1 **REVIEW**

2 **Neonicotinoid insecticides mode of action on insect nicotinic acetylcholine receptors using**
3 **binding studies**

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14

15 **Abstract**

16 Nicotinic acetylcholine receptors (nAChRs) are the main target of neonicotinoid insecticides,
17 which are widely used in crop protection against insect pests. Electrophysiological and
18 molecular approaches have demonstrated the presence of several nAChR subtypes with
19 different affinities for neonicotinoid insecticides. However, the precise mode of action of
20 neonicotinoids on insect nAChRs remains to be elucidated. Radioligand binding studies with
21 [^{3}H]-α-bungarotoxin and [^{3}H]-imidacloprid have proved instructive in understanding ligand
22 binding interactions between insect nAChRs and neonicotinoid insecticides. The precise
23 binding site interactions have been established using membranes from whole body and specific
24 tissues. In this review, we discuss findings concerning the number of nAChR binding sites
25 against neonicotinoid insecticides from radioligand binding studies on native tissues. We
26 summarize the data available in the literature and compare the binding properties of the most
27 commonly used neonicotinoid insecticides in several insect species. Finally, we demonstrate
28 that neonicotinoid-nAChR binding sites are also linked to biological samples used and insect
29 species.

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31 Keywords : insect; nicotinic receptors; neonicotinoid insecticides; binding studies.

32

33 **Introduction**

34 Nicotinic acetylcholine receptors (nAChRs) are involved in rapid neurotransmission in
35 both insect and mammalian nervous systems and play major roles in learning and memory [1–
36 3]. Because of these central roles, they are the main target of neonicotinoid insecticides which
37 are used as a chemical method worldwide to control insect pest [4]. However, this has led to
38 the evolution of resistance resulting in a reduction in effectiveness [5–8], environmental
39 concerns linked to the accumulation of these compounds and potential effects on non-target
40 insects such as pollinators [9–12]. Currently, binding studies are used to monitor and analyze
41 the mode of action of neonicotinoid insecticides on insect native nAChRs in order to understand
42 the levels of resistance. Binding studies, as well as the use of electrophysiology, have proven
43 instructive in identifying different nAChR subtypes as well as providing insights into their
44 pharmacological properties. For instance, studies using the patch clamp method demonstrated
45 that imidacloprid (IMI), the forerunner of neonicotioid insecticides, is a partial agonist of insect
46 nAChRs [13–16] while clothianidin (CLT) and acetamiprid (ACE) appear to be full agonists
47 [17]. Moreover, as it is the case with vertebrates, it is possible to identify insect α-bungarotoxin
48 (α-Bgt)-sensitive and -insensitive nAChR subtypes through binding studies [16,18–20]. α-Bgt
49 is a snake toxin commonly used in vertebrates to characterize homomeric nAChRs such as α7
50 receptors [21–23] even though several studies have demonstrated that it can bind to heteromeric
51 α9α10 and homomeric α8 receptors [24,25]. In insect species, CLT binds to both α-Bgt-
52 sensitive and -insensitive receptors expressed in the cockroach dorsal unpaired median (DUM)
53 neurons whereas, IMI and ACE may bind to α-Bgt-insensitive receptors with the finding that
54 ACE could acts as an agonist of both DUM neuron nAChR1 and imidacloprid-insensitive
55 nAChR2 subtypes [26–30]. Using electrophysiology on cockroach thoracic ganglia, it was
56 found that IMI inhibited a desensitized α-Bgt-sensitive receptor called nAChD, not usually
57 detected in binding assays with radiolabeled α-Bgt [18,31]. Despite this discrepancy between

electrophysiology and binding assays, both approaches have demonstrated the presence of several nAChR subtypes with different pharmacological profiles.

It is likely that the variation in pharmacological properties is due to the presence of several different nAChR subunits. Analysis of genome sequences have revealed that insect nAChR gene families commonly consisting of 10 to 12 nAChR subunit genes as demonstrated by the pest species *Tribolium casteneum* [32], *Nilaparvata lugens* [33], *Myzus persicae* [34], and *Acyrthosiphon pisum* [35]. nAChRs consist of five subunits arranged around a central ion channel with the combination of subunits determining the functional and pharmacological properties of the receptor. As with their vertebrate counterparts, the insect subunits have been classified into homomeric and heteromeric receptors. For instance, heterologous expression studies have demonstrated that *Drosophila melanogaster* α5 and α7 subunits can form functional homomeric nAChRs [36] which bind α-Bgt [36,37]. Thus, homomeric receptors possess five ligand binding sites for acetylcholine (ACh) while heteromeric receptors consisting of α and β subunits can present three binding sites for ACh at the interface between two different subunits. The implication that both highly conserved subunits as well as divergent subunits [38] form neonicotinoid binding sites could lead to common or species specific pharmacological properties. Whist co-immunoprecipitation studies have highlighted potential associations of several subunits [39,40] the subunit combination of insect nAChRs remains to be elucidated.

Ligand-binding interactions between neonicotinoid insecticides and insect nAChRs were studied using different insect tissues ranging from the brain tissue to the whole body. Two binding experiments are currently used: saturation and competition experiments [41]. In saturation experiments, the binding of an increasing series of radioligand concentrations is measured at equilibrium and analyzed to determine the binding constant (affinity constant K or dissociation constant K_d) and the concentration of specific binding sites for the radioligand

83 (R_T); the experimental estimation of R_T is usually designated B_{max} [41]. ^{3}H does not alter the
84 chemical structure of the radioligand and has a longer half-life compared to ^{125}I, which has a
85 higher specific activity and is particularly useful if the density of receptors is low [42]. In
86 competition experiments the binding of one or more fixed concentrations of a radioligand is
87 measured at equilibrium in the presence of increasing concentrations of a non-labeled
88 compound. The data are analyzed to determine the binding constant of the non-labeled
89 compound and the cooperativity between the non-labeled compound and the radioligand for
90 binding to the radioligand-sensitive receptors. The equilibrium inhibition constant K_i is
91 calculated from such experiments using the Cheng-Prusoff transformation [41]. According to
92 the biological samples used, these binding experiments can lead to different interpretations of
93 the results. In the present review, we summarize our current knowledge on the binding
94 experiments to characterize neonicotinoid binding sites on insect neuronal nAChRs.

95

96 ***Localization of nicotinic acetylcholine receptors by labeled α-Bgt***

97 Electrophysiological studies on insect neurons have demonstrated that they can exhibit nicotinic
98 receptors which are blocked by α-Bgt [19,43–45]. Thus, as for vertebrates, it was suggested that
99 α-Bgt binding sites preferentially reflect the expression of homomeric α5 or α7 receptors
100 [36,37]. Saturation experiments using either [^{3}H]-α-Bgt and [^{125}I]-α-Bgt highlighted the
101 presence of several α-Bgt binding sites in membrane preparations of different insect species
102 (Table 1). For example, saturable binding consistent with the presence of both high and low
103 affinity binding sites for [^{125}I]-α-Bgt was found in the aphid *M. persicae* [46]. As shown in
104 Table 1, dissociation constants and maximal binding capacities were consistent with multiple
105 receptor subtypes. The aphid *A. pisum* possessed very high affinity binding sites (with a Kd
106 value less than 0.1 nM) [37], notably higher than those of *M. persicae*, suggesting that

107 differences exist within hemiptera both in the numbers and affinities of binding sites. For
108 comparison, only one specific binding site was found in *Manduca sexta* [47], *Apis mellifera*
109 [48], and *Locusta migratoria* [49] membrane preparations from nervous tissues. The ambiguity
110 of assigning several binding sites is further apparent with *D. melanogaster*. In one study, a
111 single binding site was found [50] whilst an additional binding site with very high affinity was
112 found in another study [51], both using adult heads. The discrepancy seems to be associated
113 with the use of ^{125}I or ^{3}H isotopes suggesting that only [^{125}I]-α-Bgt is suitable to highlight the
114 very high affinity binding sites. The biological samples used can also influence the
115 quantification of binding sites. In *M. sexta* [47] and *D. melanogaster* [51], B_{max} values showed
116 a higher number of binding sites in adults compared to embryonic or larval stages. This finding
117 was not surprising since it was proposed that the expression profile of insect nAChR subunits
118 may vary according to developmental stages as shown in *D. melanogaster* [52,53], *Apis cerana*
119 *cerana* [54], *A. pisum* [35], *Cydia pomonella* [55], or *Chilo suppressalis* [56]. These results
120 highlight the presence of α-Bgt binding site in diverse insect species suggesting the expression
121 of functional α-Bgt-sensitive receptors as a common feature in insects.

122

123 ***α-Bgt as a probe to determine neonicotinoid binding sites***

124 Saturation experiments conducted on *D. melanogaster* heads showed Bmax up to 1400 fmol/mg
125 using [^{3}H]-α-Bgt [50] whereas values obtained with [^{3}H]-IMI were much lower with Bmax
126 between 126 and 980 fmol/mg [50,57]. These findings suggest that most of the nAChR binding
127 sites were α-Bgt-sensitive. The high density of these binding sites suggests that labeled α-Bgt
128 could be of particular interest to study the binding properties of other nAChR ligands such as
129 neonicotinoid insecticides, as shown in competitive experiments on native insect nAChRs
130 (Table 2). Differential results were found with commonly used neonicotinoid insecticides IMI,

131 CLT and thiamethoxam (TMX) in competition binding assays. Binding studies on *D. melanogaster* head membranes revealed that [^{3}H]-IMI and [^{3}H]-α-Bgt bind to distinct binding sites. In *D. melanogaster*, binding assays revealed that the binding capacity of [^{3}H]-IMI is not disturbed when [^{3}H]-α-Bgt is added simultaneously, demonstrating the presence of distinct binding sites for IMI and α-Bgt [50,58]. These results support the idea of weak competition of IMI for binding sites labeled with [^{3}H]-α-Bgt, as Ki values were high in *D. melanogaster* [50]. Also, there was a lack of competition between α-Bgt and [^{3}H]-IMI in the aphids *M. persicae*, *Aphis craccivora,* and *A. pisum* [35,49]. Interestingly, in the cockroach *Periplaneta americana*, it was shown that ACE, CLT, and TMX presented higher affinity for α-Bgt-binding sites than nitenpyram (NIT) and IMI, whereas dinotefuran (DTF) was a weak competitor with Ki in micromolar range [59,60]. A more recent work on *A. pisum* showed that IMI was a partial inhibitor of [^{125}I]-α-Bgt whereas CLT and TMX were able to completely inhibit the binding of labeled-α-Bgt [35]. These results suggest the presence of several nAChR subtypes that are sensitive to α-Bgt and could differently bind neonicotinoid insecticides. The presence of α-Bgt-sensitive nAChRs that are also sensitive to IMI seems to be specific to *A. pisum* and could correspond to the additional very high affinity binding sites for α-Bgt. On the contrary, CLT and TMX seem to be able to bind to both low and high α-Bgt-sensitive binding sites. Altogether, these results demonstrated that i) neonicotinoid insecticides could bind to α-Bgt-sensitive binding sites, which represents the majority of nAChRs binding sites, and ii) that all neonicotinoid insecticides do not share the same binding properties. Thus, it is important to complement these results with further binding assays using others radioligands because α-Bgt-insensitive nAChR subtypes are also present in insect nervous system.

153

154 ***High and low affinity nicotinic acetylcholine receptor binding sites with imidacloprid***

155 IMI acts as a partial agonist at insect neuronal nAChRs [13–16]. Saturable binding of [^{3}H]-IMI
156 to insect membranes reveals the presence of different [^{3}H]-IMI binding sites according to insect
157 species [59,60]. The data illustrated in Table 3 suggest dipteran and lepidopteran species have
158 a single IMI-binding site and hemipterans possess two IMI-binding sites, except for *Bemisia*
159 *argentifolii* which exhibits only one [61]. Indeed, saturable binding of [^{3}H]-IMI to *D.*
160 *melanogaster* head membranes revealed the presence of a single IMI-binding site [50,62–64].
161 Similar binding was found with membranes from housefly *Musca domestica* [61,65]. In
162 lepidopterans such as *M. sexta*, only one IMI-binding site was determined [64]. However, in
163 the orthopteran *Locusta migratoria* and in hemipteran species such as *Nilaparvata lugens*, high
164 and low affinity binding sites were identified [49,66]. Moreover, in *N. lugens*, *Nephotettix*
165 *cincticeps,* and *A. pisum*, a very high affinity binding site for IMI with a Kd value less than 0.1
166 nM was described [35,39,67]. The interpretation of one or more IMI-binding site is more
167 ambiguous in the aphids *M. persicae* and *A. craccivora* (Table 3). In *M. persicae*, some studies
168 showed the presence of only one IMI-binding site [39,65,68], whereas a supplemental high
169 affinity binding site was identified in other studies [49,64,68]. *A. craccivora* seems to possess
170 one binding site [65,68,69], but an additional high affinity binding site was also described [49].
171 These apparent discrepancies could be due to the variability of biological material as mixed
172 generations were used (Table 3). Considering the different Kd values obtained in several
173 studies, we propose that at least three nAChR subtypes with very high (Kd less than 0.1 nM),
174 high (Kd between 0.1 and 5 nM), or low (Kd more than 5 nM) affinity for IMI are present in
175 *M. persicae* and that they could be differently expressed according to developmental stages.
176 Altogether, these saturation experiments demonstrated that IMI sensitive binding sites could
177 have distinct pharmacological properties.

178

179 ***[^{3}H]-IMI as reference probe to characterize neonicotinoid binding sites***

As demonstrated with α-Bgt, competition experiments with labeled IMI-binding sites revealed several Ki values depending on the species, tissue and neonicotinoids used for the competition (Table 4). Thus, using membranes from the whole body of the aphids *M. persicae* and *A. craccivora* it was found that CLT, ACE, and NIT were powerful competitor with Ki values in nanomolar range [39,65,70]. Similar Ki values were obtained for ACE and NIT in *D. melanogaster*, *A. mellifera,* and *L. migratoria* [49,65,71] and for CLT in *A. pisum* [35]. Interestingly, THI presented an affinity for [^{3}H]-IMI-sensitive-binding sites higher than those of IMI itself in *A. mellifera*, *A. craccivora*, *M. persicae* and *M. domestica* and *L. migratoria* [49,71,72]. THI seems to be one of the most potent neonicotinoids in a wide range of insect species and could be very effective in controlling insect pests but its potency to bind nAChRs in an insect pollinator will probably limit its use in the field. Such a high affinity for [^{3}H]-IMI-sensitive-binding sites was shown for CLT in *M. domestica* and *D. melanogaster* [63] suggesting that CLT would be particularly effective against dipterans. Altogether, these results demonstrate that CLT, ACE, NIT and THI are highly competitive with IMI (Ki in nanomolar range) suggesting interaction with similar binding sites on nAChRs, as proposed in previous studies on aphids [39,70].

On the contrary, TMX was a very weak displacer with micromolar affinity for [^{3}H]-IMI-sensitive-binding sites in several insect species (Table 4) as shown in *L. migratoria* [49] and the aphids *M. persicae*, *A. craccivora* and *A. pisum* [35,39,70]. These results suggest that TMX is a non-competitive inhibitor and it was proposed that TMX is able to bind to different sites and/or in a different manner than IMI [70]. In *M. persicae*, IMI was shown to be an effective competitor of [^{3}H]-TMX whereas TMX is a weak competitor of [^{3}H]-IMI. These results suggest that TMX could bind only to one sub-population of IMI binding sites, with high affinity for IMI [66]. In *A. pisum*, it was demonstrated that TMX showed a weak binding capacity for [^{3}H]-IMI binding sites and a better potency for [^{125}I]-α-Bgt binding sites [35]. These results demonstrate

205 that TMX and α-Bgt share common binding sites, which could be correlated with the presence
206 of extra very high affinity binding sites for α-Bgt in *A. pisum*. Similar results were obtained on
207 cockroach nerve cord, where TMX inhibited [^{3}H]-α-Bgt binding with nanomolar Ki value,
208 which correlates with its insecticidal activity [57]. These results suggest that TMX could act in
209 two ways: (1) by direct binding to α-Bgt binding sites and (2) through its metabolite CLT as
210 proposed in previous works [37,73,74]. In *M. persicae*, sulfoxaflor (SFX) revealed similar
211 binding properties to TMX. In fact, SFX is a weak competitor of [^{3}H]-IMI whereas IMI is very
212 potent in inhibiting [^{3}H]-SFX binding [70,71,75]. We propose that, as previously described for
213 TMX, SFX is able to bind to high affinity binding sites for IMI. Moreover, a recent study
214 highlighted that even if high affinity binding sites for SFX were present at very low density, the
215 displacement of [^{3}H]-SFX correlated with high *in vivo* toxicity in sap-feeding pest [76]. Thus
216 the characterization of high affinity binding sites for other neonicotinoids could also be
217 toxicologically relevant and future studies should not only consider IMI binding sites to predict
218 insecticides potency. DTF was revealed to be a weak competitor of [^{3}H]-IMI and [^{3}H]-TMX in
219 *M. persicae* [70] but was able to compete with [^{3}H]-IMI in *M. domestica* [77,78]. In *P.*
220 *americana*, the presence of high-affinity binding sites for [^{3}H]-DTF [79,80] was detected and
221 DTF was able to compete with [^{3}H]-α-Bgt [57]. Thus, DTF seems to bind different nAChR
222 populations according to species.
223 Altogether, these results highlight the presence of specific pharmacological nAChR subtypes
224 according to species, the need to carefully interpret competition experiments and the relevance
225 of using probes other than [^{3}H]-IMI to study neonicotinoid binding properties.

226

227 ***How to increase the quality and highlight binding interaction between insect nAChRs and***
228 ***ligands***

229 We have demonstrated above that the use of mixed generations (adults and different
230 larval stages) and even mixed aptere/winged and male/female samples, could explain the
231 variability observed in binding data. Thus, the choice of homogenate biological material is
232 essential to ensure reproducible and comparable studies. Our point of view seems to be shared
233 by other researchers such as Kayser et al. who decided to adapt their harvest method to collect
234 mainly adult aphids by attracting them to light [70]. In *L. migratoria,* the selection of
235 homogenate sample using adult nerve chain allowed the acquisition of very reproducible results
236 in IMI saturation experiments in two different studies [49,67] (Table 3). A brief summary of all
237 data reveals that comparing findings from brain tissue, the whole body, and the nervous system
238 is likely misleading (Table 3). For example, if we use membrane preparations from the brain,
239 we expect that a large number of nAChRs are expressed because ACh is the principal
240 neurotransmitter in this organ [3]. This statement is reinforced by the IMI saturation data
241 obtained in *Drosophila*, with Bmax values ranging from 560 to 1344 fmol/mg in membrane
242 preparations from heads whereas the Bmax is only at 126 fmol/mg when using whole adults
243 (Table 3). We suppose that binding experiments conducted with the whole body could be less
244 sensitive if the nAChR populations are present at low density. Additionally, the results of
245 saturation experiments showed particularly high discrepancy in aphid species *M. persicae* and
246 *A. craccivora* (Table 3). We propose that the use of "adult heads preparation" could help to
247 improve experimental reproducibility as parthenogenetic whole individuals also carry different
248 larval stages which could express different nAChR pharmacological subtypes.

249 We can expand our analysis to experimental design. Saturation experiments were
250 performed by incubating membrane preparations with varying concentrations of [^{3}H]-α-Bgt or
251 [^{125}I]-α-Bgt which could lead to misinterpretation as these two radioligands show different
252 abilities to highlight low density binding sites. Moreover, several studies highlighted the
253 relevance of using radioligands other than IMI in competitive assays [70,71,75] and to carefully

254 interpret results. Thus to ovoid misinterpretation, one should keep in mind that competitive
255 experiments only give access to neonicotinoid binding capacity on the particular nAChR
256 subtype labeled with the radioligand. This is particularly important as other neonicotinoid
257 binding sites, even at low density, could also be toxicologically relevant as demonstrated for
258 DTF [76]. In this review, we also report that the overall majority of recent studies used IMI or
259 other neonicotinoids as radiolabeled probes whereas the first one were conducted with labeled
260 α-Bgt. We suggest that additional competitive experiments with labeled α-Bgt could help to
261 better understand comparative binding and toxicity capacity between insecticides. This would
262 be particularly interesting for neonicotinoids that have binding sites distinct from IMI as
263 demonstrated for DTF [57]. In this review, we only report saturation and competitive
264 experiments. One challenge for future studies could be to develop kinetic experiments. In these
265 experiments, the binding of radioligand is measured at incrementing series of time points and
266 analyzed to estimate association (k_{ON}) and dissociation (k_{OFF}) rate constant [41]. This would
267 enable better characterize of the mode of action of neonicotinoids, particularly at low
268 concentrations. Recently, a study conducted on hybrid nAChRs consisting of *N. lugens* α and
269 rat β subunits showed the specific action of IPPA08 (a cis-configuration neonicotinoid
270 compound) as a positive allosteric modulator for IMI binding when present at low
271 concentrations however, at high concentrations, IPPA08 was able to act as agonist of nAChRs
272 [81].

273

274

275 **Conclusion and future challenges**

276 To date, the subunit combination of insect pest native receptors remains unknown. 277 Previous studies suggest that it can be due to a lake of accessory proteins for the functional 278 expression of native receptors in *Xenopus oocytes* [36,82,83]. To overcome the lack of 279 expression, and identify subunit and/or receptor subtypes associated with the pharmacological 280 properties of neonicotinoid insecticides, binding studies with labeled neonicotinoids on insect 281 pest are currently used. However, data reported in several studies demonstrate some 282 discrepancies and suggest new challenges to highlight binding specificity and how it affects 283 species-specificity. Indeed, most of the binding assays were conducted using labeled IMI 284 because it is the first commercialized neonicotinoid worldwide, but the data obtained using 285 [^{3}H]-IMI could lead to different interpretations, according to the study. This could be due to the 286 fact that the effects of [^{3}H]-IMI does not reflect those of all neonicotinoids. For example, TMX 287 binds to different sites [70]. This may be linked to their chemical structures where IMI possesses 288 an electronegative terminal group not present in TMX [84]. The choice to use brain or whole 289 body impacts the sensitivity of the binding assay in terms of Bmax values. We propose that the 290 binding of IMI with insect nAChR subtypes should be compared according to the specific tissue 291 used. Moreover, saturation and competition binding experiments showed significant differences 292 in the number of binding sites, the displacing potencies and the mode of binding interference 293 according to the neonicotinoid used. It was hypothesized that nAChRs possess a variety of 294 binding pockets depending on the combination of receptor subunits, receptor subtype, and 295 functional state [70]. According to this hypothesis, understanding the specific ligand binding 296 interaction of each neonicotinoid according to tissue type is should be applied to exploring and 297 comparing the mode of action of the recently discovered sulfoximine and flupyrimin 298 insecticides, which also interact with nAChR subtypes [76,85]. Sulfoxaflor (SFX), a 299 sulfoximine derivative, has been shown to act as an agonist of insect nAChR subtypes, in

300 particular, SFX interacts weakly with the IMI binding site prepared from membrane
301 homogenate of whole green peach aphid *M. persicae*. It was proposed that the binding site for
302 [^{3}H]-SFX is less abundant than nAChR binding sites identified by other ligands [76]. This could
303 be due to the finding that SFX does not contain the amine nitrogen present in all previously
304 commercialized neonicotinoids but has a methyl group at the bridge between the heterocycle
305 [75]. These structural differences could lead to specific interactions with nAChRs. In addition,
306 it is possible that SFX-sensitive receptors, which are probably less expressed than other
307 receptors, will result in an overall low affinity for SFX. We propose that nAChRs binding to
308 SFX in *M. persicae* could involve additional receptor subtypes compared to IMI. Our proposal
309 is supported by the finding that the binding of several ligands using *M. persicae* is ambiguous
310 because some studies showed the presence of only one IMI-binding site [39,65,68,70], whereas
311 an additional high affinity binding site was identified in other studies depending on the tissue
312 used. Moreover, methyllicaconitine (MLA), a nAChR antagonist which is able to block α-Bgt-
313 sensitive receptors, and epibatidine, a nAChR agonist, displaced [^{3}H]-SFX with high affinity
314 [76]. Considering that MLA binds to α-Bgt-sensitive nAChRs, it could be reasonable to suggest
315 that SFX may be displaced by α-Bgt.

316 In the present review, we show that binding experiments conducted with [^{3}H]-α-Bgt and
317 [^{125}I]-α-Bgt demonstrated the existence of several low and high affinity α-Bgt binding sites.
318 Comparing the competition between α-Bgt and neonicotinoid has led to the suggestion that IMI
319 preferentially acts on α-Bgt-insensitive receptors and that other neonicotinoids such as CLT
320 and ACE act on both α-Bgt-sensitive and -insensitive receptors. Interestingly, one specific α-
321 Bgt binding site is found in some insects such as *A. mellifera* using membrane preparation from
322 the nervous system [48]. The presence of a unique binding site in bees is inconsistent with the
323 expression of several nAChR subunits differently expressed in the brain structures involved in
324 learning and memory processes [86–88]. In particular α-Bgt-sensitive receptors seem to be

325 involved in long term memory whereas mecamylamine-sensitive receptors were involved in
326 short-term memory [89]. One challenge for binding studies will be to use other labeled
327 compounds such as mecamylamine and methyllicaconitine which seems to bind to α-Bgt-
328 sensitive receptors. This point of view is of particular interest if binding studies are conducted
329 on pollinating insects such as *A. mellifera* for which several pharmacological and behavioral
330 studies have demonstrated the functional importance of nAChRs [88,89]. Moreover, the
331 expression of nAChR subunits is different according to developmental stages which may affect
332 binding studies. Indeed, we demonstrated previously that the expression of nAChR subunits
333 varies between the brain of pupae and adult honeybee. For example, Amelα5 (Apisα7-1)
334 expression was found in the outer compact Kenyon cells of the mushroom bodies of the pupae
335 and additional expression was found in the noncompact Kenyon cells and optical lobes in adults
336 [86]. The diversity of nAChRs could also be greater than what can be seen in binding
337 experiments. For example, in the cockroach *P. americana*, electrophysiological studies
338 combined with RNAi experiments on dorsal unpaired median neurons demonstrated the
339 presence of at least three nAChR subtypes that are sensitive to IMI and have different subunit
340 composition [20], whereas binding experiments only showed one binding site for IMI [57].

341 In conclusion, we highlight the complexity and diversity of neonicotinoid binding sites, which
342 reflect the diversity of nAChRs subtypes in insects. nAChRs are of particular interest as they
343 are targets of highly effective insecticides such as neonicotinoids and the recently characterized
344 sulfoximine compounds. Ongoing efforts in identifying native receptors will prove instructive
345 in characterizing the mode of action of these compounds and future approaches combining
346 binding experiments with RNAi and/or immunodepletion will likely enhance our understanding
347 of the pharmacological properties of native nAChRs.

348

349 **Table legends**

350 **Table 1.** Identification of α-bungarotoxin (α-Bgt) binding sites by saturation experiments using
351 labeled α-Bgt in different insect species.

352 Kd, dissociation constant, reflects the nAChRs' affinity for α-Bgt. Bmax corresponds to the
353 maximal binding and reflects the binding site density in the membrane preparation. The nature
354 of the biological tissues used in the experiments are also reported.

355

356 **Table 2.** Binding properties of neonicotinoid insecticides determined by competitive
357 experiments with labeled α-bungarotoxin (α-Bgt).

358 IC_{50} corresponds to the concentration of non-labeled probe needed to inhibit 50% of the specific
359 binding of the labeled probe; Ki is the inhibition constant calculated according to Cheng and
360 Prusoff formula; values are indicated ± S.E.M and with a confidence interval of 95% between
361 brackets.

362

363 **Table 3.** Identification of imidacloprid (IMI) binding sites by saturation experiments using
364 labeled IMI in different insect species.

365 Kd, dissociation constant, reflects the nAChRs' affinity for IMI. The Bmax value corresponds
366 to the maximal binding and reflects the binding site density in the membrane preparation. The
367 nature of the biological tissues used in the experiments are also reported.

368

369 **Table 4.** Binding properties of neonicotinoid insecticides determined by competitive
370 experiments with labeled imidacloprid (IMI).

371 IC_{50} corresponds to the concentration of non-labeled probe needed to inhibit 50% of the specific
372 binding of the labeled probe; Ki is the inhibition constant calculated according to Cheng and
373 Prusoff formula; values are indicated ± S.E.M. or with a confidence interval of 95% between
374 brackets.

375

376

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