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Effects of imidacloprid metabolites on habituation in honeybees suggest the existence of two subtypes of nicotinic receptors differentially expressed during adult development

D. Guez[a,*], L.P. Belzunces[b], R. Maleszka[a]

[a]*Visual Sciences, Research School of Biological Sciences, Australian National University, Canberra, ACT 0200, Australia*
[b]*Laboratoire de Toxicologie Environnementale-INRA, UMR 406, INRA-UAPV Ecologie des Invertébrés, Avignon Cedex 9 84914, France*

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Abstract

Habituation of the proboscis extension reflex (PER) in honeybees (*Apis mellifera*) is age-dependent. Very young bees (≤7 days old) require significantly less trials to abolish the response to multiple sucrose stimulations than older bees (≥8 days old). A nicotinic agonist, imidacloprid, modifies this behaviour by increasing the number of trials in ≤7-day-old bees and by decreasing it in older bees [Neurobiol. Learn. Mem. 76 (2001) 183.]. Here we tested our hypothesis that this effect is associated with a differential expression of two subtypes of nicotinic acetylcholine receptors (nAChRs). By testing the effects of six metabolites of imidacloprid, we show that two of them, olefin and 5-hydroxy-imidacloprid, modify the number of trials needed to habituate the PER in a contrasting manner. Olefin increases the number of trials in both age groups, whereas 5-hydroxy-imidacloprid decreases the number of trials, but only in 8-day-old individuals. We conclude that olefin and 5-hydroxy-imidacloprid are specific agonists of two subtypes of an nAChR that are differentially expressed during adult maturation of young honeybees. Olefin is the agonist of an nAChR expressed in both age groups, whereas 5-hydroxy-imidacloprid is the agonist of a late-onset nAChR that is activated in 8-day-old bees. The implications of this finding for the honeybee biology are discussed.

Keywords: Nicotinic receptor agonist; Neonicotinoid; Cholinergic system; Nonassociative learning; *Apis mellifera*

1. Introduction

In recent years, the involvement of neuronal nicotinic cholinergic systems in various aspects of cognitive function in animals has been the focus of intense multidisciplinary research (Levin and Simon, 1998; Lukas et al., 1999; Woolf, 1998). One particularly powerful experimental tool in these studies has been the use of both agonists and antagonists that selectively interact with the neuronal nicotinic acetylcholine receptor (nAChR). Pharmacologically distinct subtypes of nAChRs composed of heteromeric combinations of α- and β-subunits have been identified in vertebrate species (Lukas et al., 1999). Similarly, most of the nAChRs expressed in the insect central nervous system are thought to constitute heteromeric complexes; however, their in vivo subunit composition is still unknown (Chamaon et al., 2002; Huang et al., 1999, 2000). Phylogenetic comparisons suggest that the insect α-subunits evolved in parallel to the vertebrate neuronal nAChRs, whereas the insect non-α-subunits are different from vertebrate neuronal β-subunits and muscle non-α-subunits (Chamaon et al., 2002; Huang et al., 1999, 2000). In mammals, nAChR agonists have been shown to improve performance in a variety of memory tasks, whereas treatment with nAChR antagonists has been shown to impair memory functions (Levin and Simon, 1998). In the honeybee, the link between cholinergic transmission and learning and memory formation has been demonstrated by the classical olfactory conditioning of the proboscis extension reflex (PER) (Cano Lozano and Gauthier, 1998; Cano Lozano et al., 1996, 2001; Gauthier et al., 1994).

Previously, we have shown that the habituation of the PER in caged honeybees 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 individuals (4–7 days old (Guez et al., 2001). This behavioural change is most likely an important adapta-

* Corresponding author. Tel.: +61-2-6125-9701; fax: +61-2-6125-3784.
E-mail address: guez@rsbs.anu.edu.au (D. Guez).

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tion to foraging that is typically performed by older bees. Rapid habituation would be disadvantageous to foraging honeybees since they collect food from sources that are often monotonous and repeated. Treatment with a nicotinic agonist, imidacloprid (Matsuda et al., 2001), modifies the expression of this behaviour in a contrasting manner (Guez et al., 2001). To explain our findings, we proposed that: (a) two distinct populations of nicotinic receptors differing with regard to their affinity to imidacloprid and/or some of its metabolites are expressed differentially in young honeybees. One subtype, with high affinity for imidacloprid, is activated on Day 8 and is not expressed in younger bees, whereas the other subtype, with a low affinity for imidacloprid and a high affinity for one or more of its metabolites, is expressed in young honeybees regardless of age; (b) the long-term effects, observed 4 h after treatment with imidacloprid, are the result of the action of one or more of imidacloprid metabolites rather than the parent compound (Guez et al., 2001).

In an attempt to test these hypotheses, we used the same behavioural paradigm and a range of imidacloprid metabolites to investigate their effects on the PER in honeybees of 7 and 8 days of age. The results presented here support the view that two temporally separated subtypes of nAChRs are expressed in honeybees during early adult development. Our findings suggest that tightly regulated genetic switches may control the expression of molecular machinery that underlies a wide range of physiological and behavioural differences between young bees (“nurses”) and mature foragers, such as learning abilities, foraging behaviour, and biological rhythms. Further, our study suggests that two metabolites, olefin and 5-hydroxy imidacloprid, may be useful as specific nicotinic agonists to target distinct nAChRs in insects.

2. Materials and methods

Minor modifications were made to our previously described protocol (Guez et al., 2001). Bees were collected after emergence using forceps, and placed in a queenless mesh box with wooden frame (200×200×50 mm). Fresh honey was provided using 14-ml Falcon tubes with small holes pierced in the bottom. The tubes were introduced through two holes on the top of the mesh box. Each box contained around 100–150 individuals. The bees were kept in an incubator (31 °C and 80% humidity) and collected at desired ages.

The bees were placed on ice until immobile and mounted in thin-walled aluminium tubes (7 mm in diameter) using a thin strip of fabric-reinforced tape (GAFFA) with the thoraces exposed. After mounting, the bees were fed a 60% (wt/wt) sugar solution. Any bee that failed to respond to the 60% sugar solution was replaced to ensure that the fixation process did not affect the PER. After feeding, the bees were submitted to a 4-h fast before testing. During the fasting period, 1 μl of an imidacloprid metabolite was applied topically on the thorax. Imidacloprid, a chlorinated derivative of nicotine, is the first member of a new family of insecticides with high agonistic specificity for insect nicotinic receptors and low affinity to mammalian nicotinic receptors. The following metabolites of imidacloprid {1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-*N*-nitro-1*H*-imidazol-2-amine}were tested: olefin, 5-hydroxy-imidacloprid, 4,5-dihydroxy-imidacloprid, urea-imidacloprid, denitro-imidacloprid, and 6-chloro-nicotinic acid (Bayer, France) All metabolites were dissolved in DMSO (Sigma). Control bees were treated with 1 μl of DMSO. All experiments were performed at 25 °C at the same time of the day. One antenna was stimulated with a 40% (wt/wt) sugar solution at 3-s intervals. Each stimulation constituted 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 to both the restoration test and to at least one application of 40% solution were discarded and were not included in the statistical evaluation.

2.1. Statistical analysis

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

3. Results

Our initial goal was to uncover the identity of imidacloprid metabolite responsible for the effects observed 4 h after treatment, namely the increase in the number of trials required for habituation of the PER in young honeybees, regardless of age (Guez et al., 2001). Although imidacloprid is relatively stable in vitro, it is rapidly metabolised in plants, mammals, and insects, yielding a number of related compounds. Some of these metabolites, such as 5-hydroxy-imidacloprid and olefin, also have potent agonistic properties, which are distinct from that of the parent compound (Nauen et al., 1998).

Because a dose of 1 ng of imidacloprid per individual has been found to be sufficient for eliciting the maximal effect on habituation of the PER (Guez et al., 2001), we used the same amount of different imidacloprid metabolites in this study. A metabolite eliciting the same effect as seen with imidacloprid 4 h after treatment is considered to be the one responsible for that effect. Fig. 1 shows the effects of six major imidacloprid metabolites on habituation of the PER, evaluated 15 min after treatment in 7- and 8-day-old bees. Since habituation of the PER in untreated control bees has been shown to be identical to habituation in DMSO-treated control bees (Guez et al., 2001), untreated individuals were not included in this

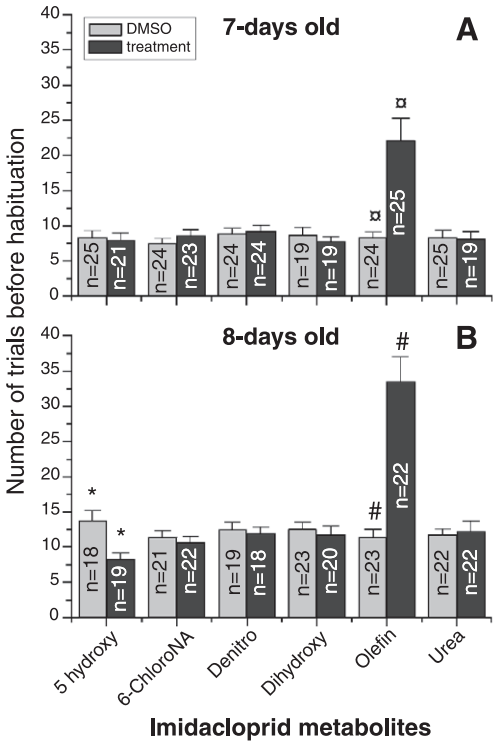

Fig. 1. Effects of six imidacloprid metabolites on habituation of the PER (1 ng per bee, 15 min after treatment). (A) Seven-day-old bees, t=4.09, ¤ P<.001. (B) Eight-day-old bees, t=3.23, *P<.01; t=6.16, $^{\#}P$<.001.

study (Guez et al., 2001). In 7-day-old bees (Fig. 1, panel A), only one of the metabolites tested, olefin, affects habituation of the PER by increasing the number of required trials. In 8-day-old bees (Fig. 1, panel B), two metabolites, 5-hydroxy-imidacloprid and olefin, elicit significant effects on the number of trials needed for habituation. However, in contrast to olefin, 5-hydroxy-imidacloprid appears to decrease the number of trials needed for habituation. Thus, olefin treatment induces a significant increase in the number of trials needed to obtain habituation of the PER in both age groups, whereas 5-hydroxy-imidacloprid decreases the number of trials only in 8-day-old bees (Fig. 1, panel A) and shows no effect in 7-day-old individuals. This finding suggests that the target of 5-hydroxy-imidacloprid is not expressed in young honeybees until Day 8. Because the target of 5-hydroxy-imidacloprid is also a nicotinic receptor (Nauen et al., 1998), this metabolite is a good candidate for a specific agonist that is effective against the nAChR subtype expressed from Day 8 onwards (Guez et al., 2001).

The effects induced by olefin 15 min after treatments in both 7- and 8-day-old bees are similar to those observed in both age groups 4 h after treatment with imidacloprid (Guez et al., 2001). This suggests that olefin, rather than the parent compound imidacloprid, is responsible for the increase in the number of trials seen in 7- and 8-day-old bees 4 h after treatment with imidacloprid. Fig. 2 shows the effects of olefin on habituation of the PER in 7- and 8-day-old bees following treatment with two doses of this metabolite (0.1

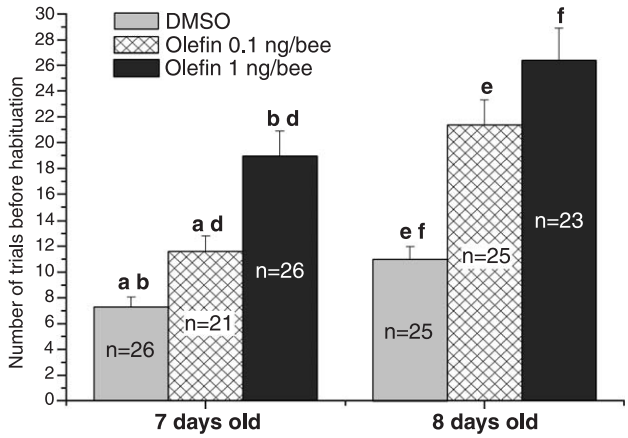

Fig. 2. Effects of olefin on habituation of PER, in 7- and 8-day-old bees 15 min after treatment. Seven days old, ANOVA F=19.070, P<.001. ^{a}P<.05, ^{b}P<.001, $^{d}P\leq$.001. Eight days old, ANOVA F=18.143, P<.001. $^{e,f}P$<.001.

and 1 ng per bee). In both cases, olefin induces a significant increase in the number of trials needed to obtain habituation. This result is in good agreement with our previous study and supports the notion that olefin is an agonist of the nAChR receptor expressed in both age groups.

The dose effect (0.1 and 1 ng of bee) of 5-hydroxy-imidacloprid in 8-day-old honeybees, observed 15 min after treatment (Fig. 3), indicates that only the higher amount (1 ng per bee) induces a significant reduction in the number of trials to achieve habituation of the PER. This is in contrast to imidacloprid, which elicits maximum reductions at 0.1 ng per bee at both times 15 min and 1 h after treatment (Guez et al., 2001). Hence, even though the 5-hydroxy metabolite appears to be a specific agonist of nAChR2, it has a lower affinity for this receptor than its parent compound, imidacloprid.

The metabolic pathway of imidacloprid is known in plants (Nauen et al., 1998) and in mammals (Thyssen and Machemer, 1999). In both cases, 5-hydroxy-imidacloprid is the precursor of olefin. To establish whether 5-hydroxy-

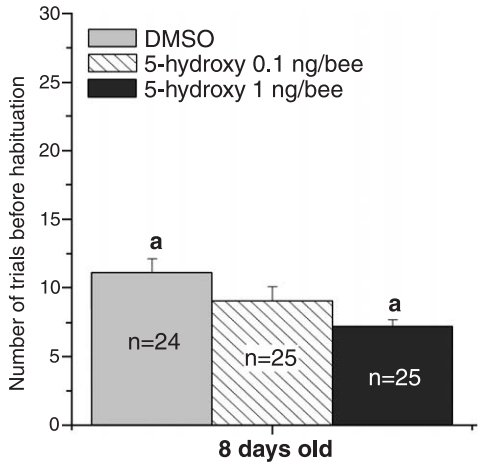

Fig. 3. Effects of 5-hydroxy-imidacloprid on habituation of PER in 8-day-old bees 15 min after treatment, ANOVA F=5.317, P<.01. ^{a}P<.01.

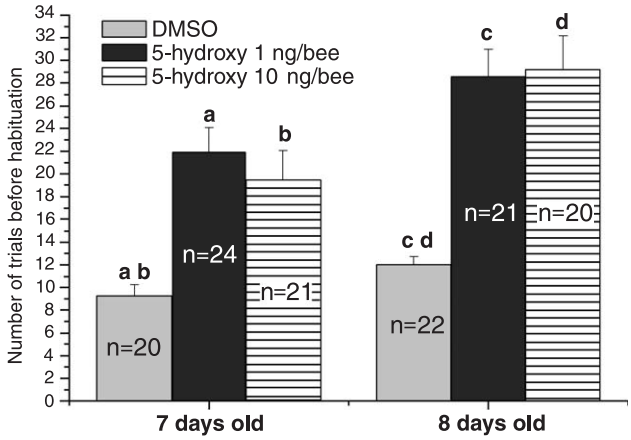

Fig. 4. Effects of 5-hydroxy-imidacloprid on habituation of PER in 7- and 8-day-old bees 1 h after treatment. Seven days old, ANOVA F=10.908, P<.001, ^{a}P<.001, $^{b}P\leq$.001. Eight days old, ANOVA F=20.783, P<.001. $^{c,d}P$<.001.

imidacloprid is also a precursor of olefin in the honeybee, we tested its effects on habituation of the PER 1 h after treatment in 7- and 8-day-old bees (Fig. 4). In both age groups, 5-hydroxy-imidacloprid induces a significant increase in the number of trials needed to obtain habituation of the PER. This result is in contrast to 15-min treatment with 5-hydroxy-imidacloprid that has no effect in 7-day-old bees, but leads to a decrease in the number of trials in 8-day-old bees (Fig. 1). These two observations suggest that the effects observed 1 h after treatment in both age groups are not due to 5-hydroxy-imidacloprid, but more likely to one of its metabolites. The obvious candidate is olefin, which elicits similar effects 15 min after treatment (Figs. 1 and 2) as those seen 1 h after treatment with 5-hydroxy-imidacloprid.

4. Discussion

As shown in the current study and in our previously published work (Guez et al., 2001), a major alteration in the honeybee cholinergic system occurs on Day 8 of adult development. We believe that the expression of a late-onset subtype of a nicotinic receptor with distinct sensitivity to imidacloprid is induced around the time when young honeybees begin the second week of their lives. We have shown previously that imidacloprid alters the number of trials needed for habituation of the PER response in a contrasting manner. In 7-day-old bees, it leads to an increase in the number of trials necessary to abolish the response (15 min, 1 h, and 4 h after treatment), whereas in 8-day-old bees, it leads to a reduction in the number of trials needed for habituation (15 min and 1 h after treatment). The effects of imidacloprid on habituation of the PER observed 4 h after treatment are similar in both age groups (Guez et al., 2001). In the present study, we provide evidence that the contrasting effects of imidacloprid are consistent with the existence of pharmacologically distinct subtypes of nAChRs that have different affinity to two metabolites of imidacloprid, olefin, and 5-hydroxy-imidacloprid. We propose that olefin is the agonist of a nAChR expressed in both age groups, whereas 5-hydroxy-imidacloprid is the agonist of a late-onset nAChR that is activated in 8-day-old bees (Fig. 5).

Although this finding is not yet integrated with molecular definition of nAChRs in the honeybee, a recent report on heterologous coexpression of nAChRs α-subunits from the aphid, *Myzus persicae*, sheds more light on the possible underlying mechanism (Chamaon et al., 2002; Huang et al., 1999, 2000). These authors examined affinity binding of nicotinic radioligands to *M. persicae* nAChR α-subunits cDNAs coexpressed with the rat β_2-subunits and found that imidacloprid selectively acts on α_2- and α_3-subunits, but not on α_1-subunit (Chamaon et al., 2002; Huang et al., 1999, 2000). Thus, it is possible that the presence of one type of α-subunit in a heteromeric receptor may influence ligand recognition of a native receptor in the honeybee. It is known from in vitro studies that ligand-binding pockets can be formed at interfaces between certain types of α- and β-subunits, but not between the others (Lukas et al., 1999). Unfortunately, knowledge of the native subunit composition of nAChRs in insects and other species is generally lacking. Evidence in vertebrates suggests that diverse nAChR recep-

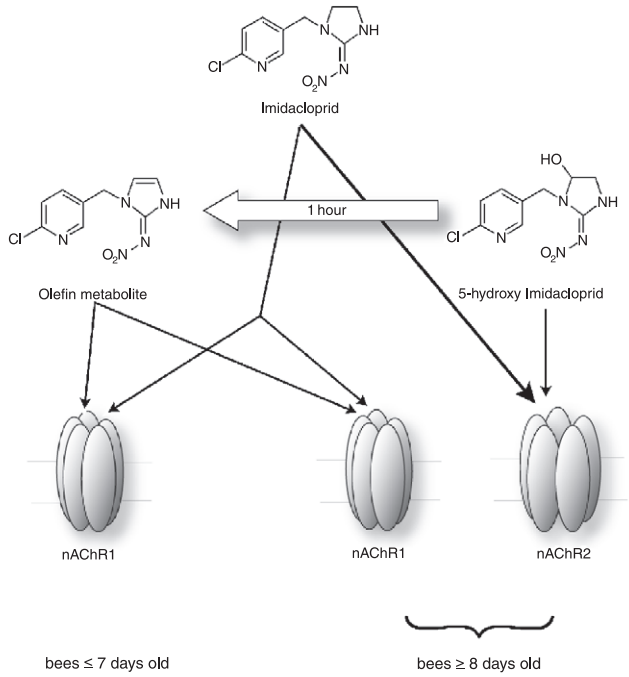

Fig. 5. A drawing illustrating the proposed interactions of imidacloprid and two of its metabolites, olefin and 5-hydroxy-imidacloprid, with the cholinergic system in the honeybee. In this model, two subtypes of a nAChR are differentially expressed during adult maturation of honeybees. nAChR1 is expressed constitutively, whereas nAChR2 is induced when the bees begin the second week of their lives. Imidacloprid and olefin act as agonists of both subtypes, although affinity of imidacloprid to nAChR2 appears to be higher than to nAChR1. By contrast, 5-hydroxy-imidacloprid interacts only with nAChR2. The large arrow indicates the temporal interval that separates the actions of 5-hydroxy-imidacloprid and olefin. This model is based on the results presented here and in Guez et al. (2001).

tor types are formed as pentamers from different combinations of genetically distinct subunits, but not from all possible combinations of subunits (Lukas et al., 1999). However, more complex subunit combinations may exist in vivo.

Given the unusual abundance of nAChRs in the honeybee brain (Bicker, 1999), cholinergic transmission is thought to play a key role(s) in the central nervous system of this insect. Acetylcholine appears to be the primary neurotransmitter in the olfactory pathway and is also found in Kenyon cells of the mushroom bodies (Bicker, 1999). Downregulation of a gene encoding acetylcholinesterase (AChE) correlates with adult maturation of worker honeybees, and treatment with the AChE inhibitor, metrifonate, improves performance in an olfactory learning assay (Shapira et al., 2001). Cholinergic transmission has also been implicated in the PER pathway (Braun and Bicker, 1992).

Our results suggest that activation of a developmentally regulated late-onset nAChR occurs during a narrow and well-defined time point of adult maturation when the young honeybees begin the second week of their lives and may be part of the dramatic changes taking place during the first 1–2 weeks of development of this insect. These changes include volumetric expansions of brain regions known as mushroom bodies (Withers et al., 1993), increases in hemolymph levels of hormones and biogenic amines (Schulz and Robinson, 2001), development of endogenous circadian rhythms (Bloch and Robinson, 2001), and differential gene expressions (Kucharski and Maleszka, 2002a,b; Toma et al., 2000). One possibility is that temporal molecular changes govern the ability of workers to perform age-related division of labour in the honeybee colonies. Young honeybee workers are typically involved in 'simple' in-hive duties, but as they grow older, they perform more complex tasks until they reach the forager status that is associated with more demanding duties outside the hive. It has been proposed that workers have different response thresholds to task-related stimuli and that these differential responses are the reason why there is a division of labour (Schulz and Robinson, 2001). Within the dark and confined space of a typical hive that is occupied by 40,000–60,000 individuals, these task-related stimuli are largely olfactory and tactile cues. Compelling evidence suggests that age-related behavioural differences among young bees are controlled by hormones, pheromones, neuromodulators, and neurotransmitters acting either individually or in concert (Morgan et al., 1998; Pankiw et al., 1998; Robinson, 1987; Schulz and Robinson, 2001; Watmough, 1997). Although in a normally functioning colony the foraging behaviour is not expressed until bees are 21 days old, changes in the colony needs or scarcity of foragers may force much younger workers to engage in foraging tasks (Schulz and Robinson, 2001). In our colonies, we frequently observe 8-day-old foragers especially during spring and summer when there is plenty of nectar and pollen available. Clearly, the individual-level genetic program is executed by default, whereas the colony-level behaviour is influenced by both environmental and internal factors. In this context, the increase in the number of trials needed to habituate the extension of the proboscis in older honeybees (slower habituation) may represent an important behavioural adjustment that could be advantageous for the foraging honeybees that often explore patchy and monotonous food sources to collect either nectar or pollen from dozens of flowers in rapid and efficient successions.

Like many other complex biological processes, the honeybee behaviour, especially at the colony level, cannot be explained by the action of a single gene product or biochemical pathway. However, the characterisation of molecules with tightly controlled expression patterns during behavioural maturation represents an important approach to an understanding of molecular mechanisms controlling behavioural maturation and age-related division of labour in the honeybee.

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