series of nervous circuits underlying different forms of sensory-motor performances.

In the fruit fly, different classes of dopaminergic neurons have been identified mediating appetitive (Burke et al., 2012; Liu et al., 2012a) and aversive (Aso et al., 2012); (Aso et al., 2010; Claridge-Chang et al., 2009; Placais et al., 2012) reinforcing functions. Thus, we posit that besides this instructive category, which we suggest to be driven by the perception and processing of a salient CS, a different class of dopaminergic neurons exist which provide a down-regulating control of responsiveness upon perception of potentially noxious stimulation.

All in all, the picture emerging from these results suggests that the dopamine/ecdysone receptor gene AmGPCR19 is a main actor in US signalling during aversive learning, while

other DA receptors (such as Amdop1, Amdop2 or Amdop3) play a role in regulating unconditioned US responsiveness.

2. Neuroanatomy of the Dopaminergic Network in the Bee Brain

The second part of this work aimed at characterising the neuroanatomy of the dopaminergic neurons in the bee brain. In this way, we hoped to understand if and how some of these neurons might convey the aversive signalling based on their connectivity with CS processing circuits (e.g. olfactory, visual). We used antibodies directed against tyrosine hydroxylase (TH). TH is a dopamine (DA) precursor in the DA biosynthesis pathway. In this way, we assured that neurons stained in this way are synthesising endogenous DA, i.e. DA-releasing neurons. To control the immunoreactivity, two differently raised commercial antibodies from two independent sources were used for the immunocytochemistry.

Our results verified one of the assumptions of our pharmacological work with respect to the dopaminergic gain-control system proposed, namely that dopaminergic neurons should exhibit an extensive and broad branching within the entire brain in order to be able to modulate different motivational components (appetitive, aversive) and sensory modalities (olfactory, visual gustatory, etc). Prior studies (Schäfer and Rehder, 1989; Schurmann et al., 1989) had reported three main putative dopaminergic clusters in the SPZ, C1-C3, whose morphology and localization was confirmed by our TH antibody. In addition to these clusters, we discovered the presence of a previously unknown dopaminergic cluster, which we termed the C4-cluster.

Our counting of the C1- and C2-clusters registered around 75 somas, each with a diameter of around 10 µm as exactly as measured in the prior works. The total number of somas we found is lower than the 100 somas (Schäfer and Rehder, 1989) and higher than the 40 somas (Schurmann et al., 1989) attributed by previous analyses; Around 140 somas could be registered in the C3-cluster, which is considerably more than the previously reported 80-90 somas reported by Schäfer and Rehder (1989) and 50 somas reported by Schurmann *et al.* (1989). Their sizes are varied, with diameters from 7 – 12 µm, in accordance with the previous reports, suggesting the existence of functionally different types of neurons, or possibly, sub-clusters. The newly discovered C4-cluster can be located dorsomedial border of the optic lobe's lobula. It contains ca. 80 somas with a diameter of 8-10 µm. With the addition

of several other smaller cell clusters, we counted around 400-450 somas altogether, which is more than the 350 somas counted by Schäfer and Rehder (1989) and the 120 somas counted by Schurmann *et al.* (1989) on each hemisphere.

The anatomical characterization performed in our work does not allow distinguishing between dopaminergic neurons that achieve a global-gain function and neurons that provide an instructive function to CS circuits (see above, section 1.3 of the Discussion) as only functional analyses of neural activity can allow making the difference. Yet, we will focus on dopaminergic neurons innervating the MBs because possible candidates of instructive neurons could be found there, as it is the case in *Drosophila* (Aso et al., 2012; Aso et al., 2010). MBs are higher-order structures associated with the presence of memory traces (see Thesis Introduction for review) and a site of CS (e.g. olfactory afferences) and US convergence so that DA neurons could provide therein the aversive US signal.

2.1. Dopaminergic Processes in the MB

The MB integrates various modalities, with afferences coming from the AL and the LH and from the optic lobes (see Introduction of this thesis). DA innervation can be found both at the level of the calyces and the vertical and medial lobes; even along the pedunculus, which consists of the axons of the Kenyon cells relaying signals from the input to the output regions (Strausfeld, 2002). The TH-ir innervations in the calyces are obvious in the lip and the collar regions, known as the input regions from olfactory and visual centres, respectively. The vertical and medial lobes are variably innervated.

The processes in the MB can be attributed to the 3 main clusters, C1-, C2- and C3. Although the polarity of these neurons could not be identified, it is obvious that the somata from these clusters project to various regions in the MBs.

The morphology of the neurons of C1-, C2-clusters resembles the A1 and A2 MB extrinsic neurons (MBEN); while some of the C3-cluster neurons resemble the A6 MBEN reported by Rybak and Menzel (1993). In the honey bee brain, the A1 and A2 MBENs are located anteriorly and in the same depth as the C1 and C2 clusters and project in the same manner into the vertical lobe. Their branches envelop the vertical lobe and project in the adjacent neuropil in the protocerebral lobe unilaterally. The fine varicose fibres are detected in both vertical and medial lobe. Interestingly, Rybak and Menzel (1993) counted an average

of 50-60 stained neurons for the A1 and the A2 neurons, a number which is relatively close to the number of somas we counted in the C1- and C2-clusters, indicating that these clusters may include these two types of MBEN, A1 and A2. Thus, these two neurons appear to be strong candidates to illustrate the morphology of the neurons of the C1- and C2-clusters at a single-cell resolution. In *Drosophila*, the homologue to the C1- and C2-clusters may be the PAM cluster with respect of the location of their somas and innervations. The neurons of the PAM cluster terminate in the horizontal lobes of the mushroom body and neuropils adjacent to it (Mao and Davis, 2009).

The honey bee's A6 MBEN is so far the only neuron which can be proposed as a model neuron for the C3-cluster. Its soma is located ventrally to the lateral calyx and it projects to the vertical lobe as well as to neuropils in the contralateral hemisphere, in addition to the ipsilateral lateral horn. Even though C3-cluster's neurons could not be defined to this extent, it is possible to sort out a part of their massive projections in this way. Looking at the number of reported A6 neurons which ranges between 60-80, we may conclude that they make up a part of the 140 C3 somas. As for its homologue in *Drosophila*, it is tempting to speculate that it may be both the PPL1- and PPL2ab-clusters together. First, the location of PPL1's somas is relatively close to the calyx and this cluster has terminals detected in the dorsal part of the fan-shaped body as one of the C3's terminals (Liu et al., 2012b; Mao and Davis, 2009). Second, PPL2ab's terminals are detectable in the calyx of the mushroom body (Mao and Davis, 2009). It is notable that C3-cluster is a big cluster consisting of different types of neurons, or possibly, sub-clusters. This, in turn, may give us the morphological information about the existence of the various functional classes of dopaminergic neurons.

In *Drosophila*, different classes of dopaminergic neurons contribute differently to aversive memory formation. Two clusters have their dense terminals in the lobes, each with a unique zonal organisation. The PAM cluster, which would correspond to the C1- and C2-clusters, mediates the aversive signal of the electric shock so that activation of these neurons contingent to an odor stimulation in thermo-sensitive mutants supports the formation of a labile aversive memory (Aso et al., 2010). On the other hand, the PPL1 cluster, which would correspond to the C3-cluster, is involved in a specific regulation of ARM levels and gates LTM (Aso et al., 2010; Claridge-Chang et al., 2009; Placais et al., 2012). Moreover, different modulatory interactions between neurons of the PAM and the PPL1 clusters tune the stability of aversive memory (Aso et al., 2012). Thus, in *Drosophila*, the different dopaminergic pathways of the PAM and PPL1 clusters that project to the MB and terminate in spatially

segregated subdomains, induce electric-shock memories of different temporal stability and interact in a combinatorial way to tune the stability of mmory components (Aso et al., 2012).

In the honey bee, the dependency of aversive olfactory SER conditioning on dopaminergic signalling has been demonstrated using pharmacological blockade (Vergoz et al., 2007). The instructive DA neurons mediating the shock signalling in this aversive conditioning could, thus, be found in the C1-, C2 and/or C3-clusters given the apparent homologies with the PAM and PPL1 clusters of the fruit fly.

2.2. Dopaminergic Neurons as Modulators of Behaviour

Besides their role as a circuitry for aversive-reinforcement signalling, dopaminergic neurons act as a more general modulatory system, generally depressing several behavioural components. For instance, dopamine decreases sucrose responsiveness (i.e. PER to increasing sucrose concentrations) when injected into the thorax. Also, injection or feeding of the DA receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN) reduces sucrose responsiveness significantly (Scheiner et al., 2002). In olfactory PER conditioning, injection of DA into the antennal lobes reduces significantly olfactory retention both after one and three conditioning trials (Macmillan and Mercer, 1987). In the case of aversive responsiveness (i.e. SER to increasing shock voltages), we showed that dopaminergic blocking induces an *increase* of shock responsiveness, thus reflecting an enhancement in shock sensitivity (Tedjakumala et al., 2014; see chapter 1 of Results). This result thus indicates that in its default mode, and besides its reinforcement-signalling role, dopaminergic signalling acts as a depressor of sting responsiveness to electric shocks so that when its effect is antagonized, responsiveness increases.

We suggested that a possible explanation for this dual function is to assume the existence of different classes of dopaminergic neurons mediating different functions: one acting as a general gain control system, with the specific role of down-regulating responsiveness and another acting as instructive neurons in aversive associative learning which mediates aversive US signalling. The latter has been discussed in the previous section (see above). We suggested that the former should exhibit extensive and broad branching within the entire brain in order to be able to modulate different motivational components

(appetitive, aversive) and sensory modalities (olfactory, visual gustatory, etc) In principle, the neural architecture of the dopaminergic circuits described in the present work provides the basis for such a broad modulation. We found dopaminergic innervations almost in all regions of the bee brain, including the visual one, a region which dopaminergic innervations were thought to be absent. Where no main clusters were found, small groups of DA neurons were identified which are distributed in the main neuropiles of the brain (see chapter 2 of the Results section). Thus, although we have no functional measurements of the role and action of these neurons, they provide a potential basis for the global modulatory action of DA neurons proposed. Further functional studies should address this point. We hope that our analyses will constitute an important anatomical reference guiding such functional hypotheses in the future.

3. Learning-Dependent Modulation of DA Receptor Expression in the MBs

In the last part of this work, we studied the molecular processes related to the aversive signalling underlying a successful aversive conditioning. We quantified aversive responsiveness of honey bees before and three days after aversive olfactory learning and showed for the first time that learning induces a long-term modification of aversive responsiveness as bees become less sensitive to shocks of intensity lower than that of the US after conditioning.

In the 2nd part of our work, we showed dense innervations of different subsets of dopaminergic neurons in the MB which might serve as neural correlates of reinforcement signalling in aversive olfactory conditioning. We thus looked closer into the MB and dissected the subpopulations of the KCs, the constitutive cells of the MB, in order to explore molecular changes at the level of DA receptors induced by aversive learning. We focused on dopaminergic receptor-gene expression in the MB due to several reasons. Firstly, these structures have been related to long-term memory formation, storage and retrieval (Busto et al., 2010; Davis, 2005, 2011; Giurfa, 2003; Heisenberg, 2003; Keene and Waddell, 2007; Menzel, 1999, 2001) and to attentional processes mediated by dopaminergic signalling (Andretic et al., 2005; Miller et al., 2011; van Swinderen and Greenspan, 2003). Secondly, the neuroanatomical part of this work revealed the dense processes of dopaminergic neurons in the MB, suggesting an intensive interaction between the cell populations. The interaction could even be detected as an increase of cAMP level in regions in the MB, traceable through dopaminergic neurons stimulation (Boto et al., 2014). Furthermore, works in *Drosophila* (see

previous section) showed how the reinforcements from various dopaminergic clusters innervating the MB could affect the aversive learning and memory performance differently (Aso et al., 2012; Aso et al., 2010; Claridge-Chang et al., 2009).

The cell-population specific analysis was performed by differentiating between inner compact (ICC) and non-compact Kenyon cells (NCC), whose cell bodies are located within the mushroom-body calyces (Farris et al., 2004) and which show heterogeneous aminergic receptor-gene expression during the lifetime of the bee (McQuillan et al., 2012). McQuillan *et al.* (2012) showed that the levels of aminergic receptor-gene expressions vary along with the age in the whole brain as well as in the Kenyon cells. The variation shows a strong correlation with the age- and task-dependency on the levels of bioamines themselves in the brain (Barron and Robinson, 2005; Schulz and Robinson, 1999; Taylor et al., 1992; Wagener-Hulme et al., 1999).

Here we found that aversive learning, but not long-term memory retrieval, induced significant variations of receptor-gene expression in Kenyon cells. As not all learners retrieved the aversive memory and some non-learners answered during the retrieval test as if they had formed such memory, no significant relationship was found between receptor-gene expression and retrieval success. On the contrary, the relationship between receptor-gene expression and learning success was highly significant for *Amdop2* and close to significance for *Amdop1*. Thus, two of the three dopaminergic receptor genes known in the honey bee exhibited a long-term increase in expression following aversive conditioning, which was concomitant of the significant long-term decrease in shock responsiveness induced by such conditioning. It is notable that both receptors are classified as the D1-like receptors and share the property of upregulating the cAMP level when activated by DA (Blenau et al., 1998; Humphries et al., 2003; Mustard et al., 2003). *Amdop3* could not be related with the decrease of shock sensitivity induced by aversive learning, thus indicating a potential different role for their corresponding receptors in the bee brain.

Remarkably, the dopamine/ecdysone receptor gene *Amgpcr19* did not exhibit any variations despite the possibility that neurons expressing this type of receptor may exist on neurons mediating the aversive US signalling (Tedjakumala et al., 2014). This was assumed based on the fact that blocking this receptor by the injection of 20E impairs the aversive conditioning in bees (Geddes et al., 2013) but leaves intact the US responsiveness to electric shock (Geddes et al., 2013; Tedjakumala et al., 2014). Neurons mediating the US signalling processed incoming signals at different stages, as in the case of VUMmx1 (Hammer, 1993). A

model of aversive signalling pathway with different processing sites would therefore receive olfactory (CS) signalling, electric shock (US) signalling and consolidate these reinforcements at another different site. It appears that the decrease in olfactory SER conditioning involving AmGPCR19 does not occur at the neurons mediating the US signalling but rather to the neurons involved in CS perception and/or in the CS-US consolidation site.

Amoa1, which has been related to appetitive sucrose signalling (Farooqui et al., 2003), was also unaffected by conditioning, as expected from the use of an aversive protocol for which octopaminergic signalling is dispensable (Vergoz et al., 2007). Aversive learning thus triggers processes leading to a stable increase in dopamine receptor-gene expression which serves as the potential basis for the modulation of behavioural responsiveness. Whether appetitive learning induces in turn a stable increase in the expression of the octopaminergic receptor gene *Amoa1* remains to be determined.

Moreover, we also found that the long-term increase induced by the aversive conditioning did not modify the appetitive reinforcement signalling. Sucrose responsiveness was quantified before and after and aversive conditioning. Despite the consistent increase of *Amdop1* and *Amdop2* neither increase nor decrease of responsiveness could be detected. This suggests that the changes are restrictive to the context of the conditioning itself. It appears that in respect of the basal signal reinforcement, there is a clear division between appetitive and aversive signalling, presented in the brain of the honey bee respectively by the OA and DA signalling. The notion of OA mediation of the basal appetitive reinforcement has been shown by OA injection in the AL or MB of honey bee. The injection can replace the presentation of sucrose involved in the appetitive signalling (Hammer and Menzel, 1998; Hammer et al., 1993). Sucrose signalling is conveyed by OA neurons and these are indispensable for the responsiveness to various concentrations/degrees of sweetness (Behrends and Scheiner, 2012). However, it is worth mentioning that sucrose responsiveness modulation by OA signalling can be undone by experience factor gathered by an individual during foraging phase, probably through an intervention of another subset of neurons retrieving the information. Works in *Drosophila* suggest also that the sweetness, but not the nutrient value, is being conveyed by OA neurons (Waddell, 2013). The assessment of the nutrient value appears to be done at a higher level, processed by DA neurons located downstream of the OA neurons (Burke et al., 2012). Taken together, these studies suggest that changes of responsiveness might be very likely context-related and experience-based. So, reinforcing a

contextually unrelated set of DA neurons would not lead to a specific selectivity of a contextually different reinforcement.

3.1. A Molecular Mechanism Accounting for the Rescorla-Wagner Model?

In the most popular model of classical conditioning, the Rescorla and Wagner model (1972), US salience directly affects learning efficiency and rate (Rescorla and Wagner, 1972). This model posits that learning will occur if events during the trial violate the expectation of the organism, saying that surprise is necessary for the increment of conditioned responses observed during the learning. The expectation itself is built up about the previous learning experiences which give a predictive value of all the stimuli present. If there has been no prior experience with a given stimulus, then the organism has no expectation about it, which leads in turn, to high potential changes of responsiveness to this stimulus if this expectation is violated. In short, if the US occurs and the organism is surprised, then it learns significantly about the CS prediction of the US. When an animal learns more about the CS-US association, the surprising effect of US delivery is progressively lost so that learning (more specifically, the variation in associative strength or the variation in how tight the CS and the US are linked) diminishes and the learning curve flattens (Rescorla and Wagner, 1972). Generally, when the existing associative strength is low, then the potential prediction change is high; on the contrary, if the existing associative strength is high, then the potential prediction change is relatively low.

This effect can be traced at the neural level in the case of the honey bee by analysing the responses of the VUMmx1 neuron (see Introduction of this thesis, p. 16), the octopaminergic neuron whose activity has been show to substitute for sucrose reward in appetitive olfactory PER conditioning (Hammer, 1993). The activity of this neuron was recorded upon olfactory differential conditioning in which one odour was rewarded with sucrose (CS+) and a different odour was non-rewarded (CS-). It was found that after differential conditioning, as expected, presentation of the CS+ alone activates VUMmx1 but presentation of the CS- alone does not (Hammer, 1993). If the US follows the presentation of the CS+, the response of VUMmx1 *to the US itself* is strongly reduced, and even inhibited. In contrast, the response of VUMmx1 *to the US itself* after the presentation of the CS- remains normal. This indicates that differential conditioning leads to different reward-related

responses, depending on whether the reward is expected (after CS+) or whether it is unexpected (after CS-). Asymptotic acquisition of CS+ may, therefore, result from a loss of reinforcing strength of the reward as predicted by the Rescorla and Wagner model (1972).At the molecular level, the increase of *Amdop2* and partially *Amdop1* may be the key mechanism underlying this decrease of responsiveness in honey bee, possibly due to their ability to predict the coming US. That DA neurons display predictive feature, has been proven in associative learning. In *Drosophila*, DA neurons have shown a prolonged activity to the punished odour in the MB after several trials (Riemensperger et al., 2005). Although our results did not show the exact predictive feature through the reinforced odour, they depict a form of prediction to the US itself which come in various voltages.

Our results showed that learning odour-shock associations induces long-lasting changes in shock sensitivity. The observed decrease of shock responsiveness to voltages lower than that used for conditioning after a successful aversive conditioning may reflect a form of error prediction, as suggested by the Rescorla and Wagner model. In other words, having learned during conditioning that a specific US value (7.5 V) is expected, lower values (0.25 - 4 V) become less attendable. The subjective value of these lower-intensity punishments was probably lowered as a consequence of punishment learning. As a consequence, the US responsiveness curve varied, yet the sensitivity for the US used during conditioning remained the same.

We have shown that an increase in specific dopaminergic receptor genes, Amdop2 and Amdop1, is associated with this variation in responsiveness. This change would thus constitute a potential molecular mechanism for this change in US subjective value, and thus, for further error predictions derived from associative learning. In this case, increasing the dopaminergic receptor genes would facilitate dopaminergic signalling at the level of the Kenyon cells studied. If these receptors act in the down-regulation of responsiveness as suggested (see above, p. 185) this would explain why bees become less sensitive to shocks of lower intensity. This effect may be accompanied by an increase of DA release by dopaminergic neurons innervating the MBs; in this case, having more DA receptors would enhance the down-regulation of responsiveness. On the other hand, DA release may remain constant and the increase of DA rceptors would be enough, *per se*, to enhance down-regulation by favouring a better capture of DA. In all cases, the increase of DA receptors provides a basis for understanding some associative effects at the molecular level. Whether the opposite effect (i.e. an *increase* of responsiveness) would occur if one provides a voltage

higher than that used for conditioning remains to be determined. The experiment is however difficult as above 7.5 V electric shocks induce important damages of the bees. Also, it is still unknown if similar changes occur after appetitive learning in the case of octopaminergic receptors. Further experiments should address these and other related questions related to the search of a molecular basis for error prediction in associative learning.

4. General Conclusions

In this work we showed that:

1. Dopamine signalling modulates electric-shock responsiveness. An augmentation of responsiveness can be observed upon blockade of dopaminergic signalling by pharmacological dopamine antagonist. This suggests their role as a general down-regulator system, contributing in regulating responsiveness to aversive stimulus, besides mediating aversive reinforcement during aversive conditioning.

2. Dopaminergic processes can be traced in all principal neuropils of the honey bee brain. Dense dopaminergic innervations are detectable in all mushroom body regions. They can also be visualised in both olfactory and, for the very first time, visual first order neuropils, respectively the antennal lobe and the optic lobe. Further work should study these processes at a functional level by recording their activity upon different forms of stimulation (e.g. electric shock and other forms of aversive stimuli) or at different stages during learning acquisition and memory formation.

3. Aversive conditioning induced a long-term cell-specific and context (aversive vs appetitive)-related modulation of receptor-gene expression. Dopaminergic gene receptor (mainly in D1-like receptor genes, *Amdop1* and *Amdop2*) expression in Kenyon cells increased after a successful aversive conditioning. This effect reflects a lowering of responsiveness to voltages lower than that used during conditioning and thus a form of reinforcement expectation akin to error prediction learning. Further work should address this hypothesis in other learning contexts for which reinforcement-related neurotransmitter receptors are known.

All in all, this work provides a novel insight in the aversive signalling pathway in honey bee, *Apis mellifera*. By means of behavioural, pharmacological, neuroanatomical and molecular approaches, it shows the relevance of dopaminergic signalling for various aspects of aversive reinforcement in the brain of the honey bee.

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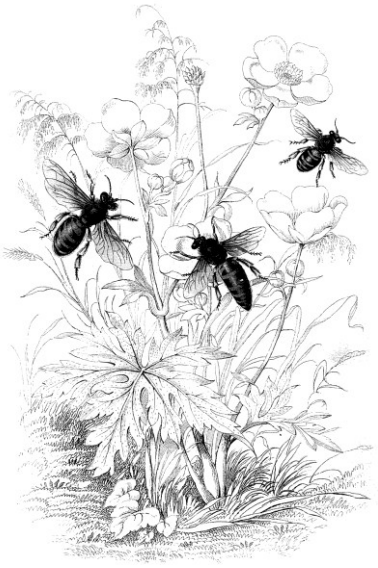
ANNEXE



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REVIEW

Rules and mechanisms of punishment learning in honey bees: the aversive conditioning of the sting extension response

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Summary

Honeybees constitute established model organisms for the study of appetitive learning and memory. In recent years, the establishment of the technique of olfactory conditioning of the sting extension response (SER) has yielded new insights into the rules and mechanisms of aversive learning in insects. In olfactory SER conditioning, a harnessed bee learns to associate an olfactory stimulus as the conditioned stimulus with the noxious stimulation of an electric shock as the unconditioned stimulus. Here, we review the multiple aspects of honeybee aversive learning that have been uncovered using Pavlovian conditioning of the SER. From its behavioral principles and sensory variants to its cellular bases and implications for understanding social organization, we present the latest advancements in the study of punishment learning in bees and discuss its perspectives in order to define future research avenues and necessary improvements. The studies presented here underline the importance of studying honeybee learning not only from an appetitive but also from an aversive perspective, in order to uncover behavioral and cellular mechanisms of individual and social plasticity.

Key words: learning, memory, conditioning, aversive conditioning, insect, honeybee, sting extension response, SER, division of labor, stimulus responsiveness, stimulus sensitivity.

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Introduction

Honeybees (*Apis mellifera*) constitute a traditional invertebrate model for the study of associative learning at the behavioral, cellular and molecular level (Menzel, 1999; Menzel, 2001; Giurfa, 2003; Giurfa, 2007). For almost a century, research on honeybee learning and memory has focused almost exclusively on appetitive learning, exploiting the fact that bees can learn about a variety of sensory stimuli or to perform certain behaviors if these are rewarded with sucrose solution, the equivalent of nectar collected in flowers (Giurfa, 2007; Avarguès-Weber et al., 2011). Since the discovery of the immense potential of this appetitive behavior by Karl von Frisch (von Frisch, 1914), researchers interested in honeybee learning have concentrated on appetitive learning. Following its establishment in 1961 (Takeda, 1961), a single Pavlovian conditioning protocol – the olfactory conditioning of the proboscis extension reflex (PER) – has been used as the unique tool to access the neural and molecular bases of learning and memory in honeybees (Bitterman et al., 1983; Giurfa and Sandoz, 2012; Matsumoto et al., 2012). This protocol relies on an appetitive response exhibited by a harnessed honeybee to the unconditioned stimulus (US) of sucrose solution delivered to its antennae and mouth parts (Bitterman et al., 1983): upon such appetitive stimulation, a hungry bee reflexively extends its proboscis searching for a food reward. After pairing a neutral odorant (the conditioned stimulus, CS) and sucrose, the bee learns the association between odorant and food and extends its proboscis in response to the odorant alone (Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012; Matsumoto et al., 2012). This protocol has been used extensively in the last five decades and has provided valuable information about the behavioral, cellular and molecular mechanisms underlying appetitive learning (Menzel, 1999; Giurfa and Sandoz, 2012).

In contrast, not much was known about the capacity of honeybees to learn aversive events in their environment. In the fruit fly *Drosophila melanogaster*, the other insect that has emerged as a powerful model for the study of learning and memory, aversive learning has been the dominant framework (Heisenberg, 2003; Davis, 2005; Margulies et al., 2005; Keene and Waddell, 2007; Busto et al., 2010). In the fruit fly, olfactory aversive conditioning consists of training groups of flies in a T-maze which allows alternated presentation of two different odors, one (CS+) paired with the US of an electric shock, and another (CS–) non-paired with the shock (Tully and Quinn, 1985). Retention is tested afterwards in a dual-choice situation as flies have to choose between the CS+ and the CS– without aversive reinforcement. Successful learning and retention result in CS+ avoidance. This behavioral protocol has allowed the dissection of aversive learning at the cellular and molecular level and identification of the cellular location of different aversive memory traces (Heisenberg, 2003; Davis, 2005; Margulies et al., 2005; Keene and Waddell, 2007; Busto et al., 2010).

Because of obvious differences in behavioral and motivational contexts, in addition to the impracticality of equating the nature and strength of the US, it has been difficult to compare appetitive and aversive learning in bees and flies, respectively, despite their fundamental contribution to the understanding of learning and

memory at multiple levels. As a consequence, the question of whether the mechanisms underlying learning and memory in these two insect models are general or rather specific has remained unanswered. Yet, in the last 5 years, a new conditioning protocol has been established in honeybees, which was conceived to fill this gap (Vergoz et al., 2007a). This protocol is the aversive conditioning of the sting extension response (SER), which is a defensive response to potentially noxious stimuli (Breed et al., 2004). This unconditioned response can be elicited by means of electric-shock delivery to a harnessed bee (Núñez et al., 1997). As no appetitive responses are involved in this experimental context, true punishment (aversive) learning could be studied for the first time in harnessed honeybees. Here we review the multiple aspects of honeybee aversive learning that have been uncovered using Pavlovian conditioning of the SER. From its behavioral principles and sensory variants to its cellular bases and implications for understanding social organization, we present the latest advancements in the study of punishment learning in bees and discuss its perspectives in order to define future research avenues and necessary improvements.

Prior studies on honeybee punishment learning

The first attempts to study aversive learning in bees used free-flying honeybee or bumblebee foragers and avoidance learning protocols (Gould, 1986; Dukas, 2001; Chittka et al., 2003; Dukas and Morse, 2003; Avarguès-Weber et al., 2010). Even though these studies have contributed to the understanding of learning and memory in bees, they all preserved an appetitive framework. Indeed, bees were trained to find food on artificial feeders and were, afterwards, confronted with different kinds of disturbance. Their behavioral responses to these disturbances were then measured. For instance, free-flying bees foraging for sucrose solution learned to avoid flower patches infested with real or robotic crab spiders (Dukas, 2001; Dukas and Morse, 2003; Ings and Chittka, 2008; Ings and Chittka, 2009), or artificial flowers when penalized either with quinine (Chittka et al., 2003; Avarguès-Weber et al., 2010) or a puff of compressed air (Gould, 1986). Aversive treatments given in a context in which a sucrose reward is also delivered induce an increase in choice accuracy in difficult perceptual discriminations (Chittka et al., 2003; Ings and Chittka, 2008; Avarguès-Weber et al., 2010). This improvement is usually achieved *via* a decrease in the speed of inspection flights, which results in accurate stimulus detection and recognition.

Electric shock had also been used, although seldom, to generate avoidance of visited food sources in free-flying honeybees (Núñez and Denti, 1970; Abramson, 1986). In a pioneer study, Núñez and Denti (Núñez and Denti, 1970) delivered an electric shock to individual bees trained to collect sucrose solution at a food source and landing on a metal plate covering the feeder. Bees reduced drinking attempts upon shock delivery and showed evidence of learning the disturbance program (i.e. the timing of the electric shock) established by the experimenters. Comparable results were later obtained by Abramson (Abramson, 1986), who showed that free-flying bees quickly learned to avoid a feeder paired with an electric shock delivered upon feeding.

Despite using aversive stimulations, all these studies have in common the impossibility of accessing the nervous system in parallel with behavioral recording because they used free-flying bees. Furthermore, they all maintain an appetitive framework as they aim to inhibit the appetitive response of food search. Thus, they pose the problem of potentially confounding frameworks in the study of associative learning. This problem is also present in a variant of olfactory PER conditioning in which, after pairing an odorant and sucrose, an electric shock is delivered to the proboscis of the bee so that it learns to retract it in response to the odorant (Smith et al., 1991).

Olfactory conditioning of the SER

Eluding the appetitive context was first possible when a non-appetitive reflex was chosen as the behavior to be conditioned. Inspired by the work of Núñez and co-workers, who used the SER to study the presence of an opioid-like system in honeybees (Núñez et al., 1997), and by the well-established protocol of olfactory PER conditioning (Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012), the protocol of olfactory conditioning of the SER was successfully established (Vergoz et al., 2007a). This protocol allowed punishment learning in bees to be studied for the first time independently of appetitive stimulations, as forward-pairing an odor with an electric shock results in bees learning this contingency and therefore extending their sting in response to the previously punished odor (Vergoz et al., 2007a).

To this end, bees are fixed individually on a metallic holder so that they build a bridge between two brass plates through which a 2 s mild electric shock (7.5 V) is delivered by a stimulator (60 Hz, AC current) (Fig. 1A). Bees treated in this way extend their sting reflexively in response to the electric shock (Burrell and Smith, 1994; Núñez et al., 1997) (Fig. 1B). Bees of a ‘paired group’ are trained with explicitly paired presentations of an odor (the CS) and the electric shock (the US) following an absolute-conditioning design (a single odorant reinforced). As a control for this kind of conditioning, an ‘explicitly unpaired group’ of bees is presented with unpaired presentations of odor and shock. Fig. 1C,D shows that bees from the paired group learn the odor–shock association and increase the conditioned SER to the punished odor during trials. In contrast, bees in the explicitly unpaired group show no significant change in responsiveness to the odor during trials. Thus, the increase of the SER observed in the paired group is due to associative learning and not to the simple experience with the odor and the shock. One hour after conditioning, bees of the paired group still remember the conditioned odor while bees of the unpaired group do not respond to the odor (Fig. 1C). Therefore, an aversive memory retrievable 1 h after learning is established in the paired but not in the explicitly unpaired group (Vergoz et al., 2007a).

Moreover, in a differential-conditioning design in which each bee acts as its own control, bees learn to extend their sting in response to an odor paired with an electric shock and not to respond to another non-reinforced odor. Bees are conditioned during six reinforced and six non-reinforced trials, presented in a pseudo-random sequence. The resulting learning curves (Fig. 1D) show that bees learn to discriminate between odors as a result of conditioning. Thus, olfactory conditioning of the SER is truly associative and does not rely on the simple exposure to the training stimuli, independently of their outcome (Vergoz et al., 2007a).

The capacity to learn about aversive outcomes of olfactory cues can enable bees to overcome hardwired appetitive responses. For instance, immobilized bees were trained to discriminate two odorants in a differential conditioning procedure in which one odor was neutral and the other was either an attractive pheromone (geraniol or citral) or an attractive floral odorant (phenylacetaldehyde). In all cases, bees developed a conditioned aversive response to the punished odor and efficiently retrieved this information 1 h later. No learning asymmetries between neutral odors and pheromones were found (Roussel et al., 2012). Thus, associative aversive learning in bees overcomes pre-programmed

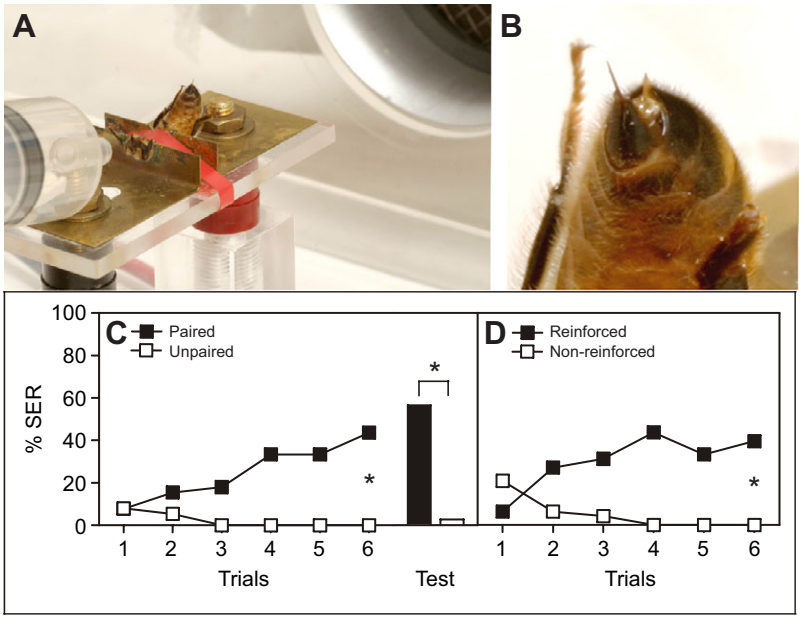

Fig. 1. (A) View of a honeybee in the experimental set-up. The bee is fixed between two brass plates set on a Plexiglas plate, with electroencephalography (EEG) cream smeared on the two notches to ensure good contact between the plates and the bee, and a girdle that clamps the thorax to restrain mobility. The bee closes a circuit and receives a mild electric shock (7.5 V), which induces the sting extension reflex (SER). An originally neutral odorant is delivered through a 20 ml syringe placed 1 cm from the antennae. Odorant stimulation lasts 5 s. The electric shock starts 3 s after odorant onset and lasts 2 s so that it ends with odorant offset. Contamination with the remains of odorants used for conditioning or pheromones is avoided *via* an air extractor, which is on continuously. (B) The SER elicited upon stimulation with an electric shock of 7.5 V. (C) Responses (SER) of two groups of bees, one trained with an odorant explicitly paired with an electric shock (N=38) and the other with the same odorant and an unpaired electric shock (N=39) during six trials. Only the bees in the paired group learned the association and extended their sting as a response to the odorant. One hour after conditioning, an olfactory aversive memory was present in the paired, but not in the unpaired, group. (D) Responses (SER) of a group of bees (N=48) trained in a differential conditioning procedure to discriminate an odorant reinforced with an electric shock and a non-reinforced odorant during 12 trials (six reinforced and six non-reinforced). Bees learned to discriminate between odorants as a result of conditioning (*P<0.0001). Modified from Vergoz et al. (Vergoz et al., 2007a).

responses as bees learn to develop a conditioned aversive response to attractant pheromones and to an attractive floral odor, thereby uncovering an impressive behavioral flexibility.

Olfactory conditioning of the SER is a true case of aversive learning

Pairing an odor with the electric shock results in the odor gradually gaining control over the SER. Because the animals are restrained in individual holders, their eventual avoidance of the punished odor cannot be assessed. In *Drosophila*, the aversive nature of differential conditioning (CS+ *versus* CS–) is clear, because after a successful conditioning the flies avoid the odor paired with the shock (CS+) and choose the safe odor (CS–) in dual-choice tests (see above). The flies learn the Pavlovian association between odor and shock, but at the same time their active choice determines whether or not they receive the shock, which suggests that operant associations are also learned in this protocol, which is presented as being exclusively Pavlovian.

In the case of olfactory SER conditioning, the term ‘aversive’ could be considered inappropriate given that no response inhibition

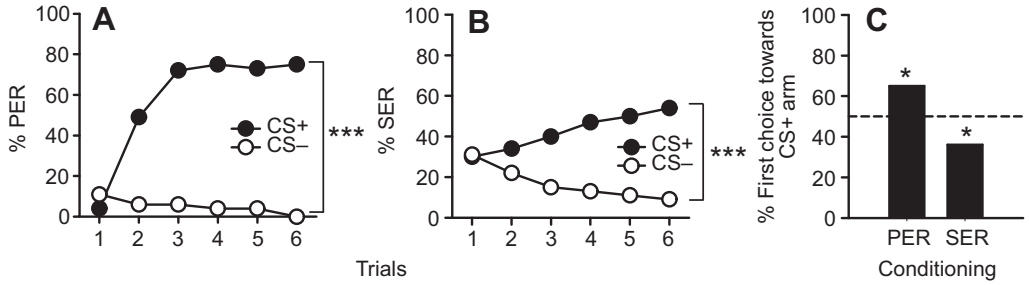

Fig. 2. (A) Appetitive conditioning. The percentage proboscis extension reflex (PER) in bees trained with an odorant explicitly reinforced with sucrose solution (CS+, N=142) and with a non-reinforced odorant (CS–). Bees learned to differentiate between CS+ and CS– in the course of training (***P<0.001). (B) Aversive conditioning. The percentage SER in bees trained with an odorant explicitly reinforced with electric shock (CS+, N=238) and with an odorant explicitly non-reinforced (CS–). Bees learned to differentiate between CS+ and CS– in the course of training (***P<0.001). (C) Orientation of honeybees in the Y-maze, 1 h after associative olfactory conditioning. The graphs show the first choice towards the arm containing the CS+, after PER conditioning (N=79) and SER conditioning (N=72). The dashed line at 50% indicates random choice between CS+ and CS– arms. After PER conditioning, honeybees showed a significant preference for the CS+. In contrast, after SER conditioning, honeybees significantly avoided the CS+ (*P<0.05). Modified from Carcaud et al. (Carcaud et al., 2009).

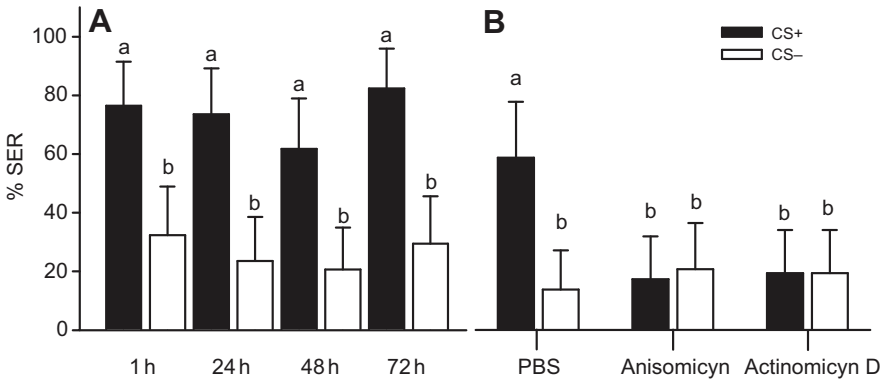

Fig. 3. (A) Memory retention after SER differential conditioning. The percentage SER (+95% confidence interval) to the CS+ and to the CS–. Four groups of bees (N=155) were trained in parallel (acquisition) and tested afterwards after different retention intervals (1, 24, 48 and 72 h post-conditioning). Each group was tested once with each odorant. Different letters indicate significant differences. All groups remembered the discrimination learned during training. (B) Dependency of late long-term memory (l-LTM; 72 h retention) on translation and transcription. Three groups of bees (N=89) were trained in parallel (acquisition) and tested 72 h after the last acquisition trial and after injection of PBS, anisomycin or actinomycin D. Each group was tested once. Different letters indicate significant differences. Only the group injected with PBS (control) remembered the discrimination learned during training; inhibition of transcription (actinomycin D) or translation (anisomycin) resulted in an absence of l-LTM. Modified from Giurfa et al. (Giurfa et al., 2009).

or avoidance is observed after successful conditioning and that the orientation behavior of bees towards the CS was never evaluated. In order to determine whether conditioned bees explicitly avoid the CS as a consequence of the odor having acquired an aversive value, Carcaud and colleagues (Carcaud et al., 2009) conditioned bees in a differential conditioning protocol (one odor punished with shock and the other not) and then released them individually in a mini Y-maze under red light (i.e. in the dark for bees) in which the two odors used as CS+ and CS– were presented (Carcaud et al., 2009). To provide a comparison with appetitive conditioning, they also conditioned bees following differential conditioning but using the appetitive PER protocol (one odor rewarded with sucrose solution and the other not). These bees were also released individually in the Y-maze and their orientation behavior towards the two odors used as CS was evaluated (Carcaud et al., 2009). The question raised was whether SER-conditioned bees would avoid the CS+ in accordance with the aversive punishment associated with it, while PER-conditioned bees would approach it in accordance with the appetitive sucrose reward associated with it.

Fig. 2 shows that both groups of bees (PER- and SER-conditioned bees) efficiently learned to discriminate between two odorants with different valence. PER-conditioned bees significantly increased the PER to the CS+ and decreased it to the CS–. SER-conditioned bees also learned to differentiate the CS+ from the CS– in the course of training and significantly increased the SER to the former and decreased it to the latter. In both cases, bees that performed correctly in the last two blocks of trials, responding only to the CS+ and not to the CS–, were tested 1 h after the end of conditioning in the Y-maze.

Once in the maze, bees that learned the appetitive discrimination preferred the odor previously paired with sucrose (Fig. 2C). In contrast, bees that learned the aversive discrimination avoided the odor previously paired with the shock, thus preferring the previously non-reinforced odor (Fig. 2C). The inhibitory, aversive nature of SER conditioning was, therefore, revealed by this avoidance behavior, which was expressed when the bees had the opportunity to freely choose between CS+ and CS– (Carcaud et al., 2009).

In these experiments, foragers captured at the hive entrance when departing from the hive were used. The possibility cannot be excluded that the same experiments performed with guards would yield a different result (i.e. bees orienting towards the odor paired with shock and exhibiting the SER) or that providing contextual stimuli such as odors from the hive or social pheromones within the Y-maze may also change the response of the bees towards the odor previously punished. Despite these particularities, the results obtained so far demonstrate that SER conditioning in honeybees is a true case of aversive conditioning (Carcaud et al., 2009).

Olfactory conditioning of the SER leads to the formation of long-term memories

Restrained honeybees showed the presence of aversive memories in retention tests performed 1 h after conditioning (Vergoz et al., 2007a; Carcaud et al., 2009). This period corresponds, in appetitive PER conditioning, to mid-term memory, which is independent of protein synthesis and thus relatively labile (Menzel, 1999). The appetitive protocol, however, leads to the formation of more stable memories, including long-term memories, which can last the entire lifetime of a bee (i.e. 2–3 weeks in the case of an active forager) if no other odors are learned that may interfere with the original learning (Menzel, 1999). One pairing of an odorant with sucrose (i.e. one conditioning trial) leads to an early long-term memory (e-LTM) that can be retrieved 24–48 h after conditioning. This e-LTM depends on translation but not on gene transcription and is not, therefore, affected by transcription inhibitors such as actinomycin D. Three conditioning trials, however, lead to a stable late long-term memory (l-LTM) that can be retrieved 72 h or more after conditioning. Unlike e-LTM, l-LTM requires gene transcription and can therefore be inhibited by actinomycin D (Menzel, 1999; Menzel, 2001; Eisenhardt, 2006; Schwärzel and Müller, 2006; Giurfa and Sandoz, 2012). Does olfactory SER conditioning also lead to the formation of different memories with different stability and persistence?

To answer this question, Giurfa and colleagues (Giurfa et al., 2009) conditioned bees in a differential conditioning protocol with spaced trials (intertrial interval of 10 min), and performed retention tests 1, 24, 48 and 72 h after training. An independent group of bees was used for each retention time. All groups learned to discriminate the CS+ from the CS– and reached comparable levels of discrimination at the end of training. After conditioning, bees responded more to the CS+ than to the CS– in all retention intervals assayed (Fig. 3A). These results show that SER conditioning leads

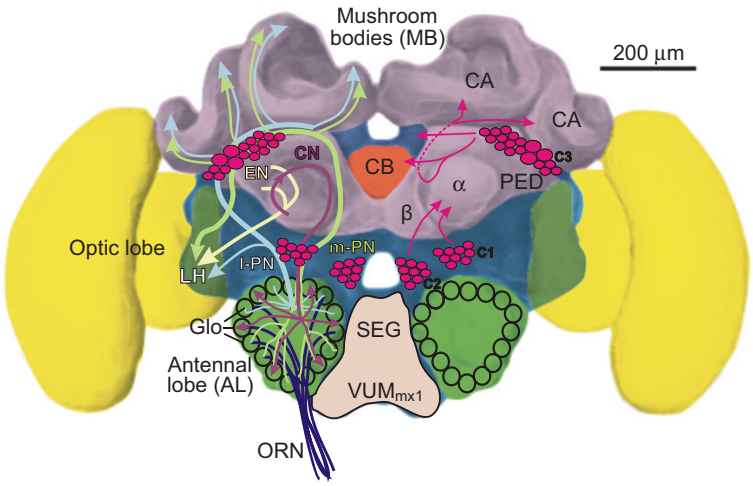

Fig. 4. Neural substrates for CS and aversive-US information in the honeybee brain. The CS pathway is shown in more detail on the left side. The antennal lobe, first-order olfactory neuropil, receives input from ~60,000 olfactory receptor neurons (ORN), which detect odorants within sensilla on the antenna. Within the AL's anatomical and functional units, the 160 glomeruli (Glo), ORNs contact ~4000 inhibitory local neurons (LN, not shown), which carry out local computations, and ~800 projection neurons (PN), which convey processed information to higher brain centers *via* different tracts. The lateral antenno-protocerebralis tract (l-PN) projects first to the lateral horn (LH) and then to the mushroom body calyces (CA), within the lips and the basal ring. The medial tract of projection neurons (m-PN) projects to the same structures, but in the reverse order. The dendrites of the Kenyon cells (KC), the mushroom bodies' (MBs') 170,000 intrinsic neurons, form the calyces (CA), while their axons form the pedunculus (PED), composed of two output lobes: the vertical (or α) lobe and the horizontal (or β) lobe, formed by two collaterals of each KC axon. Within the MBs, feedback neurons (not shown) project from the PED and lobes back to the CA, providing inhibitory feedback to the MB input regions. Extrinsic neurons (ENs) take information from the pedunculus and the lobes and project to different parts of the protocerebrum, but most conspicuously to the LH. Moreover, centrifugal neurons (CN) are thought to be involved in a retrograde modulation of antennal lobe circuits. Dopaminergic neuron clusters, C1–C3, whose activity may mediate aversive US reinforcement, are shown in red. Red arrows indicate possible dendritic arborizations/axonal projections (see Schaefer, 1989). C1 clusters are located in the inferior medial protocerebrum. The almost adjacent C2 clusters are found inferior to the α-lobe (α). Expanding themselves from the most anterior to the most posterior part of the brain, the C3 clusters are observed at the superior border of protocerebrum, below the calyces (CA) of the mushroom bodies. The C1 and C2 clusters, each consisting of around 60–70 cell bodies, send their processes ventro-medially into the α-lobes. Three main processes emanate from the C3 clusters, which consists of around 140 cell bodies; the first goes to a small most anterior region of the superior medial protocerebrum, the second goes to the central body (CB), and the third goes along the dorsal border of the α-lobe, makes a turn at the border of the CB and directly innervates the two calyces equally. Various dopaminergic cell bodies (1–10) are observed sporadically in the brain, as well as in the subesophageal ganglion (SEG). VUM_{mx1}, ventral unpaired median cell mx1.

to a robust memory that is retrievable even 3 days after training (Giurfa et al., 2009).

Such a LTM was studied with respect to its molecular basis. Specifically, the possible dependency of 3 day LTM on *de novo* protein synthesis was analyzed. The conditioning procedure was identical to that of the previous experiment. In the 2 h following conditioning, bees were injected in the brain through the ocellar tract with PBS (control group), anisomycin (a translation inhibitor) or actinomycin D (a transcription inhibitor). Retention performance measured 72 h after conditioning varied depending on treatment (Fig. 3B). Retention performance was significant in control bees injected with PBS but not in bees injected either with anisomycin or with actinomycin D, thus showing that both translation and transcription are essential events for LTM formation of the odor–shock association (Giurfa et al., 2009).

These results show that aversive learning can induce a robust and stable l-LTM that relies on protein synthesis as it depends on both translation and transcription. Bees have the capacity to remember aversive experiences long after they took place. The biological contexts in which such capacity could be applied are multiple. On the one hand, foragers could in this way avoid returning to food places in which negative experiences, or eventually unfulfilled expectations, occurred, thereby enhancing foraging efficiency. On the other hand, it may be adaptive to memorize and remember for long periods the smell of predators in order to exhibit appropriate defensive responses to them.

The neural basis of aversive learning: CS signaling

Odorants are processed at different stages in the bee brain (Fig. 4) (for a review, see Sandoz, 2011). Olfactory detection starts at the level of the antennae where olfactory receptor neurons are located within specialized hairs called sensilla. Sensory neurons endowed with molecular olfactory receptors convey information about odorants to the antennal lobe *via* the antennal nerve. Each antennal lobe consists of 165 globular structures called glomeruli (Galizia and Menzel, 2001). Glomeruli are synaptic interaction sites between olfactory receptor neurons, local inhibitory interneurons interconnecting glomeruli and projection neurons conveying processed olfactory information to higher order centers such as the lateral horn and the mushroom bodies (Kirschner et al., 2006). Mushroom bodies are considered to be higher order integration centers as they receive input from visual, gustatory and mechanosensory pathways in addition to the olfactory pathway (Strausfeld, 2002).

In naive bees, odorants are encoded at the level of the antennal lobe in terms of specific spatial patterns of glomerular activity (Joerges et al., 1997). These patterns can be visualized using optophysiological recordings (calcium imaging) of neural activity. Such recordings were coupled with differential SER conditioning to determine whether punishment learning induces changes in the neural representation of the learned odorants (Roussel et al., 2010). No differences were found between glomerular responses to the CS+ and the CS– in bees that learned the discrimination, in spite

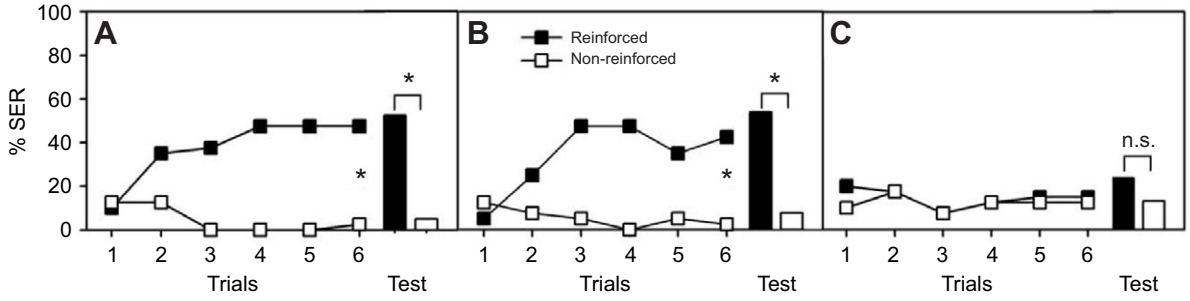

Fig. 5. The effect of octopaminergic and dopaminergic receptor antagonists on olfactory conditioning of the SER. Responses (SER) of bees trained to discriminate an odorant reinforced with an electric shock and a non-reinforced odorant during 12 acquisition trials (six reinforced and six non-reinforced). A retention test was conducted 1 h after the last acquisition trial. SER responses are shown for (A) control bees injected with Ringer solution into the brain (N=40), (B) bees injected with the octopaminergic antagonist mianserine (3.3 mmol l^{-1}) into the brain (N=40) and (C) bees injected with the dopaminergic antagonist flupentixol (2 mmol l^{-1}) into the brain (N=40). Ringer solution- and mianserine-injected bees learned to discriminate the reinforced from the non-reinforced odorant and remembered the difference 1 h later. Flupentixol-injected bees did not learn to discriminate the reinforced from the non-reinforced odorant, nor did they respond appropriately in the retention tests. Similar results were obtained with other concentrations of octopaminergic and dopaminergic antagonists. These results show that dopamine but not octopamine receptors are required for aversive olfactory learning in honeybees. Modified from Vergoz et al. (Vergoz et al., 2007a).

of the fact that in appetitive olfactory PER conditioning, changes in neural activity have been found after differential conditioning (Faber et al., 1999; Rath et al., 2011) (but see Peele et al., 2006).

A possible explanation for this lack of difference between the neural responses to the CS+ and the CS– could be that the aversive olfactory memory traces are located downstream to the antennal lobe, for instance in the mushroom bodies (Gerber et al., 2004). Another possibility relates to the timing of the neural activity recording. In this case, recordings were obtained in parallel to conditioning (i.e. during conditioning trials), taking advantage of the fact that SER conditioning enables simultaneous recording of behavioral output (sting extension) and calcium variation at the neural level. Note that such simultaneity is in principle not possible in PER conditioning because proboscis extension induces muscular activity that interferes with stable calcium-signal recordings in the brain. In the case of SER conditioning, changes in neural activity in response to the CS+ and the CS–, if any, could be only detectable some time after conditioning, upon later memory formation. Further experiments are required in which antennal lobe activity should be measured at different time intervals following conditioning. Similarly, focusing on higher order structures such as the mushroom bodies is crucial.

The neural basis of aversive learning: US signaling

In appetitive PER conditioning, octopamine mediates the reinforcing properties of sucrose reward in the bee brain (Hammer, 1993; Hammer and Menzel, 1998; Farooqui et al., 2003). Pairing an odor with injections of octopamine in the bee brain leads to olfactory learning in harnessed bees, which exhibit PER to this odor (Hammer and Menzel, 1998). In the fruit fly, where octopamine also mediates sucrose reinforcement (Schwaerzel et al., 2003), dopamine was shown to mediate the aversive properties of the electric shock reinforcement used in olfactory conditioning (Schwaerzel et al., 2003; Claridge-Chang et al., 2009; Aso et al., 2010; Aso et al., 2012). More recently, the distinction between dopaminergic and octopaminergic circuits as separate substrates for aversive- and appetitive-reinforcement signaling has been reconsidered in *Drosophila* because an interconnection between octopaminergic and dopaminergic pathways was discovered that plays a crucial role in appetitive olfactory conditioning (Burke et al., 2012; Liu et al., 2012). Specifically, a subset of dopaminergic neurons that possesses octopaminergic receptors was found, allowing them to receive signals from octopaminergic neurons signaling the presence of sucrose. These dopaminergic neurons convey the sucrose-reward signal to the mushroom bodies. Their afferences are spatially segregated from those of other subsets of dopaminergic neurons, which convey punishment signals to the mushroom bodies (Burke et al., 2012; Liu et al., 2012).

In order to establish whether dopaminergic signaling is also crucial for aversive US signaling in bees, neuropharmacological experiments were first performed in order to block this signaling and determine whether olfactory SER conditioning was possible (Vergoz et al., 2007a). Separate groups of bees were injected with Ringer solution (control), mianserine or epinastine (octopaminergic blockers), or fluphenazine or flupentixol (dopaminergic blockers) into the brain through the medium ocellus, 30 min before differential conditioning.

Bees injected with Ringer solution learned to discriminate the punished from the non-punished odor (Fig. 5A). One hour later, they remembered the aversive association and extended their sting in response to the previously punished odorant. Octopaminergic antagonists (mianserine or epinastine) did not affect performance at any of the concentrations used in these experiments. Fig. 5B shows that mianserine-injected bees learned to discriminate between the two odorants and responded with the SER only to the odorant paired with the electric shock. Retention tests also showed significant discrimination. Thus, octopaminergic antagonists did not impair aversive olfactory learning in honeybees. Dopaminergic antagonists (fluphenazine and flupentixol), in contrast, has a dramatic effect on aversive olfactory learning. Flupentixol-injected bees did not learn to discriminate between odorants. Consequently, they did not show discrimination in the tests performed 1 h later (Fig. 5C). Fluphenazine produced similar results although it was less effective. These results showed therefore that dopamine signaling, but not octopamine signaling, is necessary for aversive olfactory learning in honeybees (Vergoz et al., 2007a).

These results prompt a precise neuroanatomical characterization of dopaminergic neurons in the honeybee brain. This characterization is necessary because although immunocytochemistry studies using an antiserum against dopamine were performed 25 years ago (Schäfer and Rehder,

1989), the technique used to stain candidate dopaminergic neurons did not allow differentiation between neurons producing dopamine (true dopaminergic neurons) and neurons incorporating dopamine.

In Schäfer and Rehder's study, dopamine-like immunoreactive neurons were identified in most parts of the brain and in the suboesophageal ganglion (Schäfer and Rehder, 1989) (Fig. 4). Only the optic lobes were devoid of staining. Approximately 330 dopamine-immunoreactive cell bodies were found in each brain hemisphere plus the corresponding suboesophageal hemi-ganglion. Most of the stained cell bodies were situated within three clusters: two (C1 and C2) below the α-lobe of the mushroom body, in the inferior medial protocerebrum, and one below the lateral calyx (C3) (Fig. 4). Other stained cell bodies lie dispersed or in small groups around the protocerebral bridge, below the optic tubercles, proximal to the inferior rim of the lobula, and in the lateral and inferior somatal rind of the suboesophageal ganglion. Because of limitations of the staining technique, not all of the dendritic arborizations and axons of these neurons could be visualized, so where and how dopaminergic circuits contact the olfactory pathway remain to be determined (Schäfer and Rehder, 1989). This information is crucial for studying where the association between the odor CS and the electric shock US takes place.

In addition, a dissection of the contribution of the three dopaminergic receptors identified in the honeybee, AmDOP1 (Blenau et al., 1998), AmDOP2 (Humphries et al., 2003) and AmDOP3 (Beggs et al., 2005), to US signaling in aversive learning is necessary. AmDOP1 and AmDOP3 are related to the vertebrate D1-like and D2-like family of dopamine receptors, respectively (Blenau et al., 1998; Beggs et al., 2005), while AmDOP2 appears to be related to invertebrate octopamine receptors and constitutes a distinct 'invertebrate-type' dopamine receptor (Humphries et al., 2003). From a functional point of view, it can be referred to as a 'D1-like receptor' because it upregulates cAMP. The lack of specific pharmacological blockers of these receptors has until now precluded straightforward analyses of their role in aversive learning. Impairment of aversive learning yields conflicting evidence with respect to this topic: while pharmacological blocking with vertebrate antagonists indicated that AmDOP2 receptors are necessary for aversive learning (Vergoz et al., 2007a), analyses of transcript levels of dopaminergic receptor genes suggested that impairment of aversive learning is associated with an increase of AmDOP2 receptors (Geddes et al., 2013). More experiments are necessary to elucidate whether and how these different receptors contribute to aversive learning.

An interesting twist to the study of aversive learning and dopaminergic signaling is the discovery that 20-hydroxyecdisone (20E), a metabolite of the steroid hormone ecdysone, which intervenes in insect development and reproduction (Riddiford et al., 2000), impairs aversive but not appetitive conditioning in bees (Geddes et al., 2013). This impairment seems to be achieved in part *via* the dopamine/ecdysone receptor gene AmGPCR19, which is the honeybee ortholog of the dopamine/ecdysone receptor gene 48 (DmDopEcR) identified in *Drosophila* (Srivastava et al., 2005). Thus, exogenous 20E injection determines both a reduction in AmGPCR19 levels and a decrease in aversive learning performance, therefore indicating that aversive learning in honeybees can be modulated by ecdysteroids (Geddes et al., 2013).

SER and the division of labor: behavioral syndromes and colony social organization

As social insects, honeybees exhibit a division of labor in which different tasks are accomplished by different groups of individuals, usually of different ages (Wilson, 1971). Several models have been proposed to explain why individuals within a social insect colony are differently biased to perform distinct tasks, resulting in task specialization (Beshers and Fewell, 2001). Among these models, the response-threshold model has played an influential role in the explanation of the division of labor in social insects. It posits that differences in sensitivity to external stimuli exist between individuals and that individuals highly sensitive to a given stimulus are prospective candidates for becoming specialized in tasks involving such a stimulus (Page and Erber, 2002). For instance, the well-established difference between nectar and pollen foragers has been explained in terms of their different sensitivity to sucrose (Page and Erber, 2002; Scheiner et al., 2004). These two groups do indeed exhibit differences in sucrose responsiveness, which is assessed by quantifying appetitive PER responses along a series of increasing concentrations of sucrose solution (Pankiw and Page, 1999). The lowest concentration at which the bee starts responding with a PER defines its sucrose responsiveness threshold. Nectar foragers exhibit higher thresholds (i.e. lower responsiveness) than pollen foragers, which exhibit lower thresholds and thus higher responsiveness (Page et al., 1998). Although this difference may appear counterintuitive at first sight, the currently accepted explanation is that nectar foragers are more selective when collecting nectar, and thus only respond to the highest sucrose concentrations, which provide the highest energy gain to the colony. Sucrose responsiveness thresholds vary with multiple factors such as age, caste, sex (Pankiw and Page, 1999), foraging experience, genotype, feeding status (Pankiw and Page, 2001) and season (Scheiner et al., 2003), among others.

The plethora of studies on sucrose responsiveness has led to the general idea that this behavioral trait can explain *per se* diverse behavioral responses to stimuli as different from sugar as odors or light (Scheiner et al., 2004; Erber et al., 2006). For instance, Page and colleagues (Page et al., 2006) state that, 'Bees who are sensitive to sucrose are also sensitive to stimuli of other modalities', so that 'Sucrose responsiveness can be used as a robust indicator for general differences of processing information in the central nervous system'. This suggestion could, however, be erroneous as the behavioral traits that have been related so far to sucrose responsiveness all have an appetitive framework in common, i.e. they are related to foraging behavior. In a drastically different framework, in which stimuli possess a hedonic value different from sucrose or its related context, would stimulus sensitivities correlate with sucrose sensitivity? In other words, do bees that exhibit high responsiveness to sucrose also display high responsiveness to an aversive stimulus?

To answer this question, Roussel et al. (Roussel et al., 2009) determined whether sucrose responsiveness in forager bees correlates with responsiveness to electric shocks of varying voltage. Like PER for sucrose, SER allows direct quantification of response thresholds to a stimulus that, in this case, is fully independent of a foraging context. PER to a logarithmic series of sucrose solutions of increasing concentration were measured in a first phase, and SER to a series of shocks of increasing voltage were measured in a second phase. In another group of bees, the reversed sequence (first shock, then sucrose) was employed. Neither the responses to the electric shocks nor the responses to the sucrose solutions differed significantly between these two groups, thus showing that the order of stimulation was irrelevant.

Pooled responses are shown in Fig. 6. As expected, bees significantly increased the PER to sucrose solutions of increasing concentration and, similarly, bees significantly increased the SER

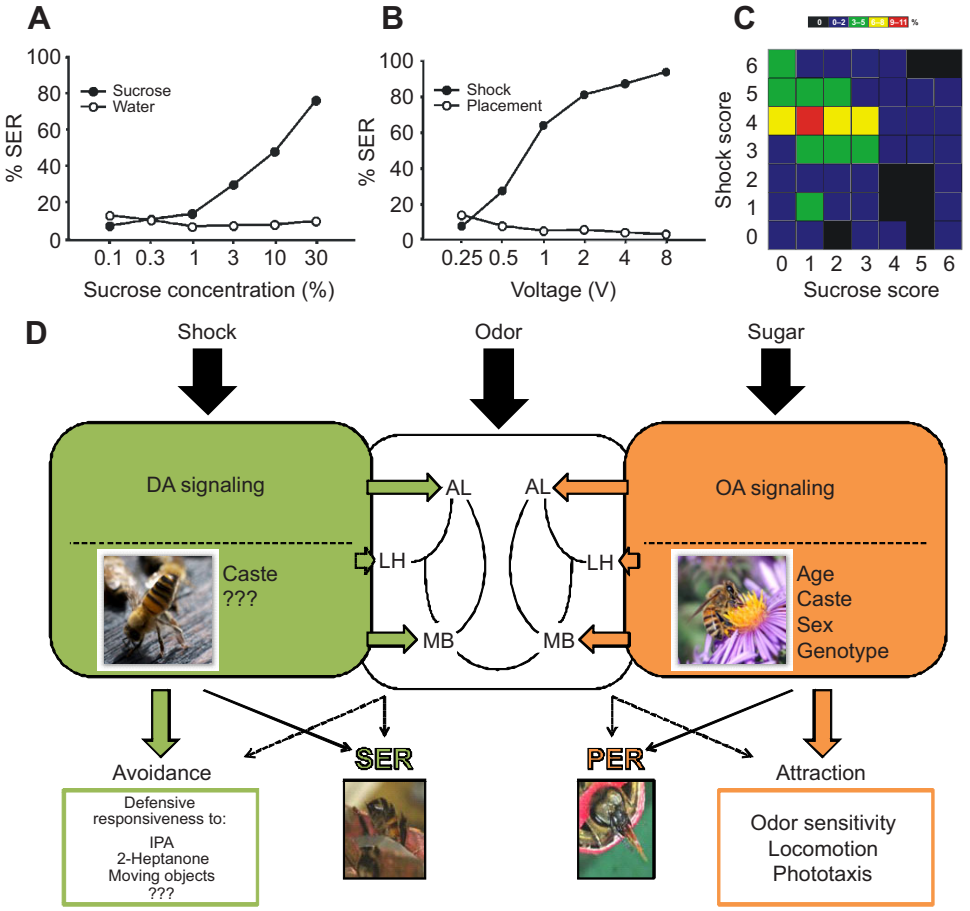

Fig. 6. Correlation between sucrose and shock responsiveness in honeybees. (A) Sucrose responsiveness. The percentage PER to a series of sucrose solutions of increasing concentration (N=198) or to the presentation of water (control) in the same bees. Bees showed an increase in their response to sucrose solution of increasing concentration. (B) Shock responsiveness of the same bees. The percentage SER to a series of shocks of increasing voltage and the same setup without shock delivery (control) in the same bees. Bees increased their responses to shocks of increasing voltage. (C) A 7×7 matrix of correlation between sucrose and shock responsiveness scores in the same bees. Scores varied from 0 (no response to any stimulus tested in the series) to 6 (responses to all six stimuli of the series). Colors assigned to each box represent the percentage of bees exhibiting a particular combination of sucrose and shock responsiveness scores. No significant correlation exists between sucrose and shock responsiveness scores (R=–0.03; t_{N-2}=–0.42; NS). A–C modified from Roussel et al. (Roussel et al., 2009). (D) Scheme of an 'appetitive' and an 'aversive behavioral syndrome' and their potential control of different aspects of bee behavior. Sucrose is a pertinent unconditioned stimulus for the former and electric shock is a pertinent unconditioned stimulus for the latter. While sucrose activates octopaminergic (OA) signaling in the bee brain, electric shock activates mainly dopaminergic (DA) signaling. Signals of these pathways convey reinforcing properties of their corresponding US to the olfactory circuit processing odor signals (AL, antennal lobe; LH, lateral horn; MB, mushroom body), thus mediating appetitive or aversive olfactory learning. Factors such as caste, age, pheromones, etc., modulate these learning processes. Unconditioned responses triggered by sucrose and shock are PER and SER, respectively, which may in turn be translated in motor performances of attraction and avoidance, respectively, towards stimuli such as odors, light, etc. IPA, isopentyl acetate.

to electric shocks of increasing voltage. The increase in PER and SER with sucrose concentration and voltage does not, however, answer the question of whether the bees responding more to concentrated sucrose are also those responding more to the highest voltages. To answer this question, both a sucrose responsiveness score and a shock responsiveness score were determined for each bee (Roussel et al., 2009). Scores were quantified as the sum of all responses made along the whole sequence of tested stimulations. For instance, a bee extending its sting at voltages from 0.5 to 8 V, i.e. in response to five out of the six voltages assayed, had a shock responsiveness score of 5 as it responded to five consecutive voltages. This bee also had a sucrose responsiveness score derived from its response to the six concentrations of sucrose solution. Scores may therefore vary from 0 (no response to any stimulus tested in the series) to 6 (responses to all six stimuli of the series) (Fig. 6C).

The results of this analysis are thus represented as a 7×7 matrix in which one axis is defined by sucrose responsiveness scores and the other axis by shock responsiveness scores (Fig. 6C). Colors assigned to each box represent the percentage of bees exhibiting a particular combination of sucrose and shock responsiveness scores. Fig. 6C shows no clear relationship between appetitive and aversive responsiveness, i.e. the correlation analysis performed on the two scores was non-significant. In other words, there is no correlation between responsiveness to two stimuli of opposing hedonic value such as sucrose and electric shock (Roussel et al., 2009).

In studies in which the PER was used, correlated responsiveness was found for stimuli that are related to the appetitive search for food in which bees engage during foraging activity (Scheiner et al., 2004; Humphries et al., 2005; Erber et al., 2006). It seems coherent that responsiveness to odors (which are characteristic of food sources) and to light (which elicits foraging flight), as well as motor

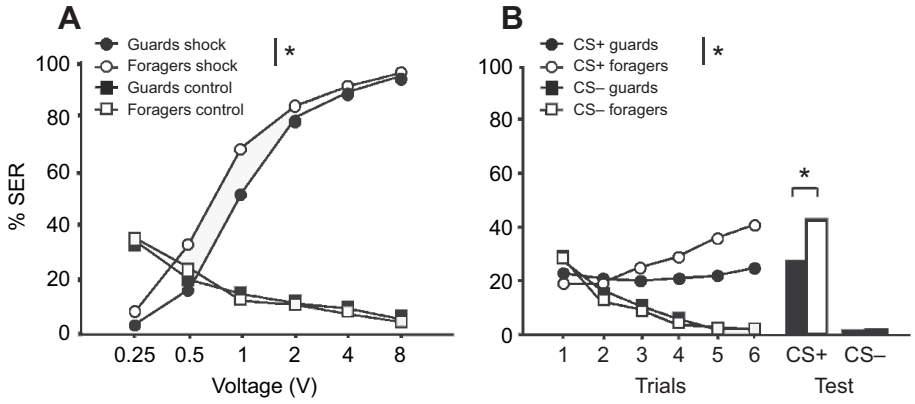

Fig. 7. Shock responsiveness and learning and retention performance of guard and nectar forager bees. (A) Guards (N=151) were less responsive to a series of shocks of increasing voltage than were foragers (N=205). SER responses of guards and foragers when placed in the setup without shock (control) are also shown. (B) The percentage SER of guards (N=105) and foragers (N=102) during differential SER conditioning (CS+ or CS–). Both groups learned to discriminate between punished and non-punished odors but foragers responded more to the CS+ and remembered it better 1 h after conditioning than did guards. *P<0.05. Modified from Roussel et al. (Roussel et al., 2009).

activity, are correlated in the same bees (Scheiner et al., 2004; Humphries et al., 2005; Erber et al., 2006). This variety of related sensitivities can be grouped in a 'foraging behavior syndrome' (Pankiw, 2005), defined as a set of correlated behaviors reflecting between-individual consistency in behavior across multiple foraging situations (Sih et al., 2004). Yet, this syndrome may just constitute a part of the complex behavioral tuning within a hive.

Several behavioral syndromes may coexist in an insect society. A 'defensive behavior syndrome' could be conceived, in which a correlated set of defensive traits could be linked to sensitivity to electric shock. For instance, responsiveness to shock could correlate with defensive responsiveness to alarm pheromone components such as isopentyl acetate (IPA), the main component of the sting pheromone (Boch et al., 1962), and 2-heptanone, an alarm substance released by mandibular glands (Shearer and Boch, 1965). Foraging and defensive syndromes would constitute independent insulated modules coexisting within the same individual and defining its tendency to act as a forager or as a defender (Roussel et al., 2009).

Shock sensitivity and olfactory SER conditioning

In Pavlovian learning, in which an animal learns that a CS acts as a predictor of the US, sensitivity to the US, which directly determines its salience for the animal, plays a crucial role in learning efficiency and rate (Rescorla and Wagner, 1972). Higher sensitivity to an US results in better learning performance as shown by studies relating sucrose sensitivity and appetitive PER conditioning; bees that are highly sensitive to sucrose show better appetitive learning performance (Scheiner et al., 1999; Scheiner et al., 2001a; Scheiner et al., 2001b; Scheiner et al., 2003; Scheiner et al., 2005). Does shock responsiveness affect olfactory aversive learning in bees in a similar way?

To answer this question, shock responsiveness scores were determined in a group of honeybee foragers (see above), which were then divided into two subgroups according to their scores: bees exhibiting the highest response selectivity and responding only to the highest shock voltages (score 1–3; 'low responsiveness group') and bees exhibiting generalized, non-selective responses to 4–6 of the voltages tested including lower ones ('high responsiveness group'). The next day, bees were trained in a differential conditioning procedure to discriminate an odor paired with a shock (CS+) from an odor not paired with a shock (CS–). Both groups of bees learned to discriminate the CS+ from the CS– and remembered this information 1 h later. Yet, the high responsiveness group showed a higher percentage of conditioned responses to the CS+ than the low responsiveness group. Responses to the CS– did not differ between groups. In the retention tests, bees of the high responsiveness group also responded more to the CS+ than did bees of the low responsiveness group while no differences were found for the CS–.

These results show that the more responsive a bee is to electric shocks, the better it learns to associate an odor with this noxious stimulus. Similarly, in the case of sucrose reinforcement, the more responsive a bee is to sucrose, the better it learns and memorizes CS–US associations in appetitive olfactory and tactile learning protocols (Scheiner et al., 1999; Scheiner et al., 2001a; Scheiner et al., 2001b; Scheiner et al., 2003; Scheiner et al., 2005). Taken together, these results underline the crucial role of US sensitivity for learning and retention performance as underlined by models of classical conditioning, where US salience directly affects learning rate (Rescorla and Wagner, 1972).

Shock sensitivity, olfactory SER conditioning and caste specialization within the hive

We have seen so far that honeybee foragers exhibit a shock responsiveness that does not necessarily correlate with sucrose responsiveness, and that their US sensitivity directly determines their learning success in olfactory SER conditioning. Do these principles apply to other honeybee castes and do castes differ from each other in terms of these variables?

To answer this question, a first study focused on a comparison between guards and foragers in terms of shock responsiveness and aversive learning. Foragers were collected upon arrival at a feeder containing sucrose solution to which they were previously trained, thus ensuring that they were real nectar foragers. Guards were collected at the hive entrance after an attack had been elicited by means of a mechanical disturbance. One day after determining the shock responsiveness scores of these two groups of bees, they were subjected to differential conditioning. Retention tests were again performed 1 h after the last conditioning trial.

Fig. 7A shows that shock responsiveness differed significantly between guards and nectar foragers, the responses of foragers to shocks being generally higher than those of guards, especially for lower voltages. Thus, guards are less sensitive to electric shocks than are nectar foragers. Fig. 7B shows that both guards and nectar foragers learned to discriminate between the CS+ and the CS– and remembered the aversive association 1 h later. Yet, although both groups responded similarly to the CS– during conditioning, at the end of training, nectar foragers responded significantly more to the CS+ than did guards. The same difference was found in the retention tests as nectar foragers remembered the CS+ significantly better than did guards (which reflected their better acquisition) but did not differ in their response to the CS–.

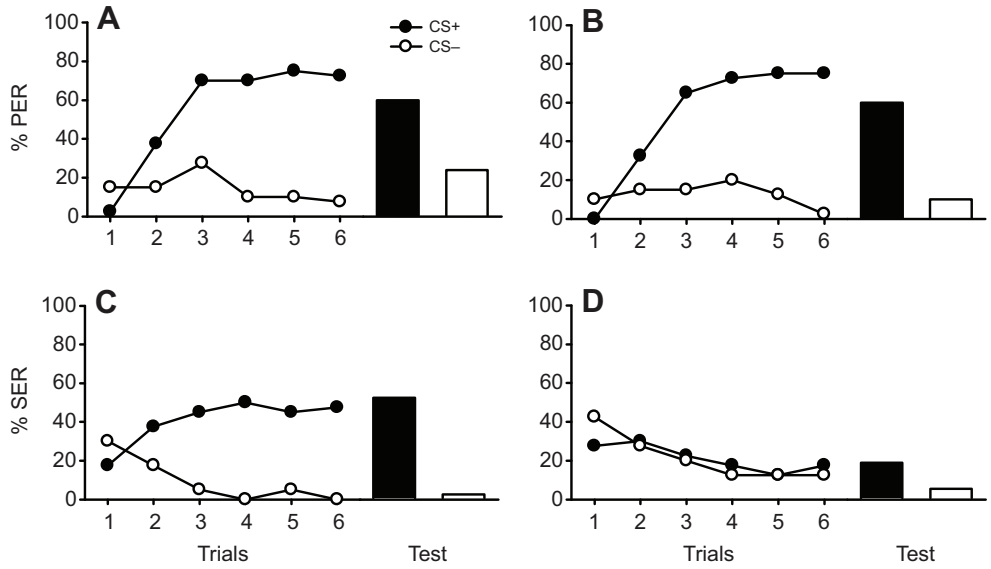

Fig. 8. Effects of queen mandibular pheromone (QMP) on appetitive learning (A,B) or aversive learning (C,D) in 6-day-old workers. (A,B) Associative olfactory conditioning of the PER in control (non-exposed) bees and bees exposed to QMP, respectively. Bees were trained to discriminate between an odorant paired with sucrose (CS+) and a non-reinforced odorant (CS–). After 12 conditioning trials (six CS+ and six CS–), control bees clearly learned to discriminate between the two odorants and remembered this 1 h after the last conditioning trial (A). QMP-exposed bees also learned to discriminate between the two odorants and remembered this 1 h after the last conditioning trial (B). (C,D) Associative olfactory conditioning of the SER in control (non-exposed) bees and bees exposed to QMP, respectively. After 12 conditioning trials, control bees learned to discriminate between the two odorants and remembered this 1 h later (C). QMP-exposed bees did not learn to discriminate between the two odorants; 1 h after the last conditioning trial, the percentage of bees responding to the two odorants was similar (D). Modified from Vergoz et al. (Vergoz et al., 2007b).

Thus, the more responsive, and presumably more sensitive, foragers are the ones learning and remembering better aversive associations. Although this result appears surprising, it may be adaptive for guards to be less sensitive, and presumably more tolerant, to noxious stimuli. Accordingly, they would assign low values to an aversive reinforcement, thus determining lower acquisition and retention performance. Such a low sensitivity of guards to noxious stimuli may indeed be adaptive for honeybees, as defensive responses are costly for the colony (especially when recruitment takes place), and defensive responses should not be triggered by any kind of aggression, but rather by situations that are potentially dangerous for the colony.

Neural-based explanations could account for the difference found between guards and foragers in shock responsiveness and aversive conditioning. Dopamine levels in the bee brain depend on age (Taylor et al., 1992; Schulz and Robinson, 1999) so that older bees have more dopamine in their brains. Foragers, which are generally older than guards, are more sensitive to shock and thus more likely to learn aversive associations than guards (Roussel et al., 2009). Nurse bees are the youngest adult members of the colony and stay in close contact with the queen. Dopamine levels are even lower in nurses than in guards and foragers (Taylor et al., 1992; Schulz and Robinson, 1999) and, as a consequence, shock sensitivity should be lower and olfactory SER conditioning less successful in these bees. In addition, nurses are exposed to queen mandibular pheromone (QMP) *via* their close contacts with the queen. QMP is a chemical blend that has priming and acute effects on social control within the colony (Sandoz et al., 2007). Among these effects, QMP induces young workers to feed and groom the queen and primes bees to perform colony-related tasks (Slessor et al., 1988; Keeling et al., 2003).

Olfactory SER conditioning of nurse bees has been studied in relation to the presence of the queen and QMP (Vergoz et al., 2007b). One of the key components of QMP, homovanillyl alcohol (HVA), bears a striking structural resemblance to dopamine. The presence of this compound within the pheromone blend suggested that dopamine function in the brain of recipient young bees might be affected by exposure to QMP (Beggs et al., 2007). Indeed, exposure to QMP, and more precisely to HVA, affected dopamine levels, levels of dopamine receptor gene expression and cellular responses to this amine in young worker bees. These results show that dopamine levels in the bee brain depend not only on age but also on contact with QMP (Beggs et al., 2007).

How does this inhibition of dopaminergic signaling affect aversive olfactory learning in young bees? To answer this question, Vergoz and colleagues (Vergoz et al., 2007b) examined the impact of QMP on associative olfactory learning in young bees (6 days old) exposed to QMP from the time of adult emergence. Bees of the same age maintained under identical conditions but without exposure to QMP were used as controls. These two groups were in turn subdivided into two groups, one trained following appetitive PER conditioning to discriminate an odor reinforced with sucrose from a non-reinforced odor (Fig. 8A,B) and another trained following aversive SER conditioning to discriminate an odor reinforced with shock from a non-reinforced odor (Fig. 8C,D) (Vergoz et al., 2007b). Both exposed and non-exposed bees learned the appetitive discrimination and showed retention 1 h later (Fig. 8A,B). Interestingly, while non-exposed young bees (Fig. 8C) learned the aversive discrimination and remembered it 1 h later, bees of the same age exposed to QMP failed to show aversive learning and retention (Fig. 8D). Thus, QMP suppresses aversive olfactory learning in young bees but leaves their appetitive learning intact (Vergoz et al., 2007b). A possible interpretation of these results is that the inhibition exerted by QMP on aversive learning increases the probability that young nurses remain in close contact

with their queen by impeding aversive experiences around her (Vergoz et al., 2007b).

The effect of QMP on the associative learning of young bees resembles that of ecdysteroid hormones like 20E when injected into adult bees (see above). Indeed, 20E impairs aversive but not appetitive learning. Moreover, greater impairment of aversive learning when bees are 2 days old correlates with higher levels of endogenous ecdysone (Hartfelder et al., 2002). Recent results have shown, in addition, that like 20E (see above), QMP impairs aversive learning, inducing a concomitant reduction of the AmGPCR19 receptor (Geddes et al., 2013). The ecdysone/dopamine signaling pathway would therefore be implied in aversive US signaling as well as in social regulation.

Taken together, these results show how aversive olfactory SER conditioning has helped in uncovering unsuspected aspects of social organization and division of labor within the hive. These articulate on specific and variable stimulus sensitivities, which in turn reflect complex regulation of biogenic amine levels and neural signaling, which determine not only distinct aversive learning performance but also different behavioral roles and syndromes (i.e. sets of correlated behaviors across situations) (see Sih et al., 2004) within the hive.

Other sensory variants of aversive SER conditioning

The experiments presented so far used olfactory SER conditioning to answer questions focusing on various topics, from learning and memory to social organization in honeybees. Besides this rich spectrum of research, SER conditioning has also recently been achieved using visual rather than olfactory stimuli as CS (Mota et al., 2011b).

In this protocol, which allows the study of visual learning and memory in intact harnessed bees in the laboratory, two visual stimuli are used as CS+ and CS–. Bees learn to discriminate between CS+ and CS– by using, for instance, chromatic cues (Fig. 9). It should be noted, however, that acquisition levels in visual SER conditioning can be lower than in olfactory SER conditioning. Further improvements are thus necessary in visual SER conditioning to enhance the efficiency of the protocol.

Despite this, the fact that the SER can be visually conditioned opens new doors for accessing the neural correlates of visual learning and memory in honeybees. Indeed, visual learning in bees has been studied for almost 100 years using almost exclusively free-flying bees conditioned to choose visual targets paired with sucrose solution (von Frisch, 1914; Avarguès-Weber et al., 2012). This approach revealed numerous aspects of an organism, the honeybee, which has emerged as a model for the study of visual processing because of its color vision and visual orientation capabilities (Menzel and Backhaus, 1991; Giurfa and Menzel, 1997; Srinivasan, 2010; Avarguès-Weber et al., 2012). Coupling behavioral measures of visual perception and invasive recordings of neural activity at the level of visual centers or pathways in the bee brain has so far been impossible. Important advances have recently been achieved in the neurophysiological study of visual processing in the bee brain thanks to the advent of *in vivo* optical imaging of visual neuron populations (Mota et al., 2011c; Mota et al., 2013). Visual SER conditioning could provide the basis for the necessary coupling between behavioral and neurophysiological measurements, thus allowing a qualitative improvement of our knowledge of the neural mechanisms underlying visual perception and their intrinsic plasticity.

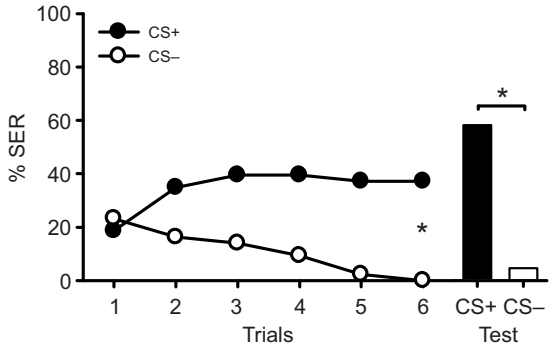

Fig. 9. Visual SER conditioning in harnessed bees: bees learn a visual discrimination based on aversive reinforcement. The percentage SER during six blocks of conditioning trials and in retention tests for bees trained to discriminate between a blue and a green light, which differed in their chromatic properties (N=43). Bees significantly increased SER to the CS+ and decreased SER to the CS– in the course of conditioning. During retention tests, they also responded significantly more to the CS+ than to the CS–. Modified from Mota et al. (Mota et al., 2011a).

Conclusion

Here, we have reviewed recent and current developments of aversive conditioning in honeybees based on the SER. This protocol, which has recently been established, gives access to punishment learning in honeybees, which have been a traditional model for studying reward learning. The fact that bees are harnessed but nevertheless exhibit learning and retention of CS–electric shock associations has opened new research avenues to uncover neural principles of associative, aversive learning in a framework that is distinct from appetitive behavioral contexts.

Starting with the demonstration that individual bees learn odor–shock associations, a pluridisciplinary body of research has been developed spanning questions on honeybee social organization, learning and memory, neurobiology and behavior. At the crossroad of these different research avenues is the olfactory SER conditioning protocol, which constitutes, in our opinion, a significant contribution to the study of different aspects of honeybee behavior. We expect through it to overcome the mono-faceted view offered for 50 years by the equivalent protocol in the appetitive domain, the olfactory conditioning of PER. The results presented in this review show that the endeavor was successful and offers further promising perspectives.

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Author contributions

M.G. and S.R.T. contributed equally to the conception, design and execution of the study, interpretation of the findings, and drafting and revising the article.

Competing interests

No competing interests declared.

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TITLE : The Role of Dopaminergic Neurons in Aversive Learning in Honeybees Apis m.

ABSTRACT :

In Pavlovian learning animals learn to associate a conditioned stimulus (CS) and an unconditioned stimulus (US). The honey bee *Apis mellifera* is a well-established model for the study of this learning form. In a novel Pavlovian protocol, bees associate an odorant (CS) with an electric shock (US) and the conditioned response produced to the learned odorant is the sting extension response. This aversive learning depends on the biogenic amine dopamine (DA), which mediates the reinforcing properties of the electric shock. Yet, the neural mechanisms and architecture underlying aversive US perception remain largely unknown. We combined behavioural, pharmacological, neuroanatomical and molecular approaches to characterize dopaminergic circuits and signalling in the bee brain.

Firstly, we studied innate aversive responsiveness and its neurotransmitter basis by quantifying the sting extension reflex (SER) to a series of increasing voltages and injecting into the brain pharmacological antagonists of candidate neurotransmitters. We found that DA and serotonin act as down-regulators of SER so that blockade of these amines increased US responsiveness. We thus provided the first evidence of the involvement of these biogenic amines in the *central control* of sting responsiveness to noxious stimuli. Our results suggested that different classes of dopaminergic neurons exist in the bee brain: an instructive class mediating aversive conditioned stimuli in associative learning and a global gain-control class regulating responsiveness to noxious stimuli.

Secondly, we characterized the neuroanatomy of DA neurons in the bee brain. We performed immunocytochemistry using an antibody against tyrosine hydroxylase, a DA precursor, and fluorescence confocal microscopy to obtain a 3D reconstruction of DA neurons. Our results confirmed prior reports showing dense innervations in the higher-order neuropil, the mushroom body (MB), which is heavily innervated by 3 relatively big clusters of DA neurons surrounding it. Smaller DA processes were also found in the protocerebral and antennal lobes. These DA neurons contact the olfactory circuit at different stages and provide the neural basis for DA involvement in olfactory signalling. We also provided the first evidence of previously unknown DA processes in the optic lobes and identified a novel DA cluster at the origin of these processes, which may provide instructive aversive signals for visual cues.

Thirdly, we studied the molecular mechanisms underlying aversive responsiveness and associative learning to analyze whether aversive learning induces variations in the expression of specific receptor genes, thereby changing the responsiveness to punishment. We quantified the SER to shocks before a differential aversive conditioning and 3 days after conditioning, after measuring the presence of aversive long-term memory. We found that aversive olfactory learning induces a long-term decrease in shock responsiveness for shocks that were lower than the US. Using laser-capture micro dissection, we collected specific populations of MB's Kenyon cells and measured long-term changes in receptor-gene expression induced by aversive learning/retrieval. We found that aversive learning (but not retrieval) promotes a specific long-term increase in the expression of the dopaminergic receptor genes *Amdop2* and partially *Amdop1*. This variation correlates with the long-term decrease in shock sensitivity resulting from learning. Our results suggest that specific molecular changes - here dopaminergic receptor expression - mediate a decrease in behavioural responsiveness to reinforcing stimuli that lose their 'surprising effect' as a consequence of conditioning.

Our studies span a broad spectrum spanning from behavioural to molecular analyses and provide a novel, integrative view of dopaminergic signalling in the bee brain. They yield new insights into the neural/molecular representation of aversive US in an insect brain.

KEYWORDS : learning, memory, conditioning, honey bee, Sting Extension Reflex (SER), dopamine

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TITRE : Le Rôle des Neurones Dopaminergiques dans l'Apprentissage Aversif chez Abeilles Apis m.

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LIEU ET DATE DE SOUTENANCE : Toulouse, le 30/09/2014

RESUME :

Dans un apprentissage Pavlovien les animaux apprennent à associer un stimulus conditionné (SC) à un stimulus inconditionné (SI). L'abeille Apis mellifera est un modèle bien établi pour étudier cette forme d'apprentissage. Dans un nouveau protocole Pavlovien, les abeilles associent une substance odorante (SC) à un choc électrique (SI) et la réponse conditionnée produite à l'odorant appris est le reflexe d'extension du dard (RED). Cet apprentissage aversif dépend de l'amine biogène dopamine (DA) qui assure la médiation des propriétés de renforcement du choc électrique. Nous avons combiné plusieurs approches pour caractériser les circuits DA et leur signalisation dans le cerveau de l'abeille.

Tout d'abord, nous avons étudié la réponse innée aversive et ses substrats en quantifiant le RED à une série de chocs de tension croissante et en injectant des antagonistes de neurotransmetteurs dans le cerveau. Nous avons trouvé que la DA et la sérotonine régulent negativement RED et le blocage de ces amines augmente la réponse. Nous avons fourni la première preuve de l'implication de ces amines biogènes dans le contrôle central de la réponse aux stimuli nocifs. Nos résultats proposent que différentes classes de neurones DA existent dans le cerveau de l'abeille: une classe « instructive » qui code pour l'aversion des stimuli conditionnés dans l'apprentissage associatif et une classe de « commande générale » qui diminue la réponse de la perception des stimuli nocifs.

Ensuite, nous avons caractérisé la neuroanatomie des neurones DA dans le cerveau de l'abeille. Nous avons pratiqué l'immunocytochimie en appliquant un anticorps dirigé contre la tyrosine hydroxylase, un précurseur de la DA, et la microscopie confocale pour reconstruire les neurones DA en 3D. Nos résultats ont confirmé des données antérieures montrant de l'innervation dense dans un neuropile d'ordre supérieur, le corps pedonculés (MB) étant fortement innervé par 3 grands groupes de neurones DA. Des processus DA étaient également trouvés dans les lobes protocerebraux et les lobes antennaires. Ces neurones sont connectés au circuit olfactif à différentes étapes et constituent la base neuronale pour l'étiquetage DA de différentes formes de signalisation olfactive. Nous avons également trouvé la première preuve de l'existence de prolongements DA dans le lobe optique et identifié leur nouveau groupe DA.

Enfin, nous avons étudié les mécanismes moléculaires qui sous-tendent la réponse aversive et l'apprentissage associatif en evaluant si l'apprentissage aversif induit des variations dans le niveau d'expression des gènes des récepteurs spécifiques ce qui modifie la sensibilité d'aversion aux stimuli aversifs. Nous avons quantifié le RED aux chocs avant un conditionnement et trois jours après le conditionnement, après la mesure de la mémoire d'aversion à long terme. Nous avons trouvé que l'apprentissage olfactif aversif induit une diminution à long terme de la réponse au choc. En utilisant la microdissection au laser, nous avons collecté des populations particulières de cellules de Kenyon puis mesuré les changements à long terme dans l'expression du récepteur du gène. Nous avons trouvé que l'apprentissage aversif seulement favorise une augmentation spécifique à long terme de l'expression des gènes du récepteur dopaminergique Amdop2 et partiellement d'Amdop1. Cette variation est corrélée avec la diminution à long terme de la réponse au choc résultant de l'apprentissage. Nos résultats proposent que les changements moléculaires spécifiques de l'expression du récepteur DA induisent une diminution de la réponse comportementale ce qui pourrait constituer une perte de l'«effet de surprise» résultant du conditionnement.

Nos études couvrant les niveaux comportementale, pharmacologiques, neuroanatomiques et moléculaire, afin de fournir une nouvelle vision intégrée de la signalisation DA dans le cerveau de l'abeille c.à.d. de la représentation neural du SI aversif dans un cerveau d'insecte.

MOTS-CLES : apprentissage, mémoire, conditionnement, abeille, Reflex d'Extension du Dart (RED), dopamine

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