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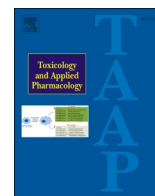
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Pharmacology and molecular modeling studies of sulfoxaflor, flupyradifurone and neonicotinoids on the human neuronal $\alpha 7$ nicotinic acetylcholine receptor

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ABSTRACT

We conducted electrophysiological and molecular docking studies using a heterologous expression system (*Xenopus* oocytes) to compare the effects of four neonicotinoids (acetamiprid, imidacloprid, clothianidin and thiamethoxam), one sulfoximine, (sulfoxaflor), and one butenolide (flupyradifurone), on human $\alpha 7$ neuronal nicotinic acetylcholine receptors (nAChRs). All neonicotinoids (except thiamethoxam), as well as the recently introduced nAChR competitive modulators, flupyradifurone and sulfoxaflor, appear to be weaker agonists than acetylcholine. Two mutations in loop C (E211N and E211P) and one mutation in loop D (Q79K), known to be involved in the binding properties of neonicotinoids were introduced to the $\alpha 7$ wild type. Interestingly, the acetylcholine and nicotine-evoked activation was not modified in human $\alpha 7$ mutated receptors, but the net charge was enhanced for clothianidin and imidacloprid, respectively. Flupyradifurone responses strongly increased under the Q79K mutation. The molecular docking investigations demonstrated that the orientations and interactions of the ligands considered were in accordance with those observed experimentally. Specifically, the charged fragments of acetylcholine and nicotine, used as reference ligands, and their neonicotinoid homologs were found to be surrounded by aromatic residues, with key interactions with Trp171 and Y210. Furthermore, the molecular docking investigations predicted the water-mediated interaction between the carbonyl oxygen of acetylcholine and the Nsp² nitrogen of the pyridine ring for nicotine (as well as for the majority of the corresponding neonicotinoid fragments) and main chain NH of L141. The docking scores, extending over a significant range of 6 kcal/mol, showed that most neonicotinoids were poorly stabilized in the $\alpha 7$ nAChR compared to acetylcholine, except sulfoxaflor.

1. Introduction

Neonicotinoid insecticides have become the fastest growing class of insecticides over the past few decades and have been banned in the European Union due to their potential adverse effects on humans and the environment (Faro et al., 2019; Han et al., 2018; Hirano et al., 2018; Zhang et al., 2018). Recent derivatives, including a sulfoximine insecticide (sulfoxaflor, SFX) (Babcock et al., 2011; Cutler et al., 2013), and a butenolide insecticide (flupyradifurone, FLU) (Montgomery et al., 2022; Nauen et al., 2015), were designed to replace neonicotinoids on the

market, leading to new polemics concerning their use. Indeed, they share their mode of action with neonicotinoids as selective agonists of nicotinic acetylcholine (ACh) receptors (nAChRs) (Nauen et al., 2015; Sparks et al., 2013; Zhu et al., 2011), and several studies have demonstrated that non-target species could be exposed to these new compounds (Al Naggar et al., 2022; Boff et al., 2021; Mundy-Heisz et al., 2022) and subsequently adversely impacted by them. Unfortunately, there is a lack of knowledge concerning their affinity with human nAChRs, which are cys-loop receptors. Among the nAChR subtypes, homomeric $\alpha 7$ receptors are highly expressed in the mammalian central

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nervous system (Beinat et al., 2016; Seguela et al., 1993). They are characterized by rapid activation, low affinity and high permeability to calcium (Millar and Gotti, 2009; Seguela et al., 1993). Several studies indicate that $\alpha 7$ nAChRs are implicated in cholinergic anti-inflammatory pathways (Han et al., 2017; Han et al., 2014; Liu et al., 2023; Wang et al., 2003). For example, the release of ACh can activate $\alpha 7$ nAChRs on various immune cells, such as macrophage and microglia, and down-regulate the release of related inflammatory factors (Han et al., 2017). Moreover, $\alpha 7$ nAChRs are known to be involved in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Parri et al., 2011; Paterson and Nordberg, 2000; Wallace and Porter, 2011), and in particular regions of the brain involved in cognitive processes (Levin et al., 2006). All these features make them an interesting model for studying the mode of action of diverse compounds that can act as agonists, antagonists and modulators of human neuronal nAChRs (Beinat et al., 2016; Chatzidaki et al., 2015; Gill et al., 2013; Han et al., 2014). We propose that they could help understand the mode of action of neonicotinoids and recently introduced insecticides on human neuronal nAChRs. Indeed, $\alpha 7$ subunit exhibits significant sequence homologies with insect subunits (See supplementary data fig. S1), and it was proposed that modifications in the current amplitudes induced by agonists can be caused by mutation of specific residues in the $\alpha 7$ nAChRs (Aldea et al., 2007; Bertrand et al., 1993; Criado et al., 2008). For example, the residues impacting the affinity of vertebrate nAChRs to neonicotinoids are located in several loops, such as in loops C and D (Shimomura et al., 2002; Shimomura et al., 2004; Shimomura et al., 2003; Toshima et al., 2009). Mutation of Q79E in loop D reduced the maximum current responses to the neonicotinoids on the chicken $\alpha 7$ receptor, whereas Q79K and Q79R mutations increased it, suggesting that the glutamine residue binds to the nitro group of the neonicotinoid insecticides (Matsuda et al., 2000; Shimomura et al., 2002). In other studies, it was demonstrated that the non-acidic (X) residue in the YXCC motif in loop C could be critical for the binding of neonicotinoid insecticides between insect and mammalian neuronal nAChR subtypes (Shimomura et al., 2004; Toshima et al., 2009). Site-directed mutations E219P in the mammalian $\alpha 4$ subunit increased the imidacloprid (IMI) sensitivity of the $\alpha 4\beta 2$ nAChRs (Shimomura et al., 2004). Therefore, it was concluded that basic residues in loop D (Shimomura et al., 2006) and X residues in the YXCC motif located in loop C (Shimomura et al., 2004), are key determinants of the selective actions of neonicotinoids (Ihara et al., 2017; Matsuda et al., 2009; Matsuda et al., 2005; Matsuda et al., 2000). Thus, in the present study, we evaluated the involvement of Q79 and E219 (Corresponding to E211 in the human $\alpha 7$ subunit) residues in the mode of action of neonicotinoids and recently introduced compounds. In particular, the involvement of insect lysine (K) in the loop D, and asparagine (N) residues at position X in the YXCC motif in loop C.

From a structural point of view, high-resolution structural information on intact and full nAChR has been scarce until recently (Gharpure et al., 2020). Progress in terms of knowledge of the structural features of nAChRs therefore relied on the structures of homologous proteins and lower-resolution electron microscopy (EM) reconstructions for a long period of time. The discovery and exploitation of the acetylcholine (ACh)-binding protein (AChBP), a synaptic protein released by glial cells in mollusks (Smit et al., 2001), has had a significant impact in the field. Indeed, AChBP is structurally homologous to the extracellular domain of nicotinic receptors, sharing 24 % sequence identity with the N-terminal region of the $\alpha 7$ nAChR subunit. The resolution of the AChBP structure opened the way for a detailed characterization of the ligand-binding domain (LBD) of nAChRs. Since then, a significant number of X-ray structures of AChBPs, mostly from *Lymanaea stagnalis* and *Aplysia californica* have been determined, with a wide range of resolutions (4.2 Å – 1.75 Å) in apo forms, and co-crystallized with several agonists of nAChRs (Brejc et al., 2001; Rucktooa et al., 2009; Shahsavari et al., 2016). With respect to neonicotinoids, the crystal structures of several complexes with a double-mutated AChBP mimicking the insect-ion-channel orthosteric site was published (Montgomery et al., 2022).

Significant progress have been made concerning the entire nAChR in the last few years, notably with the publication of cryo-EM structures of the human $\alpha 7$ nAChR in three conformational states: resting, activated and desensitized (Noviello et al., 2021). A large number of studies based on the implementation of molecular modeling approaches can now be found in the literature (Cottone et al., 2020; Oliveira et al., 2019).

Despite all of these studies, nothing is known about the comparative affinity of neonicotinoids and their latest derivatives, such as SFX and FLU, on human $\alpha 7$ neuronal nAChRs. Here, we demonstrated that neonicotinoid insecticides, (excluding thiamethoxam TMX), and their latest derivatives, acted as partial agonists of human neuronal $\alpha 7$ receptors. In addition, the Q79K mutation in loop D, and E211N and E211P mutations in loop C, impact the affinity of the latest nAChR competitive modulators SFX and FLU for $\alpha 7$ nAChRs differently. We complemented these experimental findings through molecular docking investigations, using the $\alpha 7$ nAChR structure resolved recently (Noviello et al., 2021; Prevost et al., 2023) in the activated state for the wild type, and the E211N, E211P and Q79K mutants. Overall, our set therefore included eight ligands and four $\alpha 7$ nAChRs (wild type and three mutants). In the case of SFX, as only RS and SS diastereoisomers were observed by Montgomery et al., (Montgomery et al., 2022) on the binding site of a double-mutated *Ls*-AChBP, they were the only ones investigated through induced-fit molecular docking (leading to a total of 36 ligand...receptor systems).

2. Materials and methods

2.1. Oocytes

For all experiments, distinct batches of defolliculated *Xenopus leavis* oocytes were purchased from Ecocyte Bioscience (Dortmund, Germany). They were incubated at 18 °C.

2.2. Mutagenesis analysis

We constructed three mutant cDNAs of human $\alpha 7$ neuronal nAChR from loops D and C (Fig. 1a). The human $\alpha 7$ subunit in the pcDNA3.1/Zeo vector (accession number: Y08420.1) was used as a template for mutagenesis and was named $\alpha 7$ WT receptor. The cDNAs containing the E211N, E211P and Q79K mutations were prepared as follows: 5 ng of human $\alpha 7$ WT cDNA was mixed with sense and antisense oligonucleotide primers (Table 1) using the Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, USA), according to the manufacturer's protocol. PCR conditions were: 30 s hot start at 98 °C, 25 amplification cycles (10 s at 98 °C, 20 s at 67 °C, 3 min at 72 °C) and 2 min at 72 °C. The final product was cloned into the pcDNA3.1/Zeo vector and sent to be sequenced by Eurofins Genomics Germany GmbH (Ebersberg, Germany), to confirm the mutations (Fig. 1a).

2.3. Preparation of crNAs

The validated cDNAs containing E211N, E211P and Q79K mutations were linearized with BamHI (New England Biolabs, Ipswich, USA) to obtain the corresponding crNAs. Similarly, the cDNA coding for the rat resistant to inhibitors of cholinesterase (RIC-3, accession number: NP_001108517) was cloned into the pGEM vector, and recombinant plasmid was linearized with *NheI* (New England Biolabs, Ipswich, USA). RIC-3 is a transmembrane chaperone protein which exerts a dramatic influence upon the maturation (folding and assembly) of neuronal nAChRs (Halevi et al., 2002; Halevi et al., 2003; Millar, 2008). All RNAs were prepared using the T7 mMMESSAGE mMACHINE kit (Ambion, Austin, USA) according to the manufacturer's protocol.

2.4. Oocyte injection

The oocyte was injected with 50.6 nl of crNA mix using Nanoinject II

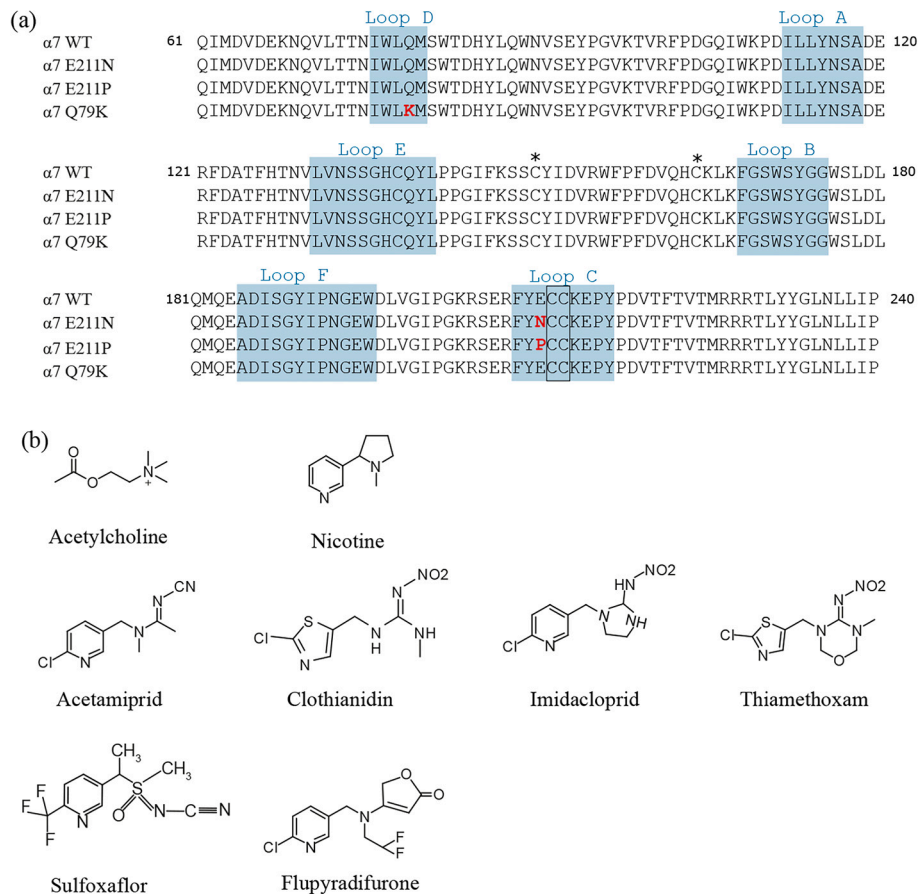


Fig. 1. Amino acid sequences of the $\alpha 7$ WT and $\alpha 7$ mutated receptors and chemical structure of all tested compounds. (a) Protein alignments of the N-terminal domain from $\alpha 7$ WT and mutants of nAChRs. The mutations are indicated in red. Mutation Q79K is in loop D and the mutations E211P and E211N are located in loop C. Loops involved in ligand binding are highlighted with blue boxes. Sites of cysteine residues involved in the cys-loop are marked with asterisks and the vicinal residues characteristic of the α -type subunit are boxed. (b) Chemical structures of acetylcholine, nicotine and all tested insecticides: clothianidin, acetamiprid, imidacloprid, thiamethoxam, sulfoxaflor and flupyradifurone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

PCR primers used for the preparation of mutant cDNAs. Letters in bold indicate changed nucleotides.

Mutation	Direction	Nucleotide sequence
E211N	Sense	GAAAGGTTCTAT a ATTGCTGCAAAAGAC
	Antisense	ACTCCTCTTGCCGGGGAT
E211P	Sense	AGGTTCTAT cc GTGCTGCAAAAG
	Antisense	TTCACCTCCTCTTGCCGGG
Q79K	Sense	ACATTTGGCTG aaa ATGTCTTTG
	Antisense	TGGTGGTTAA aa CTTGGTTCTT

(WPI, Hertfordshire, UK). They were then incubated at 18 °C in standard oocyte saline solution (SOS) composed of (in mM): NaCl 100, KCl 2.0, CaCl₂ 1.8, MgCl₂ 1.0 and HEPES 5.0, pH 7.4, supplemented with penicillin (100 U.ml⁻¹), streptomycin (100 µg.ml⁻¹), gentamycin (50 mg.ml⁻¹) and 2.5 mM sodium pyruvate (Cartereau et al., 2018; Cieslikiewicz-Bouet et al., 2020). It should be noted that the 50.6 nl of cRNA mix included 10 ng of cRNA coding for human $\alpha 7$ WT or mutated receptors ($\alpha 7$ E211N, $\alpha 7$ E211P or $\alpha 7$ Q79K), and 10 ng of the protein chaperone RIC-3 (Halevi et al., 2002; Halevi et al., 2003; Millar, 2008) supplemented with distilled water. For voltage-clamp experiments, the oocytes were recorded 1–3 days after injection (Papke and Porter Papke, 2002; Papke et al., 2018).

2.5. Voltage-clamp recordings

Electrophysiological recordings were carried out using Roboocyte 2 hardware (Multi Channel Systems MCS GmbH, Germany). Membrane currents were recorded using two microelectrodes (the voltage and current electrodes) filled with 3 M KCl, which had a resistance ranging between 0.2 and 5 M Ω (Cartereau et al., 2018). Oocytes were voltage-clamped at -60 mV and perfused with SOS at 3 mL.min⁻¹ (Papke et al., 2018). To inhibit potential muscarinic responses, SOS was supplemented with atropine at 1 µM (Papke and Porter Papke, 2002). To evaluate the effect of the experimental compound we used acetylcholine (ACh) as a control. Control responses were defined as the average of the two initial applications of ACh at 60 µM made before test applications (Papke et al., 2018). Drugs were applied for 12 s followed by a 5 min washout period (Gulsevina et al., 2019; Papke and Porter Papke, 2002). Each oocyte received these two applications before application of one or more experimental compound. At the end of the experiment the control application was reapplied (Papke and Stokes, 2010). If the oocyte response did not recover at least 75 % of the previous control recording, they were discarded, and all data were excluded (Papke et al., 2010). Indeed, some compounds can induce the residual inhibition or prolonged desensitization of nAChRs (Papke and Porter Papke, 2002).

The peak current (µA) and the net charge (µA.s) for each recording were collected at 50 KHz and filtered at 5 Hz (Brunzell et al., 2014; Papke and Lindstrom, 2020). For the determination of the net charge, data were collected between 2 s and 90 s of continuous recording (Papke

et al., 2004; Papke and Porter Papke, 2002). Analysis of the net charge is very important for human $\alpha 7$ receptors as it has been shown that for concentrations of ACh $\geq 60 \mu\text{M}$, the current amplitudes occurred before the full application of the agonist (Papke and Thinschmidt, 1998). The same parameters were applied for $\alpha 7\text{WT}$ and mutated receptors. All recordings were performed at room temperature ($21 \pm 2 \text{ }^\circ\text{C}$).

2.6. Compounds

Neonicotinoids (acetamiprid, (ACE), clothianidin (CLT), and IMI), FLU, ACh, and Nic were purchased from Sigma Aldrich (St Quentin, France) and SFX was obtained from Emanime (Latvia). Neonicotinoids, FLU and SFX stock solutions were prepared in dimethyl sulfoxide (DMSO) at 1 M, and diluted with SOS to prepare the working solutions. Tested solutions did not exceed 0.1 % DMSO, to ensure the solvent didn't affect the nAChR responses (Andersen et al., 2016; Ihara et al., 2020). A stock solution of 1 M ACh and nicotine (Nic) was prepared in water and diluted in SOS. Neonicotinoids, FLU, and SFX tested concentrations were used based on the previous set of *in vitro* studies using neuronal nAChRs (Cartereau et al., 2018). All test solutions were prepared just before the experiment.

2.7. Molecular modeling studies

2.7.1. Ligand preparation

The eight compounds (ACh, Nic, IMI, CLT, TMX, ACE, SFX and FLU) used throughout the study (Fig. 1b) were constructed from their SMILES string formulas using RD-Kit's *Embed Molecule* function with default parameters (G. Landrum et al., rdkit/rdkit: 2021032(q12021) release (2021)). These structures were then geometry optimized using an OPLS-3e force field implemented in the LigPrep utility included in the 2021-1 Schrödinger suite (Schrödinger release (2021-1) Prime, Schrödinger, LLC, New York, NY). For each processed input, different isomers and tautomers were generated and protonation states were computed at pH 7.4 using the Epikv5.5 utility (Greenwood et al., 2010; Shelley et al., 2007) (Schrödinger release (2021-1) Epik, Schrödinger, LLC, New York, NY). For neonicotinoids (IMI, CLT, TMX, ACE), the *E* isomers were retained as they were the only ones to show activity against nAChRs. For nicotine (Nic), only the (*S*)-(-) isomer was kept as it is the most naturally occurring (Barlow et al., 1986). In the case of SFX, only RS and SS were taken into account, as they were the only diastereoisomers that were cocrystallised on the binding site of a double-mutated *Ls*-AChBP (Montgomery et al., 2022).

2.7.2. X-ray crystal structure

The X-ray crystal structure of the human homomeric $\alpha 7$ nAChR in complex with epibatidine and PNU-120596 (a positive allosteric modulator) in the activated state was selected from RCSB PDB (access code:7K0X) for the present study (Noviello et al., 2021). This structure was retained as it is to our knowledge the only resolved structure of the extracellular domain of the human homomeric $\alpha 7$ nAChR. The protein structure was prepared using Maestro software from the 2021-1 Schrödinger suite (Noviello et al., 2021). The protonation states were computed using ProPka 3 and the corresponding bond orders, formal charges, and hydrogens were added to the system (Olsson et al., 2011). Tautomeric states for HIS residues and rotamers for ASN and GLN residues were inspected and adjusted manually to maximize the hydrogen bonding network. Co-crystallized water molecules were removed from the structure, except for the one present in the binding site, and hence involved in the binding mode of epibatidine. An energy minimization using OPLS-3e was performed on a constrained structure in which the hydrogens atoms were allowed to move freely while the heavy atoms had an RMSD threshold of 0.30 Å. The same protocol was used for the mutated systems, after introducing the mutation using the *Mutate Residues* utility from Maestro software. It is worth noting that for all the systems considered, the 3D model we designed corresponds to the

extracellular domain (ECD) of the human $\alpha 7$ nAChR, that contains the ligand binding domain (LBD).

2.7.3. Induced fit docking (IFD)

The molecular flexible docking was conducted using Glide software v9.0 from the 2021-2 Schrödinger suite, using the standard IFD protocol (Schrödinger release (2021-1) Induced Fit Docking protocol; Glide, Schrödinger, LLC, New York, NY; 203 Prime, Schrödinger, LLC, New York, NY). IFD allows flexibility for both the ligand and receptor and was performed using the Glide docking XP mode scoring function (GlideScore). As the $\alpha 7$ is a homomeric pentamer, it possesses five identical binding sites found between each two consecutive subunits. Thus, we selected the binding site between chain E and chain A as our target for this study. The receptor box was centered on the epibatidine bound in the binding site. All residues in a 12 Å sphere radius from ligand poses were selected and refined with Prime refinement and OPLS-3e force field and implemented in Prime code v6.5 (Jacobson et al., 2002; Jacobson et al., 2004) (Schrödinger release (2021-1) prime, Schrödinger, LLC, New York, NY). The number of Prime refinement iterations was set to 3. The IFD docking score is a crude estimation of the binding free energy ($\Delta G_{\text{binding}}$) for the ligand and some caution is required when interpreting this kind of data. It is expressed in kcal/mol and defined using the following equation:

$$\text{IFD Score} = 1.0 * \text{GlideScore} + 0.05 * \text{Prime_Energy}$$

where *GlideScore* is the score from the Glide scoring function and *Prime_Energy* is the total energy of the system. The best complexes were chosen using *IFD score*, *GlideScore* and through visual inspection.

2.8. Data and statistical analysis

Responses to compound applications were normalized using the corresponding ACh current, compensating for the varying levels of channel expression among the oocytes (Papke et al., 2004). For concentration-response relation, data derived from net charge analyses were plotted using GraphPad Prism 10.9 (Boston, MA, USA). The dose-response curves were derived from the fitted curve following the equation:

$$Y = I_{\text{min}} + \frac{(I_{\text{max}} - I_{\text{min}})}{1 + 10^{\log(\text{EC}_{50} - [X])H}}$$

where *Y* is the normalized response, *I*_{max} and *I*_{min} are the top and bottom 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 (Cartereau et al., 2018; Shimomura et al., 2002). The data were derived from net charge analyses, and were analyzed using R (v4.1.1). Statistical analyses were performed with the software GraphPad Prism 10 (Boston, MA, USA). Mann-Whitney test was applied to all results. *p* < 0.05 was the accepted minimum level of significance. The statistical test used for each figure is specified in the supplementary Information.

3. Results

3.1. Mode of action of neonicotinoids and latest derivatives FLU and SFX on human $\alpha 7$ nicotinic receptor wild-type

We first determined the dose-response curves of all insecticides and reference compounds on human neuronal $\alpha 7$ homomeric nAChRs wild type ($\alpha 7\text{WT}$) expressed in *Xenopus* oocytes. In accordance with previous studies from Papke et al. on human $\alpha 7$ nAChRs (Papke and Porter Papke, 2002; Papke and Thinschmidt, 1998), we considered the net charge as a criterion for comparing all experimental conditions (Fig. 2). The dose-response curves were fitted with the Hill equation (Fig. 2a), and the data are shown in Table 2. The *EC*₅₀ values (in μM) were $117.22 \pm$

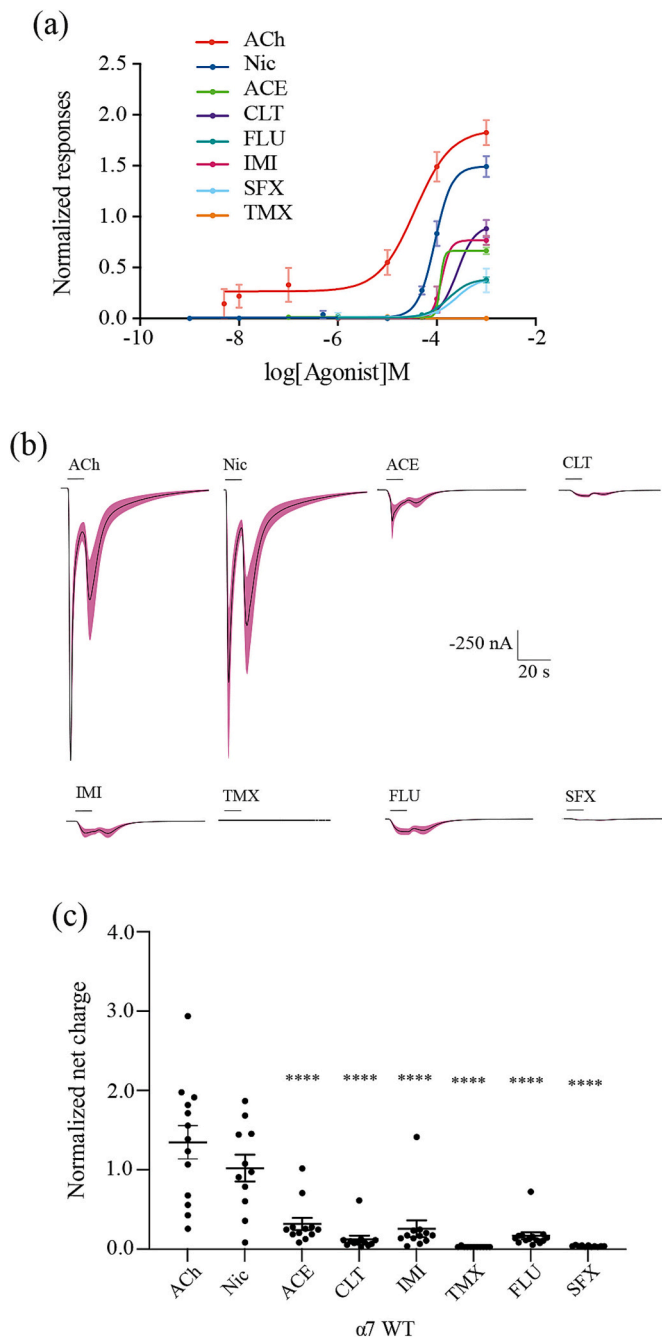


Fig. 2. Agonist actions of acetylcholine, nicotine, neonicotinoid insecticides and their derivatives on human $\alpha 7$ WT receptors. (a) Concentration response curves of acetylcholine (ACh), nicotine (Nic), neonicotinoids: acetamiprid (ACE), clothianidin (CLT), imidacloprid (IMI), thiamethoxam (TMX), and their derivatives: sulfoxaflor (SFX) and flupyradifurone (FLU), obtained on $\alpha 7$ WT nAChRs expressed in *X. laevis* oocytes. Each point plotted represents the mean \pm S.E.M of four to fifteen experiments ($n = 3-7$ batches). For each compound, the net charge was normalized to net charge obtained after application of ACh 60 μ M and data were fitted to the Hill equation. (b) Representative raw data traces for each compound used at 100 μ M. (c) Histograms representing the normalized net charge induced by 12 s application of 100 μ M of each compound. Each bar indicates the mean \pm S.E.M of eleven to thirteen experiments ($n = 5$ batches). Mann-Whitney test was used to statistically compare each normalized net charge to ACh 100 μ M. Significant differences from ACh are indicated with asterisks (**** indicates $p < 0.0001$).

Table 2
EC₅₀ and I_{max} values for each compound

	EC ₅₀ (μ M)	nH	I _{max}
ACh	36.22 \pm 13.60	1.18	1.86 \pm 0.16
Nic	91.62 \pm 7.98	2.54	1.50 \pm 0.12
ACE	117.22 \pm 116.29	12.31	0.67 \pm 0.02
CLT	258.82 \pm 190.93	2.44	0.92 \pm 0.25
IMI	125.60 \pm 55.96	4.89	0.77 \pm 0.02
TMX	0	ND	ND
FLU	172.98 \pm 69.49	2.00	0.39 \pm 0.04
SFX	252.35 \pm 178.57	1.93	0.40 \pm 0.14

Characterization of the net charge induced by the compounds applied on $\alpha 7$ WT receptors. I_{max}, EC₅₀, and nH values are reported for acetylcholine (ACh), nicotine (Nic), acetamiprid (ACE), clothianidin (CLT), imidacloprid (IMI) thiamethoxam (TMX), flupyradifurone (FLU), and sulfoxaflor (SFX). Data are the mean \pm S.E.M of eleven to thirteen experiments, and correspond to the current induced by the tested compound normalized by the net charge induced by 60 μ M ACh. ND: not determined

116.29 (ACE), 258.82 \pm 190.93 (CLT) and 125.60 \pm 55.96 (IMI), respectively. In comparison, the EC₅₀ values obtained for ACh and Nic were 36.22 \pm 13.6 and 91.62 \pm 7.98 μ M, respectively (Table 2). In order to compare our data with previous studies (Hirata et al., 2015; Ihara et al., 2014; Toshima et al., 2009), all compounds were applied at 100 μ M (Fig. 2b, c). Nic (net charge = 0.99 \pm 0.17, $n = 11$) was able to fully activate $\alpha 7$ WT receptors, whereas IMI, CLT and ACE were weak activator. In particular, ACE (net charge = 0.29 \pm 0.08, $n = 12$) and IMI (net charge = 0.23 \pm 0.11, $n = 12$) appeared to be more active than CLT (net charge = 0.10 \pm 0.05, $n = 12$). Thus, CLT, IMI and ACE acted as partial agonists on the human $\alpha 7$ WT nAChR. Interestingly, TMX, which is metabolized into CLT in insects and plants (Benzidane et al., 2010; Nauen et al., 2003), had no agonist effect even at higher concentrations (Fig. 2, Table 2). The determination of the net charges using the latest derivatives demonstrates that 100 μ M FLU was able to activate $\alpha 7$ WT receptors (net charge = 0.14 \pm 0.04, $n = 14$), whereas SFX was not (Fig. 2b, c). However, SFX and FLU appeared to have agonist effects on $\alpha 7$ WT receptors at higher concentrations, as indicated by their EC₅₀ values of 252.35 \pm 178.57 μ M and 172.98 \pm 69.49 μ M, respectively (Table 2). These results demonstrated that FLU and SFX acted as partial agonists of human $\alpha 7$ receptors, and were less efficient at activating the receptors than neonicotinoids (ACE, IMI, CLT).

3.2. Modification of neonicotinoid, flupyradifurone and sulfoxaflor activity on $\alpha 7$ nAChRs containing E211N, E211P and Q79K mutations

We evaluated the activity of each compound on mutated $\alpha 7$ receptors, including E219P mutation (corresponding to E211P) which was previously studied in the mammalian $\alpha 4\beta 2$ nAChRs (Shimomura et al., 2004; Toshima et al., 2009). As for the $\alpha 7$ mutations, TMX was not able to induce any currents (Fig. 3, Fig. 4, and Fig. 5). Results obtained for the $\alpha 7$ E211N receptor are presented in Fig. 3. We found that Nic appeared to be a full agonist (net charge = 1.13 \pm 0.15, $n = 10$) in contrast to ACh, whereas all tested insecticides (except TMX) were partial agonists. Indeed, we observed a significant decrease in the net charges induced after application of the 6 insecticides compared to ACh, with the net charge ranging from 0.54 \pm 0.19 for CLT ($n = 9$), 0.59 \pm 0.16 for IMI ($n = 9$), 0.37 \pm 0.13 for FLU ($n = 10$), 0.28 \pm 0.05 for ACE ($n = 9$) to 0.21 \pm 0.06 for SFX ($n = 10$) (Fig. 3). The results obtained for the $\alpha 7$ E211P receptor confirmed that Nic was a full agonist, and no significant difference was observed with the net charges for ACh, (0.96 \pm 0.12, $n = 13$) and Nic (0.89 \pm 0.06, $n = 9$, Fig. 4). Neonicotinoid insecticides, CLT (net charge = 0.85 \pm 0.07, $n = 13$), IMI (net charge = 0.80 \pm 0.05, $n = 13$), ACE (net charge = 0.61 \pm 0.04, $n = 13$) and the butanilide, SFX (net charge = 0.56 \pm 0.06, $n = 13$) were also able to activate the $\alpha 7$ E211P receptor as partial agonists. A very low activation was found with FLU (net charge = 0.18 \pm 0.01, $n = 13$), which can be considered as

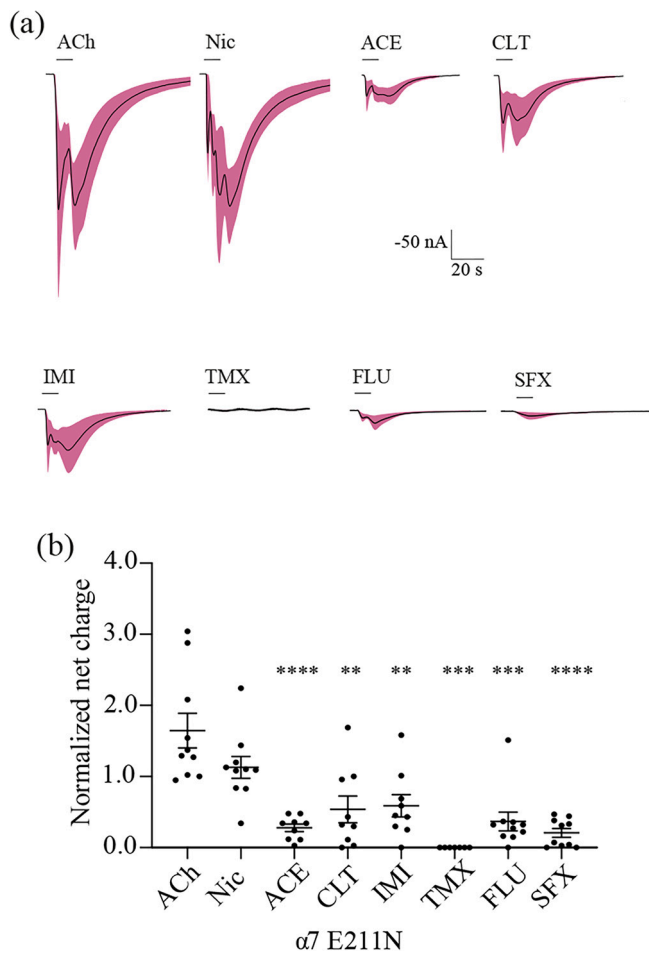


Fig. 3. Agonist actions of acetylcholine, nicotine, neonicotinoid insecticides and their derivatives on human $\alpha 7E211N$ receptors. (a) Representative raw data traces for each compound used at 100 μM . (b) Histograms representing the normalized net charge induced by 12 s application of 100 μM of each compound. Each bar indicates the mean \pm S.E.M of eleven to thirteen experiments ($n = 6$ batches). Mann-Whitney test was used to statistically compare each normalized net charge to ACh 100 μM . Significant differences from ACh are indicated with asterisks (* indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$).

a weak activator of the $\alpha 7E211P$ receptor (Fig. 4). The $\alpha 7Q79K$ receptor demonstrated more variations in activity for the tested compounds (Fig. 5a and b). Nic remained a full agonist (net charge = 2.27 ± 0.38 , $n = 15$). The neonicotinoids CLT (net charge = 1.59 ± 0.32 , $n = 10$), and IMI (net charge = 1.52 ± 0.30 , $n = 10$), as well as the butenolide FLU (net charge = 1.48 ± 0.30 , $n = 11$) also became full agonists of the $\alpha 7Q79K$ receptor. However, the significant decrease in the net charge induced by ACE (net charge = 0.51 ± 0.05 , $n = 10$) and SFX (net charge = 0.04 ± 0.17 , $n = 9$) compared with the ACh effect demonstrated that they were able to partially activate the receptors (Fig. 5a and b). Once the effect of each compound on the mutated $\alpha 7$ receptors had been compared with that of ACh, we looked to see whether the mutations had a different effect compared to that of the $\alpha 7WT$ (Table 3). Net charges were evaluated after application of ACh at 100 μM on mutated $\alpha 7$ nAChRs. For example, the net charges recorded for ACh demonstrated that there was no significant difference between $\alpha 7E211N$ receptors (net charge = 1.64 ± 0.24 , $n = 10$), $\alpha 7E211P$ receptors (net charge = 1.09 ± 0.06 , $n = 13$) or $\alpha 7Q79K$ receptors (net charge = 1.70 ± 0.15 , $n = 11$) compared to $\alpha 7WT$ receptors (net charge = 1.30 ± 0.20 , $n = 13$). These results suggested that mutated residues had no effect on the currents induced by ACh. Similarly, the responses to Nic were not significantly

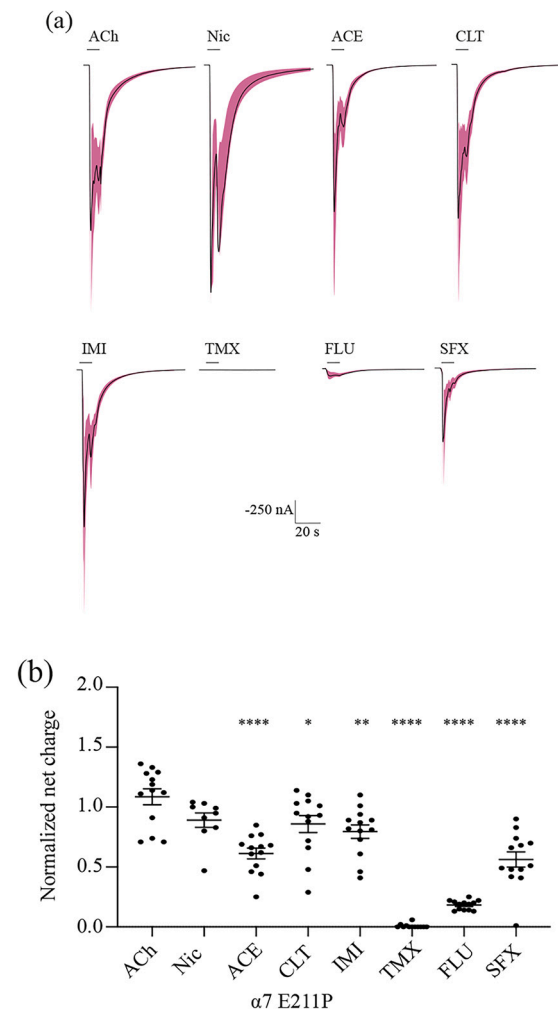


Fig. 4. Agonist actions of acetylcholine, nicotine, neonicotinoid insecticides and their derivatives on human $\alpha 7E211P$ receptors. (a) Representative raw data traces for each compound used at 100 μM . (b) Histograms representing the normalized net charge induced by 12 s application of 100 μM of each compound. Each bar indicates the mean \pm S.E.M of eleven to thirteen experiments ($n = 5$ batches). Mann-Whitney test was used to statistically compare each normalized net charge to ACh 100 μM . Significant differences from ACh are indicated with asterisks (* indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$).

different between $\alpha 7WT$, $\alpha 7E211N$ and $\alpha 7E211P$ receptors, whereas a significant increase of the response was registered with $\alpha 7Q79K$ receptors (Table 3). Interestingly, the agonist actions of CLT, IMI and FLU were higher in all mutated receptors compared to $\alpha 7WT$. The most important increases were obtained for $\alpha 7Q79K$ receptors, whereas SFX appeared more active with $\alpha 7E211N$ and $\alpha 7E211P$ receptors (Table 3).

3.3. Co-application of ACh with FLU and SFX

In a previous study, we demonstrated that application of ACh in the presence of neonicotinoids decreased ACh-evoked current amplitudes (Cartereau et al., 2018). We evaluated the effect of a co-application of FLU and/or SFX with ACh (See supplementary data, fig. S2, S3 and S4). The comparison of the net charges showed that co-application of ACh with FLU had no significant effect on $\alpha 7WT$ receptors (0.99 ± 0.05 and 1.06 ± 0.19 , for ACh and ACh + FLU respectively. $p > 0.05$, $n = 7$, supplementary data fig. S2a). A significant decrease in the net charge was observed with $\alpha 7E211N$ receptors (1.05 ± 0.03 and 0.75 ± 0.06 , for ACh and ACh + FLU respectively. $p < 0.05$, $n = 5$, supplementary data

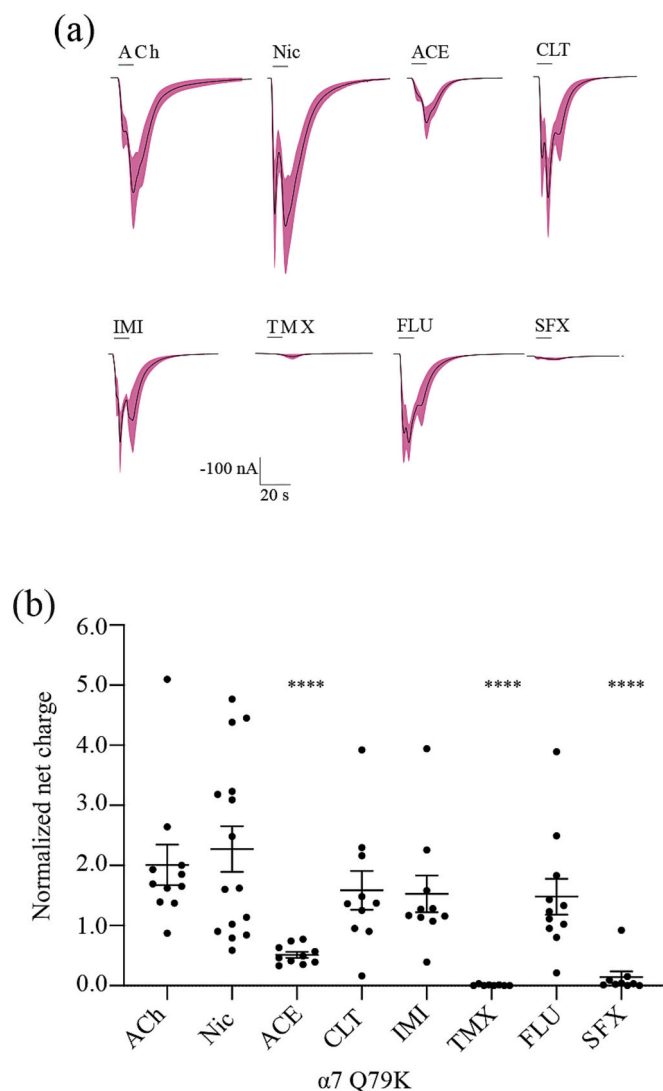


Fig. 5. Agonist actions of acetylcholine, nicotine, neonicotinoid insecticides and their derivatives on human $\alpha 7$ Q79K receptors. (a) Representative raw data traces for each compound used at 100 μ M. (b) Histograms representing the normalized net charge induced by 12 s application of 100 μ M of each compound. Each bar indicates the mean \pm S.E.M of eleven to thirteen experiments ($n = 6$ batches). Mann-Whitney test was used to statistically compare each normalized net charge to ACh 100 μ M. Significant differences from ACh are indicated with asterisks (* indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$).

fig. S2b), whereas no change was found with $\alpha 7$ E211P receptors (1.01 ± 0.05 and 1.02 ± 0.18 , for ACh and ACh + FLU respectively, $p > 0.05$, $n = 5$, supplementary data fig. S2c). But, a significant increase was found with $\alpha 7$ Q79K receptors (from 1.06 ± 0.05 to 1.50 ± 0.19 , for ACh and ACh + FLU respectively, $p < 0.05$, $n = 9$, supplementary data fig. S2d). The responses elicited by co-applying 60 μ M ACh with 1 μ M SFX demonstrated no significant change in ACh-evoked net charges for $\alpha 7$ WT, $\alpha 7$ E211N, $\alpha 7$ E211P, or $\alpha 7$ Q79K receptors (supplementary data fig. S3). Similarly, when SFX (10 μ M) and FLU (10 μ M) were co-applied together with ACh (60 μ M), we did not observe any modification in the ACh-induced activity in any receptors (supplementary data fig. S4).

3.4. Neonicotinoid, sulfoxaflor and flupyradifurone- $\alpha 7$ nAChR binding site interactions

Table 4 reports the docking results (scores, distances and valence

Table 3
Normalized net charges

Compounds	Receptors	Normalized net charge	n
ACh	$\alpha 7$ WT	1.30 ± 0.20	13
	$\alpha 7$ E211N	1.64 ± 0.24	10
	$\alpha 7$ E211P	1.09 ± 0.06	13
	$\alpha 7$ Q79K	1.70 ± 0.15	11
	$\alpha 7$ WT	0.99 ± 0.17	11
	$\alpha 7$ E211N	1.13 ± 0.15	10
Nic	$\alpha 7$ E211P	0.89 ± 0.06	9
	$\alpha 7$ Q79K	$2.27 \pm 0.38^*$	15
	$\alpha 7$ WT	0.29 ± 0.08	12
ACE	$\alpha 7$ E211N	0.28 ± 0.05	9
	$\alpha 7$ E211P	$0.61 \pm 0.04^{**}$	13
	$\alpha 7$ Q79K	$0.51 \pm 0.05^{**}$	10
	$\alpha 7$ WT	0.10 ± 0.05	12
CLT	$\alpha 7$ E211N	$0.54 \pm 0.19^*$	9
	$\alpha 7$ E211P	$0.85 \pm 0.07^{****}$	13
	$\alpha 7$ Q79K	$1.59 \pm 0.32^{****}$	10
	$\alpha 7$ WT	0.23 ± 0.11	12
	$\alpha 7$ E211N	$0.59 \pm 0.16^*$	9
IMI	$\alpha 7$ E211P	$0.80 \pm 0.05^{***}$	13
	$\alpha 7$ Q79K	$1.52 \pm 0.30^{***}$	10
	$\alpha 7$ WT	0.00 ± 0.00	11
TMX	$\alpha 7$ E211N	0.00 ± 0.00	7
	$\alpha 7$ E211P	0.00 ± 0.00	12
	$\alpha 7$ Q79K	0.00 ± 0.00	8
	$\alpha 7$ WT	0.14 ± 0.04	14
FLU	$\alpha 7$ E211N	$0.37 \pm 0.13^{**}$	10
	$\alpha 7$ E211P	$0.18 \pm 0.01^{**}$	13
	$\alpha 7$ Q79K	$1.48 \pm 0.30^{****}$	11
SFX	$\alpha 7$ WT	0.00 ± 0.00	11
	$\alpha 7$ E211N	$0.21 \pm 0.06^{**}$	10
	$\alpha 7$ E211P	$0.56 \pm 0.06^{****}$	13
	$\alpha 7$ Q79K	0.04 ± 0.17	9

Net charge recorded after application of acetylcholine (ACh), nicotine (Nic), acetamiprid (ACE), clothianidin (CLT), imidacloprid (IMI) thiamethoxam (TMX), flupyradifurone (FLU) and sulfoxaflor (SFX) at 100 μ M. For each receptor the net charges were normalized with ACh 60 μ M. Mann-Whitney test was used to statistically compare normalized net charge obtained for each receptor with the normalized net charge from the $\alpha 7$ WT. Significant differences are indicated with asterisks (* indicates $p < 0.05$, ** indicates $p < 0.01$). The n values for each condition represents the number of tested oocytes (3–7 different batches).

angles of key ligand...receptor contacts) obtained for the interactions of the eight compounds selected with $\alpha 7$ wild type nAChR using the IFD molecular docking protocol described in the methodology section, and Figs. 6 and 7 show the predicted binding modes. The results for the three mutants are reported in the supplementary data (Tables S3-S5 report the corresponding results, whereas figs. S5-S7 show the corresponding binding site interactions). The data have been ranked from top to the bottom in the table according to the ligand IFD score values, which appear for us to be the most relevant values for such comparisons. The acronyms of the ligands used in Table 4 correspond to the ones defined above. The validity of the docking protocol used was confirmed by the conformity between our results and the experimental data available. Thus, the orientation and interactions predicted by the docking for our two reference ligands, ACh and Nic (Fig. 6a and b) are similar to those observed experimentally (Walsh Jr. et al., 2018). More precisely, the water-mediated hydrogen-bond (H-bond) interaction between the carbonyl oxygen atom of ACh, or the pyridinic Nsp² nitrogen of Nic with the main chain NH of L141, is predicted by the docking. For the other fragment of the ligands, the positively charged ammonium groups of ACh and Nic are surrounded by aromatic residues and are involved in cation... π interactions with the six membered ring of the key W171 and the aromatic ring of Y210. The ligands are also in the close vicinity of the disulfide bonded cysteine residues (C212 and C213). All these observations are in accordance with those reported in experimental investigations (x-ray crystallography, electron microscopy) (Gharpure et al., 2020). It is worth noting that the scores predicted by the docking

Table 4

Docking scores and interactions of the neonicotinoids and derivatives investigated with wild type human $\alpha 7$ nAChR. The distance and valence angles of relevant interactions are also given.

Ligand	IFDS ^a	DS ^b	Interaction ^c	d(D...A) ^d	(DH...A) ^e Angle (π ... π)
ACh	-2134.0	-11.8	NH ^{MC} _{(LEU141_B)...} O _{(H2O)...}	2.83	176.3171.9
			O _(OC)	2.73	
			Ce ^{SC} _{(TRP171_A)...} N _(NMe3+)	4.45	
RS-SFX	-2133.5	-7.9	Ce ^{SC} _{(TYR210_A)...} N _(NMe3+)	4.58	169.8169.2
			NH ^{MC} _{(LEU141_B)...} O _{(H2O)...}	2.98	
			N _(Pyr)	3.50	
SS-SFX	-2133.4	-8.4	NH ^{MC} _{(LEU141_B)...} O _{(H2O)...}	2.83	176.7162.4
			N _(Pyr)	3.18	
			OH ^{SC} _{(SER172_A)...} F _(CF3)	2.95	
SR-Nic	-2133.1	-13.1	O ^{MC} _{(TRP171_A)...}	3.41	129.3
			NH _(NHMe+)		
			Ce _{(TRP171_A)...} N _(NHMe+)	3.91	
FLU	-2131.4	-9.0	NH ^{MC} _{(LEU141_B)...} O _{(H2O)...}	2.90	164.1
			N _(Pyr)		
			NH ^{SC} _{(ARG208_A)...} F _(CF2)	3.16	
CLT	-2128.8	-6.0	NH ^{MC} _{(LEU141_B)...} O _{(H2O)...}	2.82	176.1174.7
			N _(Pyr)	2.90	
			Ce ^{SC} _{(TYR217_A)...} (butenolide)	4.05	
ACE	-2128.5	-5.1	OH ^{SC} _{(TYR115_A)...}	2.80	175.6
			NH _(Nitroguanidine)		
			O ^{MC} _{(TRP171_A)...}	3.15	
IMI	-2127.6	-5.8	NH _(Nitroguanidine)		141.8
			NH ^{MC} _{(LEU141_B)...} O _{(H2O)...}	2.82	
			N _(Thiazolyl)	3.13	
TMX	-2121.1	-5.4	Ce _{(TRP171_A)...} (Thiazolyl)	4.79	88.4
			OH ^{SC} _{(SER58_B)...} N _(CN)	3.34	
			Ce _{(TRP171_A)...} (Pyr)	5.24	
ACE	-2128.5	-5.1	NH ^{MC} _{(LEU141_B)...} O _{(H2O)...}	2.81	164.4170.2
			N _(Pyr)	2.82	
			O ^{SC} _{(GLN79_B)...}	2.85	
IMI	-2127.6	-5.8	NH _(Nitroimidazolidin)		173.5
			NH ^{MC} _{(LEU141_B)...} O _{(H2O)...}	2.79	
			N _(Pyr)	3.30	
TMX	-2121.1	-5.4	Ce _{(TRP171_A)...} (Pyr)	4.83	80.1

^a Induced fit score (kcal/mol)

^b Docking score (kcal/mol) (see the methodology section for more information).

^c Groups of the ligand and of the proteins in interaction (SC: Side chain; MC: main chain; Ce: centroid of the ring (for Trp, 6-membered ring centroid).

^d Distance of interaction (in Å).

^e Interaction valence angle (in °).

for these compounds are among the best, as expected. For the three nitro-neonicotinoids (CLT, IMI, TMX), Figs. 6c, d and e, and Table 4 show that the binding features are very similar. Thus, the water-mediated interaction with the main chain of L141 is quasi systematically conserved, whatever the chemical nature of the heterocyclic fragment on this side of the binding site (chloropyridyl in IMI and chlorothiazolyl in CLT). The only exception concerns TMX, which, according to the docking does not make any polar contact with the receptor, in line with its worst score. For IMI and CLT, the push-pull fragment is stabilized in the binding pocket by H-bond interactions between NH groups of the guanidine moieties and $\alpha 7$ nAChR residues. Interestingly, in the case of CLT, these contacts involve the main chain carbonyl of W171, one of the key residues of the human $\alpha 7$ nAChR, with the other NH behaving as a H-bond donor for the side chain OH of Y115. This is not the case for IMI, for which the NH of the cyclic guanidine fragment makes a H-bond with the side chain carbonyl of Q79. For ACE (Fig. 6f), which is a cyano neonicotinoid, and does not present any NH groups, the terminal nitrogen of the nitrile group accepts a H-bond from the main chain hydroxyl of S58. For SFX, it is first worth noting that the water-mediated H-bond interactions with L141 stay within the two

predicted binding modes (Fig. 7a and b), in line with those observed in the crystallographic structure (Montgomery et al., 2022). In our models, the CF₃ group of the two isomers form van der Waals contacts with the OH group of the lateral S172 chain. Fig. 7a and b show that for the SS diastereoisomer, the CH of the epimerizable junction carbon is close to the indole ring of W171, which confirms the importance of this residue in SFX...AChBP crystallographic structures (Montgomery et al., 2022). It is also interesting to note that as in the experimental structure, our docking models show apolar contacts between the SFX pyridine fragment and loop E residues, and more specifically with the L141 isopropyl group. Lastly, for FLU (Fig. 7c), the water-mediated H-bond with the main chain of L141 had also been predicted, as observed in the only FLU...AChBP experimental structure available (Montgomery et al., 2022). In our predicted binding mode, the carbonyl group of the butenolide fragment does not appear to be involved in any H-bond interactions, whereas one of the fluorine atoms of the CF₂ groups makes van der Waals contacts with the positively charged lateral chain of R208. Another notable interaction was the π stacking between the butenolide ring and the phenol ring of Y217, one of the aromatic residues of the human $\alpha 7$ nAChR involved in agonist binding. The docking scores extend over a significant range of about 6 kcal/mol. The most stabilized structures correspond to ACh, SFX, and Nic. TMX was found to be the weakest stabilized structure. In between, neonicotinoids (IMI, CLT, ACE) and FLU, had a 3 kcal/mol score range. With respect to the mutants, no significant effects on ligand binding were observed, the docking scores being very similar to those of the wild type.

4. Discussion

In the present study, we used the net charge as critical criterion for evaluating the effect of neonicotinoids, and recently introduced compounds, on $\alpha 7$ WT and mutated receptors. It is worth noting that for WT and mutated receptors, we observed a specific double peak shape for the net charges, which highlighted a rapid desensitization of the receptors. This effect is very particular and is consistent with previous studies on human $\alpha 7$ nAChRs (Papke and Porter Papke, 2002; Papke and Thinschmidt, 1998), and could lead to some discrepancy in the results when considering the maximum currents. To illustrate this, we reported the normalized currents obtained in our experiments in supplementary data table S1. In contrast to what was interpreted from net charge data, we observed no increase in either the Nic response on $\alpha 7$ Q79K and FLU on $\alpha 7$ E211P receptors. In addition, a significant increase in ACE activity was found on $\alpha 7$ E211N and SFX on $\alpha 7$ Q79K receptors. These results confirmed the importance of considering the net charge when evaluating the binding with $\alpha 7$ nAChRs.

Originally, the neonicotinoids, butenolide and sulfoximine were designed to be selective for insect neuronal nAChR subtypes (Nauen et al., 2015). We demonstrated that the tested neonicotinoids were partial agonists of the human $\alpha 7$ nAChRs compared to ACh, except for TMX which was not able to induce currents. ACE appears to be more active on $\alpha 7$ WT than CLT and IMI. The latest derivatives of neonicotinoids on the market, SFX and FLU, were also poor agonists of human $\alpha 7$ nAChRs. Our results are in accordance with previous studies demonstrating that neonicotinoids poorly activate vertebrate neuronal nAChRs (Cartereau et al., 2018; Tomizawa and Casida, 2005; Tomizawa et al., 2000). Their chemical structure does not seem to be a determining factor in discriminating between them since they act as partial agonists of human $\alpha 7$ nAChRs. Thus, their structural chemical changes cannot be used to understand their effect on human $\alpha 7$ nAChRs. For example, IMI and its desnitro metabolite (DN-IMI) differed greatly in binding site specificity, IMI was highly potent in insects whereas DN-IMI was much more active in mammals (Tomizawa and Casida, 1999). This observation could explain the lack of effect with TMX (Cartereau et al., 2018; Loser et al., 2021). Basically, unlike other insecticides, it only acts on insect nAChRs, in contrast to CLT.

Previous studies have shown the importance of basic residues in the

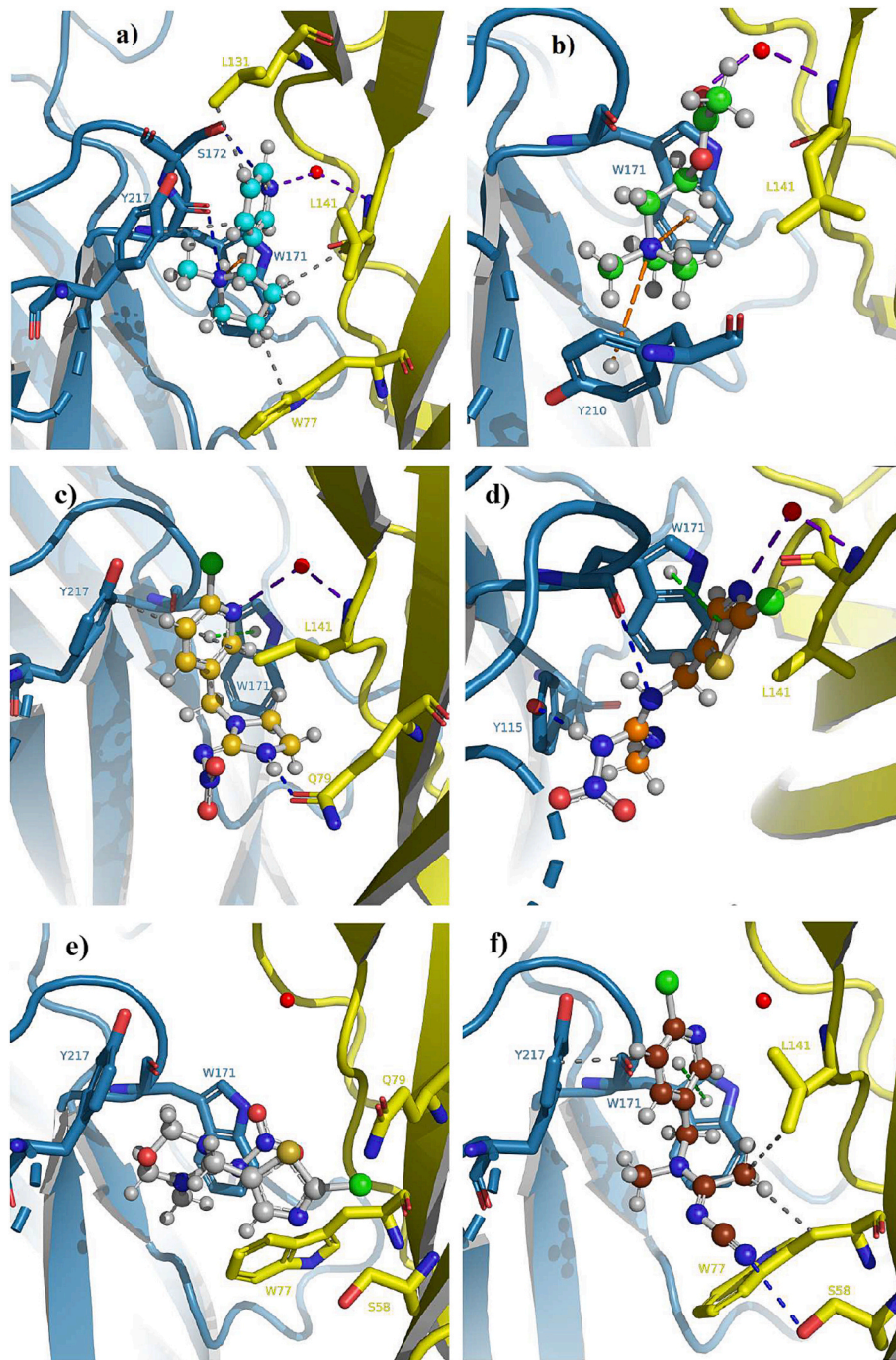


Fig. 6. Docking predicted binding modes of ACh (a), Nic (b), the nitro-neonicotinoids IMI (c) CLT (d), and TMX (e), and one cyano-neonicotinoid ACE (f), with wild type human $\alpha 7$ nAChR. The main and complementary components of the binding site are colored in blue and yellow, respectively. The ligands are shown in balls and sticks and the various kinds of interactions are indicated with dashed lines: hydrogen-bonds in blue, π stacking in green, π cation in orange and water bridges in purple. The main $\alpha 7$ nAChR residues are identified with their one letter code and numbering. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

loop D as well as non-acidic residues in the loop C for neonicotinoid selectivity (Shimomura et al., 2002; Shimomura et al., 2006; Shimomura et al., 2004). Considering the net charges, it appears that the responses of all tested insecticides, including neonicotinoids (except TMX), FLU and SFX, were enhanced by the E211P mutation in loop C. Mutation E211N induced a lower increase in the net charge for CLT, IMI, FLU and SFX compared to E211P. In particular, the response to ACE was not modified in $\alpha 7$ E211N compared to $\alpha 7$ WT receptors. In other words, the presence of proline (non-polar amino acid) in the loop C, as well as of insect nAChR subunits, such as drosophila SAD (Dm $\alpha 2$) or Tobacco

budworm *Heliothis virescens* Hvir $\alpha 2$, induces a high increase in the activity of all tested insecticides. On the other hand, when an asparagine (neutral amino acid) is present at the same position, as is the case in human and chicken $\alpha 3$ nAChR subunits, the $\alpha 7$ receptors are less sensitive to insecticides. The $\alpha 7$ WT containing the glutamic acid (negatively charged amino acid) in loop C shows the lowest insecticide activity. We propose that at this position, mammalian residues induce a different affinity for insecticides. Our hypothesis is supported above all by the findings of Shimomura et al., who observed that the P242E mutation in loop C of the drosophila SAD subunit (Dm $\alpha 2$), decreased IMI currents

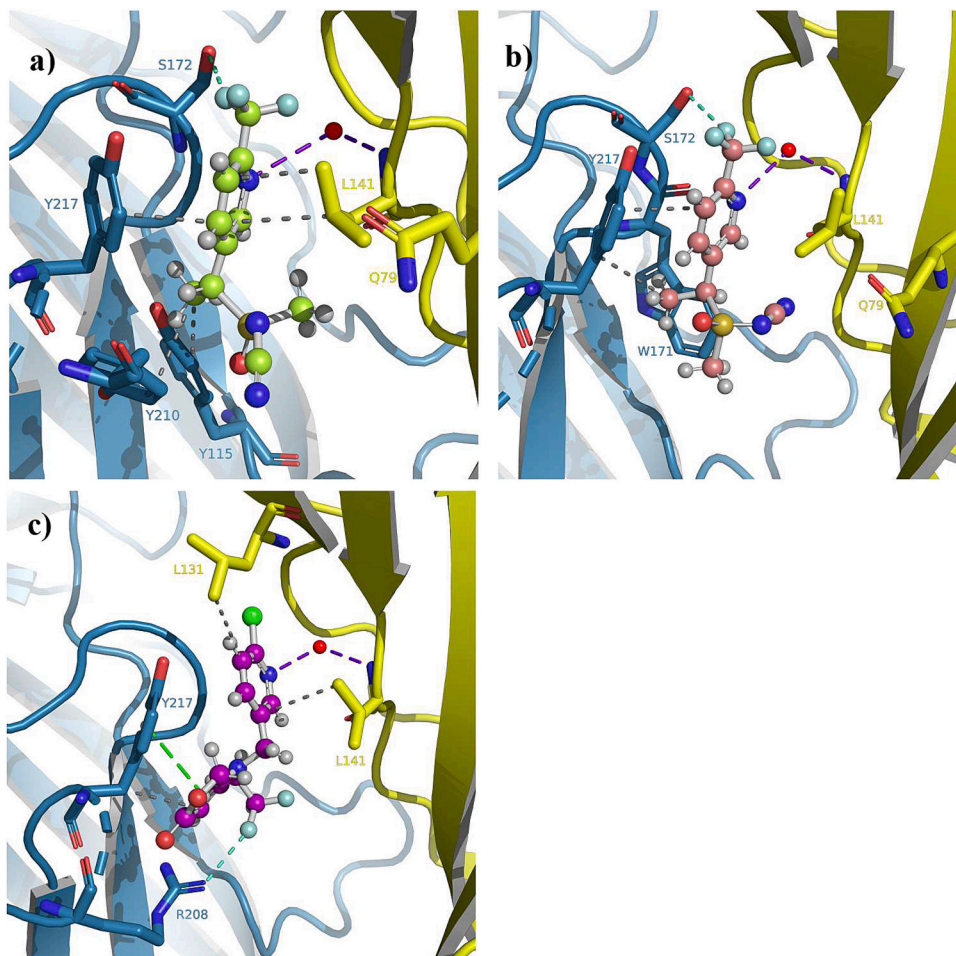


Fig. 7. Docking predicted binding modes of the two diastereoisomers of SFX taken into account in this work (RS (a) SS (b)) and docking predicted binding mode of FLU (c), with wild type human $\alpha 7$ nAChR. The main and complementary components of the binding site are colored in blue and yellow, respectively. The ligand is shown in balls and sticks and the various kinds of interactions are indicated with dashed lines: hydrogen-bonds in blue, π stacking in green, π cation in orange and water bridges in purple. The main $\alpha 7$ nAChR residues are identified with their one letter code and numbering. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compared to the wild-type receptor (Shimomura et al., 2004), confirming the functional role of the YXCC motif in loop C. Substitution of Q79K in the loop D of $\alpha 7$ nAChR greatly increased the net charge induced by ACE, CLT, IMI, and FLU, but did not significantly modify the activity of SFX compared to the $\alpha 7$ WT receptor. It was proposed that loop D basic residues were predominantly involved in determining the agonist affinity in terms of the EC_{50} of the insecticides, whereas loop C enhanced the coupling of IMI-binding and channel opening in term of the maximum response (Toshima et al., 2009). Indeed, in insects, the position 219 corresponding to the X residue in the YXCC motif of the $\alpha 4$ subunit is a proline, serine, or threonine. The E219P mutation in the YXCC motif of the loop C of the $\alpha 4$ subunit enhanced the I_{max} value of IMI for the $\alpha 4\beta 2$ nAChRs with a minimal influence on the EC_{50} value (Toshima et al., 2009). The involvement of loop C in the neonicotinoid mode of action was also demonstrated in insects using hybrid receptors composed of the insect α subunit and the mammalian $\beta 2$ subunit. Mutation of serine at position 221 to glutamate (S221E) in the Dm $\alpha 1$ subunit reduced the agonist action of IMI and thiacloprid on Dm $\alpha 1$ /chicken $\beta 2$ hybrid receptor (Hikida et al., 2018). More recently, in order to clarify the role of this serine in the selective interactions with neonicotinoids, Shimada et al., studied the effects of two mutations: S221A and S221Q on (Dm $\alpha 1$) $_3$ ($\beta 2$) $_2$ and (Dm $\alpha 1$) $_2$ ($\beta 2$) $_3$ hybrid receptors. They found that contrary to S221E, these mutations hardly lowered the affinity of the neonicotinoids (Shimada et al., 2020). On a hybrid receptor composed of *Drosophila* SAD (Dm $\alpha 2$) and chicken nicotinic $\beta 2$

subunit, the P242E mutation reduced the sensitivity of the receptor to IMI. On the contrary, mutation E219P on the vertebrate receptor $\alpha 4\beta 2$ increased the imidacloprid response (Shimomura et al., 2004). In line with all these findings, our results demonstrated that loop C and D are differently involved in the binding properties of the latest derivatives of neonicotinoids, SFX and FLU.

The mutations differently modified the activity of ACh and Nic, with the ACh-induced net charge being stable in mutated and wild-type receptors, whereas Nic-induced activity was specifically enhanced in the $\alpha 7$ Q79K receptor compared to the $\alpha 7$ WT. This leads to the hypothesis that the presence of a positively charged amino acid in the loop D could be important for Nic activity. It seems that the E211P mutation is associated with the highest increase in SFX response, whereas the Q79K mutation is associated with the highest increase in FLU activity. To further understand the specificity of receptor sensitivity to FLU and SFX, we evaluated the effect of co-application with ACh. In a previous study, we demonstrated that neonicotinoids (ACE and CLT) did not modify the ACh-evoked response in mammal $\alpha 7$ nAChRs when co-applied with ACh (Cartereau et al., 2018). We consistently failed to observe any modifications in the response of $\alpha 7$ WT. Interestingly, when observing the mutated receptors, we found the E211N mutation was related to a decrease in the ACh-evoked response when co-applied with FLU, whereas the Q79K mutation increased the ACh response potentiation with FLU. This suggests that the key amino acids in loops C and D are differently implicated in FLU's mode of action. In contrast, the co-

application of SFX did not influence the ACh response and its binding to the $\alpha 7$ receptor cancelled the previous observed effect of FLU co-application on $\alpha 7E211N$ and $\alpha 7Q79K$. Such antagonistic effect could be in line with binding sites interactions between FLU and SFX.

As discussed in the previous section, the docking results are validated from a structural point of view on the basis of the consistency observed between the predicted binding modes, and the corresponding experimental observations available from x-ray crystallography or cryo-electron microscopy structures for the human $\alpha 7$ nAChR (Noviello et al., 2021), which is directly relevant for this work or for AChBP (Montgomery et al., 2022). A direct comparison of these results with the electrophysiology measurements is of course not possible, but our results provide a qualitative structural description of the binding modes for a wide diversity (in terms of chemical structure) of agonists of the human $\alpha 7$ nAChR, including neonicotinoid insecticides and new representatives of nAChR competitive modulators (SFX, FLU). To the best of our knowledge, our results are the first report of molecular docking results for such a wide range of ligands, and for different mutants of the human $\alpha 7$ nAChR. Nevertheless, since our approach is limited to molecular docking, any analysis must be considered very cautiously, even though an induced fit protocol was used. Thus, it is worth noting that in agreement with the experimental trends, TMX appears to have the weakest binding affinity to the human $\alpha 7$ nAChR, both for the wild type and the mutants. The results obtained for SFX are not in line with the experimental electrophysiology trends, since this compound is predicted to be among the most stabilized ligands in the $\alpha 7$ nAChR binding site, which is true for the two stereoisomers considered. We will not comment further on the scores obtained due to the limitations of molecular docking. Indeed, the functional group diversity of the ligand set considered in this work requires an accurate description of the interaction, which is challenging for force fields (Vanommeslaeghe and MacKerell Jr., 2015). Furthermore, the static description inherent to molecular docking limits the potential of the method, specifically for reproducing large-scale motions that could occur upon mutation of the $\alpha 7$ nAChRs investigated. In that respect, the molecular docking results do not show any stabilization of agonist binding for the three mutants of $\alpha 7$ nAChRs considered. In our opinion, these trends simply reflect the limits of both the molecular docking, and the 3D models designed for this work. The weakness of molecular docking methodologies is that they don't take dynamic characteristics into account. Furthermore, the conformational changes induced by agonist binding/unbinding and how they are communicated to the ion channel might have a significant influence on the overall properties of the nAChR. Answering this question requires knowledge of the dynamics of the full nAChR protein and is limited by serious computing challenges. These problems are associated firstly to the large size of the systems (full receptor (Extracellular domain (ECD), transmembrane domain (TMD) and intracellular domain (ICD), and membrane bilayer) and the time scales involved, which explains why such studies remain scarce.

In conclusion, our study explores the potential human toxicity of these compounds, which is an area of great interest, and has already led to several polemics. Indeed, outside the agricultural context, some of these compounds are used as repellents to protect humans against arthropod borne-diseases (Hilton et al., 2024; Ngufor et al., 2017). It has now been established that neonicotinoid insecticides are able to contaminate soil (Pietrzak et al., 2020), food (Chen et al., 2014; Kapoor et al., 2013; Li et al., 2024), water (Luo et al., 2024; Morrissey et al., 2015), and consequently humans (Han et al., 2018; Wei et al., 2024), as well as useful insects like bees (Chen et al., 2024; Nicholson et al., 2024; Sanchez-Bayo and Goka, 2014). This toxicity seems to be applicable to new compounds acting on nAChRs such as FLU and SFX (Fang et al., 2024; Mesnage et al., 2018; Xu et al., 2022). Based on this potential toxicity, the European Union restricted the use of IMI, TMX and CLT, followed in 2019 by the US environmental Protection Agency, implementing restrictions on twelve neonicotinoid containing products. However, most countries still continue to use these compounds because

of their minimum low cost and arthropod resistance mechanisms. As neonicotinoids and associated compounds use will probably continue to increase, it remains fundamental to study their mode of action on human neuronal nAChRs. In general, it has been considered that because neonicotinoids are low activators of mammalian neuronal nAChRs, they have no potential effect for humans. One of the fundamental reasons for this is that we expected activation to be similar to that of insect neuronal nAChRs. We believe that the ability to activate human receptors is a potential criterion for cellular toxicity to neuronal nAChRs, which could in turn affect physiological processes in humans. Indeed, it was demonstrated that neonicotinoids have both analgesic and toxic effect in mammals (Tomizawa et al., 2001). We must also take into account the fact that we used a heterologous expression system (xenopus oocytes) to study the effect of these pesticides on the human $\alpha 7$ receptors. It cannot be ruled out that there could be a more pronounced, or at least different effect on human neurons. Nevertheless, this study of the three mutated receptors and their binding to neonicotinoids and more recently introduced insecticides has demonstrated that specific residues in loops D and C could help improve the binding affinities to insect neuronal receptors rather than vertebrate receptors. However, these experimental trends are not reproduced by the molecular docking results. In our opinion, this apparent contradiction is related to the inherent complexity of the nAChR system and the approximations of the molecular modeling approach mentioned above.

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Consent for publication

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CRedit authorship contribution statement

Alison Cartereau: Writing – original draft, Investigation, Formal analysis, Data curation. **Zakaria Bouchouireb:** Writing – original draft, Software, Methodology, Investigation. **Sara Kaaki:** Writing – original draft, Investigation, Formal analysis. **François Héricourt:** Investigation. **Emiliane Taillebois:** Writing – original draft, Formal analysis, Data curation. **Jean-Yves Le Questel:** Writing – original draft, Funding acquisition, Data curation, Conceptualization. **Steve H. Thany:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2024.117123>.

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