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1 Elucidating the structural basis for ligand binding and translocation in
2 conserved insect odorant receptor co-receptors

3

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13

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19

20 **This PDF file includes:**

21 Main Text
22 Figures 1 to 7
23 Supplementary Figures 1 to 10
24 Supplementary Tables 1 to 3

¹ These authors contributed equally to this work

25 **Abstract**

26 In numerous insects, the olfactory receptor family forms a unique class of heteromeric cation
27 channels. Recent progress in resolving the odorant receptor structures offers unprecedented
28 opportunities for deciphering their molecular mechanisms of ligand recognition. Unexpectedly,
29 these structures in apo or ligand-bound states did not reveal the pathway taken by the ligands
30 between the extracellular space and the deep internal cavities. By combining molecular modeling
31 with electrophysiological recordings, we identified amino acids involved in the dynamic entry
32 pathway and the binding of VUAA1 to *Drosophila melanogaster's* odorant receptor co-receptor
33 (Orco). Our results provide new evidence for the exact location of the agonist binding site and a
34 detailed and original mechanism of ligand translocation controlled by a network of conserved
35 residues. These findings would explain the particularly high selectivity of Orcos for their ligands.

36 **Main Text**

37

38 **Introduction**

39

40 Among all living multicellular organisms, insects represent more than half of all identified species
41 on Earth, thus forming the most diverse group of animals (1). Insects show a remarkable capacity
42 to adapt to a wide range of ecological niches. The rapid evolution of insect olfactory receptors
43 is thought to contribute to this adaptation (2), endowing each insect species with the ability to
44 selectively detect volatile chemicals associated with its specialized habitat and lifestyle. Therefore,
45 olfaction is a vital sense necessary for them to find food, a mate, an oviposition site and a host.
46 Moreover, the insect olfactory receptors are the main targets for the rational design of repulsive or
47 attractive compounds for protection against vector-borne species or pest control (3, 4).

48 Ground breaking studies have provided a structural description of the proteins involved in odor
49 recognition by insects (5, 6). In addition to the gustatory receptors, the repertoire of odorant
50 receptors is mainly composed of two distinct families: i) the olfactory receptors (ORs) that form a
51 complex with the highly conserved odorant receptor co-receptor (Orco) (7); and ii) the ionotropic
52 receptors (IRs) that are structurally similar to the ionotropic glutamate receptor (8). The OR/Orco
53 receptors are mainly expressed in olfactory sensory neurons (OSNs) found in insects' antennae.
54 An individual OSN typically expresses only a single type of OR (9), which defines the neuron's
55 response spectrum (10), even if non-canonical co-expressions have been observed in a
56 mosquito(11). The OR/Orco complexes are proposed to form a unique class of heteromeric cation
57 channels composed of the two related 7-transmembrane subunits. It has been shown that Orcos
58 could form homotetrameric channels (Fig. 1A), which have a different recognition spectrum than
59 ORs (12, 13).

60 Orcos seems to appear late in the evolution of insects and constitute a remarkable example of an
61 adaptive system, with a unique highly conserved signaling subunit (Orco) that can associate with
62 a large repertoire of odorant receptor subunits that diverge to recognize specific ligands (14, 15).
63 The evolution of ORs that led to the appearance of Orcos induced a total loss of odorant binding
64 for this subunit, while engendering the ability to bind few synthetic ligands, like VUAA1 (16-22). On
65 the other hand, the "ancestral" OR5 receptor from *Machilis hrabei* (MhraOR5) is activated by a
66 large set of odorants, but not by VUAA1 (6). DmelOrco and MhraOR5 share 18.3% sequence
67 identity and adopt the same tertiary fold (Fig. 1B). However, the origin of the differences in the
68 recognition spectra of the two receptors is still not fully understood.

69 To decipher the molecular mechanisms governing the response of Orcos to ligands, different
70 structure-function studies were previously employed based on site-directed mutagenesis combined
71 with two-electrode voltage-clamp (TEVC) measurements. Fig. 1C summarizes the position of
72 different residues that showed a functional impact when mutated (5, 23-26). These studies have
73 highlighted the central role of residues from helix S7 in forming a hydrophobic gate that contributes
74 to ion selectivity. Moreover, the structures of MhraOR5 in complex with two agonists, eugenol and
75 DEET, revealed the ligand binding cavity of this receptor (Figs. 1B and D) (6).

76 Despite these highly informative structural studies, several questions remain, in particular the entry
77 pathway and the binding site of ligands in Orcos. Their identification is essential for understanding
78 the high specificity of action of Orco ligands and for the rational design of new molecules for

79 attractive or repulsive applications. To identify the binding pocket and the translocation pathway of
80 VUAA1 from the extracellular space to the Orco binding site, we combined molecular modeling
81 approaches with site-directed mutagenesis and functional characterization by the TEVC technique.

82 83 **Results**

84
85 **Determination of the optimal Orco.** Olfactory receptors are notorious for weakly expressing in
86 heterologous systems, which impedes their functional characterization. Before initiating molecular
87 dynamics (MD) simulations, we searched for the optimal Orco that generates the highest response
88 to VUAA1 when expressed in *Xenopus* oocytes. Orcos from *Apocrypta bakery*, *Drosophila*
89 *melanogaster*, *Aedes albopictus* and *Culex quinquefasciatus* were functionally characterized by
90 the TEVC technique. The results (Fig. 2) clearly demonstrate that DmelOrco generated the highest
91 current amplitude in the presence of VUAA1 and it was chosen as the model for both computations
92 and experiments.

93
94 **Orcos show a conserved cavity.** A 3D model of DmelOrco was built by homology modeling using
95 the experimental structure of AbakOrco homotetramer (pdb ID: 6C70) as a template (5). The two
96 protein sequences are highly similar (76 % of sequence identity) prefiguring a high confidence in
97 the accuracy of the model of DmelOrco (27). The full protocol is detailed in the Materials and
98 Methods section. We compared it to a model obtained by AlphaFold2 (extracted from the Alpha
99 Fold Protein database) (28). Both structures show a high similarity of transmembrane segments
100 (RMSD = 0.7 Å). The largest deviation between the structures is observed at the intracellular loop 2
101 (IL2) (*SI Appendix*, Fig. S1). This loop is not resolved on the cryoEM structure of DmelOrco, which
102 suggests a high flexibility.

103 The structure of AbakOrco (6) and the homology model of DmelOrco (Figs. 1B and D), revealed a
104 cavity between helices S1 to S4 and S6 (Fig. 1C) that could play the role of the ligand binding site
105 for VUAA1 and its analogues. Interestingly, this cavity has a position similar to the ligand binding
106 site found in the structure of MhraOR5 (6) (Fig. 1B). The amino acids lining the two cavities are
107 highly conserved between DmelOrco and AbakOrco with 73% identity (Fig.1D). Notably, the cradle
108 of this pocket would be formed by the residue F83^{Dmel}, which is critical for activation by VUAA1
109 (26). In both structures and models, the access of VUAA1 to its putative binding site seems
110 hindered by constrictions of the transmembrane helices, suggesting a progression of the molecule
111 through a hidden and dynamic pathway.

112 113 **MD simulations highlight a stepwise mechanism of VUAA1 entry to the embedded binding** 114 **cavity.**

115 We studied the entry of VUAA1 molecules to their putative binding site in DmelOrco by means of
116 MD simulations. To reach this deeply embedded site, residing in the core of the transmembrane
117 helices, the molecule must transit through a path that is assumed to be dynamic since it is closed
118 in the structures of AbakOrco and MhraOR5. To identify this path, multiple MD simulations were
119 performed with several ligands to enhance the sampling of rare events such as ligand migration
120 (29, 30) and protein conformational changes (31, 32). We constructed a system containing 4
121 DmelOrco monomers with five VUAA1 molecules randomly placed in the extracellular part of the
122 simulation box. Then, 22 replicas were subjected to MD simulations, leading to a total of 88
123 simulations on DmelOrco monomers. The total simulation time is approximately 31 μ s (see
124 Materials and Methods). A constraint was applied between each VUAA1 and the top of the channel
125 pore to increase sampling speed without biasing the binding process. Thus, the ligands were free
126 to sample the extracellular region of the simulation box and to diffuse into the receptor core. The
127 migration of VUAA1 through the protein core was evaluated by the evolution of the distance
128 between the VUAA1 center of mass and the center of mass of the binding cavity (defined as the
129 center of mass of the eugenol molecule in MhraOR5, pdb: 7LID). We thus defined 4 distinct steps:
130 contact (Fig. 3A, *area a*), entry (Fig. 3A, *area b*), vestibule (Fig. 3A, *area c*) and binding site (Fig. 3A,
131 *area d*).

132 The results of our simulations revealed a predominant pathway of VUAA1 entry into the binding
133 site. From the 88 trajectories, 19 showed an entry of VUAA1 within the receptor bundle (Fig. 3A,

134 *area c*). Out of these 19 trajectories, 7 full entries into the binding pocket (Fig. 3A, *area d*) were
135 observed. The other trajectories resulted in a partial binding event, where VUAA1 remains within a
136 vestibular site, half-way to the pocket cradle (*SI Appendix*, Figs. S2).

137 In all the seven observed binding events, VUAA1 consistently enters the receptor through the same
138 gate and showed contact with residues belonging to helices S2 to S6. Interestingly, most of these
139 residues are highly conserved among various Orcos (*SI Appendix*, Dataset S1 and Fig. S2C) in
140 line with the similar action of VUAA1 observed in the majority of insect Orcos (26). The ligand does
141 not interact with helix S7, which forms the tetrameric pore, suggesting that VUAA1 acts indirectly
142 on the gate through conformational changes in Orco.

143 The migration of VUAA1 appears to be governed by stepwise hydrophobic and hydrophilic
144 interactions throughout the ingress of the ligand towards the cradle of the binding site. The four
145 steps have been identified based on the distance to the binding cavity and but also on the
146 percentage of solvation of VUAA1 (Figs. 3B and C). The first step (a) is a rapid contact (few ns)
147 VUAA1 with the extracellular side of DmelOrco and a rapid partial desolvation. The second step
148 (b) is a stabilization of the position of VUAA1 during ~500 ns and a solvation stable at ~50%. The
149 third step (c) is a rapid progress (less than 200 ns) of the molecule toward the cavity and a decrease
150 of solvation up to ~20%. The fourth and last step (d) is a position of the molecule in the cavity with
151 stable solvation around 20%. In steps (a) and (c), the desolvation of VUAA1 significantly increases,
152 playing an essential role in the progression of the molecule toward the binding site.

153 The hydrophobicity and electrostatic complementarities of VUAA1 with DmelOrco in the different
154 areas (*a* to *d*) have been evaluated in the *SI Appendix*, Table S1. Analogues of VUAA1 (VUAA0.5,
155 2, 3 and 4) were also incorporated and ranked by their EC₅₀ (18). For all ligands considered, we
156 noticed an increase in hydrophobic and electrostatic complementarity when the ligand was located
157 deeper in the protein. Furthermore, the trend in hydrophobic complementarity approached that of
158 ligand strength. Although the differences in ligand EC₅₀ were minor, these observations suggest a
159 correlation between hydrophobic complementarity in the ligand translocation pathway and ligand
160 strength of VUAA analogues.

161 During its progression toward the binding site from the *area b* to *d* (Fig. 3B), VUAA1 is mostly
162 orthogonal to the membrane plane (*area c*). In addition to the desolvation process, the flexibility of
163 the molecule appears to greatly facilitate the migration of VUAA1. Thus, VUAA1 adopts several
164 conformations to adapt to the local constraints, which allow the entrance into the protein either by
165 its pyridine or its phenylethyl moiety. However, when considering an alternative orientation of the
166 VUAA analogues in the binding site (*SI Appendix*, Table S1), we observe a decrease in electrostatic
167 and hydrophobic complementarities compared to the initial orientation. This suggests that VUAA1
168 would have a preferred orientation in the binding site.

169 In the simulations, VUAA1 is stabilized by a subset of residues and must overcome an energetic
170 barrier to reach the next metastable intermediate state. Several residues were identified as
171 interacting with VUAA1 during its penetration into DmelOrco. A comprehensive list of these
172 residues is provided as supplementary information (*SI Appendix*, Dataset S2). The initial binding
173 event occurs through a contact between VUAA1 and Y390^{S6} at the extracellular end of S6 (Fig. 3B,
174 *area a*). Starting from this position, VUAA1 makes regular contacts with the residue side chains
175 (Fig. 3B, *area b*) and undergoes a large desolvation process upon its entry into the receptor bundle
176 (Fig. 3B, *area c*). The ligand then establishes additional contacts with I79^{S2}, T80^{S2}, W150^{S3}, I181^{EL2},
177 V206^{S4}, K373^{S5} and Y397^{S6}, where it pauses for several nanoseconds (Fig. 3B, *area c*). The ligand
178 finally enters the cavity (Fig. 3B, *area d*) that was previously identified in the structures of AbakOrco
179 and MhraOR5, and in the model of DmelOrco (Fig. 1D). The final position of VUAA1 in the cavity
180 is parallel to the membrane, and it interacts with F83^{S2}, F84^{S2}, S146^{S3}, M210^{S4} and Y400^{S6}, similar
181 to the position of the eugenol molecule in the MhraOR5 structure (*SI Appendix*, Fig. S3).

182 These results further guided site-directed mutagenesis experiments combined with functional
183 assays to assess the critical role of residues identified as interacting with VUAA1 in the simulations.

184
185 ***Site directed mutagenesis and electrophysiological characterization support the entry***
186 ***mechanism of VUAA1.***

187 To experimentally assess the functional role of residues that significantly interacted with VUAA1 in
188 the simulations, different mutants were designed. The influence of the volume or the

189 physicochemical properties of their side chains was evaluated according to the response of Orco
190 to stimulation by VUAA1. To facilitate or block the translocation process of VUAA1, the residues
191 were mutated to smaller (alanine) or larger (tryptophan) residues, respectively. For disrupting
192 hydrophobic interactions or hydrogen bonds between side chains and VUAA1, mutations were
193 made in serine (small hydrophilic residue) or in phenylalanine (aromatic residue without hydroxyl
194 group), respectively. To invert the charge at position 373^{S5}, the lysine was mutated in a negatively
195 charged glutamate. The response to VUAA1 of each mutant was assessed by electrophysiological
196 recordings with the TEVC method.

197 The simulations revealed that Y390^{S6} is the first residue that has a significant interaction with
198 VUAA1, interacting at a frequency of 0.47 averaged over all entry trajectories. Y390^{S6} was mutated
199 into alanine (Y390A) and phenylalanine (Y390F) and both mutations did not show significant
200 change in the response to VUAA1 (Fig. 4). Thus, the reduction of the side chain into alanine or the
201 removal of the hydroxyl group of Y390 did not favor or abolish the action of VUAA1. Consequently,
202 neither aromaticity nor a hydroxyl group on the aromatic ring are necessary for the interaction with
203 VUAA1 in position 390. On the contrary, its mutation into serine led to a decrease in the activation
204 by VUAA1 (2.44 μ A vs 4.71 μ A for the WT). A Western-blot has been performed to verify that the
205 expression level of the Y390S mutant was similar to the WT (*SI Appendix*, Fig. S4), and the semi-
206 quantitative analysis indicated no significant differences between both constructs. This result
207 supported the role of Y390 in VUAA1 activation. In particular, the differences observed between
208 the mutants emphasize the importance of the hydrophilic character of position 390^{S6}. Introduction
209 of a serine in place of tyrosine generates a hydrophilic environment (33) that would hamper the first
210 step of desolvation process that is crucial for the entry of VUAA1, as observed in the simulations
211 (Fig. 3C).

212 When going deeper in the protein, VUAA1 has shown high frequencies of interaction with a planar
213 section of seven residues: I79^{S2}, T80^{S2}, W150^{S3}, I181^{EL2}, V206^{S4}, K373^{S5} and Y397^{S6} interacting
214 with VUAA1 (Fig. 5A) at frequencies of 0.44, 0.56, 0.68, 0.17, 0.11, 0.70 and 0.70, respectively
215 (averaged over all entry trajectories). Mutations into alanine of all seven residues did not
216 significantly affect the amplitude of activation induced by VUAA1 (Fig. 5B), indicating that the side
217 chains of these residues are not critical or involved in a limiting step for the interaction with VUAA1.
218 In contrast, mutations of the non-aromatic residues in the bulkier tryptophan significantly reduced
219 or abolished the activation by VUAA1 (Fig. 5B-I, *red dots*). Western-blot results (*SI Appendix*, Fig.
220 S4) showed a decrease of expression of T80W and V206W. These results suggest that these
221 mutations not only affected the expression level of the mutants but also the response to VUAA1. In
222 contrast, mutations I181S and I181W showed an increase of expression in Western-blot results,
223 but still a clear loss of VUAA1 activation supporting that the ability of VUAA1 to access this region
224 is critical for the channel response.

225 As these residues are pointing into the ligand pathway observed during simulations, these
226 functional results support the implication of these residues in the entry of VUAA1. Interestingly,
227 inserting the hydrophilic and shorter serine residue in place of the hydrophobic I181^{EL2} (I181S),
228 significantly reduced the amplitude of activation (1.40 vs 4.71 μ A for the WT) (Fig. 5B) as previously
229 observed with Y390S mutant. This deleterious effect of the mutation into serine is site specific since
230 the similar mutation of Y397^{S6} (Y397S) showed no significant effect on VUAA1 response (Fig. 5B).
231 Mutation of the only charged residue identified in the simulations (K373^{S5}) generated unexpected
232 responses. Thus, mutation of K373^{S5} into alanine (K373A) that profoundly modifies the physico-
233 chemical properties by reducing the size of the side chain and by removing the positive charge, did
234 not change the response to VUAA1 (Fig. 5B). Inversion of the charge by mutation of K373^{S5} into
235 glutamate (K373E) did not abolish the response but increased it (7.47 μ A), potentially by
236 decreasing the polarity of the binding cavity (*SI Appendix*, Fig. S5). Western-blot results confirmed
237 that the K373E was not overexpressed. All mutations made at position Y397^{S6} did not significantly
238 change the amplitude of activation induced by VUAA1 (Fig. 5). In the simulations, VUAA1 is in

239 transit in this section of seven residues, and move on to a deeper cavity, which would constitute
240 the binding site.

241

242 **Site directed mutagenesis and electrophysiological characterization support the binding**
243 **site of VUAA1.**

244 In the deeper section, five residues were identified in the simulations to frequently interact with
245 VUAA1 and formed a cavity suspected to be the binding site (*SI Appendix*, Dataset S2). The five
246 positions F83^{S2}, F84^{S2}, S146^{S3}, M210^{S4} and Y400^{S6} (respectively interacting with VUAA1 at a
247 frequency of 0.32, 0.02, 0.23, 0.19, 0.24, averaged on all entry trajectories) were mutated to defined
248 more precisely the cradle of the VUAA1 binding cavity (Fig. 6). Using the site-directed mutagenesis
249 approach, all the five residues were mutated in alanine and tryptophan (Fig. 6B) to reduce or
250 increase the steric hindrance of the side chains, respectively. The Western-blot results (*SI*
251 *Appendix*, Fig. S4) demonstrated that all mutants of the Fig. 6 were expressed at similar levels.

252 In contrast to previous results, mutation in alanine of two phenylalanine residues (F83A and F84A)
253 decreased the response to VUAA1 (Fig. 6B-D) with a greater extent for F84A (medians: 2.33,
254 0.68 μ A for F83A, F84A respectively vs 4.71 μ A for WT). Mutation in tryptophan induced the same
255 phenotype in position 83 (F83W) (Fig. 6B), while the mutation in serine had the same impact in
256 position 84 (F84S) (Fig. 6B). Finally, the mutation F84W did not induce a significant change
257 compared to the WT (Fig. 6B). These results indicate that these two adjacent phenylalanine
258 residues play a critical role in the activation by VUAA1, but with different characteristics. Position
259 83 must be a phenylalanine and cannot be replaced by a homologous residue like tryptophan, while
260 position 84 is more tolerant to replacement by tryptophan but much less to alanine and serine. The
261 peripheral position of F84^{S2} in the cavity could explain this selective tolerance to large hydrophobic
262 residues, while the central position of F83^{S2} in the cavity suggests a larger and more specific
263 interaction with the ligand. These results are in agreement with those of Corcoran et al. (26),
264 showing that F83^{S2} is one of the essential residues for the action of VUAA1.

265 On the opposite side of the cavity, S146^{S3} is also pointing toward the binding cavity. Mutation of
266 this hydrophilic residue induced a unique phenotype of increased response to VUAA1 when
267 mutated in alanine (medians: 7.55 μ A vs 4.71 μ A for WT). This effect is strengthened by the
268 introduction of the bulkier and more hydrophobic residue, valine (33) (median: 11.68 μ A) (Fig. 6B
269 and E). This mutation S146V showed the highest response to VUAA1 and could be used in further
270 studies to increase the amplitude of the response.

271 Mutations of M210^{S4} in shorter alanine (M210A) or leucine (M210L) residues did not change the
272 response to VUAA1 (5.72 and 6.19 μ A, respectively vs 4.71 μ A for WT), while the mutation in the
273 bulkier tryptophan induced a significant decrease in the amplitude of activation (2.12 μ A) (Fig. 6B
274 and F). Consequently, the methionine 210 that is in close proximity to F83^{S2} and F84^{S2} does not
275 specifically interact with VUAA1, but this position does not tolerate steric hindrance.

276 Mutation of Y400^{S6} in either alanine, serine or tryptophan did not significantly change the response
277 to VUAA1. Despite the high conservation of Y400, this result is consistent with the position of the
278 residue, located deeply in the core of the protein, so its mutation is unlikely to change the properties
279 of the binding cavity.

280 Concentration-effect curves performed on mutants with the most significant results (Fig. 6G and *SI*
281 *Appendix*, Table S2) showed a change in I_{max} that was either negative (for F84A and M210W,
282 1.01 and 1.50 μ A, respectively vs 3.97 μ A for WT) or positive (for S146V, 6.99 μ A vs 3.97 μ A for
283 the WT), without affecting the EC_{50} . These results suggest a dominant effect of the mutations on
284 the efficacy of VUAA1.

285 Western blot results show that mutants with a significant gain or loss of function are always
286 expressed (*SI Appendix*, Fig. S4). Gain of function means an increase of current amplitude induced
287 by VUAA1, while loss of function is a decrease or an absence of this current amplitude. Moreover,
288 the level of expression has no correlation with the mutant response to VUAA1. Finally, we
289 performed control experiments on a position, which do not interact with VUAA1 during the
290 simulations, but close to the residue S146 that is particularly sensitive to gain- and loss-of-function
291 mutations when mutated in alanine, valine and tryptophan. Leucine 141^{S2} was mutated to these

292 three types of amino-acid. Contrary to what is observed on the position S146^{S2}, these mutations
293 did not induce significant change in the channel response to VUAA1 (*SI Appendix*, Fig. S6).

294

295 **Discussion**

296

297 The simulations of VUAA1 binding onto DmelOrco were carried out with little knowledge about the
298 amino acids involved in the mechanism of binding. They indicated an entry pathway and a binding
299 site in a model of Orco that is highly conserved across species. The binding mode of VUAA1
300 depends on interactions mediated by different hydrophobic, aromatic, and hydrophilic residues
301 within the pocket. Finally, the experimental results supported the predictions made by the
302 simulations and allowed the identification of residues critical for the entry of VUAA1 and for its
303 binding in a deeply embedded cavity that is also found in the structures of AbakOrco and MhraOR5.

304

305 ***The translocation of VUAA1 through the protein is highly conserved among Orcos.***

306 The analysis of the sequence conservation reveals that the pathway followed by VUAA1 to reach
307 the binding site of DmelOrco is highly conserved (Fig. 7). As Orcos are known to recognize a
308 remarkably restricted number of ligands, the high conservation of the translocation pathway can be
309 interpreted as a molecular sieve, which filters the entrance of ligands to the binding site. These
310 residues show a high conservation in Orcos and are likely to be crucial for initiating the opening of
311 the channel upon ligand binding. In contrast, ORs that recognize a large diversity of ligands (34-
312 36) show a low conservation at similar positions. The chemical variation observed in residues that
313 line the translocation pathway in ORs allows a large diversity of odorants to diffuse inside the
314 protein and reach their binding sites.

315

316 ***Desolvation of VUAA1 is fundamental for its entry.***

317 The recent advances in structural biology have led to greater insight into the role of desolvation in
318 the thermodynamics and kinetics of binding (37-39). The importance of hydrophobic interactions
319 as a ligand-desolvation penalty or a driving force for the induced fit of receptors is a long-term
320 challenge in drug design (40, 41). In particular, it has been shown that water plays a crucial role in
321 the binding kinetics (42). The binding process of VUAA1 to Orco is accompanied by a desolvation
322 at each metastable state. The most important decrease in the number of water molecules in the
323 first solvation shell is observed when VUAA1 enters the protein. Accordingly, the mutation of the
324 hydrophobic Y397^{S6} to a hydrophilic serine decreased the response of DmelOrco to VUAA1, most
325 probably by preserving water molecules around VUAA1. Our results also suggest that I181^{EL2} could
326 be involved in the desolvation process required for entry into the transmembrane core of Orco,
327 which would explain why no continuous translocation pathway is observed in the structures of the
328 apo state of AbakOrco and MhraOR5.

329 Comparative analysis of the eugenol-bound MhraOR5 structure (pdb: 7LID) with our VUAA1-bound
330 DmelOrco model revealed a shared binding site position with a high conservation (16 amino acid
331 pocket: 50 % identity, 62.5 % similarity; 24 amino acid pocket: 33 % identity, 62.5 % similarity)
332 (Fig. 7). However, the ORs show a remarkable diversity in the binding site composition. This
333 particularity is also found in mammalian ORs, allowing for a broad detection of chemicals (43-45).

334

335 ***The polarity and volume of the ligand binding cavity influences the efficacy of VUAA1.***

336 The polarity of the binding cavity appears to have a pronounced influence on the channel response
337 to VUAA1: a decrease induces a gain of function while an increase leads to a loss of function (*SI*
338 *Appendix*, Fig. S5). We further investigate this observation by evaluating the polarity of 176 Orcos
339 from 174 species. This analysis reveals that the binding cavity of the VUAA1-insensitive MdesOrco
340 is more polar than the responsive Orcos. When Corcoran et al. (26) replaced the hydrophilic H81^{S2}
341 from MdesOrco by a more hydrophobic phenylalanine (H81F), it induced a response to VUAA1. In
342 contrast, mutations that increased the polarity of the binding cavity abolished the response to
343 VUAA1 in AsegOrco. The polarity of the cavity seems to be a good indicator to predict the response
344 to VUAA1 of a given Orco or mutant (*SI Appendix*, Fig. S5).

345 The volume of the cavity also influenced the response of Orco to VUAA1 (*SI Appendix*, Fig. S7). A
346 substantial reduction of the volume (such as the introduction of a tryptophan residue, in position

347 F83^{S2}, S146^{S3}, V206^{S4} or M210^{S4}) induced a significant decrease in the response to VUAA1. In
348 contrast, mutations that increased the volume of the cavity did not rationally induce a change in the
349 response to VUAA1. An exception was the mutation F84A, which abolished the response to
350 VUAA1, potentially due to an indirect interaction with the ligand. These results suggest that the
351 protein could fluctuate to accommodate bulky ligands such as VUAA1, as has already been shown
352 for olfactory receptors (6, 43, 44).

353

354 ***The architecture of the ligand binding site is conserved among Orcos and ORs.***

355 Once in the binding cavity, VUAA1 is stabilized by a combination of hydrophobic h-bond, Van der
356 Waals and pi-stacking interactions and does not move back into the bulk within the simulation time.
357 We compared our electrophysiological results with already published data on mutants of two ORs:
358 MhOR1 and MhraOR5 (*SI Appendix*, Table S3). In particular, our results highlight the importance
359 of two residues from segment 2 (F83 and F84) to form the binding site. Mutations made at similar
360 positions in MhOR1 (Y106^{S2A}, I107^{S2A}) and MhraOR5 (Y91^{S2A} and F92^{S2A}) result in non-
361 responsive channels.

362 The natural complex is formed by the association of Orco subunits with ORx subunits. In this
363 configuration, it has been suggested that VUAA1 acts as an allosteric ligand but it could also act
364 as orthosteric ligand on the ORx subunit. The absence of response to VUAA1 of the ancestral
365 MhraOR5, that recognizes a large diversity of odorants, suggest that VUAA1 would not act on the
366 OR subunits. A possible approach would be to co-express mutants of DmelOrco in the translocation
367 pathway or binding site that are insensitive to VUAA1 (such as I79W, T80W or V206W) with an
368 ORx and challenge with VUAA1.

369 In the final pose, VUAA1 remained in the same orientation, with the ethyl phenyl moiety located
370 between the helices S3 and S4 and the pyridine next to S2 and S5 (Fig. 7 and *SI Appendix*, Fig. S3).
371 This conclusion is strengthened by the increased sensitivity of S146^{S3} mutants (alanine and valine).
372 Interestingly, decreasing the ethyl moiety to a methyl almost abolished the response of Orco (18).
373 In contrast, the replacement of the ethyl group by an isophenyl group improved the potency of the
374 VUAA1-derivative. All together, these observations show that increasing hydrophobicity by
375 mutations or ligand modifications increases the response of Orco to its ligands.

376 To explore the potential binding modes of VUAA1, the ligand was structurally modified to VUAA2,
377 VUAA3 and VUAA4, which display significant greater potency, and in VUAA0.5, which is less potent
378 than VUAA1. The calculated hydrophobic and electrostatic matches between the ligands and the
379 receptor correlated with the functional results. We then evaluated the final orientation of VUAA1 in
380 the binding site. Because of its general shape, the cavity could only accommodate VUAA1 in two
381 directions, one of which is the opposite of the one observed in the simulations. Therefore, we
382 manually flipped VUAA1 into the cavity (*SI Appendix*, Fig. S8) and constructed the VUAA analogs.
383 Scores of both electrostatic and hydrophobic matches were inferior to those of the initial binding
384 mode (*SI Appendix*, Table S1), suggesting that the initial orientation is preferred.

385 In conclusion, this study revealed the translocation pathway and binding site of VUAA1 into
386 DmelOrco using a combination of dynamic simulations and functional characterization. The results
387 highlight the role of desolvation for the progression of the ligand, the role of the polarity of the
388 binding cavity in the efficacy of VUAA1 and the lower limit of size of the cavity for VUAA1 binding.
389 This study shows that the binding pocket location is conserved between ORs and Orcos. The
390 striking difference between the two families is the high level of sequence conservation of the
391 translocation pathway and binding pocket observed in Orco compared to the high diversity in ORs.
392 The conservation and the variability are then shared in between the two subunits forming the
393 heterodimer. This combination of the highly conserved Orcos subunit with the more versatile ORs
394 provides the insect with extremely high chemical discrimination power.

395 Orcos have been shown to play a fundamental role in insect behavior such as foraging and
396 oviposition and are thus a potential target for the development of behaviorally disruptive chemicals
397 (46, 47). Our results provide a fine description of the particular binding process, opening the way
398 to a rational design of orthosteric and allosteric modulators.

399

400 **Materials and Methods**

401

402 **In silico modelling**

403

404 *Alignment between Orcos and ORs with MhraOR5*

405 Alignment between MhraOR5 and Orcos was based on the alignment files for 176 Orcos and 361
406 ORs from Butterwick *et al.* (5). MhraOR5 was realigned with the Orcos using ClustalO (48) with
407 default settings, then optimized by hand to conserve the existing alignment. The same process was
408 applied for the ORs.

409

410 *Orco modelling*

411 The 176 Orcos tetramer models plus *DmelOrco* WT and mutants were generated by SWISS-model
412 pipeline (49) using PDB 6C70 as a template with default settings. *DmelOrco* alpha fold model
413 (version 07.01.2021) was retrieved from AlphaFold Protein structure database (50). RMSD
414 between the SWISS-model and AlphaFold model was calculated using cpptraj (51) after alignment
415 of the structures on *i)* all the sequence, *ii)* all the sequence except IL2, and *iii)* only helices.

416

417 *Cavity analysis of DmelOrco, AbakOrco and MhraOR5*

418 Detection of the pockets of the 176 Orcos plus *DmelOrco* mutants (SWISS-model), *AbakOrco* (pdb:
419 6C70) and *MhraOR5* (in APO form, pdb: 7LIC) cavities was carried out using fpocket3 (52) with
420 default settings. For each receptor, visual inspection was used to identify the pocket of interest.

421

422 *Molecular dynamics setup*

423 As IL2 is not resolved in the *AbakOrco* (pdb: 6C70) template structure, IL2 was discarded from the
424 structure of each *DmelOrco* monomer. Propka (53) was used to predict protonation states of the
425 protein at a target pH 6.5. The *DmelOrco* tetramer orientation in its membrane was determined
426 using OPM server (54). Five VUAA1 molecules were added in different orientations on the
427 extracellular side. The system was embedded into a POPC-only model membrane using
428 PACKMOL-memgen (55). The simulation box was completed using TIP3P water molecules and
429 neutralized using K⁺ and Cl⁻ ions with a final concentration of 0.15 M. The total system is made up
430 of 286736 atoms, in a 3.4.10⁶ Å³ periodic box. Molecular dynamics simulations were performed
431 with the sander and pmemd.cuda modules of AMBER18, with the ff14SB force field for the proteins
432 and the lipid14 forcefield for the membrane (56). VUAA1 parameters were generated by calculating
433 partial atomic charges with the HF/6-31G* basis set using Gaussian 09 (57). The obtained
434 electrostatic potential was fitted by the RESP program (58). The other parameters were taken from
435 the General Amber Force Field 2 (gaff2). Bonds involving hydrogen atoms were constrained using
436 the SHAKE algorithm and long-range electrostatic interactions were handled using Particle Mesh
437 Ewald. The cut-off for non-bonded interaction was set to 10 Å. Each system was first minimized
438 with the AMBER sander module, with 5000 steps of steepest descent algorithm then 5000 steps of
439 conjugate gradient with a 50 kcal·mol⁻¹·Å² harmonic potential restraint on the protein part of the
440 system. A second minimization of the same length without restraint was applied. The systems were
441 then thermalized from 100 to 310 K for 10000 steps (restraining the protein and ligands with a 200
442 kcal·mol⁻¹·Å² harmonic potential). Each system underwent 50000 steps of equilibration in the NPT
443 ensemble and 1 bar (restraining the protein and ligands with a 15 kcal·mol⁻¹·Å² harmonic potential)
444 before the production phase. During equilibration and production phase, temperature was kept
445 constant in the system at 310 K using a Langevin thermostat with a collision frequency of 5 ps⁻¹.
446 To increase sampling, all 5 VUAA1 molecules were constrained in a sphere of 45-55 Å radius,
447 centered on the center of mass of the Lys486 of the four Orco monomers (with a potential of 10
448 kcal·mol⁻¹). To avoid VUAA1 aggregation, each VUAA1's sulfur atom was constrained to be a
449 minimum of 20 Å from each other with a soft potential penalty of 5 kcal·mol⁻¹. The VUAA1 system
450 in water only was built solvating the molecule in a 20 Å TIP3P periodic box using the gaff2 and
451 tip3p forcefield parameters. The system was minimized with the AMBER sander module, with 500
452 steps of steepest descent algorithm then 500 steps of conjugate gradient, then heated
453 incrementally from 100 to 310K for 10000 steps. The first 10 nanoseconds of the production phase
454 were considered as equilibration and not taken into account for analysis. The system stability was
455 evaluated from the root mean square deviation (RMSD) evolution computed on the backbone of
456 the full system. During the 22 replicas, the receptors underwent small fluctuations (RMSD < 3Å)

457 showing that they remained correctly folded during microsecond simulations (*SI Appendix*, Fig. S9).
458 Hydration of VUAA1 was calculated using the pytraj watershell function.

459

460 *Minimum distance between VUAA1 and eugenol for all trajectories*

461 The minimum distance between VUAA1 and eugenol was calculated for all trajectories by
462 structurally aligning MhraOR5 (pdb: 7LID) on each DmelOrco monomer using the cealign pymol
463 command (59), then calculating the center of mass distance between eugenol and VUAA1 on each
464 trajectory using the mindist pytraj module (51).

465

466 *Selection of representative frames for b, c, and d*

467 Representative frames of the diffusion were obtained by dividing the prototypical trajectory into 4
468 parts according to the curve shown in Fig. 3C. For each part, a frequency analysis between VUAA1
469 and the receptor using the get_contacts module (<https://getcontacts.github.io/>) identified the critical
470 residues. These residues, plus VUAA1, were selected and used to cluster each part by kmeans
471 clustering, using cpptraj (51) with a fixed number of 4 clusters. The representative frame of the
472 largest cluster was then extracted as the representative frame of that part of the trajectory.

473

474 *Electrostatic and hydrophobic complementarity*

475 For each representative frame (b, c and d), the protein was extracted with VUAA1 which was then
476 replaced with VUAA0.5, VUAA2, VUAA3 and VUAA4 (18). For the representative frame of the
477 binding site (d), VUAA1 was also manually flipped over using the pair fitting tool in PyMol, and then
478 replaced again with VUAA0.5, VUAA2, VUAA3 and VUAA4. Each system was then minimized
479 using the AMBER sander module, with 5000 steps of steepest descent algorithm then 5000 steps
480 of conjugate gradient, while restraining the backbone of the protein with a 50 kcal·mol⁻¹ potential.
481 Hydrophobic complementarity scores for each system were calculated using the PLATINUM web
482 server (60) with default settings. Electrostatic complementarity scores for each system were
483 calculated using the Flare electrostatic complementarity tool (61).

484

485 **Chemicals**

486 VUAA1 (N-(4-ethylphenyl)-2-((4-ethyl-5-(3-pyridinyl)-4H-1, 2, 4-triazol-3-yl)thio)acetamide) (CAS
487 525582-84-7) was purchased from Sigma-Aldrich. The stock solution was 110 mM in DMSO and
488 subsequently diluted into appropriate buffer solution.

489

490 **Molecular biology**

491 All Orco gene sequences were optimized (62) for protein expression in *Xenopus laevis* oocytes
492 with the GenSmart™ Codon optimization Tool and subcloned into a pGEMHE-derived vector. The
493 wildtype gene of *Drosophila melanogaster* Orco (DmelOrco) was synthesized by Genscript and
494 subcloned with XmaI/XhoI cloning sites. Site-directed mutagenesis of DmelOrco was done by PCR
495 with the Q5® site directed mutagenesis kit (NEB) using primers optimized with the NEBase
496 Changer online tool and following the supplier's protocol. After transformation of commercial
497 competent bacteria (XL10 Gold) by standard heat-shock protocol and overnight culture in
498 ampicillin-containing LB plates, positive clones were identified by electrophoretic restriction profile
499 and external sequencing (Genewiz). DNAs of a positive clones were amplified with Qiagen
500 MidiPrep Kit and the ORF fully sequenced. For in vitro transcription, DNAs were linearized with
501 restriction enzyme NotI that cuts a unique site in the 3' region of the polyA tail. The linearized DNAs
502 were purified by the standard phenol:chloroform extraction method and transcribed into mRNA
503 using the T7 ultra mMessage mMachin kit (Thermo Fisher Scientific). mRNAs were purified with
504 the NucleoSpin RNA plus XS kit (Machery-Nagel). DNA and RNA were analyzed by agarose-gel
505 electrophoresis and quantified by spectrophotometry.

506

507 **Electrophysiological recordings**

508 *Xenopus laevis* oocytes were prepared as previously described (63). Briefly, oocytes were
509 defolliculated after surgical retrieval by type 1A collagenase over 2-3h under smooth horizontal
510 agitation. They were manually selected and incubated at 19°C in modified-Barth's solution (1 mM
511 KCl, 0.82 mM MgSO₄, 88 mM NaCl, 2.4 mM NaHCO₃, 0.41 mM CaCl₂, Ca(NO₃)₂ 0.3 mM, 16 mM

512 HEPES, pH 7.4) supplemented with 100 U·mL⁻¹ of penicillin and streptomycin and 0.1 mg·mL⁻¹ of
513 gentamycin. Each oocyte was micro-injected with the Nanoject instrument (Drummond) with 50 nL
514 of 20 ng of mRNA coding for the Orco of interest. Injected oocytes were incubated individually in
515 96-well plates for 4 days at 19°C in the same buffer. Different batches of oocytes have been tested
516 per construct. The results of the mutants of interest have been confirmed by a second set of
517 experiments comparing the amplitudes with WT in the same day and from the same batch of
518 oocytes (SI appendix Fig. S10).

519 Whole cell currents were recorded with the two-electrode voltage-clamp (TEVC) technique with the
520 HiClamp robot (MultiChannel System). The results of the mutants of interest have been confirmed
521 by a second set of experiments comparing the amplitudes with WT in the same day and from the
522 same batch of oocytes. Microelectrodes were filled with 3M KCl. The high K⁺ buffer used for
523 recordings was composed of 91 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.4.
524 Membrane voltage was clamped to -50 mV and VUAA1 was applied for 60 s. Data were extracted
525 with M. Vivaudou's programs (64) and statistically analyzed with Prism 8 (Graphpad).

526 Animal handling and experiments fully conformed to European regulations and were approved by
527 the French Ministry of Higher Education and Research (APAFIS#30915-2021040615209331 v1 to
528 CM). The animal facility was authorized by the Prefect of Isere (Authorization #E 38 185 10 001).

529

530 **Western Blots**

531 All expression experiments were assessed on 4-20% mini-Protean TGX SDS-PAGE gels (Bio-
532 Rad). All oocytes loaded on gel were from the same batch and injected as described above, with 4
533 days of incubation.

534 Oocytes were homogenized through several passes in a syringe with two sizes of needles (18g
535 then 27g) into a solubilization buffer (PBS 1X, protease inhibitor cocktail tablets) and stored at -
536 80°C. Western blots were performed by transferring proteins onto a nitrocellulose membrane using
537 the trans-blot turbo system (BioRad). Membranes were blocked with PBS 1x-1% non-fat milk
538 overnight at 4°C and incubated in primary antibody anti-Orco (1:500) and the secondary antibody
539 Goat anti-rabbit IgG HRP conjugate (1:5000 Sigma-Aldrich) for 1 hour each. The immunoblot was
540 revealed with ECL substrate kit (Abcam) and recorded on ChemiDoc (BioRad) at different times for
541 identifying the optimal exposition time without pixel saturation. Gels were stained with standard
542 Coomassie blue staining protocol and the pictures were taken with the Chemidoc apparatus.
543 Relative intensities of bands in blots and volume of lanes in gels were determined with the Image
544 Lab software (BioRad).

545 The polyclonal primary antibody anti-Orco was purchased from Genscript and designed against
546 the peptide sequence SSIPVEIPRLPIKSFYPW in the second extracellular loop (ECL2). Anti-Orco
547 was produced in rabbit and purified by antigen affinity.

548

549

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551

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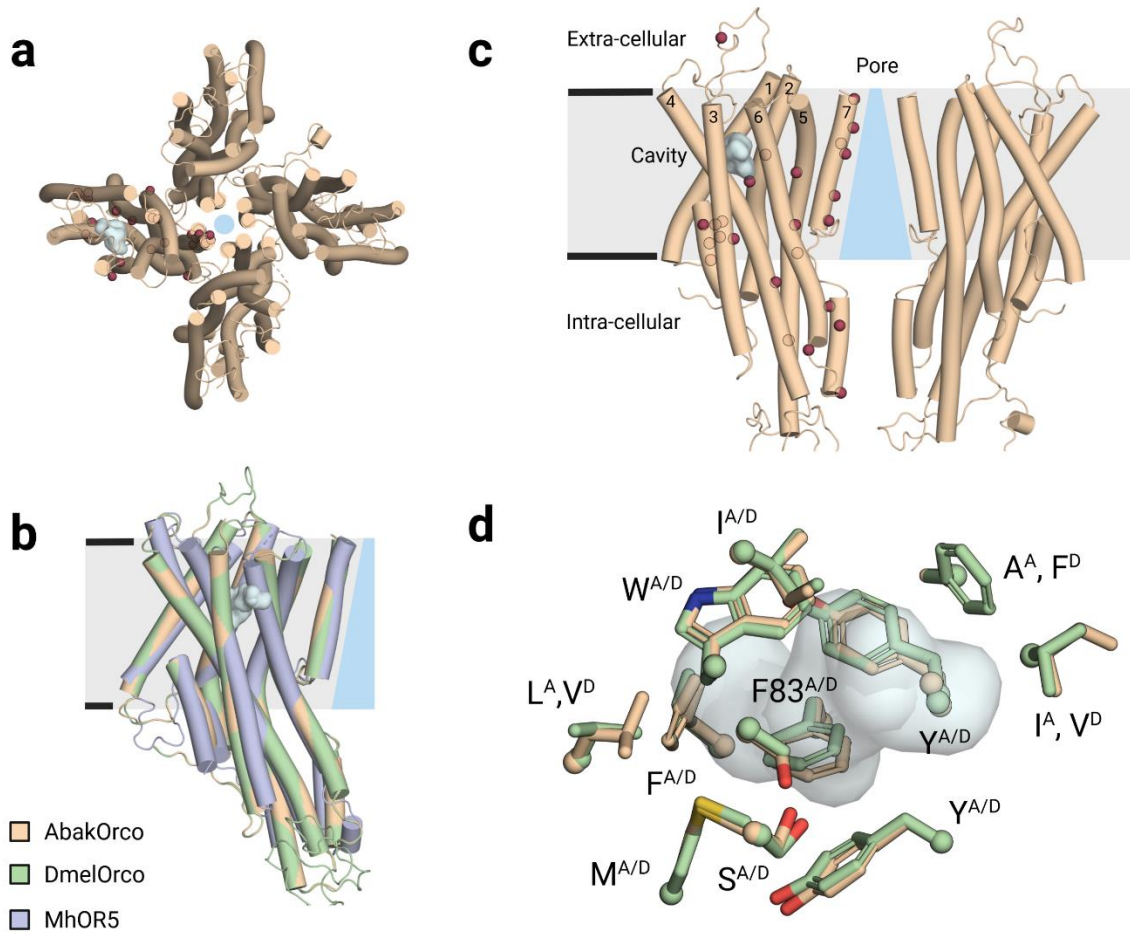
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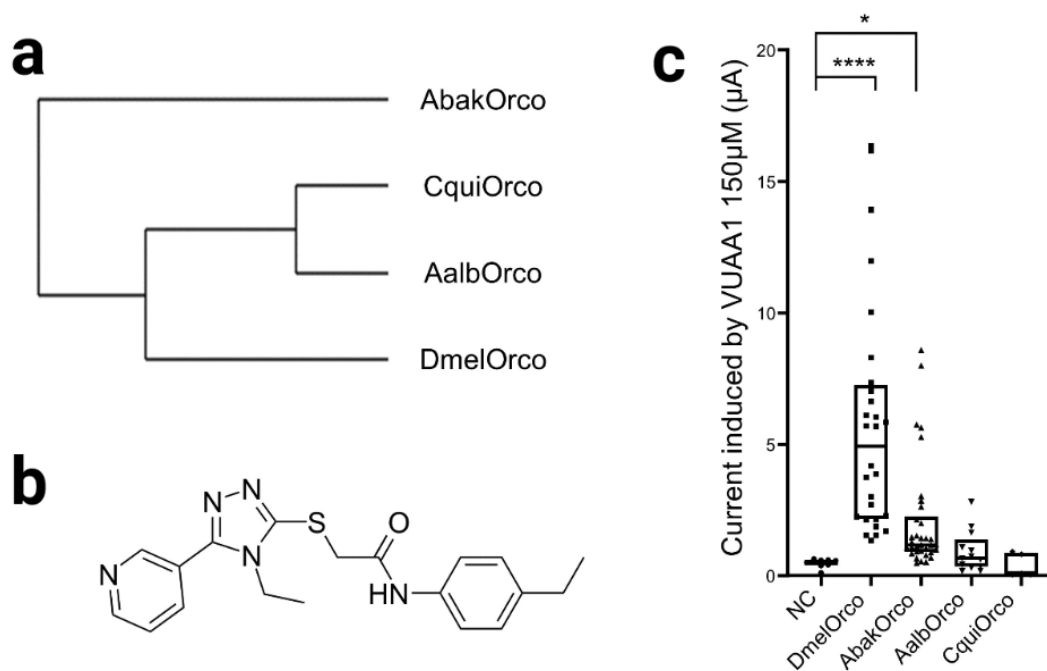
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722 **Figures and Tables**



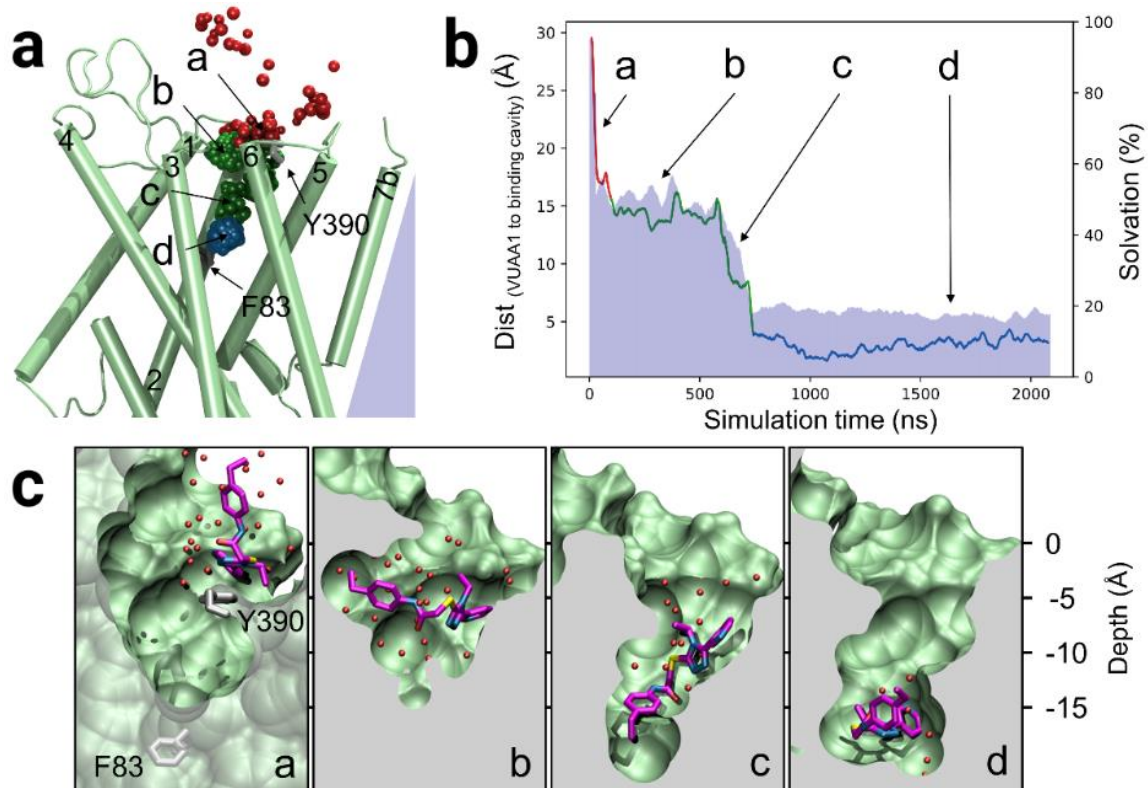
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725 **Fig. 1 | Architecture of the DmelOrco homotetramer model and comparative representations**
 726 **of cavities and mutants. (a)** Extracellular view of the cryo-electronic microscopy structure of the
 727 homotetramer of *Apocrypta bakeri* Orco (AbakOrco) (pdb: 6C70). A ligand-binding pocket (in cyan)
 728 of related Orco receptors shown on the left subunit. The central pore is symbolized by a blue circle.
 729 **(b)** AbakOrco (pdb: 6C70, beige) membrane view, superposed on *Machilis hrabei* odorant receptor
 730 5 (MhraOR5, pdb: 7LID, blue) and *Drosophila melanogaster* Orco (DmelOrco, green) homology
 731 model. Cavity analysis reveals the conserved position of a pocket (cyan) in these 3 structures.
 732 **(c)** Side view of two Orco subunits with a diagram of the channel pore (blue trapezoid). Residues
 733 shown in red spheres are equivalent to residues critical for VUAA1 response found in Orcos from
 734 point mutations that alter channel function in *Drosophila melanogaster*, *Agrotis segetum*, *Mayetiola*
 735 *destructor*, *Bombyx mori*, or *Apocrypta bakeri*. **(d)** Close view of the cavities (cyan) of DmelOrco
 736 and AbakOrco with their amino acids represented as sticks (respectively green and beige).
 737 DmelOrco and AbakOrco cavities share 73% of sequence identity (82% of similarity). The
 738 superscript letters A and D refer to AbakOrco and DmelOrco, respectively.
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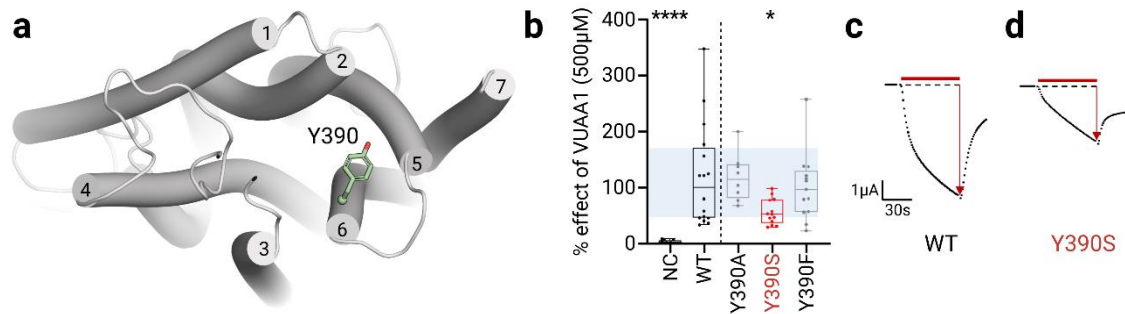
Fig. 2 | Determination of the Orco species generating the highest response to VUAA1 in TEVC recordings. (a) Phylogenetic tree of Orcos from different species. Abak: *Apocrypta bakeri*; Cqui: *Culex quinquefasciatus*, Aalb: *Aedes albopictus*, Dmel: *Drosophila melanogaster*. **(b)** Structure of VUAA1. **(c)** Current amplitudes induced by 500 μM VUAA1 on Orcos from different species expressed in *Xenopus* oocytes and recorded by TEVC. Results are median +/- SEM. P values are <0.0001 (Dmel, ****), 0.0438 (Abak, *) and 0.92 (Aalb), with NC as reference in the Kruskal-Wallis test. n= 8 Negative control, n= 28 DmelOrco, n= 34 AbakOrco, n=13 AalbOrco, n= 5 CquiOrco; NC: negative control (water-injected oocytes).



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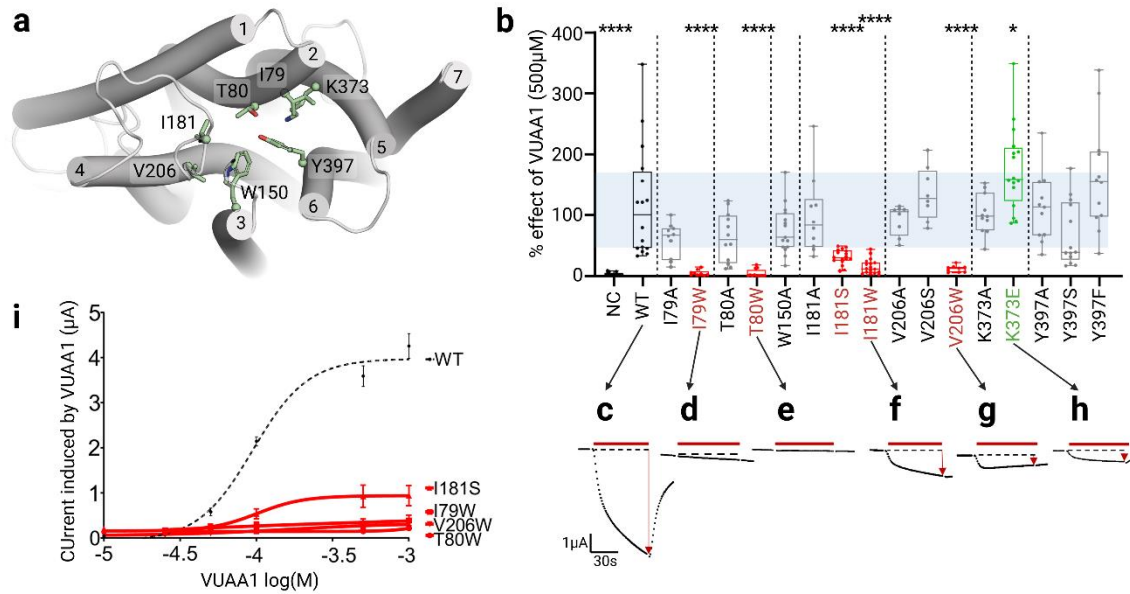
752 **Fig. 3 | Stepwise translocation of VUAA1 into DmelOrco model in MD simulations.** (a)
753 Prototypical trajectory of VUAA1 binding event. The Orco monomer is shown in green. F83 and
754 Y390 labels give their localization. The VUAA1 center of mass is represented by beads colored
755 from red to blue according to the simulation time. a, b, c and d are sequential of VUAA1 progressing
756 toward the binding site, from contact to Orco, entry, vestibule and binding to the internal cavity,
757 respectively. (b) Close view of the progression of VUAA1 inside the Orco monomer corresponding
758 to the positions a, b, c and d. Carbon atoms of VUAA1 are colored purple and the sulfur atom in
759 yellow. Carbon atoms of F83^{S2} and Y390^{S6} are in grey and water molecules found less than 3 Å
760 away from VUAA1 are represented by red spheres. (c) Evolution of the distance between the
761 VUAA1 centers of mass and the center of mass of the binding cavity (defined as the center of mass
762 of the eugenol molecule in MhraOR5, pdb: 7LID). The red curve represents the positions outside
763 of the receptor to the contact (a). The green part of the curve represents the entry and vestibule
764 events (b and c) and the blue one the sample of the binding cavity (d). The blue area shows the
765 percentage of ligand solvation during the binding process (normalized to the solvation of the ligand
766 outside the protein).

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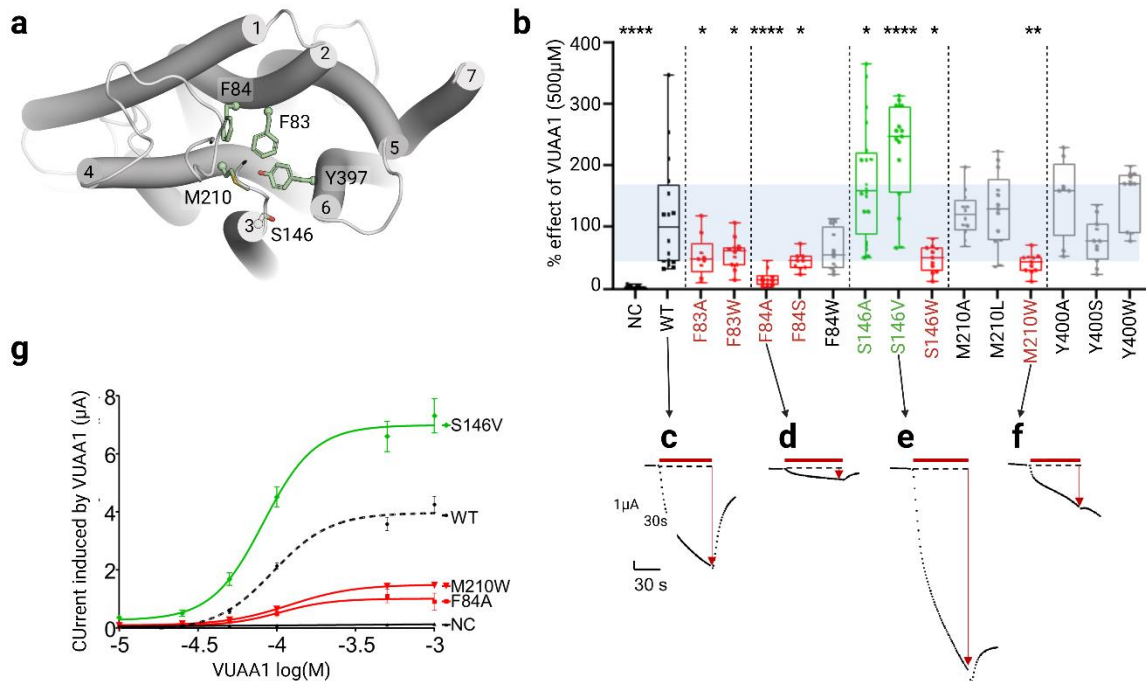
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Fig. 4 | Electrophysiological characterization of Orco mutants in the contact region. (a) Top view of the entry highlighting the position of Y390. **(b)** Boxplot showing the current induced by 500µM of the ligand VUAA1 and measured by TEVC recordings on DmelOrco WT and mutants expressed by the Xenopus oocyte. n= 8 minimum recordings from different oocytes; NC: Negative Control (non-injected oocytes). Data are analysed with one-way ANOVA with α -error= 0.05 followed by Dunn's post-hoc test, with WT used as reference for the multiple comparison test. Results from mutant showing a statistical decrease from WT are coloured in red. **(c-d)** Representative current measured on WT and mutant with statistical difference from DmelOrco WT.



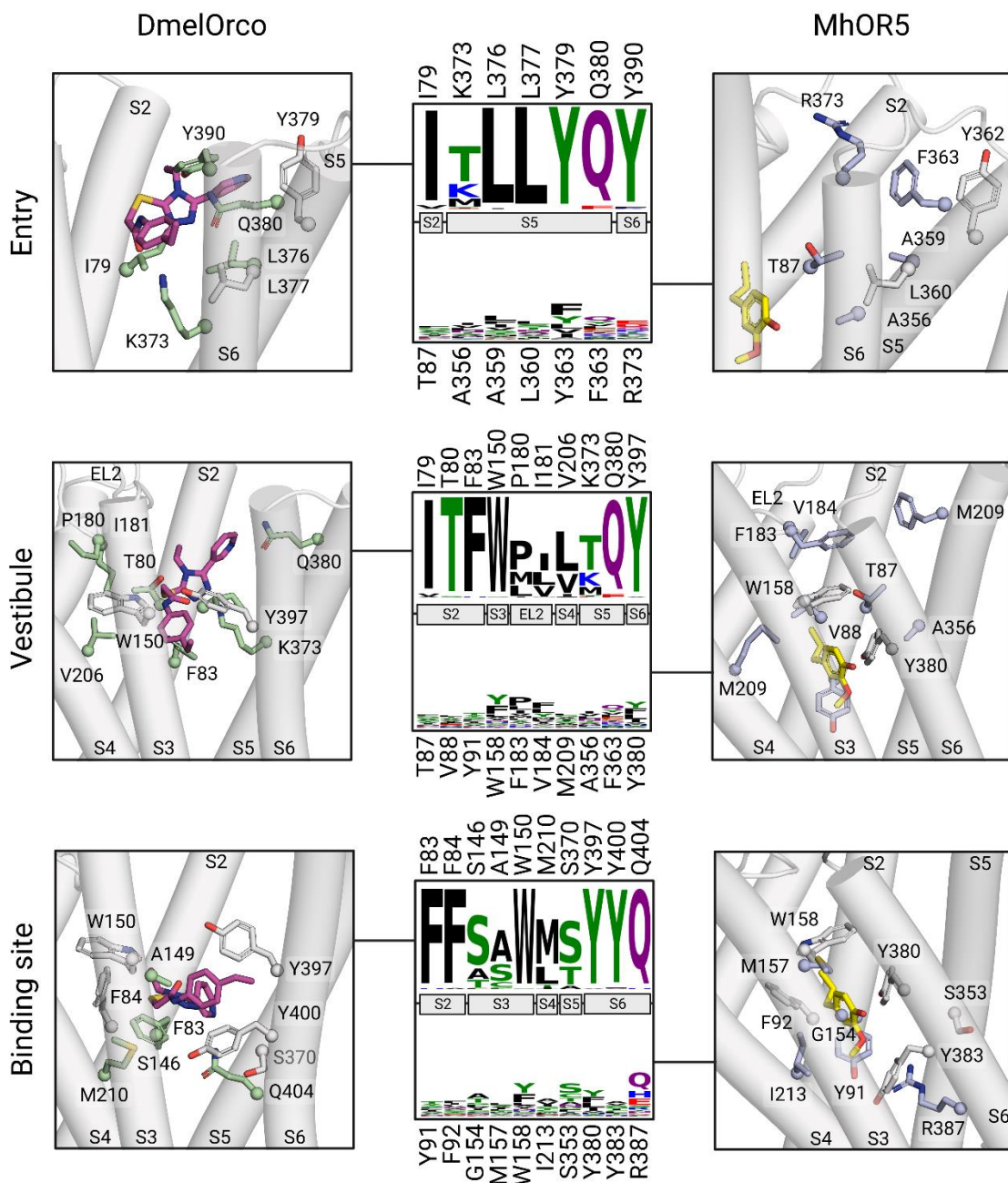
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 781 **Fig. 5 | Electrophysiological characterization of Orco mutants in the entry and vestibule**
 782 **region. (a)** Close view of the vestibule region highlighting the position of the different mutation
 783 sites. **(b)** Boxplot showing the current induced by 500µM of the ligand VUAA1 and measured by
 784 TEVC recordings on DmelOrco WT and mutants expressed in *Xenopus* oocytes. n= 8 minimum
 785 recordings from different oocytes; NC: Negative Control. Data are analysed with one-way ANOVA
 786 with α -error= 0.05 followed by Dunn's post-hoc test, with WT used as reference for multiple
 787 comparisons test. Results of mutants with statistically significant decrease or increase compared
 788 to WT are coloured in red or green, respectively. **(c-h)** Representative current measured on
 789 mutants with statistical differences from DmelOrco WT. **(i)** Dose-response curves for the mutants
 790 considered. EC₅₀ are 94.5 µM for WT, 101.9 µM for I181S, not determined for the others. n≥ 8
 791 recordings from different oocytes.

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Fig. 6 | Electrophysiological characterization of Orco mutants in the binding cavity. (a) Close view of the binding region highlighting the position of the different mutation sites. (b) Boxplot showing the current induced by 500µM of the ligand VUAA1 and measured by TEVC recordings on DmelOrco WT and mutants expressed by *Xenopus* oocyte. n= 8 minimum recordings from different oocytes; NC: Negative Control. Data are analysed with one-way ANOVA with α -error= 0.05 followed by Dunn's post-hoc test, with WT used as reference for multiple comparisons test. Results from mutant showing a statistical decrease or increase from WT are coloured in red or green respectively. (c-f) Representative current measured on mutants with statistical differences from DmelOrco WT. (g) Dose-response curves for the mutants considered. EC₅₀ are 94.5 µM for WT, 82.2 µM for S146V, 120.2 µM for M210W, 105.0 µM for F84A and not determined for NC. n≥ 7 recordings from different oocytes.



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Fig. 7 | Structural details and sequence conservation of the entry, vestibule and binding site in Orcos and ORs. The structures for DmelOrco are extracted from MD simulations, while the structure for MhOR5 is taken from pdb: 7LID. The amino acids were selected according to their frequencies of interaction with VUAA1 during MD simulations. Carbon atoms from amino acids conserved between Orcos and MhOR5 are coloured in white. Carbon atoms from VUAA1 and Eugenol are shown in purple and yellow, respectively. Residue conservation among 176 Orcos from 174 species and 361 ORs from 4 species are coloured according to their side-chain chemistry. In each frame, the upper consensus logo account for Orcos and the lower one for ORs.

821 **Supplementary Information for**

822 **Ligand of the conserved insect odorant receptor co-receptor reaches**
823 **its binding site through a dynamic translocation pathway**

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837 **This PDF file includes:**

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839 Figures S1 to S8

840 Tables S1 to S3

841 Legends for Datasets S1 to S2

842 SI References

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844 **Other supplementary materials for this manuscript include the following:**

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846 Datasets S1 to S2

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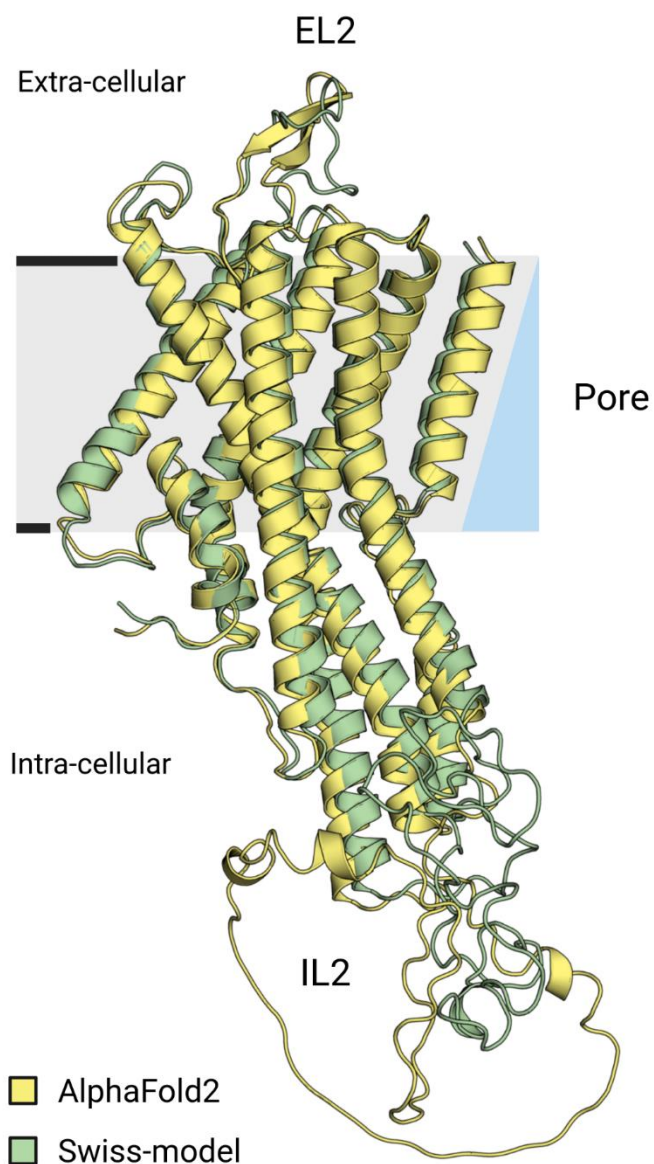
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² These authors contributed equally to this work.



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853 **Figure S1. Superposition of the AlphaFold2 DmelOrco model with the AbakOrco structure.**

854 Superposition of the experimental structure of AbakOrco homotetramer (pdb ID: 6C70) superposed

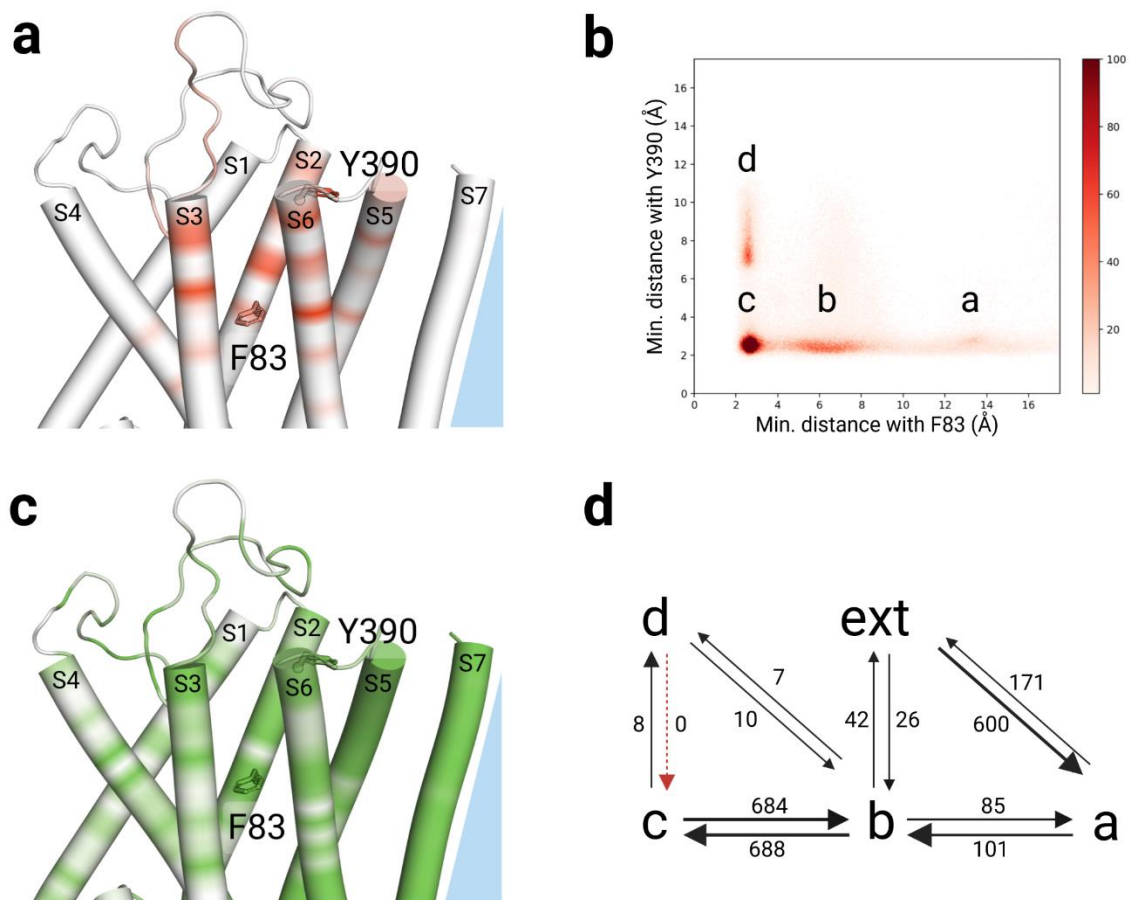
855 on the AlphaFold2 model retrieved from the Alpha Fold Protein database (in yellow). The two

856 structures have an RMSD of 0.7 Å calculated on their transmembrane segments. To orient the

857 protomer, part of the pore is shown on right as blue right-angled triangle. EL2: extracellular loop 2.

858 IL2: intracellular loop 2.

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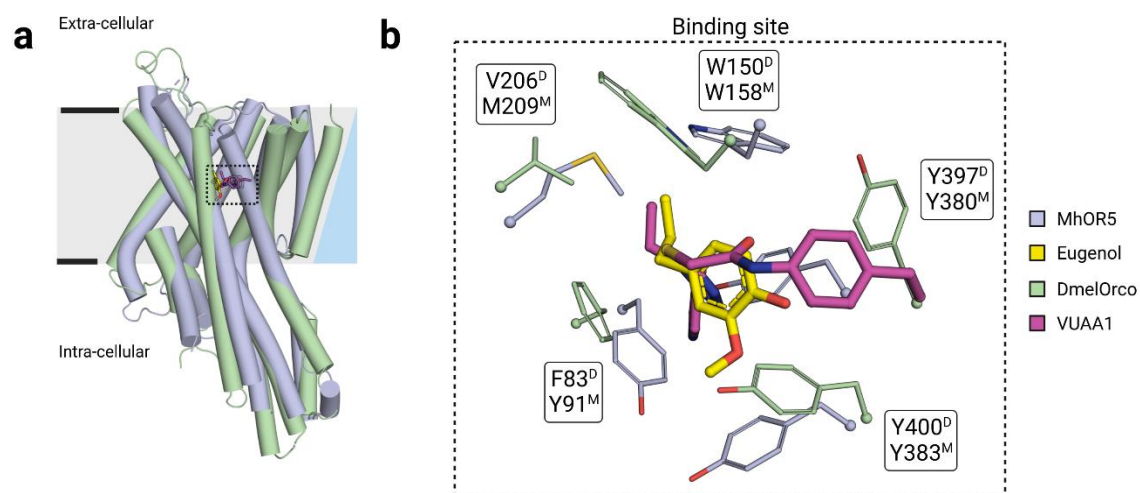


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862 **Figure S2. Summary of the interactions observed during the MD simulations between**
 863 **VUAA1 and DmelOrco. (a)** Contact frequencies of VUAA1 mapped onto the structure of
 864 DmelOrco. The color gradient account for high frequency interaction (in red) to no interaction (in
 865 white). **(b)** Contour map of VUAA1 migration as the minimum distance from F83^{S2} (distance from
 866 the entry) and minimum distance from Y390^{S6} (distance from the cradle of the cavity). The four
 867 basin allow identifying four states in the VUAA1 binding: contact a, entry b, vestibule c and
 868 binding d. **(c)** Residue conservation among 176 Orcos from 174 species mapped onto the structure
 869 of DmelOrco using a colour scale from green high conservation, to white low conservation. **(d)**
 870 Proposed mechanism of VUAA1 binding. The numbers indicate the transition from one state to
 871 another.

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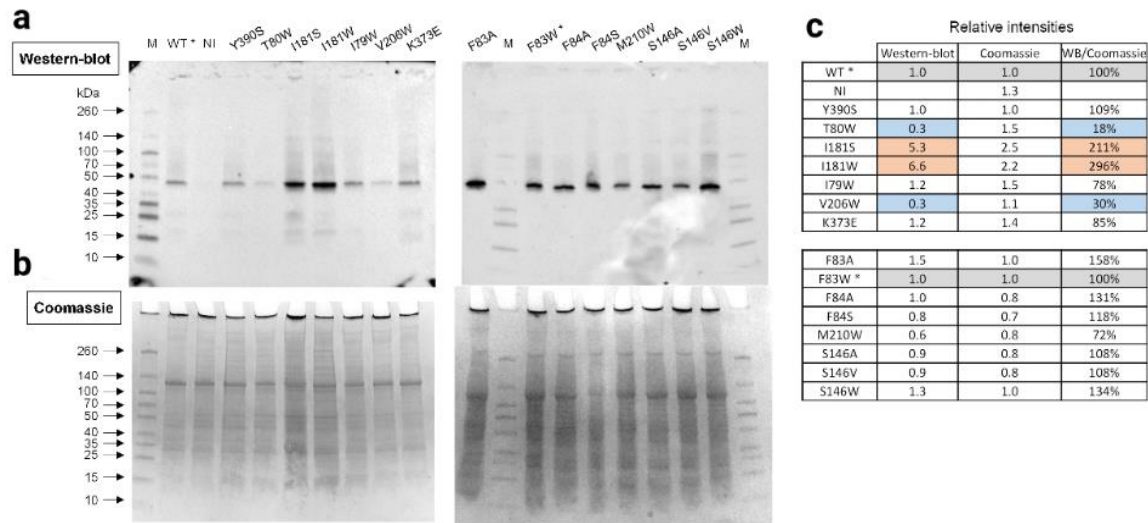
875 **Figure S3. Comparison of the binding site location predicted in DmelOrco and observed in**

876 **MhraOR5 structure. (a)** Superposition of DmelOrco (green tubes) and MhraOR5 (blue tubes), with

877 their respective ligands VUAA1 (magenta sticks) and eugenol (yellow sticks). **(b)** Close view on the

878 binding site of DmelOrco (green licorice) and MhraOR5 (blue licorice). The superscripts D or M on

the amino acids names represent DmelOrco and MhraOR5, respectively.

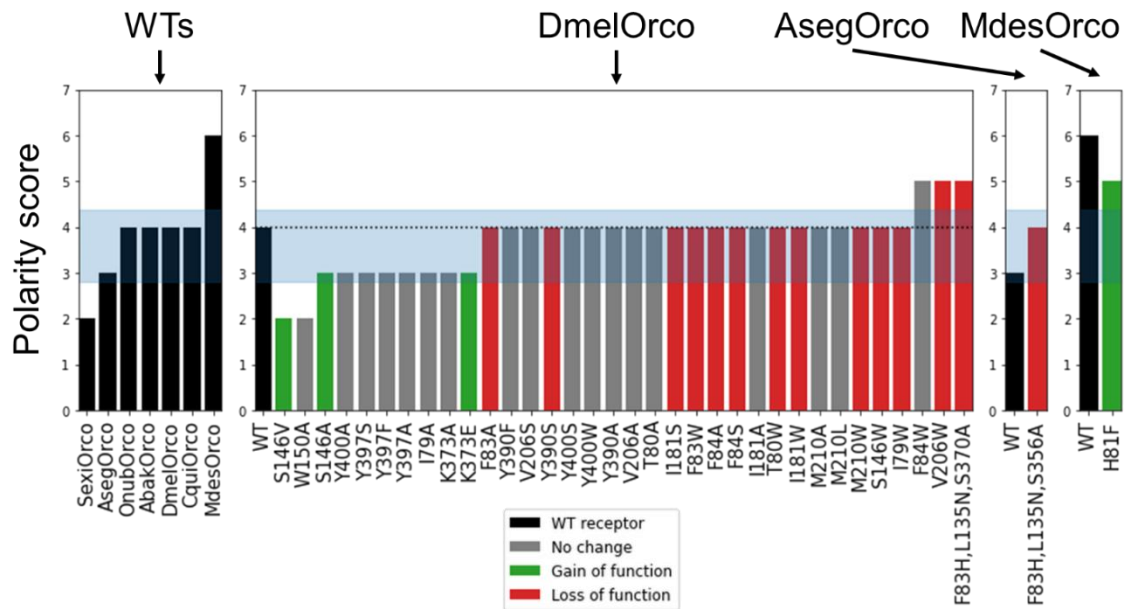


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880 **Figure S4. Western-blot analysis of DmelOrco WT and mutant expression in Xenopus**
 881 **oocytes. (a)** Western blots with a polyclonal primary antibody directed against DmelOrco. The
 882 constructs indicated above the lanes were expressed in Xenopus oocytes and the samples are
 883 crude membrane extracts. M: ThermoScientific Spectra Multicolor Broad Range Protein Ladder;
 884 WT*: wild-type used as reference for determining the relative intensities of bands of the first blot
 885 and gel, while the mutant F83W* was used for the second blot and gel. **(b)** Coomassie blue –
 886 stained SDS PAGE gels (4-20%). **(c)** Values of relative intensities of the bands corresponding to
 887 Orco (Western-blot column) in panel A and to the lanes (Coomassie) in panel B. Corrected
 888 intensities of bands relative to the intensities of lanes are indicated in the column WB/Coomassie.

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892 **Figure S5. Evolution of the polarity score of DmelOrco's mutants and from other species**

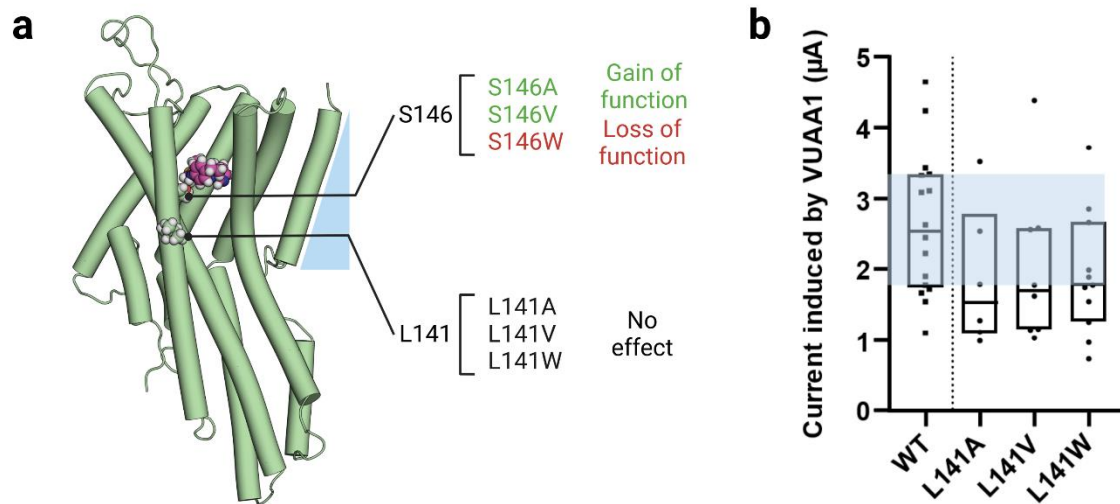
893 **computed with fpocket.** On the left, the results for WT Orco; for clarity, only the VUAA1-

894 responsive and VUAA1-non-responsive Orcos are shown. SexiOrco and MdesOrco are the

895 receptors with the lowest and highest scores, respectively. The central panel gathers the results

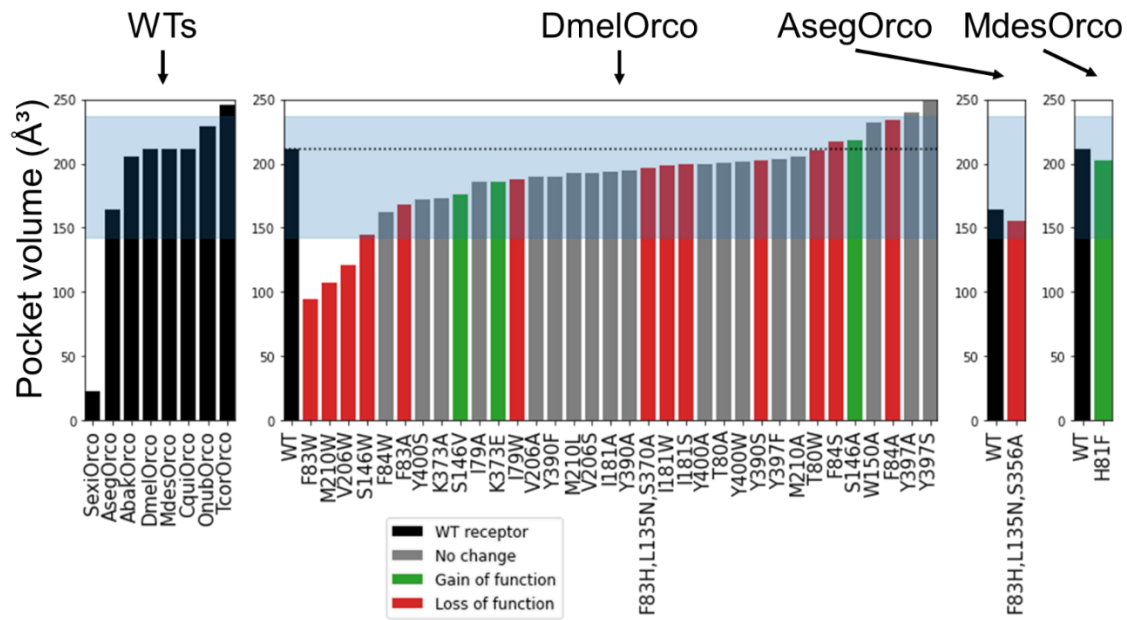
896 for DmelOrco mutants. The two right panel gathers results for AsegOrco and MdesOrco. The blue

897 bands account for the mean and standard deviation among 176 Orcos from 174 species.



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899 **Figure S6. Characterization of mutations at the position L141 that is not involved in the**
900 **binding site nor in the translocation pathway of VUAA1. (a)** Location of the L141 in the model
901 of DmelOrco. For comparison, S146 was also depicted. The carbon atoms from the residue are in
902 pale green, while the ones of VUAA1 are in purple. **(b)** Boxplot showing the current induced by
903 500 μM of the ligand VUAA1 and measured by TEVC recordings on DmelOrco WT and mutants
904 expressed by the *Xenopus oocyte* ($n \geq 5$). P values are 0.290, 0.396, 0.192 for L141A, L141V and
905 L141W, respectively. Data are analysed with one-way ANOVA with α -error= 0.05 followed by
906 Dunn's post-hoc test, with WT used as reference for multiple comparisons test.
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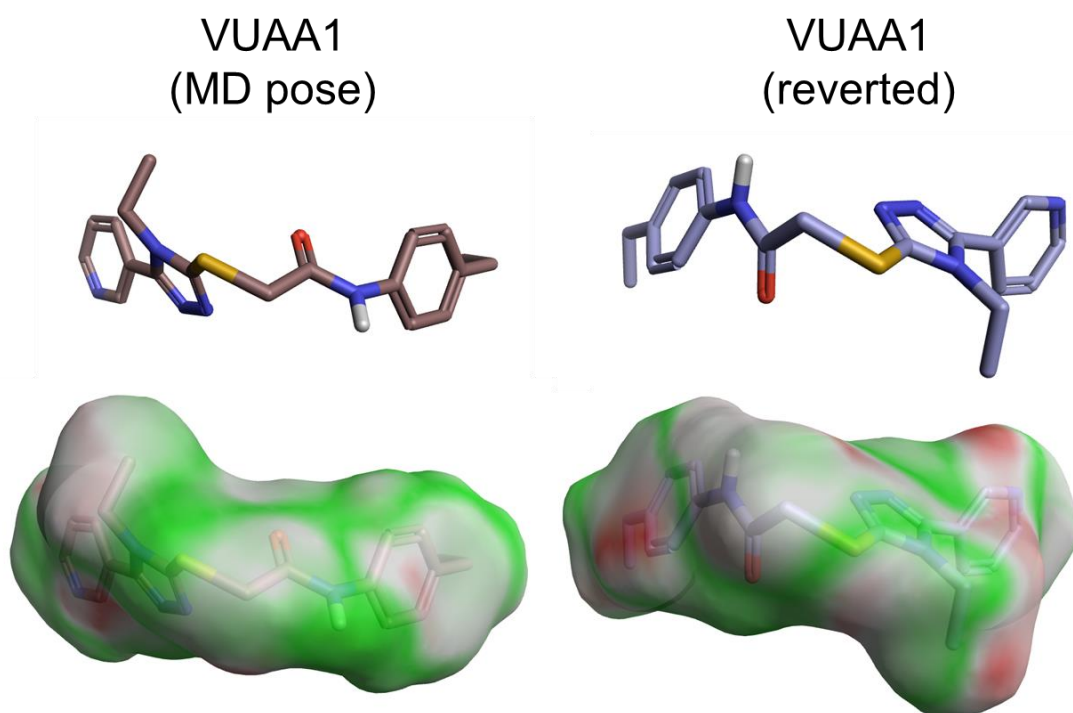


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909 **Figure S7. Evolution of the binding cavity volume of DmelOrco's mutants and from other**
 910 **species computed with fpocket.** On the left, the results for WT Orcos. For more clarity, only the
 911 VUAA1-responsive and VUAA1-non-responsive Orco are shown. SexiOrco and TcorOrco are the
 912 receptors with the lowest and highest volume, respectively. The central panel gathers the results
 913 for DmelOrco mutants. The two right panel gathers results for AsegOrco and MdesOrco. The blue
 914 bands account for the mean and standard deviation among 176 Orcos from 174 species.

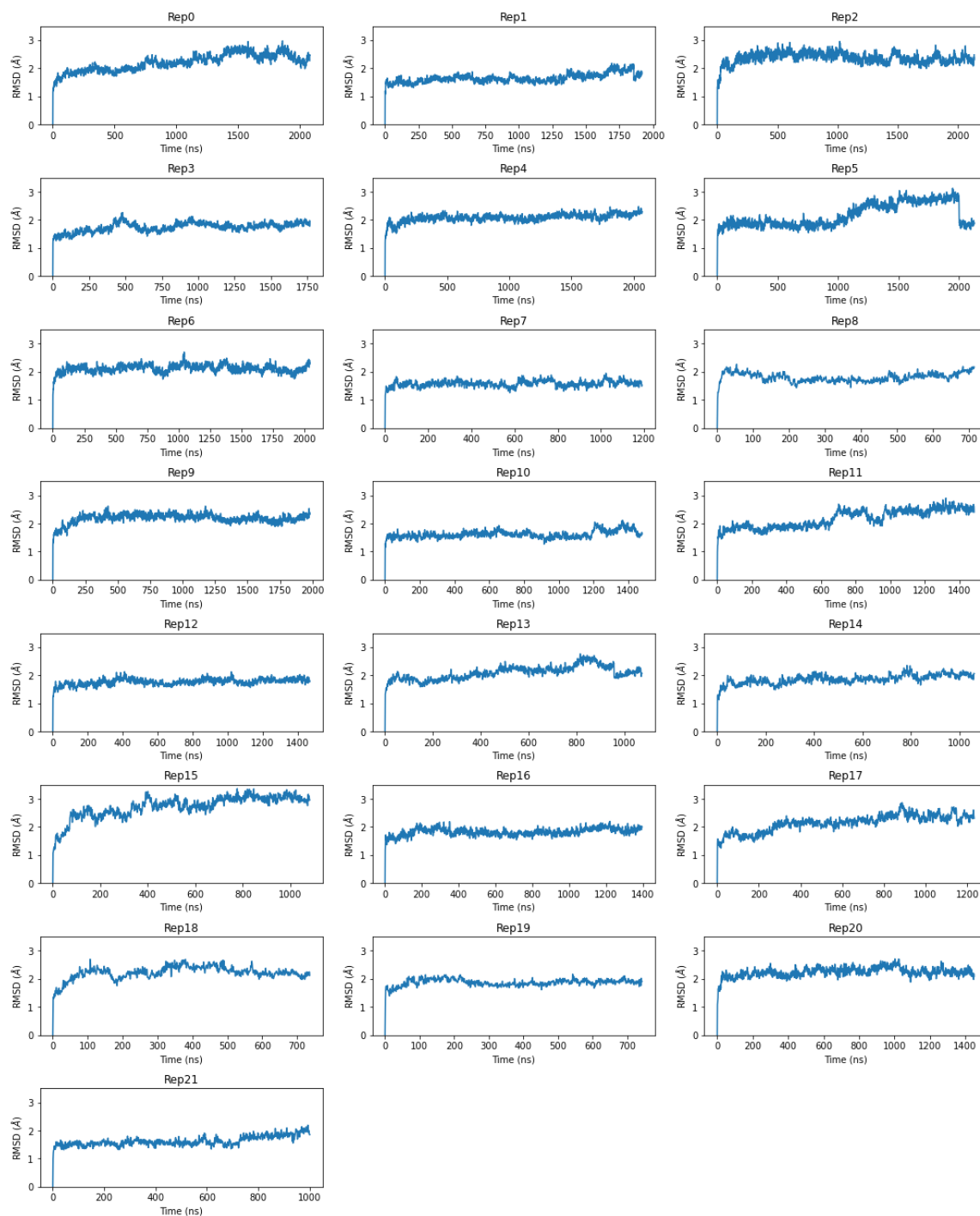
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918 **Figure S8. Comparison of the orientation of VUAA1 in the binding site obtained during the**
919 **MD simulations (MD pose) and the manually reversed orientation.** The electrostatic
920 complementarity of the ligand to the protein appears as a surface. The areas where the protein-
921 ligand electrostatics are favorable or unfavorable are colored from green to red.



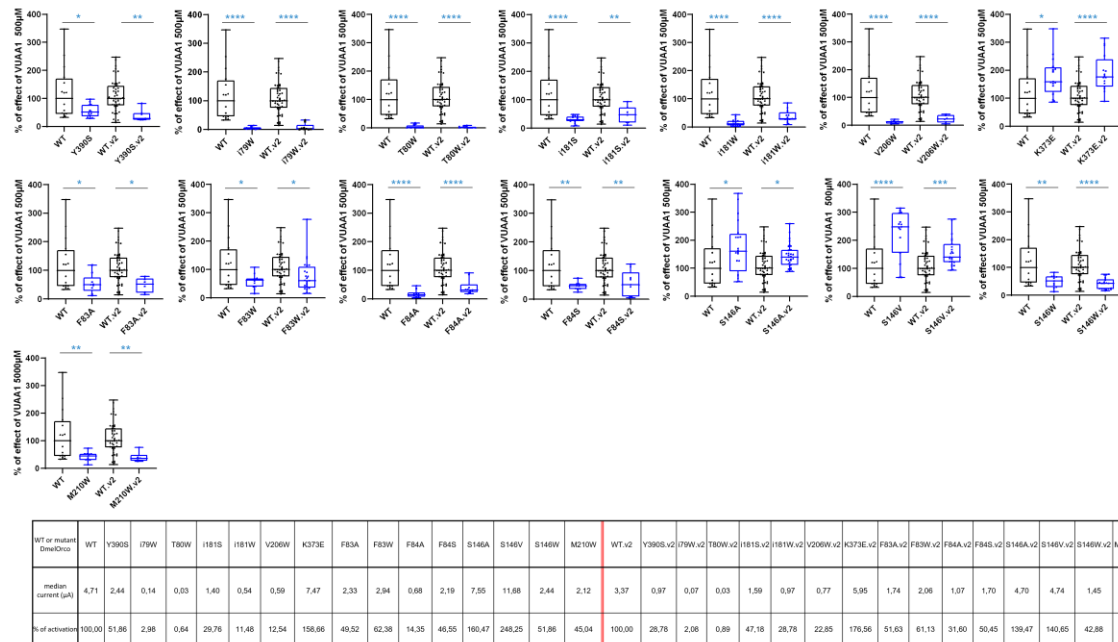
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Figure S9. Stability of the DmelOrco model in 22 replicas. The RMSD is computed for the receptor backbone (CA, C, N atoms) with respect to the initial model structure.



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Figure S10. Relative responses to 500 μ M VUAA1 of mutants (blue bars) compared to WT (black bars) in 2 different sets of experiments. Percentages are calculated based on the median of WT current amplitudes as 100%. The experiments in the 2nd set (noted -v2) have been performed in the same day and with the same batch of oocytes as WT-v2. The number of recordings are between 6 to 31 for mutants-v2 and 49 for WT-v2. P values are symbolized over bars as blue stars, each star being one decimal. Data are analysed with one-way ANOVA with α -error= 0.05 followed by Dunn's post-hoc test, with WT used as reference for multiple comparisons test.

936 **Table S1 | Electrostatic and hydrophobic complementary between the series of VUAA1**
 937 **analogues and different stable locations in the protein.** The location are defined according to
 938 Figure 3: entry (b), vestibule (c) and binding site (d). The analogues have been ranked according
 939 to the EC₅₀ measured by Tailor *et. al.* on AgamOrco/AgamOR65 (1). The reversed ligand account
 940 for the reversed orientation described in Figure S7.

Location	Ligand (EC ₅₀)	Hydrophobic complementarity	Electrostatic complementarity
Entry (b)	VUAA0.5	0.37	0.26
	VUAA1	0.41	0.29
	VUAA2	0.44	0.31
	VUAA3	0.46	0.33
	VUAA4	0.45	0.32
Vestibule (c)	VUAA0.5	0.61	0.28
	VUAA1	0.62	0.30
	VUAA2	0.63	0.31
	VUAA3	0.64	0.29
	VUAA4	0.66	0.29
Binding site (d)	VUAA0.5 (110 μM)	0.65	0.33
	VUAA1 (37 μM)	0.64	0.34
	VUAA2 (9.2 μM)	0.64	0.36
	VUAA3 (8.4 μM)	0.71	0.36
	VUAA4 (2.1 μM)	0.72	0.36
Binding site (d) with reversed ligand	VUAA0.5	0.58	0.28
	VUAA1	0.58	0.31
	VUAA2	0.58	0.31
	VUAA3	0.65	0.30
	VUAA4	0.64	0.32

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942 The electrostatic complementarity of the VUAA series in the binding site show little differences in
 943 EC values, although it shows a correct trend (pearson correlation of -0.93). These small differences
 944 are in the same order of magnitude with a previous study from Bauer et al.(2) In their study, the 6
 945 ligands are discriminated based on an EC score with subtle EC variations (lig18: IC50 = 2nM,
 946 EC = 0.425; lig23: IC50 = 447nM, EC = 0.385).

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948 **Table S2 | EC₅₀ and I_{max} of VUAA1 from WT and mutated DmelOrco.** ND means Not
949 Determined due to the absence of plateau. Separation lines differentiate WT from mutants in the
950 vestibule (Figure 5) and the binding site (Figure 6), in this respective order. Within separation lines,
951 mutants are ranked in descending order of I_{max}, which is measured in concentration-effect curves
952 and slightly lower than the I_{max} measured in single-concentration experiments.

	EC₅₀ (μM)	I_{max} (μA)
WT	94.5	3.97
I181S	101.9	0.94
I79W	ND	0.65
V206W	ND	0.33
T80W	ND	0.23
S146V	82.2	6.99
M210W	120.2	1.50
F84A	105.0	1.01

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955 **Table S3 | Comparison of the mutation effects on MhraOR5, MhraOR1 eugenol-induced**
 956 **channel response and DmelOrco VUAA1-induced channel response.** A, D, I, na and nt mean
 957 Abolished, Decreased, Increased, non-affected and not tested, respectively. Results for MhraOR5
 958 and MhraOR1 are taken from ref(3). Each line corresponds to the same position in the three
 959 considered proteins MhraOR5, MhraOR1 and DmelOrco. Alignment between the sequences was
 960 taken from ref(2).

	MhraOR5	Effect on Eugenol	Effect on DEET	MhraOR1	Effect on Eugenol	DmelOrco	Effect on VUAA1
Ligand	T87A	D		L102A	n.a	I79A	na
diffusion	L379A	na		L398A	n.a	G399	nt
	V88A	D		T103	A	T80A	na
	Y91A	D		Y106A	A	F83A	D
	F92A	A		I107A	A	F84A	D
	S151A	A		S166A	A	S146A	I
	G154A	A		G169A	I	A149	nt
	W158A	A		W173A	A	W150A	na
Binding site	M209A	A	D	L227A	D	V206A	na
	M209V	D	I			V206S	n.a
	M209L	D	D			V206W	A
	I213A	A	A	M231A	A	M210A	na
	I231M	D	A			M210W	D
	L379A	na		L398A	na	G399	nt
	Y380A	A		Y339A	A	Y400A	na
	Y383A	A		C402A	na	A403	nt

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963 **Dataset S1 (Dataset S1.xlsx).** Sequence alignment of different Orco. The known mutations are
 964 indicated in color on the sequence.

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966 **Dataset S2 (Dataset S2.xlsx).** Contact frequencies between VUAA1 and amino-acids from Orco
 967 during the MD simulations. The first sheet gathers the contact frequencies for the simulations in
 968 which the ligand sampled the binding site. The second sheet gather the frequency when the ligand
 969 visits cavity b, c or d but do not reach the binding site.

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977 agonist. *ACS Chem. Biol.* **7**, 1647-1652 (2012).

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980 optimize binding and selectivity of protein–ligand complexes. *J. Med. Chem.* **62**, 3036-3050
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