Assessing species-specific neonicotinoid toxicity using cross-species chimeric nicotinic acetylcholine receptors in a *Drosophila* model

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Running title: Chimeric nAChRs for neonicotinoid toxicity in *Drosophila*

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Abstract

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels and the main mediators of synaptic neurotransmission in the insect brain. In insects, nAChRs are pivotal for sensory processing, cognition and motor control, and are the primary target of neonicotinoid insecticides. Neonicotinoids are potent neurotoxins and pollinators such as honey bees are more sensitive and affected by extremely low sub-lethal doses. The pentameric nAChR channel is made up either of five α-subunits constituting five ligand-binding sites or a mixture of two to three α and β subunits constitute two to three ligand-binding sites. Of particular note, the honey bee nAChRα8 subunit is converted into a β subunit (nAChRβ2) in *Drosophila*, raising the question whether this α to β conversion makes flies less sensitive to neonicotinoids. To investigate species-specific aspects of neonicotinoid toxicity we CRISPR-Cas9 engineered a cross-species chimeric nAChR subunit by swapping the ligand-binding domain in *Drosophila* of nAChRβ2 with honey bee nAChRα8. Toxicity assessment by neonicotinoid thiamethoxam revealed significantly impaired motor functions in climbing and flight assays when comparing the α8/β2 chimeric channel to wild type or a β2 knock-out. However, both the α8/β2 chimeric channel and the β2 knock-out showed the same increased survival after neonicotinoid exposure compared to wild type flies. Combinatorial exposure to neonicotinoids also did not reveal differences. These findings highlight the critical role of nAChR subunit composition in motor control and demonstrate how subtle structural differences can profoundly impact motor function and pesticide response, offering new insights into the molecular mechanisms of neurotoxicity across species.

Introduction

Pesticides are indispensable for protecting global agricultural yields by reducing crop losses caused by pests[1]. Among these, neonicotinoids are highly effective due to their broad-spectrum efficacy and systemic activity, which enable integration into plant tissues for targeted pest control, and versatility in application methods, such as seed coating, to reduce environmental contamination[2,3]. Neonicotinoids exhibit high toxicity to insects, but not to vertebrates, making them safer alternatives to other classes of chemical[4,5]. However, neonicotinoids do not distinguish between pests and beneficial insects, and harm beneficial insects like honey bees, which are critical pollinators for both wild flora and agricultural crops[6–8]. The widespread decline of pollinators, partially attributed to pesticide exposure, poses significant ecological and economic challenges, highlighting the need for pest-control strategies with reduced non-target effects.

Honey bees, wild bees and other pollinators are adversely affected by extremely low sub-lethal doses of neonicotinoids (2 ng/ml compared to the LC50 dose of 4.28 µg/ml) impairing foraging behaviour, cognition, navigation and weakening of the immune system, and reducing reproductive success and genetic diversity within a colony[6,9–20]. Neonicotinoids, which target cholinergic neurotransmission, inhibit mushroom body Kenyon cell activity[21] and disrupt memory and olfactory sensory neuron activity in honey bees (*Apis mellifera*) and fruit flies (*Drosophila melanogaster*)[21–24].

The sensitivity of insects to neonicotinoids varies considerably between species, with honey bees being highly susceptible and *Drosophila* more resistant. For example, the 24-hour lethal concentration 50% (LC50) of thiamethoxam (TMX) is 3.13 µg/ml for adult *Drosophila*[25], whereas for honey bees it is 4.28 µg/ml[20]. This variation arises from differences in the repertoire of cytochrome P40 detoxification genes. *Drosophila* encodes 85 of these neonicotinoid-metabolising enzymes, whereas honey bees have only 46[26,27]. Additionally, honey bees have

69 fewer antioxidant gene paralogs than *Drosophila*, which has an expanded antioxidant defence
70 system. Since neonicotinoids can induce oxidative stress, this weaker antioxidant capacity may
71 contribute to the greater sensitivity of bees compared to *Drosophila*[28].

72 Another reason contributing to this species-specific susceptibility could be the
73 composition of nicotinic acetylcholine receptors (nAChRs). nAChRs are essential for
74 neurotransmission, synaptic plasticity, and neuronal development, making prominent targets
75 for neonicotinoid pesticides[29–32]. These pentameric cys-loop ligand-gated cation channels
76 consist of α and β subunits forming homo- or heteropentamers. Ligand binding pockets, located
77 at α-α or α-β interfaces, consist of loops A, B, and C from an α subunit and loops D, E, and F
78 from the adjacent α or β subunit[33–35]. Importantly, β subunits lack the ability to form functional
79 homopentamers due to the absence of loops A, B, and C[33,35]. Consequently, the subunit
80 composition of nAChRs receptor affect their properties and determines susceptibility to
81 agonists, such as acetylcholine or neonicotinoids (Fig. 1a).

82 Insects have relatively small nAChR gene families. *Drosophila melanogaster* encodes
83 10 subunits (Dα1-Dα7 and Dβ1-Dβ3), while the honey bee *Apis mellifera* encodes 11 (Amelα1-
84 Amelα9 and Amelβ1-Amelβ2)[36]. α subunits are characterised by the presence of two adjacent
85 cysteines in loop C of the ligand-binding domain (LBD), whereas β subunits lack these
86 residues[35]. Notably, *Drosophila* encodes fewer α subunits compared to *Apis mellifera*. Instead,
87 the *Drosophila* β2 subunit exhibits striking conservation with the honey bee Amelα8 subunit
88 indicating a subunit conversion (Fig. 1b). However, the absence of loop C in *Drosophila* β2,
89 which is essential for ion channel gating, highlights a structural divergence that may contribute
90 to species-specific functional properties and differences in neonicotinoid susceptibility. Further,
91 specific amino acid substitutions in nAChR subunits are known to impact insecticide
92 susceptibility. For instance, *Drosophila* mutants lacking certain nAChR subunits or carrying

93 the natural variant R81T in the β1 subunit from aphids exhibit a 10- to 100-fold increase in
94 resistance to neonicotinoids[37–39].

95 Here, we examine the evolutionary divergence and functional implications of nAChR
96 subunits in *Drosophila melanogaster* and *Apis mellifera*. Phylogenetic analysis reveals that the
97 *Drosophila* β2 subunit has evolved from an ancestral α subunit. To investigate whether this
98 subunit conversion contributes to neonicotinoids sensitivity, we employed CRISPR-Cas9 gene-
99 editing technology to generate chimeric *Drosophila* flies expressing a chimeric nAChR
100 receptor. Specifically, we replaced the ligand-binding domain (LBD) of the *Drosophila* β2
101 subunit with that of the honey bee α8 subunit, providing a model to explore cross-species
102 receptor functionality. Behavioural and survival assays revealed significant impairments in
103 motor functions and altered sensitivity to insecticides, highlighting how structural differences
104 in receptor subunits can influence pesticide response. Our results provide insights into the
105 evolutionary trajectories of nAChR subunits and their roles in mediating neurotoxic compound
106 interactions, establishing a valuable *Drosophila* model for investigating cross-species pesticide
107 toxicity.

108

109 **Results**

110 **Species-specific specialization of *Drosophila* β2 subunit during evolution**

111 Insect *nAChRs* are classified into divergent subunits without clear orthologous
112 relationships between species, alongside a subset of genes that are highly conserved across
113 taxa[40]. Among these conserved subunits is the nAChRα8 subunit; however, it is absent in
114 certain Dipteran species, including *D. melanogaster*. Instead, the *Drosophila* β2 subunit
115 exhibits striking similarity to the honey bee α8 subunit, with 75% overall sequence identity and
116 92% identity within the LBD indicating that α8 converted in to the β2 subunit (Fig. 1b and
117 Supplementary Fig. 1). Alphafold structural modelling further confirmed conservation of the

118 protein secondary structure of these two subunits, particularly in the LBD, with the exception
119 of the loop C (Fig. 1d)[41].

120 Next, to investigate whether *Drosophila* β2 represents a divergent lineage from other β
121 subunits or shares a closer evolutionary relationship with α subunits, we conducted a
122 phylogenetic analysis of all nAChR subunits in *D. melanogaster* and *A. mellifera*. Strikingly,
123 the analysis revealed that *Drosophila* β2 clusters within a clade predominantly composed of α
124 subunits, including α1–α4 and α8, indicating that *Drosophila* β2 is more closely related to α
125 subunits than to other β subunits (Fig. 1c). Interestingly, the honey bee β2 subunit exhibits
126 significant divergence and does not closely group with any other subunits, suggesting a
127 divergent evolutionary origin.

128

129 ***nAChR* subunits exhibit developmental dynamics and cell-specific distribution**

130 To investigate the expression patterns of *nAChR* subunits, we analysed tissue specific
131 expression data in adults, taking into account the enrichment values providing the gene
132 abundance measure in a tissue relative to that in the whole fly[42]. The majority of *nAChR*
133 subunits exhibits higher expression levels in females than in males (Fig. 2a). In both sexes,
134 *nAChR* subunits are predominantly expressed in the head, brain and CNS, and thoraco-
135 abdominal ganglion, with *Drosophila β2* being the most highly expressed subunit in the brain
136 and CNS. In other tissues, however, the levels of expression differ between sexes, with
137 *Drosophila β2* and *α7* being the most expressed subunits in the thoraco-abdominal ganglion of
138 males and females, respectively. Furthermore, only two subunits, *Drosophila α4* and *β3*, are
139 expressed across all tissues, with *Drosophila β3* showing notable expression in the heart.
140 Specific differences were observed between sexes. For instance, in the male salivary glands,
141 carcass, and crop, only *Drosophila α1*, *α2*, and *α3* were expressed, while *α4*, *β1*, and *β3* were
142 restricted to females.

143 Further, we investigated whether the expression of *nAChR* subunit changes during
144 development. Larval tissue analysis revealed that *Drosophila α3* is not expressed in any tissue
145 (Supplementary Fig. 2a), suggesting it is dispensable during development. In contrast,
146 *Drosophila β3* was expressed in all larval tissues, with the garland cells showing the highest
147 expression. The larval brain and CNS were the only tissues expressing all *nAChR* subunits, with
148 *Drosophila β3* showing the highest levels. Overall, *nAChR* subunit expression is relatively low
149 in most tissues in both larvae and adults (Fig. 2a and Supplementary Fig. 2a).

150 To refine the spatial expression of *β2* in *Drosophila* cells in more detail, we analysed
151 expression in single cells[43]. In whole adult flies, *β2* was found to be expressed in various cell
152 types, including neurons, sensory neurons, glial cells, as well as male germline and reproductive
153 system, however, its expression is not ubiquitous (Fig. 2b). Notably, a prominent cluster of
154 neurons classified as adult fly head CNS cells strongly expressed *β2*, suggesting it may have a
155 significant role in neuronal circuits[23,44]. Since *β2* displayed the highest expression levels in the
156 brain and CNS, we further analysed single cell expression data for the larval brain and whole
157 adult fly head. In adults, *β2* expression was observed in distinct neuronal populations,
158 contrasting with the broader expression of the neuronal marker *elav* (Fig. 2c-e)[45]. In contrast,
159 larval *β2* was colocalised with *elav* in the majority of CNS neurons (Supplementary Fig. 1b-
160 d)[46].

161 To validate the expression patterns, we used *nAChRβ2*$^{[2A\text{-}GAL4]}$ allele which has a *T2A-*
162 *GAL4* sequence fused at the 3' end of the gene, resulting in GAL4 expression as a separate
163 protein under the control of *nAChRβ2* regulation[47]. Therefore, we analysed both larval and adult
164 brains using nuclear *UAS-Histone2B-YFP* or membrane-tethered *UAS-mCD8-GFP* reporters,
165 co-staining neurons with anti-*elav* antibody. Consistent with our observations from the single
166 cell expression analyses, *β2* expression was restricted to specific neuronal populations,
167 confirming its non-ubiquitous distribution (Fig. 2f-k and Supplementary Fig. 1e-j).

168 Furthermore, we noticed enhanced *β2* expression in Kenyon cells, particularly when a
169 membrane-bound GFP reporter was used (Fig. 2i-k)[44].

170 Together, these findings reveal that nAChR subunit expression is dynamically regulated
171 during development and exhibits sex- and cell-specific specialisation, highlighting the
172 functional diversity of these receptors in neural circuits and other tissues. Furthermore, the
173 observed developmental shift in β2 expression, from widespread larval CNS expression to more
174 specialised adult neuronal populations, reflects the transition from simpler larval neural circuits
175 to the more specialised and complex adult CNS.

176

177 **Chimeric *Amelα8/Dβ2* ligand-binding domain flies are viable with impaired motor**
178 **functions**

179 Since the two adjacent cysteines in the loop C are essential for ligand binding in
180 nAChRs, we hypothesised that this difference may influence neonicotinoid susceptibility,
181 making honey bees more vulnerable. Therefore, we generated chimeric *nAChRβ2/α8*
182 ($nAChR\beta2^{Am\alpha8LBD}$) flies, where the *β2* LBD coding region in *Drosophila* was swapped with the
183 *Amelα8* LBD coding sequence using CRISPR-Cas9 genome engineering (Fig. 3a and
184 Supplementary Fig. 3). Successful recombinant flies were marked with *white+* selection
185 marker, disrupting the LBD, therefore resulting in a null allele ($nAChR\beta2^{nullA}$). To restore the
186 open reading frame (ORF) to generate a fly stock expressing the chimeric *nAChRβ2/α8* subunit
187 ($nAChR\beta2^{Am\alpha8LBD}$), the selection marker was then excised by PiggyBac transposase. Flies were
188 validated by polymerase chain reaction (PCR), Sanger sequencing, and subsequently by whole
189 genome sequencing.

190 To normalise the genetic background, we used trans-heterozygotic flies for the
191 $nAChR\beta2^{attP}$ allele ($nAChR\beta2^{nullB}$) in which the coding region was replaced with an *attP* site,
192 *3xP3-RFP* and a *loxP* site[47], together with the $nAChR\beta2^{nullA}$ allele. The chimeric

193 *nAChRβ2*^{Amα8LBD} mutants, as well as *nAChRβ2*^{nullA} and *nAChRβ2*^{nullB} flies are fully viable when
194 crossed with a chromosomal deficiency (n = 453, 275, 421, respectively).

195 To assess motor abilities, we performed negative geotaxis assays (Fig. 3b) and evaluated
196 flight ability (Fig 3c). In negative geotaxis assays the climbing ability of LBD swap mutant
197 (*nAChRβ2*^{Amα8LBD}/*nAChRβ2*^{nullB}) was greatly reduced ($p = 7.5*10^{-7}$) (Fig. 3b) compared to wild
198 type (WT). We did not detect significant differences in the *β2* null mutant
199 (*nAChRβ2*^{nullA}/*nAChRβ2*^{nullB}) nor the heterozygous mutant controls.

200 In flying ability assays, we observed the same, namely the LBD swap mutant
201 (*nAChRβ2*^{Amα8LBD}/*nAChRβ2*^{nullB}) were impaired ($p = 0.0066$) (Fig. 3c) compared to control flies,
202 but not the *β2* null mutant (*nAChRβ2*^{nullA}/*nAChRβ2*^{nullB}) or heterozygote mutant controls.

203

204 **Chimeric *Amelα8/Dβ2* ligand-binding domain flies display resistance to insecticides**

205 To investigate the impact of substituting the *Drosophila* β2 LBD with the honey bee
206 Amelα8 LBD on neonicotinoid susceptibility, we selected two well-characterised
207 neonicotinoids, TMX and imidacloprid (IMI), both of which exhibit high toxicity to
208 pollinators[5,39]. These compounds differ slightly in chemical structure and receptor specificity,
209 with TMX being one of the most potent full agonists of *Drosophila* and honey bee α1, α2, β1
210 and β2 combinations when heterologously expressed in frog oocytes and assayed by patch-
211 clamp. In contrast, IMI shows a stronger affinity for *Drosophila* β2-containing receptors
212 compared to TMX[39,48]. Both compounds target multiple nAChR subunits, enhancing their
213 versatility broad-spectrum toxicity[39].

214 In addition, we tested flupyradifurone (FPF), a systemic butanolide compound that is
215 chemically distinct from neonicotinoids and represents a novel alternative to these compounds
216 in pest management[49]. While FPF is not primarily targeted at *Drosophila* β2 subunits[39], its
217 broad-spectrum activity and demonstrated toxicity to non-target pollinators, including bees[50,51],

218 make it an important candidate for assessing cross-resistance and potential off-target effects
219 resulting from altered nAChR subunit composition.

220 First, we conducted survival analysis testing a range of TMX concentrations on WT
221 flies (Supplementary Fig. 4a, b). Fly viability was measured over 6 days. Based on the survival
222 curves, three different TMX concentrations were used for the toxicity experiments.

223 At 10 μM TMX, wild type flies had 43.75% survival after 144 h, while
224 *nAChRβ2*$^{Am\alpha8LBD}$/+, *nAChRβ2*^{nullA}/+, *nAChRβ2*^{nullB}/+, *nAChRβ2*^{nullA}/*nAChRβ2*^{nullB}, and
225 *nAChRβ2*$^{Am\alpha8LBD}$/*nAChRβ2*^{nullB} flies remained viable (Fig. 4a). At 20 μM, survival rates
226 declined progressively across all genotypes, with *nAChRβ2*^{nullA}/*nAChRβ2*^{nullB} mutants showing
227 the highest survival rates, followed by *nAChRβ2*$^{Am\alpha8LBD}$/*nAChRβ2*^{nullB} flies retaining more than
228 90% viability on day 6. This contrasted with *nAChRβ2*^{nullA}/+ (71.25%), *nAChRβ2*^{nullB}/+ (60%),
229 *nAChRβ2*$^{Am\alpha8LBD}$/+ (35%), and WT (11.25%) (Fig. 4b). At 40 μM, similar trend was observed,
230 although the viability of all flies was decreased below 50%. *nAChRβ2*^{nullA}/*nAChRβ2*^{nullB}
231 mutants retained by 48.75% day 6, followed by *nAChRβ2*$^{Am\alpha8LBD}$/*nAChRβ2*^{nullB} (41.25%),
232 *nAChRβ2*^{nullA}/+ (35%), *nAChRβ2*^{nullB}/+ (16.25%), *nAChRβ2*$^{Am\alpha8LBD}$/+ (3.33%), while WT flies
233 showed 0% survival by day 4 (Fig. 4c). Interestingly, apart from the trans-heterozygous
234 β^{nullA}/β^{nullB} flies, all genotypes experienced a sharp drop in viability between the 1st and 2nd day
235 of treatment, particularly *nAChRβ2*$^{Am\alpha8LBD}$/+ dropping from 62.5% to 30% and WT failing from
236 79% to 20%.

237 While both neonicotinoids and butenolides act as nAChR agonists, bind to the same site
238 and cause prolonged activation, they vary in the binding dynamics, receptor subtype
239 preferences, and potential to develop resistance[52]. To assess whether combining neonicotinoids
240 and butanolides results in additive or synergistic toxicity, we subjected flies to a mixture of
241 TMX (20 μM) with either FPF (20 μM) or IMI (20 μM). In nature, pollinators and insects are
242 frequently exposed to multiple insecticides rather than a single compound, making it crucial to

243 understand potential interactions and cross-resistance effects[53,54]. The TMX concentration was
244 chosen based on prior survival analyses, where double mutant flies retained > 90% viability
245 (Fig. 4b), while WT flies showed significant susceptibility. We tested chimeric
246 *nAChRβ2*^{Amα8LBD}/*nAChRβ2*^{nullB} flies and *nAChRβ2*^{nullA}/*nAChRβ2*^{nullB} mutants to determine
247 whether altered receptor composition influences responses to dual insecticide exposure. Flies
248 were treated for six days, and viability on day six was analysed as a log-fold change relative to
249 WT control and TMX-only treatment (Fig. 4d). Notably, exposure to TMX in combination with
250 either FPF or IMI resulted in a slight increase in survival rates. However, the combined
251 treatment did not significantly alter overall insecticide susceptibility. As expected,
252 *nAChRβ2*^{Amα8LBD}/*nAChRβ2*^{nullB} flies and *nAChRβ2*^{nullA}/*nAChRβ2*^{nullB} mutants flies exhibited
253 significantly higher resistance compared to WT across all conditions.

254 Taken together, our findings demonstrate that *nAChRβ2*^{nullA}/*nAChRβ2*^{nullB} null mutants
255 as well as the LBD swap mutants *nAChRβ2*^{Amα8LBD}/*nAChRβ2*^{nullB} flies exhibit higher survival
256 rates upon exposure to TMX, reinforcing the role of *Drosophila* β2 in neonicotinoid
257 susceptibility. Further, exposure to combined neonicotinoid and butanolide treatments did not
258 significantly alter overall lethality compared to TMX treatment on its own, indicating that sub-
259 lethal doses of these nAChR-targeting insecticides do not act synergistically in this context.
260 The observed resistance of chimeric and β2^{null} mutants further highlights the functional
261 divergence of Amelα8 in modifying nAChR-mediated insecticide susceptibility.

262

263 **Discussion**

264 The agricultural industry relies on pesticides to protect crops and maintain a stable food
265 supply for a growing population. Among these, neonicotinoids are the most widely used
266 insecticides due to their high efficacy and systemic action, reducing the need for repeated
267 applications. However, residues in nectar and pollen pose a significant risk to pollinators.

268 Despite their well-documented toxicity to bees and other beneficial insects, neonicotinoids
269 remain the dominant insecticides worldwide[55], highlighting the urgent need to mitigate their
270 ecological impact.

271 Beyond their effects on pollinators, neonicotinoids exhibit cross-species variability in
272 toxicity, with *Drosophila melanogaster* showing notably higher resistance than honey bees,
273 despite both species having homologous nAChRs[48]. The mechanisms underlying this
274 differential sensitivity remain unclear but are likely influenced by differences in receptor
275 composition, metabolism, or detoxification pathways[26,28]. To investigate species-specific
276 differences in neonicotinoid sensitivity, we established a *Drosophila* model where we swapped
277 the LBD of *D. melanogaster* β2 with honey bee Amelα8 subunit. The LBD swap mutant did
278 not reveal increased sensitivity to neonicotinoids compared to the *Drosophila* β2 null mutant.
279 However, the LBD swap mutant displayed motor function deficits indicating that this chimeric
280 receptor is functional. Minor structural changes in receptor subunits can profoundly impact
281 pharmacological properties[33,35,38,39] and could account for the altered neonicotinoid responses
282 observed in LBD chimeric mutant flies.

283 Previous studies in honey bees revealed tissue-specific expression of nAChR subunits,
284 including α2, α8 and β1 in Kenyon cells, while α7 was restricted to antennal lobe neurons[56]. A
285 similar functional diversity is observed in *Drosophila*, where nAChR subunits like α1 and α6
286 contribute to dendrite morphogenesis and synaptic transmission within larval visual circuits[57].
287 The absence of α3 expression in *Drosophila* larvae, combined with reduced nAChR subunit
288 expression during early developmental stages, suggests that receptor composition undergoes
289 developmental reprogramming to support the formation of complex adult neural circuits. This
290 dynamic tissue-, sex-, and stage-specific expression underscores the diverse roles of nAChRs,
291 including potential adaptations for behaviours such as reproduction[58,59]. Interestingly, in the
292 cockroach *Periplaneta americana*, subchronic exposure to sublethal doses of imidacloprid

293 induces a significant decrease in α2 mRNA expression, which correlates with a reduced
294 sensitivity to the insecticide[60], highlighting potential adaptive changes in nAChR subunit
295 composition even in fully developed insects.

296 Furthermore, high *Drosophila* β2 expression levels in CNS-specific neuronal
297 populations, including Kenyon cells, suggest its involvement in higher-order neuronal functions
298 such as learning and memory[44]. Similarly, honey bee α8 is enriched in honey bee mushroom
299 bodies[36,61,62], suggesting a conserved role for *Drosophila* β2 and honey bee α8 in regulating
300 mushroom body function across insect taxa, despite species-specific specialisations. Notably,
301 it has been shown that exposure to neonicotinoid insecticides affects cell activity in mushroom
302 body, and impairs learning and memory in insects, including *Drosophila* and honey bees[21–23,63],
303 further highlighting the critical role of nAChRs in cognitive functions and suggesting that
304 disruption of these receptors can have detrimental effects on neuronal processes related to
305 behaviour and learning.

306 Pharmacological and genetic studies have identified α1, α2, β1, and β2 as the primary
307 mediators of neonicotinoid toxicity[40,48,64]. Here, we generated a cross-species chimeric nAChR
308 using CRISPR-Cas9 genome engineering to evaluate neonicotinoid sensitivity in a *Drosophila*
309 model. Our study reveals that the *Drosophila* β2 conversion of honey bee α8 is not a main
310 determine of neonicotinoid sensitivity requiring to expand this approach to the other main
311 mediators of neonicotinoid toxicity. Our study establishes a framework for investigating
312 molecular determinants of neonicotinoid sensitivity of insects and vertebrates nAChR using a
313 cross-species chimeric nAChR *Drosophila* model. This model can contribute to the design of
314 next generation of insecticides for enhanced species-specific responses with implications for
315 pollinator conservation and pesticide safety for vertebrates including humans.

316

317 **Materials and methods**

318 **Fly stocks, genetics, immunostaining of tissues and imaging**

319 *D. melanogaster* CantonS and w^{1118} were used as the wild type control. *nAChRβ2*^{attP}
320 (BDSC 84545) and *nAChRβ2*$^{[2A\text{-}GAL4]}$ (BDSC 84666) stocks were described previously [47] and
321 were together with the chromosomal deficiency (BDSC 24996) from Bloomington. Fly crosses
322 were maintained at 25°C in plastic vials containing 10 ml of a standard cornmeal/yeast-rich
323 medium (1% agar, 2% yeast, 7% dextrose, 8% cornmeal w/v and 2% Nipagin from a 10%
324 solution in ethanol) with a 12:12 h light–dark cycle.

325 Third instar wandering larvae and adult brains from the progeny of *UAS-Histone2B-*
326 *YFP/+;nAChRβ2*$^{[2A\text{-}GAL4]}$*/+* and *UAS-mCD8-GFP/+;nAChRβ2*$^{[2A\text{-}GAL4]}$*/+* were dissected in
327 phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBT (PBS with 0.1%
328 TritonTM X-100 (Sigma-Aldrich, T8787)) for 30 min, followed by washes in PBT 3 × 15 min.
329 Samples were incubated overnight at 4°C with primary mouse anti-*elav* antibodies (MAb 7D,
330 1:20)[65], followed by secondary antibodies conjugated with Alexa Fluor 546 again overnight at
331 4°C. Samples were counterstained with DAPI (1:1000), mounted in Vectashield (Vector Labs),
332 scanned with Leica SP8, and processed using FIJI.

333

334 **Sequence analysis and single cell expression data visualisation**

335 Amino acid sequences were aligned using ClustalW with Megalin (DNAstar) or with
336 MAFFT[66] and phylogenetic trees were generated in NGPhylogeny.fr[67–69] and visualised using
337 iTOL[70].

338 Raw whole-genome sequencing reads were assessed for quality using FastQC, followed
339 by adapter trimming and quality filtering with Trim Galore. High-quality reads were aligned to
340 the *Drosophila melanogaster* (dm6) and *Apis mellifera* (Amel_HAv3.1) reference genomes
341 using BBMap. Mapping quality and coverage were evaluated, and alignments were visualized
342 using IGV (Integrative Genomics Viewer).

343 Single cell expression data was visualised as t-distributed stochastic neighbour
344 embedding (tSNE) from the 10x Stringent dataset in Scope and ASAP[71].
345
346 **Generation of chimeric *nAChRα8β2* flies**
347 Two single guide RNAs (sgRNAs) flanking the ligand binding domain *Dβ2* were
348 designed using PlatinumCRISPr[72] (Supplementary Fig. 3a, b) and cloned into *pUC-3GLA* using
349 the following primers: nACHr8 sgRNA left F1
350 (AAGATATCCGGGTGAACTTCGCTTATTGGAGCTAGGAAAGGTTTTAGAGCTAGA
351 AATAGC) and nACHr8 sgRNA right R1
352 (GCTATTTCTAGCTCTAAAACCACATGGCACAATCAAATTCGACGTTAAATTGAAA
353 ATAGG) as described previously[72]. Chimeric nAChR subunits were constructed by combining
354 sequences of *nAChRα8* from *A. mellifera* and *nAChRβ2* from *D. melanogaster* in three steps
355 (Supplementary Fig. 3c-e). Fragments used for generating the chimeric nAChR subunit were
356 synthesised using PCR with primers Bee alpha8 F2
357 (GGGAGTGGTCATTGCCATCTCAACCCTATATAAATTTG) and Bee alpha8 R2
358 (GGAATGTAATACCCACGCAAGGAATTATTAAG), followed by Bee alpha8 F1
359 (GCCAGTGAATTCGAGCTCGGTACCGCAGCTAGCGAAGCAAATCCTGACACAAAG
360 AGACTTTATGATGAC) and Bee alpha8 R1
361 (TTTACGCAGACTATCTTTCTAGGGTTAACCGTATAGAATAATGTTTTTCTTCGCAT
362 TG) for Amelα8 ligand binding domain, Dmel nAChRb left F1
363 (CGGCCAGTGAATTCGAGCTCGGTACCGATCCTTTTAGATAAAACATTTAGGAGCT
364 ATC) and nAChRsgmut left R1
365 (CTCTTTGTGTCAGGATTTGCTTCGAAACTCACTGGAGCCGTGAGAGGAGGGGTA
366 ATTCAGGGGAAAAACAGAGAAAAATGCC) for Dβ2 left homology arm, and
367 nAChRsgmut right F1

368 (ATTTTACGCATGATTATCTTTAACGTACGTCACAATATGATTATCTTTCTAGGGTT
369 AATCTGATTGTGCCCTGCGTAGCTTTAACATTCC) and Dmel alpha8 right R1
370 (GCATGCCTGCAGGTCGACTCTAGAGGATCCTGATAGTTGCTGCTGCGAATGCGG
371 AGCTG) for Dβ2 right homology arm, ensuring sgRNA sites in both homology arms are
372 mutated. Mutations were introduced based on conservation of the region between closely
373 related *Drosophila* species analysed using the UCSC genome browser
374 (https://genome.ucsc.edu) as described previously[73]. Fragments were then cloned stepwise into
375 the *pUC19 pBac w+* [accession number: PV267745] containing *white* + marker, disrupting the
376 LBD, therefore resulting in $nAChR\beta2^{null}$ flies. The selection marker was flanked with inverted
377 terminal repeats inserted at a TTAA PiggyBac motifs for the later scarless excision by PiggyBac
378 transposase.

379 The *pUC19 pBac w+ Amelα8/Dβ2* plasmid was treated with the nicking endonuclease
380 *Nb.Bts*I (NEB, R0707S) prior injection. Transgenic lines were generated by injection into a
381 GFP nosCas9 carrying flies (kindly provided by FlyORF). Positive transformants were
382 identified by red eye colour.

383 For *Drosophila* whole genome sequencing, large fragment DNA was extracted with the
384 Quick-DNA Tissue/Insect Miniprep Kit (Zymogene) according to the manufacturers'
385 instructions but replacing the BashingBead lysis step with cracking flies in liquid nitrogen
386 followed by gentle homogenization with a pestle in the provided BashingBead Buffer. Illumina
387 sequencing was done by Novogene and the sequence submitted to GEO [PRJNA1233772].

388

389 **Locomotive assessment**

390 Negative geotaxis experiments were done as previously described[74]. Briefly, two to five
391 day-old flies of both sexes were collected with CO_2 anaesthesia and grouped in sets of 20. Flies
392 were allowed to recover for a day and then placed in two inverted fly vials (19 cm). Flies were

393 tapped to the bottom, and their climbing behaviour was recorded on video. Every 5 s for a total
394 of 30 s, the distance climbed by each fly was measured and recorded. The data was collected
395 for four biological replicates.

396 For flying ability assessment, two to five day-old flies of both sexes were collected with
397 CO_2 anaesthesia and grouped in sets of 10. Flies were allowed to recover for a day before the
398 flying test. Then, flies were tapped to a flat surface, and their ability to fly was recorded on
399 video. Flies were observed over a 30 s period. At the end of 30 s, the number of flies that
400 successfully flew away was recorded. The data was collected for four biological replicates.

401

402 **Insecticide toxicity assay**

403 Two to five day-old flies of both sexes, grouped in sets of 20, were exposed to various
404 concentrations of thiamethoxam (Sigma-Aldrich, 37924), or a combination of 20 μM of
405 thiamethoxam with 20 μM imidacloprid (Thermo Fisher, 466752500) or 20 μM flupyradifurone
406 (MCE, HY-145295). The insecticides were dissolved in 0.01% acetone and added on top of the
407 standard fly food, and flies were added to the vials after 24 h. Exposure concentrations ranged
408 from 1.25 μM to 80 μM for survival curve analysis (Supplementary Fig. 4) and from 5 μM to
409 40 μM for testing all genotypes. Flies were exposed for 6 days, and survival was assessed every
410 24 h.

411

412 **Statistical analysis**

413 Statistical analyses were carried out using GraphPad Prism 9, applying one-way
414 ANOVA for behavioural assays or two-way ANOVA for the toxicity assays followed by
415 Tukey's test for pairwise comparisons. The data represent a minimum of three independent
416 replicates conducted on separate days.

417

418 **Acknowledgments**

419 We thank the Bloomington stock centre and FlyORF for fly lines, FlyORF for *Drosophila*
420 injection service for embryo injections, and Al Handler for the PiggyBac plasmid.

421

422 **Authors' contributions**

423 A.L. performed all experiments and analysed data. M.S. and J.J.L.H. conceptualised the project.
424 A.L. wrote the manuscript and M.S. and J.J.L.H. reviewed it. All authors read and approved the
425 final manuscript.

426

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429 M.S. and J.J.L.H.

430

431 **Availability of data and materials**

432 The data generated or analysed during this study are included in the supplementary information
433 in the supplementary information files. Whole genome sequencing data have been deposited at
434 GEO under the accession number PRJNA1233772. The sequence for the *pUC19 pBac w+* has
435 been deposited in GenBank under the accession number PV267745 and the plasmid is available
436 from Addgene and the European plasmid repository.

437

438 **Declarations:**

439 **Ethics approval and consent to participate**

440 Not applicable.

441

442 **Consent for publication**

443 Not applicable.

444

445 **Competing interests**

446 The authors declare no competing interests.

447

448 **Figure Legends**

449 **Figure 1: Highly conserved *Drosophila* β2 subunit originated from an alpha subunit in**
450 **other insects**

451 (a) Schematic representation of homo- and heteropentameric nAChR receptors in insects
452 exhibiting different compositions of α (blue) and β (pink) subunits, with their active ligand
453 binding sites represented by ligands (cyan).

454 (b) Amino acid alignment of *D. melanogaster* β2 and honey bee Amelα8 subunits. Identical
455 amino acids are marked as red, complementary amino acid substitutions are labelled orange,
456 and non-complementary amino acid substitutions remained unmarked. The ligand binding
457 domain is underlined.

458 (c) Molecular phylogenetic analysis of all nAChR subunits present in *Drosophila* and honey
459 bee with two major α subunits clades highlighted with light blue and pink (subunits highly
460 conserved with mammalian α7). Species codes are Dm: *D. melanogaster* Amel: *A. mellifera*.

461 (d) Structural alignment of *D. melanogaster* β2 (blue) and honey bee α8 (pink) with all amino
462 acid differences in *D. melanogaster* ligand binding domain indicated (cyan).

463

464 **Figure 2: *Drosophila β2* is highly expressed in adult *Drosophila* brain and CNS but not in**
465 **every cell**

466 (a) Expression levels of *nAChR* subunits in female and male adult fly tissues.

467 (b) Visualisation of single cell expression for the *nAChRβ2* gene indicated in red with
468 annotation of the major cell clusters exhibiting *Dβ2* expression.

469 (c-e) Visualisation of single cell expression for *nAChRβ2* (c) and neuronal marker *elav* (d) or
470 both (e).

471 (f-k) Expression of *Dβ2* (f, i), *elav* (g, j) and merged (h, k) in adult *Drosophila* brains with
472 nuclear (f, h) and membrane-bound (i, k) *UAS* reporters. Scale bars are 20 µm.

473

474 **Figure 3: Chimeric *nAChRα8β2* flies exhibit locomotor defects**

475 (a) Schematic *Dβ2* gene model with the ligand binding domain swap indicated with coding
476 exons marked in pink. The *white+* selection marker was incorporated into the *nAChRβ2* gene
477 along with the honey bee *nAChRα8* LBD encoding fragment, generating a $nAChR\beta2^{null}$ allele.
478 The reading frame can be restored by excising the PiggyBac transposon.

479 (b) Climbing activity was assessed by negative geotaxis and is shown as means with the
480 standard error from four biologically independent groups (n=20). Statistically significant
481 differences are indicated by asterisks (****$p \leq 0.0001$).

482 (c) Flies of the indicated genotypes were tested for their flight ability shown as means with the
483 standard error from four biologically independent groups (n=10). Statistically significant
484 differences are indicated by asterisks (**$p \leq 0.01$).

485

486 **Figure 4: The α8 ligand binding domain does not sensitize flies to neonicotinoids**

487 (a-c) Flies viability exposed to 10 µM (a), 20 µM (b) and 40 µM (c) thiamethoxam measured
488 every 24 h for 6 days is shown as mean with standard error from four biologically independent
489 groups (n = 20).

490 (d) Survival rate of $nAChR\beta2^{Am\alpha8LBD}/nAChR\beta2^{nullA}$ and $nAChR\beta2^{nullA}/nAChR\beta2^{nullB}$ mutants
491 compared to WT flies subjected to different insecticides combinations. The log2 fold-change

492 shown as mean with standard error from four biologically independent groups (n = 20)
493 represents the viability of flies after six days of treatment with thiamethoxam (TMX) or TMX
494 mixed with imidacloprid (IMI) or flupyradifurone (FPF) in equimolar ratios. Statistically
495 significant differences are indicated with letters (a = *$p \leq 0.5$, b = **$p \leq 0.01$, c = ***$p \leq 0.001$,
496 d = ****$p \leq 0.0001$).
497

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665

Figure 1

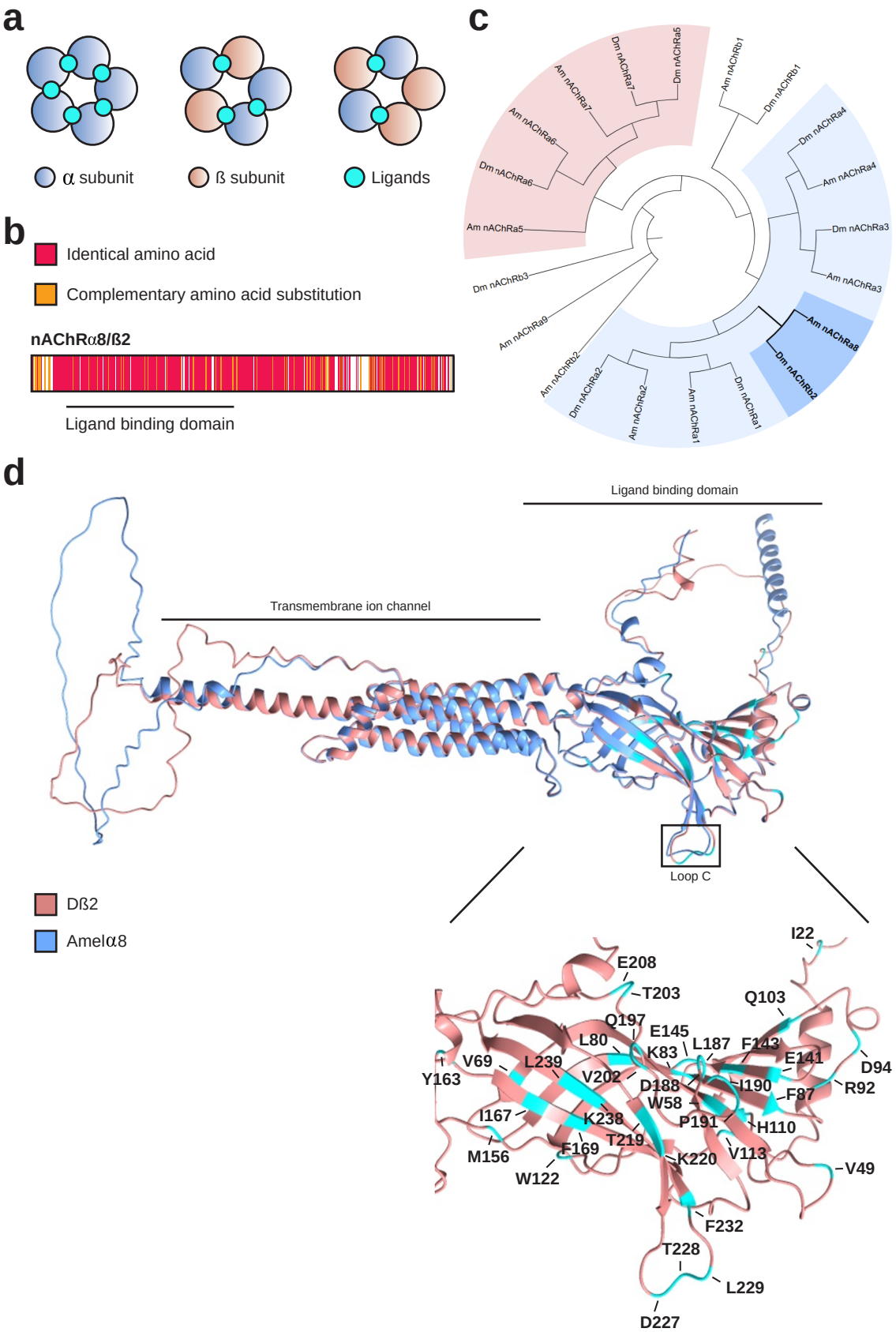

a
α subunit
ß subunit
Ligands
b
Identical amino acid
Complementary amino acid substitution
nAChRα8/ß2
Ligand binding domain
c
Dm nAChRa5
Dm nAChRa7
Am nAChRa7
Am nAChRa6
Dm nAChRa6
Am nAChRa5
Am nAChRb1
Dm nAChRb1
Dm nAChRa4
Am nAChRa4
Dm nAChRa3
Am nAChRa3
Dm nAChRb3
Am nAChRa9
Am nAChRb2
Dm nAChRa2
Am nAChRa2
Am nAChRa1
Dm nAChRa1
Am nAChRa8
Dm nAChRb2
d
Ligand binding domain
Transmembrane ion channel
Loop C
Dß2
AmelΔ8
I22
E208
T203
Q103
Q197
E145
L80
K83
L187
F143
E141
D94
V69
L239
Y163
V202
D188
I190
R92
F87
W58
I167
K238
P191
H110
T219
M156
F169
K220
V113
V49
W122
F232
T228
L229
D227

Figure 2

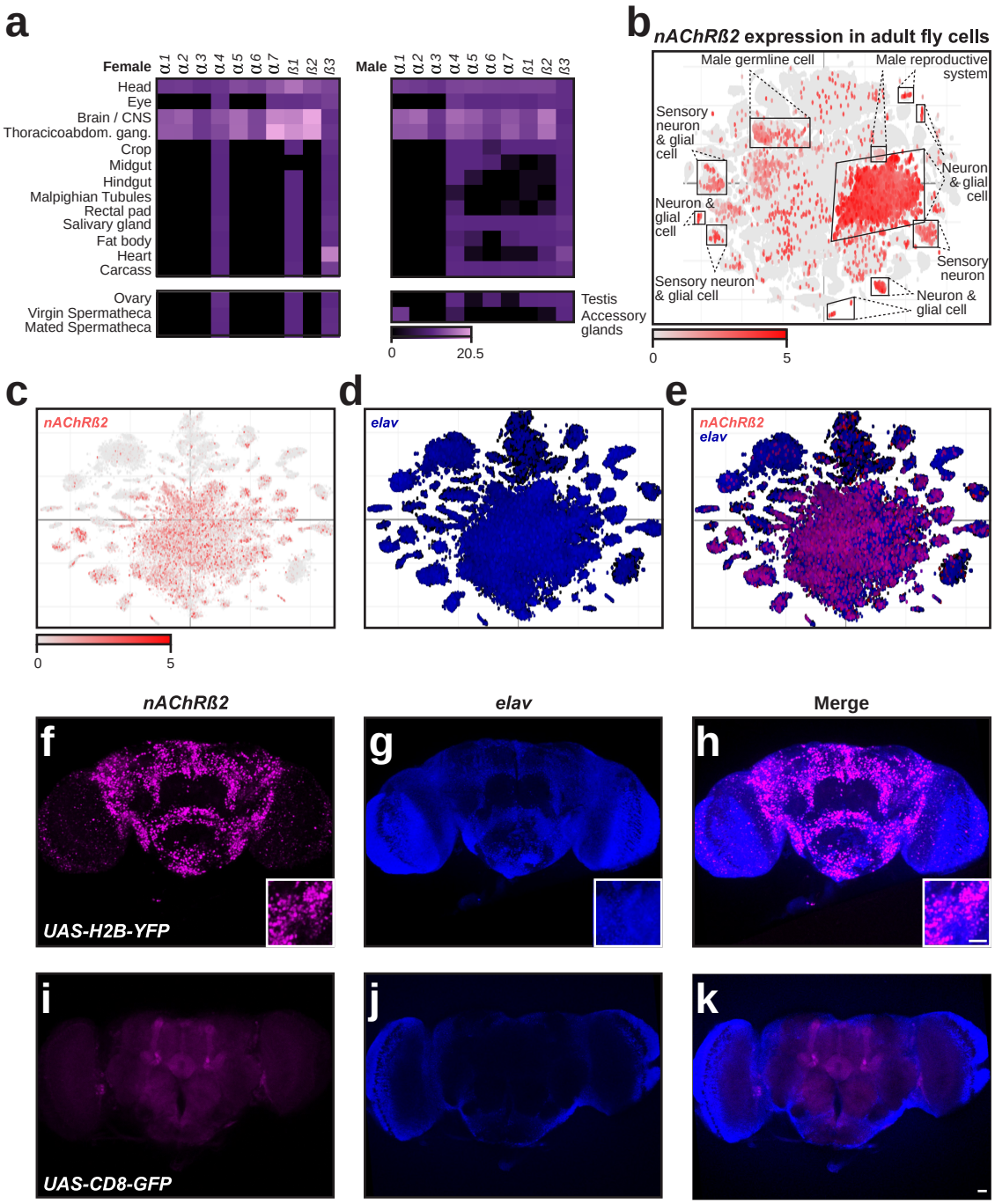

a
Female
α1 α2 α3 α4 α5 α6 α7 ß1 ß2 ß3
Head
Eye
Brain / CNS
Thoracicoabdom. gang.
Crop
Midgut
Hindgut
Malpighian Tubules
Rectal pad
Salivary gland
Fat body
Heart
Carcass
Ovary
Virgin Spermatheca
Mated Spermatheca
Male
Testis
Accessory glands
0
20.5
b
nAChRß2 expression in adult fly cells
Male germline cell
Male reproductive system
Sensory neuron & glial cell
Neuron & glial cell
Neuron & glial cell
Sensory neuron
Sensory neuron & glial cell
Neuron & glial cell
0
5
c
nAChRß2
0
5
d
elav
e
nAChRß2
elav
nAChRß2
elav
Merge
f
UAS-H2B-YFP
g
h
i
UAS-CD8-GFP
j
k

Figure 3

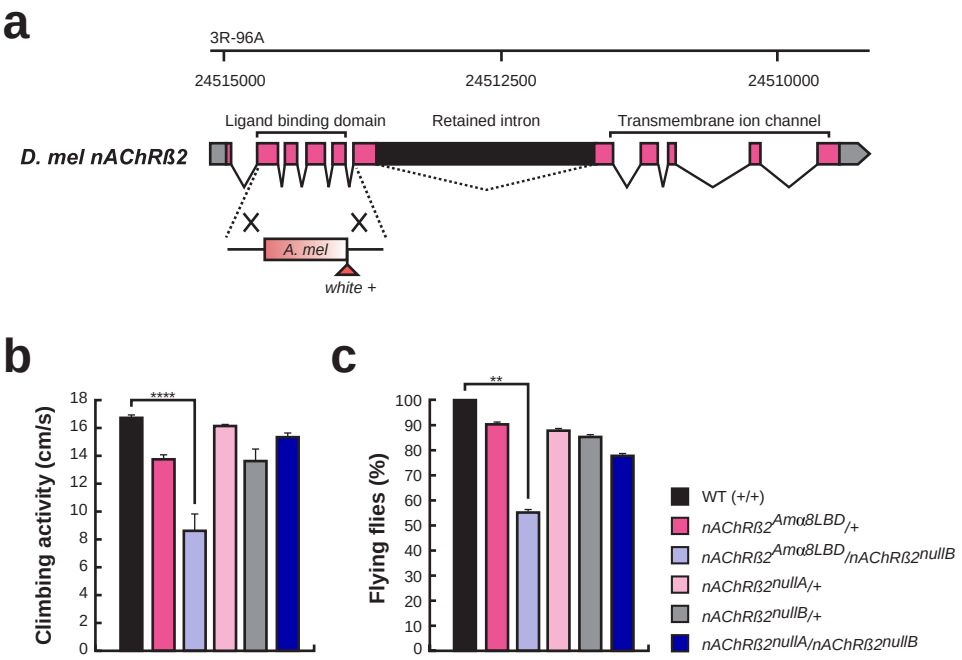
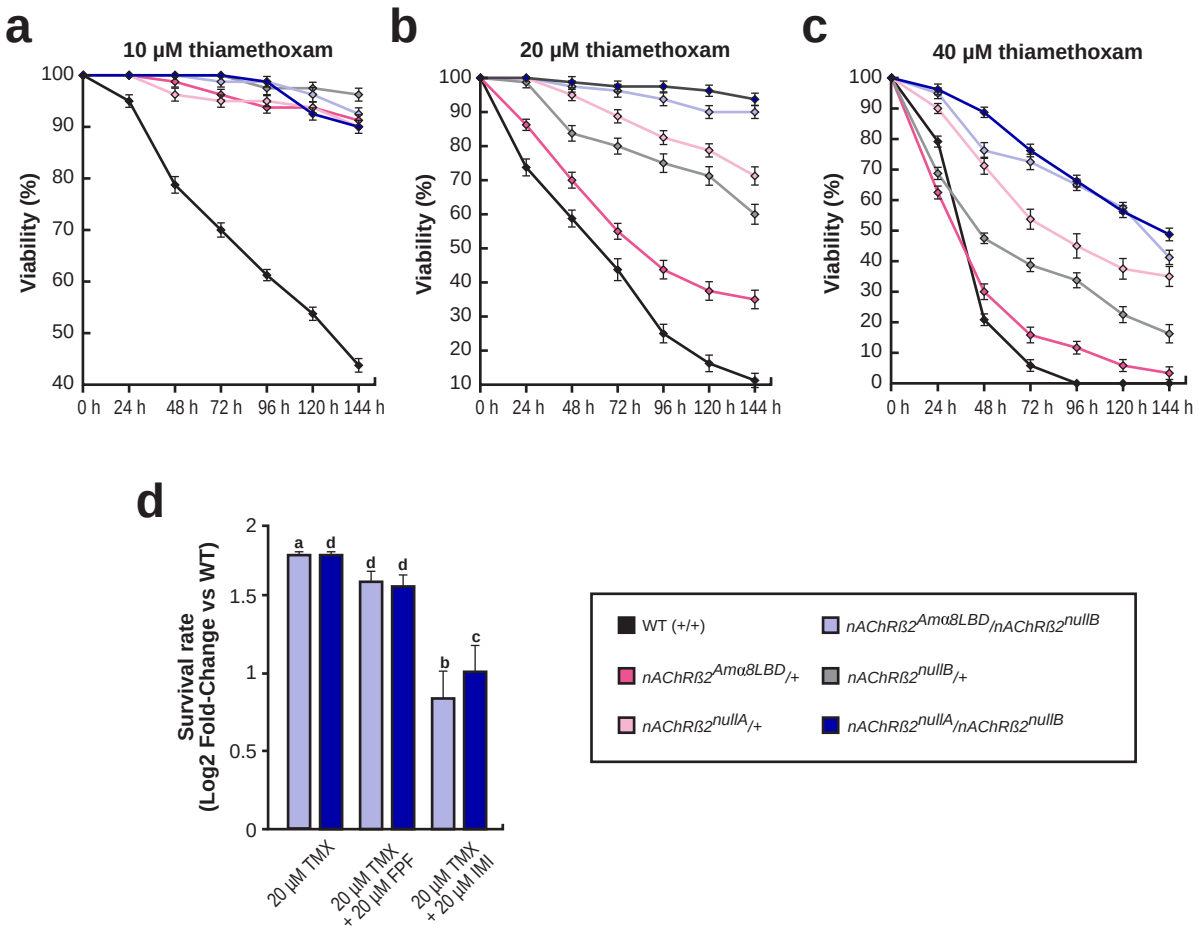

a
3R-96A
24515000
24512500
24510000
Ligand binding domain
Retained intron
Transmembrane ion channel
D. mel nAChRß2
A. mel
white +
b
Climbing activity (cm/s)
****
c
Flying flies (%)
**
WT (+/+)
nAChRß2Amα8LBD/+
nAChRß2Amα8LBD/nAChRß2nullB
nAChRß2nullA/+
nAChRß2nullB/+
nAChRß2nullA/nAChRß2nullB

Figure 4


a
10 µM thiamethoxam
Viability (%)
100
90
80
70
60
50
40
0 h
24 h
48 h
72 h
96 h
120 h
144 h
b
20 µM thiamethoxam
Viability (%)
100
90
80
70
60
50
40
30
20
10
0 h
24 h
48 h
72 h
96 h
120 h
144 h
c
40 µM thiamethoxam
Viability (%)
100
90
80
70
60
50
40
30
20
10
0
0 h
24 h
48 h
72 h
96 h
120 h
144 h
d
Survival rate
(Log2 Fold-Change vs WT)
2
1.5
1
0.5
0
a
d
d
d
b
c
20 µM TMX
20 µM TMX
+ 20 µM FPF
20 µM TMX
+ 20 µM IMI
WT (+/+)
nAChRß2Amα8LBD/nAChRß2nullB
nAChRß2Amα8LBD/+
nAChRß2nullB/+
nAChRß2nullA/+
nAChRß2nullA/nAChRß2nullB