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Effects of NMDA receptor antagonists on olfactory learning and memory in the honeybee (*Apis mellifera*)

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Abstract

In contrast to vertebrates the involvement of glutamate and *N*-methyl-D-aspartate (NMDA) receptors in brain functions in insects is both poorly understood and somewhat controversial. Here, we have examined the behavioural effects of two noncompetitive NMDA receptor antagonists, memantine (low affinity) and MK-801 (high affinity), on learning and memory in honeybees (*Apis mellifera*) using the olfactory conditioning of the proboscis extension reflex (PER). We induced memory deficit by injecting harnessed individuals with a glutamate transporter inhibitor, L-*trans*-2,4-PDC (L-*trans*-2,4-pyrrolidine dicarboxylate), that impairs long-term (24 h), but not short-term (1 h), memory in honeybees. We show that L-*trans*-2,4-PDC-induced amnesia is 'rescued' by memantine injected either before training, or before testing, suggesting that memantine restores memory recall rather than memory formation or storage. When injected alone memantine has a mild facilitating effect on memory. The effects of MK-801 are similar to those of L-*trans*-2,4-PDC. Both pretraining and pretesting injections lead to an impairment of long-term (24 h) memory, but have no effect on short-term (1 h) memory of an olfactory task. The implications of our results for memory processes in the honeybee are discussed.

Keywords: MK-801; Memantine; Glutamate; Associative learning; Mushroom bodies; Long-term memory; Proboscis conditioning

1. Introduction

The role of the amino acid L-glutamate in the central nervous system (CNS) of mammals is well established. Glutamate is considered to be the major excitatory neurotransmitter in the brain, but it also plays crucial roles in the development of the nervous system, including cell differentiation and synapse formation (Danbolt, 2001). Acting through *N*-methyl-D-aspartate (NMDA) receptors, glutamate is integrally involved in eliciting persistent changes in synaptic function resulting in learning and memory (Milner et al., 1998). By contrast, the involvement of glutamate in specific brain functions in insects and other invertebrates is both poorly understood and controversial (Kucharski et al., 2000; Maleszka, 2000; Sinakevitch et al., 2001) in spite of the fact that glutamate-like immunoreactivity has been reported in identified neuronal populations of insect brains (Bicker, 1999; Bicker et al., 1988; Schürmann et al., 2000; Sinakevitch et al., 2001). In addition, high concentrations of glutamate, which rise and fall with age, have been found in the brains of honeybees (Fuchs et al., 1989). Recently, we showed that pretraining injections of a glutamate transporter inhibitor L-*trans*-2,4-PDC (L-*trans*-2,4-pyrrolidine dicarboxylate) impair long-term (24 h) associative olfactory memory in the honeybee (Maleszka et al., 2000). This result suggested a role for glutamatergic transmission in memory processing in this organism and prompted us to investigate the effects of glutamate receptor antagonists on behaviour in the honeybee. Here we have evaluated the effects of two NMDA antagonists commonly used in mammalian studies, namely memantine and MK-801 on associative memory.

Memantine is a low-affinity, noncompetitive antagonist of the NMDA receptor that shows great promise in the treatment of neurological disorders such as Alzheimer's dementia and Parkinson's disease (Parsons et al., 1999; Reisberg et al., 2003; Rogawski, 2000). In comparison with high-affinity channel blocking NMDA receptor antagonists, low-affinity noncompetitive antagonists have a reduced

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tendency to cause neurobehavioural side effects in laboratory animals and in humans, and consequently, are clinically well tolerated (Palmer and Widzowski, 2000; Parsons et al., 1999). Memantine binds and blocks open NMDA channels more rapidly than closed channels. This 'use-dependence' property is considered as particularly desirable in enhancing the utility of this class of drugs since NMDA receptors would only be blocked when necessary (Parsons et al., 1999; Rogawski, 2000).

MK-801 or dizocilpine is a potent, high-affinity, non-competitive NMDA antagonist, that has long been used in vertebrates to investigate the effects of NMDA receptors in learning and memory (see Riedel et al., 2003, for review). In the vertebrate field a general tenet is that MK-801 hinders memory formation (Riedel et al., 2003). In insects, it was recently shown that MK-801 was able to eliminate the stimulatory effect of NMDA on cockroach juvenile hormone biosynthesis (Chiang et al., 2002); the effect of this drug on learning and memory, however, remains unclear.

We trained both drug-treated and control bees in an olfactory paradigm and then tested their ability to remember the learning task either after 1 h (short-term memory) or after 24 h (long-term memory). In this report we show that treatment with memantine has either no significant effect on memory (young bees), or slightly improves memory in older bees. However, this drug restores long-term memory impairment induced in honeybees by injections with L-*trans*-2,4-PDC. Memantine reverses this experimentally induced amnesia regardless of whether it is injected before training, together with L-*trans*-2,4-PDC, or injected before testing 24 h after L-*trans*-2,4-PDC. We also show that MK-801 and L-*trans*-2,4-PDC block memory recall in much the same way, in spite of their differing modes of action. Taken together our results suggest that these three glutamatergic drugs affect memory recall rather than memory formation in this insect.

2. Materials and methods

2.1. Organism

Individual frames of brood comb were removed from an experimental hive and placed in an incubator maintained at a constant 32 °C. Newly emerged bees were collected from these frames every day, thus ensuring that the experiments were carried out only on bees of known ages.

2.2. Chemicals

Memantine (1-amino-3,5-dimethyl-adamantane hydrochloride), MK-801 (5-methyl-10,11-dihydro-5 H-dibenzo[a,d]cyclo-hepten-5,10-imine maleate) and limonene were purchased from Sigma. L-*trans*-2,4-PDC was purchased from Tocris.

2.3. Training and drug administration

The training protocol employed by Bitterman et al. (1983) was adopted for the present study. The main modification in our protocol is the use of two odours, limonene and vanilla, associated with a rewarding and nonrewarding stimulus, respectively. Thus, our paradigm consisted of three learning sessions involving paired presentations of two odours, one closely following the other. To facilitate handling during training and the administration of pharmacological agents, individual bees were first anaesthetised on ice, and then secured in thin-walled aluminium tubes (7 mm in diameter) using thin strips of fabric-reinforced tape (GAFFA). The bees were mounted in these tubes so as to leave the head and antennae free to move, while also leaving the dorsum of the thorax exposed. Any bee that seemed sluggish was discarded before training. Bees were fed on 1 M sucrose solution via a syringe fitted with a 19-gauge needle once a day. The tubes holding the bees were then arranged in a Perspex rack and placed in an incubator overnight, to allow the bees to become accustomed to their new conditions. All bees were 6 days old when mounted, 7 days old when trained and 8 days old when tested, except when stated otherwise.

Bees were injected with the pharmacological agent(s) of interest according to the protocol employed by Maleszka et al. (2000). Injections were carried out 1 h before either a training session, a test session or both, depending on the experimental condition. Injections were carried out using a 25 μl Hamilton syringe with a repeating dispenser. Typically, 1 μl of 20 mM L-*trans*-2,4-PDC (3 ng/bee) in Bee Ringer (Bicker, 1995), 10 mM memantine in Bee Ringer (2 ng/bee), 10 mM MK-801, in Bee Ringer (3.3 ng/bee), or Bee Ringer alone was introduced into the thorax (1 bee = 100 mg). Training consisted of teaching the bees to associate one odour (conditioned stimulus) with an unconditioned stimulus. Limonene (4 μl/ml) in 1 M sucrose solution was the rewarding stimulus, while natural vanilla (4 μl/ml) in saturated NaCl solution was used as the nonrewarding stimulus. During each training session, the bee was first allowed to smell the rewarding stimulus for 5 s, following which one antenna was touched with the stimulus, leading to the extension of the proboscis and the tasting of the sugar reward. This was repeated with the nonrewarding stimulus. Each of these conditioning trials was repeated three times at 6-min intervals. A small exhaust fan positioned behind the bees was constantly employed throughout the duration of the experiment to remove any lingering odours. The test for the short-term retention of associative learning was carried out on the same day, 1 h following training. The test for the long-term retention of associative memory was carried out the next day, by presenting first the nonrewarding and then the rewarding stimulus to the bees, and noting the presence or absence of proboscis extension. Those subjects that withheld their proboscis when presented with the non-

rewarding stimulus (vanilla), and then extended the proboscis when presented with rewarding stimulus (limonene) were scored as having responded correctly. Bees responding to the nonrewarding stimulus or to both stimuli were considered to have responded incorrectly. A small proportion of bees (10–15%) not responding to either stimulus and then unable to extend the proboscis when stimulated with sucrose were discarded from subsequent analyses because it was impossible to determine their learning status.

3. Results

Fig. 1 shows the results of the first experiment that was designed to test the effectiveness and possible side effects of memantine injected into the thorax of honeybees of different ages, namely 4, 7 and 8 days old. These age groups represent very young individuals (4 day old) that typically perform very poorly in the PER paradigm under standard conditions and older bees (7–8 days old) that perform significantly better under the same conditions (Maleszka and Helliwell, 2001). We chose 7- and 8-day-old bees following our finding that major changes in the honeybee cholinergic system occur at the beginning of the second week of its life (Guez et al., 2003). Age-related changes in the concentrations of acetylcholine, glutamate and GABA in the honeybee brain have also been reported by other authors (Fuchs et al., 1989). We reasoned that such shifts in neurotransmitter levels might underlie behavioural responses. As illustrated in Fig. 1, 10 mM memantine (2 ng/bee) does not impair the long-term memory of PER conditioning in any of the tested age groups. In fact, a small but statistically significant improvement is seen in 7-day-old bees following the administration of memantine.

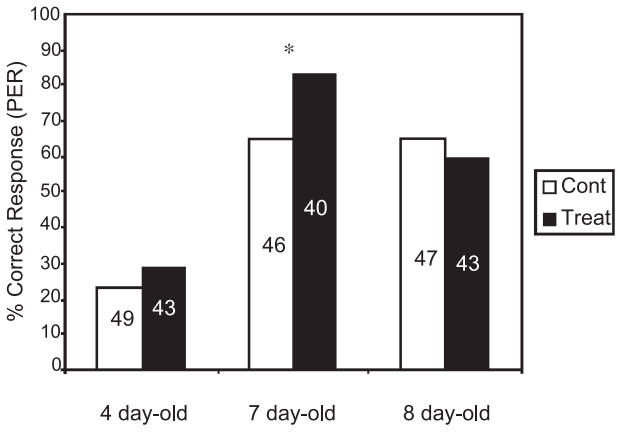

Fig. 1. Long-term memory of an associative olfactory task tested 24 h after training in bees of various ages injected with 1 μl of 10 mM memantine 1 h prior to training. The percentage of correct responses was evaluated by the proboscis extension reflex (PER) conditioning. The labels under the *x* axis indicate treatment conditions. The control in all cases was Bee Ringer. The numbers on the bars give the number of bees tested in each condition. * $P<.05$ (χ^2 test).

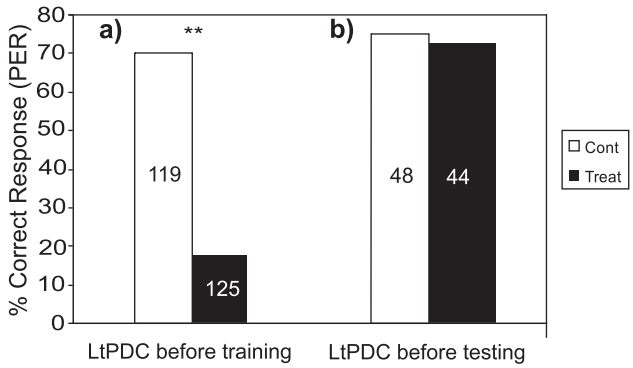

Fig. 2. Long-term memory of PER conditioning in bees treated with 20 mM L-*trans*-2,4-PDC before training (results from Maleszka et al., 2000) or before testing. ** $P<.01$. Bees were trained when 7 days old, and tested when 8 days old. Other details as in Fig. 1.

Further investigations into the effects of memantine on honeybees with pharmacologically induced amnesia were carried out in a set of experiments shown in Fig. 2. Experimental amnesia was induced by pretraining injections with 20 mM L-*trans*-2,4-PDC, a potent inhibitor of glutamate transport that causes a significant impairment of long-term (24 h) associative memory in classically conditioned honeybees (Fig. 2a; Maleszka et al., 2000). Injections of L-*trans*-2,4-PDC 1 h before testing have no effect on memory (Fig. 2b). This last result suggested either (a) that L-*trans*-2,4-PDC was affecting memory formation or storage rather than recall, or (b) that the kinetics of L-*trans*-2,4-PDC in the bee were such that there was a significant time delay between the administration of the drug and its effect.

Bees were then treated with 20 mM L-*trans*-2,4-PDC in combination with varying concentrations (0–10 mM) of memantine. Fig. 3 shows that memantine acted in a dose-dependant manner, and was able to bring about a significant

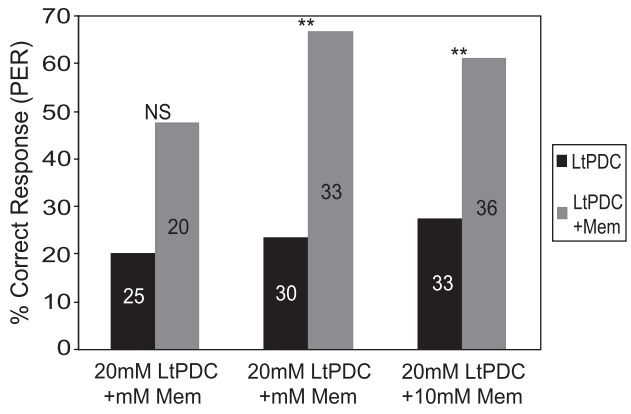

Fig. 3. Dependence of the level of PER conditioning on the concentration of memantine administered in conjunction with 20 mM L-*trans*-2,4-PDC before training. The control (dark bars) in all cases was 20 mM L-*trans*-2,4-PDC. Bees were trained when 7 days old, and tested when 8 days old. **($P<.01$) or N.S. (no significant difference) (χ^2 tests). Other details as in Fig. 1.

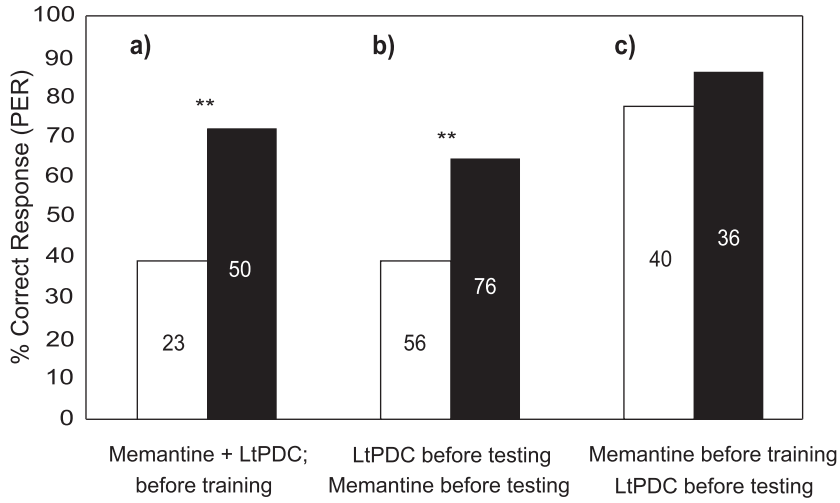

Fig. 4. Long-term memory of PER conditioning in bees treated with L-*trans*-2,4-PDC and memantine in various combinations, or alone. Control (white bars) for experiment (a), 20 mML-*trans*-2,4-PDC before training; control for experiment (b), 20 mM L-*trans*-2,4-PDC before training and Bee Ringer before testing; control for experiment (c), Bee Ringer before training and 10 mM L-*trans*-2,4-PDC before testing. Bees were trained when 7 days old, and tested when 8 days old. $**P<.01$ (χ^2 test). Other details as in Fig. 1.

improvement in the performance of bees even at a dosage as low as 5 mM.

The next series of experiments was carried out to determine which step in the memory pathway (memory formation, storage or recall) was being affected by the two drugs. Once again, the administration of 10 mM memantine in conjunction with 20 mM L-*trans*-2,4-PDC prior to training restored the percentage of correct responses to normal levels (Fig. 4a). An injection of 10 mM memantine 1 h before a test was also able to bring about a dramatic improvement in the performance of bees treated with L-*trans*-2,4-PDC before training on the previous day (Fig. 4b). This suggested that it was memory recall that was being acted upon by memantine. A reversal of the sequence of pharmacological intervention (memantine before training and L-*trans*-2,4-PDC before testing), however, had no effect on the responses of the animals (Fig. 4c).

To distinguish between the two possibilities arising from the experiment reported in Fig. 2b, another group of bees was treated with L-*trans*-2,4-PDC 1 h after training, followed by 10 mM memantine 1 h before testing the following day (Fig. 5). The control bees were only treated with L-*trans*-2,4-PDC. L-*trans*-2,4-PDC was administered 1 h after training to rule out the possibility that either memory formation or storage was being affected, and also to give the drug sufficient time (approximately 24 h) to have an effect before the bees were tested. The performance of the control bees was reduced to the levels seen in previous experiments, while an injection of memantine before testing was able to raise it back to normal. Thus, it was the recall of long-term memory that was being acted upon by both drugs.

To determine whether other commonly used NMDA receptor antagonists also affect olfactory memory in this insect, the high-affinity NMDAR antagonist MK-801 was administered to bees both before training and testing. The recall of long-term (24 h) memory was impaired by MK-801 in much the same way as by L-*trans*-2,4-PDC (Fig. 6b and c). In addition, short-term (1 h) memory was not affected by pretraining injections of MK-801 (Fig. 6a). This finding shows that MK-801 has no effect on brain faculties needed for sensory perception, acquisition of learning task or short-term memory, but impairs long-term memory of associative olfactory learning.

Finally, we evaluated the effects of both antagonists MK-801 and memantine, on the honeybee neuromuscular

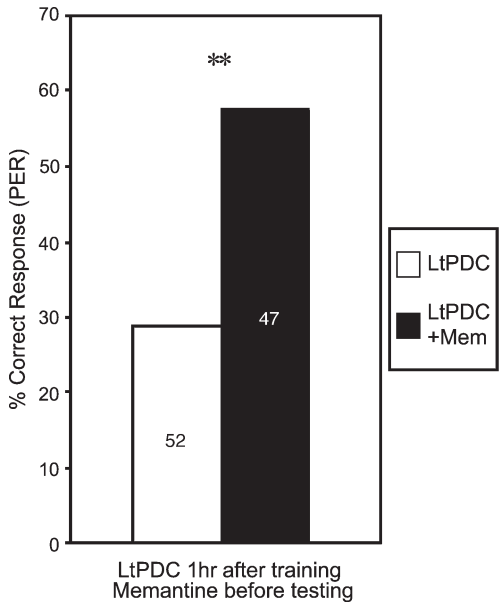

Fig. 5. Long-term memory of PER conditioning in bees treated 20 mM L-*trans*-2,4-PDC 1 h after training, and 10 mM memantine 1 h before testing. Control bees were given 20 mM L-*trans*-2,4-PDC after training and Bee Ringer before testing. Other details as in Fig. 3.

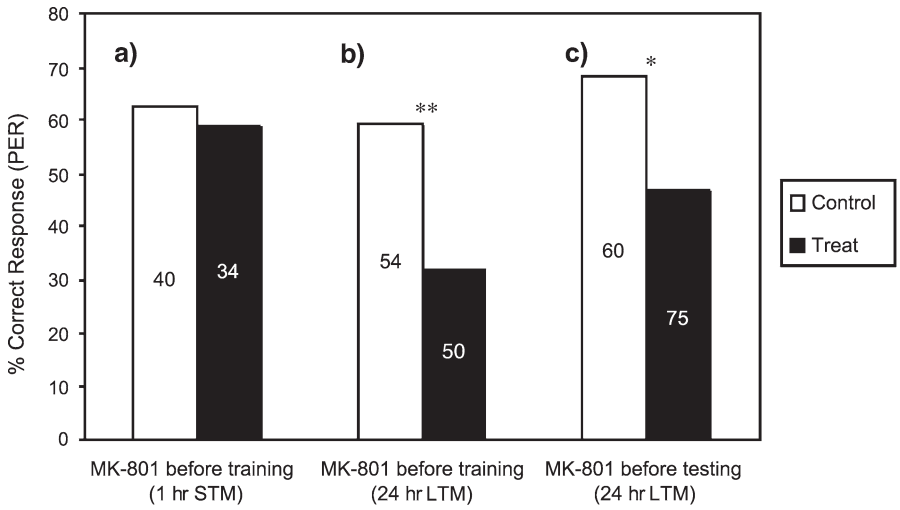

Fig. 6. Short-term (a) and long-term memory (b and c) of PER conditioning in bees treated with 10 mM MK-801. (a) The drug was administered before training, and bees were tested 1 h later. (b) The drug was administered before training, and bees were tested 24 h later. (c) The drug was administered before testing, which was carried out 24 h after the training session. Control bees in all cases were given Bee Ringer. LTM = long-term memory, STM = short-term memory. Other details as in Fig. 4.

system. The highest concentrations tested were 20 mM (6.7 ng/bee) for MK-801 and 50 mM (10 ng/bee) for memantine. The relative mobility of bees can be easily assessed by observing mounted individuals; normal (and untreated) bees are seen to vigorously move their antennae and forelegs. Judging from the relative mobility of drug-injected and control subjects, both MK-801 and memantine have no significant effects on locomotor activities of honeybees.

4. Discussion

In vertebrates, much of the brain's neuronal activity is controlled by the various functional states of glutamate receptors that translate the concentration profile of neurotransmitter into a defined time course of ion flow across the postsynaptic membrane (Milner et al., 1998). In insects, a growing body of evidence supports the notion that glutamate is also used for synaptic communication in the central pathways in addition to its well-established role at the neuromuscular junction (Petersen et al., 1997). Genomic sequencing has revealed highly conserved genes encoding both ionotropic and metabotropic glutamate receptors in insects (Ultsch et al., 1993, Parmentier et al., 1996, Völkner et al., 2000, GenBank AAP94623) and glutamate-like immunoreactivity has been detected in insect brains, including the honeybee brain (Bicker, 1999; Bicker et al., 1988; Sinakevitch et al., 2001). In accord with these findings our recent molecular and pharmacological studies on a glutamate transporter in the honeybee have provided more clues to the functional role of the glutamatergic system in the insect brain. The honeybee gene AmEAAT encoding a putative orthologue of the mammalian glutamate transporter subtype EAAT-2 is expressed in two regions of the brain, namely in the optic lobes and in a subset of Kenyon cells of the mushroom bodies, and high levels of AmEAAT message are found in pupal stages, possibly indicating a role for glutamate in the developing brain (Kucharski et al., 2000). At the behavioural level, injections of a glutamate transporter inhibitor, L-*trans*-2,4-PDC, impair long-term, but not short-term, associative olfactory memory (Maleszka et al., 2000). In this report we show that treatment of honeybees with two noncompetitive NMDA receptor antagonists, memantine (low-affinity) and MK-801 (high-affinity) leads to behavioural effects consistent with the role of this class of receptors in memory recall. Memantine restores L-*trans*-2,4-PDC-induced memory impairment in honeybees, regardless of whether it is injected before training or before testing. L-*trans*-2,4-PDC, too, is able to induce amnesia in bees under the same conditions, provided there is a sufficiently long delay between administration and testing. We also demonstrate that MK-801 is able to induce memory deficits in honeybees when administered before both training and testing. This suggests that memantine, L-*trans*-2,4-PDC and MK-801 affect memory recall rather than memory acquisition or storage. The results of the L-*trans*-2,4-PDC and MK-801 experiments also suggest that the recall of long-term memory is impaired by any kind of disruption of the relevant signalling pathways, be it overstimulation of the NMDA receptors (L-*trans*-2,4-PDC), or blockade of these receptors by high-affinity antagonists (MK-801).

In mammals, high-affinity NMDA receptor antagonists appear to have differential effects on various types of memory. Under physiological conditions, conventional inhibitors of NMDA receptors suppress long-term potentiation (LTP) and impair learning and memory (Izquierdo, 1994, Riedel et al., 2003). On the other hand, investigations on memory functions in humans after NMDA-receptor

blockade, including treatment with memantine, suggest that NMDA-receptor antagonists have differential effects on memory functions. For example, a recent study has shown that recognition performance for objects was impaired under memantine, whereas performance on face recognition was not affected (Rammsayer, 2001). According to the current mammalian model, memantine improves cognition by ensuring a sufficient signal to noise ratio under conditions of increased tonic activation (noise) of NMDA receptors (Parsons et al., 1999). Memantine acts as a neuroprotective agent in mammals, but also can reverse NMDA-induced deficits in synaptic plasticity, both at the neuronal (LTP) and behavioural (learning) level (Parsons et al., 1999). The improvement in the PER performance in 7-day-old bees following memantine treatment resembles the positive symptomatologic effects of this drug on learning seen in some experiments with mammals. Although the reason for this cognitive improvement is not entirely clear, some experimental data suggest that memantine can reduce the synaptic noise and in fact enhance learning, in particular in those animals that perform poorly in learning tasks (Parsons et al., 1999). The honeybee performance in the PER paradigm is age-dependent and maximum responses are typically not achieved until the age of 6–7 days (Maleszka and Helliwell, 2001; Ray and Ferneyhough, 1997). This is likely to result from a combination of factors that differentiate between younger and older bees, such as sugar thresholds, brain development and gene expression. Recent evidence from our lab has shown that a major change in the cholinergic system occurs in the honeybee brain when they begin the second week of their lives (Guez et al., 2003). Whether a similar change occurs in other neurotransmitter systems, or alternatively whether the improvement in the PER conditioning, induced by memantine in 7-day-old bees, reflects an interplay of several neurotransmitter and modulatory systems remains to be established. In this context it is noteworthy that the levels of several neurotransmitters in the adult bee brain undergo age-related changes. For example, the concentrations of both acetylcholine and glutamate in the bee brain gradually increase until Days 6 and 10, respectively, and then drop to much lower levels (Fuchs et al., 1989).

Our data so far are most consistent with the idea that memantine and/or MK-801-sensitive receptors in the honeybee are involved in memory recall. Although direct evidence is still lacking, it is expected that these receptors belong to the NMDA family and have similar properties to their mammalian counterparts. This notion is supported by our recent sequencing and preliminary characterization of a honeybee cDNA encoding a highly conserved protein showing 70% similarity to the mammalian NMDA subtype 1 (GenBank AAP94623). The honeybee NMDAR1 messenger is enriched in the central brain (GenBank AAP94623) that includes paired neuropil called the mushroom bodies (MB), which is widely accepted as a learning and memory hub in insects (Menzel, 2001; Menzel and Giurfa, 2001). Further, our results are in good agreement with both histochemical and in situ hybridisation data showing that a defined area of the MB neuropil stains with antibodies against glutamate and with specific probes for a highly conserved glutamate transporter (Bicker, 1999; Kucharski et al., 2000; Sinakevitch et al., 2001). Interestingly, our data imply that the MB neurons involved in memory recall and acquisition are clearly separate. This notion is reminiscent of two recent molecular studies in *Drosophila* demonstrating that synaptic output from the MB is required for olfactory memory recall, but not for its acquisition or storage (Dubnau et al., 2001; McGuire et al., 2001). It is conceivable that this output in the fly is also glutamatergic.

Like in other animals, memory formation in the honeybee following a 3-trial classical conditioning is a dynamic, multiphase process that involves several brain regions and a sequence of events leading from a transient, interruptible memory trace to long-lasting, stable memory (Menzel, 2001; Menzel and Giurfa, 2001). The involvement of antennal lobes and octopamine in the initial stages of this process, and mushroom bodies in later stages is well established (Menzel, 2001). Other neurotransmitters, in particular acetylcholine, have also been implicated in memory processes in the honeybee (Lozano et al., 2001; Shapira et al., 2001). Although the molecular mechanisms underlying memory processing in the honeybee appear to be highly conserved (Müller, 2002), it has been suggested that the temporal dynamics of memory stages are adjusted to foraging behaviour in this insect. For example, the existence of two types of long term memory, one that is protein synthesis-independent (intervals 1–3 days) and the other that can be blocked by protein synthesis inhibitors (intervals >3 days) may be related to flowering periods of plants in a patch (Menzel, 2001). The paradigm used in this study employed a 6-min interval between learning sessions and is expected to lead to a protein synthesis-dependent long-term memory that lasts for days (Müller, 1996). It is noteworthy that in mammals, NMDA receptor-induced phosphorylation of the transcription factor CREB and expression of its target genes is an essential step in memory consolidation (Ghosh, 2002).

In conclusion, our study provides strong, albeit indirect, support for the notion that glutamatergic transmission is an integral part of memory in the honeybee. Our data so far are most consistent with the idea that memantine- and MK-801-sensitive putative NMDA receptors in the honeybee are involved in memory recall. Given that it is very difficult to distinguish between memory formation, storage and retrieval, our experimental design offers a convenient way to study these processes separately. Finally, since some of the commonly used mammalian NMDA receptor antagonists (D-AP5 and CNQX) have been ineffective in insects (Oleskevich, 1999), the successful usage of two noncompetitive antagonists, memantine and MK-801, in the honeybee suggests that these drugs may prove to be valuable tools in pharmacological studies in insects.

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