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Application of *In Silico* Methods to Support Experimental Data: Interactions of Antidepressants with Nicotinic Acetylcholine Receptors

Katarzyna M. Targowska-Duda[1,*], Hugo R. Arias[2] and Krzysztof Jozwiak[1]

[1]*Department of Chemistry, Laboratory of Medicinal Chemistry and Neuroengineering, Medical University of Lublin, 20-093 Lublin, Poland*

[2]*Department of Medical Education, California Northstate University College of Medicine, Elk Grove, CA 95757, USA*

Abstract: Over the last decades, several computational (*in silico*) methods have been developed and applied to test pharmacological hypotheses. An important hypothesis is that the therapeutic activity of many commonly used antidepressants may be partially mediated through the inhibition of different nicotinic acetylcholine receptors (nAChRs). This is based on pathologic conditions where the activity of the cholinergic system is exacerbated compared to the adrenergic system. Different *in silico* methods, including comparative/homology modeling, molecular docking, and molecular dynamics simulations, have been employed to study the interactions between several classes of antidepressants with distinct nAChR subtypes. More specifically, these methods were used to structurally characterize the antidepressant binding sites and to better understand their inhibitory mechanisms. This review focuses on computational methods that were found important in explaining and supporting several experimental results concerning the interaction of antidepressants with different nAChR subtypes. Among the studied antidepressants are norepinephrine selective reuptake inhibitors [e.g., (-)-reboxetine] as well as less selective antidepressants such as dopamine/norepinephrine reuptake inhibitors [e.g., (±)-bupropion and its derivatives], tricyclic antidepressants (e.g., imipramine), and (±)-mecamylamine and its enantiomers.

Keywords: Antidepressants, *in silico*, molecular docking, molecular modeling, molecular dynamics, nicotinic acetylcholine receptors, noncompetitive antagonists.

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are membrane embedded proteins member of the Cys-loop ligand-gated ion channel (LGIC) superfamily that includes the type 3 serotonin (5-HT), type A and C γ-aminobutyric acid, and glycine receptors (reviewed in [1-3]). nAChRs are pentameric assemblies of separate subunits oriented around a centrally located pore permeable to cations. In vertebrates, seventeen nAChR subunits have been identified (α1–α10, β1–β4, γ, δ, and ε) which can co-assemble to generate diverse nAChR subtypes. All these subunits are found in humans and other mammalian species, except α8 which has been identified only in avian species [4]. These subunits can form homomeric (i.e., containing only the α7, α8, or α9 subunit) or heteromeric pentamers (i.e., containing different subunits such as α3β4, α4β2, and α1β1γδ). These receptors are heterogeneously distributed in the central nervous system (CNS), while in the periphery, they mediate synaptic transmission at the neuromuscular junction and ganglia [4, 5]. Presynaptic nAChRs regulate the release of several neurotransmitters, such as acetylcholine (ACh), serotonin (5-hydroxytryptamine; 5-HT), norepinephrine (NE), dopamine (DA), glutamate, and γ-aminobutyric acid (GABA) [6]. In this regards, nAChRs are involved in several physiological functions, including cognition and memory process, muscle contraction, and pain (reviewed in [5]). nAChRs are also found in non-neuronal cells (*e.g.*, keratinocytes, epithelia, and macrophages), where they modulate the anti-inflammatory cholinergic pathway [5] and angiogenesis [7].

Based on their important functional activities, nAChRs are promising pharmaceutical targets for CNS disorders such as schizophrenia, attention deficit hyperactivity disorder, anxiety and depression, Tourette´s syndrome, nicotine and drug addiction, and Alzheimer's and Parkinson's disease [8, 9].

MOLECULAR STRUCTURE OF THE NACHR AND POTENTIAL LIGAND BINDING SITES

The nAChR structure is one of the most extensively studied from the LGIC superfamily. A very important reason for it, at least at the beginning, was the possibility of obtaining large quantities of nAChRs in its native lipid membrane environment prepared from electric organs of *Torpedo* electric rays. The *Torpedo* nAChR (i.e., α1β1γδ) is structurally and functionally analogous to the human muscular nAChR subtype (*i.e.*, α1β1δγ/ε, embryonic/adult). The cryo-electron microscopy studies detected for the first time important structural domains and the helical organization of the transmembrane region of the nAChR [10]. The nAChR molecular structure was refined down to a resolution of ~4 Å (PDB id: 2BG9). Each subunit consists of

*Address correspondence to this author at the Laboratory of Medicinal Chemistry and Neuroengineering. Medical University of Lublin, W. Chodzki 4a Street, 20-093 Lublin, Poland; Tel: (+48) 756 48 63;
E-mail: katarzyna.duda@umlub.pl

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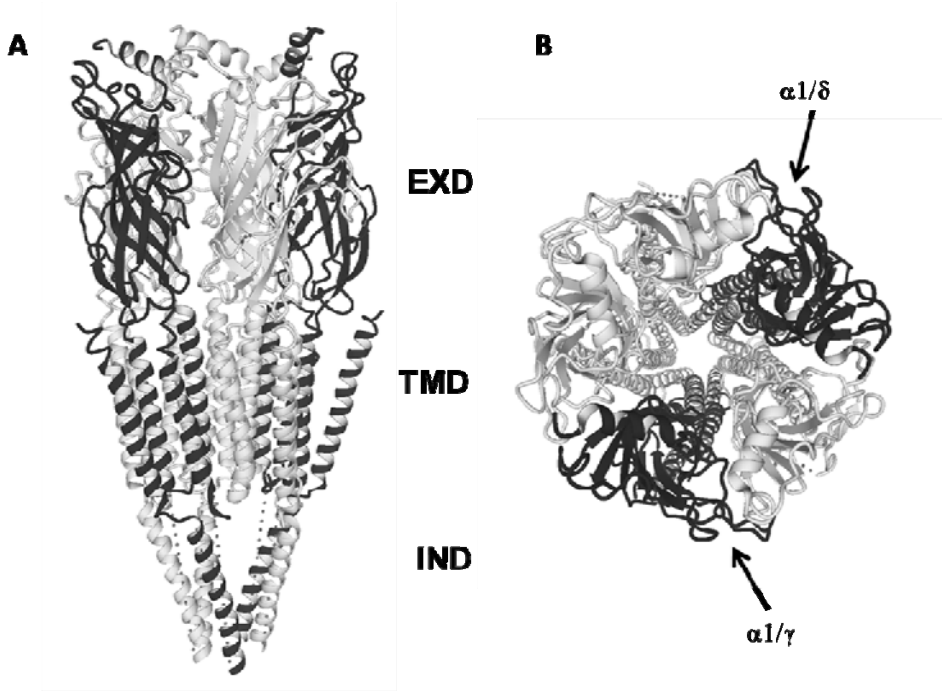

Fig. (1). The molecular structure of the *Torpedo* nAChR obtained by cryo-electron microscopy at 4 Å resolution (modified from [10]). (A) Side view of the receptor showing the extracellular (EXD), transmembrane (TMD), and intracellular (IND) domains, where two α1 subunits are depicted in black and the other three (i.e., β1, γ, and δ) are shown in grey. (B) Top view of the nAChR showing the EXD containing the agonist binding sites at the α1/γ and α1/δ subunit interfaces (see arrows). The ion pore is visible in the center of the structure.

three domains (Fig. **1**): the N-terminal extracellular domain where β-sheet folding arrangements dominate, the membrane domain which consists of four transmembrane α-helices (M1–M4), and the intracellular domain, with lengths that depend on the subunit type. This model was used to build the homology models of neuronal and muscular nAChR subtypes presented in the current review. Interestingly, a recently published paper presents the conformational changes underlying the receptor activation by ACh using ACh-sprayed and freeze-trapped postsynaptic membranes [11]. Based on these results, the closed (PDB id: 4AQ5) and open (PDB id: 4AQ9) *Torpedo* nAChRs were obtained at a resolution of ~6.2 Å.

Among the transmembrane segments, M2 is relatively more hydrophilic and thus, it tends to face the center of the ion channel. Considering that each subunit has one M2 segment, the ion channel is formed by five M2 segments. The amino acid sequences in M2 are highly conserved among different subunits and species, forming a series of amino acid rings exposed to the center of the pore and distributed along the axis of the channel (see Table **2**). For example, in the *Torpedo* nAChR, the amino acid rings are named: outer or extracellular (position 20'), nonpolar (position 17'), valine (position 13'), leucine (position 9'), serine (position 6'), threonine (position 2'), intermediate (position -2'), and cytoplasmic or inner (position -5'). Although the ion channel is very much conserved among species, differences are also apparent among nAChR subunit sequences (see Table **1**), producing variations in the nAChR ion channel structure. For instance, the α7 nAChR has the polar rings (positions 2' and 6') switched: the serine ring occurs at position 2' and the threonine ring at position 6' (see Table **2**). On the other hand, the α4 subunit carries an important change: several Phe residues are located at position 13', becoming the valine/phenylalanine ring. This single difference dramatically changes the overall binding properties of the channel in docking simulations. Thus, the pattern of interactions between the channel and the ligand depends on the amino acid sequence of each subunit in the M2 region.

The extracellular portion of the nAChR carries the binding sites for the endogenous neurotransmitter ACh (i.e., orthosteric sites) and other agonists (e.g., nicotine, epibatidine, and cytisine) as well as for competitive antagonists [1, 9, 12, 13]. Agonists trigger a series of conformational changes in the receptor, including local structural changes, the gating process comprising selected domains from the extracellular portion, and finally the opening of the intrinsic cation channel. This activation, in turn, causes membrane depolarization that finally produces the respective physiologic function (e.g., neurotransmitter release, muscle contraction, etc.). There are many different types of ligands that can modulate the pharmacological activity mediated by agonists. Competitive antagonists (e.g., α- and κ-bungarotoxins, methyllycaconitine, dihydro-β-erythroidine, and α-conotoxins) inhibit the activity of agonists by a simple mechanism where the ligand directly occupies the agonist binding sites [9, 14]. Allosteric ligands, on the other hand, can modulate the activity of agonists not by interacting with the orthosteric sites but with allosteric loci. Several types of modulators have been described based on their pharmacological mechanisms. Noncompetitive antagonists (NCAs) are represented by a large number of structurally different compounds that inhibit nAChR function by binding to allosteric sites located in general in

Table 1. Amino Acid Sequence Alignment of the M2 Segments from Several nAChR Subunits

Subunit	-5'	-4'	-3'	-2'	-1'	1'	2'	3'	4'	5'	6'	7'	8'	9'	10'	11'	12'	13'	14'	15'	16'	17'	18'	19'	20'
hα3	D	C	G	E	K	V	T	L	C	I	S	V	L	L	S	L	T	V	F	L	L	V	I	T	E
hα4	E	C	G	E	K	I	T	L	C	I	S	V	L	L	S	L	T	V	F	L	L	L	I	T	E
hα7	D	S	G	E	K	I	S	L	G	I	T	V	L	L	S	L	T	V	F	M	L	L	V	A	E
hα1	D	S	G	E	K	M	T	L	S	I	S	V	L	L	S	L	T	V	F	L	L	V	I	V	E
Tα1	D	S	G	E	K	M	T	L	S	I	S	V	L	L	S	L	T	V	F	L	L	V	I	V	E
hβ2	D	C	G	E	K	M	T	L	C	I	S	V	L	L	A	L	T	V	F	L	L	L	I	S	K
hβ4	D	C	G	E	K	M	T	L	C	I	S	V	L	L	A	L	T	F	F	L	L	L	I	S	K
hβ1	D	A	G	E	K	M	G	L	S	I	F	A	L	L	T	L	T	V	F	L	L	L	L	A	D
Tβı	D	A	G	E	K	M	S	L	S	I	S	A	L	L	A	L	T	V	F	L	L	L	L	A	D
Tδ	E	S	G(G)	E	L	M	S	T	A	I	C	V	L	L	A	Q	A	V	F	L	L	L	T	S	Q
hδ	D	S	G	E	K	T	S	V	A	I	S	V	L	L	A	Q	S	V	F	L	L	L	I	S	K
Tγ	Q	A	G	Q	K	C	T	L	S	I	S	V	L	L	A	Q	T	I	F	L	F	L	I	A	Q
hγ	K	A	G(G)	Q	K	C	T	V	A	I	N	V	L	L	A	Q	T	V	F	L	F	L	V	A	K
hε	Q	A	G(G)	Q	K	C	T	V	S	I	N	V	L	L	A	Q	T	V	F	L	F	L	I	A	Q

The M2 segments are helical, thus every 3rd–4th residue is exposed to the center of the channel forming amino acid rings. The position of the amino acid rings (in gray) are labeled using the prime nomenclature corresponding to the sequence from Asp238 (-5') to Glu262 (20') in the *Torpedo* nAChR α1-subunit. h, human; T, *Torpedo*.

Table 2. Inhibitory Potency of Structurally Different Antidepressants at Different Human nAChR Subtypes

nAChR Subtype	Antidepressants	Method	IC_{50} [a] μM	Reference
hα4β2	Amitriptyline	Ca^{2+} influx fluorimetry	2.2±0.6	[21]
	Imipramine	Ca^{2+} influx fluorimetry	5.4±1.2	[21]
	Doxepin	Ca^{2+} influx fluorimetry	6.8±1.6	[21]
	(±)-Mecamylamine	Ca^{2+} influx fluorimetry Electrophysiology	3.0±0.7 2.5±0.6	[21] [27]
	(-)-Reboxetine	Ca^{2+} influx fluorimetry	16.0±1.0	[28]
	(±)-Bupropion	$^{86}Rb^{+}$ efflux	12.0±1.1	[23]
hα4β4	(±)-Bupropion	$^{86}Rb^{+}$ efflux	14.0±1.1	[23]
hα7	Imipramine	Ca^{2+} influx fluorimetry	7.8±1.4	[29]
	(±)-Mecamylamine	Electrophysiology	6.9±1.6	[27]
hα3β4	Amitriptyline	Ca^{2+} influx fluorimetry	1.8±0.4	[22]
	Imipramine	Ca^{2+} influx fluorimetry	2.3±1.2	[22]
	Doxepin	Ca^{2+} influx fluorimetry	6.8±1.6	[22]
	(±)-Mecamylamine	Ca^{2+} influx fluorimetry Electrophysiology	3.0±0.7 0.64±0.09	[22] [27]
	(±)-Bupropion	$^{86}Rb^{+}$ efflux	1.51±0.32[b]	[30]
hα3β4*	(±)-Bupropion	Ca^{2+} influx fluorimetry $^{86}Rb^{+}$ efflux	0.82±0.10[b] 1.8±1.1	[31] [23]

Table 2. contd….

nAChR Subtype	Antidepressants	Method	IC_{50} [a] µM	Reference
hα3β2	(±)-Mecamylamine	Electrophysiology	3.6±1.2	[27]
hα1β1γδ	(±)-SADU-3-72	Ca^{2+} influx fluorimetry	1.4±0.2	[32]
	(±)-Bupropion	$^{86}Rb^{+}$ efflux	7.9±1.0	[23]
		$^{86}Rb^{+}$ efflux	10.5±1.0	[30]
		Ca^{2+} influx fluorimetry	23.4±4.5	[33]
hα1β1εδ	(±)-SADU-3-72	Ca^{2+} influx fluorimetry	4.1±0.7	[32]
	(±)-Bupropion	Ca^{2+} influx fluorimetry	24.4±3.3	[33]

[a] IC_{50} is the required drug concentration to produce 50% inhibition of agonist-activated AChRs.
[b]The native nAChR can have additional subunits. h, human.
*Additional subunits that may be present in the assembled receptor

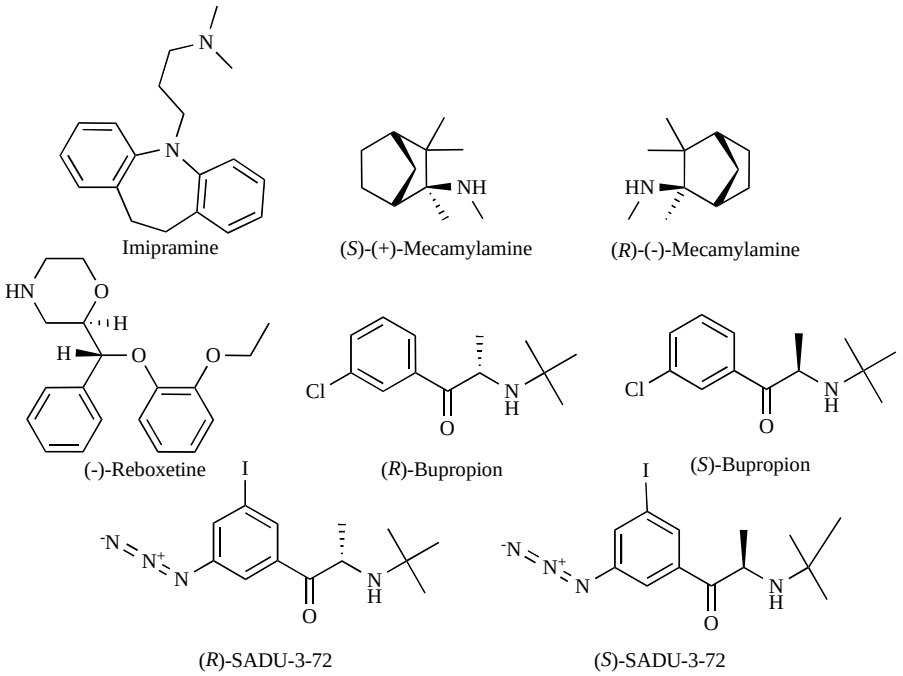

Fig. (2). Chemical structures of the antidepressants described in this review, including imipramine, (*S*)-(+)- and (*R*)-(-)-mecamylamine, (*R*)- and (*S*)-bupropion, (*R*)- and (*S*)-SADU-3-72, and (-)-reboxetine.

the ion channel lumen [15]. The allosteric inhibition mediated by NCAs is similar to that produced by negative allosteric modulators (NAMs). The main difference is that whereas NCAs bind preferably to luminal sites, producing the so-called open-channel blockade, NAMs bind to non-luminal sites, including those at the extracellular, transmembrane-extracellular junction, and transmembrane domains, producing other types of inhibitory mechanisms [8]. Positive allosteric modulators (PAMs), on the other hand, enhance the activity elicited by agonists by increasing the gating process (type I) and/or by decreasing desensitization (type II). The allosteric modulation of neuronal nAChRs emerges as a promising mechanism for the development of novel therapies. In particular, the allosteric modulation mediated by antidepressants will be presented in this review

LINK BETWEEN NEURONAL NACHRS AND DEPRESSION

Depressed mood states have been associated with the hyperactivity or hypersensitivity of the cholinergic system compared to the adrenergic system (i.e., the so-called cholinergic-adrenergic hypothesis for depression) [16, 17]. Over the past decades, increasing data support the idea that the therapeutic activity of many commonly used antidepressants may be partially mediated through inhibition of neuronal nAChRs [16, 17]. Several studies have shown that structurally different antidepressants behave as NCAs of several neuronal-type nAChRs (reviewed in [9, 16-18]). Table **2** shows the inhibitory potency (IC_{50}) of structurally and functionally different antidepressants studied in several laboratories. Among them we can name tricyclic antidepressants (TCAs), including imipramine, amitriptyline, doxepin, nortriptyline, and desipramine [19-22]; norepinephrine selective reuptake inhibitors (NSRIs), including (-)-reboxetine [20] and nisoxetine (not in clinical use); and DA/NE reuptake inhibitors such as (±)-bupropion [23, 24]. Fig. (**2**) shows the chemical structures of only the antidepressants that are described in this review.

(±)-Mecamylamine, an unspecific NCA of nAChRs, decreases depression-like symptoms in Tourette's patients

with comorbid bipolar disorder or major depressive disorder [25]. In addition, mecamylamine is an effective antidepressant in patients who have been unresponsive to SSRIs (reviewed in [26]). Based on the numerous clinical and preclinical studies, it is established that decreasing the activity of specific nAChR subtypes can positively affect mood disorders (reviewed in [26]). Despite the fact that a variety of antidepressants has been used to treat the symptoms of depression for many years, it is still unclear how they work at the molecular and neuronal levels.

Mounting data support the role of nAChRs in depression and in the clinical activity of antidepressants. Coincident with the antidepressant activity elicited by nicotine [34], a higher rate of smokers in depressed patients compared to that in the general population was reported [35]. In addition, nicotinic agonists (e.g., nicotine [36]) as well as competitive antagonists (*e.g*., dihydro-β-erythroidine) and NCAs (*e.g.*, amitriptyline and mecamylamine) potentiate the activity mediated by antidepressants such as TCAs (e.g., imipramine) and SSRIs (e.g., citalopram) [37, 38]. Behavioral studies also show that nicotine enhances the effects mediated by SSRIs and NSRIs, possibly reflecting nicotine's facilitating effects on the release of 5-HT and NE [39]. These data support the view that nAChRs are essential pharmacological targets for the therapeutic activity mediated by antidepressants.

The evidence regarding the relationship between the modulation of nAChRs by antidepressants and mood control might be connected with the pathophysiological activity of nAChR subtypes in specific brain areas confirmed by many behavioral studies presented below. α4β2 and α7 nAChRs are the most abundant subtypes in the CNS, whereas α3β4 nAChRs are expressed in the habenulo-interpeduncular pathway [40, 41]. The α4β2, α3β4, and α7 nAChR subtypes are targets for the clinical activity of antidepressants [18, 42, 43] (Table **2**). Interestingly, β4-containing nAChRs are implicated in nicotine addiction [34, 36, 39], anxiety-like behavior [44], and in the antidepressant activity of (±)-bupropion [45], (reviewed in [18, 46]). Recently published evidence shows that partial agonists and agonists of α4β2 nAChRs present antidepressant activity in animal studies [47, 48]. There is evidence showing that subjects with major depressive disorder (MDD) as well as recovered patients have significantly lower β2*-nAChR availability across all brain regions compared with matched healthy subjects [49]. The lower availability of β2-containing nAChRs, determined *in vivo* by using [^{125}I]5-I-A-85380 single photon emission computed tomography (SPECT), was explained by high extracellular ACh levels in depressed patients, consistent with the cholinergic hypothesis of depression [16, 17]. Moreover, the antidepressant activity of SSRIs (e.g., citalopram) and SNRIs (*e.g*., reboxetine) can be enhanced by selective α4β2 or α7 nAChR agonists, suggesting that both nAChR subtypes are targets for these antidepressants [50]. Additional behavioral studies indicate that the antagonistic activity of (±)-mecamylamine on nAChRs produces antidepressant-like effects, and that these effects are dependent on both β2 and α7 subunits [51]. Based on the preclinical evidence it can be suggested that drugs targeting nAChRs may represent an important new approach to the treatment of depression [20].

A better understanding of the interaction of antidepressants with these nAChR subtypes is crucial to develop more specific, and subsequently, safer antidepressants. The interactions of structurally different antidepressants with human (h) neuronal (*i.e.*, α3β4, α4β2), embryonic (hα1β1γδ) and adult (hα1β1εδ) muscle subtypes, and *Torpedo* (Tα1β1γδ) nAChRs are presented in the current review.

IN SILICO METHODS

Over the last decade, several computational (*in silico*) methods have been developed and applied to study, at the molecular level, the pharmacological interaction of ligands with different receptor binding sites [52]. More specifically, comparative/homology modeling, molecular docking, and molecular dynamics simulations, have been employed to study ligand–receptor interactions. The identification of biomolecular moieties involved in the interaction with specific receptor domains and/or sites is a key step to understand the molecular mechanisms underlying its specific pharmacological activity and subsequently, serves as the basis for the development of more specific and safer drugs.

This review focuses on the application of *in silico* approaches to explain the experimental results on the interaction between a variety of nAChRs subtypes and structurally different antidepressants such as TCAs, NSRIs, and DA/NE reuptake inhibitors, as well as mecamylamine [14, 15, 28, 33]. In this regard, the first step involves the construction of the nAChR target by molecular homology [21, 22, 28, 31-33], using the *Torpedo* nAChR model (Fig. **1**) as a structural template. The antidepressants under study were built in the protonated and neutral state, and subsequently docked in the nAChR model [21, 22, 28, 31-33]. Finally, to verify whether the ligand orientation is stable within each binding site, molecular dynamics simulations were performed [28, 31-33]. Each section from this review, presented below, describes the rationale for the application of *in silico* methods to explaining and supporting experimental results concerning with the interaction of several classes of antidepressants with different nAChR subtypes.

NON-OVERLAPPING INTERACTIONS BETWEEN TRICYCLIC ANTIDEPRESSANTS AND MECAMYLAMINE

TCAs have been widely used as the first choice for the clinical treatment of major depressive and mood disorders [53]. TCAs act primarily by blocking the 5-HT and/or NE transporters, resulting in elevation of the synaptic concentration of these neurotransmitters, and therefore an enhancement of the serotonergic and adrenergic neuronal pathways. There is also evidence indicating that TCAs inhibit both muscle [54] and neuronal [19, 21, 22] nAChRs by noncompetitive mechanisms (Table **2**). Other members of the Cys-loop LGIC superfamily are inhibited by TCAs as well [55].

The binding domain for TCAs was first characterized on *Torpedo* nAChRs by employing photolabeling, radioligand, and molecular modeling approaches [56]. The photoaffinity labeling studies using [^{3}H] 2-azidoimipramie indicate that TCAs bind to the M2 segment only in the desensitized state

Table 3. Binding Affinity of Structurally Different Antidepressants for nAChRs at Different Conformational States

Radioligand	nAChR Subtype	Compound	Conformational State				Reference
			Resting/α-BTx-Bound		Desensitized/agonist-Bound		
			K_i [a] (μM)	n_H [b]	K_i [a] (μM)	n_H [b]	
[^{3}H]Imipramine	hα4β2	Imipramine	0.93±0.09	0.87±0.07	0.97±0.10	0.84±0.07	[21]
	hα3β4		0.68±0.08	0.75±0.05	0.83±0.08	0.71±0.05	[22]
	Tα1β1δγ		3.8±0.4	0.82±0.08	0.85±0.05	0.96±0.05	[56]
	hα4β2	(±)-Mecamylamine	135±19	0.53±0.05	209±34	0.58±0.06	[21]
	hα3β4		239±64	0.72±0.04	161±23	0.56±0.05	[22]
	hα4β2	(-)-Reboxetine	38±3	0.77±0.05	18±1	0.87±0.05	[28]
[^{3}H]TCP	Tα1β1δγ	(±)-Bupropion	5.1±0.3	1.20±0.08	2.0±0.1	1.01±0.06	[33]
		(±)-SADU-3-72	0.75±0.04	0.91±0.04	0.89±0.05	0.99±0.05	[32]
		Imipramine	6.4±0.4	1.06±0.06	0.7±0.1	1.03±0.05	[54]
		Amitriptyline	3.4±0.3	1.09±0.08	0.8±0.1	1.01±0.03	[54]
		Doxepin	15.8±1.4	1.08±0.09	5.3±0.3	0.99±0.06	[54]

[a] The inhibition constant represents the affinity of the antidepressant for the particular radioligand binding site(s).
[b] Hill coefficient (index of ligand binding cooperativity).
h, human; T, *Torpedo*.

[56]. [^{3}H]TCP competition binding results also indicate that imipramine binds to this site with higher affinity when the receptor is in the desensitized state compared to the resting state [56] (Table **3**). Finally, molecular modeling and docking results suggest that TCAs and PCP/TCP interact with overlapping sites located in the middle of the nAChR ion channel [56].

(±)-Mecamylamine (Inversine) was launched in the 1950s as one of the first oral antihypertensive agents [57]. Although the therapeutic target was originally the ganglionic nAChR (i.e., α3β4), additional evidence indicates that (±)-mecamylamine also inhibits other nAChR subtypes (Table **2**). Interestingly, this inhibition seems to be related with the antidepressant [22, 42, 58], anti-addictive [59], and pro-cognitive [60, 61] properties that this drug possesses.

Since (±)-mecamylamine and TCAs are structurally different drugs (see Fig. **2**) but both inhibit nAChRs (Table **2**), it was necessary to determine whether they impede nAChR function by similar inhibitory mechanisms and by binding to homologous sites. Based on the radioligand competition results, TCAs inhibit [^{3}H] imipramine binding to hα4β2 and hα3β4 nAChRs with affinities ~100-fold higher than that for (±)-mecamylamine (Table **3**). However, these ligands block nAChR ion flux with inhibitory potencies in the same concentration range (Table **2**). In this regard, the use of computational tools such as molecular modeling and molecular docking was essential to explain this dichotomy.

Fig. (**3**) shows the differences in the binding site locations between imipramine and (*R*)-(-)-mecamylamine [same results were obtained for (*S*)-(+)-mecamylamine] at the hα4β2 nAChR [21]. The docking results indicate that imipramine, in the neutral and protonated states, and neutral mecamylamine interact with an overlapping domain located between the serine (position 6') and valine (position 13') rings, whereas the positively charged amino group of (*R*)-(-)-mecamylamine interacts by coulombic forces with the negatively charged carboxylic group from the α4-Glu261 residue located at the outer ring (position 20'). Protonated (*R*)-(-)-mecamylamine also interacts by van der Waals contacts with the nonpolar (position 17') and valine rings. Considering that mecamylamine is protonated at physiological pH [21, 22], the most important pharmacological interaction is that in the protonated state close to the extracellular mouth. In this regard, the binding of imipramine should not interfere with the binding of mecamylamine, as it was observed in the radioligand binding results (Table **3**).

The same basic result (*i.e.*, non-overlapping sites for mecamylamine and imipramine) was observed for the hα3β4 nAChR [22]. The modeling results for neutral mecamylamine at the hα3β4 nAChR are in agreement with point mutation studies on the activated hα3β4 nAChR ion channel, where the double mutation in the β4 subunit, Ser→Phe (position 6') and Ala→Thr (position 10'), decreases the inhibitory activity of (±)-mecamylamine [62]. Based on the example presented here (Fig. **3**), we proposed that imipramine directly blocks the hα4β2 and hα3β4 ion channels, whereas protonated mecamylamine can be first attracted to the extracellular channel's mouth by electrostatic interactions before binding to a luminal locus in its neutral state, to finally blocking ion flux.

PHOTOAFFINITY LABELING RESULTS WITH [^{125}I]SADU-3-72 ARE SUPPORTED BY MOLECULAR DOCKING STUDIES

Bupropion has been used for long time as an antidepressant (Wellbutrin) as well as in the pharmacotherapy for smoking cessation (Zyban) [58, 63].

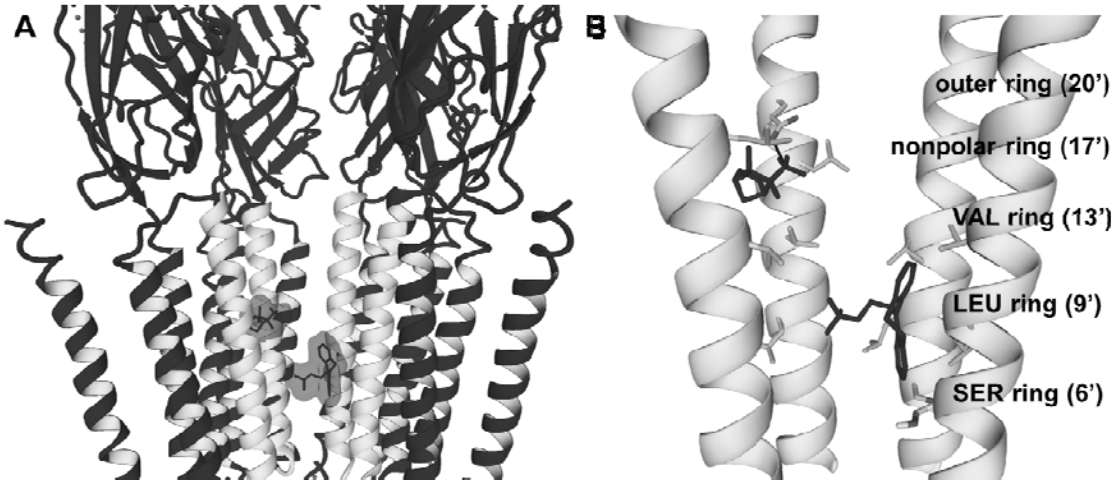

Fig. (3). Molecular docking of imipramine and (*R*)-(-)-mecamylamine (in black), both in the protonated state, within the hα4β2 nAChR ion channel model (modified from [21]). (**A**) Side view of the non-overlapping binding sites for each ligand. Imipramine interacts with the middle portion of the ion channel, whereas (*R*)-(-)-mecamylamine binds closer to the extracellular mouth. (**B**) Molecular details of the interactions presented in (**A**). Imipramine (lower location) interacts mainly by van der Waals contacts with a domain formed between the serine (position 6') and valine (position 13') rings, whereas the positively charged amino group of (*R*)-(-)-mecamylamine (upper location) interacts electrostatically with the negatively charged carboxylic group of α4-Glu261 at the outer ring (position 20'), and additional van der Waals interactions occur at the nonpolar (position 17') and valine rings. One β2-M2 segment is removed for clarity. Residues involved in ligand binding are presented in stick mode, whereas ligands are rendered either in the stick/surface (**A**) or stick mode (**B**).

Bupropion can also be used for the treatment of atypical depression, which is associated with interpersonal deficits (i.e., rejection sensitivity and social avoidance) [64].

The pharmacological [*e.g.*, inhibitory potency (Table **2**) and binding affinity (Table **3**)] and behavioral (e.g., antidepressant, locomotor, and anti-addictive) activities of bupropion, its natural metabolites and isomers, as well as a large variety of synthetic bupropion derivatives have been studied by *in vitro* and *in vivo* approaches [23, 32, 65, 66]. These studies open the possibility of developing novel bupropion derivatives with improved clinical profiles important for their antidepressant and anti-smoking activities (reviewed in [46]). Although bupropion is classified as a dual DA/NE reuptake inhibitor, it also inhibits several neuronal nAChR subtypes (Table **2**).

The aim of this particular work was to determine the pharmacological properties of (±)-SADU-3-72, a photoreactive derivative of (±)-bupropion, in muscle-type nAChRs, with the intention of using this compound as a photoaffinity probe to characterize the bupropion binding sites in neuronal nAChRs [32]. In this regard, the docking of bupropion enantiomers and its analog SADU-3-72 with the ion channel of different muscle nAChR subtypes was performed to determine their binding sites and to support additional experimental results (*i.e.*, Ca^{2+} influx, receptor state-dependent radioligand binding, and photoaffinity labeling study) [32]. Ca^{2+} influx results indicate that (±)-SADU-3-72 is 17- and 6-fold more potent than (±)-bupropion in inhibiting hα1β1γδ and hα1β1εδ nAChRs, respectively (Table **2**). From the [^{3}H]imipramine competition binding results it was evident that (±)-bupropion displays higher affinity for the desensitized nAChR compared to that for the resting nAChR [33], whereas (±)-SADU-3-72 has high affinity [even higher than (±)-bupropion] for both conformational states [32] (Table **3**). This evidence suggests that although (±)-bupropion discriminates between the desensitized and resting states better than (±)-SADU-3-72, the latter is a superior probe for the resting state.

The application of computational techniques helps to identify the domains involved in SADU-3-72 binding compared to that for bupropion. Docking simulations of either bupropion or SADU-3-72 enantiomers (see structures in Fig. **2**), in the protonated and neutral state, to the *Torpedo*, hα1β1γδ, and hα1β1εδ nAChR ion channels, indicate that both ligands interact *via* two different binding modes. Bupropion (Figs. **4A,B**), SADU-3-72 (Fig. **4C,D**) and PCP/TCP bind to overlapping sites located in the middle portion of the ion channel, in a cavity formed between the serine and valine rings [26, 33]. However, a second binding site located between the outer (position 20') and valine (position 13') rings was additionally suggested for SADU-3-72 (Figs. **4C,D**) [32].

The molecular modeling data indicate that the photoreactive azide group (possessing negative and positive charges) at the aromatic ring of SADU-3-72 enantiomers (see structure in Fig. **2**) enhances hydrogen bond formation and polar interactions with polar residues compared to bupropion enantiomers. Moreover, in the *Torpedo* nAChR model, the azido group of (*S*)-SADU-3-72 presents higher probability of forming a hydrogen bond with α1-Ser248 (serine ring) (Fig. **4D**) compared to bupropion (Fig. **4B**) [33]. In the case of the human muscle nAChR ion channels, two hydrogen bonds were found for (*S*)-SADU-3-72, one between its azido group and γ-Asn257 (serine ring) and another between its carbonyl group and α1-Thr263 (position 10') [32]. Interestingly, the latter residue also forms a hydrogen bond with bupropion. Based on the [^{125}I]-SADU-3-72 photoaffinity labeling results, (±)-bupropion may discriminate between two non-overlapping sites depending on the nAChR conformational state [67]. In the resting state, [^{125}I]-SADU-3-72 labeled the middle portion of the *Torpedo* nAChR ion channel, more specifically at the leucine ring

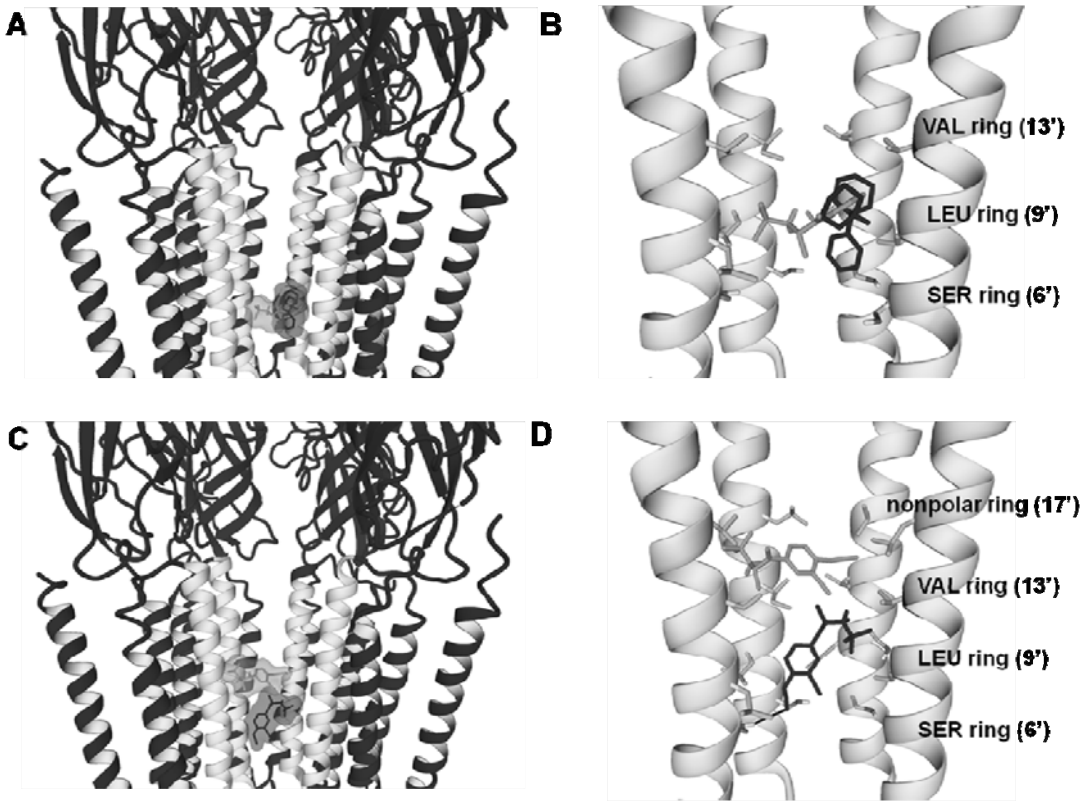

Fig. (4). Molecular docking of (*S*)-SADU-3-72, (*R*)-bupropion, and PCP, in the protonated state, within *Torpedo* nAChR ion channels. (**A**) Side view of the overlapping binding sites for (*R*)-bupropion (gray) and PCP (black) in the middle of the ion channel. (**B**) Molecular details of the interaction presented in (**A**). (*R*)-Bupropion and PCP interact by van der Waals contacts with the valine (VAL), leucine (LEU), and serine (SER) rings. (**C**) Side view of two binding modes for (*S*)-SADU3-72: one closer to the extracellular mouth (gray) and another located in the middle of the ion channel (black). (**D**) Molecular details of the interactions presented in (**C**). In the middle of the ion channel (black), (*S*)-SADU-3-72 interacts by van der Waals contacts with the SER, LEU, and VAL rings, and through the formation of a hydrogen bond (see black arrow) between its azido group and the hydroxyl group of α1-Ser248 (SER ring). In addition, (*S*)-SADU-3-72 interact by van der Waals contacts with the nonpolar (position 17') and VAL rings (gray). The δ subunit is removed for clarity. See Figure 3 for other details.

[67], which concurs with our docking results (Fig. **4**), whereas in the desensitized state, [^{125}I]-SADU-3-72 also labeled α1-Tyr-213 was located in a non-luminal site above the extracellular mouth.

The computational studies also supported the concept that certain particular structural features of SADU-3-72 are responsible for the more potent activity compared to (±)-bupropion (Table **2**). In particular, the large halogen (iodine) group of SADU-3-72 at position 3' (see the structure in the Fig. **2**) makes this compound more hydrophobic than bupropion, increasing its polarizability and consequently van der Waals interactions with the nonpolar, valine, and leucine (position 9') rings [32]. The computational calculations of lipophilicity (i.e., logP value) and polarizability [32] support the above hypothesis.

These results indicate that the use of *in silico* methods helps to locate ligand binding sites and to characterize the noncompetitive inhibitory mechanisms mediated by bupropion and SADU-3-72 enantiomers. Based on these findings, a potential inhibitory mechanism for (±)-bupropion and (±)-SADU-3-72 can be proposed: (±)-SADU-3-72 first binds to the resting nAChR ion channel with high affinity, probably at the extracellular mouth of the ion channel, and this interaction may induce nAChR desensitization [32]. Subsequently, (±)-SADU-3-72 interacts deeper within the middle portion of the ion channel lumen, a domain shared by (±)-bupropion and PCP. The combination of these events is observed as a decrease in ion flux activity.

(-)-REBOXETINE BINDS TO LUMINAL AND NON-LUMINAL SITES AT THE HUMAN α4β2 NACHR

Racemic reboxetine (Edronax, Norebox) was the first commercially available NSRI developed for the treatment of depressive disorders [20]. Behavioral studies show that nAChR agonists enhance the antidepressant activity mediated by reboxetine [48]. Reboxetine also attenuates nicotine self-administration in rats [68], suggesting additional anti-addictive properties. These results suggest that neuronal nAChRs could be potential targets for the beneficial actions elicited by NSRIs.

The Ca^{2+}-influx and voltage-clamp results indicate that (-)-reboxetine does not inhibit hα4β2 nAChRs *via* interaction with the orthosteric sites but by a noncompetitive mechanism and in a dose-response manner [28] (Table **2**). Based on the radioligand displacement experiments, we can infer that although (-)-reboxetine interacts with the [^{3}H] imipramine binding site with relatively low affinity, it discriminates between the resting and desensitized states (Table **3**). The fact that the calculated n_H values are close to unity (Table **3**) indicates that (-)-reboxetine inhibits [^{3}H]imipramine binding

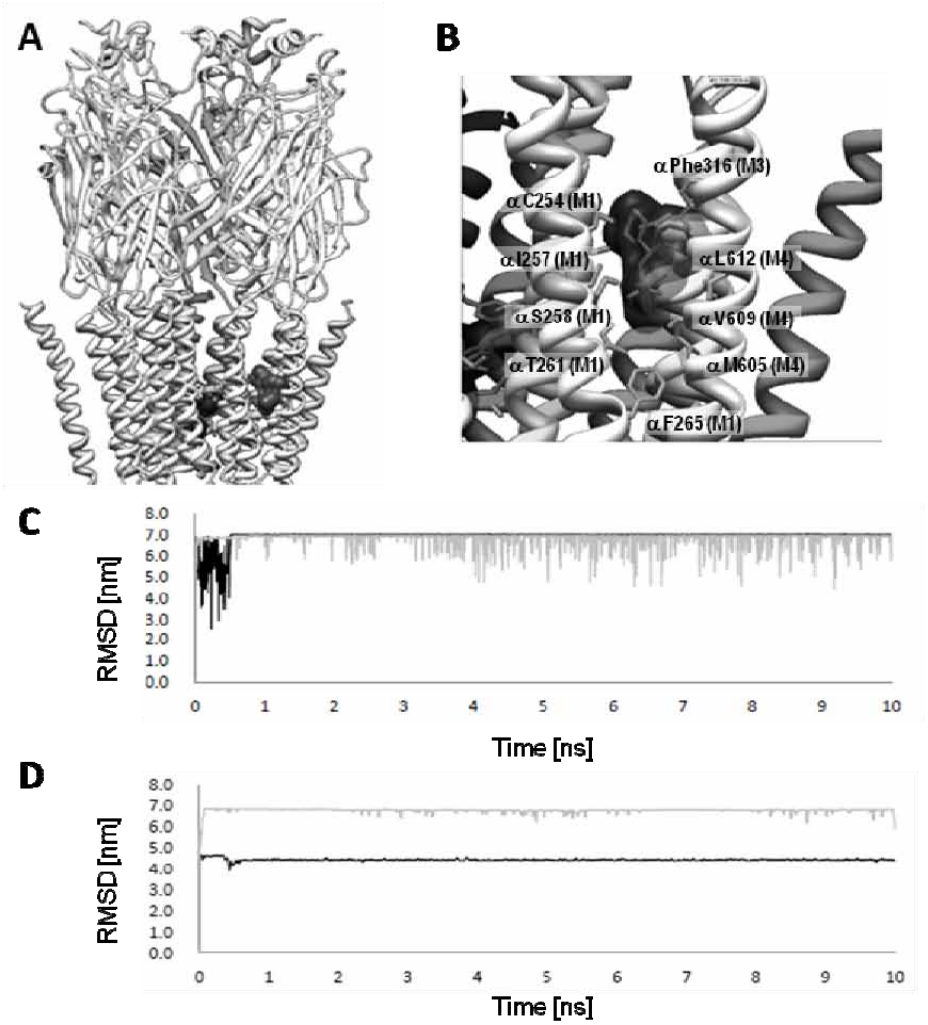

Fig (5). Molecular docking and molecular dynamics of (-)-reboxetine to the hα4β2 nAChR model (modified from [28]). (**A**) Side view of the luminal (center location) and non-luminal (right location) binding sites for (-)-reboxetine. (**B**) Molecular details of the non-luminal site. (-)-Reboxetine is docked within the α-helix bundle from the α4 subunit. The residues involved in binding are presented in stick mode whereas ligands are rendered in the stick/surface mode. (**C**,**D**) Molecular dynamics of (-)-reboxetine and imipramine binding to the (C) luminal and (D) non-luminal sites. The RMSD (root mean square deviation) values of (-)-reboxetine (grey line) and imipramine (black line) are obtained during 10-ns of MD simulation.

in a noncooperative manner, suggesting that these ligands bind to overlapping sites [28]. In this regard, molecular docking techniques were applied to determine the (-)-reboxetine binding mode at the hα4β2 nAChR. The docking results suggest the existence of several putative binding sites for (-)-reboxetine, in two distinct regions of the hα4β2 nAChR [28]. One site is located in the middle of the ion channel, between positions 6' and 14', exactly the same domain for imipramine interacting with the hα4β2 nAChR ion channel (see Fig. **3**) [28]. These results support the previous radioligand competition experiments indicating that (-)-reboxetine also binds to the same binding domain as that for TCAs [11].

The other non-luminal site where both (-)-reboxetine and imipramine bind is located within the α4 subunit transmembrane helix bundle (Fig. **5**). At this position these drugs do not block the channel directly but they can presumably alter the luminal structure of the channel, decreasing ion flux. In this regard, it seems that (-)-reboxetine might allosterically promote changes in the channel properties with more potency, including desensitization, than a direct blocking of the channel [28].

USE OF MOLECULAR DYNAMICS TO VALIDATE THE INITIAL DOCKING RESULTS

Molecular dynamics (MD) simulations (*e.g*., see Figs. **5C**,**D**) were key to validate the initial docking experiments in terms of ligand binding stability. The intermolecular conformational changes and the translation of the whole molecule in the docking site during the MD simulation are defined by the root mean square deviation (RMSD) [28]. MD simulations (*i.e*., 10 ns) of the (-)-reboxetine and imipramine conformers show that they slightly depart from their original docked positions at their respective luminal and non-luminal sites (see Figs. **5C**, **D**) [28]. In the luminal site, imipramine remains stable to the end of the MD simulation, whereas (-)-reboxetine is less stable than imipramine (see Fig. **5C**). In the non-luminal site, imipramine changes slightly from its original docking site but it maintains the new orientation without any major deviation, whereas (-)-

reboxetine deviates more from the original docking site but it remains there in a more stable way that in the ion channel lumen (see Fig. **5D**). In this regard, it seems that (-)-reboxetine might allosterically promote changes in the channel properties with more potency, including desensitization, than a direct blocking of the channel.

The MD results on bupropion and SADU-3-72 enantiomers indicate that the molecules slightly change positions compared to the starting pose (*i.e*., original docked positions) but the complexes were stable during the simulations. In the case of human muscle and *Torpedo* nAChR ion channels, the interaction of each SADU-3-72 enantiomer with nearby pore-lining residues (between the valine and serine rings) is stable during the 15-ns simulation. In the case of *Torpedo* nAChRs, the interaction of (*S*)- and (*R*)-SADU-3-72 with the nonpolar ring (position 17') also remained stable, suggesting that this ring is also important for SADU-3-72 binding at this receptor [32].

CONCLUSIONS

The application of *in silico* methods (i.e., comparative/homology modeling, molecular docking, and molecular dynamics simulations) in combination with experimental techniques (e.g. receptor state-dependent radioligand binding, and Ca^{2+}-influx and electrophysiological experiments) facilitated a better understanding of the molecular interactions of each presented antidepressant with several nAChRs subtypes. Furthermore, these results expanded the current knowledge on the noncompetitive inhibitory mechanisms elicited by antidepressants on nAChRs that might be significant to further studies on the Cys-loop LGIC superfamily. Finally, the combination of functional and structural studies will facilitate the development of novel drugs for the therapy of depression.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

Ach	=	acetylcholine
α-BTx	=	α-bungarotoxin
CCh	=	carbamylcholine
CNS	=	central nervous system
DA	=	dopamine
EXD	=	extracellular domain
FST	=	forced swim test
GABA	=	γ-aminobutyric acid
serotonin	=	5-hydroxytryptamine (5-HT)
IC_{50}	=	ligand concentration that inhibits 50% binding
K_i	=	inhibition constant
LGIC	=	ligand-gated ion channel
MD	=	molecular dynamics
MDD	=	major depressive disorder
nAChR	=	nicotinic acetylcholine receptor
NAMs	=	negative allosteric modulators
NCAs	=	noncompetitive antagonists
NE	=	norepinephrine
NSRIs	=	norepinephrine selective reuptake inhibitors
PAMs	=	positive allosteric modulators
PCP	=	phencyclidine
SADU-3-72	=	2-(*N-tert*-butylamino)-3'-iodo-4'-azidopropiophenone
SSRIs	=	serotonin selective reuptake inhibitors
TCAs	=	tricyclic antidepressants
[^{3}H]TCP	=	[piperidyl-3,4-^{3}H(*N*)]-(*N*-(1-(2-thienyl)cyclohexyl)-3,4-piperidine)
TMD	=	transmembrane domain

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