Available online at www.sciencedirect.com
ScienceDirect
Neurobiology of Learning and Memory 86 (2006) 164–174
Neurobiology of Learning and Memory
www.elsevier.com/locate/ynlme

Involvement of α-bungarotoxin-sensitive nicotinic receptors in long-term memory formation in the honeybee (*Apis mellifera*)

Monique Gauthier [a,*], Matthieu Dacher [b], Steeve Hervé Thany [c], Claudia Niggebrügge [d], Patrice Déglise [e], Pierre Kljucevic [a], Catherine Armengaud [a], Bernd Grünewald [d]

[a] *Centre de Recherches sur la Cognition Animale, CNRS/Université Paul Sabatier Toulouse III, 4R3, 118 route de Narbonne, 31062 Toulouse Cedex 09, France*
[b] *School of Life Science, Arizona State University, P.O. Box 874501, Tempe, AZ 85287-4501, USA*
[c] *Université d'Angers, UFR Sciences, 2, Bd Lavoisier, 49045 Angers Cedex 01, France*
[d] *Institut für Biologie—Neurobiologie, Freie Universität Berlin, Königin Luise Strasse 28-30, D-14195 Berlin, Germany*
[e] *Laboratoire de Neurobiologie des Canaux Ioniques, Faculté de Médecine secteur Nord, Bd Pierre Dramard, 13916 Marseille Cedex 20, France*

Received 13 December 2005; revised 16 February 2006; accepted 22 February 2006
Available online 17 April 2006

Abstract

In the honeybee *Apis mellifera*, multiple-trial olfactory conditioning of the proboscis extension response specifically leads to long-term memory (LTM) which can be retrieved more than 24 h after learning. We studied the involvement of nicotinic acetylcholine receptors in the establishment of LTM by injecting the nicotinic antagonists mecamylamine (1 mM), α-bungarotoxin (α-BGT, 0.1 mM) or methyllycaconitine (MLA, 0.1 mM) into the brain through the median ocellus 20 min before or 20 min after multiple-trial learning. The retention tests were performed 1, 3, and 24 h after learning. Pre-training injections of mecamylamine induced a lower performance during conditioning but had no effect on LTM formation. Post-training injections of mecamylamine did not affect honeybees' performances. Pre-training injections of MLA or post-training injection of α-BGT specifically induced LTM impairment whereas acquisition as well as memory retrieval tested 1 or 3 h after learning was normal. This indicates that brain injections of α-BGT and MLA did not interfere with learning or medium-term memory. Rather, these blockers affect the LTM. To explain these results, we advance the hypothesis that honeybee α-BGT-sensitive acetylcholine receptors are also sensitive to MLA. These receptors could be essential for triggering intracellular mechanisms involved in LTM. By contrast, medium-term memory is not dependent upon these receptors but is affected by mecamylamine.

Keywords: Mecamylamine; Methyllycaconitine; α-Bungarotoxin; Nicotinic receptors; Learning; Memory; Honeybee; Insect

1. Introduction

Acetylcholine (ACh)[1] is the major excitatory neurotransmitter in the insect central nervous system and insect neurons express several subtypes of nicotinic acetylcholine receptors (nAChRs, for reviews: Gundelfinger & Schulz, 2000; Sattelle et al., 2002, 2005; Tomizawa & Casida, 2001, 2003). Since the subunit compositions of most native insect neuronal nAChRs are largely unknown, the combination of neuropharmacological and behavioural experiments is an important method for distinguishing functional subtypes of neuronal receptors. As in other insects (Colhoun, 1963) ACh and nAChRs are widespread in the honeybee brain (Huang & Knowles, 1990; Kreissl & Bicker, 1989; Scheidler, Kaulen, Bruning, & Erber, 1990). Several evidences indicate that olfactory neurons (sensory and projection neurons) of the honeybee are cholinergic (Kreissl & Bicker, 1989; Oleskevich, 1999; Scheidler et al., 1990). Since

* Corresponding author. Fax: +33 561 558 444.
E-mail address: gauthiem@cict.fr (M. Gauthier).

[1] *Abbreviations used:* ACh, acetylcholine; α-BGT, α-bungarotoxin; CR, conditioned response; CS, conditioned stimulus; DHE, dihydro-β-erythroidine; LTM, long-term memory; MLA, methyllycaconitine; MTM, medium-term memory; nAChRs, nicotinic acetylcholine receptors; PER, proboscis extension response; UR, unconditioned response; US, unconditioned stimulus.

1074-7427/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.nlm.2006.02.003

the honeybee is a model system for studying olfactory learning and memory formation (Bitterman, Menzel, Fietz, & Schafer, 1983; Menzel, 1999) we combined behavioural and pharmacological experiments to analyze the role of the nAChRs in olfactory memory in the honeybee.

In vertebrates, the neuronal nAChRs consist of several subtypes and may be pharmacologically classified into α-bungarotoxin (α-BGT)-sensitive and α-BGT-insensitive receptors (Karlin, 2002; Paterson & Nordberg, 2000; Sharples & Wonnacott, 2001). The α-BGT-insensitive nAChRs are made up of combinations of α2-6 and β2-4 peptidic subunits whereas the α7-9 subunits are involved in the α-BGT sensitive branch. Apart from their sensitivity to α-BGT, the specific subunit combinations show distinct differences in their pharmacological profiles. Indeed, mecamylamine is a relatively selective antagonist of the α3β4 nicotinic receptor (Sharples & Wonnacott, 2001; Yokotani, Okada, & Nakamura, 2002), dihydro-β-erythroidine (DHE) is an antagonist of the nAChR subtype constituted by α4β2 subunits (Sharples & Wonnacott, 2001; Yokotani et al., 2002) and methyllycaconitine (MLA) is a specific antagonist of α7 containing nicotinic receptor (Davies et al., 1999). Besides, ligand binding studies have shown that high affinity α-BGT sites bind ACh and nicotine with low affinities and low affinity α-BGT sites have high affinities for nicotine and ACh (Clarke, Schwartz, Paul, Pert, & Pert, 1985; Sharples & Wonnacott, 2001).

For insects nAChRs, α-BGT-sensitive and insensitive subtypes have been described in several species: cockroach (Buckingham, Lapied, Corronc, & Sattelle, 1997; Courjaret & Lapied, 2001; Salgado & Saar, 2004), locust (Hermsen et al., 1998), moth (Fickbohm & Trimmer, 2003), and fruitfly (Lansdell & Millar, 2000; Raymond-Delpech, Matsuda, Sattelle, Rauh, & Sattelle, 2005; Sattelle et al., 2002, 2005; Zhang, Tomizawa, & Casida, 2004). Genes encoding for α- and β-subunit proteins have been identified in various insect species (reviews: Gundelfinger & Schulz, 2000; Sattelle et al., 2002, 2005) but the subunit compositions of native nAChRs remained unknown. The biophysical and pharmacological properties of the ionic currents through α-BGT-sensitive nAChRs were analyzed on cultured neurons from honeybee pupae (Barbara, Zube, Rybak, Gauthier, & Grünewald, 2005; Déglise, Grünewald, & Gauthier, 2002; Goldberg, Grünewald, Rosenboom, & Menzel, 1999; Wüstenberg & Grünewald, 2004). Among the several tested antagonists, DHE and MLA were the most potent inhibitors of the ACh-induced current, followed by mecamylamine. We have recently cloned four α-subunits in the honeybee that are differentially expressed in different somata clusters, including Kenyon cells of mushroom bodies and cells surrounding antennal lobes (Thany, Lenaers, Crozatier, Armengaud, & Gauthier, 2003; Thany, Crozatier, Raymond-Delpech, Gauthier, & Lenaers, 2005). Both structures are important for olfactory information processing and olfactory learning. Antennal lobes are the first relay station in the insect brain for olfactory information and antennal lobe projection neurons transmit olfactory information to the mushroom bodies. These paired neuropiles are one of the higher integrative structures in the insect brain, involved in olfactory learning and memory in honeybees (Cano Lozano, 1997; Cano Lozano, Armengaud, & Gauthier, 2001; Déglise, Dacher, Dion, Gauthier, & Armengaud, 2003; Erber, Masuhr, & Menzel, 1980; Hammer & Menzel, 1998; Mauelshagen, 1993; Menzel, 1999).

The type of memory formed during learning in the honeybee depends upon the number of learning trials during the training phase. Both single- and multiple-trial learning lead to the formation of medium-term memory (MTM) after an initial short-term memory, but after multiple-trial learning MTM fades and is replaced 24 h after learning by long-term memory (LTM; Menzel, 1999). LTM is not formed after single-trial learning; thus MTM is the final consolidated form of memory after single-trial learning whereas it is LTM in multiple-trial learning. Using single-trial olfactory learning, we have shown that the nAChRs are involved during acquisition and retrieval processes in the honeybee. Brain injections of mecamylamine before single-trial learning session impaired the learning of the odour as a predictive signal for reward. Furthermore, mecamylamine injected before the retention test transiently blocked memory retrieval of the previously learned odour (Cano Lozano, 1997; Cano Lozano et al., 2001; Cano Lozano, Bonnard, Gauthier, & Richard, 1996). Similar results were obtained with global brain injections of the nicotinic antagonist hexamethonium (Cano Lozano, 1997). Unexpectedly, α-BGT injections under the same experimental conditions had no significant effects on olfactory memory (Cano Lozano, 1997), which indicates that α-BGT sensitive receptors are not essential for olfactory MTM induced by single-trial learning procedure. Similar experiments conducted with an antennal mechanosensory learning protocol (Erber, Kierzek, Sander, & Grandy, 1998) supported this conclusion (Dacher, Lagarrigue, & Gauthier, 2005).

The aim of the current experiments was to assess the involvement of different subtypes of nAChRs in insect olfactory memory. We induced the formation of olfactory LTM by conditioning honeybees with a multiple-trial learning and we tested the effects of mecamylamine, MLA, and α-BGT on acquisition and memory retention.

2. Materials and methods

2.1. Animals

Experiments were conducted during two consecutive spring and summer seasons in Toulouse, France. Honeybees (*Apis mellifera*) were caught at the top of the hive in plastic cages. At the laboratory they were anaesthetized with CO_2 and individually fixed in small plastic tubes with a drop of wax–rosin mixture onto the dorsal part of the thorax and the head. The antennae and the mouth parts were free to move. After being fed with sucrose (sucrose solution 1.17 M), honeybees underwent a 3 h food deprivation period before the experiment started. For each bee, the experiment lasted for 2 days. Animals were restrained throughout the experiment, fed to satiation with sucrose solution (1.17 M) at the end of the 1st day to survive until the 2nd day. At the end of experiments, honeybees were deep frozen for sacrifice.

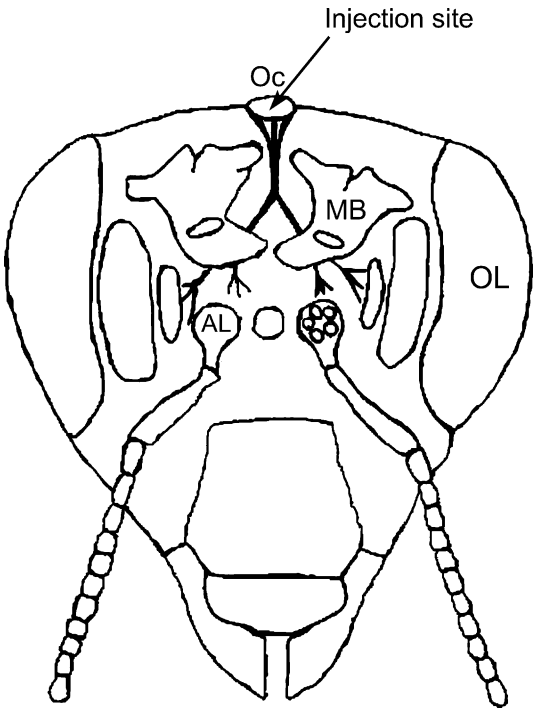

Fig. 1. Injection site and way of the drugs into the brain. The site of injection is the median ocellus (Oc) pointed by the arrow. The drugs follow the way of the large neurons of the tract that arborise in the posterior protocerebral neuropile (behind the mushroom bodies, MB) and the optic lobes (OL) and of the small neurons that reach the antennal lobes region (AL).

2.2. Drug injections

A volume of 0.3 μl of the drug or saline solution was injected into the median ocellus and the median ocellar nerve (Mercer & Menzel, 1982) (Fig. 1). The site of injection allows the drug to follow the main ocellar tracts (Goodman, 1981). Water-soluble substances injected by this method spread rapidly across the entire brain as was demonstrated using control experiment with Lucifer Yellow dye (Menzel, Heyne, Kinzel, Gerber, & Fiala, 1999). Thus, in our experimental conditions, the drug injected can affect both the mushroom bodies and the antennal lobes, which are the brain structures involved in olfactory learning.

To perform the injection, the lens of the median ocellus (located at the head vertex) was cut 1 h before the beginning of the experiments. The solution was injected either with a pulled glass capillary connected to a microinjector (WPI PV820 Pneumatic PicoPump; MLA experiments) or using a 1-μl microsyringe (Hamilton Microliter) slightly introduced into the cut ocellus (mecamylamine and α-BGT experiments). MLA and α-BGT were injected at a concentration of 10^{-4} M, mecamylamine at 10^{-3} M. Table 1 summarizes the drugs used and the experiments conducted.

The chemicals were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). They were dissolved in a bee saline solution (pH 7.2, final osmolarity: 327.5 mosmol L^{-1}), containing 2.7 mM KCl, 0.9 mM $CaCl_2$, 153.8 mM NaCl, and 11.7 mM sucrose. Furthermore, the bee saline was also injected alone in some animals (control groups) to assess potential unspecific effects of surgery and injection. These saline control groups are important, because the honeybee performance is rather variable and the behaviour of the control animals vary across the experiments.

2.3. Behavioural experiments

The olfactory conditioning of the proboscis extension response (PER) in the honeybee consists of temporal pairing of an odour (conditioned stimulus, CS) with a sugar stimulation of the antennae and the proboscis (unconditioned stimulus, US), which induces the PER (unconditioned response, UR). After training the CS presentations alone are able to elicit the PER (conditioned response, CR; Bitterman et al., 1983).

Animals were placed in a constant airflow directed toward the antennae of the bee and the odour was added to this airflow during 6 s (CS). Three seconds after odour onset, both antennae were touched for 6 s by a drop of sugar water (sucrose solution, 1.17 M; US). Hence, there was an overlap of 3 s between the CS and the US. The sucrose stimulation elicited the PER. The animals were allowed to drink the sucrose solution during 3 s. Three CS/US pairing trials were applied following this procedure with an inter-trial interval of 1 min. The retrieval tests consisted of presentations of the CS alone. The rate of bees showing the conditioned response was used as a measure for the successful association between CS and US. We tested retention repeatedly in each animal at 1, 3, and 24 h after the last learning trial (see Table 1). We assume that the drug injections do not decrease the PER rate during the 24 h test via increasing extinction, because extinction of the PER was only observed after several presentations of the CS alone at short intervals (Sandoz & Pham-Délègue, 2004; Stollhoff, Menzel, & Eisenhardt, 2005).

The retention level in each group was evaluated as the CR rate (see Section 3 for details). At the end of each experiment, the UR to antennal sugar stimulation was tested in those honeybees that did not respond to the odour presentation during the retention tests. This was done to be sure that the treatments did not impair the motor component of the proboscis extension or the sucrose perception. Only bees which showed UR were included in the study.

2.4. Time of injections

Injections were performed 20 min before or 20 min after training. Pre-training injection was aimed to study the effects of the drug on acquisition and early consolidation processes. Post-training injection could interfere with late consolidation processes or have a very delayed effect on retrieval. The duration of the drug effects was previously evaluated in experiments consisting in injecting the drug into the brain 20 min before retrieval testing and performing retrieval tests at different time intervals after injection (Cano Lozano et al., 1996; Dacher et al., 2005). We found in these conditions that mecamylamine was active from 30 min to less than 1 h; α-BGT and MLA had no effect on retrieval. Thus, we assumed that under a 20-min pre-training injection the whole learning session remains within the drug activity duration (30 min).

2.5. Statistical analyses

In all our experiments, we determined whether or not an animal released a PER to the odour presentation. Thus, we obtained binary data. The graphs show the PER rate, which is the proportion of honeybees displaying a PER to the odour during a retention test or during the odour stimulus of a learning trial. The sample size for each group is provided on each graph. The results were analysed using R 2.0 (R Development Core Team, 2004). All tests were two-tailed and the significance level was

Table 1
Drugs and experiments

Injections	Target	Time of injection	Retrieval tests after training
Mecamylamine (10^{-3} M)	α-BGT insensitive nAChR	20 min before (Fig. 2) or 20 min after (Fig. 4) training	1, 3, and 24 h
α-BGT (10^{-4} M)	α-BGT sensitive nAChR	20 min before (Fig. 3) or 20 min after (Fig. 4) training	1, 3, and 24 h
MLA (10^{-4} M)	α-BGT sensitive nAChR	20 min before (Fig. 5A) or 20 min after (Fig. 5B) training	1 or 3 h, and 24 h

set to .050. As we have binary data, Fisher's exact tests (not to be confused with the Fisher–Snedecor test used in ANOVA) were computed to compare the PER rate between the different groups (i.e., performance in the saline injected group vs. the drug injected group).

3. Results

3.1. Mecamylamine but not α-BGT reduces PER rate during acquisition

In a first experiment, mecamylamine was injected 20 min before training. The learning session consisted of three pairings of a coffee odour as the CS with a sucrose reward. Each honeybee was tested for memory retrieval 1, 3, and 24 h after training (Fig. 2). The acquisition curves of mecamylamine- and saline-injected animals differed. The response rates of mecamylamine-treated honeybees were lower during the third learning trial (Fisher's exact test; 1st learning trial: $p = .999$; 2nd learning trial: $p = .715$; 3rd learning trial: $p = .020$). During the retrieval tests mecamylamine-injected animals seemed to perform worse, but the effects were not significant (Fisher's exact test; 1 h retrieval test: $p = .999$; 3 h retrieval test: $p = .272$; 24 h retrieval test: $p = .142$). These animals formed a LTM as shown by high response level at the 24 h delay.

In a second experiment, α-BGT was injected 20 min before a three-trial olfactory learning with citral as CS. Retrieval tests were performed 1, 3, and 24 h after training in each animal (Fig. 3). Saline and α-BGT injected animals did not respond differently during acquisition and retrieval (Fisher's exact test; 1st learning trial: $p = .999$; 2nd learning trial: $p = .668$; 3rd learning trial: $p = .133$; 1 h retrieval test: $p = .658$; 3 h retrieval test: $p = .825$; 24 h retrieval test: $p = .490$).

3.2. Treatment with α-BGT but not mecamylamine impairs LTM

In a third experiment, the effects of a 20-min post-training injection of mecamylamine or α-BGT (Fig. 4) were compared; the saline control group was common for both groups. Animals underwent three-trial conditioning with coffee odour as CS and retention tests were performed 1, 3, and 24 h after learning in each animal. The learning curves prior to injections did not differ between the three groups (Fisher's exact test; 1st learning trial: $p = .999$; 2nd learning trial: $p = .463$; 3rd learning trial: $p = .759$). The retrieval performances evaluated 1 and 3 h after learning were equivalent between these groups (Fisher's exact test; 1 h retrieval test: $p = .151$; 3 h retrieval test: $p = .528$). By contrast, the PER rate during the 24 h test decreased in the α-BGT injected honeybees as compared to the saline- or mecamylamine-injected animals (Fisher's exact test; $p = 1.4 \times 10^{-4}$). This experiment indicates that α-BGT but not mecamylamine injections specifically affect LTM. It also indicates that mecamylamine induced no detectable memory effects 40 min after injection (1 h retention test) or at longer delays.

3.3. MLA impairs LTM

In this experiment, MLA or saline was injected 20 min before a three-trial learning session (Fig. 5A). We used lavender or citronella as CS; although during acquisition citronella-conditioned animals performed better than lavender-conditioned bees (data not shown), both odours led to similar effect, so the results were pooled. Each honeybee was tested twice, firstly at either a delay of 1 or 3 h and

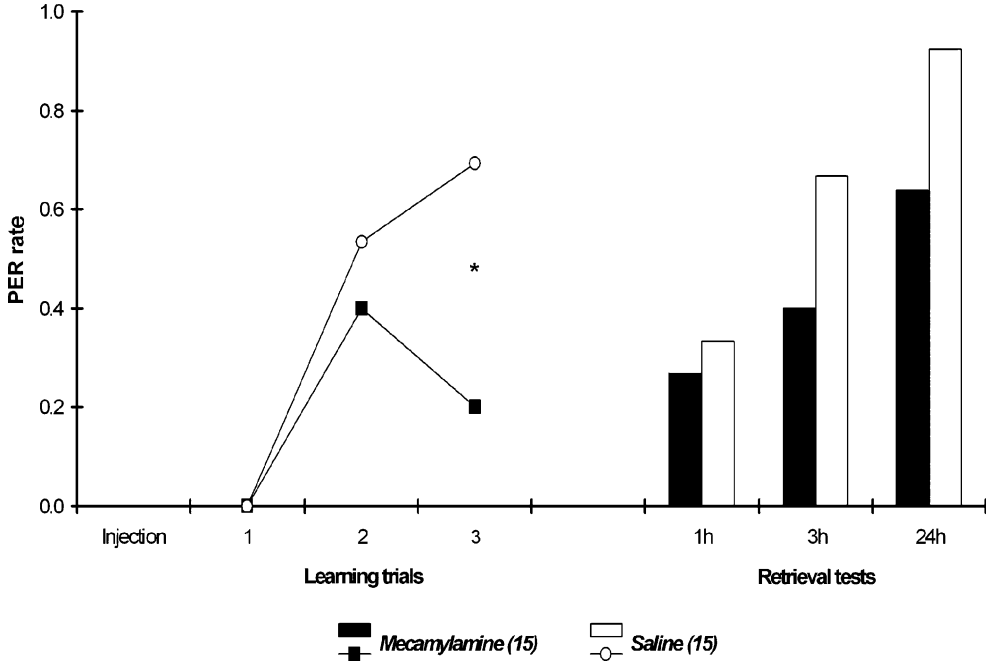

Fig. 2. Mecamylamine effects on multiple-trial learning. This figure shows the PER rates during three-trial learning and subsequent retrieval tests performed 1, 3, and 24 h after learning. Animals were injected with either mecamylamine or saline 20 min before learning. Numbers in parentheses indicate the number of animals used. *The two groups are different (Fisher's exact test, $p < .050$).

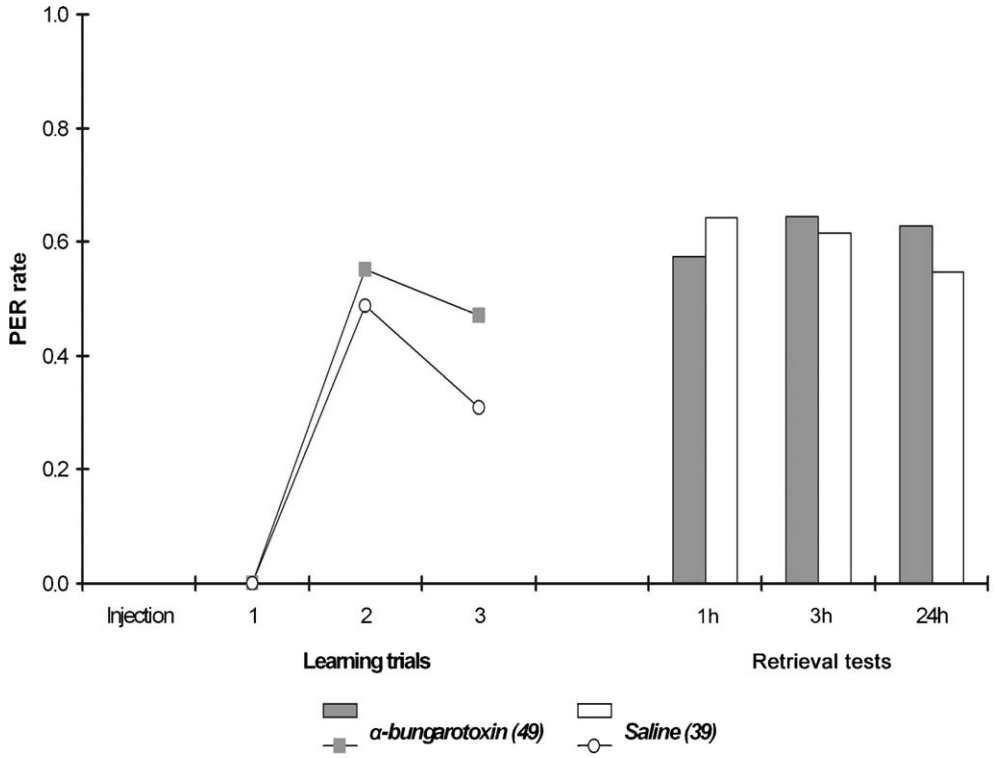

Fig. 3. Effects of α-BGT injection 20 min before multiple-trial learning. This figure shows the PER rates during three-trial learning and subsequent retrieval tests performed at 1, 3, and 24 h after learning. Animals were injected either with α-BGT or saline 20 min before learning. Numbers in parentheses indicate the number of animals.

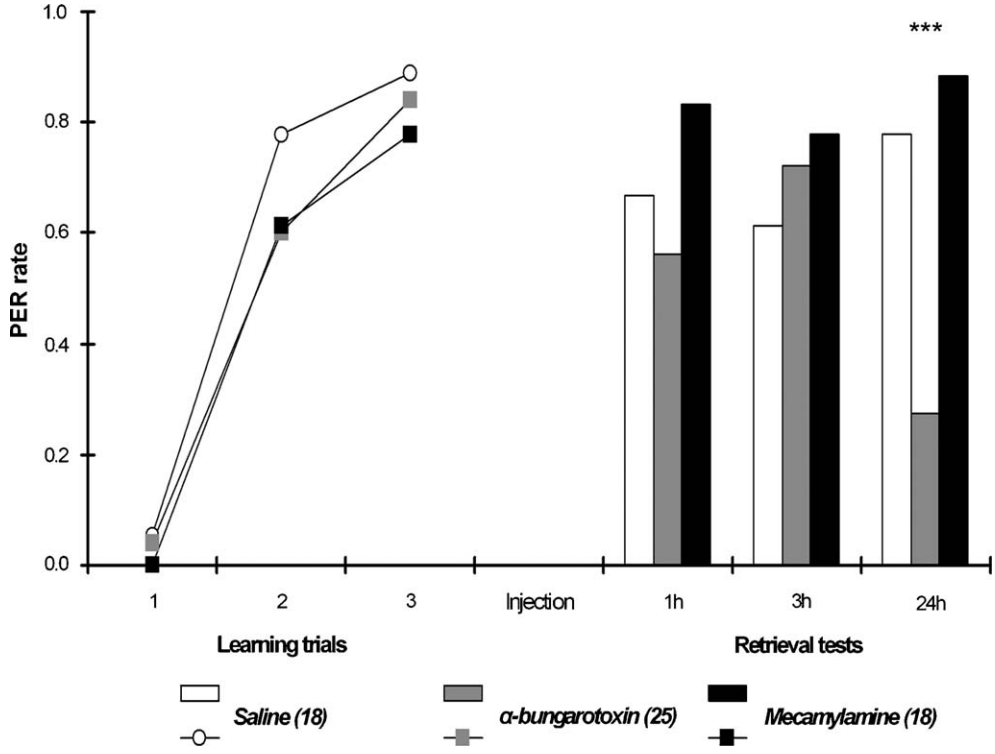

Fig. 4. Comparison of α-BGT and mecamylamine injections. This figure displays the PER rates during three-trial learning and subsequent retrieval tests performed 1, 3, and 24 h after learning. Animals were injected either with α-BGT, mecamylamine or saline 20 min after learning. Numbers in parentheses are the number of animals used. ***The three groups are different (Fisher's exact test, $p < .001$).

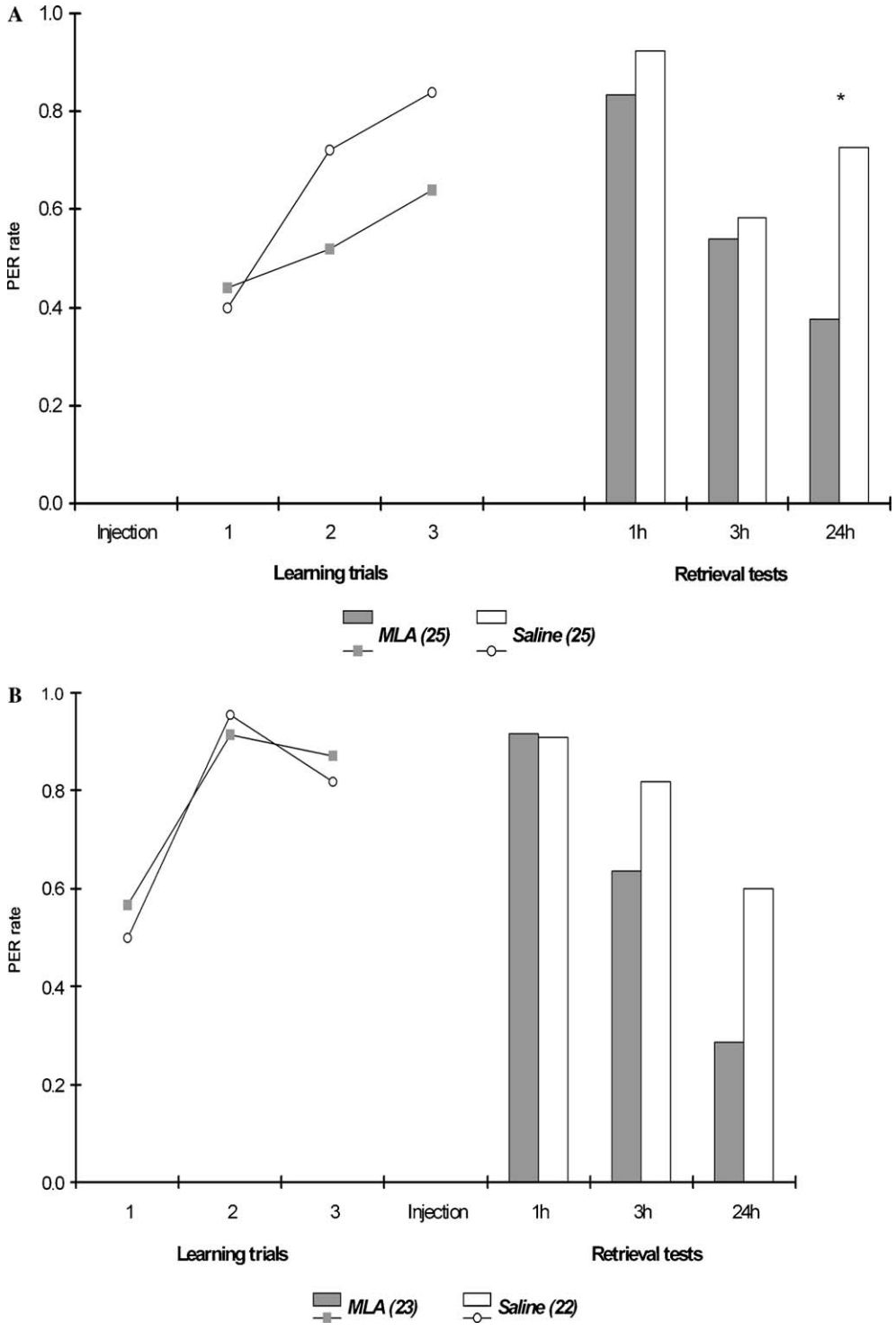

Fig. 5. Effects of MLA injections before (A) or after (B) multiple-trial learning. This figure displays the PER rates during three-trial learning and subsequent retrieval tests performed 1 or 3 h and again 24 h after training. Animals were injected with either MLA or saline 20 min before (A) or 20 min after (B) learning. Numbers in parentheses indicate the number of animals used. *The two groups are different (Fisher's exact test $p < .050$).

secondly at a delay of 24 h. Hence, retention tests were performed 1, 3, and 24 h after training. Pre-training injections of MLA did not affect the acquisition rate (Fisher's exact test; 1st learning trial, $p = .999$; 2nd learning trial, $p = .244$; 3rd learning trial, $p = .196$). Animals of all groups had high levels of spontaneous PER to the odour during the first conditioning trial. The retention rates at 1 and 3 h after training were similar (Fisher's exact test; 1 h retrieval test, $p = .593$; 3 h retrieval test, $p = .999$). However, MLA-treated animals showed a significant decrease of PER rate during the test 24 h after learning ($p = .021$).

In another experiment, MLA was injected 20 min after the last training trial (Fig. 5B) using the same protocol and the same odours as above. Both saline- and MLA-injected groups showed a high conditioning rate and a high spontaneous response rate to the odour. No difference was determined between the performances of the two groups during acquisition (Fisher's exact test; 1st learning trial, $p = .768$; 2nd learning trial, $p = .999$; 3rd learning trial, $p = .699$). MLA injection did not affect the retention rate tested 1 and 3 h after training (Fisher's exact test; 1 h retrieval test, $p = .999$; 3 h retrieval test, $p = .635$). At the 24 h test the MLA-injected bees showed a strong (though not significant) decrease of PER rate (Fisher's exact test; $p = .062$). From these results we conclude that MLA specifically impairs LTM, which is induced by multiple-trial learning. Since MLA does not prevent MTM formation (as can be seen during 1 and 3 h retrieval tests) it does not affect the acquisition process.

4. Discussion

4.1. Interpreting the effects of the drugs

All used nicotinic antagonists were repeatedly proven to block nAChR function in vitro (Barbara et al., 2005; Déglise et al., 2002; Goldberg et al., 1999; Wüstenberg & Grünewald, 2004). Nevertheless, we show here that they do not completely abolish olfactory learning or memory retention. We observed a LTM reduction after injections of MLA and α-BGT, whereas mecamylamine impaired performance during acquisition. In addition, our observations confirm that low concentrations of nicotinic antagonists do not abolish olfactory perception nor affect sucrose sensitivity or the PER itself, as was shown for mecamylamine (Cano Lozano, 1997; Cano Lozano et al., 2001, 1996). If mecamylamine or any other substance used interfered with olfactory or sucrose perception, the animals would not have been able to learn when the injections were done before learning. Therefore, the drugs probably do not affect nicotinic receptors of antennal lobe networks involved in olfaction and of neurons of the suboesophageal ganglion supporting sucrose perception. The nicotinic receptors of the mushroom bodies are probably the main target of the drugs.

Previous data showed that injections of the nicotinic antagonist mecamylamine into the brain impaired single-trial olfactory learning and temporarily blocked retrieval of the learned odour, whereas α-BGT had no effect on olfactory retrieval and MTM (Cano Lozano, 1997; Cano Lozano et al., 2001, 1996). On the other hand, our results indicate that α-BGT but not mecamylamine blocks LTM.

Furthermore, we attempted experiments with DHE (10^{-4} M) as this drug is an antagonist of α-BGT insensitive nAChRs. Pre-training injection led to memory increase. Post-training injection induced a transient decrease of memory retrieval at short delays, reminiscent of the blocking effect of mecamylamine (unpublished data). No impairment of LTM was noticed. These observations suggest that inside the class of α-BGT insensitive nAChRs, different receptors subtypes could support different memory mechanisms.

The functional effects of various similar drugs (such as nicotinic antagonists) on behaviour cannot be interpreted straightforward. Indeed, we can only make indirect inferences on the underlying neural processes and compare in vivo effects with in vitro studies, that cannot unravel the effects of the drugs on memory. For instance, a 10^{-4} M concentration of MLA used in our study is quite high for vertebrates and lead to unspecific effects (Paterson & Nordberg, 2000; Sharples & Wonnacott, 2001). For adult honeybees this kind of data is no yet available.

4.2. Different nicotinic antagonists affect different memory phases

Pre-training injections of mecamylamine decreased performance during learning but retrieval performance in the same animals tested later was intact. Post-training injections of mecamylamine had no effect. One possible way to explain these findings is to assume that mecamylamine acts on retrieval (Cano Lozano, 1997; Cano Lozano et al., 2001, 1996; Dacher et al., 2005). Thus, it would impair the performance during the conditioning phase, by blocking the retrieval processes requested each time the honeybee is submitted to the CS. This would be responsible for the weak performance of the 3rd conditioning trial. The mecamylamine effects are time-limited (less than 1 h: Cano Lozano, 1997; Cano Lozano et al., 2001, 1996; Dacher et al., 2005) and this could explain the fact that 20 min post-training injection did not impair the 1 h retrieval performance.

Alternatively, mecamylamine may rather act on short-term memory. It would be consistent with a complete recovery of the MTM tested 1 h after learning. However, this hypothesis is unlikely if we consider that MTM and/or LTM are formed through processes initiated with short-term memory (Menzel, 1999).

On the contrary, post-training α-BGT injections led to memory impairment at 24 h. Post-training injections of MLA yielded similar results as α-BGT, but the effect closely failed to reach significance; thus α-BGT and MLA impaired LTM. Moreover, pre-training injection of MLA impaired the PER rate during memory recall. This effect can be explained by an impairment of LTM acquisition, of LTM consolidation or of LTM retrieval.

If MLA or α-BGT had a 24 h-delayed effect on LTM retrieval the drugs would affect the LTM performance when injected either 20 min before or 20 min after learning. This is the case for MLA but not for α-BGT, which affects LTM only when injected after learning. Moreover, a 24 h-delayed effect of α-BGT and MLA on retrieval processes was not observed with antennal tactile learning (Dacher & Gauthier, unpublished; Dacher et al., 2005). One other explanation for α-BGT and MLA effects on LTM could be an impairment of acquisition and/or consolidation

processes. As α-BGT had an effect only 20 min after learning, the drug can interact with a late phase of consolidation processes. MLA had an effect when injected before learning and thus can affect both processes of acquisition and consolidation. Because interfering with acquisition would probably also affect MTM (which is not observed with MLA) it is likely that MLA rather acts on memory consolidation. Therefore, if one interprets the effects of MLA and α-BGT on 24 h performance as an effect on LTM formation, MLA could affect early processes of consolidation whereas α-BGT would affect later consolidation process.

In summary, despite the fact that MLA and α-BGT have different biochemical properties (slow association and dissociation binding kinetics for α-BGT and rapid and reversible inhibition of nAChRs for MLA) they share similar effects on LTM. We propose as a working hypothesis that this effect is linked to their action on the same subtype of nAChRs. Previous results indicated that ocellar injections of mecamylamine impair retrieval processes (Cano Lozano, 1997; Cano Lozano et al., 2001, 1996; Dacher et al., 2005), whereas we report here that ocellar injections of MLA and α-BGT block LTM. A specific involvement of α-BGT and MLA but not mecamylamine in honeybee LTM-formation was also found using antennal tactile learning (Dacher & Gauthier, unpublished; Dacher et al., 2005). Hence, our results can be interpreted assuming that different types of nAChRs are present in the honeybee brain. One type of nAChRs, sensitive to MLA and α-BGT, may be involved in the cellular mechanisms of LTM and another type, α-BGT-insensitive (blocked by mecamylamine), may be involved in retrieval processes.

4.3. Discrepancies between pharmacology and patch-clamp data: A developmental effect?

This study uncovered several discrepancies between behavioural pharmacological experiments and in vitro patch-clamp experiments of nicotinic receptors (Barbara et al., 2005; Déglise et al., 2002; Goldberg et al., 1999; Wüstenberg & Grünewald, 2004). Mecamylamine, MLA, and α-BGT are potent inhibitors of nicotinic currents as has been revealed with patch-clamp experiments on cultured mushroom bodies Kenyon cells of honeybee pupae. MLA completely blocked the ACh-induced current at concentration lower than 100 nM, but did not discriminate between different receptor subtypes (Wüstenberg & Grünewald, 2004). Similarly, a complete block of the ACh-induced current was obtained with α-BGT in mushroom body Kenyon cells (Déglise et al., 2002). These findings suggested that only one nAChR subtype is expressed by Kenyon cells, though in another set of experiments α-BGT only partially inhibited currents through nicotinic receptors on cultured pupal Kenyon cells (Goldberg et al., 1999) and antennal lobe neurons (Barbara et al., 2005). Thus, there is a mismatch between the effects of α-BGT (or MLA) and mecamylamine tested electrophysiologically in vitro and behaviourally in vivo.

This discrepancy may be explained by developmental differences in the expression of nAChR subunits. Indeed, most patch-clamp recordings were performed in neurons from late pupal stages (immature bees) but not from adult bees (mature bees used in the experiments reported here). Consequently we suggest that cultured neurons from pupae do not express the same nAChRs subtypes as adult cells do. Two populations of honeybee nAChRs have been described in neurons isolated from adult antennal lobes (Nauen, Ebbinghaus-Kintscher, & Schmuck, 2001). Behavioural studies suggested the existence of two distinct subtypes of nicotinic receptors in the honeybee (Guez, Belzunces, & Maleszka, 2003; Guez, Suchail, Gauthier, Maleszka, & Belzunces, 2001). These receptors have different affinities to the insecticide imidacloprid and are differentially expressed at the various ages of the animals. Thany et al. (2005) detected Apis α7-2 mRNA in pupal and adult Kenyon cells bodies lying outside each calyx, whereas Apis α7-1 mRNA is located in Kenyon cells lying inside calyces only in adults. Owing to these discrepancies between nicotinic receptors sub-units, the existence of different nicotinic receptor sub-units across different Kenyon cell populations and/or antennal lobe neurons and/or during different developmental stages is rather likely. Interestingly, vertebrate MLA and α-BGT-sensitive nAChRs are made of α7 subunits (Karlin, 2002; Paterson & Nordberg, 2000; Sharples & Wonnacott, 2001). The vertebrate α7 subunits are phylogenetically old (Gundelfinger & Schulz, 2000; Le Novère & Changeux, 1995; Le Novère, Corringer, & Changeux, 2002; Ortells & Lunt, 1995; Sattelle et al., 2005; Tsunoyama & Gojobori, 1998) and exhibit high homologies with insect sequences coding for α7 subunits (see for the honeybee Thany et al., 2005).

4.4. Linking nicotinic receptors to cellular events related to LTM

We suggest as a working hypothesis that multiple-trial learning triggers intracellular events involved in LTM formation through repeated stimulation of α-BGT-sensitive receptors but not α-BGT insensitive receptors (Fig. 6). Like vertebrates neuronal nAChRs (Adams, Stevens, Kem, & Freedman, 2000; Haberberger, Henrich, Lips, & Kummer, 2003; Rathouz, Vijayaraghavan, & Berg, 1996; Shoop, Chang, Ellisman, & Berg, 2001; Si & Lee, 2001; Smith, Hoffman, David, Adams, & Gerhardt, 1998; Vijayaraghavan, Pugh, Zhang, Rathouz, & Berg, 1992), a subpopulation of honeybee α-BGT-sensitive nAChR may trigger an influx of Ca^{2+} ions into the cell (Bicker, 1996; Bicker & Kreissl, 1994; Goldberg et al., 1999). Intracellular Ca^{2+} may in turn act as a key 2nd messenger for the triggering of intracellular cascades leading to LTM. In insects the enzyme NO synthase is activated by Ca^{2+} ions (Muller, 1994; Muller & Bicker, 1994) and NO is specifically involved in LTM formation (Dacher & Gauthier, unpublished; Muller, 1996). Since in vitro α-BGT-sensitive nAChRs can trigger NO synthesis in insects (Bicker, 1996; Zayas, Qazi, Morton, & Trimmer, 2002), α-BGT- and MLA-sensitive nAChRs may

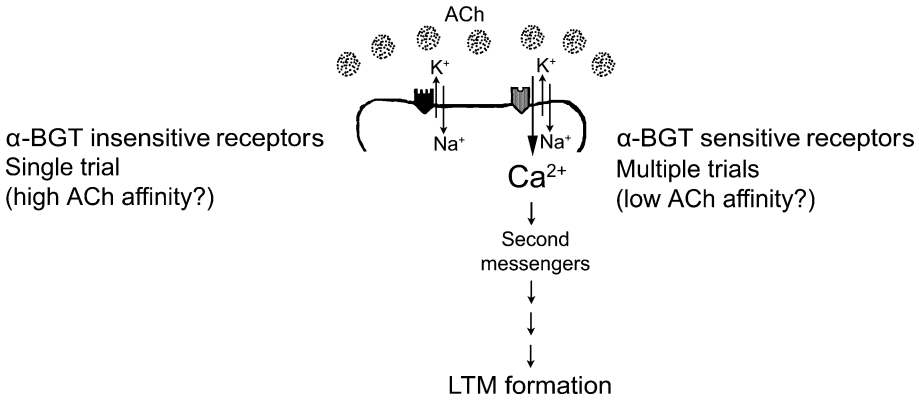

Fig. 6. Summary of the suggested working hypothesis. The α-BGT-sensitive nAChRs (grey receptor on the right, low affinity for ACh?) need several trials to be activated and possess high permeability to calcium ions. Thus, they trigger calcium-dependent cellular events linked to LTM formation. On the other hand, α-BGT-insensitive nAChRs (black receptor on the left, higher ACh affinity?) may be involved during single-trial learning and retrieval (as well as during multiple-trial learning). The two receptors types are depicted on the same neuronal membrane only for convenience and no hypothesis is made concerning their localization in neurons or brain.

specifically trigger the NO release involved in LTM formation during multiple-trial learning.

Acknowledgments

We are particularly grateful to Guillaume Barbara, Maud Bernardet, Amina Gharbi, Jérôme Martin, Sebastian Watzl, and Anna Wersing for participating in some experiments. We also thank Dr. Valérie Raymond-Delpech for helpful comments on discussion. This work was supported by a DAAD/MAE PROCOPE Grant, the Region Midi-Pyrénées (Grant 99002824) and a French Agriculture Ministry Grant to M. Dacher and S.H. Thany (Grant 407 ONIFLHOR).

References

Adams, C. E., Stevens, K. E., Kem, W. R., & Freedman, R. (2000). Inhibition of nitric oxide synthase prevents alpha 7 nicotinic receptor-mediated restoration of inhibitory auditory gating in rat hippocampus. *Brain Research, 877*, 235–244.

Barbara, G., Zube, C., Rybak, J., Gauthier, M., & Grünewald, B. (2005). Acetylcholine, GABA and glutamate induce ionic currents in cultured antennal lobe neurons of the honeybee, *Apis mellifera*. *Journal of Comparative Physiology A, 191*, 823–836.

Bicker, G. (1996). Transmitter-induced calcium signaling in cultured neurons of the insect brain. *Journal of Neuroscience Methods, 69*, 33–41.

Bicker, G., & Kreissl, S. (1994). Calcium imaging reveals nicotinic acetylcholine receptors on cultured mushroom body neurons. *Journal of Neurophysiology, 71*, 808–810.

Bitterman, M. E., Menzel, R., Fietz, A., & Schafer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *Journal of Comparative Psychology, 97*, 107–119.

Buckingham, S., Lapied, B., Corronc, H., & Sattelle, F. (1997). Imidacloprid actions on insect neuronal acetylcholine receptors. *Journal of Experimental Biology, 200*, 2685–2692.

Cano Lozano, V. (1997). Etude du rôle du système cholinergique dans les processus de mémorisation chez l'abeille. (Study of the role of the cholinergic system in the memorization processes in the honeybee). Ph.D. Thesis, Université Paul Sabatier Toulouse III.

Cano Lozano, V., Armengaud, C., & Gauthier, M. (2001). Memory impairment induced by cholinergic antagonists injected into the mushroom bodies of the honeybee. *Journal of Comparative Physiology A, 187*, 249–254.

Cano Lozano, V., Bonnard, E., Gauthier, M., & Richard, D. (1996). Mecamylamine-induced impairment of acquisition and retrieval of olfactory conditioning in the honeybee. *Behavioral Brain Research, 81*, 215–222.

Clarke, P. B., Schwartz, R. D., Paul, S. M., Pert, C. B., & Pert, A. (1985). Nicotinic binding in rat brain: autoradiographic comparison of [^{3}H]acetylcholine, [^{3}H]nicotine, and [^{125}I]alpha-bungarotoxin. *Journal of Neuroscience, 5*, 1307–1315.

Colhoun, E. (1963). The physiological significance of acetylcholine in insects and observations upon other pharmacological active substances. *Advances in Insect Physiology, 1*, 1–46.

Courjaret, R., & Lapied, B. (2001). Complex intracellular messenger pathways regulate one type of neuronal alpha-bungarotoxin-resistant nicotinic acetylcholine receptors expressed in insect neurosecretory cells (dorsal unpaired median neurons). *Molecular Pharmacology, 60*, 80–91.

Dacher, M., Lagarrigue, A., & Gauthier, M. (2005). Antennal tactile learning in the honeybee: effect of nicotinic antagonists on memory dynamics. *Neuroscience, 130*, 37–50.

Davies, A. R., Hardick, D. J., Blagbrough, I. S., Potter, B. V., Wolstenholme, A. J., & Wonnacott, S. (1999). Characterisation of the binding of [^{3}H]methyllycaconitine: a new radioligand for labeling alpha 7-type neuronal nicotinic acetylcholine receptors. *Neuropharmacology, 38*, 679–690.

Déglise, P., Dacher, M., Dion, E., Gauthier, M., & Armengaud, C. (2003). Regional brain variations of cytochrome oxidase staining during olfactory learning in the honeybee (*Apis mellifera*). *Behavioral Neuroscience, 117*, 540–547.

Déglise, P., Grünewald, B., & Gauthier, M. (2002). The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. *Neuroscience Letters, 321*, 13–16.

Erber, J., Masuhr, T., & Menzel, R. (1980). Localization of short-term memory in the brain of the bee, *Apis mellifera*. *Physiological Entomology, 5*, 343–358.

Erber, J., Kierzek, S., Sander, E., & Grandy, K. (1998). Tactile learning in the honeybee. *Journal of Comparative Physiology A, 183*, 737–744.

Fickbohm, D., & Trimmer, B. (2003). Antisense inhibition of neuronal nicotinic receptors in the tobacco-feeding insect, *Manduca sexta*. *Archives of Insect Biochemistry and Physiology, 53*, 172–185.

Goldberg, F., Grünewald, B., Rosenboom, H., & Menzel, R. (1999). Nicotinic acetylcholine currents of cultured Kenyon cells from the mushroom bodies of the honey bee *Apis mellifera*. *Journal of Physiology, London, 514*, 759–768.

Goodman, L. J. (1981). Organisation and physiology of the dorsal ocellar system. In H. Autrum (Ed.), *Handbook of sensory physiology* (Vol. VII/6C, pp. 504–580). Berlin: Springer-Verlag.

Guez, D., Belzunces, L., & Maleszka, R. (2003). Effects of imidacloprid metabolites on habituation in honeybees suggest the existence of two subtypes of nicotinic receptors differentially expressed during adult development. *Pharmacology Biochemistry and Behavior, 75*, 217–222.

Guez, D., Suchail, S., Gauthier, M., Maleszka, R., & Belzunces, L. (2001). Contrasting effects of imidacloprid on habituation in 7- and 8-day-old honeybees (*Apis mellifera*). *Neurobiology of Learning and Memory, 76*, 183–191.

Gundelfinger, E., & Schulz, R. (2000). Insect nicotinic acetylcholine receptors: genes, structure, physiological and pharmacological properties. In F. Clementi, D. Fornasari, & C. Gotti (Eds.), *Handbook of experimental pharmacology* (pp. 497–521). Berlin: Springer-Verlag.

Haberberger, R. V., Henrich, M., Lips, K. S., & Kummer, W. (2003). Nicotinic receptor alpha 7-subunits are coupled to the stimulation of nitric oxide synthase in rat dorsal root ganglion neurons. *Histochemistry and Cell Biology, 120*, 173–181.

Hammer, M., & Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learning & Memory, 5*, 146–156.

Hermsen, B., Stetzer, E., Thees, R., Heiermann, R., Schrattenholz, A., Ebbinghaus, U., et al. (1998). Neuronal nicotinic receptors in the locust *Locusta migratoria*. Cloning and expression. *Journal of Biological Chemistry, 273*, 18394–18404.

Huang, Z. Y., & Knowles, C. (1990). Nicotinic and muscarinic cholinergic receptors in honeybee (*Apis mellifera*) brain. *Comparative Biochemistry and Physiology C, 97*, 275–281.

Karlin, A. (2002). Emerging structure of the nicotinic acetylcholine receptors. *Nature Review Neuroscience, 3*, 102–114.

Kreissl, S., & Bicker, G. (1989). Histochemistry of acetylcholinesterase and immunocytochemistry of an acetylcholine receptor-like antigen in the brain of the honeybee. *Journal of Comparative Neurology, 286*, 71–84.

Lansdell, S. J., & Millar, N. S. (2000). The influence of nicotinic receptor subunit composition upon agonist, alpha-bungarotoxin and insecticide (imidacloprid) binding affinity. *Neuropharmacology, 39*, 671–679.

Le Novère, N., & Changeux, J. P. (1995). Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells. *Journal of Molecular Evolution, 40*, 155–172.

Le Novère, N., Corringer, P. J., & Changeux, J. P. (2002). The diversity of subunit composition in nAChRs: evolutionary origins, physiologic and pharmacologic consequences. *Journal of Neurobiology, 53*, 447–456.

Mauelshagen, J. (1993). Neural correlates of olfactory learning paradigms in an identified neuron in the honeybee brain. *Journal of Neurophysiology, 69*, 609–625.

Menzel, R. (1999). Memory dynamics in the honeybee. *Journal of Comparative Physiology A, 185*, 323–340.

Menzel, R., Heyne, A., Kinzel, C., Gerber, B., & Fiala, A. (1999). Pharmacological dissociation between the reinforcing, sensitizing, and response-releasing functions of reward in honeybee classical conditioning. *Behavioral Neuroscience, 113*, 744–754.

Mercer, A., & Menzel, R. (1982). The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee *Apis mellifera*. *Journal of Comparative Physiology, 145*, 363–368.

Muller, U. (1994). Ca^{2+}/calmodulin-dependent nitric oxide synthase in *Apis mellifera* and *Drosophila melanogaster*. *European Journal of Neuroscience, 6*, 1362–1370.

Muller, U. (1996). Inhibition of nitric oxide synthase impairs a distinct form of long-term memory in the honeybee, *Apis mellifera*. *Neuron, 16*, 541–549.

Muller, U., & Bicker, G. (1994). Calcium-activated release of nitric oxide and cellular distribution of nitric oxide-synthesizing neurons in the nervous system of the locust. *Journal of Neuroscience, 14*, 7521–7528.

Nauen, R., Ebbinghaus-Kintscher, U., & Schmuck, R. (2001). Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis mellifera* (Hymenoptera: Apidae). *Pest Management Science, 57*, 577–586.

Oleskevich, S. (1999). Cholinergic synaptic transmission in insect mushroom bodies in vitro. *Journal of Neurophysiology, 82*, 1091–1096.

Ortells, M. O., & Lunt, G. G. (1995). Evolutionary history of the ligand-gated ion-channel superfamily of receptors. *Trends in Neuroscience, 18*, 121–127.

Paterson, D., & Nordberg, A. (2000). Neuronal nicotinic receptors in the human brain. *Progress in Neurobiology, 61*, 75–111.

R Development Core Team. (2004). *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing. Available from: <http://www.R-project.org>.

Rathouz, M. M., Vijayaraghavan, S., & Berg, D. K. (1996). Elevation of intracellular calcium levels in neurons by nicotinic acetylcholine receptors. *Molecular Neurobiology, 12*, 117–131.

Raymond-Delpech, V., Matsuda, K., Sattelle, B., Rauh, J., & Sattelle, D. (2005). Ion channels: molecular targets of neuroactive insecticides. *Invertebrate Neuroscience, 5*, 119–133.

Salgado, V. L., & Saar, R. (2004). Desensitizing and non-desensitizing subtypes of alpha bungarotoxin-sensitive nicotinic acetylcholine receptors in cockroach neurons. *Journal of Insect Physiology, 50*, 867–879.

Sandoz, J.-C., & Pham-Délègue, M.-H. (2004). Spontaneous recovery after extinction of the conditioned proboscis extension response in the honeybee. *Learning & Memory, 11*, 586–597.

Sattelle, D. B., Culetto, E., Grauso, M., Raymond, V., Franks, C. J., & Towers, P. (2002). Functional genomics of ionotropic acetylcholine receptors in *Caenorhabditis elegans* and *Drosophila melanogaster*. *Novartis Found Symposium, 245*, 240–257.

Sattelle, D. B., Jones, A. K., Sattelle, B. M., Matsuda, K., Reenan, R., & Biggin, P. C. (2005). Edit, cut and paste in the nicotinic acetylcholine receptor gene family of *Drosophila melanogaster*. *Bioessays, 27*, 366–376.

Scheidler, A., Kaulen, P., Bruning, G., & Erber, J. (1990). Quantitative autoradiographic localization of [^{125}I]alpha-bungarotoxin binding sites in the honeybee brain. *Brain Research, 534*, 332–335.

Sharples, C., & Wonnacott, S. (2001). Neuronal nicotinic receptors. *Tocris Reviews, 19*, 1–12.

Shoop, R. D., Chang, K. T., Ellisman, M. H., & Berg, D. K. (2001). Synaptically driven calcium transients via nicotinic receptors on somatic spines. *Journal of Neuroscience, 21*, 771–781.

Si, M. L., & Lee, T. J. (2001). Presynaptic alpha7-nicotinic acetylcholine receptors mediate nicotine induced nitric oxidergic neurogenic vasodilation in porcine basilar arteries. *Journal of Pharmacology and Experimental Therapeutics, 298*, 122–128.

Smith, D. A., Hoffman, A. F., David, D. J., Adams, C. E., & Gerhardt, G. A. (1998). Nicotine-evoked nitric oxide release in the rat hippocampal slice. *Neuroscience Letters, 255*, 127–130.

Stollhoff, N., Menzel, R., & Eisenhardt, D. (2005). Spontaneous recovery from extinction depends on the reconsolidation of the acquisition memory in an appetitive learning paradigm in the honeybee (*Apis mellifera*). *Journal of Neuroscience, 25*, 4485–4492.

Thany, S. H., Crozatier, M., Raymond-Delpech, V., Gauthier, M., & Lenaers, G. (2005). Apis α2, Apis α7-1 and Apis α7-2: three new neuronal nicotinic acetylcholine receptor α-subunits in the honeybee brain. *Gene, 344*, 125–132.

Thany, S. H., Lenaers, G., Crozatier, M., Armengaud, C., & Gauthier, M. (2003). Identification and localization of the nicotinic acetylcholine receptor alpha3 mRNA in the brain of the honeybee, *Apis mellifera*. *Insect Molecular Biology, 12*, 255–262.

Tomizawa, M., & Casida, J. E. (2001). Structure and diversity of insect nicotinic acetylcholine receptors. *Pest Management Science, 57*, 914–922.

Tomizawa, M., & Casida, J. E. (2003). Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annual Review of Entomology, 48*, 339–364.

Tsunoyama, K., & Gojobori, T. (1998). Evolution of nicotinic acetylcholine receptor subunits. *Molecular Biology and Evolution, 15*, 518–527.

Vijayaraghavan, S., Pugh, P. C., Zhang, Z. W., Rathouz, M. M., & Berg, D. K. (1992). Nicotinic receptors that bind alpha-bungarotoxin on neurons raise intracellular free Ca^{2+}. *Neuron, 8*, 353–362.

Wüstenberg, D. G., & Grünewald, B. (2004). Pharmacology of the neuronal nicotinic acetylcholine receptor of cultured Kenyon cells of the

honeybee, *Apis mellifera*. *Journal of Comparative Physiology A, 190*, 807–821.

Yokotani, K., Okada, S., & Nakamura, K. (2002). Characterization of functional nicotinic acetylcholine receptors involved in catecholamine release from the isolated rat adrenal gland. *European Journal of Pharmacology, 446*, 83–87.

Zayas, R. M., Qazi, S., Morton, D. B., & Trimmer, B. A. (2002). Nicotinic-acetylcholine receptors are functionally coupled to the nitric oxide/cGMP-pathway in insect neurons. *Journal of Neurochemistry, 83*, 421–431.

Zhang, N., Tomizawa, M., & Casida, J. E. (2004). Drosophila nicotinic receptors: evidence for imidacloprid insecticide and α-bungarotoxin binding to distinct sites. *Neuroscience Letters, 371*, 56–59.