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Molecular targets of neurotoxic insecticides in *Apis mellifera*

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Abstract: The intensive use of insecticides, combined with other factors such as habitat loss, might explain worldwide decrease of insect populations documented in the past twenty years. However, due to the involvement of pest species in crop destruction and in vector-borne diseases, insecticides will probably continue to be required still for decades. The most commercially successful insecticides are neurotoxicants acting on ion channels of the central nervous system affecting thereby cellular excitability and synaptic transmission, and causing insect paralysis and fatality. In this article, we provide an overview of the insecticides acting on voltage-gated sodium channels, GABA-gated chloride channels and nicotinic acetylcholine receptors. We summarize the current knowledge on those ion channels from the honeybee *Apis mellifera* and discuss the possible mode of action of neurotoxic insecticides.

1. Introduction

The vast majority of the >900,000 insect species play an unsuspected, underestimated or understudied role in ecosystem processes[1]. However, some of them, such as pollinators, attract so much attention that the more emblematic of them, the honeybee *Apis mellifera*, has become undoubtedly the symbol of biodiversity[2]. About 1% of insect species are considered as pests because they destroy crops and serve as vectors for many devastating diseases such as malaria. To fight these pest species (including mites and ticks in addition to insects) we use more and more sophisticated insecticides (and acaricides). Without these insecticides, it is estimated than 70% of crop yields could have been lost due to pests, although the global population, and consequently the demand for food production, is projected to increase to exceed 9 billion by 2050. Moreover, about 229 million cases of malaria occurred in 2019 and there were around 409,000 deaths, mostly children under 5 years of age, and synthetic insecticides are expected to remain a vital component of vector-borne disease control. Therefore, insecticides will probably continue to play a role in pest management for decades.

Insecticides differ in their mode of action but those targeting excitable cells of the central nervous system dominate the insecticide market for several reasons, including their high efficiency[3]. The principal neurotoxic insecticides act on only six molecular target families in the insect nervous system, namely voltage gated Na^+ channel, GABA and glutamate-gated chloride channels, nicotinic acetylcholine receptors (these four target families are the subject of the present review), ryanodine receptors and acetylcholine esterase. Ryanodine receptors are Ca^{2+} channels mediating the controlled release of Ca^{2+} ions from intracellular store involved, for instance, in excitation-contraction coupling in muscle cells. Acetylcholine esterase is the enzyme that catalyzes the hydrolysis of acetylcholine in the synaptic cleft of cholinergic synapses.

In the United States, people can be exposed to approximately 40,000 formulation of pesticides, which include insecticides, herbicides, fungicides and rodenticides. With the over 85,000 chemicals registered by the US Environmental Protection Agency, these compounds constitute only a part of the human chemical “exposome”[4]. The use of pesticides is not without risks for human health since individuals can be exposed to insecticides either actively through occupational exposure or passively through non-occupational exposure. Millions of unintentional acute pesticide poisoning occur each year worldwide resulting in 11,000 deaths[5]. Moreover, there is a significant association between insecticide exposure and neurodegenerative diseases (such as Parkinson’s disease) or the risk of developing certain cancers[6,7]. But evidently, one the major concern raised by intensive use of insecticides is their detrimental effects on non-target insect species. In the past 20 years, worldwide decrease in the number, range and species diversity of insects has been documented. The main reasons explaining insect decline are habitat loss, climate change, agriculture (and the use of pesticides) and pollution[8]. More specifically for honeybees, colony collapse disorder observed since 2006 manifests by winter loss of around 30% of managed colonies. In addition to the above mentioned factors, this sudden decline of honeybee colonies have been linked to parasites (such as the mite *Varroa destructor* or fungi of the genus *Nosema*), viral infections, and poisoning by neonicotinoids[9].

Seventy percent of the major commodity crops directly used for human consumption are dependent on pollinators. Pollination of crops is provided by over 25,000 species of bees worldwide and by other insects such as flies, butterflies or wasps, or by vertebrates such as bats or birds[1]. However, *Apis mellifera* is the main species responsible for bee pollination providing roughly 50% of global crop pollination[10]. Owing to their importance in pollination and the other benefits that they provide (honey, wax…), the health of honeybees is closely scrutinized.

This review provides an overview of the current knowledge on the mechanism of action of the insecticides that target the voltage gated sodium channels, the GABA- and glutamate-gated chloride channels and the nicotinic acetylcholine receptors. We summarize the data that our team at the IBMM and other groups worldwide collected on the functional properties and insecticide sensitivity of those channels from the honeybee *Apis mellifera*.

2. Voltage-gated Sodium Channels

Voltage-gated sodium channels (NaVs) initiate and propagate action potentials in neurons and other excitable cells. In mammals, they are crucial for neuronal firing and heart rhythmicity. NaVs are transmembrane proteins that open their pore in response to membrane depolarization (a process called activation) allowing Na^+ ion influx for few milliseconds. The repolarization of the membrane to its resting value is made possible by the rapid closing of the channel pore through an intrinsic fast-inactivation mechanism. Mammalian NaVs are made of a NaVα subunit (240-280kDa), and one or two regulatory NaVβ subunits (30kDa) that influence trafficking and electrophysiological properties of the NaVα subunits. The mammalian genome includes a family of nine genes encoding NaVα subunits (NaV1.1-9) and a family of four genes encoding the NaVβ subunits (NaVβ1-4). NaV1.1, 1.2 and 1.6 are strongly expressed in the central nervous system whereas NaV1.4 and 1.5 are abundant in skeletal and cardiac muscles, respectively. The NaVβ subunits are expressed in a broad range of cells, including non-excitable cells, where they are critical in many signaling pathways. The NaVα subunit comprises four homologous domains (DI-IV), in which each domain possesses six transmembrane helices (S1-S6), surrounding a central ion pore. S1-S4 helices of each domain form a voltage sensor domain where charged residues (essentially arginine residues) in the S4s are important for voltage sensing (Fig. 1). The S5 and S6 helices and the re-entrant “P-loop” located between them form the pore domain. Some of the amino acids of the P-loop determine the high selectivity of NaVs for Na^+ ions. The transmembrane helices and the intracellular loop connecting DIII to DIV domains, which is involved in the fast-inactivation mechanism, exhibit high amino acid sequence identity. Conversely, the two other intracellular loops (connecting DI to DII, and DII to DIII) and the cytoplasmic amino and carboxy terminus are more divergent. Due to the fundamental role played by NaVs in cellular excitability, numerous (several hundred) inherited or *de novo* mutations in the genes encoding NaVα or NaVβ subunits are the cause of human pathologies such as epilepsy, cardiac arrhythmias and chronic pain syndromes. Anticonvulsants such as phenytoin and carbamazepine, antiarrhythmics such as quinidine and flecainide or local anesthetics such as lidocaine are NaV blockers[11]. These molecules bind to the “local anesthetic site” in the central cavity of the channel pore and interact with amino acid residues highly conserved among the different NaVα subunits[12,13]. Most of insect species, including *Apis mellifera*, have a single gene encoding a NaVα subunit (NaV1)[14]. Insect NaV1 is associated with a TipE (for Temperature induced paralysis phenotype, locus E) or TipE Homologue (TH1-4) subunit that, similarly to mammalian NaVβ subunit, modulates NaV1 electrophysiological properties[14]. However, TipE is not homologous to mammalian NaVβ, suggesting a separate evolutionary pathway[15]. Conversely, the overall structure and the amino acid sequence of insect NaV1 subunit are very similar to those of mammalian NaVα subunits, particularly in the four homologous domains. Voltage-gated Na^+ currents have been recorded in *Apis mellifera* neurons from antennal lobes that are responsible for the processing of olfactory information[16], and from the mushroom bodies that are essential for memory storage and retrieval[17]. Conversely, cardiac and skeletal muscles are devoid of voltage gated Na^+ currents[18].

NaVs are targets of naturally occurring toxins from animals and plants, and of synthetic insecticides that prolong channel opening (DDT and pyrethroids) or that block the channel pore (oxadiazines and semicarbazones) (Fig. 1). By stabilizing the open conducting state of sodium channels, DDT and pyrethroids cause repetitive firing or membrane depolarization in the nervous system whereas by blocking sodium channels, SCBIs lead to nerve conduction block.

2.1. DDT

The insecticidal activity of DDT (dichlorodiphenyltrichloroethane) was discovered in 1939 by P. Müller who received the Nobel Prize in 1948 for his work. DDT was efficiently used to fight a typhus epidemic in Naples in December 1943 by killing the body lice responsible for transmitting the disease. DDT appeared to be the ideal insecticide because it is very toxic to pest insects and moreover it is cheap to produce and it retains its toxicity for a prolonged period of time. DDT allowed the eradication of malaria in the entire European continent in 1975. However, owing to their environmental hazards (persistency in water and soil, and killing of beneficial insects) and possible carcinogenic risks to human, DDT and derivatives (methoxychlor…), like other organochlorine insecticides (dieldrin, lindane, see below) were banned in most countries in early 1970s. Nevertheless, DDT is one of the 12 insecticides, and the only organochlorine compound, still recommended by World Health Organization for use in indoor spraying for vector-borne disease control.

2.2. Pyrethroids

Pyrethroids are synthetic analogues of naturally occurring insecticidal pyrethrins, originally found in the chrysanthemum flowers. Their insecticidal activities have been known since the Middle Ages and plant extracts were used to protect people from household pests [19]. Pyrethrins are registered as pesticides and, in 2014, more than 2,000 commercial products containing pyrethrins were found worldwide[20]. However, their instability in air and light limited their general use in agriculture[19]. Structural modifications of pyrethrins aimed at increasing stability and insecticidal properties produced the first synthetic pyrethroid insecticides, the most successful of which being allethrin (Fig. 1) invented at the United States Department of Agriculture in 1949. It was followed in 1963 by tetramethrin (Sumitomo Chemical), and permethrin (Rothamsted Experimental Station) in 1973... Today, 42 different pyrethroid insecticides with varying chemical structure or relative composition of stereoisomers are available[21]. Pyrethroids are among the most used insecticides, accounting for about 16% of the worldwide insecticide market[22]. Pyrethroids are classified into two categories (Type I and Type II) based on their distinct poisoning symptoms and chemical structures. Type II pyrethroids (deltamethrin, fenvalerate…) have a cyano group at the α benzylic position and are highly toxic, whereas Type I pyrethroids (allethrin, permethrin…) do not contain a cyano group and are less toxic (Fig. 1)[23]. Insect NaV1 channels are over 100-fold more sensitive to pyrethroids than mammalian channels[24]. Nevertheless, some pyrethroids such as lambda-cyhalothrin have high acute and chronic high toxicity to mammals[25]. One of the major concerns caused by pyrethroids is their toxicity to pollinators. Moreover, due to their intensive use in agriculture and for control of vector-borne diseases, many insects, including mosquitoes, have developed pyrethroid resistance[26].

2.3. Oxadiazines and semicarbazones

Characterization of the mode of action of oxadiazines and semicarbazones demonstrates that these insecticides inhibit NaVs without cross-resistance to DDT and pyrethroids. They are classified as sodium channel blocker insecticides (SCBIs). Their history began in the 1970s with the development of pyrazoline compounds with high insecticidal efficacy but their chronic toxicity to mammals and their unacceptable persistence in the soil have prevented their use as commercial insecticides[27]. Studies at DuPont to overcome these limitations led to the discovery of oxadiazines, including indoxacarb (Fig. 1). This latter insecticide differs from its SCBI predecessors in that it is a pro-insecticide which is metabolically activated within insects to form a more active compound, called DCJW[28]. Such bio-activation also occurs in mammals but less efficiently and indoxacarb is metabolized into non-toxic compounds. Further research at Nihon Noyaku Company in the early 1990s led to the discovery of the only other member of the SCBI class of insecticides to be commercialized, metaflumizone (Fig. 1). This insecticide features a semicarbazone structure that is an open-ring version of pyrazolines. Despite the restricted use of SCBIs in agriculture, pest insects such as the diamondback moth *Plutella xylostella* and the leafminer *Tuta absoluta*, two destructive crop pests, have developed indoxacarb and metaflumizone resistance[29,30].

2.4. *Apis mellifera* voltage-gated Sodium and voltage-gated Calcium channels.

In 2015, with Dr. Mohamed Chahine (University of Laval), we cloned the cDNA encoding the honeybee NaV1 subunit[14]. As expected, the overall structural organization and the amino acid sequence of honeybee NaV1 were highly similar to those of mammalian NaVα subunits. We identified genetic variations that led to insertion or deletion of 9-11 amino acid stretches in the cytoplasmic amino-terminus and in the intracellular loops connecting DI to DII and DII to DIII. Since, in mammalian channels, these loops are often associated with protein-protein interactions or post-translational modifications, we speculated that these features might diversify honeybee NaV1 functional properties. We also identified and cloned the honeybee TipE and TH1-4 subunits. These subunits appear to share a common ancestor and analysis of their amino acid sequence indicates that TH3 and TH4 are more closely related to each other than to the three other subunits TipE, TEH1 and TEH2. It is widely considered that NaVs would have evolved from voltage-gated calcium channels (CaVs), a closely related family of proteins that share structural features with NaVs, but are highly selective for Ca^{2+} ions instead of Na^{+} ions[15]. The honeybee genome contains a family of three genes encoding CaVα subunits (Cav1, Cav2 and Cav3). NaV and CaV channels selectivity is governed mainly by a cluster of four amino acids located at equivalent position in the 4 homologous domains, making a so-called selectivity filter (Fig. 1). In CaV channels, the selectivity filter is highly electronegative being made only of negatively charged amino acids, including aspartic (D) and glutamic (E) acids (i.e. EEEE in CaV1 and CaV2, and DDEE in CaV3), whereas in NaV1, it contains neutral and positively charged amino acids, including alanine (A) and lysine (K) (i.e. DEKA). In *Apis mellifera*, besides NaV and CaV channels, there is a protein that exhibits a high similarity with NaV1 but in which the cluster of amino acids at the selectivity filter (DEEA) appears to be a hybrid of the sequences found in NaVα and CaVα subunits. This protein was initially thought to be a Na^{+} channel and was named NaV2 (also known as DSC1 for Drosophila Sodium Channel 1) but functional analysis has revealed that this channel is highly selective for Ca^{2+} ions and, as a consequence, we renamed it CaV4. In addition to NaV1 and ancillary subunits, we also cloned the four honeybee CaVα subunits and their regulatory subunits, namely CaVβ (1 gene) and CaVα2δ (three genes)[31–33]. Indeed, it has been suggested that Cav channels might be, in addition to NaV, the target of neurotoxic insecticides[34]. NaV1, as well as CaV3 and CaV4, elicit robust voltage dependent Na^{+} and Ca^{2+} currents when expressed in a heterologous expression system such as *Xenopus laevis* oocytes, whereas CaV1 and CaV2 expression is not satisfactory for functional studies. Expression failure of insect proteins in heterologous expression system, especially channels and receptors is, unfortunately, rather common. This can sometimes be overcome by the co-expression of chaperone proteins, but such CaV chaperone proteins have not been identified yet, which delays the functional studies of CaV1 and CaV2. In electrophysiological experiments, pyrethroids induce the prolongation of channel opening. This can be visualized with suitable conditioning protocols and the induction of a “tail” current. Both permethrin (Type I pyrethroid) and fenvalerate (Type II pyrethroid) induce tail currents with honeybee NaV1, but these currents are larger and decrease more slowly with fenvalerate than with permethrin and moreover, the apparent affinity of fenvalerate for NaV1 is an order of magnitude higher than that of permethrin[14]. In a first set of experiments, we observed no detectable effect of allethrin and permethrin (both of Type I) on Ca^{2+} currents recorded in *Xenopus laevis* oocytes that expressed honeybee CaV3 channels[35]. However, additional experiments are required to decipher the effects of pyrethroids on honeybee CaVs. Indeed, a recent *in vivo* study suggests that deltamethrin (Type II) might affect differentially honeybee CaV1 and CaV3 channels[36]. Moreover, we recently found that chloranthraniliprole, a compound of the diamide class of insecticides, initially thought to target exclusively the ryanodine receptors, induced a dose-dependent block of Ca^{2+} currents recorded in isolated honeybee muscle fibers and in antennal lobe neurons[37].
Contrary to pyrethroids that prolong channel openings, SCBIs slowly inhibit NaVs. Rather than occluding the channel pore, SCBIs trap NaVs in an inactivated, non-conducting state. SCBIs share this mode of action with local anesthetics that similarly block inactivated Na^{+} channels and have lower affinity to channels in the resting state. Due to their slow kinetics of association, SCBIs bind Na^{+} channels in the slow inactivated state, a unique property whereby Na^{+} channels close after a prolonged depolarization. Recovery from slow inactivation requires seconds to minutes, whereas recovery from fast inactivation takes only tens of milliseconds. We found that the semicarbazone metaflumizone specifically reduces the recovery from the slow inactivated state of the honeybee NaV1 maintaining thereby the channel in a closed non-conducting state[38].

2.5. Insecticide resistance and docking in NaV

The effect produced by pyrethroids on insects resembles a “knock-down”, and the development of resistance to these compounds was designated as “knock-down resistance” (kdr). A reduced sensitivity to DDT or pyrethroids resulting from kdr mutations in NaVα can virtually be found in any crop pests and disease vectors. Different kdr mutations can confer higher resistance to Type I than to Type II

pyrethroids and vice versa, and an additional mutation can synergize with the kdr mutation to confer even stronger pyrethroid resistance (a phenotype known as “super kdr”). So far, more than 50 mutations associated with pyrethroid resistance have been reported in NaVα in diverse pest species[39]. The resistance occurs when there is an alteration at the insecticide binding site but all the mutations identified in insensitive pests are not implicated in direct interactions with insecticide compounds. Indeed, several mutations do not affect insecticide potency on their own, but only when associated with another substitution, suggesting allosteric effects. In the absence of molecular structure for arthropod sodium channels with insecticides, several homology models have been built, including a honeybee NaV1 model with permethrin and fenvalerate[14]. On the basis of experimental results and molecular homology modeling, two different binding sites have been suggested. In the first one, the pyrethroid binding pocket is formed by the IIS4-S5 linker (i.e. the fragment linking the 4th segment to the 5th one within the homologous domain II) and by the IIS5, IIS6 and IIIS6 segments[40]. In a second model, there are two binding pockets (Pyr 1 and Pyr 2) symmetrically formed by the IS4-S5 linker, IS5 and IIS6 and by the IIS4-S5 linker, IIS5 and IIIS6 (Fig.1)[41]. In this model, both sites should be simultaneously occupied by DDT or pyrethroids to produce maximal effect. Interestingly, both models predicts that the pyrethroids binding pocket is exposed to lipids and it turns out that some of the residues on the transmembrane helices involved in insecticide binding are not conserved between vertebrates and invertebrates. This may explain the difference in pyrethroid potency between mammalian and insect NaVs.
Functional studies in heterologous expression systems have demonstrated that SCBIs interact similarly in mammalian and in insect NaVs (stabilization of the slow inactivated state). However, the different mammalian NaVα subunits exhibit a specific pattern of sensitivity to SCBI compounds. Electrophysiological experiments have shown that metaflumizone is able to reduce the ability of lidocaine to cause mammalian sodium channel block, suggesting that local anesthetics and SCBIs share a similar binding site on NaVs[42]. Mutagenesis experiments have identified critical amino acids for channel block by SCBIs in IVS6 and it is precisely in this segment that first mutations were found in SCBI resistant pests[29,30]. Molecular modeling suggests that the binding site for SCBIs overlaps only partially that of local anesthetics in the inner cavity of NaVα subunit, beneath the selectivity filter, and involves specific residues in IIS6 that are not implicated in local anesthetic binding[43]. The recent Cryo-Electron Microscopy (cryo-EM) reconstructions of the coackroach NaV1[44] and of the human NaV1.5 bound with the antiarrhythmic drugs flecainide and quinidine [12,13] pave the way for the elaboration of more precise homology-based models of insect NaV bound with SCBIs.

3. GABA-gated chloride channels

Fast inhibition in the mammalian central nervous system is governed by the binding of γ-aminobutyric acid (GABA) to ionotropic GABA ($GABA_A$) receptors. $GABA_A$ receptors are pentameric chloride channels belonging to the Cys-loop superfamily of ligand-gated ion channels (LGIC). GABA released from synaptic vesicles mediates Cl^- influx through $GABA_A$ receptors into post synaptic neurons leading to hyperpolarization of the cell membrane decreasing thereby the probability of occurrence of action potentials. Mammalian $GABA_A$ receptors are composed of various combination of 19 different subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3) typically constructed with two copies of a α subunit, two copies of a β subunit, and one copy of either γ or another subunit. Subunit combination and arrangement determine the biophysical and pharmacological properties of the assembled $GABA_A$ receptor, and experimental evidences suggest that only a few dozen of combinations exist *in vivo*. The $(\alpha1)_2(\beta2)_2\gamma2$ receptor subtype is the most prevalent combination, contributing to more than the half of all the $GABA_A$ receptors, and the subunits are arranged $\gamma2\beta2\alpha1\beta2\alpha1$ counterclockwise around the central pore, as viewed from the cell exterior. Each subunit (52-59kDa) contains a large extracellular domain (ECD) with a disulfide bridge (hence “Cys-loop”), four transmembrane helices (termed M1-4) and a short carboxy terminus (Fig. 2). The chloride-permeable pore is delineated by the M2 segments of all five subunits. The intracellular loop between M3 and M4 is the site of various protein-protein interactions as well as post-translational modifications that modulate receptor activity. Several different epileptic syndromes have been associated with mutations in $GABA_A$ receptor subunits and the dysfunction of GABA mediated neurotransmission is involved in diverse disorders of the central nervous system such as anxiety and mood disorders, and neurodevelopmental disorders such as autism and schizophrenia[45]. $GABA_A$ receptors are distinguished by their rich pharmacology, which comprises compounds with anti-anxiety, analgesic, sedative and anesthetic properties. Indeed, most general anesthetics such as phenobarbital and etomidate, and classical benzodiazepine drugs such as diazepam (= Valium) and alprazolam (= Xanax) act through positive modulation of $GABA_A$ receptors to dampen neuronal activity.
Similarly to mammals, GABA is the principal neurotransmitter mediating fast inhibitory neurotransmission in insects. Genomic and phylogenic analysis have revealed the existence of at least 4 different genes encoding $GABA_A$ subunits, namely the RDL (for Resistance to Dieldrin), GRD (GABA and glycine-like receptor of Drosophila), LCCH3 (ligand-gated chloride channel homologue 3) and CG8916 (also known as LCCH-14A) subunits[46]. All these subunits belong to the insect LGIC family and share similar structural features with mammalian Cys-loop receptors. Genomes of insect species comprises a specific number of genes encoding $GABA_A$ subunits. For instance, *Apis mellifera* has one whereas the silkworm *Bombyx mori* has three genes encoding RDL subunits. Certain other arthropod species, like the pea aphid *Acyrthosiphon pisum* or the two-spotted spider mite *Tetranychus urticae* are devoid of genes encoding for GRD, LCCH3 and CG8916 subunits. In heterologous expression system, *Drosophila melanogaster* RDL forms homo-pentameric GABA-gated chloride channel reminiscent of mammalian $GABA_A$ receptors[47], although mammalian subunits rarely form functional homo-pentamer receptors. On the other hand, *Drosophila melanogaster* GRD, LCCH3 and CG8916 subunits are unable to form functional receptors when expressed alone in heterologous system but when expressed together, GRD + LCCH3 (but not GRD + CG8916) form GABA-gated cation channels. However such GABA-gated cationic currents have not been recorded in insect cells so far[48]. Conversely, GABA-gated chloride currents have been recorded for instance in *Apis mellifera* neurons from mushroom bodies and from antennal lobes[49,50], and RDL plays a central role in olfactory associative learning and regulation of sleep[51,52].

Vertebrate or invertebrate $GABA_A$ receptors are the target sites of plant-derived ligands such as bicuculline, picrotoxin and muscimol, which are widely used for studying $GABA_A$ functions *in vivo*. Bicuculline is a competitive antagonist of $GABA_A$ receptors whereas muscimol is an orthosteric agonist that was used to reduce anxiety and induce sleep but that may also be a potent psychoactive drug. On the other hand, picrotoxine (the structure of which can be related to that of aldrin and dieldrin) is a non-competitive channel blocker, which was useful as an insecticide and for treating epilepsy. Insect $GABA_A$ receptors are the target of different synthetic insecticides acting either as channel blocker (organochlorides and phenylpyrazoles) or allosteric modulators (meta-diamides, isoxazolines and avermectins) (Fig. 2). Organochlorides and phenylpyrazoles are non-competitive antagonists (NCAs) that block channel pore and do not interact at the orthosteric GABA binding site. Meta-diamides are negative allosteric modulators that also block $GABA_A$ receptors but at a site distinct from that of NCAs and conversely, avermectins are positive allosteric modulators.

3.1. Organochlorides (HCH and cyclodienes) and phenylpyrazoles

Lindane is a first generation insecticide that exhibits similar potency against insect and mammalian $GABA_A$ receptors. Hexachlorocyclohexanes (HCH) were known since the 19th century and lindane (γHCH) was named according to its inventor Teunis Van der Linden in 1912. Lindane was intensively used as insecticide to treat seed and livestock. As DDT, the use of lindane was gradually banned from many countries beginning early 1970s. However, lindane is still used today for fruit and vegetable crops and also in lotions, creams and shampoos for the control of lice and mites in humans.
The cyclodienes aldrin and dieldrin were first synthesized in 1948 by Julius Hyman Company. These organochlorides are effective by contact or ingestion and were mainly used until 1990s for the protection of wood and structures against attack by termites. Aldrin is not toxic to insects by itself, it is oxidized in insects (and also in human body!) to form dieldrin which is the active compound. Dieldrin is still used in some tropical countries in housing sprays to control vector-borne diseases.
Fipronil (Fig. 2), the only commercial member of the phenylpyrazole class of insecticides, was invented by Rhone-Poulenc in 1987. Fipronil, widely known under its trade name Regent, was once regarded as a safer alternative to organochlorine insecticides (DDT, lindane). Indeed, fipronil was shown to be more efficient on insect $GABA_A$ receptors than on mammalian receptors. But fipronil is metabolized both in insects and mammals to sulphone fipronil, which is more persistent and less selective for insect GABA receptors[53]. In addition to $GABA_A$ receptors, fipronil acts as a potent channel blocker of glutamate-gated chloride receptors (GluCls), which are also members of the LGIC receptor family. Influx of chloride ions through GluCl produces hyperpolarization in muscle cells and neurons upon activation by glutamate, similarly to GABA when acting on $GABA_A$ receptors[54]. Interestingly, GluCls are found only in invertebrates and might thus be of special interest for the development of insecticides. Besides $GABA_A$, the additional effect onto GluCl might contribute to the insecticidal activity of phenylpyrazoles.
It is now well documented that fipronil and/or its metabolites have neurotoxic, hepatotoxic, reproductive adverse effects in vertebrates[55] and that they are detrimental also to benefic pollinators[56]. Nowadays, fipronil is the main active ingredient of treatments used in fighting tick and flea infestations in dogs and cats. Resistance to cyclodienes and phenylpyrazoles have been found in numerous insect species including crop devastating species like the brown planthopper *Nilaparvata lugens* and the green peach aphid *Myzus percicae*, and disease vectors like *Aedes aegypti*[57].

3.2. Meta-diamides, isoxazolines and avermectins

Flubendiamide is an organo-fluorine insecticide that was invented at Nihon Nohyaku Company in the early 2000s. Flubendiamide (like chloranthraniliprole mentioned above) is a member of the diamide class of insecticides that target the ryanodine receptors. Meta-diamides have a unique chemical structure generated via structural modifications of flubendiamide at Mitsui Chemicals leading to the discovery of the first member of this new insecticide class, broflanilide (Fig. 2)[58]. Broflanilide is probably metabolized in insects to desmethyl-broflanilide. The insecticidal potencies of broflanilide and desmethyl-broflanilide are similar, but electrophysiological experiments have demonstrated that the activity of desmethyl-broflanilie is 1,000 times higher than that of broflanilide on insect $GABA_A$ receptors[59]. Moreover, meta-diamides are considerably more potent against insect than mammalian $GABA_A$ receptors[60]. Broflanilide has been registered in Australia, China and Canada in 2020 and the Arthropod Pesticide Resistance Database (www.pesticideresistance.org) does not report any case of resistance to this compound up to now[57].
The isoxazoline class of insecticides originates, like the meta-diamides, from structural modifications of diamides realized at Nissan Chemical in the early 2000s. These compounds are highly selective for insect receptors although they do not target specifically the $GABA_A$ receptors. Indeed, isoxazolines are also antagonists of GluCl but with a weaker efficiency than on $GABA_A$[61]. The isoxazoline insecticides, which include fluralaner (Fig. 2), lotilaner and fluxametamide, are used as veterinary drugs to control of ticks and fleas, and fluxametamide has a great potential to be used in crop pest control.
The mechanism of action of meta-diamides and isoxazolines differs from that of NCAs since these compounds are still efficient on insects displaying resistance to dieldrin and fipronil[62]. However, the different potencies of meta-diamides and isoxazolines towards $GABA_A$ and GluCl[63] suggest a different binding site for these compounds on their target receptors.
Avermectins and milbemycins, a class of drugs with compounds targeting, among others, Cys-loop receptors, are characterized by another mode of action. Indeed, these macrocyclic lactones are positive allosteric modulators of LGICs but not antagonists like the NCAs, the meta-diamides and the isoxazolines. The identification of avermectins (the “wonder drug” ivermectin being the most known[64]) was the result of a close collaboration in the 1960s and 1970s between Satoshi Omura at the Kitasato Institute and William C. Campbell at Merck, which were both awarded by the Nobel Prize in 2015. Avermectins and milbemycins were isolated from fermentation of the soil bacterium *Streptomyces avermitilis* and *Streptomyces hygroscopicus*, respectively. They attracted considerable attention because of their high potency as anthelmintic drugs and ivermectin was used to rescue hundreds of millions of people from

the parasitic diseases onchocerciasis (river blindness) and lymphatic filariasis (elephantiasis)[65]. Other macrocyclic lactones such as abamectin were used to regulate insects like mites or cockroaches and for crop protection[66].
In the absence of endogenous agonist, ivermectin and analogs open glutamate-gated chloride channels, therefore acting as activators. In the presence of endogenous agonist, avermectins act as allosteric modulators, potentiating the response to low doses of glutamate (*i.e.* doses < EC_{50}), but inhibiting the response to high ones (doses >EC_{50})[67]. Activation of glutamate-gated receptor by ivermectin is much slower than seen with glutamate and, once opened, the channel remains in this state for a long time. Avermectins do not specifically target glutamate-gated receptor but modulates other LGICs (GABA-, acetylcholine-, glycine-, histamine-, and pH-gated receptors) and moreover, they activate ATP-gated P2X receptor and G-protein-gated inwardly rectifying K^+ (GIRK) channel[68]. The effects produced by avermectins on $GABA_A$ are similar to those on GluCl but require higher concentrations. The extensive use of ivermectin in livestock treatments has led to resistance in parasitic nematodes and there is also concern regarding helminth parasiting humans. Until now however, studies have failed to identify mutations in the genes encoding glutamate-gated receptor subunits in parasitic nematodes and rather suggest that ivermectin resistance is multigenic in nature[69].

3.3. *Apis mellifera* GABA- and Glutamate-gated chloride channels

Dieldrin was used to localize in fruit-fly genome the locus associated with cyclodiene resistance and identify, at the molecular level, the RDL $GABA_A$ receptor subunit[70]. In addition to RDL, the sequencing of the honeybee genome by an international consortium in 2006 allowed the identification of a family of genes encoding LGICs. Besides those encoding nicotinic acetylcholine receptor (nAChR, which are cationic permeant channels, see below) and $GABA_A$ receptor (RDL, GRD, LCCH3 and CG8916) subunits, honeybee genome contains one gene encoding GluCl, one gene encoding pH-sensitive chloride channel subunit and two genes encoding histamine-gated chloride channel subunits[71]. All these subunits share the overall structural organization of Cys-loop receptors. The orthosteric binding site is located at the ECD, at the interface between two adjacent subunits, which are noted to as the principal or (+) and the complementary or (-) side of the binding site (Fig. 2). The (+) side of the binding site is made of three loops (A, B and C) whereas the (-) side comprises four loops (D, E, F and G)[72]. The amino acid sequence found in these loops govern agonist specificity in each LGICs. In a mammalian typical $GABA_A$ receptor $(\alpha1)_2(\beta2)_2(\gamma1)$, there are two binding sites at the $\beta(+)/\alpha(-)$ interfaces and channel opens upon GABA binding to just one of them. However simultaneous occupancy of both sites is dramatically more efficient than the single occupancy of any of the two binding sites.
We[73], and others[74,75], cloned the honeybee RDL subunit and showed that RDL forms, similarly to fruit-fly RDL, homopentamer chloride-permeant channel. Therefore, there are five potential binding sites in the homomeric RDL channels but whether they are similarly efficient or whether they act cooperatively is still unknown. Alternative splicing at the ECD leads to *Drosophila melanogaster* RDL variants with specific sensitivity to $GABA_A$ agonists, in particular GABA and muscimol[76]. Similar *Apis mellifera* variants exist also but have not been characterized so far[71]. Conversely, three honeybee variants resulting from differential splicing at the M3-M4 intracellular loop were studied. They share a similar sensitivity to GABA and more importantly to the phenylpyrazole fipronil[74], although splicing at M3-M4 linker in other species affects fipronil sensitivity[77]. However, the honeybee variants display specific sensitivity to the neonicotinoid imidacloprid[74], which is an insecticide known to target nAChRs (see below). In addition to alternative splicing, RNA editing is a post-translational mechanism that leads to the substitution of particular amino acids and can profoundly affect protein functions. One out of the four substitutions identified in the *Drosophila melanogaster* RDL, lying at the ECD, reduces the sensitivity to fipronil[78] whereas another one, located in M1 has no effect[79]. Moreover, RNA editing in *Anophele gambia* RDL affects action of ivermectin[80]. However, none such substitution in *Apis mellifera* RDL has been reported so far. Honeybee RDL receptor is highly sensitive to fipronil and to the isoxazoline fluralaner, but functional studies report different sensitivity towards avermectins and cyclodienes[73–75], and further investigations are needed to elucidate these discrepancies. It is also unknown whether avermectins may act as allosteric activators on *Apis mellifera* RDL in the presence of low doses of GABA. Pharmacological properties of RDL expressed in *Xenopus laevis* oocytes differ substantially from those of native GABA-gated currents recorded in honeybee antennal lobe neurons[49]. One can speculate that these differences result from splicing, editing or coexpression of an unknown subunit[81] and it is therefore important to investigate all these possibilities.
In addition to RDL, we also cloned GRD, LCCH3 and CG8916, the three others $GABA_A$ subunits. As reported for *Drosophila melanogaster* and for the ectoparasite mite *Varroa destructor*, honeybee GRD and LCCH3 fail to reconstitute functional receptors when expressed alone in heterologous expression system[48,82]. However, when expressed together, they form GABA-gated cationic receptor displaying a pharmacology distinct from that of RDL receptors. The M2 transmembrane segments of the five subunits of Cys-loop receptors delineate the channel pore (Fig. 2), and bear amino acids that govern ion selectivity. Indeed, RDL M2 amino acid sequence differs from that of GRD and LCCH3 subunits. Contrary to voltage-gated channels (see above), a stretch of amino acids acting as a selectivity filter is lacking in LGICs, and ion selectivity seems rather to be controlled by many charged residues distributed along the channel pore[83]. Recent structures obtained for mammalian LIGCs[84,85] will undoubtedly help to understand molecular events at work for selecting anion or cation in the various LGICs. The GRD amino-acid sequence at M2 helice resembles that of CG8916 and it has been suggested recently that rice striped stem borer *Chilo suppressalis* CG8916 can reconstitute functional cationic receptors when co-expressed with LCCH3[46]. Therefore, it will be interesting to compare the pharmacology of these two potential GABA-mediated cationic receptors in honeybee.
Little is known about the functional role of glutamate-gated chloride channels in insect physiology. Nevertheless, genes encoding these receptors have been identified in many species. As mentioned above, there is a single gene encoding GluCl in honeybee[71], but the genome of certain arthropods may include up to six different genes. Alternative splicing in honeybee GluCl ECD produces three variants with specific amino acid sequence at the orthosteric binding site [86]. This splicing is found in other species such as *Musca domestica* or *Bombyx mori*. An additional alternative splicing has been found at the M3-M4 loop in the mosquito *Anopheles gambiae* and the moth *Plutella xylostella*. GluCls from numerous species have been successfully expressed in heterologous expression system but, so far,

none study has reported the biophysical and pharmacological properties of honeybee GluCl, leaving a gap in the analysis of the effects produced by insecticides on *Apis mellifera*. The affinity for glutamate of GluCl variants from *Musca domestica* or *Bombyx mori* that differ at ECD is similar[87,88], suggesting that the glutamate binding site is marginally affected. *Musca domestica* GluCl is 180 times more sensitive to ivermectin than RDL, and conversely for sensitivity to fipronil[67,89]. Thus, in insects, GluCl is probably the principal target of avermectins whereas RDL is the principal target of phenylpyrazoles. Ivermectin potency is not affected by splicing at M3-M4 in *Anopheles gambiae* or *Bombyx mori* GluCl[88,90]. However, abamectin is more efficient on one of the three varaints of *Plutella xylostella* GluCl than on the two others [91], suggesting that various avermectins might affect specifically the GluCl variants found in different species. *Musca domestica* GluCl is potently blocked by fluxametamide and fluralaner[92,93], but lotilaner, another isoxazoline, does not affect the human louse *Pediculus humanus* GluCl[94], suggesting also species specificity for isoxazolines.

3.4. Insecticide resistance and docking in ligand-gated chloride channels.

The gene encoding RDL was identified in field-collected strains of *Drosophila melanogaster* resistant to dieldrin and the substitution of an, otherwise conserved, Ala at M2 (position known as A2', Alanine numbered from the first residue of the M2 segment, Fig. 2) into Ser was showed to confer insensitivity to, not only cyclodienes, but also picrotoxin and fipronil, demonstrating the similar mode of action of these compounds[47,95]. Other mutations at A2' into Ser, Gly, or Asn were found in numerous other species resistant to NCAs[96] and A2' mutation were sometimes combined to an additional mutation of T51' at M3 that can by itself affect NCA potency and synergizes with A2' substitution[97]. A2' and T6' were often replaced by other amino acids in certain RDL subunits from *Varroa destructor*[82], *Bombyx mori*[98] or *Plutella xylostella*[99]. Although substitution at T6' has never been described in NCA field-isolated resistant species, T6' mutation in *Drosophila melanogaster* RDL suppresses picrotoxin antagonism[100], suggesting that this threonine plays a critical role in the formation of NCA binding site in RDL[101]. In molecular modelling of RDL subunits from different species (including *Apis mellifera*) fipronil is erected in the channel pore surrounded by the five M2 segments, the trifluoromethyl group being oriented toward the intracellular compartment[75,102,103]. The binding site of fipronil is located between L9' (which defines the narrowest point of the pore) and P-2', with A2' and T6' critically involved in direct interaction with phenylpyrazole[75]. Lack of conservation of these two residues probably explains the weaker sensitivity of mammalian $GABA_A$ for fipronil compared to insect RDL[104].
Meta-diamides are similarly efficient on a *Drosophila melanogaster* RDL mutant harboring substitutions at both A2' and T6' than on wild type receptor. Conversely, it was found that mutations at G36', in the M3 helix, critically affect broflanilide blockade while sparing NCA antagonist potency. This demonstrates the different mode of action of these compounds[59,105]. Molecular models of *Drosophila melanogaste*r RDL suggest that the meta-diamide binding pocket is located at the entrance of an inter-subunit transmembrane cavity wedged between the M3 and M1 helices of two consecutive subunits, proximal to the extracellular side of the phospholipid bilayer (Fig. 2)[105,106]. In addition to G36', meta-diamide binding pocket seems to involved critical residues of the M1 segment[105,106]. The meta-diamide binding pocket is reminiscent to that of the general anesthetic binding site in mammalian $GABA_A$ receptor defined by Cryo-EM[107]. In a typical $GABA_A$ receptor, etomidate binds into a cavity at $\beta(+)/\alpha(-)$ interface below de GABA binding site and similarly to GABA at orthosteric site, $GABA_A$ receptor can accommodate etomidate at each of the two α-β interfaces.
The X-ray structure of *Caenorhabditis elegans* GluCl with ivermectin reveals a binding pocket that resembles that of $GABA_A$ receptors[108]. Ivermectin fits deeply at subunit interfaces embedded between the M3 and M1 helices and makes important interactions with residues of M2. These interactions stabilize the open state of the channel and increase the time the pore channel stays open[68]. G36' (in M3) contributes to ivermectin binding site and any other residue than Gly at this position would affect binding site conformation or its access[109]. G36'E and G36'D substitutions have been found in resistant strains of *Tetranychus urticae* and G36'E mutation abolished abamectin response in electrophysiological experiments[110]. Other substitutions close to G36', for instance P23'[111], A30'[112], I31'[113] and L39'[92] have been linked to avermectin sensitivity in arthropods but it is unlikely that all of them affect directly the binding site.
Abamectin was docked in a molecular model of *Apis mellifera* RDL and the compound interacts with residues at M1, M2 and M3 helices (including G36' and F39')[75]. Interestingly, the same residues are also involved in the interaction with the isoxazoline fluralaner when docked in the same RDL model suggesting that isoxazolines and meta-diamides might share a similar mode of action. The F39' in RDL is replaced by L39' in *Musca domestica* GluCl and the single exchange L39'F is sufficient to confer the isoxazoline sensitivity of RDL to GluCl[92]. More work remains to be done to clearly decipher the interactions of the various insecticides that affect GABA- and Glu-gated chloride channels, for instance, it will be interesting to compare the meta-diamide docking in RDL and in GluCl since these compounds affect specifically $GABA_A$ receptors.

4. Nicotinic Acetylcholine receptors

In mammalian brain, acetylcholine (ACh) regulates the release of both excitatory and inhibitory neurotransmitters through the activation of the ionotropic family of nicotinic ACh receptors (nAChRs). These receptors that permeate mainly Na^+ and Ca^{2+} ions are widely expressed throughout the nervous system. Their presynaptic or preterminal localization influences many synaptic and non-synaptic circuit components. Nicotinic ACh receptors are also found at the mammalian neuromuscular junction where they mediate neuromuscular synaptic transmission. Muscle and neuronal nAChRs belong to the Cys-Loop family of receptors and share structural features with other family members including a large ECD that carries the ACh binding site (Fig. 3). Mammalian genome contains a family of genes that encode 5 muscle (α1, β1, γ, δ and ε) and 12 neuronal (α2-10, β2-4) nAChR subunits. Muscle nAChR consists of two α1 subunit and 3 non-α subunits (β1, δ and either γ or ε) whereas neuronal nAChRs can assemble as homo- or heteropentamers.

Each α subunit harbors a pair of adjacent Cys linked by a disulfide bridge whereas the β subunits do not have those Cys. Homopentameric receptors are thought to have five identical binding sites and heteropentameric receptors have two binding sites which are located at the α(+)/β(-) interfaces between two neighboring subunits. Three nonconsecutive binding sites in homopentamer and two binding sites in heteropentamer have to be occupied by endogenous agonist to trigger efficient receptor activation. As for $GABA_A$, stoichiometry and subunit arrangement define functional and pharmacological receptor properties. For instance, one of the most abundant neuronal nAChR contains the α4 and β2 subunits and these subunits can assemble as $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ to produce receptors with specific properties including a different Ca^{2+} permeability. Native nAChRs are composed by a relatively restricted number of subunit combinations suggesting that subunit arrangement is a tightly regulated process. Nicotinic AChRs are considered promising therapeutic targets for treatment of neurodegenerative diseases. Indeed, dysfunction of cholinergic neurotransmission contributes to the neuropathology of neurodegenerative diseases and genetic polymorphism of nAChR genes might be implicated in the pathogenesis of sporadic cases of Alzheimer's disease. Galantamine, used for the treatment of Alzheimer's disease, is an acetylcholinesterase inhibitor that enhances cholinergic signaling. This drug is also a positive allosteric modulator of nAChRs potentiating thereby cholinergic transmission. Mutations in the gene encoding nAChRs have been found in patients suffering autosomal dominant nocturnal frontal lobe epilepsy and congenital myasthenic syndromes[114,115]. Of course, nAChRs are implicated in nicotine addiction which involves physiological changes in nAChR function and expression[116].
As in mammals, nicotine is an agonist of insect nAChRs. However, insect nAChRs are mainly located in the central nervous system and ionotropic glutamate receptors (iGluRs) mediate synaptic transmission at the neuromuscular junction. The number of genes encoding nAChR subunits is smaller in insects than in mammals. There are 10 and 11 genes encoding nAChR subunits in *Drosophila melanogaster* (7 are α and 3 are β) and *Apis mellifera* (9 are α and 2 are β), respectively. Phylogenic analysis supports the distribution of the nAChR subunits into seven groups, among which the α7-group contains the insect α5, α6 and α7 subunits which share high sequence homology with mammalian α7[117]. The other insect nAChR subunits do not have such homology with the mammalian subunits. Curiously, the *Drosophila melanogaster* β2 subunit is the only "β"subunit of the β2 group which contains otherwise α8 subunits from other insect species suggesting a species-specific specialization during evolution. Moreover, insects possess at least one divergent subunit (β3 in *Drosophila melanogaster*, and α9 and β2 in *Apis mellifera*) characterized by their short M3-M4 intracellular loop as well as the absence of an extracellular carboxy terminus [118]. Nicotinic AChRs are expressed in several regions of insect brain involved in sensory and cognitive processes but the functional role played by the various nAChR subunits is considerably less understood for insects than for mammals[119,120]. Molecular analysis revealed that honeybee Kenyon cells express only the α2, α8 and β1 nAChRs subunits whereas antennal lobe neurons express the supplementary α7 subunit. Since Ach-gated currents with distinctive properties can be recorded in these two neuron populations, it is likely that different nAChR combinations play specific physiological functions. Moreover, a recent study demonstrated that *Drosophila melanogaster* α1 and α6 critically support dendrite morphogenesis and synaptic transmission in larval visual system[121]. The lack of expression of insect nAChRs in heretologous expression system explains partly why knowledge on nAChR functions is still scarce. This deprives us of a valuable mean to develop biochemical and pharmacological tools needed for studying these receptors. Moreover, this prevents also the assessment of insecticides targeting nAChRs with various subunit combinations and/or from different species. Indeed, nAChRs are the target of several classes of insecticides that act as competitive modulators at the orthosteric binding site (neonicotinoids, sulfoximines, butenolides, mesoionics and pyridylidenes), as channel blockers (nereistoxin analogues), or as allosteric modulators (spinosyns and GS-ω/κ-HXTX-Hv1a).

4.1. nAChR competitive modulators.

Nicotine or tobacco extracts were used for centuries to control crop pests but with poor efficiency, little species-specificity and moreover, they were hazardous to people. Compounds structurally related to nicotine (nicotinoids such as epibatidine) have never been exploited as commercial insecticides. In the 1970s, the optimization of nitromethylene compounds with insecticidal activity by Shell leads to the discovery of nithiazine. Although a potent insecticide targeting nAChRs, its instability prevented its use for crop protection but it was a lead compound for the development of the neonicotinoids. The first compound of this class, imidacloprid, was invented by Nihon Bayer Agrochem in 1991, and it was followed by 6 other compounds: nitenpyram (Sumimoto, 1995), acetamiprid (Nippon Soda, 1996), thiametoxam (Syngenta, 1998), dinotefuran (Mitsiu, 2002), thiacloprid (Bayer CropSciences, 2000) and clothianidine (Bayer/ Takeda Chemical Industries, 2002)[122]. In 2018, neonicotinoids represented 25% of global insecticide market with thiametoxam being the leading product. Neonicotinoids show diverse actions on nAChRs acting as partial (for instance imidacloprid) or super-agonist (clothianidine) when compared to the endogenous agonist ACh[123]. However, the relationship between toxicity and nAChR agonism has not been demonstrated[122]. Neonicotinoids harboring a cyanoimine (NCN) substituent (such as thiacloprid or acetamiprid) are generally considered less toxic than compounds that have a nitroimine (NNO_2, such as imidacloprid and clothianidine) or nitromethylene ($CHNO_2$, such as nitenpyram) substituent (Fig. 3). Neonicotinoids are water-soluble and are distributed systemically throughout the growing plant, reaching the nectar and the pollen of crop species and wild flowers exposed to field treatment. Field-realistic doses of neonicotinoids have sub-lethal impact impairing learning and memory, locomotion, circadian rhythms and sleep, which may contribute to the dramatic losses of pollinators[124]. Based on these concerns, neonicotinoids have been largely banned in most countries and acetomiprid is the last compound approved in the European Union. Intensive use of neonicotinoids has led to resistance that often involves metabolic changes characterized by overexpression or duplication of genes coding for detoxification enzymes and more rarely target alteration.
Other compounds such as sulfoxaflor (sulfoximines)[125] and flupyradifurone (butenolides)[126] targeting nAChRs were developed in early 2000's to circumvent the concerns raised by neonicotinoids towards pollinators and growing cases of resistance in crop pests. Sulfoxaflor (Dow AgroSciences) was invented on the basis of the sulfoximine functionality whereas flupyradifurone (Bayer CropScience)

was inspired by the natural product stemofoline, isolated from the medicinal plant *Stemona japonica* and known as a potent nAChR agonist. Both sulfoximines and butenolides are usually not considered as neonicotinoids because their chemical structure only partially overlaps with that of neonicotinoids[127]. However, they are nAChR agonists acting at the orthosteric site and show an appreciable cross-resistance with neonicotinoids[128]. Unfortunately, recent studies suggest that these novel insecticides might be detrimental also for beneficial insects[129].
Contrary to the neonicotinoids, sulfoximines and butenolides, the mesoionic insecticides have a very weak nAChR agonism and act rather as competitive antagonist[130]. Triflumezopyrim (DuPont Crop Protection), the first commercial member of the mesoionic class to be commercialized in 2016, originated with byproducts of a fungicide optimization program[131]. Based on their unique chemical structure, their high level insecticidal activity and low toxicity towards pollinators, other mesoionic insecticides (dicloromezotiaz) are expected to appear in the next future. The new pyridylidene compounds (flupyrimin, Meji Seika Pharma) seem to have a similar mode of action [132].

4.2. nAChR channel blockers

Nereistoxin is a natural neurotoxin isolated from the marine annelid worm *Lumbriconereis heteropoda*, which has a high insecticidal activity and serves as a model for the development of synthetic derivatives including cartap (Takeda Chemical Industries). It is generally accepted that nereistoxin analogs are non-competitive analogs [3]. However, only few studies have analyzed their precise mode of action because of the low commercial success and the limited use of these insecticides (for instance they are not allowed in European Union).

4.3. nAChRs allosteric modulators

Spinosyns are natural or synthetic macrocyclic lactones characterized by a mode of action on nAChRs which is reminiscent to that of avermectins on GluCl. Indeed, spinosyns act as nAChR agonist at a site distinct from orthosteric site and synergizes with ACh[122]. These compounds are derivatives of spynosyns isolated from the actinomycete soil bacterium *Saccharopolyspora spinosa*. Spinosyns include spinosad (a mixture of natural spinosyns) and spinetoram (a mixture of semi-synthetic spynosins), which were commercialized both by Dow AgroSciences, in 1997 and in 2007, respectively[133]. It has been suggested that avermectins and spinosyns might share a similar binding site on GluCl and nAChR, respectively[134].
The venoms of spiders, scorpions or other animals are a tremendous source of toxins that might be used either as bio-pesticides or serve as lead compounds for the development of novel molecules[135]. Voltage gated channels and LGICs are targeted by toxins from a wide variety of species but today there is a single toxin commercialized as an insecticide: GS ω/κ HxTx Hv1a (Vestaron). This compound derives from toxins isolated from the venom of the funnel-wed spider *Hadronyche versuta*, which includes ω-hexatoxin-Hv1a (ω-HxTx-Hv1a, also known as ω-atracotoxin-Hv1a or ω-ACTX-Hv1a) and κ-hexatoxin-Hv1c (κ-HxTx-Hv1c, also known as Janus-faced-atracotoxin-Hv1c or J-ACTX-Hv1c)[136]. Since ω-HxTx-Hv1a and κ-HxTx-Hv1c have been described to specifically act on voltage gated Ca^{2+} channels[137] and Ca^{2+}-activated K^+ channels[138], respectively, it was thought that GS-ω/κ-HxTx-Hv1a might target also these channels. However, recent studies suggest that this insecticide is rather a positive allosteric modulator of nAChRs, acting similarly to spinosyns but at a different site[139].

4.4. Apis mellifera nAChRs

Genome sequencing projects have allowed the identification of nAChR genes in several insect species, including *Drosophila melanogaster*, *Apis mellifera*, and some crop pests and disease vectors. Although they possess a variable number of divergent subunits, insect nAChR subunits share similar features like the existence of α4 and α6 splice variants that differs at the ECD, or α6 RNA editing [117,140]. Unfortunately, the lack of expression in heterologous system prevents the analysis of the consequences of these modifications on functional properties and pharmacology. Moreover, very little is known on the stoichiometry (and arrangement) of native nAChRs although this is a prerequisite for studying physiologically relevant receptors. Lack of expression can sometimes be overcome by the co-expression of insect nAChR subunits with a vertebrate β subunit. Studies utilizing such “hybrid” receptors have shown that α8-containing receptors display higher sensitivity to dinetofuran than those with α1 or α3[141], and that α1/β subunit ratio influence the efficacy of imidacloprid and thiacloprid, but not clothianidine[142]. Efficient expression of *Apis mellifera* nAChRs has been obtained in a recent study when co-expressing three chaperone proteins: RIC-3, UNC-50 and TMX-3[143]. It has thereby been demonstrated that picomolar concentration of neonicotinoids, below the threshold for agonist actions, can inhibit ACh-mediated responses. This suggests that the insecticidal activity of neonicotinoids might be linked to an antagonist rather than agonist activity on nAChRs. Only few nAChRs have been studied so far but these results pave the way for future investigations including other subunit combinations and/or other chaperone proteins such as NACHO that is required for the functional expression of mammalian α7[144]. Indeed, the three chaperone proteins RIC-3, UNC-50 and TMX-3 do not allowed the functional expression of all honeybee nAChR subunit combinations and the identification of other chaperone proteins seems to be necessary (unpublished results).

4.5. Insecticide resistance and docking in nAChRs

X-ray structures of human α4β2[85] and α7[145] nAChRs, with nicotine and epibatidine, respectively, have recently been obtained. This obviously opens new avenues for the understanding of the mode of action of insecticides targeting these receptors because such structures with insect nAChRs are still lacking. Before these high resolution structures, an insight of the molecular architecture of nAChR has been provided by the ACh Binding Protein (AChBP) from *Lymnaea stagnalis* and *Aplysia californica*[146]. AChBP forms soluble pentamers that are homologous to the ECD of LGIC and X-ray structures obtained for AChBP with imidacloprid, clothianidine and

thiacloprid have revealed the critical role played by residues in loop B (W_{143}) and C (Y_{185}) from de principal (+) side and in loop D (Q_{55}) and E (L_{112}) in the complementary (-) side of the binding site. All of these residues are conserved in insects except Q_{55} which is replaced by a basic residue (R or K) in β subunits or an acidic residue (E) in α subunits. Crystallization of a mutated AChBP ($Q_{55}R$) reveals that the introduced R_{55} interacts with the nitro groups of imidacloprid and clothianidine and with the cyano group of thiacloprid suggesting the importance of this residue for the potency of neonicotinoids towards insect nAChRs[123]. Its mutation in *Drosophila melanogaster* α1 ($E_{78}K$) or β1 ($R_{81}T$) abolishes agonist and also antagonist activity of neonicotinoids on nAChRs[143,147]. Coincidently, $R_{81}T$ mutation was identified in resistant populations of the aphids *Myzus persicae* and *Aphis gossypii*[148,149]. In the other hand, laboratory selection of a resistant strain has identified a mutation at a conserved Tyr residue in the α1 and α3 nAChR subunits of *Nilaparvata lugens*[150]. This Tyr residue is not involved in direct interaction with neonicotinoids, but its mutation might affect the mobility of the neighboring Trp (W_{143}) in the loop B, crucial for neonicotinoid binding[151]. Genetic invalidation of the *Drosophila melanogaster* nAChR subunits highlighted the involvement of α1, α2, β1 and β2 in neonicotinoid-mediated toxicity, whereas resistance toward spinosyns was remarkably conferred only by the knock-out of the α6 subunit[152]. In fact, single point mutation or genetic alteration in the gene encoding the nAChR α6 subunit have been found in pests resistant to spinosoid insecticides[153,154].

Conclusions

Functional expression in heterologous expression system greatly enhances our understanding of honeybee voltage-gated and ligand-gated ion channels and allows the precise analysis of the effects produced by insecticides that target those membrane proteins. Moreover, heterologous expression allows to compare the effects produce by insecticides on the same target but from different species (i.e. pest versus beneficial) in similar conditions. This is especially important for the development of novel insecticides that might be less toxic towards beneficial insects and one can speculate that such analysis will be required in the next future before the commercialization of any new phytosanitary products.
To overcome concerns raised by insecticide resistance, agrochemical companies develop compounds with novel mode of action, which either act on new targets (such as TRP channels or G-protein coupled receptor) or at a site different from known insecticides (such $GABA_A$ competitive antagonist). Moreover, due to their high specificity, toxins are a promising source of insecticidal peptides that might target only one pest species leaving beneficial insects unaffected[155,156].
We can expect that in the near future many structure of insect channels and receptors will be solved by X-ray crystallography or cryo-EM[44]. Meanwhile, homology modelling, docking and molecular dynamic are precious tools to decipher the action of insecticides and to understand how target resistance may arise. Computational methods may also allow the design of new compounds or the optimization of existing molecules in the coming years[157].

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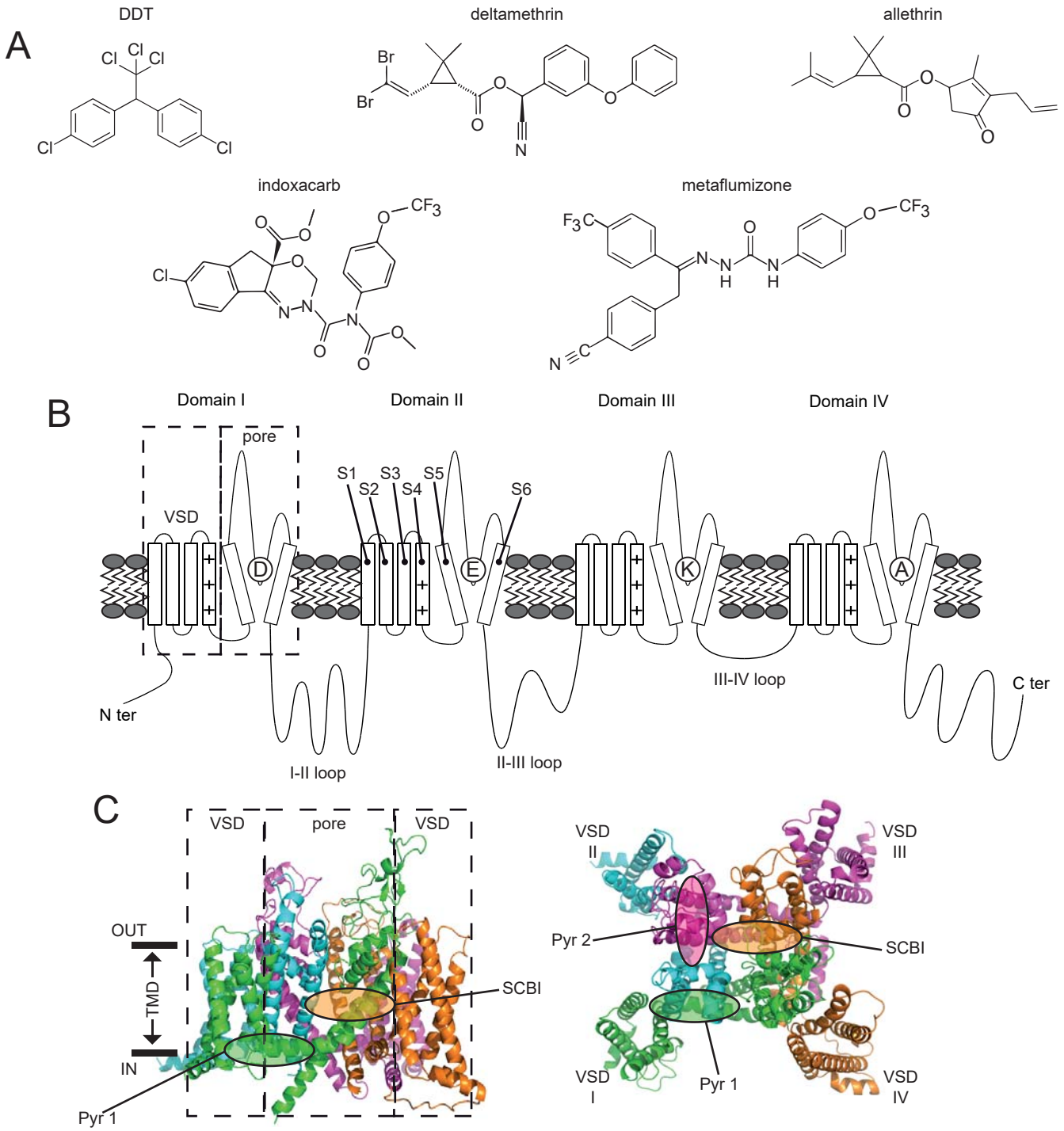

Figure 1. Insect voltage gated sodium channel (NaV) as insecticide target. A) Some insecticides targeting NaV. B) Schematic representation of the NaV1 subunit. C) Side view (left) and view from the extracellular side of Apis mellifera NaV1 homology model in which the binding sites for the Sodium Channel Blocker Insecticides (SCBI) and for pyrethroids (Pyr1 and Pyr2) are indicated. VSD : Voltage sensing domain, TMD : Transmembrane Domain

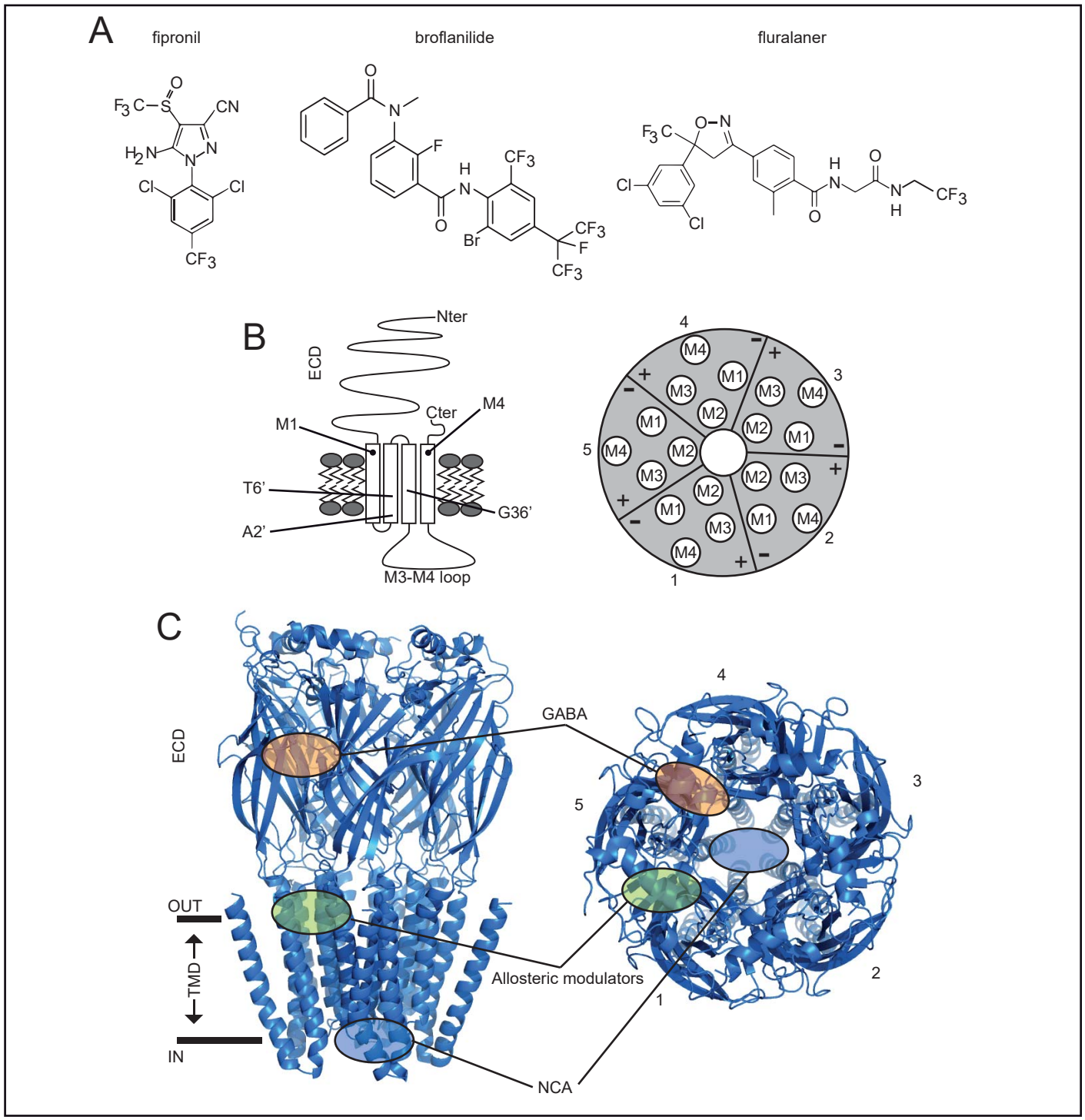

Figure 2. Insect GABA-gated chloride channel (GABAA) as insecticide target. A) Some insecticides targeting GABAA. B) Schematic representation of a GABAA subunit (left) and subunit arrangement in homopentameric receptor (right). C) Side view (left) and view from the extracellular side of Apis mellifera GABAA homology model in which the binding sites for the Non-Competitive Antagonists (NCA), allosteric modulator insecticides and endogenous ligand (GABA) are indicated. ECD : Extra Cellular Domain. TMD : Transmembrane Domain.

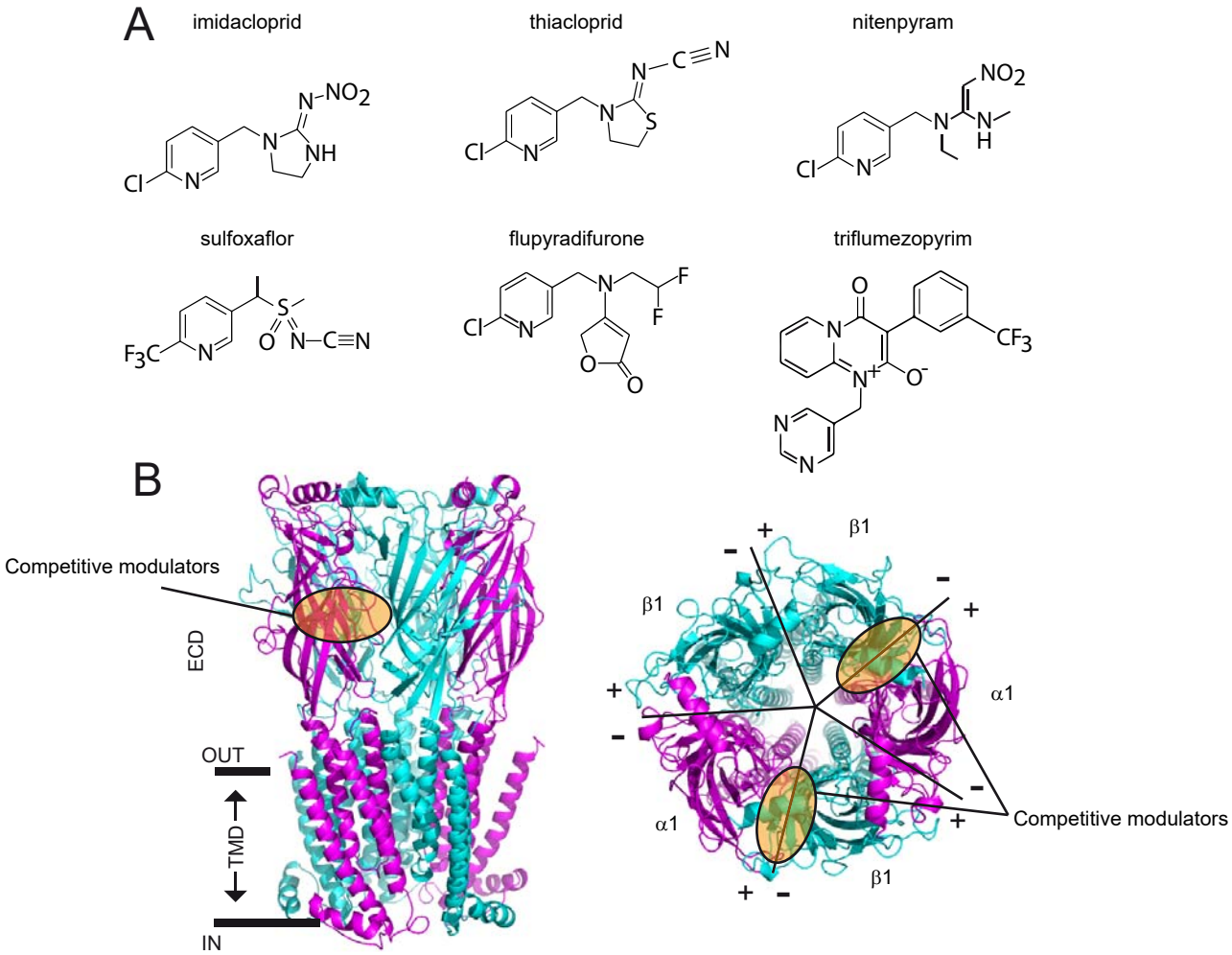

Figure 3. Insect nicotinic acetylcholine receptor (nAChR) as insecticide target. A) Some insecticides targeting nAChR. B) Side view (left) and view from the extracellular side of 1 1 nAChR homology model in which the binding sites for competitive modulator antagonists are indicated. ECD : Extra Cellular Domain. TMD : Transmembrane Domain.