Review

Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honeybees

Martin Giurfa[1,2,4] and Jean-Christophe Sandoz[3]

[1]*Université de Toulouse, UPS, Research Centre for Animal Cognition, 31062 Toulouse Cedex 9, France;* [2]*CNRS, Research Centre for Animal Cognition, 31062 Toulouse Cedex 9, France;* [3]*Laboratory Evolution, Genomes and Speciation, CNRS, 91198 Gif-sur-Yvette, France*

The honeybee *Apis mellifera* has emerged as a robust and influential model for the study of classical conditioning, thanks to the existence of a powerful Pavlovian conditioning protocol, the olfactory conditioning of the proboscis extension response (PER). In 2011, the olfactory PER conditioning protocol celebrates 50 years since it was first introduced by Kimihisa Takeda in 1961. Here, we review its origins, developments, and perspectives in order to define future research avenues and necessary methodological and conceptual evolutions. We show that olfactory PER conditioning has become a versatile tool for the study of questions in extremely diverse fields in addition to the study of learning and memory and that it has allowed behavioral characterizations, not only of honeybees, but also of other insect species, for which the protocol was adapted. We celebrate, therefore, Takeda's original work and prompt colleagues to conceive and establish further robust behavioral tools for an accurate characterization of insect learning and memory at multiple levels of analysis.

Classical conditioning (Pavlov 1927) is a form of conditioning in which a subject learns to associate a neutral stimulus (the "conditioned stimulus," or CS), which does not originally elicit a behavioral response, with a stimulus of biological significance (the "unconditioned stimulus," or US), which elicits an innate, often reflexive, response. Through this association, the originally neutral stimulus acquires the capacity to elicit a conditioned response.

Decades of research on animal learning and memory have established some invertebrates (e.g., the sea hare, *Aplysia californica*, the fruit fly, *Drosophila melanogaster*, the honeybee, *Apis mellifera*) as standard models for the study of classical conditioning (Giurfa 2007b; Menzel et al. 2007). This success can be attributed to the fact that these animals can learn nonassociative as well as Pavlovian and operant associations and possess relatively simple nervous systems that allow retracing of these phenomena to the cellular and molecular levels in different kinds of laboratory preparations. Among these invertebrates, the honeybee, *Apis mellifera*, has emerged as a robust and influential model for the study of classical conditioning (Hammer 1993, 1997; Menzel 1999, 2001; Giurfa 2003, 2007a). This is mainly due to the existence of a powerful Pavlovian conditioning protocol, the olfactory conditioning of the proboscis extension response (PER) (Takeda 1961; Bitterman et al. 1983), which has been repeatedly used for the study of appetitive learning and memory at multiple levels of analysis, from behavioral to molecular ones.

This year, 2011, constitutes a jubilee year for researchers interested in honeybee learning and memory: The olfactory PER conditioning protocol celebrates 50 years, reaching an age of maturity since it was established in 1961 by a Japanese researcher, Kimihisa Takeda (Takeda 1961), inspired by the pioneering work of his supervisor, Matsutaro Kuwabara (Kuwabara 1957). Kuwabara took the first steps toward the establishment of this protocol (see below) by noticing that associative learning could be studied in harnessed bees using a sucrose solution as the US and colored lights as the CS (see below). Later, Takeda adapted Kuwabara's protocol in order to use odors as CS. Without knowing that dozens of laboratories all over the world would adopt this olfactory protocol and that hundreds of scientific papers during the next five decades would be based on it, Takeda established the foundations of a conditioning protocol that, even though it was not perfected until 20 years later (Bitterman et al. 1983), became one of the most robust and reliable tools to date for the study of invertebrate learning and memory. We, therefore, considered that it is more than appropriate to honor this powerful conditioning tool and review its origins, developments, and perspectives in order to define future research avenues and necessary methodological and conceptual evolutions.

The protocol

In the current and standardized version of the protocol (Felsenberg et al. 2011), honeybee workers are individually harnessed in small tubes from which only the head protrudes (Fig. 1A). Once harnessed, bees are usually rested for 2–12 h so that they become habituated to the experimental situation and to increase their feeding motivation. In such conditions, hungry harnessed bees extend their proboscis (PER) if their antennae, tarsi, or mouthparts are contacted with sugar solution (the unconditioned stimulus) presented in a pipette or on a toothpick. Odorants used as conditioned stimuli are delivered to the antennae of the bee using syringes containing a filter paper soaked with a few microliters of the chemical substance, or even better, by means of a controlled olfactometer delivering a constant clean airflow to the bee in which a short pulse of odorant can be injected through a valve system controlled by a computer. This is probably one of the reasons for the success of this protocol: It admits a high-tech expensive version, but also a low-cost version accessible to everyone. During a conditioning trial, the bee receives a so-called forward pairing of CS and US: A few seconds after odorant onset (usually 3 sec, an inter-stimulus interval ensuring best retention performances) (see Menzel et al. 1993), the sugar solution (US) is delivered to

[4]Corresponding author.
E-mail giurfa@cict.fr.
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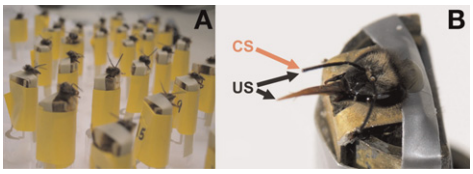

Figure 1. Conditioning of the proboscis extension response in restrained honeybees (Takeda 1961). (*A*) Honeybees placed individually in metal holders are awaiting conditioning. Small pieces of tape restrain the bees without harming them, so that only the antennae and mouthparts can freely move. (*B*) Conditioning of the proboscis extension response on restrained bees. The PER is a response shown by bees when their antennae, tarsi, or mouthparts are contacted with sucrose solution. During conditioning, an odor (conditioned stimulus) is presented in temporal association with sucrose solution to the antennae and to the proboscis (unconditioned stimulus). After conditioning, the odor CS, which initially did not evoke any response, triggers the PER.

the antennae and then to the proboscis, allowing the bee to lick the solution (Fig. 1B). Through this pairing, bees learn to associate the CS with the US, and as a consequence, they exhibit conditioned PER to future presentations of the odor alone (Takeda 1961; Bitterman et al. 1983).

The origins

Long before research on PER conditioning started, it was well-known that the proboscis extension response 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.

Later, a Japanese researcher, Matsutaro Kuwabara, who had worked with Karl von Frisch, realized that this appetitive response could be conditioned using visual stimuli as CS (Kuwabara 1957). Interestingly, this idea was probably irrelevant for von Frisch himself, as, when 10 years later he published his seminal book, *The dance language and orientation of bees* (von Frisch 1967), he did not mention Kuwabara's work despite describing that touching gustatory receptors with sugar solution elicits the extension of the proboscis in various insect species (von Frisch 1967).[5] Even more astonishing, Takeda's work on olfactory PER conditioning (Takeda 1961) was already available when von Frisch's book was published. Yet, von Frisch made no mention of it.[6]

Kuwabara reported that the proboscis extension response of immobilized honeybees could be conditioned using colored lights as CS and sucrose solution delivered to the tarsi as US (Kuwabara 1957). However, Kuwabara's work did not receive broad attention as shown by the fact that almost 50 years passed before other researchers published results on honeybee visual conditioning using Kuwabara's method (Hori et al. 2006, 2007). This lack of popularity was probably due to the fact that Kuwabara's results could not be reproduced for many years. It was only a few years ago that other Japanese researchers realized that the critical procedural aspect to follow was to cut the antennae of the bees prior to conditioning (Hori et al. 2006, 2007). Indeed, Kuwabara wrote "en passant" 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. Kuwabara 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. He, therefore, 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 what an experimenter wants. 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, 2007) may be related to this fact. Indeed, it has been recently shown that antennae deprivation reduces sucrose responsiveness when measured through tarsal stimulation (de Brito Sanchez et al. 2008), probably leading to a reduction of US value and of acquisition and retention performances.

Takeda's original protocol and results

We do not know if these criticisms were the basis for Takeda's attempt to use odors as CS. To introduce his study, Takeda briefly mentioned Kuwabara's work and wrote that "it seems valuable to compare the characteristics of these responses[7] in an invertebrate (which has a relatively simple nervous system) with the well-known phenomena in mammals, using classical conditioning methods" (Takeda 1961). In this way, he introduced the comparative dimension of honeybee conditioning, which has been extremely inspiring in the last five decades for studies of invertebrate learning and memory (for recent reviews, see Menzel and Giurfa 2001; Giurfa 2007b; Menzel et al. 2007).

Takeda clipped bees by the wings, and then delivered odorants by means of capillaries placed close to the bees' antennae. Sucrose solution (1.5 M) was delivered to the tarsi to elicit PER and then to the proboscis so that the bees could drink the solution. Today, we know that stimulation of the tarsi was not the best choice as sucrose sensitivity is much lower on the tarsi than on the antennae (de Brito Sanchez et al. 2008). In addition, since the bees were only immobilized by the wings, they probably exhibited permanent movements, thus rendering sucrose delivery difficult. This aspect was later improved by inserting the entire body of the bee within a container tube from which only the head protrudes, a method introduced by Vareschi (1971) (see below) (Fig. 1A).

As was common practice 50 years ago, Takeda did not report any acquisition, retention, or extinction curves, nor did he provide any statistical analysis of PER responses. He only presented tables with the raw data of single bees. For some of the bees, data were not complete, so that it would not be appropriate to pool them with those of bees that completed the whole conditioning sequence; yet, when considering only data from the latter type, it is possible to represent acquisition curves from Takeda's original work (Fig. 2A,B). The sample sizes are extremely low ($n = 5$ for bees conditioned to hydroxycitronellal [Fig. 2A]; and $n = 7$ for bees conditioned to citral [Fig. 2B]) compared to today's standard ($n > 40$). However, clear acquisition can be seen for both odorants paired with sucrose.

Despite data paucity, lack of statistics, absence of controls, representative sample sizes, and strict protocols,[8] this work laid down the experimental principles and the scientific questions that would serve as a basis for future, more controlled research

[5]See von Frisch 1967, p. 517. Von Frisch mentions therein Kuwabara's work on chemoreception but omits mentioning the conditioning experiments performed by this researcher.
[6]As for Kuwabara, von Frisch only mentions Takeda's work on hygro- and chemoreception but omits mentioning the conditioning experiments performed by this researcher.

[7]He meant "conditioned responses."
[8]For instance, Takeda would present bees with the same test situation—e.g., respond to a novel, nonconditioned odor—after different numbers of conditioning trials without verifying that the acquisition plateau reached by all the bees before the test was the same.

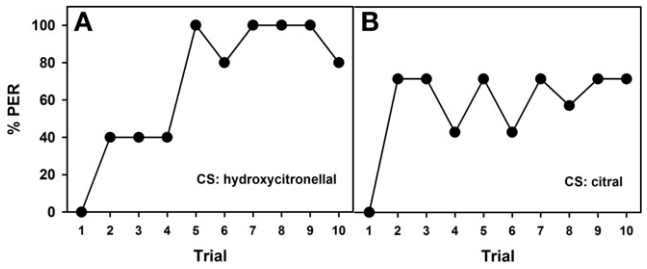

Figure 2. First acquisition performances in an olfactory PER conditioning experiment, as reported by Takeda (1961). (*A*) Percentage of bees presenting the PER to the odor in the course of a 10-trial associative conditioning procedure with hydroxycitronellal as CS ($n = 5$ bees). Within five associative trials, the CS, which did not initially trigger any response, induces PER in all the bees. (*B*) Performance of bees in a similar experiment with citral as CS ($n = 7$ bees). Here, performance reaches its maximum after a single trial, saturating at ~70% responses.

on honeybee learning and memory. Takeda's paper introduced data on extinction learning (including spontaneous recovery), stimulus generalization and discrimination, conditioned inhibition, and second-order conditioning.

Extinction is a form of learning in which animals learn that a previous reinforced CS is no longer reinforced (Pavlov 1927). Takeda's experiments showed—with the limitations already mentioned—that extinction occurred after 10 or more unrewarded presentations of the odor CS. He verified that the unconditioned response to sucrose still occurred when bees were no longer responding to the CS, thus precluding motor fatigue or sensory adaptation. He also showed spontaneous recovery following extinction and concluded that spontaneous recovery is a general phenomenon, observable both in mammals and in the bee. Takeda also addressed conditioned inhibition, another learning form studied by Pavlov (1927). In a first phase, animals learn to respond to a CS1 paired with the US (CS1+). Then, in a second phase, they experience trials with a nonreinforced CS1/CS2 compound (CS1CS2−) interspersed with the CS1+ trials. In this case, animals learn to inhibit their response to CS1 in the compound due to the presence of CS2, which acts as a conditioned inhibitor, while they keep responses to CS1 intact when it is presented alone (CS1-only trials). Takeda took care to choose as CS1 and CS2 two odors for which generalization was—after his experiments—nonexistent and showed that conditioned inhibition can take place in bees. In yet another experiment, he provided evidence for second-order conditioning (also studied by Pavlov [1927]). In such a procedure, a CS (CS2) is learned through its association with another CS (CS1) (Fig. 3B) which has been previously paired with the US (Fig. 3A). Figure 3 shows evidence of second-order conditioning from seven bees, even if, as in most of Takeda's experiments, controls were absent, a fact acknowledged by Takeda himself (see p. 177 of his work).

When discussing his work, Takeda pointed out two important facts that would become fundamental research avenues for several generations of researchers: (1) the necessity of exploring learning phenomena in an across-species manner; and (2) the fact that bees should be endowed with memory as the information gained through learning could be retrieved even two days after the training (Takeda 1961).

The protocol perfected: Bitterman and Menzel's study

Randolf Menzel is probably the researcher who has provided the richest insights into the biology of learning and memory in honeybees using the olfactory conditioning of PER (Menzel 1999). Menzel became aware of this protocol through the experiments carried out by Ekkehard Vareschi, a researcher at the Max Planck Institute of Behavioral Physiology in Seewiesen. Vareschi was interested in olfactory discrimination and generalization in honeybees. He used the PER conditioning protocol to ask bees about similarities and differences between conditioned odors and other odors (Vareschi 1971). Vareschi had already made important modifications to Takeda's conditioning protocol, like placing each bee within an individual tube and standardizing stimulations by developing a motorized carousel that applied odor and sucrose stimuli automatically. However, it was a seminal paper by Randolf Menzel, Jeff Bitterman and coworkers which definitively established PER conditioning as a standard protocol for studying learning and memory processes in bees (Bitterman et al. 1983). In this paper, the authors not only provided a standard controlled protocol for carrying out PER conditioning experiments but, most importantly, reconciled this approach with the current state-of-the-art methods in the domain of experimental psychology. For the first time, experiments with adequate sample sizes included all the necessary experimental controls, backed up by adequate statistical tests, for proving that this conditioning is associative in nature and is based on Pavlovian—and not on operant—associations. They thus showed that the CS-US association is formed only when CS and US are presented in close temporal association (paired group) but not when they are presented separately (unpaired group) (Fig. 4). In addition, they used an omission experiment to prove that performance is subtended by the CS-US association and not by the formation of an operant association between the response itself (PER) and the reward. The experiment clearly showed that, even when reward was omitted, when the bee extended the proboscis to the odor CS, conditioning takes place. Thus PER conditioning depends on CS-US associations, the hallmark of classical conditioning.

PER conditioning and the study of olfactory perception

One of the central questions in sensory neuroscience is how animals perceive the world. In bees, researchers have attempted to understand the rules governing olfactory perception, as, for instance, the characteristics of odor molecules determining if they would be perceived as similar or dissimilar by the bees. Initially, free-flying bees were used, as in experiments in which Karl von

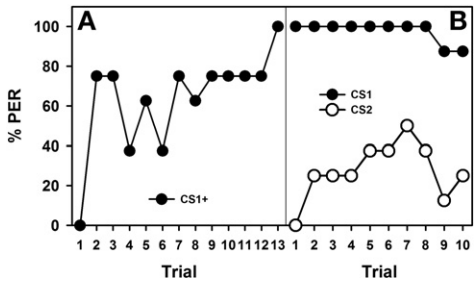

Figure 3. First observation of second-order conditioning in honeybees, as reported by Takeda (1961). The experiment consists of two phases. (*A*) In the first phase, bees learn to associate an odorant (CS1) with sucrose solution. In the course of training, performance to the CS1 increases from 0% to 100% ($n = 7$ bees). (*B*) In the second phase, a novel odorant (CS2) is now associated with the previously reinforced odorant (CS1). Bees start responding to CS2 after a few CS2-CS1 associations. This shows that CS1, after its association with sucrose, acted as a second-order reinforcement for CS2.

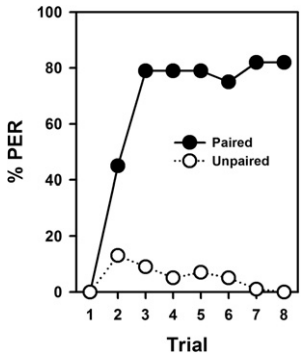

Figure 4. Demonstration of the associative quality of PER conditioning, as reported by Bitterman et al. (1983). The performances of two groups of bees are compared. Bees from the paired group receive CS-US presentations in close temporal association (forward pairing, 3 sec). Bees from the unpaired group receive the same number of CS and US presentations, but both stimuli are temporally dissociated. Paired bees, but not unpaired bees, show an increase in PER responses in the course of training, reaching ~80% responses within three trials. This experiment demonstrates that only the close temporal association between CS and US, but not the presentations of CS or US alone, allows successful conditioning.

Frisch trained bees to visit an artificial feeder presenting several essential oils (odor mixtures) (von Frisch 1919). Von Frisch observed for the first time that after learning one odor associated with sucrose solution, bees preferred this odor over others, showing clear odor discrimination, but also tended to visit other odors that were, at least to the human nose, similar to the rewarded one. Thus, bees generalized their learned preference to novel, perceptually similar stimuli. However, such experiments on free-flying bees did not allow the high throughput necessary for a precise understanding of olfactory perception. It was the advent of PER conditioning which allowed for the first time the precise study of perceptual relationships among odorants, on a much broader scale in terms of the number of tested odorants and animals (Kriston 1971; Laska et al. 1999). Vareschi (1971) pioneered these studies using a differential PER conditioning approach, with one rewarded odor (CS+) and 27 nonrewarded odors (CS−) presented in between CS+ trials. His work demonstrated the extraordinary discrimination capacities of bees as they differentiated the odors from >95% of the 1816 tested odor pairs. To understand perceptual relationships among odorants, later studies applied generalization procedures rather than discriminative ones. In this case, bees are first conditioned to a given odorant (CS) and are then presented with novel odorants without any reinforcement. This approach provides a fine measure of perceptual similarity relationships among odorants. The perceived similarity between the CS and each novel odorant is measured as the level of response to this odorant relative to the CS. With this approach, generalization among odors varying according to identified chemical features could be tested. Aliphatic odor molecules are especially interesting as they can be described using two main chemical characteristics: their chemical group and the length of their carbon chain. Olfactory generalization studies using PER conditioning showed that bees generalize more often between odors with similar carbon chain lengths or belonging to the same functional group (Smith and Menzel 1989). Recently, Guerrieri et al. (2005b) used olfactory PER conditioning to study systematically the generalization behavior of bees in the case of 16 odorants, presenting all combination of four possible functional groups (primary and secondary alcohols, aldehydes and ketones) and four chain lengths (6–9 carbons). More than 2000 bees were used to provide a complete generalization matrix among these 16 odorants (Fig. 5). Multidimensional scaling techniques applied to this behavioral matrix allowed the construction of a putative olfactory perceptual space for these odors in honeybees. A striking observation was that the first dimension in this space was the molecules' chain length, while the two other dimensions were determined by their chemical group. This result showed that these chemical dimensions are somehow encoded in the brain of honeybees and determine their behavior (Guerrieri et al. 2005b). These studies show that the honeybee constitutes a key model for the study of the neural basis of olfactory perception. For more than a decade, optical imaging has allowed odor-evoked activity maps to be measured in the olfactory pathway of the bee brain, especially at the level of the first olfactory center, the antennal lobe (Joerges et al. 1997). This structure is made of 165 glomeruli which each receive input from one type of sensory neuron carrying a distinct type of receptor protein. Odors elicit patterns of activity in a subset of glomeruli, according to a code that is conserved between individual bees (Galizia et al. 1999). However, it was unclear how these neural activity patterns recorded in the bee brain related to the perceptual quality of odors. Thanks to PER conditioning and the derived behavioral generalization matrix, Guerrieri et al. (2005b) demonstrated a significant correlation between similarity measures among odors in the behavior and in the neurophysiological recordings. Thus, calcium signals in the bee brain could, to some extent, allow prediction of the bees' generalization behavior with odorants. This research now needs to be extended to other brain regions downstream from the antennal lobe, such as the mushroom bodies, where neural activity maps can be recorded (Faber and Menzel 2001; Szyszka et al. 2005; Yamagata et al. 2009) and their predictive power with respect to behavioral PER measures can be assessed. PER conditioning has

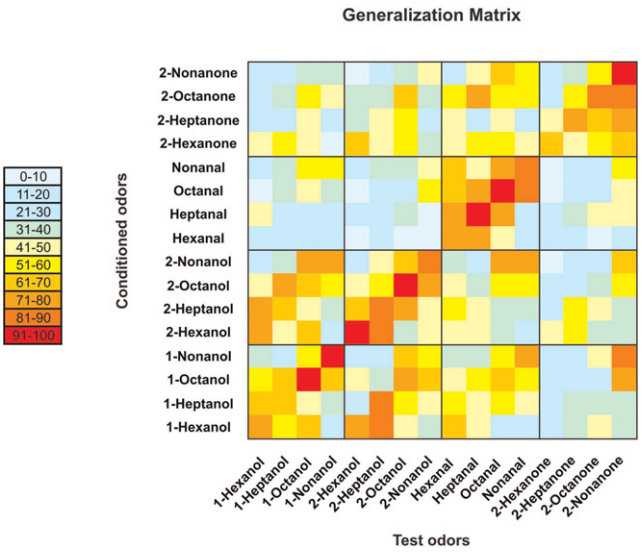

Figure 5. Use of PER conditioning for measuring perceptual relationships among odorants (Guerrieri et al. 2005b). In a generalization experiment, 1457 bees were trained with a particular odorant (CS) and were afterward tested with a panel of novel odorants. The more bees respond to a novel odorant, the more perceptually similar to the CS this odorant is considered to be. Using such a generalization experiment on a big scale, it was possible to measure the perceptual similarity among all possible pairs of 16 aliphatic odorants. The table uses a color-coded graphic display grouping the level of responses in 10 10% response categories (red: maximal response; light blue minimal response) in order to present the amount of generalization between any two of these odors. Odors used for conditioning are presented vertically, while odors used in the generalization tests are presented horizontally. Bees respond preferentially to the learned odorant (main diagonal), but also to other—perceptually similar—odorants.

thus provided key reference data for studying the relevance of activity maps in different brain regions for shaping bees' olfactory behavior.

Psychological studies

Olfactory PER conditioning arose, in Takeda's own words, as a protocol to determine whether psychological phenomena studied in vertebrates could be generalized across species, beyond vertebrates. Inspired by Pavlov's tradition (Pavlov 1927), Takeda attempted to reproduce the basic features of protocols that had attracted the attention of experimental psychologists. Extinction, spontaneous recovery, second-order conditioning, and conditioned inhibition were some of the topics he addressed in his paper, with a double objective: to show that these phenomena could be found in an invertebrate with the same basic features as in vertebrates, and to establish the honeybee as a model for this kind of psychological research.

Since then, many studies have used olfactory PER conditioning in bees to study problems of experimental psychology. These studies have revealed what Takeda already concluded in his work, namely the generality of certain learning phenomena, but also shed new light on the mechanisms underlying behavioral performances. Studies on compound processing[9] illustrate well this point and are of major interest, as the strategies used to process such stimuli are a matter of intense debate (Rowe 1999; Pearce and Bouton 2001). In experimental psychology, two main approaches have been proposed so far for explaining compound processing: "elemental" (Rescorla 1972, 1973; Rescorla and Wagner 1972; Whitlow and Wagner 1972) and "configural" (Pearce 1987, 1994) approaches. The former assumes that animals are able to extract the elemental composition of the compound, while the latter postulates that animals process a compound as a whole new configuration, independently of its individual components.

Olfactory PER conditioning was used to choose between these different models in honeybees in the case of olfactory compound stimuli (Deisig et al. 2001, 2002, 2003). Patterning experiments, in which bees had to discriminate between single odors and a mixture made out of them (the compound), were used to this end. "Negative patterning" (elements rewarded vs. compound nonrewarded) and "positive patterning" (elements nonrewarded vs. compound rewarded) discriminations of different complexity were solved by bees, and results showed that the strategy used was neither purely elemental (i.e., the total associative strength [V] of a compound AB results from the mere summation of the associative strengths of its elements A and B, i.e., $V_{AB} = V_A + V_B$, [Rescorla and Wagner 1972]) nor purely configural (i.e., a compound AB constitutes a novel configural unit, different from its components, which acquires associative strength independently [Pearce 1987, 1994]). Rather, the bees' performance was consistent with a corrected version of the unique cue theory (Rescorla 1972, 1973; Whitlow and Wagner 1972), which assumes that a compound AB is processed as the sum of the single elements A and B, plus a stimulus U which is unique to the compound and results from the joint presentation of A and B (i.e., $V_{AB} = V_A + V_B + V_U$) (Deisig et al. 2003). Although elemental associations are also invoked in the unique cue theory, it constitutes a nonelemental approach, as problem solving cannot be explained on the pure basis of the physically present elements of a compound. The correction incorporated into this theory in the case of bees is related to the reduced salience of components in the olfactory compound due to an interference between them (Deisig et al. 2003). Neurobiological studies on olfactory compound processing at the level of the antennal lobe using calcium imaging (see above) have supported this conclusion (Deisig et al. 2006, 2010).

Another psychological phenomenon which received wide attention in PER conditioning studies also involves olfactory compound processing: The phenomenon of "blocking" has been intensively studied in vertebrates because it invalidates the notion that contiguity of the CS and the US is sufficient for establishing an association between them (Kamin 1968). In a blocking experiment, there is a first phase (or preconditioning phase), in which subjects are presented with a single conditioned stimulus A paired with an US. In the second phase (or compound-conditioning phase), subjects experience a compound consisting of stimuli A and B, paired with the US. Subjects in a "control group" are presented with a novel odor N paired with the US in the first phase and with the compound AB paired with the US in the second phase. Finally, in the "test phase," subjects in both groups are presented with B alone. Blocking occurs if B elicits weaker responding in the "block" than in the "control group" despite the fact that B was identically paired with the US in both groups. It is then said that learning about stimulus A "blocks" subsequent learning about stimulus B when A and B are trained in compound.

In the case of olfactory PER conditioning, a controversy existed concerning the existence of blocking. While blocking was reported in some cases (Smith and Cobey 1994; Smith 1997; Hosler and Smith 2000), its existence could not be confirmed in other cases (Gerber and Ullrich 1999). Confounding factors like a lack of balance between groups or an insufficient number of odorants and bees tested per group led Guerrieri and coworkers (Guerrieri et al. 2005a) to readdress the question of blocking in the olfactory domain in honeybees. They assayed all 24 possible combinations of the four odors chosen for their experiments, which stand for A, B, and N, in a balanced design involving ~1000 bees. Blocking was found in only four out of 24 cases, so that it was concluded that blocking is not a consistent phenomenon in the olfactory domain. This study and that of Ullrich and Gerber on the same topic (Gerber and Ullrich 1999) were particularly interesting because they showed that subtle modifications in experimental parameters such as inter-trial intervals, balance (vs. lack of balance) of the odorants' role between experimental and control groups, odorant identity, number of conditioned bees, and appropriate statistical thresholds of significance in multiple group experiments, among others, may lead to radically different conclusions, some of which may be misleading. It highlighted the power of PER conditioning for carrying out large-scale experimental psychological studies and the value of the honeybee as a model to answer relevant questions on learning theories.

Ecological studies

PER conditioning has also brought substantial knowledge on how honeybees learn floral odors in an ecological context. Natural floral odors encountered by bees while foraging for nectar and/or pollen are not single molecules but are complex mixtures containing tens to hundreds of different components (Knudsen et al. 1993). Honeybees are, thus, constantly confronted by the problem of discriminating among complex odor blends and also of recognizing the same floral source despite variations in blend composition (Wright and Schiestl 2009). Because it allows a good control over the stimuli and the experience of the tested animal, PER conditioning was used to ask which features honeybees learn within a complex floral mixture. Bees were thus conditioned with floral extracts (Le Métayer et al. 1997; Pham-Delègue

[9]We use the term "compound stimulus" in the sense of a stimulus composed of two or more elements. The term refers to a psychological perspective and thus to the question of how this kind of stimulus is processed by the animal.

et al. 1997; Wright et al. 2002) or with synthetic mixtures containing 6–14 components (Pham-Delègue et al. 1993; Wadhams et al. 1994; Blight et al. 1997; Reinhard et al. 2010). A general finding of these experiments is that when bees learn a mixture and are afterward tested with the individual components, they respond most to only some of the mixture's components. Such components have been coined key-compounds (Pham-Delègue et al. 1993; Blight et al. 1997). To test bees' responses to all the components of a complex mixture, an elegant approach consisted of coupling chemical analysis techniques with PER conditioning (Wadhams et al. 1994; Le Métayer et al. 1997). Honeybees were first conditioned with a floral extract as CS. After conditioning, the same extract was run through a gas-chromatograph (GC), which separates the individual constituents of a mixture so that they elute from the GC's capillary column at different times after injection. About half of the effluent of the column was directed to the restrained bees, and their PERs were recorded throughout the GC run (Le Métayer et al. 1997). Thus, biologically active compounds in the mixture could be identified and their importance confirmed in PER experiments using the pure substances (Blight et al. 1997). These authors finally demonstrated that a mixture of three key-compounds [phenylacetaldehyde, linalool, and (E,E,)-α-farnesene] could mimic bees' responses to the highly complex oilseed rape extract. Nevertheless, in considering these results, one has to be cautious, as, in some cases, they may present confounding factors. For instance, the effluents from gas chromatography may arrive at the bee's antennae always in the same sequence, causing thereby sequential extinction effects. Careful experimental designs should be able, nevertheless, to control for this caveat.

What determines that an odorant will be a key-compound within a mixture? It does not only depend on its relative quantity in the mixture or on its individual salience for bees but seems to strongly depend upon the identity of the other components in the mixture (Laloi et al. 2000; Reinhard et al. 2010). The rules governing such mixture-specific interactions between mixture components are still not known, but broad scale systematic PER conditioning experiments with varying mixture compositions will be instrumental for solving this question.

Neurobiological studies

Dissection of honeybees' olfactory memory phases

One of the crucial contributions of PER conditioning to invertebrate neuroscience was that it permitted a careful dissection of appetitive olfactory memories in bees, as well as the elucidation of some of their key molecular actors, thanks to the fact that pharmacological injections and uncaging experiments could be coupled with controlled PER conditioning procedures (Menzel 1990, 2001; Menzel and Müller 1996).

As for other classical (Pavlovian) protocols, olfactory memory acquired after PER conditioning is dependent on variables such as the kind of CS, US intensity (i.e., the amount and/or quality of sucrose solution received during conditioning), the number of conditioning trials, and the inter-trial interval (Menzel et al. 2001). Trial spacing is the dominant factor both for acquisition and retention performance. Generally, massed trials (i.e., trials succeeding each other in a fast sequence) lead to lower memory performances compared to spaced trials (i.e., trials separated in time). Longer inter-trial intervals lead to better acquisition and higher retention. Several studies on olfactory memory dynamics (for review, see Menzel 1999) showed that memories in bees pass through an early consolidation phase and are fragile before consolidation is completed. Transfer from short-term memory (STM) to long-term memory (LTM) via mid-term memory (MTM) is not a purely sequential process but also includes parallel processes (Menzel 1999).

At least five types of olfactory memory phases were identified (Fig. 6). After a single conditioning trial, responses to the CS are high shortly (1–2 min) after conditioning, then decrease, showing a "dip" ~3 min, and are high again after 7 min, until ~1 d, when performance definitively decays (Fig. 6). Two different memory phases are thought to underlie this performance: In the first minutes after conditioning, performance depends on STM, which is mostly nonassociative (because of sensitization from the US). While STM decays after 2–3 min, a consolidation process leads to a highly odor-specific MTM, which lasts ~1 d. This consolidation process is characterized by a prolonged activity of the cAMP-dependent protein kinase A (PKA) (Müller 2000). Different memory phases, which rely on different cellular actors, are established after multiple conditioning trials (Fig. 6). In this case, performance does not decay after 1 d but remains very high for several days or even several weeks. After an initial STM phase (which may include two forms, early and late STM) (Fig. 6), consolidation leads to a different MTM phase (multiple-trial MTM), which is characterized by a selective increase in Ca^{2+}/calmodulin-dependent protein kinase C (PKC) activity (Grünbaum and Müller 1998). In the day's range, performance is controlled by two LTM phases formed in parallel. The early phase (e-LTM) depends on translation of already existing mRNA and is predominantly induced by massed training (short inter-trial intervals, usually 1 min). The late phase (l-LTM) is critically dependent on transcription and is only formed after spaced training (long inter-trial intervals, usually 10 min). Therefore, depending on the number and the distribution of associative events, multiple memory forms are formed: short forms depending on short-term cellular modifications, later forms depending on the production of new proteins.

Olfactory memory phases were found to correspond to the temporal dynamics of foraging activities in the field (Menzel 1999) so that early components of memory can be related to the

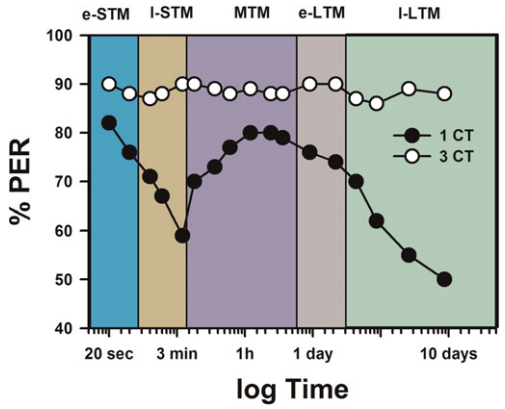

Figure 6. Olfactory memory phases in honeybees. Retention performance (measured as PER percentage: %PER) depending on trial number and the respective underlying memory phases (after Menzel 2001). Note the log scale for the abscissa. (Black dots) A single CS-US association (1 CT; black dots) supports good retention performance for ~1 d. A dip in retention, sometimes observed at 3 min, is interpreted as the transition between the decaying nonassociative short-term memory (STM), early (e-STM) and late (l-STM), and the purely associative mid-term memory (MTM) phase. (White dots) Three (or more) conditioning trials (3 CT; white dots) with 10-min inter-trial intervals 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). Multiple trials presented in a massed fashion (short inter-trial intervals typically of 1 min) give rise to e-LTM but not to l-LTM (not shown).

fast succession of experiences that a bee gathers while foraging within a patch or when moving between close patches. In the same way, mid-term memory corresponds, because of its intrinsic dynamics, to the intervals occurring between foraging bouts. Finally, long-term memory relates to foraging bouts that are spaced in time and which may occur on different days (Menzel 1999).

Neural bases of CS and US processing

A fundamental advance made possible by olfactory PER conditioning was the opportunity of tracing CS and US pathways in the honeybee brain and studying in an integrative way the neural circuits underlying Pavlovian learning (Fig. 7), a task that has been rendered easier through the establishment of a standard atlas of the bee brain (see http://www.neurobiologie.fu-berlin.de/beebrain/) (Rybak et al. 2010).

In the case of the "CS processing pathway," odorants are processed at different stages in the bee brain (Fig. 7). 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. As indicated above, each antennal lobe is constituted of 165 globular structures called glomeruli. 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. The latter are considered to be higher-order integration centers as they receive input from visual, gustatory, and mechanosensory pathways in addition to the olfactory pathway.

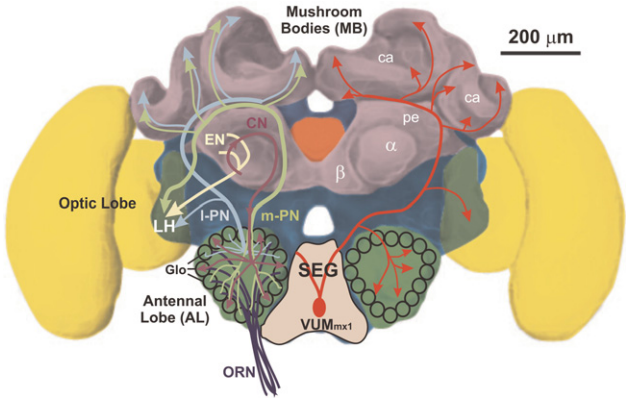

Figure 7. Neural pathways for CS and US information in the honeybee brain. CS pathway (*left* side): The antennal lobe (AL), first-order olfactory neuropil, receives input from ~60,000 olfactory receptor neurons (ORN) which detect odorants within sensilla on the antenna. Within the AL's anatomical and 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 antennoprotocerebralis tract (l-PN) projects first to the lateral horn (LH) and then to the mushroom body (MB) 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 (KCs), the MBs' 170,000 intrinsic neurons, form the calyces, while their axons form the pedunculus and the MBs' 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 pedunculus and lobes back to the calyces, 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. US pathway (*right* side): Gustatory sensory neurons on the antennae, tarsi, and mouthparts detect sucrose reinforcement and project to a first relay, the subesophageal ganglion (SEG). A single identified octopaminergic neuron, VUM-mx1, was shown to represent reinforcement during appetitive conditioning. This neuron has its dendrites in the SEG, where it gets (probably indirect) input from sucrose receptor neurons and projects to different regions of the brain, converging with the olfactory pathway in three areas, the AL, the MB calyces, and the LH.

Neural activity at the different stages of this CS processing pathway has been measured using a variety of recording techniques including electrophysiological and optophysiological methods (Mauelshagen 1993; Joerges et al. 1997; Abel et al. 2001; Szyszka et al. 2005; Yamagata et al. 2009; Denker et al. 2010). In many cases, myogram recordings of the muscle M17 (Rehder 1987), a muscle which controls the proboscis extension, were used to monitor the bees' responses under experimental conditions that do not allow free movements of the proboscis. Although some parts of the CS pathway are still only superficially characterized (for instance, the lateral horn), and we are only beginning to understand temporal aspects of odorant processing at several stages of this pathway (Szyszka et al. 2005; Fernandez et al. 2009; Yamagata et al. 2009; Denker et al. 2010), an integrative view of the CS circuit is already available.

The first studies coupling PER conditioning and neural interference combined single-trial conditioning with cooling-induced retrograde amnesia (through locally applied cold needles inserted into various brain regions) in order to determine the specific contribution of the different stages of the olfactory circuit (antennal lobes, mushroom bodies, lateral horn) to the memory phases described above. Retrograde amnesia was induced if the antennal lobes were locally chilled within a minute after single-trial conditioning and if the mushroom bodies were chilled within 5–7 min after conditioning (Menzel et al. 1974; Erber et al. 1980). No retrograde amnesia effect was observed after chilling the lateral horn. Since the retrograde amnestic effect of cooling the mushroom bodies was similar to that of the whole animal for longer intervals (>1 min) but was less pronounced for short intervals (<1 min), it was concluded that the mushroom bodies play an essential role in the consolidation process during lSTM, whereas the antennal lobes may be more related to eSTM (Menzel and Müller 1996). These findings constituted the first convincing evidence showing the involvement of the mushroom bodies in memory formation. As such they exerted a fundamental influence in further works which later confirmed this involvement in the fruit fly *Drosophila melanogaster* (Heisenberg 2003; Davis 2005).

Olfactory PER conditioning studies have further been used to determine whether antennal lobes and mushroom bodies are able to independently establish olfactory memory traces. Olfactory processing at the level of the antennal lobe has been intensively studied, and these studies have established that odorants are encoded as odor-specific spatiotemporal patterns of glomerular activity (Joerges et al. 1997). How does learning modify neural activity in this neuropil? PER conditioning has been instrumental for answering this question. By performing calcium imaging experiments shortly after PER conditioning, Faber and colleagues (Faber et al. 1999) found that olfactory differential conditioning (A+ vs. B−) induces an increase in the intensity of the glomerular activation pattern for the rewarded odorant A. No change was recorded in the pattern of the nonrewarded odorant B. In addition, a decorrelation of the patterns of odors A and B was found, suggesting that their discriminability was improved (Faber et al. 1999). This conclusion was recently confirmed and extended by Rath and coworkers (Rath et al. 2011), who also employed calcium imaging to measure antennal lobe activity 2–5 h after differential conditioning. They found that the response patterns to A and B

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 also been applied in the case of studies performed at the level of the mushroom bodies. Recordings of Kenyon cells, the constitutive neurons of the mushroom bodies, 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 and due to the probable action of inhibitory recurrent neurons (Szyszka et al. 2005). These responses can be modified by associative learning, as shown by PER conditioning studies coupled to calcium imaging recordings. While repeated stimulation with an odor leads to a nonassociative decrease in the response strength of Kenyon cells, the pairing of an odor with sucrose induces an associative prolongation of Kenyon-cell responses. After conditioning, Kenyon-cell responses to a rewarded odor (CS+) recover from the decrease induced by repetition, while the responses to a nonrewarded odor (CS−) decrease further. The spatiotemporal pattern of activated Kenyon cells changes for both odors when compared with the response before conditioning, but the change is stronger for the CS− (Szyszka et al. 2008).

Besides calcium imaging, PER conditioning has also been coupled to molecular interference methods. A recent study has shown, for instance, that the NMDA receptor, which acts as a sensor of coincident activity between neural inputs and whose activation during learning is considered important for various forms of memory, also contributes to memory differentiation in bees (Mussig et al. 2010). When the expression of the NR1 subunit of the NMDA receptor was inhibited in the mushroom bodies using RNA interference, an impairment of both MTM and e-LTM was found, while l-LTM was left intact (for further analyses coupling PER conditioning and molecular interferences, see Schwärzel and Müller 2006).

The question of how olfactory representations are modified by associative learning still requires intensive studies which should analyze changes at the different stages of the olfactory pathway and at different intervals post-conditioning in order to address different memory phases. Moreover, the effect of different conditioning protocols of varying complexity should also be considered.

In the case of the "US processing pathway," our knowledge is only partial, at least in neuroanatomical terms. Concerning the reinforcing function of the US pathway, our knowledge is so far restricted to a unique neuron which is thought to mediate the sucrose reward in the honeybee brain. This neuron, is called VUM_{mx1} (abbreviation for "ventral unpaired median neuron 1 of the maxillary neuromere") and responds with long-lasting spike activity to sucrose solution delivered to the antennae or to the proboscis (Hammer 1993). The neural processes of VUM_{mx1} arborize symmetrically in the brain and converge with the olfactory pathway at three sites: the antennal lobes, the calyces, which are multimodal input areas of the mushroom bodies, and the lateral horns. Such a convergence is particularly remarkable in the case of a neuron coding for sucrose solution as it provides a neuroanatomical basis for CS-US associations. That VUM_{mx1}, indeed, constitutes the neural representation of the US in olfactory PER conditioning was shown through an elegant substitution experiment performed by Hammer (1993). He showed that behavioral learning of an olfactory stimulus can be induced by substituting the sucrose reward in PER conditioning with an artificial depolarization of VUM_{mx1} immediately after olfactory stimulation (forward pairing). If depolarization, and thus spike activity, preceded olfactory stimulation (backward pairing), no learning was observed. The same forward-backward effect was seen when sucrose was used as the reward under similar experimental conditions. These results thus showed that VUM_{mx1} activity constitutes a neural correlate of the US in associative olfactory learning.

VUM_{mx1} belongs to a group of octopamine-immunoreactive neurons (Kreissl et al. 1994). Octopamine, a biogenic amine usually associated with increased levels of arousal and behavioral facilitation in invertebrates (Libersat and Pflüger 2004; Huber 2005), had been shown to increase responsiveness of bees to olfactory stimuli (Mercer and Menzel 1982). This finding was of particular interest to Hammer and Menzel who hypothesized that octopamine acts as the neurotransmitter necessary and sufficient to substitute for the sucrose reward (Hammer and Menzel 1998). Indeed, pairing an odorant with injections of octopamine, as a substitute for sucrose, into the mushroom bodies or the antennal lobes (but not the lateral horn) lobe produced a lasting CS-octopamine-pairing-specific increase of proboscis extension (Hammer and Menzel 1998). Thus, octopamine signaling via VUM_{mx1} is sufficient to substitute for sugar reinforcement in honeybees. This conclusion was confirmed by silencing octopaminergic receptor expression in the honeybee antennal lobe using double-stranded RNA (Farooqui et al. 2003). This treatment inhibited olfactory acquisition and recall but did not disrupt odorant discrimination. This result underlines the fact that appetitive reinforcer function in the bee brain is subserved by octopamine, which acts as a positive value system, i.e., as a system allowing ordering, prioritizing, and assigning a "good" label to odorants (Giurfa 2006). The elucidation of the US pathway in honeybees is a good example of the numerous possibilities granted by PER conditioning for studying olfactory learning: It critically allowed coupling an olfactory learning protocol with electrophysiology, pharmacological injections, and RNA interference.

Alternative forms of PER conditioning

US variations

Olfactory PER conditioning has been attempted using pollen instead of sucrose as the appetitive US (Grüter et al. 2008). An important proportion of bees showed unconditioned PER upon contact of their antennae with bee-collected pollen. Furthermore, bees readily learned to associate an odor with pollen and increased their responses to that odor following associative learning (Grüter et al. 2008). Yet, the reinforcing mechanism of pollen remains unclear as, besides proteins, pollen also contains carbohydrates and water. The relative amounts vary greatly between species, and bee-collected pollens usually contain more sugars, so that learning after odor-pollen pairings might be due, in part, to the presence of carbohydrates.

CS variations

Different kinds of sensory stimuli have replaced odors as CS and have been paired with sucrose solution in order to generate alternative forms of PER conditioning. Successful attempts include mechanosensory and thermal stimuli. Less successful attempts include visual stimuli.

Different protocols have been developed which exploit the principle that harnessed honeybees can associate a "mechanosensory stimulation" of their antennae or specific antennal movements with a reward of sucrose solution delivered to the proboscis. In one of these protocols, the bee is rewarded when its frequency of antennal contacts with an object (a plate close to the bee's head) exceeds a certain threshold (Kisch and Erber 1999). As a result of this operant conditioning, bees increase their frequency of antennal contacts with the reinforced object. In another protocol, bees are rewarded after scanning with their antennae the surface of a given object in order to learn its texture properties (Erber

et al. 1998; Scheiner et al. 1999, 2001). The associations established in this form of conditioning are probably both operant and classical. In a third variant, bees are rewarded whenever their antennae, left, right, or both, are mechanically stimulated by the experimenter (Giurfa and Malun 2004). In this case, and contrary to the two previous protocols, the antennal response of the bees is not crucial for obtaining the reward, so that bees learn a Pavlovian association between mechanosensory stimulation and sucrose reward.

These forms of mechanosensory learning have been rarely coupled with invasive methods, so that the neural pathways underlying mechanosensory stimulus representation in the bee brain are unclear. The involvement of nicotinic pathways in tactile memory formation and retrieval processes was studied by injecting into the bee brain various nicotinic antagonists (Dacher et al. 2005). It was shown that nicotinic receptors are involved in tactile memory formation and retrieval. Nicotinic antagonists had different effects depending on the injection period, thus suggesting different pharmacological bases underlying different tactile memory phases.

"Thermal stimuli" (per radiation or contact) have also been used as the CS in order to create temperature-sucrose associations in honeybees. The rationale underlying these experiments is that bees are exposed to thermal stimuli when collecting food outside and receiving food rewards inside the nest and that, in both contexts, there is an opportunity for them to associate warmth with food rewards. Menzel and coworkers (Menzel et al. 2001) showed that bees readily learn to associate thermal stimuli with a sucrose solution reward and that longer inter-trial intervals promote better retention performances. This finding was confirmed by Hammer and coworkers (Hammer et al. 2009) who showed, furthermore, that bees can be trained to discriminate between temperatures above (warm) and below (cold) ambient air temperature and that temperature differences as small as 1°C can be discriminated (Hammer et al. 2009).

The readiness exhibited by harnessed bees to associate mechanosensory and thermal stimuli with sucrose reward contrasts with the impossibility to observe, in the same experimental conditions, conditioned responses to "visual stimuli" that have been paired with sucrose reward. After having praised the versatility of PER conditioning, it is, nevertheless, necessary to acknowledge that it has failed to provide a robust paradigm for the study of visual learning and memory. As mentioned above (see The Origins), and for reasons so far unknown, intact, harnessed bees only exhibit conditioned PER to visual cues associated with sucrose if their antennae have been ablated beforehand (Kuwabara 1957; Hori et al. 2006, 2007). This procedure results in bees that are less responsive to the US, as shown through tarsal stimulation (de Brito Sanchez et al. 2008), probably leading to impaired acquisition and retention performances.

Yet, intact bees do perceive colors under harnessing conditions, as shown by attempts to develop bimodal blocking (Gerber and Smith 1998) and recent occasion-setting experiments (Mota et al. 2011a). In the first case, a pretrained color did not block odor when delivered in a compound but, on the contrary, facilitated olfactory learning (Gerber and Smith 1998). Despite the facilitatory effect exerted by the color, it did not elicit responses per se after compound training, similarly to what has been reported for intact bees in which color conditioning of PER was unsuccessfully attempted. In the second case, bees were able to use different colors to disambiguate olfactory information and to respond or not with PER to an odor that was reinforced in some trials and not reinforced in others (Mota et al. 2011a). However, the color anticipating that the odorant would be reinforced never elicited PER responses per se, so that it did not seem to have been directly associated with the US despite being obviously perceived. This feature qualifies colors as occasion setters (Schmajuk and Holland 1998), although an alternative interpretation would suggest that the association between color and US did, indeed, occur but that PER does not provide, in this experimental context, the appropriate behavioral readout for it.

The perspectives: Looking into the future

Fifty years of olfactory PER conditioning have been extremely fruitful as they allowed questions to be addressed that covered neurobiological, psychological, and ecological domains, among others. Yet, some gaps still need to be filled.

In terms of the neural analyses of CS and US processing, a more integrative view of the US pathway is still missing. Although gustatory receptor neurons tuned to sucrose have been located within specialized sensilla on the antennae, mouth parts, and tarsi (Whitehead and Larsen 1976; Whitehead 1978; Haupt 2004; de Brito Sanchez et al. 2005, 2008; de Brito Sanchez 2011), little is known about the neural circuits allowing these receptor neurons to convey US information to the central level and, more specifically, to VUM_{mx1}. This circuit is probably localized in the subesophageal ganglion, which is the first synaptic relay in the gustatory pathway (Altman and Kien 1987; Schröter et al. 2006). Similarly, CS processing has been studied in terms of the processing from olfactory receptors to the mushroom bodies, yet little is known about the possible recoding of sensory input by mushroom bodies based on their experienced value. In particular, if and how mushroom bodies change via feedback neurons (e.g., the ALF-1 neuron) (see Kirschner et al. 2006), neural processing in the antennal lobe, which provides input to the mushroom body and other parts of the brain, remains to be determined.

Although important data have been acquired on learning-dependent plasticity in the CS pathway, many aspects remain unexplored. Thus, even if key CS-processing stages such as the antennal lobes and the mushroom bodies have been studied in terms of their learning-dependent plasticity using olfactory conditioning of PER (see above and reviews in Menzel 1999; Giurfa 2007a), other CS processing sites such as the lateral horn remain unknown in terms of how odors are represented therein and whether learning induces functional and/or structural changes in this region. Downstream processing from olfactory receptors to mushroom bodies, including extrinsic mushroom body neurons (Mauelshagen 1993; Okada et al. 2007; Strube-Bloss et al. 2011), has been analyzed by means of different approaches that have been combined with PER conditioning. For instance, recent electrophysiological recordings have shown that mushroom body extrinsic neurons change their odor response spectra as a consequence of olfactory PER conditioning by losing or gaining sensitivity for specific odors (Strube-Bloss et al. 2011). While bees show a conditioned PER after a few acquisition trials, no short-term effects are observed in neuronal activity of extrinsic neurons. Yet, associative plastic changes occur during retention 3 h after conditioning: While some neurons change their odor response spectra by newly establishing and/or losing odor sensitivity, other neurons increase and/or decrease their odor response to the learned odors but do not change their odor response spectra. Thus, the ensemble activity of extrinsic mushroom body neurons predicts the associative value of the stimulus and may provide the prerequisite for the expression of the learned behavior (Strube-Bloss et al. 2011).

In behavioral terms, one must acknowledge that most PER measurements have been historically limited to recording a dichotomic variable (extension or lack of extension of the proboscis), thus precluding finer analyses on response intensity, latency, etc., which may reveal richer aspects of learning. The recording of

electromyograms of the muscles involved in proboscis extension, for instance, muscle M17 (Rehder 1987; Smith and Menzel 1989) or other muscles (Gauthier and Richard 1992), allowed a more graded measure of the animal's response. This technique can be efficiently used to measure a neural correlate of behavioral responses when movements of the proboscis should be prohibited, such as when coupled with other electrophysiological (Hammer 1993; Okada et al. 2007; Denker et al. 2010; Strube-Bloss et al. 2011) or imaging recordings (Faber et al. 1999; Hähnel and Menzel 2010). A few attempts have also been made using video recordings and subsequent analysis of PER parameters (Hosler and Smith 2000). Novel video analysis technologies may provide on-line measurements of PER dynamics and could be incorporated to enrich our behavioral quantifications and overcome long post-recording frame-by-frame analysis.

Following the theoretical framework developed as a result of 50 years of PER conditioning, a novel protocol was recently established to enrich the spectrum of learning studies in the honeybee: the conditioning of the sting extension response (SER) (Vergoz et al. 2007; Mota et al. 2011b). In its original olfactory version, each bee is restrained in an individual harness such that a bridge is built between two metallic plates through which a mild electric shock is delivered (Vergoz et al. 2007). Bees stimulated in this way exhibit an unconditioned, defensive reaction, the SER (Núñez et al. 1997). Using an odor CS paired with this electric shock US, it is possible to condition the SER so that bees learn to extend their sting in response to the CS (Vergoz et al. 2007). Moreover, the consequence of SER conditioning is an avoidance response toward the odorant previously punished if bees have the possibility of freely choosing between the conditioned odorant and a nonpunished odorant in a Y-maze (Carcaud et al. 2009).

Olfactory SER conditioning has been coupled with invasive methods such as calcium imaging of antennal lobe activity (Roussel et al. 2010) and neuropharmacological use of aminergic and protein-synthesis blockers (Vergoz et al. 2007; Giurfa et al. 2009) to study the neural bases of aversive olfactory learning. It has been found that SER conditioning induces the formation of protein-synthesis-dependent LTM (Giurfa et al. 2009) and that dopamine substitutes for electric shock reinforcement (Vergoz et al. 2007). Thus, in the same way as the appetitive reinforcer function in the bee brain is subserved by octopamine, the aversive reinforcer function is subserved by dopamine.

These results raise expectations about the breakthroughs that SER conditioning may bring to the field of learning studies in honeybees. Such expectations are supported by a unique achievement that PER conditioning was unable to provide: the possibility of using visual stimuli as CSs and establishing thereby a visual version of SER conditioning (Mota et al. 2011b). It is, indeed, possible to condition SER by pairing a visual stimulus (CS+) with an electric shock punishment (US) and a different visual stimulus (CS−) with the absence of shock in intact harnessed bees (Mota et al. 2011b). Bees with intact antennae learn the discrimination between CS+ and CS− by using chromatic cues, achromatic cues, or both. This visual conditioning protocol does not require, therefore, injuring the experimental subjects and opens new doors for accessing the neural correlates of visual learning and memory in honeybees.

More importantly, due to the limitations of PER conditioning in the case of visual CSs (see above), SER conditioning will allow multimodal learning in harnessed honeybees to be analyzed. The fact that bees' antennae are kept intact allows bees to be conditioned with compound stimuli made of both visual and olfactory cues. In addition, the harnessing situation offers the possibility of accessing the honeybee brain with a variety of invasive techniques to understand the neural bases of bimodal (visual-olfactory) learning, a goal that has remained elusive until now. All these important novel opportunities brought by SER conditioning were only made possible by the theoretical and technical progress provided by the advent of PER conditioning.

Conclusion

When, 50 years ago, Kimihisa Takeda produced his seminal paper on olfactory PER conditioning, he probably did not imagine the rich and diverse spectrum of research approaches that his protocol would later inspire. Olfactory PER conditioning has become a versatile tool for the study of questions, not only in the field of comparative experimental psychology (e.g., Chandra and Smith 1998; Hellstern et al. 1998; Deisig et al. 2001, 2002, 2003), as originally planned by Takeda, but also in diversified fields such as olfactory perception (e.g., Vareschi 1971; Guerrieri et al. 2005b; Reinhard et al. 2010), neurobiology of olfaction and olfactory learning (e.g., Hammer 1993; Stopfer et al. 1997; Faber et al. 1999; Sandoz et al. 2003; Rath et al. 2011), molecular bases of memory (for review, see Menzel 1999; Schwärzel and Müller 2006), social bases of behavior in bees (e.g., Chaline et al. 2005; Arenas and Farina 2008), and floral ecology (Wright et al. 2002, 2005), to cite only a few examples. The basic premises of olfactory PER conditioning have also been adapted in other species, such as bumblebees (Laloi et al. 1999; Riveros and Gronenberg 2009), stingless bees (McCabe et al. 2007; McCabe and Farina 2009, 2010), moths (Fan et al. 1997; Fan and Hansson 2001; Daly et al. 2004), and even ants, which do not have a proboscis but whose mouthpart movements can also be conditioned (Guerrieri and d'Ettorre 2010; Guerrieri et al. 2011).

The choice of examples provided in this article is necessarily incomplete, as it would be impossible to cite all the extensive literature on PER conditioning produced since Takeda's original work. Yet, these few examples illustrate well how a rather simple research tool has exerted an extraordinary influence in the field of insect learning and memory. Few behavioral protocols to date have reached such a privileged status in the invertebrate learning literature: The olfactory conditioning in the T-maze (for review, see Davis 2005), the visual conditioning in the flight simulator (for review, see Heisenberg et al. 2001), in the fruit fly *Drosophila melanogaster*, and the mechanosensory conditioning of defensive gill withdrawal responses in the sea hare *Aplysia californica* (Byrne 1987; Byrne et al. 1991; Kandel 2001) are further examples of such seminal protocols.

In its 50th anniversary year, olfactory PER conditioning has reached maturity and recognition beyond the frontiers that framed its origins. Even its limitations have been inspirational for the arousing of novel conditioning protocols addressing different learning forms in bees. We celebrate, therefore, Takeda's original work (Takeda 1961) and prompt colleagues to conceive and establish further comparable and robust behavioral tools for an accurate characterization of insect learning and memory at multiple levels of analysis.

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