

## Full Length Article

# Mode of action of sulfoxaflor on $\alpha$ -bungarotoxin-insensitive nAChR1 and nAChR2 subtypes: Inhibitory effect of imidacloprid

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## ABSTRACT

Cockroach neurosecretory cells, dorsal unpaired median (DUM) neurons, express two distinct  $\alpha$ -bungarotoxin-insensitive nicotinic acetylcholine receptor subtypes, nAChR1 and nAChR2 which are differently sensitive to the neonicotinoid insecticides and intracellular calcium pathways. The aim of this study is to determine whether sulfoxaflor acts as an agonist of nAChR1 and nAChR2 subtypes. We demonstrated that 1 mM sulfoxaflor induced high current amplitudes, compared to acetylcholine, suggesting that it was a full agonist of DUM neuron nAChR subtypes. Sulfoxaflor evoked currents were not inhibited by the nicotinic acetylcholine receptor antagonist d-tubocurarine (dTC) which reduced nAChR1. But, sulfoxaflor evoked currents were reduced in the presence of 5  $\mu$ M mecamylamine which is known to reduce nAChR2 subtype. Interestingly, when 1  $\mu$ M imidacloprid was added in the extracellular solution, sulfoxaflor-induced currents were significantly suppressed. Moreover, when extracellular calcium concentration was increased, bath application of 1  $\mu$ M imidacloprid partially reduced sulfoxaflor activated currents when nAChR1 was inhibited with 20  $\mu$ M dTC and completely suppressed sulfoxaflor currents when nAChR2 was inhibited with 5  $\mu$ M mecamylamine. Our data demonstrated therefore that sulfoxaflor activates both nAChR1 and nAChR2 subtypes.

## 1. Introduction

Insect nicotinic acetylcholine receptors (nAChRs) play a central role in the synaptic transmission and they are the main targets of several insecticides including neonicotinoids (Ihara and Matsuda, 2018; Taillebois et al., 2018; Tomizawa and Casida, 2003, 2005) and sulfoximine derivatives (Cutler et al., 2013; Watson et al., 2011, 2017). They are ligand gated ion channels composed of five subunits which form homomeric or heteromeric receptors. The pharmacological properties of their native forms are currently studied using cockroach neurons expressing nAChR subtypes (Salgado, 2016; Salgado and Saar, 2004; Tan et al., 2007). It was found that neurons from the thoracic ganglia expressed two types of  $\alpha$ -bungarotoxin-sensitive receptors: the desensitized subtype called nAChRD and nondesensitized (nAChRN) receptors. The nAChRN subtypes are inhibited by the nAChR antagonist methyllicaconitine (MLA) while the nAChRD subtypes are inhibited by neonicotinoid insecticides such as imidacloprid (IMI) (Salgado and

Saar, 2004). Using cockroach terminal abdominal ganglia, it was found that the cockroach neurosecretory cells, called dorsal unpaired median (DUM) neurons, expressed two  $\alpha$ -Bgt-insensitive nAChR subtypes. nAChR1 was blocked by d-tubocurarine (dTC) and was sensitive to IMI while nAChR2 was blocked by mecamylamine (MECA) and was insensitive to protein kinases C (PKC) 1 and 2, respectively (Courjaret et al., 2003; Courjaret and Lapied, 2001; Thany et al., 2008). In addition, increasing extracellular calcium concentration resulted to an increase of nicotine evoked current amplitudes through nAChR2. This increase occurred when nAChR1 was blocked with dTC (Thany et al., 2008). Moreover, pressure ejection of IMI on DUM neuron nAChRs induced only a monophasic current-voltage curve compared to that induced with acetamiprid (ACE) and clothianidin (CLT) which evoked biphasic curves (Bodereau-Dubois et al., 2012). Thus, it was suggested that ACE and CLT were able to activate both nAChR1 and nAChR2 whereas IMI activated only nAChR2 subtype (Bodereau-Dubois et al., 2012).

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Sulfoximine insecticides such as sulfoxaflor (SFX) form a new class of active compounds which exhibited structure-activity relationships different from neonicotinoid ones (Babcock et al., 2011). They have been included in the 4C subgroup (nAChR competitive modulators) by the IRAC committee while neonicotinoids are in the 4A subgroup (Sparks et al., 2013). They are used for the control of sap-feeding insects such as *Myzus persicae*, *Aphis gossypii*, *Bemisia tabaci* and *Nilaparvata lugens* for which a strong resistance against neonicotinoid insecticides was found (Sparks et al., 2013). Indeed, SFX is a potential new alternative over the current neonicotinoids such as IMI because of the lack of cross-resistance with neonicotinoids (Wang et al., 2017). Like neonicotinoids, sulfoximine potent insecticidal activity was associated to their efficacy at nAChR subtypes (Watson et al., 2011). Thus, it was found that SFX evoked very high amplitude currents through the activation of *Drosophila melanogaster*  $\text{D}\alpha 2/\beta 2$  hybrid receptor expressed in *Xenopus laevis* oocytes. But, SFX displaced [ $^3\text{H}$ ]IMI from *Myzus persicae* nAChR membrane preparations with weak affinity (Watson et al., 2011). These studies demonstrated that the mode of action of SFX on insect nAChRs needs to be further explored. Indeed, it was not clear if it can activate similar or distinct insect neuronal nAChRs with IMI (Cutler et al., 2013; Watson et al., 2011). In the present study, we evaluated the mode of action of SFX on cockroach DUM neurons. In particular, we were interested to evaluate its effect on the known  $\alpha$ -Bgt-insensitive nAChR1 and nAChR2 subtypes.

## 2. Materials and methods

### 2.1. Insects

The experiments were carried out on DUM neuron cell bodies isolated from the dorsal midline of the terminal abdominal ganglion of the nerve cord from adult male cockroaches *Periplaneta americana*.

### 2.2. Cells isolation

DUM neurons were isolated following enzymatic treatment and mechanical dissociation as previously described (Lapied et al., 1990). Cells were incubated overnight at 29 °C before electrophysiological experiments. Currents were recorded using the patch-clamp technique in the whole-cell recording configuration (Lapied et al., 1990). The Petri dish containing isolated cell bodies was placed onto the inverted microscope (CK2: Olympus), and continuously bathed with the standard extracellular solution (in mM: NaCl 200, KCl 3.1,  $\text{MgCl}_2$  4,  $\text{CaCl}_2$  5, sucrose 50, HEPES 10, pH 7.4 adjusted with NaOH) using a gravity perfusion system positioned at a distance of 150  $\mu\text{m}$  from the cell body.

### 2.3. Patch clamp recordings

Signals were recorded using an Axopatch 200B amplifier (Axon instruments, Foster City, CA), connected to a computer with the pClamp software control (pClamp 10.0, Axon Instruments). The liquid junction potential between the bath and the internal pipette solution was compensated before the formation of a gigaOhm seal ( $> 5 \text{ G}\Omega$ ). Patch pipettes were pulled from borosilicate glass capillary tubes (GC 150T-10; Clark Electromedical Instruments, Harvard Apparatus) and had resistance of 2  $\text{M}\Omega$  when filled with the standard pipette solution (osmolarity : 227.5) containing (in mM) : NaCl 10;  $\text{MgCl}_2$  1;  $\text{CaCl}_2$  0.5; HEPES 10; ATPMg 1; EGTA 10; K aspartate 160; KF 10; and adjusted to

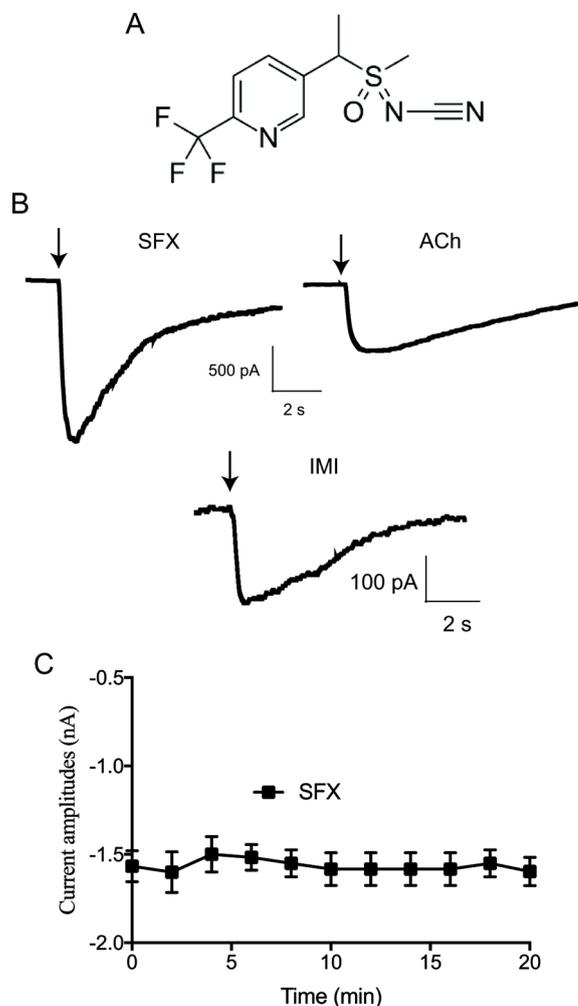
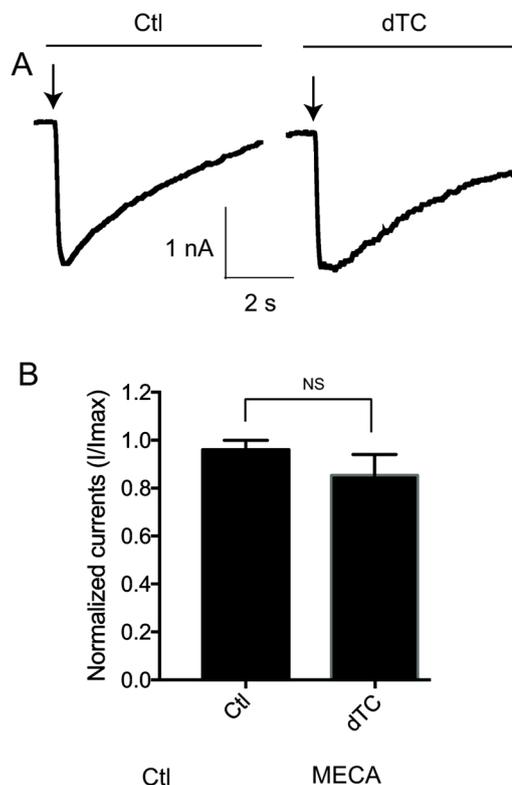
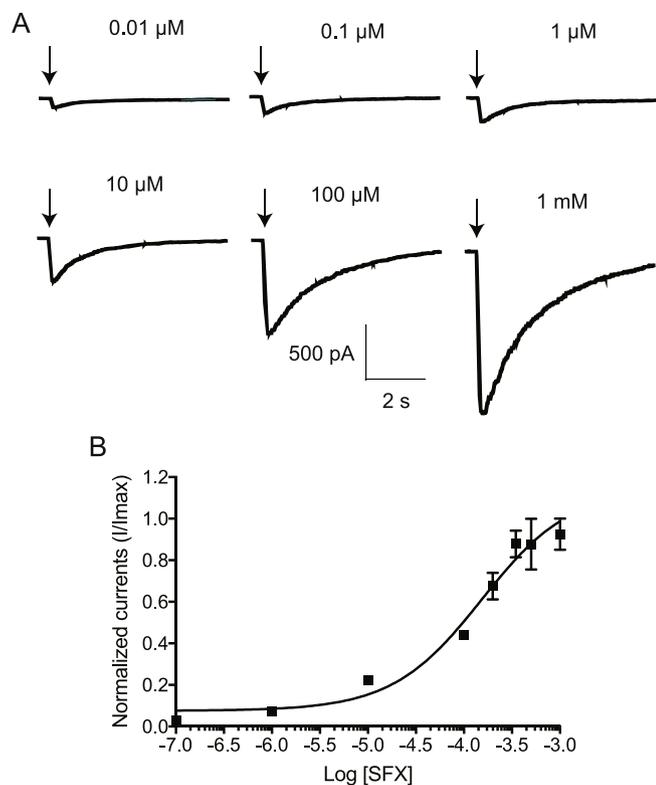


Fig. 1. (A) Chemical structure of sulfoxaflor (SFX) prepared according to literature data (Arndt et al., 2015). (B) Currents represent the effects of 1 mM sulfoxaflor (SFX), acetylcholine (ACh) and imidacloprid (IMI) on DUM neuron nAChRs. Arrows indicate 300 ms pulse application (15 psig). (C) Successive applications of 1 mM SFX (300 ms pulse application) recorded at  $-50 \text{ mV}$  holding potential. Each point represents mean  $\pm$  SEM of  $n = 12$  cells.

pH 7.4 with KOH. SFX was applied by pneumatic pressure ejection (15 psig, 300 ms, Miniframe, Medical System Corporation, USA) through a glass micropipette (resistance 2.0  $\text{M}\Omega$  when filled with agonist) positioned in solution at a distance of 150  $\mu\text{m}$  from the isolated cell body. All currents were recorded under bath application of 0.5  $\mu\text{M}$   $\alpha$ -Bgt to block  $\alpha$ -Bgt-sensitive nAChRs (Courjaret and Lapied, 2001). Currents were filtered using the Clampfit software.

### 2.4. Compounds

All compounds were purchased from SIGMA Aldrich France, except for SFX which was prepared according to data previously published (Arndt et al., 2015).



**Fig. 2.** Effect of sulfoxaflor on DUM neuron nAChRs. (A) Representative responses of sulfoxaflor on DUM neurons. Arrows indicate pulse application of 300 ms sulfoxaflor. (B) Dose-response curve for sulfoxaflor. Data are mean  $\pm$  SEM of  $n = 8$  independent experiments.

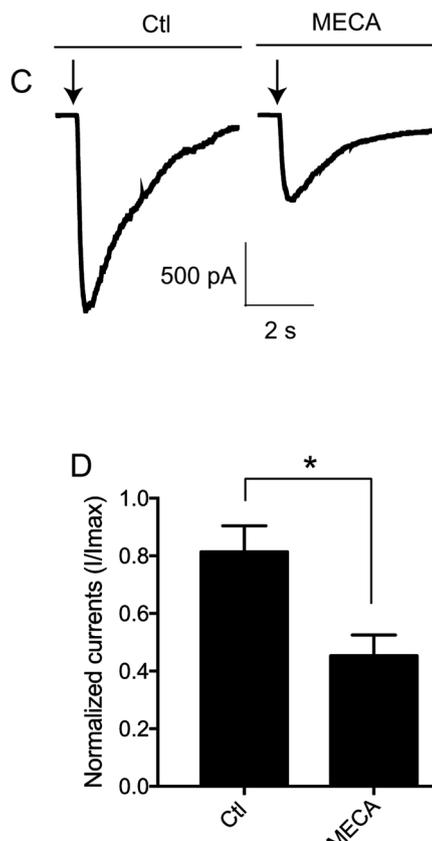
2.5. Statistical analysis

For the statistical analysis, one way ANOVA and Bonferroni post-hoc test were employed, using the Prism program (GraphPAD Software, San Diego, CA). For each data,  $I/I_{max}$  were normalized according to the  $I_{max}$  recorded in the same experimental condition. In the results, ‘n’ represents the number of recorded cells. Normalized data were fitted by use of GraphPad Prism (GraphPad Software, UK) to the following equation :  $y = I_{min} + (I_{max} - I_{min}) / (1 + 10^{-(\log(EC_{50} - X)/H)})$  where  $y$  is the normalized response,  $I_{max}$  and  $I_{min}$  are the maximum and the minimum responses,  $H$  is the Hill coefficient,  $EC_{50}$  is the concentration giving half the maximum response and  $X$  is the logarithm of the compound concentration.

3. Results

3.1. Effect of sulfoxaflor on DUM neurons  $\alpha$ -bungarotoxin-insensitive nicotinic acetylcholine receptors

Previous studies demonstrated that DUM neurons expressed two distinct  $\alpha$ -Bgt-insensitive nAChRs, named nAChR1 and nAChR2 (Courjaret et al., 2003; Courjaret and Lapied, 2001; Thany et al., 2008). Thus, all experiments were performed under bath application of 0.5  $\mu$ M  $\alpha$ -Bgt to inhibit  $\alpha$ -Bgt-sensitive nAChRs (Bodereau-Dubois et al., 2012; Courjaret et al., 2003; Courjaret and Lapied, 2001). Pressure application of 1 mM SFX (300 ms pulse duration) induced a mean inward current of  $-1.56 \pm 0.21$  nA at a holding membrane potential of  $-50$  mV. The amplitude of the SFX-evoked currents was higher compared to 1 mM ACh, suggesting that SFX was a full activator of  $\alpha$ -Bgt-insensitive nAChRs expressed on cockroach DUM neurons (Fig. 1). Note that, no desensitized currents were found during successive applications of 1 mM SFX (Fig. 1C). Using the equation describes above, we



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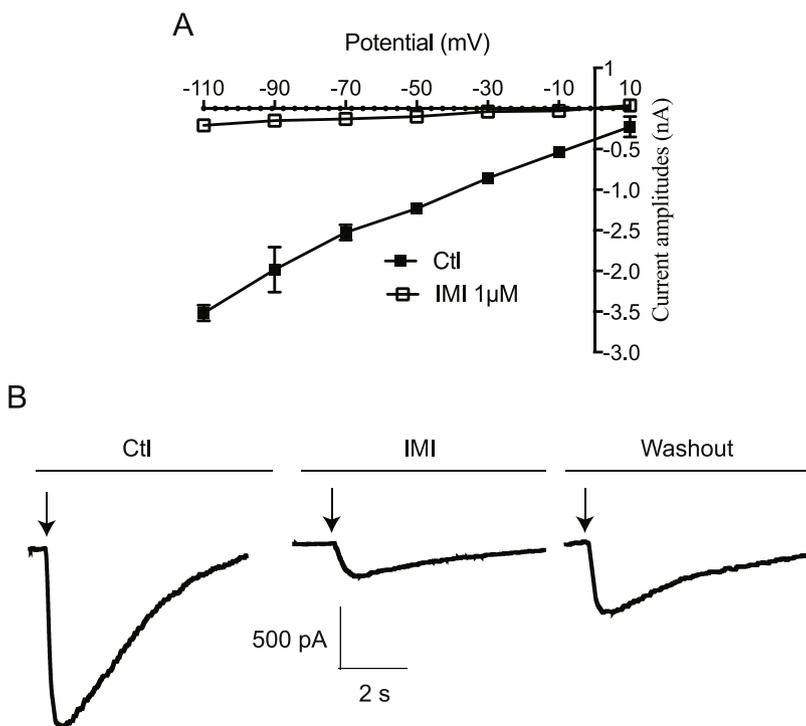
**Fig. 3.** The inhibitory effects of the nAChR antagonists, d-tubocurarine (dTC) and mecamylamine (MECA), on the responses evoked by 1 mM SFX. (A and B) Representative currents after 300 ms pulse application (arrows) of 1 mM SFX during control condition (Ctl) and bath application of 20  $\mu$ M dTC. Histograms represent the mean SFX-induced currents recorded at  $-50$  mV holding potential under 20  $\mu$ M dTC. In each case, data are mean  $\pm$  SEM of  $n = 8$  cells. NS : non significant. (C and D) Examples of SFX-induced currents under control condition (Ctl) and bath application of 5  $\mu$ M MECA. Arrows indicate 300 ms pulse application of 1 mM SFX. (D) Histograms summarizing the effect of 1 mM SFX under control condition (Ctl) and bath application of 5  $\mu$ M MECA. Each histogram represents means  $\pm$  SEM of  $n = 10$  cells. \*  $p < 0.05$ .

found that successive applications of SFX at different concentrations induced a concentration-dependent response with an  $EC_{50}$  value of  $153.7 \pm 10.2 \mu$ M and a Hill coefficient of 0.9 (Fig. 2). As illustrated in Fig. 3, currents measured in the absence and presence of 20  $\mu$ M dTC are not significantly different. Currents are respectively of  $-1.44 \pm 0.08$  nA and  $-1.43 \pm 0.1$  nA ( $p > 0.05$ ,  $n = 12$ , Fig. 3A and B). However, significant reduced current amplitudes were found when 5  $\mu$ M MECA was applied in the bath. As such, currents were reduced from  $-1.63 \pm 0.08$  nA under control condition to  $-0.87 \pm 0.05$  nA under bath application of 5  $\mu$ M MECA ( $p < 0.05$ ,  $n = 10$ , Fig. 3C and D). These results suggested that dTC was not able to inhibit SFX current amplitudes, leading to the assumption that SFX activated only nAChR2

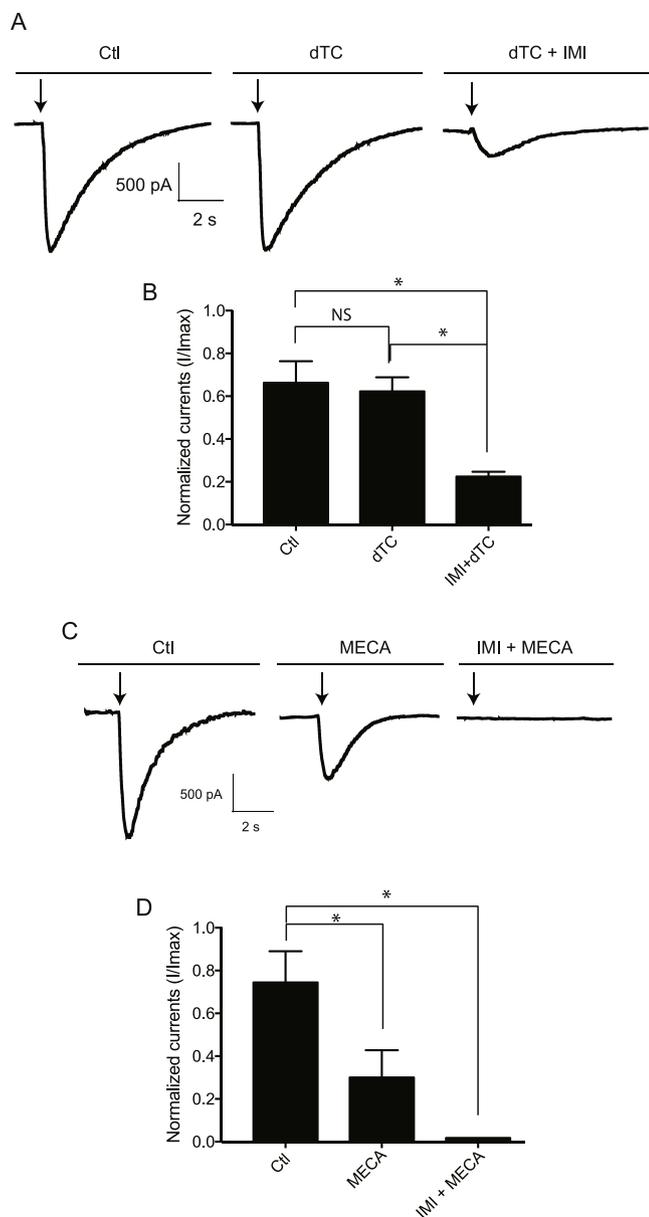
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### 3.2. Imidacloprid reduces sulfoxaflor activated current through nAChR1 and nAChR2 subtypes

To test further the involvement of nAChR1 and nAChR2 subtypes on SFX evoked currents, 1  $\mu$ M IMI was applied in the bath during pulse application of 1 mM SFX (300 ms pulse duration). Indeed, it was suggested that IMI acted preferentially on nAChR1 subtype (Courjaret and Lapied, 2001). Note that at 1  $\mu$ M, IMI was not able to elicit any current, even when applied on DUM neurons (Data not shown). But, it strongly reduced currents evoked by SFX at different holding potentials (Fig. 4). Indeed, the currents induced by 1 mM SFX at different holding potentials were strongly reduced in the presence of bath application of 1  $\mu$ M IMI (Fig. 4A). At  $-50$  mV holding potential, currents were reduced from  $-1.61 \pm 0.8$  nA to  $-0.35 \pm 0.03$  nA ( $p < 0.05$ ,  $n = 8$ , Fig. 4B) and SFX-induced currents were partially reversed after 20 min washout (Fig. 4B). These results demonstrated that IMI was able to inhibit SFX evoked current amplitudes. Nevertheless, we noted a residual current when 1  $\mu$ M IMI was applied in the bath. This current could suggest a partial blocking effect or the presence of nAChRs insensitive to IMI. Thus, we studied the involvement of nAChR1 and nAChR2 subtypes. As previously shown (see Fig. 3) when nAChR1 was blocked with 20  $\mu$ M dTC, SFX currents were not reduced. But, co-application of both 1  $\mu$ M



**Fig. 4.** Effect of SFX on DUM neuron nAChRs. (A) Current-voltage relationships of SFX evoked current amplitudes plotted as a function of steady-state holding potentials 5 min after establishing the whole-cell configuration. Values are means  $\pm$  SEM. (B) Currents induced by 1 mM SFX under control condition (Ctl) and during bath application of 1  $\mu$ M Imidacloprid (IMI). The reduction by 1  $\mu$ M IMI was partially reversible after washout.



**Fig. 5.** Effect of SFX on  $\alpha$ -bungarotoxin insensitive nAChR1 and nAChR2 under bath application of 1  $\mu$ M IMI. Data were recorded using the same cells. Thus, we found that (A) 20  $\mu$ M dTC has no effect on SFX-induced currents but currents are strongly reduced when we added 1  $\mu$ M imidacloprid (IMI). (B) Histograms summarize data obtained in the same conditions. Data are means  $\pm$  SEM. \*  $p < 0.05$ . In each case,  $n = 10$  cells. Using the same conditions, (C) bath application of 5  $\mu$ M MECA strongly reduced SFX evoked currents which were completely inhibited by under bath application of 1  $\mu$ M IMI. (D) Histograms summarize data obtained with MECA. Data are means  $\pm$  SEM. \*  $p < 0.05$ . In each case,  $n = 12$  cells.

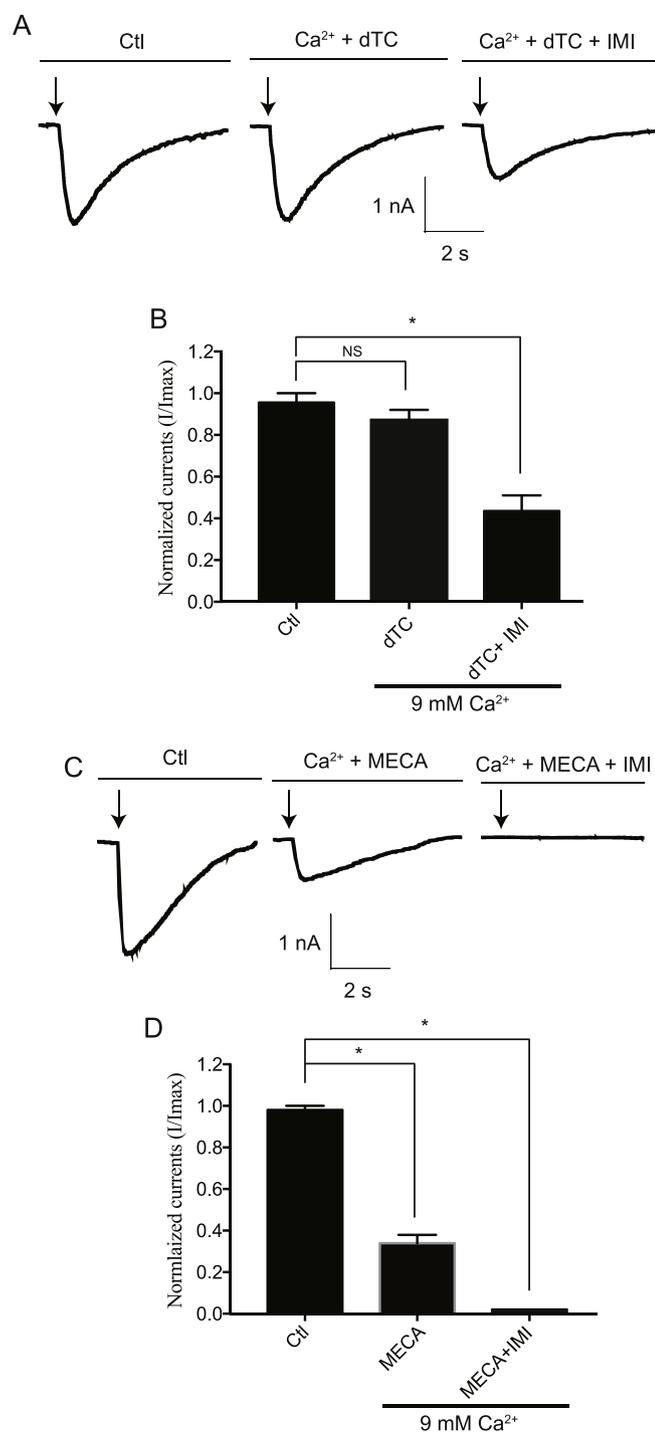
IMI and 20  $\mu$ M dTC reduced currents amplitudes to  $-0.436 \pm 0.2$  nA (Fig. 5A). We suggested that it existed at least two nAChR2 subtypes, IMI-sensitive and -insensitive which were activated by SFX. Under similar conditions, when nAChR2 was blocked with 5  $\mu$ M MECA, we found a significant decrease of SFX evoked current amplitudes and currents were completely blocked when we added 1  $\mu$ M IMI in the bath (Fig. 5B). Our data demonstrated that SFX was able to activate nAChR1 subtypes. We proposed that IMI could also activate SFX evoked currents through the activation of both nAChR1 and nAChR2 subtypes.

### 3.3. Effect of extracellular calcium concentration on SFX evoked currents

In a second series of experiments we used a cell preparation with 9 mM  $Ca^{2+}$ . We previously demonstrated that when nAChR1 was blocked with 20  $\mu$ M dTC, increasing extracellular  $Ca^{2+}$  concentration strongly increased nicotine current amplitudes (Thany et al., 2008). We found that, when extracellular  $Ca^{2+}$  concentration was increased from 5 mM (normal saline solution) to 9 mM  $Ca^{2+}$ , SFX-induced currents were not increased: currents were  $-1.52 \pm 0.16$  nA and  $-1.43 \pm 0.11$  nA under control and 9 mM  $Ca^{2+}$  solution (data not shown). These data suggested that SFX evoked currents on DUM neuronal nAChRs were not sensitive to extracellular  $Ca^{2+}$  increase, compared to nicotine (Thany et al., 2008). Moreover, no significant effect on SFX evoked current amplitudes was found during bath application of 20  $\mu$ M dTC used to inhibit nAChR1 (currents were  $-1.56 \pm 0.09$  nA and  $-1.41 \pm 0.08$  nA, respectively). However, SFX evoked current were reduced when 1  $\mu$ M IMI was applied in the bath. Currents were reduced from  $-1.56 \pm 0.09$  to  $-0.47 \pm 0.12$  nA ( $p < 0.05$ ,  $n = 9$ , Fig. 6A and B), and were not reversed after washout (data not shown). When nAChR2 was inhibited with 5  $\mu$ M MECA, SFX evoked currents were strongly reduced (currents were  $-0.49 \pm 0.04$  nA,  $p < 0.05$ ,  $n = 8$ , Fig. 6C and D). In addition, when 5  $\mu$ M MECA was co-applied with 1  $\mu$ M IMI currents were completely inhibited ( $p < 0.05$ ,  $n = 8$ , Fig. 6C and D). Our data confirmed that SFX was able to activate both nAChR1 and nAChR2 subtypes.

### 4. Discussion

The effects of the sulfoximine insecticide, SFX, on cockroach DUM neurons were tested in the present study. DUM neurons were chosen because they expressed different  $\alpha$ -Bgt-sensitive and -insensitive nAChR subtypes, more precisely,  $\alpha$ -Bgt-insensitive nAChR1 and nAChR2 which were differently sensitive to IMI (Courjaret and Lapied, 2001). Our studies provide evidence that SFX is able to activate DUM neuronal nAChRs and is a full agonist of DUM neuronal nAChRs. We also found that SFX evoked currents were not blocked by bath application of dTC but they were reduced in the presence of MECA which was known to block nAChR2 (Courjaret et al., 2003; Courjaret and Lapied, 2001). The finding that IMI blocked SFX evoked current amplitudes suggested that SFX activated both nAChR1 and nAChR2 subtypes. This finding was confirmed when SFX was tested in bath solution containing higher  $Ca^{2+}$  concentration. Indeed, we previously demonstrated that nicotine evoked current amplitudes were affected by an increase of extracellular calcium concentration when nAChR1 was blocked by dTC (Thany et al., 2008). Moreover, our data suggested that SFX was able to activate DUM



**Fig. 6.** Effects of SFX on  $\alpha$ -bungarotoxin-insensitive, nAChR1 and nAChR2, under bath application of 9 mM  $\text{Ca}^{2+}$ . (A) Currents induced by 1 mM SFX are not affected by 20  $\mu\text{M}$  dTC but they are reduced by bath application of 1  $\mu\text{M}$  IMI. (B) Histograms illustrating the effect of bath application of dTC and IMI. Histograms are means  $\pm$  SEM of  $n = 8$  cells. Data were obtained using the same cell. (C) Effect of 5  $\mu\text{M}$  MECA on SFX evoked currents. Currents are reduced under bath application of 5  $\mu\text{M}$  MECA and completely reduced when 1  $\mu\text{M}$  IMI was added in the bath. (D) Histograms represent the means  $\pm$  SEM of  $n = 8$  cells. \*  $p < 0.05$ . NS : no significant.

neuronal  $\alpha$ -Bgt-insensitive nAChRs which are sensitive to IMI but not blocked by dTC. Thus, at least more than three  $\alpha$ -Bgt-insensitive nAChR subtypes may be sensitive to SFX. The mode of action of SFX on nAChR1 and nAChR2 subtypes may reflect their subunit combinations. Indeed, Sun et al, demonstrated that cockroach DUM neuronal  $\alpha$ -Bgt-

insensitive nAChR1 was potentially composed of Pa $\alpha$ 3, Pa $\alpha$ 8 and Pa $\beta$ 1 subunits whereas, Pa $\alpha$ 1, Pa $\alpha$ 2 and Pa $\beta$ 1 may contribute to nAChR2 (Sun et al., 2017). Considering the number of subunit combinations to form functional receptors, we propose that both nAChR1 and nAChR2 subtypes could reflect the presence of several nAChR populations which are activated by SFX. Thus, nAChR1 could include only IMI-sensitive nAChR subtypes which represents low currents induced by SFX whereas nAChR2 could include nAChR2 subtypes-sensitive and -insensitive to IMI. We propose that the effect of SFX on nAChR1 may be due to this complex subunit combination. This finding was also in accordance with data demonstrating that SFX elicited higher current amplitudes on hybrid drosophila D $\alpha$ 2 $\beta$ 2 than D $\alpha$ 1 $\beta$ 2 receptors, expressed on *Xenopus oocytes* (Watson et al., 2011; Zhu et al., 2011). Our data also point out that SFX may interact with IMI-sensitive receptors as found by Cutler et al. who demonstrated that IMI displaced the tritiated analogue of SFX ( $[^3\text{H}]$ -methyl-SFX) with a  $K_i$  value in the pM range leading to the assumption that SFX interacted specifically with the high-affinity IMI binding site present in a subpopulation of the total nAChR pool (Cutler et al., 2013) which is in accordance with previous studies demonstrating that resistance of field populations of *Nilaparvata lugens* and *Bemisia tabaci* to sulfoxaflor was significantly correlated with all neonicotinoids, including IMI (Liao et al., 2019, 2017; Longhurst et al., 2013). Here, we advance the hypothesis that the correlation between SFX and neonicotinoids might be due to the same mode of action at insect neuronal nAChRs. In final, the present work add informations to our understanding on the mode of action of SFX on insect nAChRs.

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