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Mini Review

Novel GABA receptor pesticide targets

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ABSTRACT

The γ-aminobutyric acid (GABA) receptor has four distinct but overlapping and coupled targets of pesticide action importantly associated with little or no cross-resistance. The target sites are differentiated by binding assays with specific radioligands, resistant strains, site-directed mutagenesis and molecular modeling. Three of the targets are for non-competitive antagonists (NCAs) or channel blockers of widely varied chemotypes. The target of the first generation (20th century) NCAs differs between the larger or elongated compounds (NCA-IA) including many important insecticides of the past (cyclodienes and polychlorocycloalkanes) or present (fiproles) and the smaller or compact compounds (NCA-IB) highly toxic to mammals and known as cage convulsants, rodenticides or chemical threat agents. The target of greatest current interest is designated NCA-II for the second generation (21st century) of NCAs consisting for now of isoxazolines and meta-diamides. This new and uniquely different NCA-II site apparently differs enough between insects and mammals to confer selective toxicity. The fourth target is the avermectin site (AVE) for allosteric modulators of the chloride channel. NCA pesticides vary in molecular surface area and solvent accessible volume relative to avermectin with NCA-IBs at 20–22%, NCA-IAs at 40–45% and NCA-IIs at 57–60%. The same type of relationship relative to ligand-docked length is 27–43% for NCA-IBs, 63–71% for NCA-IAs and 85–105% for NCA-IIs. The four targets are compared by molecular modeling for the *Drosophila melanogaster* GABA-R. The principal sites of interaction are proposed to be: pore V1' and A2' for NCA-IB compounds; pore A2', L6' and T9' for NCA-IA compounds; pore T9' to S15' in proximity to M1/M3 subunit interface (or alternatively an interstitial site) for NCA-II compounds; and M1/M3, M2 interfaces for AVE. Understanding the relationships of these four binding sites is important in resistance management and in the discovery and use of safe and effective pest control agents.

1. GABAergic pesticides

Our continuing ability to control pests that compete for food and fiber and transmit disease is dependent on the discovery of new compounds and biochemical targets that circumvent cross-resistance patterns and give a fresh start in pesticide management to maintain effective control [1]. Any novel target is therefore a valuable contribution not only to science but also to human welfare. The γ-aminobutyric acid (GABA) receptor (GABA-R) is the target for many insecticides, acaricides, anthelmintics and rodenticides of widely varied structures [2–8]. The extracellular and transmembrane domains also have multiple targets for other antagonists, agonists and modulators of various types. There are two recent insecticide chemotypes added to this list, i.e. the isoxazolines and meta-diamides which do not appear to have target site cross-resistance with any other type of insecticide and are therefore of special importance and the focus of this review (Fig. 1; Table 1).

2. GABA receptor

2.1. Structure and function

GABA is the principal inhibitory neurotransmitter in the insect and mammalian nervous systems [5–11]. Ionotropic GABA-Rs are ligand-gated chloride channels consisting of five heteromeric subunits in mammals (usually two α subunits. two β subunits and an

Abbreviations: ave, avermectin; AVE, ave target; BPB, benzamidophenylbenzamide; *Dm*, *Drosophila melanogaster*; EBOB, 4'-ethynyl-4-*n*-propylbicycloorthobenzoate; flu, fluralaner; GABA, γ-aminobutyric acid; GABA-R, GABA receptor; $GABA_AR$, GABA-R of mammalian brain; IRAC, Insecticide Resistance Action Committee; mDA, meta-diamide; NCA, non-competitive antagonist; NCA-IA, NCA-IB, NCA-II, three NCA targets; RDL, insect GABA-R; TBPS, 4-*t*-butylbicyclophosphorothionate.

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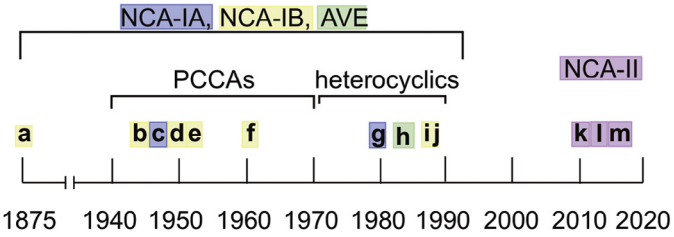

Fig. 1. Chronology of GABAergic noncompetitive antagonist and allosteric modulator pesticides. Year for discovery or first introduction: **a** picrotoxinin 1875, **b** lindane 1945, **c** TETS 1949, **d** dieldrin 1949, **e** toxaphene 1951, **f** α-endosulfan 1961, **g** TBPS 1979, **h** avermectin 1985, **i** fipronil 1988, **j** EBOB 1988, **k** flu 2010, **l** mDA 2013, **m** BPB 2013.

additional subunit) and presumably the homopentameric RDL subunit [12] in insects arranged around a central pore. The amino acid sequences and genomics are well characterized [10], and the individual subunits can be expressed in different combinations [13]. Each subunit has four transmembrane segments (M1, M2, M3 and M4) with both the N- and C- terminals located extracellularly. The receptor on binding to GABA changes conformation opening the pore allowing chloride anions to pass leading to inhibitory action.

2.2. Target site models

Most insect GABA-R studies involve *Musca domestica* or *Drosophila melanogaster* (*Dm*) head membranes or expressed RDL homopentamer receptor [6,12,14]. Mammalian brain membranes extensively used are rat, mouse and human with apparently very similar or identical results. The expressed mammalian receptor requires the β_3 subunit for high insecticide radioligand binding activity, which is modulated for sensitivity and specificity by α and γ subunits. The expressed $\alpha_1\beta_3\gamma_2$ receptor is typically used for binding assays and the $\alpha_1\beta_2\gamma_2$ for molecular modeling with respect to selective toxicity. The human expressed β_3 homopentamer is of special interest because it is similar in sensitivity and inhibitor specificity to the native housefly or RDL receptor [13,15,16]. The target site models are based on 1) mutants or sensitivity determinants in defining the relevant receptor locus, 2) the X-ray structure of the receptor or of a homologous anionic Cys-loop receptor, and 3) molecular dynamics to optimize the definition of ligand-binding site interactions. A large number of site-directed mutations have been examined for the β_3 homopentamer and RDL GABA-R to aid in locating the binding sites.

Although we reported a partial β_3 homopentamer model in 2006 [16], *de novo* generation of a new homology model was most prudent given the recent X-ray structures with high sequence homology including: PDB ID 2VL0, a ligand-gated ion channel from *Erwina chrysanthemi* [17]: 3RHW, a glutamate-gated chloride channel (GluCl) from *Caenorhabditis elegans* [18] in complex with ivermectin; and most recently 4COF, a human $GABA_AR$-β_3 homopentamer with benzamidine bound to the neurotransmitter site [11]. We built homology models of the *Dm* RDL (Uniprot P25123) using both 3RHW and 4COF as templates. Computational work presented here was done on the 3RHW-based model before 4COF was published. Preliminary results using the 4COF-based model are presented in the Supplementary Information. The 3RHW template is presumed to be in the open pore state whereas the 4COF template is in the closed state. Models were built using both Prime (version 3.6, Schrödinger, LLC, New York, NY, 2014) [19] and the Swiss-Model server [20–22]. Sequence alignment was based on BLAST with small manual adjustments. The M3/M4 intracellular connecting loop was replaced with 12 glycine residues based on other studies suggesting that a glycine-rich loop gives a stable geometry [10,23,24]. Models were refined using both Prime and Macromodel and a consensus homology structure was developed from this series of steps. Molecular dynamics simulations were performed using Desmond [25] including both a full membrane model surrounding the transmembrane helices and a water box encompassing the entire pentameric homology model with membrane. Each of three ligands (fipronil, TETS, R-fluralaner) was individually placed in the pore of this homology model + membrane + water box and subjected to 24 ns of molecular dynamics simulation. An additional 24 ns molecular dynamics run was performed with no ligand in the pore, but with 5 ivermectin units in the transmembrane interstitial binding region. Additionally, we performed docking calculations using Glide [19]. The receptor for Glide calculations was derived from the fully mature molecular dynamics refined, ligand-bound structures.

Table 1
Properties of GABA receptor pesticide targets[a].

Property	NCA-IA	NCA-IB	NCA-II			AVE
			isoxazoline	Meta-diamides		
				mDA-7	BPB-1	
Radioligand	[^{3}H]EBOB	[^{35}S]TBPS	[^{3}H]flu	-	[^{3}H]BPB-1	[^{3}H]ave
Molecular size (%)						
Relative to ave (%)[b]	36–40	23–26	59–60	58–60	57–58	100
Extended length (Å)[c]	12–13	5–8	20	16	20	19
Receptor example	*Musca*	mammal	*Musca*	*Spodoptera*	*Musca*	*Musca*
Response to						
GABA	inhib	inhib	inhib	-	inhib	no
Glutamate	-	-	-	-	poor	-
Fipronil	inhib	inhib	poor	-	enhance	no
Cyclodiene	inhib	inhib	no	-	enhance	no
Avermectin	inhib	-	inhib	-	inhib	inhib
Inhibitor SAR[d]	yes	yes	yes	yes	yes	yes
Temp coeff	positive	negative	-	-	-	-
Resistance						
IRAC classification	**2A, 2B**[e]	no	no	no	no	**6**[e]
Rdl dieldrin	yes	no	no	no	no	no

[a] References given at appropriate sections in the text.
[b] Range of surface area and solvent accessible volume for each chemotype (See Supplementary Material Table S1 and Fig. S2).
[c] Extended length in the RDL GABA-R pore from this report.
[d] Structure–activity relationships (SAR) as inhibitors of radioligand binding, ^{36}Chloride uptake or GABA response predicts toxicity (see Fig. 5 and Section 6.2).
[e] IRAC classification numbers considered in the text.

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3. First generation non-competitive antagonists

3.1. NCA-IA site

3.1.1. Structures

The first generation or 20th century GABAergic insecticides, designated here as non-competitive antagonist (NCA) Type IA (NCA-IA) (Fig. 1), consist of many major commercial compounds (Fig. 2 and Supplementary Material Fig. S1). The botanical picrotoxinin was reported in 1875 to control pest insects. The polychlorocycloalkanes (lindane and toxaphene) and cyclodienes (e.g. dieldrin and α-endosulfan) were introduced in 1945–1961 and the phenylpyrazole fipronil in 1988. A large number and variety of heterocyclics including 4-*t*-butylbicyclophosphorothionate (TBPS) and 4'-ethynyl-4-*n*-propylbicycloorthobenzoate (EBOB) were examined in the author's laboratory in the 1980s [2–4]. NCA-IA binding assays first used the radioligand [^{3}H]dihydropicrotoxinin [26–28], which was soon replaced by [^{35}S]TBPS [29] and [^{3}H]EBOB [30]. [^{3}H]α-Endosulfan [31] and particularly [^{3}H]BIDN [32] proved to be the most useful of the polychlorocycloalkanes for this target. Photoaffinity probes have been identified [33,34] but not used in structural assignment of the NCA-IA binding site.

3.1.2. NCA action, resistance and binding sites

The polychlorocycloalkanes, cyclodienes and fiproles are NCAs and inhibit the binding of diagnostic NCA-IA radioligands indicated above and inhibit GABA-induced neuroactivity and GABA-induced chloride flux [35–38] (Table 1). Resistant strains and mutants were particularly important in defining the NCA-IA target site. In a seminal study ffrench-Constant and colleagues [14] established that housefly resistance to dieldrin is attributable to a GABA-R A2'S mutation, which was then found to confer low sensitivity of the binding site [39]. It was soon recognized that broad cross-resistance to polychlorocycloalkanes was conferred by this A2'S mutation [40] but to varying degrees for dieldrin, α-endosulfan, fipronil and other NCA-IA compounds. Site-directed mutagenesis, cysteine scanning and molecular modeling of the NCA-IA target established the importance of interactions with channel-lining residues A2', T6' and L9' [6,7,16,41], a conclusion which is essentially unchanged when updated based on the docking positions for fipronil and EBOB (Fig. 2) in our current models. Diazepam and phenobarbital are effective antidotes for NCA-IAs [42].

3.2. NCA-IB site

NCA-IB compounds were serendipitous discoveries in the late 20th century (Fig. 1) as toxicants for mammals in studies on resins used in wool impregnation (TETS) [43], oxidation of phosphorothionates [42,44,45] and thermal degradation of phosphorus flame retardants [46]. TETS [47], TBPS [29] and its oxon analog (TBPO) [45,48] are highly toxic to mammals (mouse ip LD_{50} values 0.03–0.3 mg/kg) acting as convulsants and considered to be chemical threat agents [49,50]. They are small cage compounds (Type B or NCA-IB cage convulsants) with lower receptor potency relative to their toxicity than larger or more elongated compounds (NCA-IA, insecticides) [51]. TETS was developed and later banned as a rodenticide [50]. The NCA-IB target can be assayed with [^{35}S]TBPS [52,53] or [^{3}H]EBOB [30] for *Musca* and with [^{35}S]TBPS or [^{14}C]TETS for mammalian brain [29,50]. There is no cross-resistance of NCA-IBs to dieldrin in RDL houseflies [54]. Molecular dynamics modeling with the human $\alpha_1\beta_2\gamma_2$ receptor positions TETS with TBPS deep in the channel in the 1' to 2' region [50] as also found here for the RDL GABA-R (Fig. 2). [^{35}S]TBPS binding in mammalian brain membranes is inhibited by GABA, fipronil and cyclodienes. The toxicity of NCA-IBs to *Musca* has a negative temperature coefficient in contrast to the positive coefficient for more elongated (Type A) compounds [55]. Their toxicity to mice is ameliorated by phenobarbital and diazepam [42]. The chemical threat status of TETS focused attention on candidate antidotal agents with special attention to diazepam, midazolam, propofol, allopreganolone and the NMDA antagonist MK-801 [50,56].

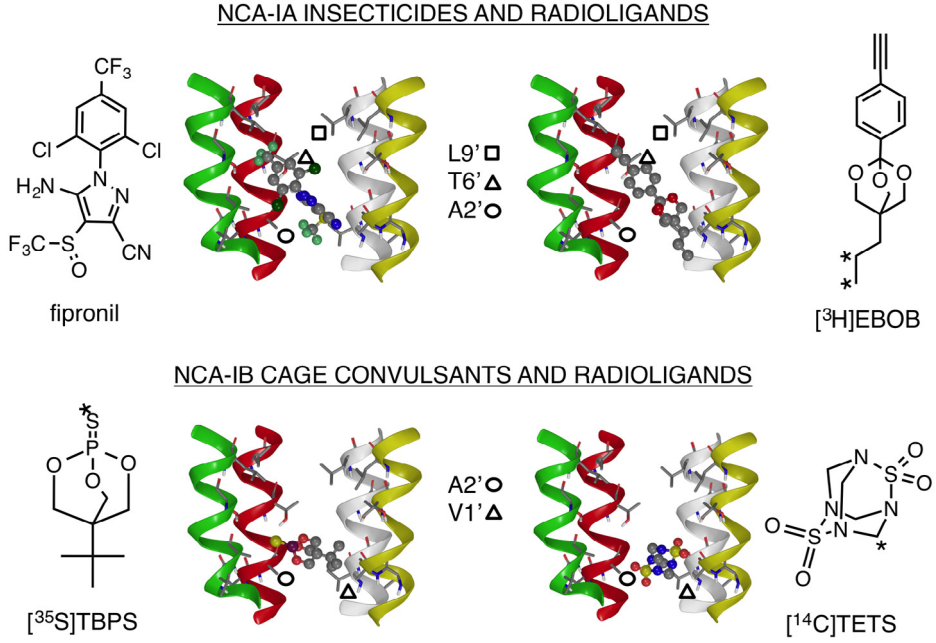

Fig. 2. First generation non-competitive antagonists (NCA-I) showing example radioligands (asterisks indicate labeling positions) and proposed *Dm* RDL GABA-R binding sites (homology model based on 3RHW) for NCA-IA compounds (fipronil and EBOB) and NCA-IB compounds (TBPS and TETS). Four of the 5 individual M2 transmembrane pore helices are shown in the binding vicinity. One M2 helix has not been displayed to provide a view into the pore.

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4. Second generation non-competitive antagonists: NCA-II site

4.1. Structures

The last 5 years has been an exciting time in studies of the GABAergic insecticides with the announcement of two second generation or 21st century chemotypes acting as NCAs and designated here as NCA-II (Figs. 1 and 3). The research in every case started from phthalic and anthranilic diamides, which are activators of insect ryanodine receptors, and ended up with insecticides of a completely different mode of action. Researchers at Nissan Chemical Industries in Japan and DuPont in the United States discovered insecticidal isoxazolines optimized to fluralaner (flu) and afoxolaner, respectively, as current commercial compounds. Scientists at Mitsui Chemicals Agro in Japan discovered the meta-diamides including benzamidophenylbenzamides (BPBs).

4.2. NCA action

Several types of evidence establish that isoxazolines [57–66] and meta-diamides (including mDA-7 [24,67] and BPB-1 [68]) are NCAs but at a different high affinity site(s) than those for compounds acting at the NCA-IA, NCA-IB and AVE targets (Table 1). There is subnanomolar [^{3}H]EBOB IC_{50} for some NCA-II compounds and apparent K_d for NCA-II radioligands [^{3}H]flu and [^{3}H]BPB-1 for a high affinity site(s) but with a portion of lower affinity sites. [^{3}H]BPB-1 binding is allosterically inhibited by micromolar GABA. Isoxazoline and meta-diamide chemotypes inhibit GABA-induced currents in housefly GABA-Rs expressed in *Xenopus* oocytes but are less potent or failed to inhibit L-glutamate-induced currents in inhibitory L-glutamate receptors. Fipronil is a weak inhibitor of [^{3}H]flu binding but strongly stimulates [^{3}H]BPB-1 binding. Ave is a potent inhibitor of both [^{3}H]flu and [^{3}H]BPB-1 specific binding.

4.3. Resistance and NCA-II binding site

There is no NCA-II target site cross-resistance with the NCA-IA insecticides, but three mutations [M3/G336M (homologous to G319M in *Spodoptera littoralis* SL-RDL), M1/I277F and M1/L281C] in the RDL GABA-R reduce its sensitivity to meta-diamides [24,67]. Homology modeling herein showing a clean overlay of isoxazolines and meta-diamides suggests the NCA-II localization is in the pore T9' to S15' region (Fig. 3), an area which is adjacent to the AVE M2/M3, M1 interstitial subunit region. The mutations mentioned above occur in this interstitial subunit area and thus might change the shape of the pore, affecting meta-diamide binding. It has also been suggested that the NCA-II target site is directly in this interstitial region, a new location perhaps bridging the pore and the AVE site [24]. Our modeling does not eliminate the possibility of this alternate NCA-II site (Supplementary Material Fig. S3). It is conceivable that the NCA-II enters in the pore and then migrates to the interstitial region or vice versa. As further speculation perhaps the meta-diamides trigger closing or stabilize the closed state. The NCA-II site(s) defined by *Musca* binding assays with [^{3}H]flu [58,66] and [^{3}H]BPB-1 [68] is probably the same target for isoxazolines and meta-diamides [66].

5. Allosteric modulator (AVE)

The macrocyclic lactone ave (Fig. 4) was first used as an antiparasitic drug in 1981 and as an agricultural pesticide in 1985 [69] and several analogs and derivatives (such as emamectin benzoate, lepimectin and milbemectin) are also important commercial compounds [1]. Ivermectin was the essential agent in greatly reducing the incidence of river blindness in millions of people by controlling the schistisome vector [69]. Ave is a positive allosteric modulator of several ligand-gated channels including GABA- and glutamate-gated chloride channels and the α7-nicotinic receptor [70–73]. The GABA-R target for ave is designated here as AVE. [^{3}H]Ave is very effective as a radioligand for both insects and mammals in defining AVE action [55,73]. [^{3}H]Ave binding in *Musca* is not inhibited by GABA, fipronil or cyclodienes but is by flu. The *C. elegans* glutamate-gated chloride channel was important in structural definition of the RDL AVE site although a muscle glutamate receptor may be involved in contributing to or the cause of the toxicity [69–75]. Decreased binding is conferred by *in silico* mutations to A/Q6 and B1/S58 [75]. The ave binding site appears to be in an interstitial region between M2/M3 of one subunit and M1 of an adjacent subunit, a site which is proximal to the L9' to S16' pore region. Interactions modulate chloride flux at low ave concentrations and block the channel at high levels.

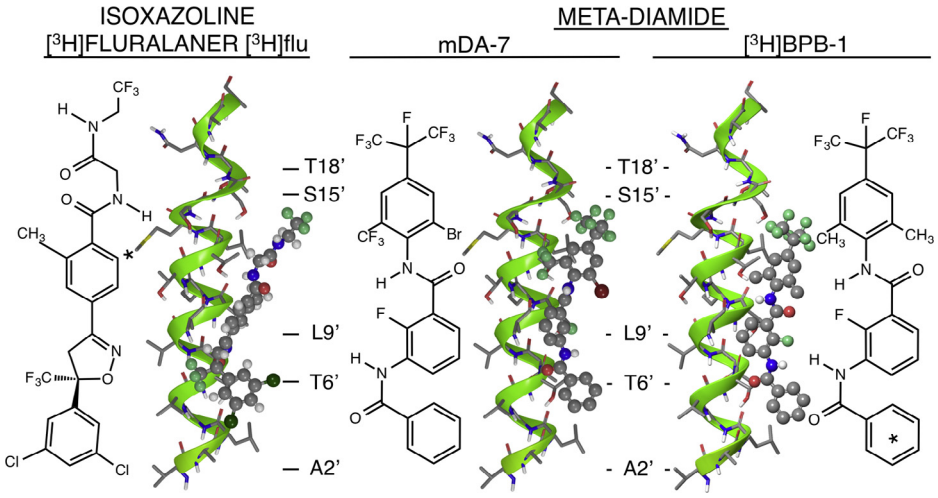

Fig. 3. Second generation isoxazoline and meta-diamide non-competitive antagonists (NCA-II) and their proposed *Dm* RDL GABA-R binding sites (homology model based on 3RHW) displayed with single M2 transmembrane pore helix (1 of 5 M2 helices in the channel pore). Asterisks indicate ^{3}H labeling positions of radioligands. Afoxolaner is the fluralaner analog with CF_3 replacing one Cl and naphthyl replacing tolyl. Meta-diamide BPB-1 (68) is also referred to as mDA-1 (67).

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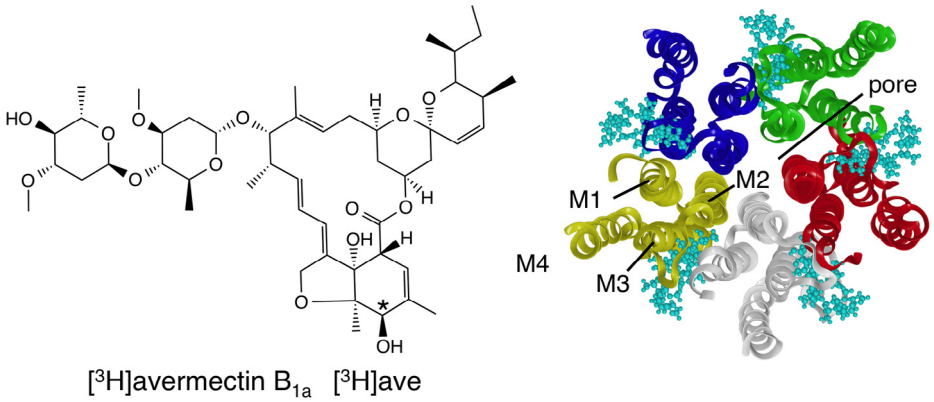

Fig. 4. Allosteric modulator ave showing radioligand [^{3}H]avermectin B_{1a} and *Dm* RDL GABA-R binding sites (homology model based on 3RHW). Abamectin is avermectin B_{1a} (docking position illustrated in cyan) and B_{1b} (methyl replaces ethyl substituent). Ivermectin is 22,23-dihydroavermectin.

6. Four distinct binding sites

6.1. Radioligand binding assays

Radioligand binding studies play an important role in defining GABAergic pesticide action. The best radioligand for each binding site is the pesticide itself or an arguably relevant analog, highly potent (i.e. high affinity and percent-specific binding) and available or attainable at adequate specific activity (normally ^{3}H, ^{32}P or ^{35}S analyzed by liquid scintillation counting but recently ^{14}C quantified by accelerator mass spectroscopy [50]). The radioligands considered here for the four GABAergic targets (Table 1) meet most or all of these requirements. The receptor source must be relevant (nerve, cell, membrane or expressed GABA-R) and readily available (culture or purchase) as the sensitive native material perhaps supplemented by less sensitive versions from selected resistant strains or from expressed receptor with low sensitivity from site-directed mutagenesis.

6.2. Toxicological relevance

The relevance of a target site assay involving radioligand binding or physiological response (e.g. ^{36}chloride uptake or GABA induced signals) is normally established by structure–activity relationships in which the *in vitro* potency for a series of compounds should be correlated with or predictive of their toxicity (using a CYP450-inhibiting synergist when appropriate). This structure–activity relationship criterion is met for NCA-IA and NCA-IB compounds assayed as either [^{35}S]TBPS binding (Fig. 5A) or ^{36}chloride uptake (Fig. 5B) in mammalian brain membranes. Inhibition of housefly membrane NCA-IB [^{35}S]TBPS binding by a series of cyclodiene insecticides also follows the same potency trend as their injected toxicity to houseflies [52]. The most extensive data set is for NCA-IA compounds of many chemotypes assayed with housefly head membranes and toxicity to houseflies with piperonyl butoxide synergist clearly establishing a target assay–toxicity correlation involving widely varied structures (Fig. 5C) [35]. The same housefly systems with [^{3}H]ave and ave analogs also validate the relevance of assays of the AVE site (Fig. 5D) [55,76]. The data sets are smaller and of different types for the NCA-II compounds with the isoxazolines based on binding assays and GABA response (Fig. 5E and 5F) [61,66] and the BPBs on radioligand results (Fig. 5G) [68]. Despite the diversity of *in vitro* assays and toxicity criteria it is clear that the targets being measured are relevant to the toxicity.

6.3. Overlapping and coupled targets

The proposed relationship of these four binding sites in the *Dm* RDL GABA-R (Fig. 6 and Supplementary Material Figs. S4 and S5) indicates the overlapping and coupled nature of the targets. The pesticides differ greatly in size and physiochemical properties. The molecular surface area and solvent accessible volume of the ligands increase in the order of those for NCA-IB < NCA-IA < NCA-II < ave (Table 1, Supplementary Material Table S1 and Fig. S2). Their extended length in the GABA-R pore of the illustrated model is 5–8 Å for NCA-IB, 12–13 Å for NCA-IA and 16–20 Å for NCA-II (Table 1). The number of halogens for compounds illustrated here is zero for NCA-IB and AVE, 0–6 chlorines and 0–6 fluorines for NCA-IA, and 0–2 chlorines or bromines and 6–11 fluorines for NCA-II. [^{3}H]EBOB specific binding in *Musca* is strongly and fully inhibited by NCA-IA and NCA-IB compounds but only partially by NCA-II and ave insecticides. The NCA-IB [^{35}S]TBPS or [^{14}C]TETS binding site interactions appear to follow the same pattern for NCA-IA, NCA-IB and ave compounds. [^{3}H]Flu binding in *Musca* is insensitive or poorly sensitive to NCA-IA compounds but very sensitive to aves. In agreement, [^{3}H]ave binding is sensitive to NCA-II flu but NCA-IA and NCA-IB compounds are not inhibitors [59,66] (Table 1). It is not known to what extent if any these binding site interactions for different GABAergic agents noted here *in vitro* might be applicable to toxicity changes *in vivo*.

6.4. Selective toxicity

GABA-R target site sensitivity and specificity play important roles in selective toxicity between pest strains, insect species and non-target organisms. The receptor preparations considered here are generally mammalian brain membranes and expressed $GABA_AR$ β_3 homopentamer compared with *Musca* and *Dm* head membranes and expressed RDL GABA-R. NCA-IA compounds are only moderately selective between insects and mammals and their GABA-Rs although lindane is somewhat more selective than the cyclodienes [13]. The selectivity is improved with fiproles [77] optimized from a great variety of insecticidal heterocycles [4,7]. NCA-IB compounds are highly toxic cage convulsants to mammals leading to their use as rodenticides and importance as chemical threat agents. The low potency of NCA-IIs in mammalian brain and β_3 homopentamer $GABA_AR$ binding assays [58,66] and use in animal health considered later indicate favorable selectivity but little information is available on these new NCAs used directly as radioligands with

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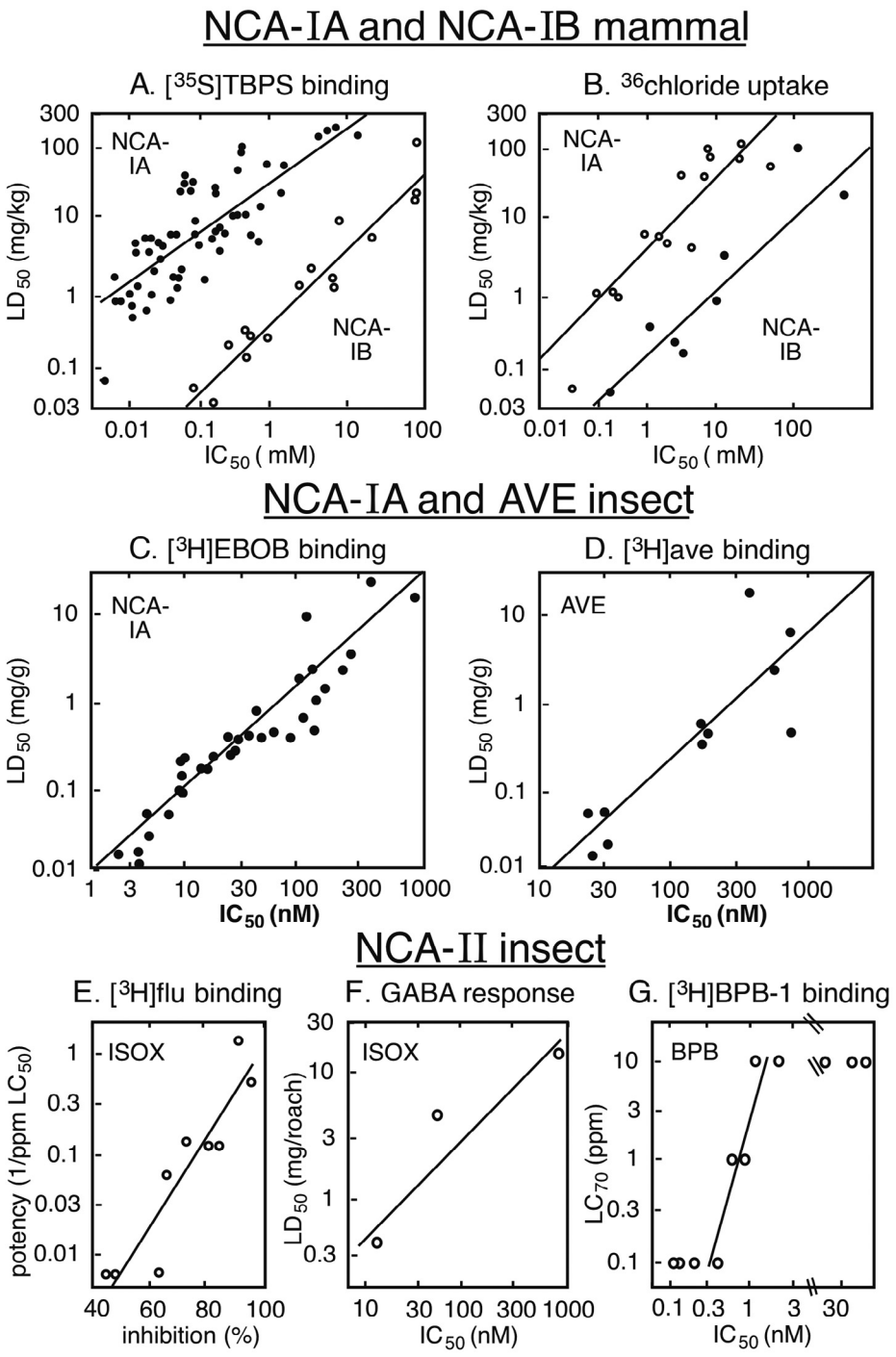

Fig. 5. Correlation for GABAergic agents as inhibitors of GABA-R radioligand binding or GABA response and toxicity. A. NCA-IA insecticides and NCA-IB cage convulsants as inhibitors of [^{35}S]TBPS binding in rat brain membranes and ip LD_{50} in mice. NCA-IA: r = 0.77, n = 60; NCA-IB: r = 0.96, n = 15. Adapted from reference 51. B. NCA-IA insecticides and NCA-IB cage convulsants as inhibitors of ^{36}chloride uptake in rat brain membranes and ip LD_{50} in mice. NCA-IA: r = 0.90, n = 15; NCA-IB: r = 0.92, n = 8. Adapted from reference 51. C. NCA-IA insecticides as inhibitors of [^{3}H]EBOB binding in housefly head membranes and topical LD_{50} to houseflies with CYP450-inhibiting synergist (piperonyl butoxide). r = 0.89, n = 34. Adapted from reference 35. D. AVE insecticides as inhibitors of [^{3}H]ave binding in housefly head membranes and topical LD_{50} to houseflies with CYP450-inhibiting synergist (piperonyl butoxide). r = 0.83, n = 11. Adapted from reference 55. E. NCA-II isoxazoline insecticides as inhibitors of [^{3}H]flu binding in *Apis mellifera* head membranes and potency (1/ ppm LC_{50}) to *Empoasca fabae* r = 0.84, n = 9. Data from references 61 and 66. F. NCA-II isoxazoline insecticides as inhibitors of GABA response of thoracic neurons and toxicity in *Periplaneta americana.* n = 3. Data from reference 61. G. NCA-II meta-diamide insecticides as inhibitors of [^{3}H]BPB-1 binding in housefly head membranes and dietary ppm LC_{70} to *Spodoptera littoralis* larvae. Toxicity data are given as high, medium or low shown as <0.1, 0.1 and 10 ppm respectively. n = 11 with deletion of *N*-methyl proinsecticides from the data set of Ozoe et al [68].

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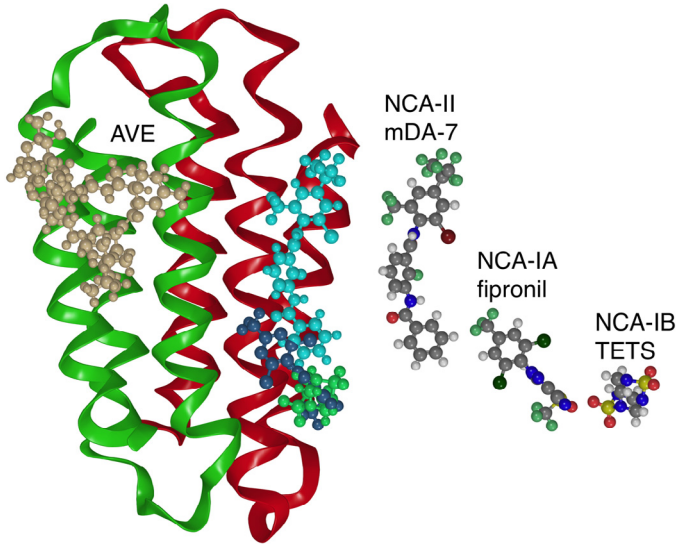

Fig. 6. Four unique proposed *Dm* RDL GABA-R binding sites (homology model based on 3RHW). Left to right, one subunit in green (front) is displayed with helices M3, M1, M2: second subunit in red is left to right M3, M2, M1. NCA-IA, NCA-IB and NCA-II pesticides in the channel pore shown together (interactions with M2) and also shown separately maintaining the same relative positions. Ave at the interface of two transmembrane subunits. Amino acid residues are identified in Supplementary Material Fig. S4.

mammalian GABA-Rs. There is extensive human toxicology data from the use of ivermectin for schistosomiasis control, and toxicity problems are largely avoided by the low doses required for human therapy and when employed as insecticides for pets and farm animals [69].

6.5. Cross-resistance

Resistance following pesticide selection of pest populations or site-directed mutagenesis is indicated when the strain survives a normally lethal dose or has reduced sensitivity in neuroactivity, chloride flux or binding assays (Table 1). Genomic definition of the mutations conferring resistance then allows monitoring their incidence in field populations [78]. The Insecticide Resistance Action Committee (IRAC) [1] classifies GABAergic insecticides on the basis of recommendations for use to avoid cross-resistance into Categories **2A** (organochlorines and cyclodienes, i.e. endosulfan and chlordane), **2B** (phenylpyrazoles or fiproles, i.e. fipronil and ethiprole) and **6** (macrocyclics such as abamectin, emamectin benzoate, lepimectin and milbemectin). The new isoxazolines and metadiamides for now have no IRAC classification. These NCA-II chemotypes are not cross-resistant with categories **2A, 2B** and **6** (i.e. compounds acting at the NCA-IA and AVE targets) which therefore makes them of special interest.

7. Prospects

The prospects for GABAergic pesticides can be projected from the rate at which new compounds have been introduced (Fig. 1) and the amounts used. More than three billion pounds of NCA-IA insecticides were used in the past seven decades. There was no target site cross-resistance of the NCA-IA compounds to DDT, organophosphate or any other major insecticide chemotypes. Use of NCA-IAs drastically declined in the late 20th century with problems of resistance, persistence and environmental toxicology. Their effectiveness was due in part to their long persistence, but this ultimately proved to be a major reason for their demise. The Stockholm Convention on Persistent Organic Pollutants [79] in 2001 banned most organochlorine and cyclodiene insecticides (NCA-IA), reducing but not eliminating their use. However the GABAergic insecticides are still very important with the 2012 world end use value for fipronil of $688 million and for abamectin of $938 million [80]. Insecticide target rankings in 2012 world sales were led by the nicotinic receptor (37%) then the sodium channel and GABA-R (each 16–17%) and the ryanodine receptor and acetylcholinesterase (each 10–11%) with 9% other targets [81]. A 2012 compilation considering the chronology and numbers for introduction of the current insecticides gave the GABA-R targeting compounds as only 1.7% of the total with half of them introduced by 1955 [81]. Newly introduced GABAergic insecticides are flu and afoxolaner used for flea and tick control on cats and dogs [58,62–65]. There are also important crop pests highly sensitive to NCA-IIs [24,60,82] indicating possible expanded use on optimization. Just as NCA-IA was recognized over time as the target for an increasing variety of insecticides [16] so the NCA-II target may ultimately be the binding site for more than the two chemotypes considered here. The NCA-IIs appear to have fewer limitations of target site cross-resistance and selective toxicity. No detailed reports are available yet on the metabolism, persistence or environmental toxicology of the new NCA-II insecticides but for now there is optimism that they can at least partially replace the NCA-IAs as safe and effective pest control agents.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.pestbp.2014.11.006.

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