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Contrasting Effects of Imidacloprid on Habituation in 7- and 8-Day-Old Honeybees (*Apis mellifera*)

David Guez,*,† Séverine Suchail,* Monique Gauthier,‡ Ryszard Maleszka,† and Luc P. Belzunces*

**Laboratoire de Toxicologie Environnementale, INRA, Unité de Zoologie, Site Agroparc, 84914 Avignon Cedex 9, France; †Research School of Biological Sciences, Australian National University, Canberra ACT 0200, Australia; and ‡Laboratoire de Neurobiologie et Comportement, Université Paul Sabatier (Toulouse 3), 118 Route de Narbonne, 31062 Toulouse Cedex, France*

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We examined the effects of sublethal doses (0.1, 1, and 10 ng per animal) of a new neonicotinoid insecticide, Imidacloprid, on habituation of the proboscis extension reflex (PER) in honeybees (*Apis mellifera*) reared under laboratory conditions. In untreated honeybees, the habituation of the proboscis extension reflex is age-dependent and there is a significant increase in the number of trials required for habituation in older bees (8–10 days old) as compared to very young bees (4–7 days old). Imidacloprid alters the number of trials needed to habituate the honeybee response to multiple sucrose stimulation. In 7-day-old bees, treatment with Imidacloprid leads to an increase in the number of trials necessary to abolish the response, whereas in 8-day-old bees, it leads to a reduction in the number of trials for habituation (15 min and 1 h after treatment), and to an increase 4 h after treatment. The temporal effects of Imidacloprid in both 7- and 8-day-old bees suggest that 4 h after treatment the observed effects are due to a metabolite of Imidacloprid, rather than to Imidacloprid itself. Our results suggest the existence of two distinct subtypes of nicotinic receptors in the honeybee that have different affinities to Imidacloprid and are differentially expressed in 7- and 8-day-old individuals.

Key Words: neonicotinoid; chloronicotinyl; nicotinic acetylcholine receptor; *Apis mellifera*; nonassociative learning; behavior; Hymenoptera.

INTRODUCTION

Nicotinic cholinergic systems have been implicated in various aspects of cognitive function in animals including attention, learning, and memory formation (Levin & Simon,

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Address correspondence and reprint requests to Luc P. Belzunces or David Guez, Laboratoire de Toxicologie Environnementale, INRA, Unité de Zoologie, Site Agroparc, 84914 Avignon Cedex 9, France. Fax: +33 (0)432 72 2602. E-mail: belzunce@avignon.inra.fr; guez@rsbs.anu.edu.au.

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1998; Woolf, 1998). In mammals, nicotinic agonist treatment can improve performance on a variety of memory tasks, whereas nicotinic antagonist treatment can impair memory functions (Levin & Simon, 1998). In invertebrates, the components required for the functioning of cholinergic synapses have been identified in many species including a popular behavioral model organism, the honeybee (Kreissl & Bicker, 1989; Osborne, 1996). Histochemical studies with antibodies against nicotinic acetylcholine receptors revealed the existence of cholinergic synapses in various regions of the honeybee brain, in particular in the putative learning center, the mushroom bodies, and the antennal lobes (Kreissl & Bicker, 1989). The involvement of cholinergic transmission in the proboscis extension reflex pathway, habituation, and memory formation in the honeybee has also been documented (Braun & Bicker, 1992; Gauthier, Cano-Lozano, Zaoujal, & Richard, 1994; Kreissl & Bicker, 1989; Lozano, Bonnard, Gauthier, & Richard, 1996; Lozano & Gauthier, 1998). More recently, studies on acetylcholine-induced currents in cultured mushroom body neurons revealed two distinct populations of nicotinic receptors, distinguished by their sensitivity to α-bungarotoxin (Goldberg, Grunewald, Rosenboom, & Menzel, 1999).

Imidacloprid {1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-*N*-nitro-1*H*-imidazol-2-amine}, a chlorinated derivative of nicotine, is the first member of a new family of insecticides (neonicotinoids) with a high potency against insects and a relatively low toxicity to noninsect species, including mammals (Elbert, Nauen, Cahill, Devonshire, Scarr, Sone, & Steffens, 1996; Leicht, 1996). Imidacloprid acts by activating receptors that are more common in insects than in other animals (Bai, Lummis, Leicht, Breer, & Sattelle, 1993; Matsuda, Buckingham, Freeman, Squire, Baylis, & Sattelle, 1998; Tomizawa, Otzuka, Miyamoto, Eldefrawi, & Yamamoto, 1995). Binding experiments in houseflies (Liu, Lanford & Casida, 1993) and electrophysiological studies in the cockroach (Buckingham, Lapied, Corronc, & Sattelle, 1997) show that Imidacloprid activates both nicotinic α-bungarotoxin-sensitive and α-bungarotoxin-insensitive receptors, as well as a "mixed" nicotinic and muscarinic cholinergic receptor (Buckingham et al., 1997). Some metabolites of Imidacloprid, such as 4-hydroxy Imidacloprid and Olefin-Imidacloprid, also have insecticidal properties which are distinct from those of the parent compound (Nauen, Tietjen, Wagner, & Elbert, 1998).

Our initial goal was to investigate the effects of Imidacloprid on nonassociative learning in honeybees. In addition, we were curious to determine whether Imidacloprid elicits differential effects on nonassociative learning in honeybees of different ages following recent findings that associative olfactory learning in this insect is age-dependent (Ray & Ferneyhough 1997, 1999).

We investigated the effects of Imidacloprid on habituation, the simplest form of nonassociative learning that is characterized by a decline in a behavioral response to a repeated nonnoxious stimulus. We used the proboscis extension reflex (PER), an appetitive component of the honeybee feeding behavior, which is elicited by touching one antenna with a droplet of sugar solution (Bitterman, Menzel, Fietz, & Schafer, 1983; Braun & Bicker, 1992), to quantify the responses of honeybees treated with Imidacloprid.

Here we show that habituation in the honeybee is age-dependent and a significant increase in the number of trials required for habituation occurs between young honeybees (4–7 days old) and older individuals (8–10 days old). Furthermore, we show that Imidacloprid has a differential effect on habituation in 7- and 8-day-old honeybees, suggesting

that major alterations in the biochemical composition of the cholinergic system occur at this stage of the honeybee development.

MATERIALS AND METHODS

Insect Preparation and Treatment

To obtain worker honeybees of known age a single frame of capped brood was removed from a hive and incubated in darkness at 31°C (80% humidity). Emerged adult insects were transferred to a honey frame and collected randomly at desired times using a pair of tweezers. Each queenless box (580 × 485 × 100 mm) contained up to 150 bees. Bees were placed on ice until immobile and mounted in thin-walled aluminium tubes (7 mm inner diameter) using a thin strip of fabric-reinforced tape (GAFFA) with the thoraces exposed. After mounting the bees were fed a 60% (w/v) sugar solution. Any bee that failed to respond to the 60% sugar solution was replaced to ensure that the proboscis extension reflex was not affected by the fixation process. After feeding, the bees were submitted to a 4-h fast before testing. During the fasting period the bees were treated by topical application (thorax) of 1 μl of Imidacloprid (a gift from Bayer AG France Inc.) in DMSO solution (0.1, 1, 10 ng/bee) or DMSO alone, 15 min, 1 h or 4 h before testing. All experiments were performed at 25°C at the same time of the day. One antenna was stimulated with a 40% (w/v) sugar solution at 3-s intervals. Each stimulation constitutes one trial. The criterion for habituation was three consecutive trials without proboscis extension. Applying a 60% sugar solution to the contralateral antenna restored the reflex. Bees that did not respond both to the restoration test and to at least one application of 40% solution were discarded and were not included in the statistical evaluation.

Statistical Analysis

The Systat 9.0 package from SPSS Inc. was used for data analysis. Data sets were analyzed using an Anova test on log-transformed data followed by the LSD Fischer post hoc test when suitable. Results are expressed as mean ± SE (data from at least three independent experiments). In all cases p values less than .05 were considered as significant.

RESULTS

In a strong, full hive colony, honeybees begin to forage 2–3 weeks after emergence. However, adult bees are sufficiently mature to begin flying 5–7 days after emergence (Capaldi & Dyer, 1999) and under certain conditions (low number of foragers) their behavioral maturation from nurses to foragers can be accelerated (Fahrbach & Robinson, 1995). We also observed that young bees in our colonies (Canberra) initiate their short orientation flights when they are 7–8 days old and we occasionally find “precocious” foragers that are 8 days old. Furthermore, in an initial habituation experiment with honeybees of different ages, reared in a laboratory incubator, we found a significant difference in the number of trials required for abolishing the PER response between honeybees representing two age groups, 4 to 7 days old and 8 to 10 days old. As shown in Fig. 1, the younger bees (4–7 days old) require on average only 7 trials before any visible

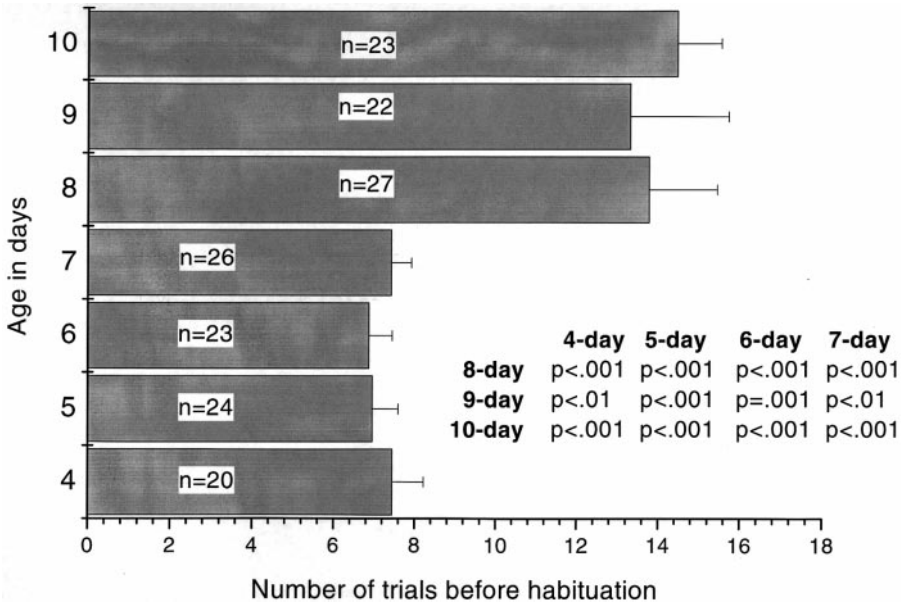


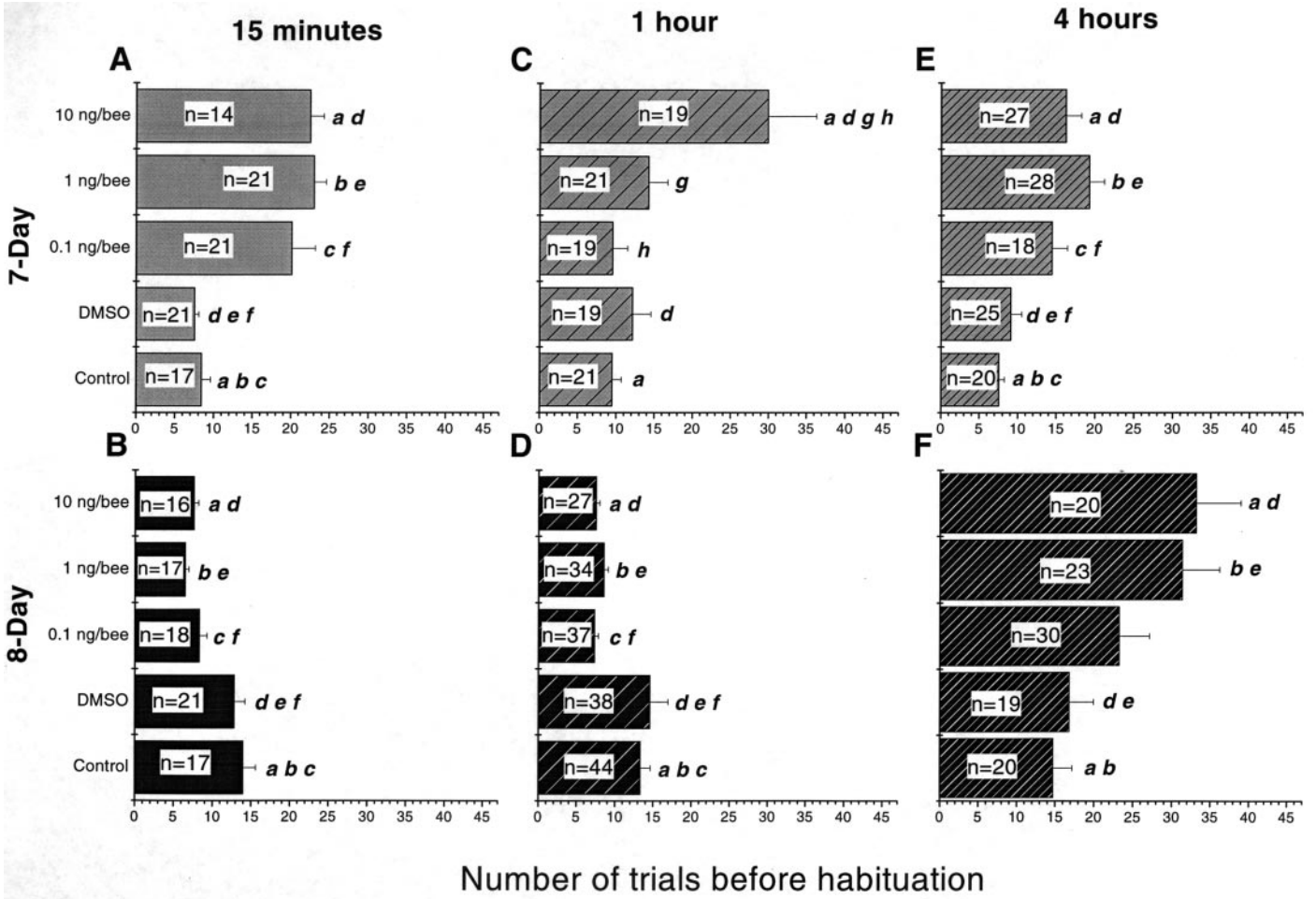
FIG. 1. Number of trials required for habituation of the proboscis extension reflex (PER) in untreated 4- to 10-day-old bees. Anova $F = 10.792$, $p < .001$, the results of the pairwise comparisons are tabularized in the figure.

response is abolished, whereas in older bees (8–10 days old), on average 14 trials are necessary to achieve habituation. Clearly, an important change affecting this behavior seems to take place between 7 and 8 days of the bee's life. This finding prompted us to test the effects of Imidacloprid on habituation in 7- and 8-day-old bees.

Figure 2 shows the contrasting effects of Imidacloprid treatment on habituation. Fifteen minutes after the application of Imidacloprid, all doses used led to an increase in the number of trials needed for habituation in 7-day-old bees (Fig. 2A) and to a decrease in the number of trials for habituation in 8-day-old bees (Fig. 2B). In both 7- and 8-day-old bees no significant difference is observed in doses of 0.1, 1, and 10 ng/bee, suggesting that 0.1 ng/bee is sufficient to saturate the Imidacloprid target. The solvent (DMSO) has no significant effect on habituation.

One hour after the application of Imidacloprid only the highest dose of the insecticide (10 ng/bee) significantly increases the number of trials needed to habituate 7-day-old bees (Fig. 2C). At lower concentrations (0.1 and 1 ng/bee) the insecticide has no apparent effect on habituation in bees at 7 days of age. In 8-day-old bees, 1 h after treatment, a significant decrease in the number of trials needed for habituation is clearly detectable at

FIG. 2. Number of trials required for habituation of PER after Imidacloprid treatment (0.1, 1, and 10 ng/bee) in 7- and 8-day-old bees. **(A)** Seven-day-old bees, 15 min after treatment, Anova $F = 28.268$, $p < .001$, ***a,b,c,d,e,f***, $p < .001$. **(B)** Eight-day-old bees, 15 min after treatment, Anova $F = 7.8$, $p < .001$, ***b,e***, $p < .001$, ***a,c,d,f***, $p < .01$. **(C)** Seven-day-old bees, 1 h after treatment, Anova $F = 4.903$, $p < .01$, ***h***, $p < .001$, ***a,d***, $p < .01$, ***g***, $p < .05$. **(D)** Eight-day-old bees, 1 h after treatment, Anova $F = 8.476$, $p < .001$, ***a,c,f***, $p < .001$, ***b,d,e***, $p < .01$. **(E)** Seven-day-old bees, 4 h after treatment, Anova $F = 11.248$, $p < .001$, ***a,b,d,e***, $p < .001$, ***c,f***, $p < .01$. **(F)** Eight-day-old bees, 4 h after treatment, Anova $F = 4.877$, $p < .01$, ***a,b,e***, $p < .01$, ***d***, $p < .05$.


15 minutes
1 hour
4 hours
A
C
E
B
D
F
7-Day
8-Day
10 ng/bee
1 ng/bee
0.1 ng/bee
DMSO
Control
n=14 a d
n=21 b e
n=21 c f
n=21 d e f
n=17 a b c
n=19 a d g h
n=21 g
n=19 h
n=19 d
n=21 a
n=27 a d
n=28 b e
n=18 c f
n=25 d e f
n=20 a b c
n=16 a d
n=17 b e
n=18 c f
n=21 d e f
n=17 a b c
n=27 a d
n=34 b e
n=37 c f
n=38 d e f
n=44 a b c
n=20 a d
n=23 b e
n=30
n=19 d e
n=20 a b
0 5 10 15 20 25 30 35 40 45
Number of trials before habituation

all concentrations tested (Fig. 2D). This result is similar to that observed 15 min after treatment in 8-day-old bees (Fig. 2B) and we conclude that this effect is due to a direct action of Imidacloprid. It also implies that the putative target in 8-day-old bees has a higher affinity for Imidacloprid than in 7-day-old bees (compare Figs. 2C and 2D).

Four hours after treatment, all the doses of Imidacloprid (0.1, 1, and 10 ng/bee) lead to an increase in the number of trials for habituation in 7-day-old bees (Fig. 2E). In 8-day-old bees the observable effect is a significant increase in the number of trials required for habituation at the concentrations of 1 and 10 ng/bee (Fig. 2F). However, considering the time after exposure and the fact that this effect is reversed in 8-day-old bees (compare 15 min and 1 h after treatment with 4 h after treatment, Figs. 2B, 2D, and 2F), the increase is most likely due to the action of a metabolite of Imidacloprid, rather than to Imidacloprid itself. This notion is supported by the fact that 0.1 and 1 ng of Imidacloprid has no significant effect on habituation in 7-day-old bees 1 h after treatment, but elicits a significant increase in the number of trials needed for habituation 4 h after treatment (compare 1 h after treatment with 4 h after treatment, Figs. 2C and 2E).

DISCUSSION

We show that habituation of the proboscis extension reflex in honeybees reared in an incubator is age-dependent and a significant increase in the number of trials required to achieve habituation occurs between 7 and 8 days of age. Treatment with a nicotinic agonist, Imidacloprid, alters the number of trials needed to habituate the PER response. These findings cannot be attributed to daily variation in performance since bees were tested on several different days and they clearly indicate that significant changes in certain brain functions occur at this time of adult development. Age-dependent learning has also been demonstrated for the 1-trial classical conditioning of the PER, whereby honeybees younger than 6–7 days cannot be consistently trained to learn an associative task (Ray & Ferneyhough, 1997, 1999). Together with previous findings showing that an increase in the mushroom body neuropil (putative learning center) begins around the time at which orientation flights take place (Fahrbach & Robinson, 1995), our results add more weight to the notion that profound molecular changes trigger behavioral maturation in honeybees. Although the precise mechanisms involved in these changes are not understood, it has been argued that they are controlled by both experience-expectant and experience-dependent components (Fahrbach et al., 1998).

The use of Imidacloprid in this study to modify habituation in the honeybee sheds more light on the nature of the underlying molecular mechanism. Imidacloprid treatment leads to an increase in the number of trials for habituation in 7-day-old bees (15 min, 1 h, and 4 h after treatment, Figs. 2A, 2C, and 2E) and to a decrease in the number of trials for habituation in 8-day-old bees (15 min and 1 h after treatment, Figs. 2B and 2D). We think that the effects observed 15 min and 1 h after treatment are due to the direct action of Imidacloprid, whereas, 4 h after treatment the behavior is more likely modified by one or more of the metabolites of Imidacloprid. The physicochemical properties of various metabolites of Imidacloprid suggest that the long-term effects on habituation in the honeybee may be elicited by either 4-hydroxy Imidacloprid, 5-hydroxy Imidacloprid or, Olefin-Imidacloprid (Nauen, Reckmann, Armborst, Stupp, & Elbert, 1999; Nauen et al., 1998). Recent studies on some of the metabolites of Imidacloprid strongly support the notion

that Olefin-Imidacloprid, rather than Imidacloprid itself, elicits the effect observed 4 h after treatment (Guez et al., manuscript in preparation). Although the target of Olefin-Imidacloprid is also a nicotinic receptor(s) (Nauen et al., 1998) our results suggest that in the honeybee this receptor has biochemical properties different from those of the high-affinity Imidacloprid nicotinic receptor.

Our findings are best explained by assuming the existence of two distinct subtypes of nicotinic acetylcholine receptors (nAChR1 and nAChR2) with different affinities to Imidacloprid and its metabolite(s). We hypothesize that subtype nAChR1, with a high affinity to one or more of the metabolites of Imidacloprid, and with a low affinity to Imidacloprid, is expressed predominantly in 7-day-old (and younger) bees. The second subtype, nAChR2, with a high affinity to Imidacloprid, is activated at Day 8 and coexists with nAChR1.

In accordance with our finding, Benke and Breer (1989) suggested the existence of different subtypes of acetylcholine receptors within the insect nervous system differentiated by their pharmacological properties. This notion has been corroborated by a number of independent studies. In the locust, distinct neuronal acetylcholine receptors have been identified on the same cell as synaptic and extrasynaptic isoforms (Tareilus, Hanke, & Breer, 1990). In other insects, including the honeybee, different subpopulations of acetylcholine receptors with distinct conductance levels and gating kinetics may occur in the same cell (Beadle, Horseman, Pichon, Amar, & Shimahara, 1989; Goldberg et al., 1999; Leech & Sattelle, 1992; Tareilus et al., 1990). Finally, in a recent study, Wiesner and Kayser (2000) have identified two subpopulations of nAChRs with different affinities to Imidacloprid in the locust and two species of aphids. These results are consistent with our idea that two nAChR receptors with different pharmacological properties are differentially expressed in the adult brain of honeybee and add more weight to the hypothesis that a major change(s) in the cholinergic system occurs during the development of an adult brain in this insect.

The biological significance of these molecular changes for the honeybee remains to be established. The workers reared in an incubator are devoid of colony context and are not exposed to the stimuli that are involved in the normal development of adult honeybees, such as the queen's pheromones. For example, honeybees reared in an incubator show a much faster development of PER conditioning than honeybees reared in a full hive colony, suggesting that the behavioral role within the colony is an important factor influencing olfactory learning (Ray & Ferneyhough, 1997). One possibility is that molecular switches are executed as default signals, whereas the proper behavioral development is also controlled by social and/or environmental factors. Clearly, more studies are required to understand the precise mechanism(s) involved in these changes and their phenotypic implications. In this context, analyses of simple behaviors, such as habituation, may be particularly useful in advancing our understanding of molecular and cellular changes underlying behavioral modifications.

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