Full Length Article

Subchronic exposure to sublethal dose of imidacloprid changes electrophysiological properties and expression pattern of nicotinic acetylcholine receptor subtypes in insect neurosecretory cells

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A B S T R A C T

Neonicotinoids are the most important class of insecticides used in agriculture over the last decade. They act as selective agonists of insect nicotinic acetylcholine receptors (nAChRs). The emergence of insect resistance to these insecticides is one of the major problems, which limit the use of neonicotinoids. The aim of our study is to better understand physiological changes appearing after subchronic exposure to sublethal doses of insecticide using complementary approaches that include toxicology, electrophysiology, molecular biology and calcium imaging. We used cockroach neurosecretory cells identified as dorsal unpaired median (DUM) neurons, known to express two α-bungarotoxin-insensitive (α-bgt-insensitive) nAChR subtypes, nAChR1 and nAChR2, which differ in their sensitivity to imidacloprid. Although nAChR1 is sensitive to imidacloprid, nAChR2 is insensitive to this insecticide. In this study, we demonstrate that subchronic exposure to sublethal dose of imidacloprid differentially changes physiological and molecular properties of nAChR1 and nAChR2. Our findings reported that this treatment decreased the sensitivity of nAChR1 to imidacloprid, reduced current density flowing through this nAChR subtype but did not affect its subunit composition (α3, α8 and β1). Subchronic exposure to sublethal dose of imidacloprid also affected nAChR2 functions. However, these effects were different from those reported on nAChR1. We observed changes in nAChR2 conformational state, which could be related to modification of the subunit composition (α1, α2 and β1). Finally, the subchronic exposure affecting both nAChR1 and nAChR2 seemed to be linked to the elevation of the steady-state resting intracellular calcium level. In conclusion, under subchronic exposure to sublethal dose of imidacloprid, cockroaches are capable of triggering adaptive mechanisms by reducing the participation of imidacloprid-sensitive nAChR1 and by optimizing functional properties of nAChR2, which is insensitive to this insecticide.

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1. Introduction

Neonicotinoids are the most important class of insecticides used in agriculture over the last decade and are effective against some crop pests such as aphids, thrips and whiteflies. Imidacloprid was the first product of this class of insecticides to be commercialized in 1991 and it was used in foliar application and seed treatments (Tomizawa and Casida, 2003). Neonicotinoids act as selective agonists of insect nicotinic acetylcholine receptors (nAChRs) (Tomizawa and Casida, 2005), which belong to the “cys-loop” superfamily of ligand-gated ion channels (French-Constant et al., 2016). These receptors are composed of five subunits (Jones et al., 2007), each subunit possesses four transmembrane domains (M1-M4), an extracellular amino-terminal domain involved in agonist binding and a large cytoplasmic loop between M3 and M4 containing several phosphorylation sites (Dupuis et al., 2012). Subunits were classified into two groups α and non α or β, depending on the presence or not of two adjacent cysteine residues in the extracellular domain, which play an important role for acetylcholine binding (Jones et al., 2007). In insects, several nAChR subunits have been cloned and the sequencing of the entire insect genome has revealed the existence of approximately ten different
nAChR subunit genes (Jones and Sattelle, 2010) suggesting a huge number of hypothetical nAChR subtypes. Combinations of nAChR subunits result in distinct receptors, with their own electrophysiological and pharmacological properties, which thereby influence sensitivity to neonicotinoids (Lansdell and Millar, 2000; Millar and Lansdell, 2010). In addition, previous studies have shown that neonicotinoid efficacy on nAChR subtypes depends on electropharmacological properties and many cellular and molecular factors such as conformational state, membrane potential, subunit composition and calcium-dependent phosphorylation/phosphorylation process (Courjaret and Laped, 2001; Bodereau-Dubois et al., 2012; Calas-List et al., 2013; List et al., 2014; Salgado, 2016; Sun et al., 2016).

However, despite this specific activity, one major problem, which may threaten the use of neonicotinoids is the emergence of insect resistance to these insecticides (Bass et al., 2015; Ffrench-Constant et al., 2016). In the case of neonicotinoids, resistance observed in several insect species was initially attributed to metabolic mechanisms through modifications of the detoxification enzyme expressions. Latter, target-site resistance to neonicotinoids was also described (Liu et al., 2005; Slater et al., 2012; Casida and Durkin, 2013; Bass et al., 2015) and finally, very recent studies have suggested that quantitative changes in nAChR subunits may also contribute to target-site resistance to neonicotinoids (Zhang et al., 2015).

In the general context of the effectiveness of pest insect resistance management, our aim is to use cockroaches as model to better understand physiological changes appearing after subchronic exposure to sublethal dose of a neonicotinoid, imidacloprid. Although previous studies have explored the effects of sublethal doses of neonicotinoids in insects, they were mainly focused on the behavioral effects (e.g., locomotor activity and impair olfactory learning and memory), especially in non-target insects, such as honey bees (Aliouane et al., 2009; Blacquière et al., 2012; Tan et al., 2015; Mengoni Goñalons and Farina, 2015). Up to date, there are no data related to the effects of subchronic exposure to sublethal dose of neonicotinoids on both physiological and molecular features of insect nAChRs. For that purpose, cockroach Periplaneta americana neurosecretory cells identified as dorsal unpaired median (DUM) neurons, known to express two distinct α-bgt-insensitive nAChR subtypes named nAChR1 and nAChR2 (Courjaret and Laped, 2001; Bodereau-Dubois et al., 2012), have been used. Previous findings have reported that nAChR subtypes present different pharmacological properties. Although nAChR1 is sensitive to the neonicotinoid imidacloprid, nAChR2 is insensitive to this insecticide, whereas the insect has never been exposed to this insecticide (Courjaret and Laped, 2001). Furthermore, we have demonstrated that the uncommon conformational state of nAChR2 (i.e., open at the resting state and closed upon cholinergic agonist application) (Courjaret and Laped, 2001; Courjaret et al., 2003; Bodereau-Dubois et al., 2012) is responsible for the different neonicotinoid sensitivity observed in these two nAChR subtypes. Consequently, because cockroach neuronal preparations together with DUM neurons are commonly used as biological models for vertebrates and invertebrates to study the mode of action of neurotoxic insecticides (Pelhate et al., 1990), these interesting features make DUM neuron nAChR1 and nAChR2 subtypes a suitable model to explore the influence of subchronic exposure to sublethal dose of imidacloprid on both physiological and molecular properties of insect nAChRs. Our study reports that the subchronic exposure of cockroaches Periplaneta americana to sublethal dose of imidacloprid, differently affect electropharmacological properties and subunit expression pattern of DUM neuron nAChR1 and nAChR2 subtypes, which thereby impact their physiological functions. These results provide additional information that may contribute to better understand the mechanisms underlying the development of insect resistance to insecticides.

2. Materials and methods

All experiments were performed on adult male cockroaches Periplaneta americana taken after the last-instar nymph stage from our laboratory stock colony, which are maintained under standard conditions (29 °C, photo-cycle 12 h light/12 h dark).

2.1. Exposure to imidacloprid

Imidacloprid (Sigma-Aldrich, Saint Quentin Fallavier, France) was resuspended in dimethyl sulfoxide (DMSO) to obtain a stock solution at 100 mg ml⁻¹. Subsequent dilutions of imidacloprid were prepared in sucrose syrup (10% sucrose solution w/v) for the cockroach exposure experiments. Cockroaches were deprived of access to water for 48 h. Insects were then exposed to imidacloprid by ingesting 10 μl of sucrose syrup containing the different doses of imidacloprid ranging from 0.01 μg to 30 μg/cockroach. Control experiments were performed under the same experimental conditions without imidacloprid. Mortality rate was assessed 48 h after the treatment. We used 30–40 cockroaches per dose. For subchronic exposure to sublethal dose experiments, 30 cockroaches were daily and orally exposed ad libitum 30 days to the highest dose of imidacloprid that did not produce significant mortality. Control groups were similarly treated without imidacloprid.

2.2. Electrophysiological recordings

2.2.1. Cell preparation

Patch-clamp recordings were performed on DUM neuron cell bodies isolated from the midline of the terminal abdominal ganglion (TAG) of the nerve cord of the treated and non-treated adult male cockroaches. The TAG were removed from the nerve cord and placed in cockroach saline containing 200 mM NaCl, 3.1 mM KCl, 5 mM CaCl₂, 4 mM MgCl₂, 10 mM HEPES and 50 mM sucrose. pH was adjusted to 7.4 with NaOH. Isolation of DUM neuron cell bodies was performed under sterile conditions after enzymatic digestion and mechanical dissection, as previously described (Laped et al., 1989). DUM neuron cell bodies were maintained at 29 °C for 24 h before electrophysiological experiments were carried out.

2.2.2. Whole-cell recording

Nicotine- and imidacloprid-induced currents were recorded by using the patch-clamp technique in the whole-cell recording configuration under voltage-clamp mode, at a steady-state holding potential of −50 mV except when otherwise stated. Input membrane resistances were recorded under current-clamp condition in response to a hyperpolarizing current pulse (150 pA in amplitude and 300 ms in duration). Signals were recorded with an Axopatch 200A patch-clamp amplifier (Axon Instruments), digitized and acquired using a MiniDigidata 1440 analog-digital converter (Axon Instruments). Currents were treated with axoscope 10.2 software (Axon Instruments). Patch pipettes were pulled from borosilicate glass capillary tubes (GC150F-10; Clark Electromedical Instruments, Harvard Apparatus Edenbridge, UK) using a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Company, Novato, U.S.A.). Pipettes had resistances ranging from 1 to 1.5 MΩ when filled with internal pipette solution (see composition below). The liquid junction potential between bath and internal solutions was always corrected before the formation of a gigahm seal (>1 GΩ). Ionic currents induced by nicotine and imidacloprid were recorded with software control pClamp
2.2.3. Solution and agonist applications

Solution superfusing the cells contained 200 mM NaCl, 3.1 mM KCl, 5 mM CaCl₂, 4 mM MgCl₂, 10 mM HEPES buffer, pH was adjusted to 7.4 with NaOH. To inhibit the ionic currents induced by the activation of the α-bgt-sensitive mixed acetylcholine receptors (Lapiéd et al., 1990), 0.5 μM α-bgt was added to the extracellular solution. Internal pipette solution contained: 160 mM K⁺/D-gluconic acid, 10 mM NaCl, 1 mM MgCl₂, 0.5 mM CaCl₂, 10 mM KF, 3 mM ATP Mg, 10 mM EGTA, 20 mM HEPES, pH was adjusted to 7.4 with KOH. Imidacloprid stock solution (1 M) was prepared in DMSO and then diluted in the extracellular solution to obtain the different concentrations used. The highest concentration used in the electrophysiological recordings of DMSO was 0.1%. Nicotine stock solution (100 mM) was directly prepared in the extracellular solution and then diluted to obtain the different concentrations used. Nicotine and imidacloprid were applied by a gravity perfusion valve controller system (VC–6 M, Harvard apparatus, 1 s in duration) controlled by Pclamp software (flow rate of perfusion: 0.5 ml/min). The perfusion tube was placed within 100 μm from the isolated neuron cell body.

2.2.4. Curve fitting and data analysis

Currents were expressed as current density (pA/pF). Each current was normalized to the cell membrane capacitance, determined from the capacitive current elicited by a 3 mV depolarizing voltage pulse.

The dose–response curve was fitted according to the Hill equation:

\[ y = I_{\text{max}} \times (I_{\text{min}} - I_{\text{max}})/(1 + 10^{\log(EC_{50}) - X \times nH}) \]

Where \( Y \) is the normalized response, \( I_{\text{max}} \) and \( I_{\text{min}} \) are the maximum and minimum current values, respectively, \( nH \) is the Hill coefficient and \( EC_{50} \) is the concentration that produces 50% of the maximal agonist-induced current. Results were expressed as means ± SEM.

2.3. Calcium imaging

For calcium imaging experiments, DUM neuron cell bodies were isolated from the TAG of treated and non-treated adult male cockroaches, as already described above. The cells were washed two times in saline and incubated in the dark with 5 μM Fura-2 pentakis (acetoxy-methyl) ester (Fura-2 AM) (Sigma-Aldrich, Saint Quentin Fallavier, France) in the presence of 0.1% pluronic acid F68 (Sigma-Aldrich, Saint Quentin Fallavier, France) for 1 h at 37 °C. Pluronic acid is a nonionic surfactant used as a stabilizer of cell membrane protecting from membrane shearing to facilitate uptake of Fura-2 AM. After loading, cells were washed two times in saline. The glass coverslips were then mounted in a recording chamber (Warner Instruments, Hamden, CT, USA) connected to a gravity perfusion system allowing drug application. Imaging experiments were performed with an inverted Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan) equipped with epifluorescence. Excitation light was provided by a 75-W integral xenon lamp. Excitation wavelengths (340 nm and 380 nm) were applied using a Lmdha DG4 wavelength switcher (Sutter instrument, Novato, CA, USA). Images were collected with an Orca-R2 CCD camera (Hamamatsu photonics, Shizuoka, Japan) and recorded on the computer with Imaging Workbench software (version 6, Indec BioSystems, Santa Clara, CA, USA). Experiments were carried out at room temperature. Intracellular calcium level was expressed as the ratio of emitted fluorescence (340/380 nm).

2.4. qPCR experiments

To study nACHr subunit expression levels after imidacloprid subchronic exposure, quantitative PCR was performed on the terminal abdominal ganglia. Ganglia were removed from the nerve cord and stored at −80 °C until RNA extraction. Total RNAs were extracted from non-treated and treated cockroaches using Nucleospin RNA kit (Macherey Nagel, Düren, Germany) and following the manufacturer’s instructions. 500 ng of purified RNA was reverse transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Relative cockroach mRNA subunit expression was quantified by quantitative real-time PCR (qPCR) on a Chromo4 Real-Time PCR system (Biorad, Hercules, CA, USA) and normalized to the expression level of the housekeeping gene actin. The sequences of primers used are indicated in Table 1. Primer sets were designed based on the Periplaneta americana nACHr subunit sequences published on GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Each reaction of qPCR was carried out with 5 μl of a 20-fold dilution of cDNA, between 0.25 and 1 μM of each primer, 10 μl of MESA GREEN qPCR MasterMix Plus for SYBR® Assay 1 Low ROX (Eurogentec, Seraing, Belgium). The optimized qPCR programs consisted in initial step at 95 °C for 5 min followed by 40 cycles of denaturation step of 15 s and a hybridization step of 1 min. Relative mRNA expression levels were calculated according to the 2⁻ΔΔCt method (Pfaffl, 2001).

2.5. Statistical analysis

For whole cell recording, calcium imaging and qPCR experiments, statistical analysis were performed with Mann-Whitney test (p < 0.05) using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA, USA).

To compare the estimated EC₅₀ values of the curve fitting, data were analyzed with extra sum-of-squares F-test (p < 0.05) using GraphPad Prism version 5.00 (GraphPad Software, La Jolla, CA, USA).

In these cases, statistical analysis were considered as significant for *p < 0.05 and **p < 0.01.

3. Results

3.1. Determination of the sublethal dose of imidacloprid

Adult male cockroaches were fed individually with imidacloprid or solution containing the same percentage of solvent (control groups) and the mortality rate was assessed at 48 h (n = 30–40 cockroaches for each dose). Imidacloprid caused dose-dependent mortality at doses ranging from 0.01 μg to 30 μg per cockroach (Fig. 1). The LD₅₀ was estimated to be 2.57 ± 0.08 μg/cockroach 48 h after treatment. The highest dose, which did not produce any mortality was estimated to be 0.025 μg/cockroach and was chosen as the sublethal dose for the subchronic exposure experiments. Cockroaches treated with this dose of imidacloprid only exhibit

<table>
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<th>Table 1</th>
<th>Sequence of primers used for amplification by real-time quantitative PCR.</th>
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<tr>
<td>N° Accession</td>
<td>Forward Primer (5’ → 3’)</td>
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<tr>
<td>α1</td>
<td>JQ585634.1</td>
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<tr>
<td>α2</td>
<td>JQ585635.1</td>
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<td>α3</td>
<td>JQ585937.1</td>
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excitatory and trembling movements. During our experiments, no mortality was observed at 30 days subchronic exposure.

3.2. Effects of subchronic exposure to sublethal dose of imidacloprid on the electropharmacological properties of α-bungarotoxin-insensitive nAChR1 expressed in DUM neurons

Whole cell patch-clamp experiments were performed on short-term cultured DUM neurons isolated from non-treated and treated cockroaches during 30 days with 0.025 μg/cocroach of imidacloprid. Previous studies reported that adult DUM neuron cell bodies expressed both α-bgt-insensitive and α-bgt-sensitive nAChR, with “mixed” nicotinic-muscarinic pharmacology (Lapied et al., 1990; Grolleau et al., 1996). Consequently, application of α-bgt (0.5 μM) used in all experiments allows us to study in isolation the effects of cholinergic ligands on α-bgt-insensitive nAChR subtypes, named nAChR1 and nAChR2, which differ from each other by their sensitivity to imidacloprid. Although nAChR1 is sensitive to imidacloprid, nAChR2 is insensitive to this insecticide (Courjaret and Lapied, 2001; Sun et al., 2016). Application of imidacloprid (10 μM) onto isolated DUM neuron cell body induced a transient inward current at a holding membrane potential of −50 mV (Fig. 2A). However, the inward current amplitude was smaller in DUM neurons isolated from treated cockroaches than in control. The maximum peak current amplitudes, expressed as current density, were plotted against the logarithm of the non-cumulative doses of imidacloprid in both experimental conditions, treated and non-treated cockroaches (Fig. 2B). We observed that the imidacloprid concentration–response curve obtained after subchronic exposure was significantly shifted to the right in a parallel manner compared to control, with no significant change in the maximum response to imidacloprid (1 mM). The EC_{50} values estimated for imidacloprid from treated and control groups were 11.3 μM and 0.6 μM, respectively (Fig. 2B). The EC_{50} of the treated group was then nineteen fold higher than for control group (p < 0.05). When current density was plotted against the steady-state holding potentials, we observed a similar monophasic aspect of the curves between −10 and −70 mV in both treated and control groups (n = 4 to 7 cells; Fig. 2C). However, a significant decrease of the current density elicited by 1 μM imidacloprid was observed for treated cockroaches compared to control group (p < 0.05). Based on previous findings indicating that imidacloprid only acts on nAChR1 (Courjaret and Lapied, 2001; Bodereau-Dubois et al., 2012; Sun et al., 2016), these results showed that the subchronic exposure to imidacloprid decreased the sensitivity of nAChR1 to imidacloprid, an effect associated with a reduction of the current density.
3.3. Effects of nicotine on nAChR1 and nAChR2 expressed in DUM neurons after subchronic exposure of cockroaches to imidacloprid

We then explored the effect of nicotine on nAChR1 and nAChR2 expressed in DUM neurons isolated from treated and non-treated cockroaches. Application of nicotine (10 μM) induced a transient inward current at a holding membrane potential of −50 mV, exhibiting very similar amplitudes (Fig. 3A). DUM neurons isolated from non-treated cockroaches were exposed to various concentrations of nicotine (n = 3 to 7 cells; Fig. 3B). Mean values for current density were plotted against the logarithm of the non-cumulative concentration of nicotine. The sigmoid curve corresponding to the best fit according to the Hill equation gave an EC50 value estimated for nicotine at 7.5 μM. The data of nicotine were compared with the concentration-dependent effect of nicotine in the presence of 50 μM d-tubocurarine (d-TC), known to specifically block nAChR1 (Bodereau-Dubois et al., 2012). The concentration-dependent semi-logarithmic curve indicated that nicotine that interacted with nAChR2 produced less than maximal effect compared to control condition with a higher estimated EC50 value at 10.2 μM.

These results were compared to the concentration-response curves obtained with application of nicotine on non-treated and treated cockroaches. As illustrated in Fig. 3C, subchronic exposure did not produce significant difference compared to control condition. The estimated EC50 values were 7.5 μM and 12.4 μM for control and treated cockroaches. Among other possibilities, the very similar concentration-response curves established in both conditions might suggest an optimization of nAChR2 versus nAChR1 since our previous investigation indicated that nAChR1 sensitivity is strongly decreased in treated cockroaches. To verify this hypothesis, the DUM neurons isolated from treated cockroaches were pretreated with d-TC (50 μM) (n = 3 to 7 cells; Fig. 3D). The relatively well superimposed concentration-response curves (EC50 12.4 μM and 33.3 μM in control and with d-TC, respectively) obtained in both experimental conditions suggest an optimization of nAChR2 functions induced by subchronic exposure to sublethal dose of imidacloprid.

Current density obtained after application of nicotine (10 μM) was plotted against the steady-state holding potentials (n = 4 to 7 cells; Fig. 4A). Under control conditions, a biphasic curve was obtained confirming the existence of both nAChR1 and nAChR2 (Courjaret and Lapied, 2001). After subchronic exposure, an increase of current density was observed in the physiological potential range between −30 and −70 mV. It is interesting to mention that similar results were obtained when DUM neurons were exposed to 50 μM d-TC, as previously reported (n = 4 to 7 cells; Fig. 4B, Bodereau-Dubois et al., 2012). The resulting increase in current density reflecting an inhibition of spontaneous outward potassium conductance indicates that nAChR2 is more open before application of nicotine. According to these data, it is strongly suggested that mainly nAChR2 is functional in DUM neurons isolated from treated cockroaches. This was confirmed by additional set of experiments illustrating that the current-voltage relationships established in control in the presence of d-TC and in treated cockroaches were very similar in the same physiological potential range between −30 and −70 mV (Fig. 4C).

We also measured the input membrane resistance, reflecting the conformational state of nAChR1 and nAChR2. Under current-clamp conditions and in presence of 0.1 μM TTX to inhibit spontaneous sodium-dependent action potentials, we compared input membrane resistance before and after application of nicotine.

![Fig. 3](image-url) Effect of nicotine on DUM neuron nAChRs isolated from non-treated and treated cockroaches. (A) Typical examples of steady-state recordings of inward currents elicited by nicotine (10 μM) at a holding potential of −50 mV obtained in whole-cell voltage-clamp mode on DUM neuron cell bodies isolated from non-treated and treated cockroaches. (B-D) Superimposed semi-logarithmic concentration-response curves for the nicotine-induced currents expressed as current density (pA/pF) on DUM neuron cell bodies under different experimental conditions indicated on the graph. Solid lines represent the best fit according to the Hill equation. Nicotine-induced currents were recorded at a holding potential of −50 mV. For all experiments, data are means ± SEM (n = 3 to 7 cells). When indicated, DUM neuron cell bodies isolated from treated and non-treated cockroaches were pretreated with 50 μM d-tubocurarine (d-TC).
Comparative current-voltage relationships of nicotine-induced current expressed as current density (pA/pF) plotted as a function of different steady-state holding potentials under different experimental conditions indicated on each graph. For all experiments data are means ± SEM (n=4 to 7 cells). When indicated, DUM neuron cell bodies were pretreated with 50 µM d-tubocurarine (d-TC). (D) Comparative histogram illustrating the DUM neuron cell body input membrane resistance recorded after application of nicotine (10 µM) under different experimental conditions as indicated below each bar. The input membrane resistances were measured under current-clamp condition by applying a hyperpolarizing current pulse (150 pA in amplitude and 350 ms in duration, membrane potential of −70 mV). Data are means ± SEM (n=5 to 7 cells), *p < 0.05 (Mann-Whitney test).

(10 µM) on DUM neurons isolated from treated and non-treated cockroaches by applying a hyperpolarizing current pulse (150 pA in amplitude and 350 ms in duration). As illustrated in Fig. 4D, the decrease in the input membrane resistance after application of nicotine was less important on DUM neurons isolated from treated cockroaches (35.4 ± 7.7%, n = 7) than in control (63.6 ± 8.1%, n = 5) (*p < 0.05). Taken together, these results confirm that the participation of nAChR1 is reduced whereas nAChR2 contribution is more important after subchronic exposure of cockroaches to sublethal dose of imidacloprid.

3.4. Effect of subchronic exposure of cockroaches to sublethal dose of imidacloprid on steady-state resting calcium level in DUM neurons

As nAChR1 and nAChR2 have been previously reported to be regulated by intracellular calcium rise and calcium-dependent phosphorylation/dephosphorylation process (Courjaret and Lapied, 2001; Courjaret et al., 2003; Bodereau-Dubois et al., 2012; Calas-List et al., 2013) additional experiments using calcium imaging were performed to evaluate a putative evolution of the steady-state resting calcium level after subchronic exposure. As illustrated in Fig. 5, a significant increase was observed in resting calcium level in DUM neurons isolated from treated cockroaches compared to control (from 0.29 ± 0.01 (n = 12) to 0.39 ± 0.04 (n = 13), respectively, *p < 0.05).

3.5. Effects of subchronic exposure to sublethal dose of imidacloprid on nAChR2 subunit expression

To determine if the modified electro-pharmacological properties of nAChR1 and nAChR2 could be linked to changes in the nAChR subunit expression, qPCR experiments were performed on the terminal abdominal ganglia of non-treated and treated cockroaches (n=6 to 7 replicates). A recent study demonstrated that the nAChR1 was associated to α3, α8 and β1 subunits whereas the nAChR2 was composed of α1, α2 and β1 subunits (Sun et al., 2016). After subchronic exposure, we demonstrated that the nAChR1 subunit expression was not change compare to control (Fig. 6A). By contrast, a significant increase of α1 by approximately 1.5-fold (**p < 0.01) and a significant decrease of α2 (1.3 fold less; *p < 0.05) involved in the subunit composition of nAChR2 were observed (Fig. 6B).
4. Discussion

In this study, we examined according to our experimental conditions the impact of subchronic exposure to oral sublethal dose of a neonicotinoid, imidacloprid, on the electrophysiological properties and molecular pattern expression of the cockroach α-bgt-insensitive nicotinic acetylcholine receptor subtypes, nACHR1 and nACHR2 expressed in DUM neurons. Nevertheless, different effects observed in other experimental conditions (i.e. shorter or longer time exposure, lower doses) cannot be excluded and complementary studies should additionally be performed.

Our findings reported that there are differential effects of the treatment on nACHR1 and nACHR2 subtypes including an ongoing compensatory nACHR functions as a response to changes induced by imidacloprid intoxication. Although previous studies showed that sublethal doses of neonicotinoids induced various negative effects on insects such as development and life traits, particularly in bees (Desneux et al., 2007; Henry et al., 2012), aphids (Shi et al., 2011; Miao et al., 2014) and flies (Hu et al., 1998), there is no data related to the participation of complex downstream signaling pathways, which are initiated, in part, with an increase in the steady-state resting intracellular calcium level after subchronic exposure to sublethal doses of imidacloprid. In fact, one of the key components participating in specifically changing the nACHR1 and nACHR2 physiological functions induced by imidacloprid is the calcium-dependent intracellular signaling pathways involved in the regulation of both nACHR1 and nACHR2 (Courjaret and Lapied, 2001; Courjaret et al., 2003; Bodereau-Dubois et al., 2012; Calas-List et al., 2013; List et al., 2014; Mannai et al., 2016).

4.1. Subchronic exposure affects sensitivity of nACHR subtypes to imidacloprid

We show in this study that subchronic exposure reduces sensitivity of nACHR1 to imidacloprid and decreases imidacloprid-induced inward current amplitude. Different hypotheses could be proposed. First, these effects could be explained by changes in nACHR subunit composition, known to be closely related to neonicotinoid sensitivity (Shimomura et al., 2002, 2006; Li et al., 2010; Frenche-Constant et al., 2016; Sun et al., 2016). Modification of nACHR subunit expression after chronic exposure to neonicotinoid insecticides has also been observed in the pea aphid Acrystosiphon pisum (Taillebois et al., 2014). However, we also report from our results that the pattern expression of α3, α8 and β1, known to be involved in the subunit composition of nACHR1 (Sun et al., 2016) was not affected by subchronic exposure of sublethal dose of imidacloprid. The second hypothesis concerns the involvement of the calcium-dependent phosphorylation/dephosphorylation process that regulates nACHR functions, which thereby influence sensitivity to insecticides (Thany et al., 2007). An interesting point of the subchronic exposure to sublethal dose of imidacloprid could be a close link between the effects observed and the steady-state resting calcium level increased in DUM neurons isolated from imidacloprid-treated cockroaches. Previous investigations reported that i) DUM neuron nACHR1 targeted by imidacloprid was regulated by both cAMP/PKA cascade and okadaic acid-sensitive protein phosphatase PP1/2A (Courjaret and Lapied, 2001) and ii) calcium-dependent phosphorylation/dephosphorylation process had fundamental consequences in the mode of action of imidacloprid on nACHR1. Based on these data, the decrease of nACHR1 sensitivity to imidacloprid associated with a reduction of the inward current amplitude observed in treated cockroaches might be the result of the participation of the calcium-dependent cAMP/PKA cascade since a decrease of the imidacloprid-induced current amplitude was already observed when intracellular concentration of cAMP was increased (Courjaret and Lapied, 2001). We previously demonstrated that an increase in intracellular calcium concentration resulted in the formation of the calcium-calmodulin complex that activated adenylylate cyclase. Increased CAMP concentration via the calcium-calmodulin-sensitive adenylate cyclase activation rendered nACHR1 less sensitive to imidacloprid resulting in the decrease of the current amplitude induced by imidacloprid (Courjaret and Lapied, 2001). Because it is known that the binding of neonicotinoids depends on receptor openings, the cAMP/PKA-dependent phosphorylation process that modulated nACHR1 openings thus decreases the efficiency of imidacloprid. Based on these findings and our results indicating that high steady-state resting calcium level was observed in DUM neuron cell bodies isolated from treated cockroaches, we suggest that high intracellular calcium concentration plays a crucial role through activation of the cAMP/PKA cascade in the decrease in nACHR1 sensitivity to imidacloprid. An additional interesting point is the current-voltage relationship that illustrates an increase in nicotine-induced current amplitude, compared to control condition. Similar results were previously obtained when the experiments were performed in the presence of d-TC (an antagonist of nACHR1), which allows the study in isolation of the effects of nicotine and other cholinergic agonists on nACHR2 (Bodereau-Dubois et al., 2012). This might suggest that the main inward current was flowing through nACHR2. Finally and based on these studies, the well superimposed current-voltage relationships between −30 and −70 mV obtained in control with d-TC and in treated cockroaches (without d-TC) demonstrates that the subchronic exposure to sublethal dose of imidacloprid strongly
reduced the participation of nAChR1. This is confirmed by the measure of the membrane resistance value reflecting the selective conformational state of nAChR2, which is higher in DUM neurons isolated from treated cockroaches than in control. In fact, the inward current measured after nAChR2 activation results from an inhibition of an outward spontaneous potassium current, as previously described (Courjaret and Laped, 2001; Thany et al., 2008; Bodereau-Dubois et al., 2012). In this case, the higher resistance value measured in DUM neurons isolated from treated cockroaches, which is closely related to the loss of spontaneous potassium conductance, is a specific response of nAChR2 functional property (Bodereau-Dubois et al., 2012).

4.2. Subchronic exposure differently modifies nAChR1 and nAChR2 properties

Subchronic exposure to imidacloprid also affects nAChR2 function. However, these effects were different from those on nAChR1. In fact the uncommon conformational state of nAChR2 (i.e., open at the resting state and closed upon cholinergic agonist application) (Courjaret and Laped, 2001; Courjaret et al., 2003; Thany et al., 2008) seems to be responsible for the different imidacloprid sensitivity observed in these two nAChR1 and nAChR2. In addition, previous results obtained in DUM neurons revealed that different cellular and molecular factors including transmembrane potential, conformational state, subunit composition and calcium-dependent signaling pathways modulate the sensitivity of nAChR2 to neonicotinoid insecticides such as clothianidin, acetamiprid and imidacloprid (Bodereau-Dubois et al., 2012; Calas-List et al., 2013; Mannaï et al., 2016; Sun et al., 2016). In the light of these findings, it is tempting to suggest that the increased steady-state resting calcium level observed in treated cockroaches is sufficient to modulate nAChR2 physiological functions and to reduce sensitivity to neonicotinoid insecticides, as previously described (Bodereau-Dubois et al., 2012). In treated cockroaches, the increase in both current amplitude and membrane resistance value is consistent with the strong reduction of the participation of imidacloprid-sensitive nAChR1 compensated by an optimization of the imidacloprid-insensitive nAChR2 functions. In fact, the inward currents mediated by nAChR1 and nAChR2 differ from each other on the basis of their voltage dependence, ionic permeability, conformational state, subunit composition and sensitivity to cholinergic ligands and neonicotinoid insecticides (Courjaret and Laped, 2001; Bodereau-Dubois et al., 2012; Calas-List et al., 2013; Sun et al., 2016). DUM neurons receive excitatory input via the release of acetylcholine, which generates depolarization through nAChR1 and nAChR2 activation. In treated cockroaches, we demonstrate that the strong reduction of nAChR1 participation seems to be compensated by an optimization of nAChR2 functional properties. The increased inward current amplitude observed after nAChR2 activation, which results, in fact, from an inhibition of an outward spontaneous potassium conductance is closely related to the increase of the input membrane resistance, reflecting conformational state changes that indicate that nAChR2 is more open before application of nicotine. These results seem to be in line with molecular experiments, which clearly indicate that the pattern expression of nAChR2 subunits, α1, α2 and β1, previously identified (Sun et al., 2016) is modified in treated cockroaches.

All together, these results strongly suggest that subchronic exposure to sublethal dose of imidacloprid, which reduces the participation of imidacloprid-sensitive nAChR1, allows cockroaches to be well adapted to this treatment by optimizing imidacloprid-insensitive nAChR2 to maintain optimum physiological functions. In conclusion, the data provided here contribute to novel information regarding mechanisms by which subchronic exposure to sublethal dose of insecticide may generate complex cellular and molecular factors to overcome insecticide treatment efficacy.

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