Latent inhibition (LI) is a decrement in learning performance that results from the nonreinforced pre-exposure of the to-be-conditioned stimulus, in both vertebrates and invertebrates. In vertebrates, LI development involves dopamine and serotonin; in invertebrates there is yet no information. We studied differential olfactory conditioning of the proboscis extension response in the honeybee *Apis mellifera*, and we compared LI in individuals treated with antagonists of biogenic amines (dopamine, octopamine, and serotonin). An antagonist of octopamine receptors and two antagonists of serotonin receptors showed LI disruption. We thus provide evidence that serotonin would participate in the regulation of LI in honeybees.

[Supplemental material is available for this article.]

Are the neural mechanisms underlying LI conserved across species? To answer this question, comparative studies dissecting the role of brain areas and neurotransmitters in a broad spectrum of species are necessary. We addressed this goal by studying the signaling pathways underlying LI in an invertebrate species, the honeybee (*Apis mellifera*). We focused on octopamine (OA), DA, and 5-HT, which are known to modulate invertebrates’ behavior in various ways (Kravitz 1988; Bicker and Menzel 1989; Erber et al. 1993; Roeder 1999; Blenau and Baumann 2001; Kravitz and Huber 2003; Giurfa 2006; Scheiner et al. 2006; Schröll et al. 2006; Mizunami and Matsumoto 2010) and which have been related to different forms of behavioral plasticity in honeybees (Giurfa 2007). Harnessed honeybees can be trained in a Pavlovian conditioning protocol in the laboratory so that they learn to associate a given odorant (CS) with sucrose solution (US) (Takeda 1961; Bitterman et al. 1983). After a few repeated acquisition trials, most individuals extend their proboscises to the odorant that predicts the sucrose reward, thus displaying a conditioned response. Some neurobiological and molecular aspects of associative learning have been unraveled using this protocol (Giurfa and Sandoz 2012). Octopamine, DA, and 5-HT modulate learning and memory in different ways (Scheiner et al. 2006). Manipulation of OA neurotransmission interferes with the response threshold to sucrose and the acquisition of the CS–US association, as OA signaling is thought to represent US reinforcement in the bee brain (Kreissl et al. 1994; Hammer and Menzel 1995, 1998; Menzel 1999; Menzel et al. 1999; Farooqui et al. 2003; Pankiw and Page 2003). Thus, possibly in LI the unrewarded odor pre-exposure would lead to a reduction of OA signaling during subsequent conditioning. DA, on the other hand, inhibits the retrieval of appetitive olfactory memories (Mercer and Menzel 1982), and has been related to the representation of an aversive US reinforcement in the bee brain (Vergoz et al. 2007). Thus, LI might rely on an activation of aversive signaling by repeated, unrewarded CS pre-
exposure, which should be overcome by subsequent appetitive conditioning. Finally, 5-HT injections proved to reduce the rate of conditioned responses (Mercer and Menzel 1982; Menzel 1999), so that unrewarded CS pre-exposure may also induce an increase of 5-HT signaling, which would then affect negatively subsequent appetitive conditioning. To test these hypotheses, we used pharmacological treatments with antagonists (henceforth indicated with the sign “−”) of these biogenic amines under a LI protocol based on olfactory conditioning of the proboscis extension reflex (PER).

First, we studied the effect of flupentixol (DA−) and mianserin (OA−) on LI. A PBS-injected group acted as control. We evaluated odor discrimination learning of injected bees trained to respond to a rewarded odor (CS+) but not to a nonrewarded odor (CS−) after having been exposed, or not, to linalool (Supplemental Table S1; Supplemental Material). Drugs were injected before odor pre-exposure (see Fig. 1 and Supplemental Material for details).

The PBS group showed significant reduced acquisition during differential conditioning in pre-exposed bees but not in unexposed ones (Fig. 1A). A two-way repeated measures ANOVA (RM-ANOVA) revealed significant changes between pre-exposed and unexposed bees for factors pre-exposure (F(1,78) = 9.62, P = 0.003) and trials (F(4,312) = 36.27, P < 0.001), but not for their interaction (F(4,312) = 1.05, P = 0.37). Therefore, this analysis indicates that PBS-treated bees, when pre-exposed to an odor that was used afterward as CS+, exhibited a reduced learning rate in comparison to unexposed bees. This result reproduces the LI effect (Fernández et al. 2009) in experimental conditions in which bees receive a brain injection through the median ocellus (see Supplemental Material for details). Injection of Flupentixol (DA−) yielded results that were similar to those of the PBS group (Fig. 1B). Bees exposed to the CS+ also showed impaired learning compared with unexposed bees, so that significant effects were found for all sources of variation (pre-exposure: F(1,78) = 7.73, F(4,312) = 0.002; trials: F(4,312) = 21.82, P < 0.001; pre-exposure × trials: F(4,312) = 2.54, P = 0.04). Thus, blocking the dopaminergic system does not affect the occurrence of LI. Contrarily, bees injected with mianserin (OA−) achieved a good learning rate despite pre-exposure (Fig. 1C), as their acquisition level was similar to that of unexposed bees (pre-exposure: F(1,78) = 0.34, P = 0.56; trials: F(4,312) = 20.255, P < 0.001). The interaction between trials and pre-exposure was not significant (F(4,312) = 0.42, P = 0.79), thus suggesting that mianserin injection counteracted the effect of the pre-exposed CS.

Second, to verify these effects, we studied the effect of two other antagonists of dopamine and octopaminergic signaling: fluphenazine and epinastine, respectively (Supplemental Table S1). Hence, we evaluated odor discrimination learning of bees injected with PBS, fluphenazine (DA−) or epinastine (OA−) after having been exposed or not to linalool (Fig. 2A–C). Fluphenazine-injected, pre-exposed bees showed decreased learning compared with unexposed bees, so that significant effects were found for all sources of variation (pre-exposure: F(1,74) = 45.28, P < 0.001; trials: F(4,296) = 33.08, P < 0.001; pre-exposure × trials: F(4,296) = 7.6, P < 0.0001). Thus, we again found a significant LI effect in control bees, showing that CS pre-exposure reduces the acquisition of the CS+ in a subsequent differential conditioning assay. Blocking the dopaminergic system did not affect LI expression (Fig. 2B). Indeed, fluphenazine-injected, pre-exposed bees showed decreased learning compared with unexposed bees injected with that same drug. Therefore, differences were found for all factors (pre-exposure: F(1,74) = 14.32, P < 0.0001; trials: F(4,296) = 31.82, P < 0.001; and pre-exposure × trials: F(4,296) = 1.40, P = 0.04). On the other hand, blocking the octopaminergic system with epinastine did not reveal the effect promoted by mianserin as in previous results. Indeed, pre-exposed bees injected with epinastine showed a reduced learning rate, thus showing normal LI, which was absent in unexposed bees (Fig. 2C). Significant effects were found for pre-exposure (F(1,74) = 4.21, P = 0.04) and trials (F(4,296) = 19.33, P < 0.001), but not for their interaction (F(4,296) = 1.37, P = 0.24).

Possibly the discrepant effects on LI expression obtained after mianserin and epinastine injection could rely on the lower affinity of mianserin for octopaminergic receptors (Roeder 2005) and on a possible effect of this drug on an alternative signaling system whose blocking would determine LI suppression. It has been reported in fruit flies that mianserin blocks serotonin receptors, at least at a high dosage (Colas et al. 1995). With this in mind, we

**Figure 2.** Effects of fluphenazine (DA−) and epinastine (OA−) on latent inhibition (experimental series II). Percentage of bees that extended the proboscis (% PER) during five pairs of trials (five reinforced: black symbols, and five nonreinforced: white symbols) for pre-exposed bees (circles) and unexposed bees (triangles): bees injected with (A) PBS, (B) 1.9 mM fluphenazine (DA−), or (C) 4 mM epinastine (OA−). Asterisks indicate significant intergroup differences for learning performance: (**) P < 0.001, (*) P < 0.05. Sample sizes are indicated in brackets.
performed another experimental series blocking serotoninergic signaling (Supplemental Table S1). Like in previous results, the PBS-injected group showed LI after odor exposure (Fig. 3A). Consequently, significant differences were found for the factors pre-exposure ($F_{(4,88)} = 15.52, P < 0.0002$) and trials ($F_{(4,352)} = 40.83, P < 0.001$), but not for their interaction ($F_{(4,352)} = 1.04, P = 0.38$). Ketanserine and methysergide, both 5-HT antagonists, suppressed LI in odor-exposed bees, which showed no difference with unexposed bees (Fig. 3B,C). Neither the effect of pre-exposure (ketanserine: $F_{(1,88)} = 4.04, P = 0.15$; methysergide: $F_{(1,88)} = 0.94, P = 0.33$) nor the interaction between trials and pre-exposure (ketanserine: $F_{(4,352)} = 1.32, P = 0.26$; methysergide: $F_{(4,352)} = 0.87, P = 0.48$) (Fig. 3B,C) were significant. Only the factor trials had a significant effect (ketanserine: $F_{(4,352)} = 3.32, P = 0.001$; methysergide: $F_{(4,352)} = 4.84, P < 0.001$), which was consistent with the increasing learning response in both exposed and nonexposed bees along trials. Blocking the serotoninergic system thus rescued the decrease in learning rate shown by odor-exposed bees, thus suppressing LI.

Finally, to confirm that our behavioral protocol did indeed induce LI we performed control experiments aimed at ruling out alternative explanations (Chandra et al. 2000) of our behavioral effect. One might be an impairment of peripheral odorant detection, such as sensory adaptation to the odorant used both for pre-exposure and later conditioning. This explanation is difficult to sustain as Chandra et al. (2000) have shown that a reduced performance during conditioning caused by CS-pre-exposure did not cause changes in olfactory-receptor responses of the trained bees’ antennae, quantified by means of electroantennograms. Furthermore, a previous study using continuous volatile exposure (Fernández et al. 2009) showed LI even several hours after exposure. Since recovery from adaptation takes place over much shorter delays (in the minute range) (Zufall and Leinders-Zufall 2000), adaptation is an unlikely explanation for the reduced learning performance observed several hours after exposure, or even 20 min later as in the present study.

Other alternative explanations for the observed learning impairment would involve habituation or conditioned inhibition. We have examined the former possibility by performing conditioning after an extended delay (from 20 min to 4 h) (see Supplemental Material for details) following pre-exposure. Such a long delay precludes effects of habituation as responses recover after prolonged intervals. Yet, we still observed a reduction of odor learning for pre-exposed bees despite the prolonged 4-h delay (Fig. 4A). In addition, the possible occurrence of conditioned inhibition was tested by performing a negative summation test for conditioned inhibition (see Supplemental Material for details; Chandra et al. 2000). The rationale of this test is that the presentation of a mixture of any new odor with a preconditioned CS should reduce the response to the latter because of overshadowing (Smith 1998). By comparing responses to mixtures where this new odor had been used or not for pre-exposure, we expected to observe a further reduction produced by the mixture containing the pre-exposed odor with the preconditioned odor, if conditioned inhibition would be taking place. Indeed, such a negative summation is a defining characteristic of conditioned inhibition but not of latent inhibition (Rescorla 1969). We thus preconditioned bees with phenylacetaldehyde (phe), then pre-exposed them to linalool (lio) following our typical procedure, and finally tested them with two mixtures, one made of the preconditioned odor and the pre-exposed odor (phe + lio), and the other made of a novel odor limonene (limo) and the preconditioned odor (phe + limo). Bees displayed similar response levels to both mixtures, irrespective of their containing the pre-exposure odor (Fig. 4B). Hence, we ruled out any role of conditioned inhibition as a possible cause for the learning impairment observed after pre-exposure in our conditions. In summary, instead of sensory adaptation, habituation, and conditioned inhibition, we conclude that our results correspond to bona fide LI.

Our work focused on biogenic amines as possible mediators of the LI effect and used a neuropharmacological approach aimed at blocking octopaminergic, dopaminergic, and serotoninergic signaling in order to establish the effect of such blocking on LI expression. Therefore, we propose here a first analysis of the neurotransmitter systems involved in LI in an invertebrate model of learning and memory. Our experiments exclude the fact that the drugs injected selectively induce motor deficits in PER because both pre-exposed and unexposed bees were injected and differed, nevertheless, in LI occurrence in several cases. This is important when testing the effect of drug injection as it precludes the possibility that the analysis reflects the action of a given antagonist on.

**Figure 4.** Evaluation of possible occurrence of habituation and conditioned inhibition following pre-exposure. (A) Percentages of bees that extended the proboscis (% PER) during five pairs of trials (five reinforced: black symbols, and five nonreinforced: white symbols) for pre-exposed bees (circles) and unexposed bees (triangles), after an extended delay (4 h) following pre-exposure. Results show a reduction of odor learning (circles) despite the prolonged delay for pre-exposed bees ($F_{(1,460)} = 5.72, P = 0.02$; repeated measure ANOVA). Asterisks indicate significant intergroup differences for learning performance: (*) $P < 0.05$. (B) Percentages of bees that extended the proboscis (% PER) during the test in the negative summation experiment toward a mixture of two odors: preconditioned odor (phenylacetaldehyde, phe) + either the pre-exposed odor (linalool, lio) or a novel odor (limonene, limo). Response levels to both mixtures did not differ significantly (NS, nonsignificant differences; McNemar test, $P = 0.7893$). Sample sizes are indicated in brackets.

**Figure 3.** Effects of ketanserine (SHT-) and methysergide (SHT-) on latent inhibition (experimental series III). Percentage of bees that extended the proboscis (% PER) during five pairs of trials (five reinforced: black symbols, and five nonreinforced: white symbols) for pre-exposed bees (circles) and unexposed bees (triangles): bees injected with (A) PBS, (B) 1 mM ketanserine (OA-), or (C) 1 mM methysergide (OA-). Asterisks indicate significant intergroup differences for learning performance: (**$P < 0.01$; (NS) nonsignificant differences. Sample sizes are indicated in brackets.
a pathway controlling only PER, irrespective of the possible association with the paired odorant.

Our results argue against the hypothesis that LI would rely on an activation of aversive signaling by repeated, unrewarded CS pre-exposure, which should afterward be overcome by subsequent appetitive conditioning. If the repeated presentation of an odorant without reinforcement would be perceived as an aversive event, the pre-exposure phase might activate dopaminergic signaling. Indeed, DA inhibits the retrieval of appetitive olfactory memories (Mercer and Menzel 1982) and has been related to the representation of aversive US reinforcement in the bee brain (Vergoz et al. 2007). In this case, antagonists of DA would have inhibited such activation so that pre-exposed bees should show no LI. This was not the case and DA antagonist-injected bees that had been pre-exposed to the CS showed LI. Similarly, our results suggest that OA signaling does not mediate LI. Blocking OA signaling affects the response threshold to sucrose and the acquisition of odorant–sucrose associations as OA mediates appetitive US reinforcement in the bee brain (Kreissl et al. 1994; Hammer and Menzel 1995, 1998; Menzel 1999; Farooqui et al. 2003; Pankiw and Page 2003). Thus, a possible mechanism for LI might be that previous unrewarded presentations of the odorant CS would lead to a reduction of OA signaling during subsequent conditioning. Our results did not provide clear evidence supporting this idea. As mentioned before, we obtained different effects after mianserin and epinastine injections (both OA antagonists), probably due to their different affinities and specificities (Roeder 1999, 2005). Thus, the rescuing effect of mianserin might be due to its known antagonist effect on 5-HT receptors (Colas et al. 1995; Tierney 2001; Il-han et al. 2010). Consistently, we could avoid a learning deficit promoted by the CS pre-exposure by using ketanserin and methysgeride (5HT5), thus suppressing LI like with mianserin. We propose that LI could be the consequence of increased levels of 5-HT, resulting from repeated unrewarded CS exposure, which would be associated with inhibitory (or reduced excitability) states and thus with a tendency to impair CS–US associations. This is consistent with previous studies, where 5-HT injections were shown to reduce conditioned responses during acquisition (Mercer and Menzel 1982; Menzel 1999). Our results pave the way for future investigation in the LI phenomenon.

Investigations of the processes underlying LI development in vertebrates have shown that the pre-exposed stimulus retards acquisition in both excitatory and inhibitory conditioning (Rescorla 1969, 1971; Reiss and Wagner 1972; Mackintosh 1975; Moore and Stickney 1980; Schmajuk and Moore 1989). It was proposed that exposure to nonreinforced CS decreases the attention (or associability) to that stimulus without affecting its associative strength (Wagner and Rescorla 1972; Mackintosh 1975; Lubow et al. 1981). This would be consistent with the lack of effect on LI after interfering specifically with OA or DA signaling, which can modulate CS–US associations. Thus, through pre-exposure to nonreinforced odorants, bees may learn to ignore them because they result irrelevant or without consequence. Attention is a multidimensional cognitive process that includes the ability to select and focus on one aspect of the environment while ignoring others (James 1890; Gaddes and Edgell 1994) and it is thought to rely on integrative higher-order brain centers. In vertebrates, the hippocampus has been shown to be involved in LI (Chamizo 2006; Lubow and Weiner 2010). Possibly, like in vertebrates, higher-order centers in the insect brain might be also involved in LI development. Candidate neurons are the mushroom bodies (MBs), which are also involved in learning and memory amongst others process (Menzel and Erber 1978; Hammer and Menzel 1995; Menzel 1999; Heisenberg 2003; Giurfa 2007). They exhibit extensive 5-HT (Schürmann and Klemm 1984) and OA innervations (Hammer 1993; Schröter et al. 2007, Sinakevitch et al. 2011), and the highest density of binding sites for these biogenic amines (Erber et al. 1993). Reversible blocking of MB function via procaine injection (Devaud et al. 2007) may allow testing this hypothesis: Blocking MB would significantly affect LI expression.

Our study shows that it is possible to study the widespread behavioral phenomenon of LI at both the behavioral and the neural levels. Further studies should dissect the fine mechanisms, temporal dynamics, and neural substrates of LI in honeybees and thereby provide an integrative view on how stimulus exposure affects subsequent learning about that stimulus.

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