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Brief Report

Using RNA interference targeting a nicotinic acetylcholine receptor subunit to counteract insecticide accommodation mechanisms: example of the β1 subunit in the imidacloprid-accommodated American cockroach, *Periplaneta americana*

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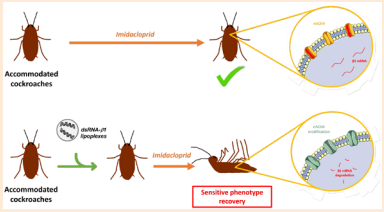
Supplementary material

Insecticide accommodation and resistance are limiting factors to the much-needed increase in agricultural production. Various physiological and cellular modifications, such as the changes of insecticide molecular targets, have been linked to these events. Thus, a previous study demonstrated that the imidacloprid accommodation set up by the cockroach *Periplaneta americana* after an exposure to a sublethal dose of this insecticide involves functional alterations of two nicotinic acetylcholine receptor (nAChR) subtypes. As RNA interference (RNAi) is one of the most promising strategies for controlling pest insects, we evaluated, in this study, the use of RNAi that targets the β1 nAChR subunit to counteract the imidacloprid accommodation phenomenon in cockroaches. Interestingly, we showed that ingestion of dsRNA-β1 increased the sensitivity to imidacloprid of accommodated cockroaches. Thus, we have demonstrated for the first time that RNAi that targets an nAChR subunit can counteract the accommodation mechanism to insecticide targeting nAChRs set up by an insect.



Keywords: RNA interference, nicotinic acetylcholine receptor subunit, insecticide resistance, pest control, imidacloprid.

Introduction

Contemporary agriculture is facing a new challenge that is to ensure a quality production able to meet the growing food needs. Until now, the crop protection and the increase of their yields was mainly based on the use of pesticides such as insecticides. However, several decades of widespread insecticide use have led to the appearance of resistant insect populations through selection pressure.[1)] Therefore, as agriculture evolves towards ecological crop protection, developing new tools to counteract the resistance mechanisms set up by the insects and to reduce the doses of insecticide used on crops is crucial to enable a safer and more sustainable agricultural production. Moreover, the exposure of insecticides, even at sublethal doses, can lead to accommodation in insect pests in a relatively short time.[2)] This effect could also contribute to the emergence of insecticide resistance. Thus, it is necessary to take into account both accommodation and resistance phenomena to develop novel pest management strategies. One of the most promising strategies is the use of RNA interference technology (RNAi).[3)] RNAi, unlike most of the traditional technologies of insect pest control, offers a powerful control over the specificity of the control agents. Recently, some studies have shown that a single double-stranded RNA (dsRNA) treatment of the insect can improve its sensitivity to neonicotinoid insecticides such as imidacloprid or thiamethoxam.[4,5)]

Neonicotinoids (IRAC classification ID 4A) are one of the major classes of insecticide[6)] that act as nicotinic acetylcholine receptor (nAChR) agonists in the insect central nervous system.[7)] Among all the neonicotinoids, imidacloprid was the top-selling insecticide for many years.[7)] Insect resistance mechanisms to neonicotinoids were attributed to modifications of the

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detoxification enzyme activity, target-site mutations or quantitative changes in nAChR subunits.[6] Indeed, several studies have focused on assessing the involvement of nAChR subunits in imidacloprid sensitivity and resistance of insects.[8–10] In particular, scientists investigated the involvement of β1 subunit.[11–14] In the American cockroach *Periplaneta americana*, the β1 subunit is part of at least two α-bungarotoxin-insensitive nAChR subtypes named nAChR1 and nAChR2.[15] These nAChR subtypes are found on dorsal unpaired median neurons (DUM neurons) that are commonly used as biological model to study the mode of action of insecticides and differ in their sensitivity to imidacloprid.[16] A previous study carried out on cockroaches that were exposed to a sublethal dose of imidacloprid for 30 days demonstrated that they were accommodated to this insecticide.[8] This reduced sensitivity involves a downregulation of the expression of the α2 subunit which composes nAChR2 with α1 and β1 subunits.[8] Using patch-clamp recordings on DUM neurons, it was demonstrated that this exposure diminished the sensitivity of the DUM neurons to imidacloprid and this decrease could be linked to changes of these two nAChR subtypes.[17] Thus, nAChR subunits seem to be key elements to target with RNAi for pest control. Moreover, to date, no study has been carried out on the use of RNAi on accommodated insects which therefore present physiological and cellular modifications that may be different from those observed in resistant insects.

Since a unique treatment of dsRNA targeting an nAChR subunit of the cockroach is able to alter its expression but also its sensitivity to imidacloprid[8] and the β1 subunit is part of both nAChR1 and nAChR2, the aim of this study is to evaluate the use of RNAi targeting this subunit to counteract the accommodation mechanism set up by the cockroach after an exposition to a sublethal dose of imidacloprid. First, we demonstrated by qPCR experiments that a single ingestion of dsRNA targeting β1 subunit (dsRNA-β1) induced a decrease in its expression in non-accommodated and imidacloprid accommodated cockroaches. In addition, acute imidacloprid intoxication tests coupled with dsRNA-β1 ingestion were performed. The ingestion of dsRNA-β1 did not modify the sensitivity of non-accommodated cockroaches but restored the sensitivity to imidacloprid of accommodated cockroaches. For the first time, we have shown that RNAi targeting an nAChR subunit can counteract the accommodation mechanism to an insecticide acting on nAChRs.

Materials and methods

1. Insects

All the experiments were performed on non-accommodated and imidacloprid accommodated adult male American cockroaches *P. americana* which were obtained according to the protocol described previously.[8,17] The cockroaches were reared under standard conditions in our laboratory at 28°C and with a photocycle of 12 hr light/12 hr dark.

2. dsRNA synthesis

To obtain dsRNA that targets the cockroach β1 nAChR subunit (dsRNA-β1), a specific fragment (100 bp) of the coding sequence of this subunit (accession No. MW201213) was amplified by PCR. This amplification was done using a high-fidelity Novagen™ KOD Hot Start DNA polymerase (Merck, Darmstadt, Germany) with specific primers each containing the T7 RNA polymerase promoter sequence (ds_β1, Supplemental Table S1). In parallel, control dsRNA targeting the bacterial β-galactosidase (dsRNA-control) were produced. To do so, a specific fragment of 100 bp of the bacterial β-galactosidase coding sequence was amplified from the recombinant vector PCR-Blunt_β1 with a primer set (ds_control, Supplemental Table S1). After a purification of the PCR products with the Nucleospin® Gel and PCR Clean-up kit (Macherey Nagel, Düren, Germany), an *in vitro* transcription reaction was performed using the MEGAscript T7 kit (Ambion, Grand Island, NY, USA). The resulting transcripts were treated with DNAse then purified with the NucleoSpin® miRNA kit (Macherey Nagel, Düren, Germany). Finally, the resulting dsRNA were denatured at 95°C for 5 min and re-hybridized at room temperature for 90 min. The dsRNA were stocked at −20°C until use.

3. dsRNA treatment

All experiments were performed on non-accommodated and accommodated cockroaches orally treated with dsRNA. To do this, dsRNA lipoplex solutions containing 0.25 μg of dsRNA-β1 or dsRNA-control mixed with 1 μL of Escort IV® transfection reagent (Sigma-Aldrich, Saint Quentin Fallavier, France) and 5% glucose solution up to 10 μL were prepared according to the protocol previously described[8] and then incubated at room temperature for 30 min. Finally, cockroaches were fed with 10 μL of these solutions according to the ingestion protocol described by Huang *et al.* (2018).[18]

4. qPCR experiments

Quantitative PCR (qPCR) experiments were performed on CFX Connect™ Real-Time PCR detection system (Biorad, Hercules, CA, USA) to evaluate the relative expression level of nAChR subunits in the terminal abdominal ganglia (TAG) of non-accommodated and accommodated dsRNA-treated cockroaches. To do this, 96 hr after dsRNA ingestion (dsRNA-β1 or dsRNA-control), the TAG which contain DUM neurons were dissected and total RNA were extracted using the Nucleospin® RNA kit (Macherey Nagel, Düren, Germany) according to the manufacturer's protocol, followed by a sodium/acetate precipitation. Then, 500 ng of purified RNA were reverse transcribed using RevertAid H Minus First Strand cDNA Synthesis® kit (Thermo Fisher Scientific, Illkirch, France). After a RNase H treatment (Thermo Fisher Scientific, Illkirch, France) of 20 min at 37°C followed by an inactivation step at 65°C for 10 min, the cDNA were diluted at 1/20 and stocked at −20°C. Each reaction of qPCR was done in duplicate and carried out in 20 μL containing 5 μL of a 20-fold dilution of cDNA, 1 μM of each primer set (Supplemental Table S2) and 10 μL of 2X Takyon No Rox SYBR® Master Mix Blue dTTP (Eurogentec, Seraing, Belgium).

The qPCR program consisted of a first step at 95°C for 5 min followed by 40 cycles comprising a denaturation step of 15 sec at 95°C and either an annealing step of 20 sec at the optimal temperature followed by an elongation step of 40 sec at 72°C for α5, β3, β4 and β6 nAChR subunits or a combined annealing-elongation step of 1 min at the optimal temperature for actin and other nAChR subunits (Supplemental Table S2). In qPCR amplification reactions, the efficiency of each specific primer set was between 98% and 102%. To validate specificity of each primer sets, melting curve analyses were carried out after the qPCR reactions. Relative mRNA expression levels of all the nAChR subunits on dsRNA-treated cockroaches were determined according to the $2^{-\Delta\Delta Ct}$ method[19] for which actin gene was used as the housekeeping gene in agreement with previous studies[8,17] and the expression level of each nAChR subunit obtained in the dsRNA-β1 condition was normalized with its counterpart in the control condition (dsRNA-control).

5. Insecticide exposure

Toxicological tests were all carried out on the two different groups named respectively non-accommodated and accommodated cockroaches which were obtained according to the protocol described previously.[8,17] Then, 96 hr after a unique ingestion of dsRNA lipoplexes, an acute intoxication to imidacloprid was performed at the lethal dose 50 (LD_{50}, 2.5 μg/cockroach), which was determined previously and in accordance with their protocol.[8] Mortality rate was recorded 48 hr after imidacloprid exposure.

6. Statistical analysis

All data were statistically analysed with a nonparametric Mann–Whitney test using GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla, CA, USA). Statistical analyses were considered as significant for $*p<0.05$ and $**p<0.01$.

Results

1. Effect of the ingestion of dsRNA targeting the β1 nAChR subunit on the expression level of nAChR subunits

First of all, the effect of dsRNA targeting the β1 nAChR subunit was assessed on the expression of the β1 subunit but also on all the other subunits identified in the cockroach *P. americana*. Thus, non-accommodated and accommodated cockroaches were treated by ingestion of 250 ng of either dsRNA-β1 or dsRNA-control. The quantification by qPCR experiments of the nAChR subunit transcript level was performed on the TAG of the dsRNA-treated cockroaches 96 hr after this unique dsRNA ingestion (Fig. 1A). Therefore, we confirmed that dsRNA targeting the β1 nAChR subunit were able to specifically decrease β1 mRNA expression. Indeed, a significant decrease of the expression level of the β1 subunit by 23% was found in non-accommodated cockroaches treated with dsRNA-β1 compared to those treated with dsRNA-control ($n=8$ with 6 TAG per n, $p<0.01$, Fig. 1B) and by 14% in accommodated cockroaches ($n=6$ with 6 TAG per n, $p<0.05$, Fig. 1C). Moreover, since no significant change in the transcript level of the other nAChR subunits was observed, the specificity of the dsRNA-β1 was validated (Supplemental Fig. S1–S2). These results indicate for the first time that a single ingestion of 250 ng of dsRNA targeting the cockroach β1 subunit effectively and significantly reduces the expression of this subunit without observing off-target effect on the expression level of other nAChR subunits.

2. Effect of the ingestion of dsRNA-β1 on the sensitivity to imidacloprid of cockroaches

To evaluate the potency of dsRNA-β1 to modulate the imidacloprid sensitivity of the cockroach, acute intoxication experiments to imidacloprid at LD_{50} were carried out on non-accommodated and accommodated cockroaches which have ingested 250 ng of dsRNA (dsRNA-control or dsRNA-β1) (Fig. 2A). First, no change in mortality was observed in non-accommodated cockroaches treated with dsRNA-control compared to those treated with dsRNA-β1 (Fig. 2B). When the mortality rate of the non-accommodated cockroaches which were treated with dsRNA-control was compared to that of the cockroaches exposed to a sublethal dose of imidacloprid for 30 days which were also treated by dsRNA-control (accommodated cockroaches), this rate was reduced from 50 to 32% respectively ($n=5$ with 10 to 15 cockroaches per n, $p<0.05$, Fig. 2B). Thus, in accordance with a previous study,[8] cockroaches that were exposed for 30 days to a sublethal dose of imidacloprid were well accommodated and less sensitive to this insecticide. Surprisingly, whereas the mortality rate of accommodated cockroaches which were treated with dsRNA-control was 32%, the mortality rate of accommodated cockroaches which were treated with dsRNA targeting β1 subunit significantly increased to 55% ($n=5$ with 10 to 15 cockroaches per n, $p<0.01$, Fig. 2B). Furthermore, no significant change in mortality rate was observed between cockroaches treated with dsRNA-β1 and exposed or not to a sublethal dose of imidacloprid for 30 days (Fig. 2B) demonstrating that accommodated cockroaches treated with dsRNA-β1 shown the same imidacloprid sensitivity as non-accommodated cockroaches. All together, these results demonstrated for the first time that a unique ingestion of dsRNA-β1 did not affect the sensibility of non-accommodated cockroaches to this insecticide but allowed the accommodated cockroaches to revert to a sensitive phenotype.

Discussion

Until now, pest insect control has been mainly focused on chemical insecticides. However, the appearance of insects less sensitive to insecticides and environmental concerns calls for the need to develop new strategies to control pests. Beyond being used to study gene function, RNAi has emerged as a promising most species-specific and eco-friendly alternative to manage pests. In this study, we have investigated the use of RNAi that targets an nAChR subunit to counteract insecticide accommodation. The nAChR1 and nAChR2 appear to play a role in the development of accommodation mechanism to imidacloprid in

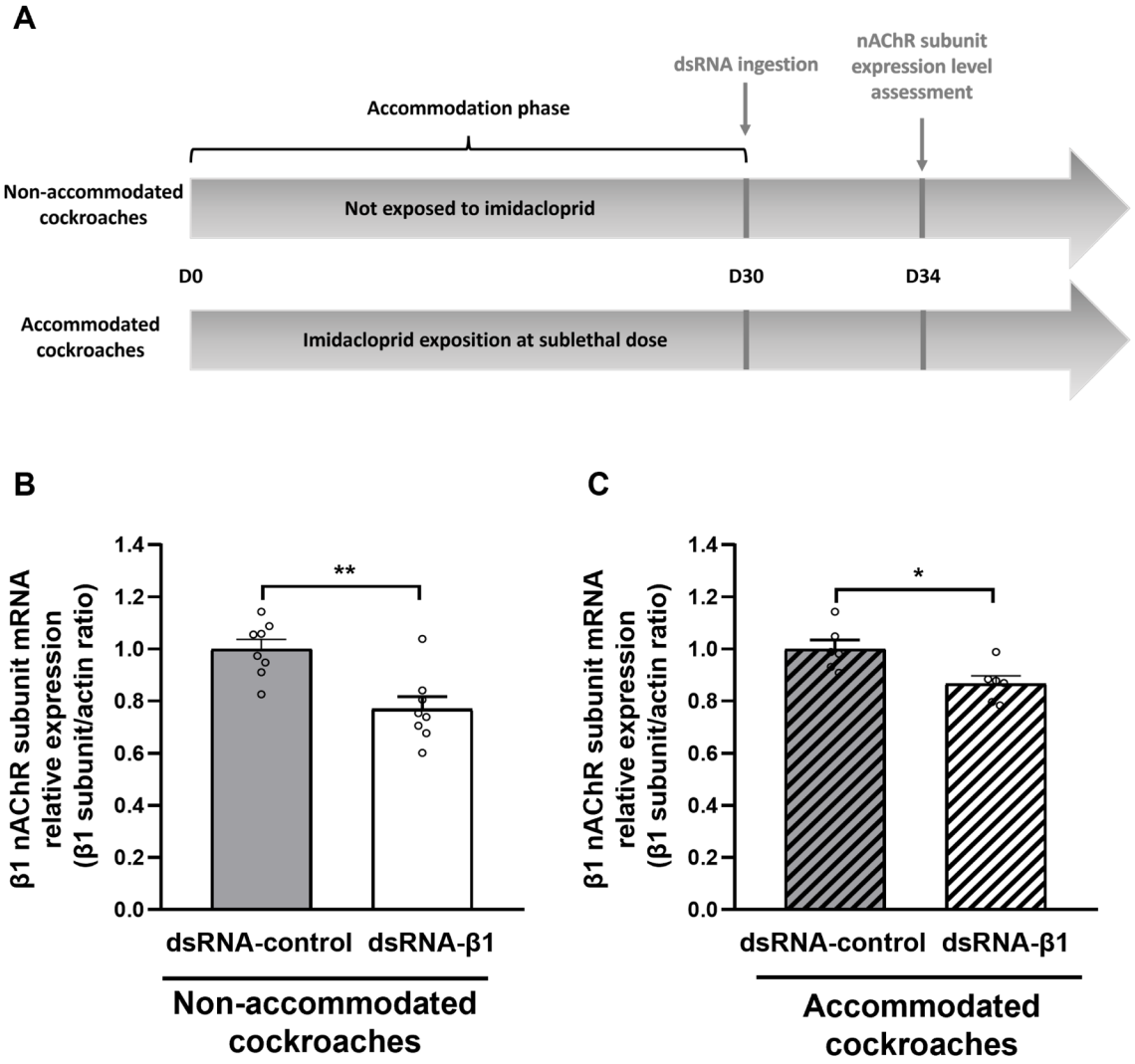

Fig. 1. Effect of the ingestion of dsRNA (dsRNA-control or dsRNA-β1) on the β1 nAChR subunit mRNA expression level in non-accommodated and accommodated cockroaches. (A) Schematic representation of the time course of the experiments. Cockroaches were exposed to either a sublethal dose of imidacloprid for 30 days (Accommodated cockroaches) or its solvent DMSO (Non-accommodated cockroaches). Then, each cockroach ingested 250 ng of either dsRNA-control or dsRNA-β1. The qPCR experiments were performed 96 hr after dsRNA ingestion (D34) on the terminal abdominal ganglia of non-accommodated (B) or accommodated cockroaches (C). Data were normalized to the expression of the housekeeping gene actin with the $2^{-\Delta\Delta Ct}$ method (* $p<0.05$, ** $p<0.01$, dsRNA-control *vs.* dsRNA-β1, nonparametric Mann–Whitney test; $n=6$ to 8 with 6 TAG per n). Data are shown as the mean±S.E.M..

the American cockroach *P. americana* after exposure to a sublethal dose of this insecticide.[17] Thus, we have shown that targeting the β1 nAChR subunit found in these two nAChR subtypes with dsRNA allowed to decrease effectively and specifically its expression. These results also suggest the implication of systemic RNAi in the cockroach as it has been shown in other insect species.[20–23] However, this mechanism is still not fully described in insects. In a second time, acute toxicity experiments to imidacloprid on accommodated cockroaches showed, for the first time, that the ingestion of dsRNA-β1 can counter this phenomenon.

Surprisingly, the ingestion of dsRNA-β1 did not involve change in the sensitivity of non-accommodated cockroaches to imidacloprid. Indeed, it is well-known that the decrease sensitivity to imidacloprid can be caused by target alteration and enhanced metabolism. Target-site mutation in β1 nAChR subunit and reduction of nAChR subunit expression such as β1 subunit have been reported in many imidacloprid resistant insects[11–13,24,25] suggesting that the β1 subunit play an important role in the sensitivity of insects to this insecticide. This has been confirmed by the use of dsRNA that targets the Boβ1 subunit of *Bradysia odoriphaga* which induced a decrease in sensitivity to imidacloprid.[14] Moreover, a strain of *Drosophila melanogaster* mutated on the Dβ1 subunit sequence was also less sensitive to imidacloprid.[26] In addition, the reduced expression of the β1 subunit was described in third instar larvae of *Bradysia odoriphaga* that survived acute intoxication with imidacloprid[14] and in resistant strain of the aphid *Aphis gossypii*.[12] However, such

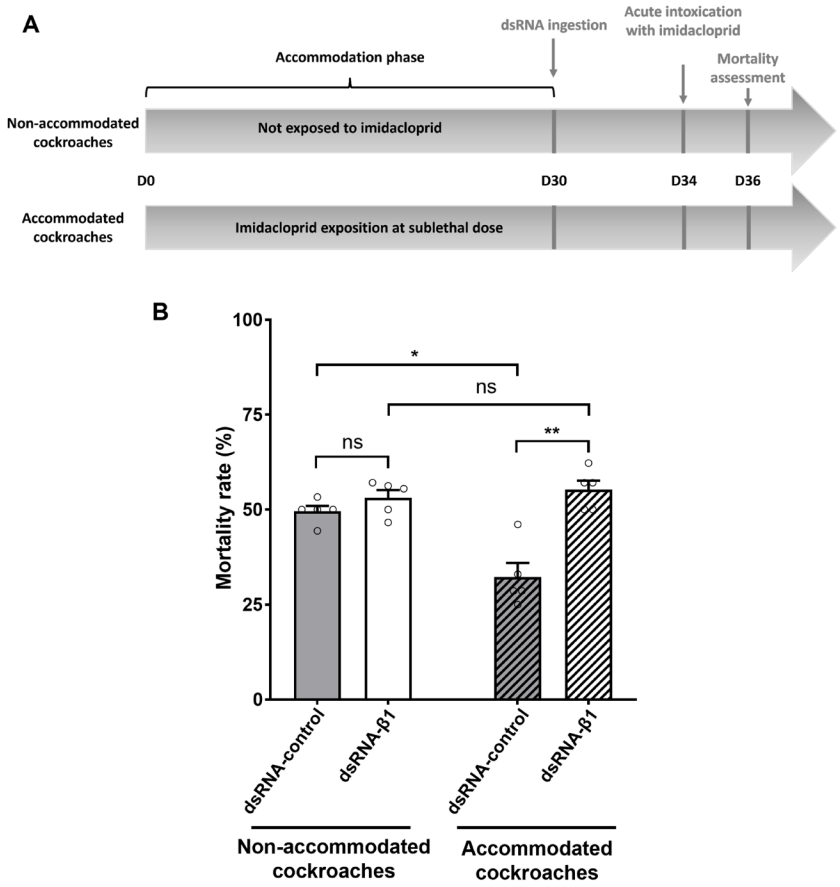

Fig. 2. Effect of dsRNA targeting β1 subunit on the sensitivity to imidacloprid of accommodated or non-accommodated cockroaches. (A) Schematic representation of the time course of the experiments. Cockroaches were exposed to either a sublethal dose of imidacloprid for 30 days (0.025 μg/day/cockroach; Accommodated cockroaches) or its solvent DMSO (Non-accommodated cockroaches). Then, each cockroach ingested 250 ng of either dsRNA-control or dsRNA-β1 (D30). An acute intoxication with imidacloprid at LD_{50} (2.5 μg/cockroach) was performed 96 hr after dsRNA ingestion (D34). (B) Mortality was assessed 48 hr after acute imidacloprid exposure (D36). Data are shown as the mean±S.E.M. (ns: non-significant, * $p<0.05$, ** $p<0.01$, nonparametric Mann–Whitney test; $n=5$ with 10 to 15 cockroaches per n).

change of the β1 subunit expression was not observed in imidacloprid accommodated cockroaches which were exposed to a sublethal dose of imidacloprid during 30 days[17] suggesting that the β1 subunit does not have the same impact on sensitivity to imidacloprid in cockroaches as in other insects. Since cockroach nAChR1, which is composed of the α3, α8 and β1 subunits,[15] is sensitive to imidacloprid[16] but the ingestion of dsRNA-β1 did not induce a change in sensitivity to imidacloprid in non-accommodated cockroaches, we suggest that other imidacloprid-sensitive nAChR subtypes that are not composed of the β1 subunit could exist. This hypothesis is also supported by the fact that the cockroach has one of the largest families of nAChR subunits[27] and that the physiological role of many of them is still unknown.

The ingestion of dsRNA-β1 by accommodated cockroaches to imidacloprid resulted in a return to a sensitivity comparable to that of non-accommodated cockroaches. These results suggest that maintaining the expression level of β1 subunit is necessary for the cockroach to resist another imidacloprid intoxication. Moreover, since the ingestion of dsRNA-β1 did not affect the expression of the other nAChR subunits, the hypothesis of nAChR compensation is difficult to consider. In addition, it was suggested that the functionality of nAChR2 was increased in accommodated cockroaches.[17] The increase in nAChR2 functionality would be sufficient to allow cockroaches to accommodate to imidacloprid. This receptor subtype is composed of α1, α2 and β1 subunits[15] and is insensitive to imidacloprid. Thus, altering the expression of the β1 subunit could at least impact the assembly of nAChR2 and allow a return of the cockroach to a sensitive phenotype.

Finally, the use of dsRNA targeting β1 subunit can be considered for other insects that have a larger economic impact, such as the pea aphid *Acyrthosiphon pisum*. Indeed, aphid larvae that survived an acute intoxication with imidacloprid showed a significant increase in the expression of the β1 subunit, suggesting its involvement in resistance to this insecticide.[28]

In this study, we demonstrated for the first time that using dsRNA targeting an nAChR subunit can counteract the accommodation mechanism to imidacloprid. These results could contribute to develop sustainable insecticide resistance management

strategies based on RNAi that targets an nAChR subunit.

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Electronic supplementary materials

The online version of this article contains supplementary materials (Supplemental Figures S1–S2 and Tables S1–S2), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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