

1 **Fentanyl binds to the μ -opioid receptor via the lipid membrane and transmembrane helices**

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9

10 **Abstract**

11 Overdose deaths from synthetic opioids, such as fentanyl, have reached epidemic proportions
12 in the USA and are increasing worldwide. Fentanyl is a potent opioid agonist, that is less well
13 reversed by naloxone than morphine. Due to fentanyl's high lipophilicity and elongated
14 structure we hypothesised that its unusual pharmacology may be explained by a novel binding
15 mode to the μ -opioid receptor (MOPr).

16 By employing coarse-grained molecular dynamics simulations and free energy calculations,
17 we determined the routes by which fentanyl and morphine access the orthosteric pocket of
18 MOPr.

19 Morphine accesses MOPr via the aqueous pathway; first binding to an extracellular vestibule,
20 then diffusing into the orthosteric pocket. In contrast, fentanyl takes a novel route; first
21 partitioning into the membrane, before accessing the orthosteric site by diffusing through a
22 ligand-induced gap between the transmembrane helices.

23 This novel lipophilic route may explain the high potency and lower susceptibility of fentanyl
24 to reversal by naloxone.

25

26 **Introduction**

27 The synthetic opioid agonist, fentanyl, has been in medicinal use for over 50 years as a
28 powerful, fast-acting analgesic and for induction of sedation and general anaesthesia.
29 However, since 2014 fentanyl and fentanyl analogues (fentanyls) have increasingly appeared
30 in the illicit drug market in North America (1, 2); this has been associated with a dramatic rise
31 in the number of acute opioid overdose deaths involving fentanyls (3), with the ease of
32 synthesis and transport making fentanyls attractive to suppliers of illicit opioids (4).
33 Concerningly, there are increasing reports that fentanyl overdose requires higher doses of the
34 antagonist naloxone to reverse, compared to heroin (4-10). Indeed, we have recently shown
35 that naloxone reverses respiratory depression induced by fentanyl in mice less readily, than
36 that induced by morphine (11). This finding is at odds with classical receptor theory, as under
37 competitive conditions the degree of antagonism depends only on the affinity and
38 concentration of the antagonist, not the potency of the agonist (12). Fentanyls, therefore, are
39 an increasing public health concern, and exhibit a unique pharmacology which is yet to be
40 fully understood.

41 In *in vitro* radioligand binding and signaling experiments there is a discrepancy between the
42 relative potencies of fentanyl and morphine in experiments performed in membrane
43 homogenate and intact cell systems. In membrane homogenate experiments fentanyl and
44 morphine exhibit similar affinity of binding to the m opioid receptor (MOPr), both in the
45 absence and presence of Na^+ ions (13-15), whilst in membrane homogenate studies of
46 receptor activation using GTP γ S binding the potency of fentanyl has been reported to be less
47 than 2 fold greater than that of morphine with fentanyl having equal or only slightly greater
48 agonist efficacy (14-16). In marked contrast, in intact cell studies of MOPr signaling (cyclic

49 AMP inhibition, G protein activation and arrestin translocation) fentanyl is some 5 to 50 fold
50 more potent than morphine (16-20). We propose that this discrepancy may be explained by
51 the unusual properties of fentanyl. Firstly, fentanyls are highly lipophilic compared to other
52 opioid ligands (21, 22) and may therefore in intact cells interact with, or even partition into,
53 the lipid bilayer thus increasing the concentration of drug in the vicinity of the receptor (23-
54 25). Secondly, fentanyls have an elongated chemical structure with a central protonatable
55 nitrogen and 6 rotatable bonds, compared to the rigid ring structure of morphine, naloxone
56 and other morphinan compounds (Supplementary Fig 1A). This flexible structure may
57 facilitate a novel binding process, distinct from that of morphinan opioid agonists and the
58 antagonist naloxone.

59 MOPr, which mediates the pharmacological effects of fentanyl (11), is a G protein-coupled
60 receptor (GPCR) found primarily at the plasma membrane. The MOPr structure follows the
61 general architecture of a class A GPCR (26-28), with a deep aqueous binding pocket for
62 orthosteric ligands which is shielded from the extracellular milieu by three extracellular loops
63 (ECLs) and from the lipid bilayer by the seven transmembrane helices (TMDs). X-ray crystal
64 structures and cryo-electron microscopy structures of the MOPr reveal small molecule
65 morphinan ligands (26, 27) and the peptide DAMGO (28) bind within this orthosteric site via
66 a key interaction between the protonated amine of the ligand and D147^{3,32} (residues are
67 labelled according to the Ballesteros-Weinstein system (29)) (Supplementary Fig 2).

68 It is generally assumed that GPCR ligands bind to the orthosteric site directly from the
69 extracellular aqueous phase (30-33). However, studies of sphingosine-1-phosphate,
70 cannabinoid (CB2), protease activated (PAR1), and purinergic (P2Y1) receptors have shown
71 that some highly lipophilic ligands are able to access the orthosteric pocket by diffusing

72 through the lipid membrane and the receptor transmembrane helices (34-37). Therefore,
73 based on fentanyl's high lipophilicity, elongated structure, and differing potencies dependent
74 on the membrane environment, we hypothesised that fentanyl may bind to the MOPr in a
75 non-canonical fashion via the lipid bilayer.

76 Long timescale all-atom molecular dynamics (MD) simulations have been used to capture
77 small molecule ligands binding to GPCRs (30, 32, 33). However, capturing a rare event such as
78 ligand binding usually requires millisecond timescale simulations using specially designed
79 machines (38). In addition, fentanyl has many rotatable bonds and therefore many degrees
80 of freedom which can pose sampling problems. Coarse-grain (CG) MD can be utilised to
81 overcome these sampling issues (39-41). In CG MD, rather than representing each individual
82 atom as a defined bead, groups of atoms are represented as a single bead which describes
83 the overall properties of the chemical group. This lower resolution representation results in
84 fewer beads and fewer degrees of freedom to describe a system, meaning the conformational
85 landscape can be sampled more efficiently, and rare events such as ligand binding can be
86 more readily captured (42, 43). To determine how fentanyls and morphinans access and bind
87 within the orthosteric site, we employed unbiased CG MD simulations of membrane-
88 embedded MOPr solvated in water, ions and opioid ligands.

89 **Results**

90 We built molecular systems of the MOPr (26, 44, 45) (PDB: 4DKL) in a solvated membrane
91 using the coarse grained MARTINI 2.2 force field (see Methods for details) (Supplementary
92 Fig 1B). We added 6 molecules of either protonated fentanyl, neutral fentanyl, protonated
93 morphine or neutral morphine (Supplementary Fig 1C and D) to the solvent and ran 3 - 6

94 independent repeats of 1 - 5 μ s, unbiased CG MD simulations to allow the ligands to bind to
95 the MOPr (Supplementary Table 1).

96 We first characterised how the protonated and neutral forms of fentanyl and morphine
97 interacted with the phospholipid membrane. In all simulations, fentanyl and morphine rapidly
98 diffused from the solvent to interact with the phospholipid bilayer. Both the protonated and
99 neutral fentanyl molecules fully partitioned into the membrane (Fig 1A), with the neutral form
100 of the ligand penetrating deeper into the bilayer centre (Fig 1C and Supplementary Fig 3A). In
101 contrast morphine interacted only with the phosphate head groups at the lipid-solvent
102 interface (Fig 1B), and neither the protonated nor neutral form of the ligand partitioned into
103 the bilayer (Fig 1C).

104 To further quantify the propensity for fentanyl and morphine to partition between the
105 aqueous and lipid phase, we performed steered MD and umbrella sampling to calculate the
106 free energy change (ΔG) of membrane partitioning. Steered MD uses an external force to
107 “pull” the ligand away from the center of the membrane (46), creating a trajectory of the
108 ligand moving between the lipid and aqueous solvent from which umbrella sampling can be
109 performed to extract potential of mean force (PMF) profiles. Using these PMFs, ΔG can be
110 calculated as the free energy difference between the ligand residing in the bilayer center
111 verses the aqueous solvent. The resulting ΔG values are shown in Fig 1D, and the PMF profiles
112 in Supplementary Fig 3B-E.

113 The calculated ΔG for membrane partitioning for the protonated and neutral forms of
114 fentanyl were $-50.3 \pm 6.0 \text{ kJmol}^{-1}$ and $-66.1 \pm 4.1 \text{ kJmol}^{-1}$, respectively. Whereas, the values
115 for morphine showed a much smaller free energy difference (protonated; $-20.6 \pm 0.3 \text{ kJmol}^{-1}$,
116 neutral; $-27.3 \pm 0.3 \text{ kJmol}^{-1}$). The spontaneous membrane partitioning exhibited by fentanyl

117 in the unbiased CG simulations, along with this greater free energy change in partitioning
118 between the lipid and the aqueous solvent, suggests that fentanyl has a greater propensity
119 to concentrate in the cell membrane, compared to morphine.

120 **Fentanyl binds to MOPr via the lipid phase and the transmembrane helices**

121 For the remaining analyses, we focus on the simulations of the protonated ligands, as the
122 charged species is required to form the canonical amine – D147^{3,32} salt bridge essential for
123 opioid ligand binding within the orthosteric pocket.

124 In the CG MD simulations fentanyl molecules in the lipid bilayer appeared to congregate
125 around MOPr (Fig 1A). We therefore constructed ligand density maps across all the fentanyl
126 simulations (Fig 2A), using the VMD VolMap tool (47). Fentanyl molecules cluster around the
127 receptor helices in the upper leaflet of the membrane, with densities determined on the lipid-
128 facing sides of the TM1/2, TM6/7 and TM7/1 interfaces.

129 Most notably, we also observed fentanyl diffusing through MOPr to the orthosteric binding
130 pocket via a novel lipophilic pathway (see Supplementary Movie 1). Snapshots from the MD
131 simulation (Fig 2C and Supplementary Fig 4A) show fentanyl first partitioning into the lipid
132 bilayer, then interacting with a ligand-induced gap (Supplementary Fig 4C and D) at the TM6/7
133 interface, and finally accessing the orthosteric site by diffusing through this gap in the MOPr
134 helices. The fentanyl molecule took 3 μ s to diffuse across the receptor TM domains to the
135 orthosteric site (Fig 2B).

136 The TM6/7 interface and the gap induced by the fentanyl molecule is shown in Fig 2D. This
137 interface comprises hydrophobic and polar residues from TM6 and 7, as well as ECL3.
138 Specifically, the relatively small side chains of L305^{6,60}, T307^{ECL3}, I308^{ECL3} and P309^{ECL3} allow

139 formation of a pore through which the phenethyl group of fentanyl (represented by the F1,
140 F2 and F3 beads, see Supplementary Fig 1D) can access the receptor orthosteric pocket.
141 Meanwhile, the aromatic side chain of W318^{7,35} stabilises the position of fentanyl's N-phenyl-
142 propanamide (represented by the F7, F8 and F9 beads, see Supplementary Fig 1D).

143 **Morphine binds to MOPr via the aqueous phase and an extracellular vestibule site**

144 During the unbiased CG simulations, we observed morphine spontaneously binding to the
145 MOPr via the canonical aqueous pathway (see Supplementary Movie 2). Ligand density maps
146 show a density for a morphine molecule in the extracellular portion of the MOPr; above and
147 within the orthosteric binding site (Fig 3A). Plotting the distance between the charged Qd
148 bead of morphine and the side chain bead of D147^{3,32} shows that the ligand rapidly diffuses
149 from the aqueous solvent to interact with the extracellular surface of MOPr within the first
150 50 ns of the CG simulation (Fig 3B). Morphine maintains stable interactions with this
151 extracellular site for 4.2 μ s, before finally moving deeper into the orthosteric binding pocket.
152 Figure 3C and Supplementary Fig 4B show snapshots of morphine travelling along this
153 canonical aqueous binding pathway, with it initially binding to the extracellular vestibule site
154 and then finally binding within the orthosteric pocket.

155 The extracellular vestibule site is shown in Fig 3D, comprising primarily polar or charged
156 residue side chains in ECL2 and the extracellular ends of TMs 5, 6 and 7. This extracellular
157 vestibule site (48) appears to be a conserved feature of small molecule binding to Class A
158 GPCRs, having previously been highlighted in MD simulations of the β 1 and β 2 adrenergic
159 receptors (32), M3 muscarinic receptor (33), adenosine A_{2A} receptor (42) and oliceridine
160 binding to the MOPr (30).

161 **Calculation of the relative binding energies in the aqueous and lipophilic access routes**

162 Next, we sought to further characterize the aqueous and lipid binding pathways by calculation
163 of the free energy of binding ($\Delta G_{\text{binding}}$) for each ligand in each pathway.

164 Starting from the final frames of the simulations where fentanyl (Fig 2C) or morphine (Fig 3C)
165 bound in the orthosteric site, steered MD simulations were performed to recreate the
166 aqueous and lipid binding routes for each ligand. Ligands were “pulled” from the orthosteric
167 site along either the aqueous or lipid access route, generating a trajectory from which starting
168 conformations for umbrella sampling could be generated. The resulting PMF profiles are
169 presented in Fig 4, along with the calculated $\Delta G_{\text{binding}}$ values for each ligand in each binding
170 pathway. Here, $\Delta G_{\text{binding}}$ represents the free energy difference between the ligand-bound
171 MOPr and the unbound ligand residing in either the aqueous solvent (Fig 4A and B) or the
172 lipid membrane (Fig 4C and D).

173 The PMF profiles for morphine and fentanyl binding via the aqueous pathway are shown in
174 Fig 4A and B, respectively. The calculated $\Delta G_{\text{binding}}$ for each ligand is similar ($-58.7 \pm 5.7 \text{ kJmol}^{-1}$
175 for morphine, $-60.1 \pm 3.7 \text{ kJmol}^{-1}$ for fentanyl), suggesting that both ligands can bind via this
176 aqueous route with similar ease. In the profile for morphine binding a small local minimum
177 can be seen between 1.0 - 1.3 nm, indicating the extracellular vestibule site identified in the
178 unbiased MD simulations (Fig 2D). In the profile for fentanyl binding no small local minimum
179 indicative of binding to the extracellular vestibule is apparent.

180 The PMF profiles for morphine and fentanyl binding via the lipid pathway are shown in Fig 4C
181 and D. For morphine, the PMF profile follows a steep curve, with a calculated $\Delta G_{\text{binding}}$ of -45.3
182 $\pm 1.8 \text{ kJmol}^{-1}$. In contrast, the fentanyl $\Delta G_{\text{binding}}$ is significantly lower ($-14.4 \pm 0.8 \text{ kJmol}^{-1}$), with
183 two local minima at 0 – 0.8 nm and 1.1 – 1.5 nm, corresponding to the orthosteric site and
184 the TM6/7 interface (Fig 2D) on the lipid-facing side of the helices, respectively.

185 **Comparison of free energy landscapes for morphine and fentanyl**

186 In order to compare the full binding pathways from solvent to MOPr for fentanyl and
187 morphine, we used the data from the PMF analyses in Fig 1D and 4 to construct free energy
188 landscapes for both ligands in their protonated forms as they interact with MOPr (Fig 5).
189 Figure 5A shows a thermodynamic cycle for each ligand, where ΔG_1 is free energy of transfer
190 between the receptor and the membrane, as measured in Fig 4 C and D, ΔG_2 is between the
191 membrane and solvent, as per Fig 1D, ΔG_3 is the energy of moving in the solvent (assumed to
192 be 0 kJ mol⁻¹) and ΔG_{direct} represents the aqueous pathway from solvent to orthosteric binding
193 site in the receptor explored in Fig 4A and B. From this, we can state that:

194
$$\Delta G_{\text{direct}} = \Delta G_1 + \Delta G_2 + \Delta G_3 = \Delta G_1 + \Delta G_2$$

195 As can be seen in Fig 5B, this indeed holds up, and the energies we have obtained here agree
196 whether measured for the direct binding route or the indirect route, via the membrane.
197 Importantly, whilst the overall binding energy for each ligand is very similar, the primary
198 difference is the increased preference of fentanyl to partition into the lipid membrane (Fig
199 5C) where it can access the lipophilic access route. This suggests that fentanyl may favour this
200 indirect, lipid access route, whereas morphine, which does not penetrate into the lipid,
201 favours the “canonical” pathway, binding directly from the aqueous solvent.

202 **Discussion**

203 Here, we applied CG MD simulations to study the interactions of both fentanyl and morphine
204 with the MOPr. Using a combination of unbiased MD simulations and free energy calculations,
205 we demonstrate that fentanyl exhibits a marked preference to access the MOPr orthosteric
206 site via a novel binding route through the lipid membrane and MOPr transmembrane helices

207 (Fig 6). Whereas, morphine accesses the orthosteric pocket by diffusing directly from the
208 aqueous solvent and an extracellular vestibule site. Free energy calculations show that whilst
209 fentanyl can also bind to the MOPr via the canonical aqueous route, fentanyl's preference for
210 the lipid access route is driven by its high lipid solubility; fentanyl rapidly partitions into the
211 lipid membrane and clusters around the MOPr. In contrast, morphine only diffuses as far as
212 the lipid surface. Once positioned at the TM6/TM7 lipid-facing interface, fentanyl interacts
213 with hydrophobic and aromatic residues to induce formation of a gap through which it gains
214 access to the MOPr orthosteric site.

215 By using CG representation of our MOPr-ligand systems, we have captured ligand binding to
216 the MOPr in a truly unbiased fashion, without the need for very longtime scale simulations
217 (32) or use of any external potential or metadynamics approach (31). Combining our unbiased
218 CG trajectories with steered MD and umbrella sampling to calculate the free energy
219 landscapes of the binding events has proved a powerful tool for interrogating opioid ligand
220 binding pathways.

221 Due to the lower resolution of the coarse-grained MD employed in this study, the two ligands
222 represent multiple "fentanyl" or "morphinan" molecules. It is likely that other fentanyl
223 analogues with high lipophilicity also exhibit lipid phase binding to the MOPr, for instance
224 carfentanil, sufentanil and ohmefentanyl. The size of the fentanyl-induced gap between TM6
225 and 7 would suggest that fentanyl's ability to bind via the lipid is a property of both its high
226 lipophilicity and the elongated, flexible structure. Morphine, which is less lipid soluble, does
227 not penetrate into the lipid far enough to access the gap, and is therefore unlikely to favour
228 this binding pathway.

229 It is increasingly appreciated that GPCR ligands can bind to a variety of topographically distinct
230 sites within the receptor structure (49). Allosteric binding pockets have been identified within
231 the extracellular vestibule (50, 51), lipid-facing portions of the transmembrane helices (52),
232 and the intracellular G protein coupling region (53), spanning the entire bilayer (54). Similarly,
233 metastable sites populated as ligands bind and unbind between the orthosteric pocket and
234 the aqueous phase have been identified, and it seems likely that for Class A GPCRs which
235 recognise small molecule ligands, some of these sites may be conserved. Indeed, the
236 extracellular vestibule to which morphine initially binds in our MD simulations appears to be
237 analogous to the vestibule sites in the β 1 and β 2 adrenergic (32) and M3 muscarinic receptors
238 (33). In extensive, all-atom simulations of MOPr in the presence of a high concentration of
239 oliceridine, the ligand was also observed to bind to sites in the extracellular portion of the
240 receptor (30).

241 A lipid phase binding route has been proposed for other GPCRs; notably rhodopsin and the
242 CB2 cannabinoid, sphingosine-1-phosphate, PAR1 and P2Y1 receptors (34-37, 55), though not
243 so far for the MOPr which has evolved to recognise non-lipophilic peptide ligands. Like
244 fentanyl at MOPr, 2-arachidonoylglycerol and vorapaxar are reported to access the
245 orthosteric pocket via the TM6/7 interfaces of the CB2 and PAR1 receptors, respectively (34,
246 35). Particularly, in simulations of vorapaxar unbinding from the PAR1 receptor, the ligand
247 also exits via a gap formed by TM6/7 and ECL3, towards the extracellular side of the receptor
248 (35). Similar to the MOPr, this gap is lined by small hydrophobic and polar residues and an
249 aromatic residue in position 7.35 (tryptophan in MOPr, tyrosine in PAR1). In the CB2 receptor,
250 the ligand entry gap is further towards the intracellular side of TM6 and 7 (34).

251 Interestingly, the novel binding mode we observe here may be specific to the MOPr over the
252 δ -opioid receptor (DOPr). Fentanyl is highly selective for MOPr over the DOPr (56). Where the
253 residues lining the TM6/7 gap in the MOPr are largely small polar, hydrophobic or aromatic
254 side chains, in the DOPr ECL3 contains two positively charged and bulky arginine residues
255 (R291 and R292) which may impede fentanyl binding by both repulsion of the protonated
256 nitrogen and steric hinderance.

257 This novel mechanism of interaction with MOPr is of great importance to our understanding
258 of the pharmacology of fentanyl.

259 Firstly, by concentrating fentanyl in the lipid membrane, the apparent concentration around
260 the receptor is markedly increased, as the membrane acts as a reservoir. This high local
261 concentration increases the likelihood of receptor association. Therefore, whilst morphine
262 and fentanyl have very similar binding energies for MOPr, the actual likelihood of fentanyl
263 binding would be far higher, and this might well explain the increased potency of fentanyl
264 over morphine, particularly in cells where a complete, intact cell membrane is present.

265 Secondly, once fentanyl has partitioned into the bilayer it will switch from 3D diffusion in the
266 solvent to 2D, lateral diffusion in the membrane (57). This reduction in dimensionality results
267 in fentanyl having a greater chance of finding the receptor target, compared to morphinan
268 ligands exhibiting 3D diffusion in the aqueous phase. Similarly, the membrane may also serve
269 to organise the fentanyl molecules at a depth and orientation which favours MOPr binding
270 through the TM6/7 interface (54).

271 Our identification of the TM6/7 metastable interface on the outside of the MOPr helices also
272 invites the possibility that fentanyl exhibits “exosite” binding and re-binding, as described by
273 Vauquelin & Charlton (58). Unlike morphinan ligands which bind and unbind via the aqueous

274 phase, fentanyl is not free to diffuse away from MOPr and instead binds to the “exosite”
275 TM6/7 interface. From here, fentanyl can then rapidly and efficiently rebind to the orthosteric
276 site.

277 The mechanisms outlined here may also explain the poor reversibility of fentanyls by the
278 morphinan antagonist naloxone. Naloxone has similar lipid solubility to morphine and is
279 therefore unlikely to concentrate in the bilayer or have access to the lipid phase binding route
280 and TM6/7 exosite (Fig 6). It would therefore only compete with fentanyl for binding via the
281 aqueous route, not the lipophilic route. Whilst naloxone can still compete with fentanyl to
282 occupy the orthosteric pocket, fentanyl can remain bound to the TM6/7 exosite and thus
283 rapidly rebind to the orthosteric site once naloxone has dissociated. A similar phenomenon
284 has been demonstrated for the lipophilic β 2 adrenergic receptor agonist, salmeterol, where
285 the ligand may be retained in the lipid membrane allowing reassertion of its agonist effects
286 after wash-out (59, 60).

287 Fentanyl and other synthetic opioid agonists are driving the current opioid overdose epidemic
288 in the United States (61). Fentanyl’s rapid onset and high potency are compounded by poor
289 naloxone-reversibility, making the risk of fentanyl overdose high. Only by understanding fully
290 how fentanyl interacts with and activates MOPr will we be able to develop better antagonists.

291 We have previously shown that fentanyl-induced respiratory depression in mice is poorly
292 reversed by naloxone compared to that by morphine. In light of these MD data, this is hardly
293 surprising given that naloxone has low lipid solubility. However, we have also shown that the
294 more lipophilic antagonist diprenorphine is better able to antagonize the effects of fentanyl
295 (11). This might suggest that diprenorphine can at least access the entry point in the TM
296 domains to block fentanyl access. Whilst elucidating how diprenorphine and other lipophilic

297 ligands interact with the MOPr requires further study, the development of lipophilic MOPr

298 antagonists may prove highly beneficial in combatting fentanyl overdose.

299

300 **Methods**

301 **System set-up**

302 The MOPr model was taken from the inactive, antagonist-bound crystal structure (26) (PDB:
303 4DKL), with the T4 lysozyme and ligands removed, and the missing intracellular loop 3
304 modelled using Insight II, as described in (45). The protein structure coordinates were then
305 converted to coarse-grained MARTINI 2.2 representation using the *martinize* script (62). The
306 secondary structure was constrained using an elastic network between backbone (BB) beads;
307 elastic bonds with a force constant of 100 kJ mol⁻¹nm⁻² were defined between BB_i-BB_{i+4} helix
308 atoms, BB_i-BB_{i+10} helix atoms, and BB atom pairs with low root mean square fluctuation and
309 highly correlated motion as determined from all-atom MD simulations. All MD simulations
310 were run using GROMACS 2019.2 (63).

311 To parameterise morphine and fentanyl in MARTINI, firstly, 1 μs all-atom MD simulations of
312 fentanyl or morphine in water and 0.15 M NaCl were conducted under the Amber ff99SB-ildn
313 force field (64). Ligands were parameterised using acpype/Antechamber and the General
314 Amber Force Field (65). Atom-to-bead mapping for morphine and fentanyl was then created
315 as shown in Supplementary Fig 1A and B, respectively. The CG ligands were then solvated in
316 water and 0.15 M NaCl, energy minimized for 10000 steps using the steepest descents
317 algorithm, box dimensions and temperature equilibrated, and then production MD was run
318 for 1 μs. Bond lengths and angles were measured and compared to the all-atom simulations,
319 to determine appropriate mapping and bonded terms.

320

321

322 **Unbiased CG simulations**

323 The CG MOPr model was then embedded in a POPC:POPE:cholesterol lipid bilayer (ratio 5:5:1)
324 using the *insane* script (66), and solvated in water, 0.15 M NaCl and 6 molecules of opioid
325 ligand. The starting size of the system box was 15 x 15 x 15 nm³. Systems were first energy
326 minimized over 50000 steps using the steepest descents algorithm, then equilibrated under
327 NVT ensemble and then NPT ensembles, before production MD simulations were run at 310
328 K with a 10 fs timestep. The temperature and pressure were controlled by the V-rescale
329 thermostat and Parrinello-Rahman barostat, respectively. Simulations were performed for up
330 to 5 μs; the exact simulation lengths for each ligand are shown in Supplementary Table 1.

331 All simulations were analysed using the GROMACS suite of tools (63). Unless otherwise stated,
332 all analyses were performed using the entire production trajectories. Data were plotted in
333 GraphPad Prism v8, and images made in VMD (47).

334 **Free energy calculations**

335 For the membrane/solvent partitioning calculations, systems were set up with small (5 x 5 x
336 10 nm³) membrane patches containing 32 POPE, 32 POPC and 6 cholesterol molecules, and
337 solvated in 0.15 M NaCl. One molecule of either protonated fentanyl, neutral fentanyl,
338 protonated morphine or neutral morphine was placed in the bilayer center. The systems were
339 minimized for 50000 steps, keeping the ligand restrained. To generate the starting
340 conformations for umbrella sampling, steered MD simulations were performed. Ligands were
341 pulled from the bilayer center into the solvent (46), in a direction defined by the vector
342 between the centers of mass of the ligand and the PO4 lipid beads, at a rate of 0.1 nm ns⁻¹
343 and a force constant of 1000 kJ mol⁻¹ nm⁻².

344 For the ligand binding calculations, the final frames from the unbiased CG simulations with
345 morphine or fentanyl bound in the orthosteric pocket were taken as the starting
346 conformations. All other unbound ligands were removed, and the receptor-ligand complex
347 was re-embedded in a smaller lipid bilayer (10 x 10 x 10 nm³). Steered MD simulations were
348 performed to generate the starting conformations for umbrella sampling. In each case,
349 separate simulations were performed to pull morphine or fentanyl from the orthosteric
350 pocket along a) the aqueous / extracellular route, and b) the lipophilic / transmembrane
351 domain route. The reaction coordinate was defined as the distance between the center of
352 mass of the ligand and the receptor. Ligands were pulled at a rate of 0.1 nm ns⁻¹ and a force
353 constant of 1000 kJ mol⁻¹ nm⁻², with a 1000 kJ mol⁻¹ nm⁻² position restraint on 4 backbone
354 beads (D114^{2.50}, D147^{3.32}, N150^{3.35} and S154^{3.39}) of the MOPr to prevent translation or rotation
355 of the receptor.

356 The starting conformations for umbrella sampling were extracted from these steered MD
357 trajectories at 0.05 nm intervals along the reaction coordinate, generating ~80 umbrella
358 sampling windows for each calculation. Each was subjected to 1 μ s MD simulations, with a
359 harmonic restraint of 1000 kJ mol⁻¹ nm⁻² to maintain the separation between the centers of
360 mass of the ligand and PO4 beads (membrane partitioning calculations) or protein (ligand
361 binding calculations). The PMFs were then extracted using the Weighted Histogram Analysis
362 Method (WHAM) in GROMACS (67). PMFs were plotted as the average profile with statistical
363 error calculated from bootstrap analysis. For the ligand binding calculations, $\Delta G_{\text{binding}}$ for each
364 ligand in each binding pathway was calculated as the difference between the ligand-bound
365 and final unbound states.

366

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542

543 **Acknowledgements**

544 The work described in this paper was supported by a grant from the Medical Research Council

545 (MR/S010890/1) to GH, EK and SJC and was carried out using the computational facilities of

546 the Advanced Computing Research Centre, University of Bristol <http://www.bris.ac.uk/acrc/>.

547 We thank Roseanna Jackson of Slowe Club for artwork.

548 **Author contributions**

549 KJS and RAC designed and performed the MD simulations. KJS, RAC and RBS analysed the

550 data. GH, EK, SJC and KJS conceived the study. All authors contributed to and approved the

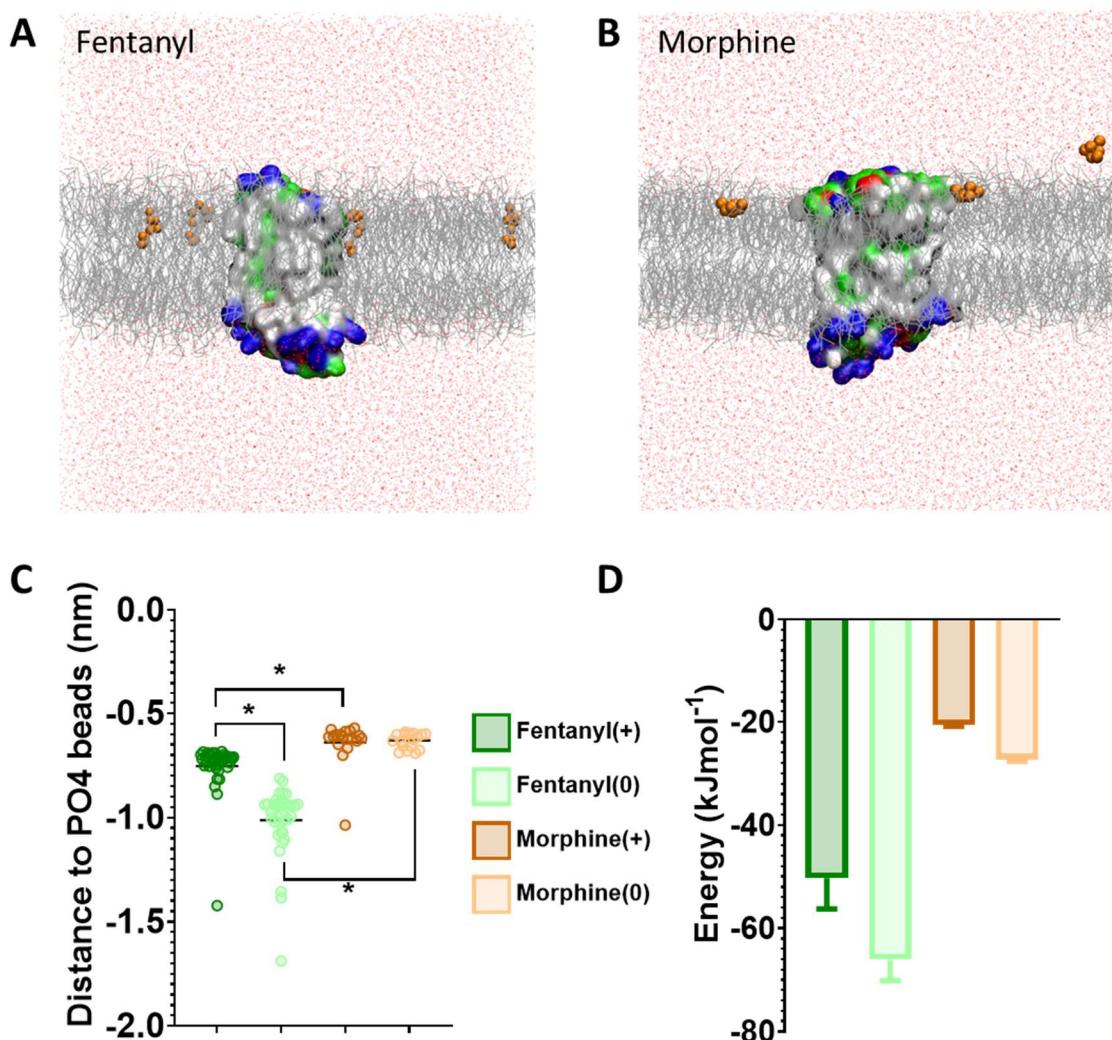
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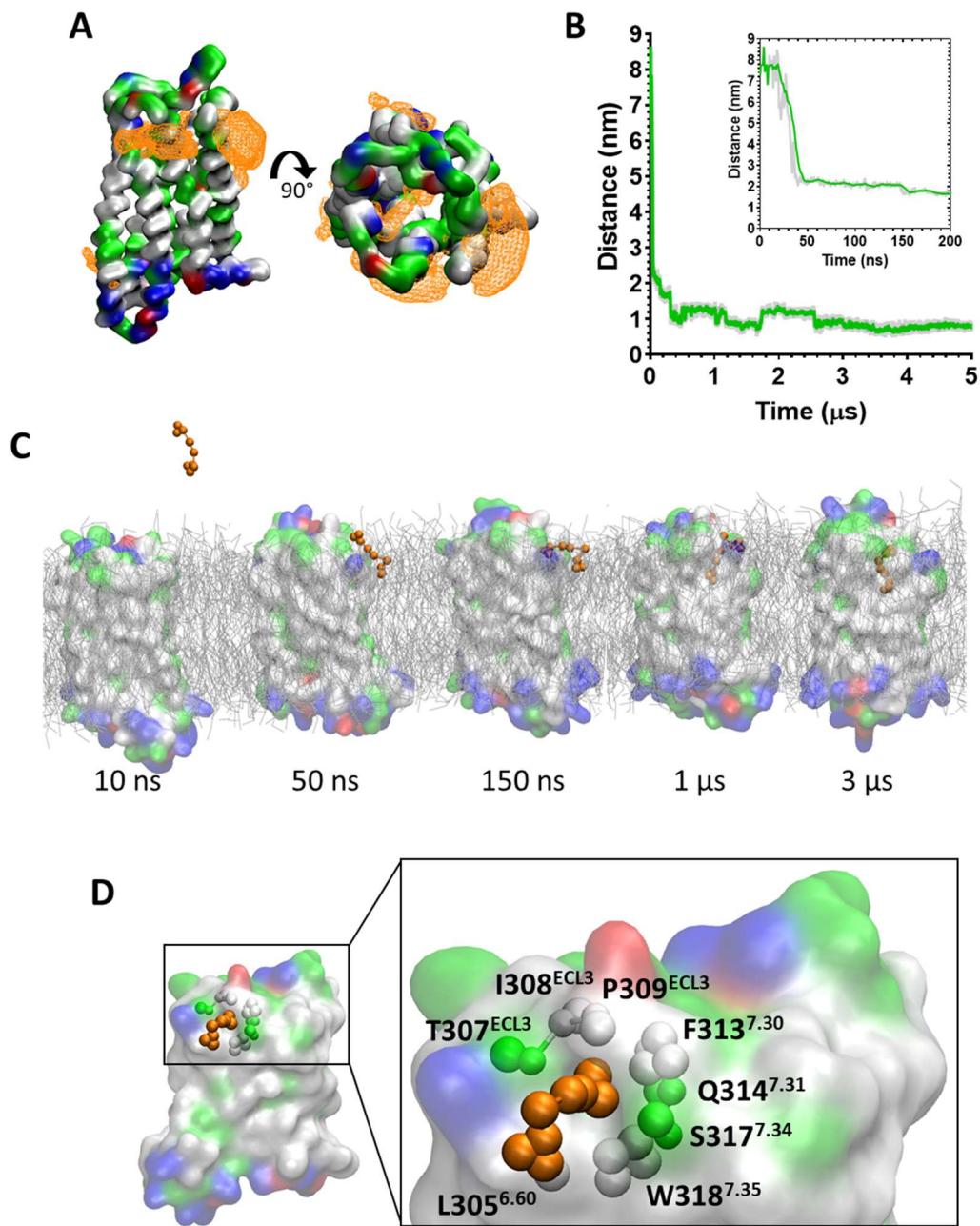
552 **Competing Interests**

553 The authors declare no competing interests.

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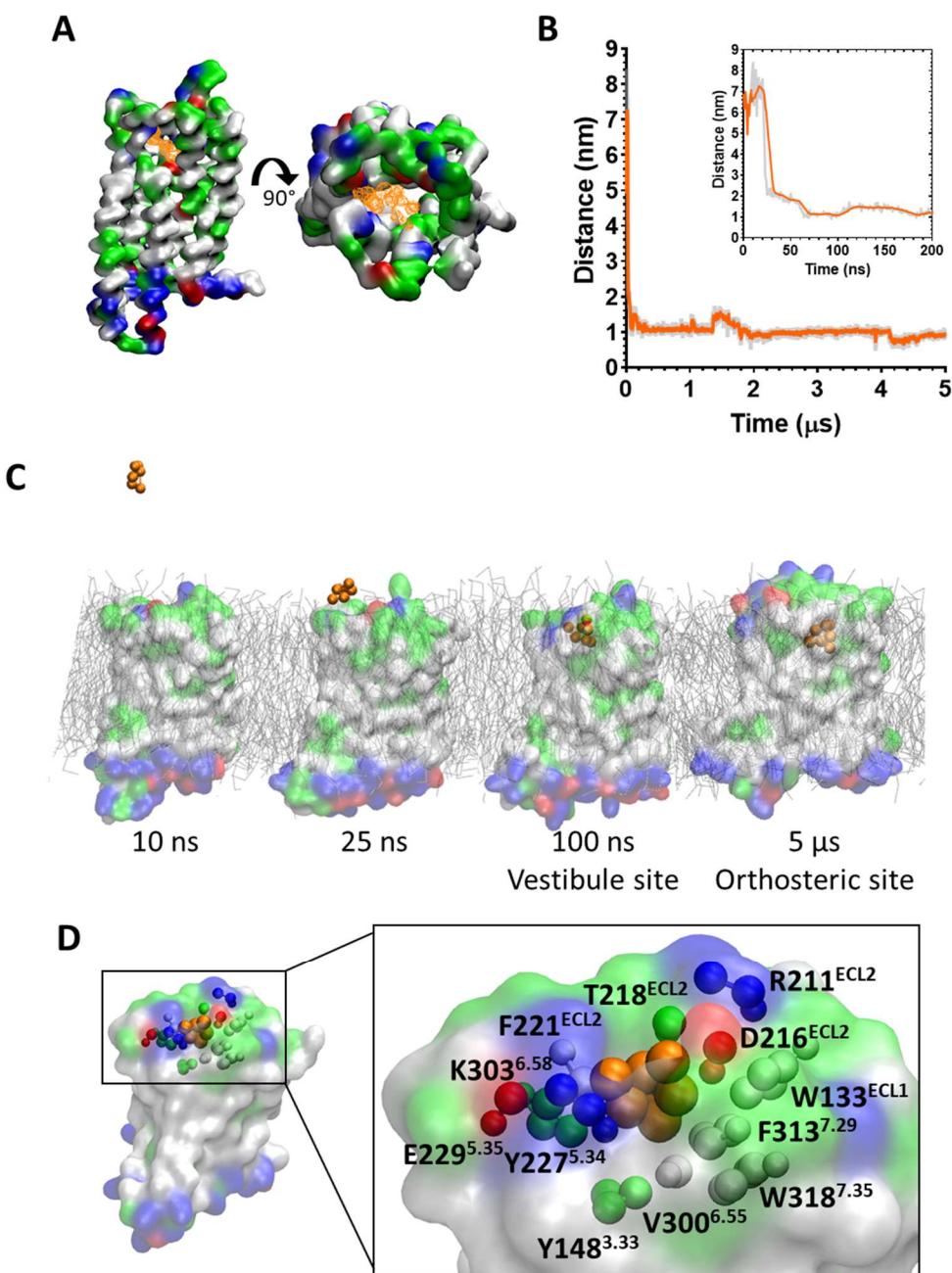
555 **Figures**





574 **Figure. 2. Fentanyl binds to the MOPr from the lipid phase, via a gap between TM6 and TM7**
575 **A.** Ligand density maps averaged over the 5 μ s simulation, show fentanyl densities around the
576 receptor transmembrane domains and within the orthosteric pocket (orange). The protein is
577 coloured according to residue properties (hydrophobic; grey, polar; green, acidic; red, basic;
578 blue). **B.** Distance between the Qd bead of fentanyl and the SC1 bead of D147^{3.32} over the
579 entire 5 μ s and in the first 200 ns (inset). Data are presented as the raw values (grey) and
580 moving average over 10 frames (green). **C.** Snapshots from the unbiased simulation of fentanyl
581 binding to MOPr. Fentanyl moves from the aqueous solvent into the lipid bilayer, then
582 interacts with the MOPr transmembrane domains and induces the formation of a gap
583 between TM6 and 7, through which fentanyl accesses the orthosteric site. **D.** Fentanyl at the
584 TM6/7 interface. Fentanyl is depicted as orange beads, and the residues comprising the lipid
585 entry gap as coloured beads.

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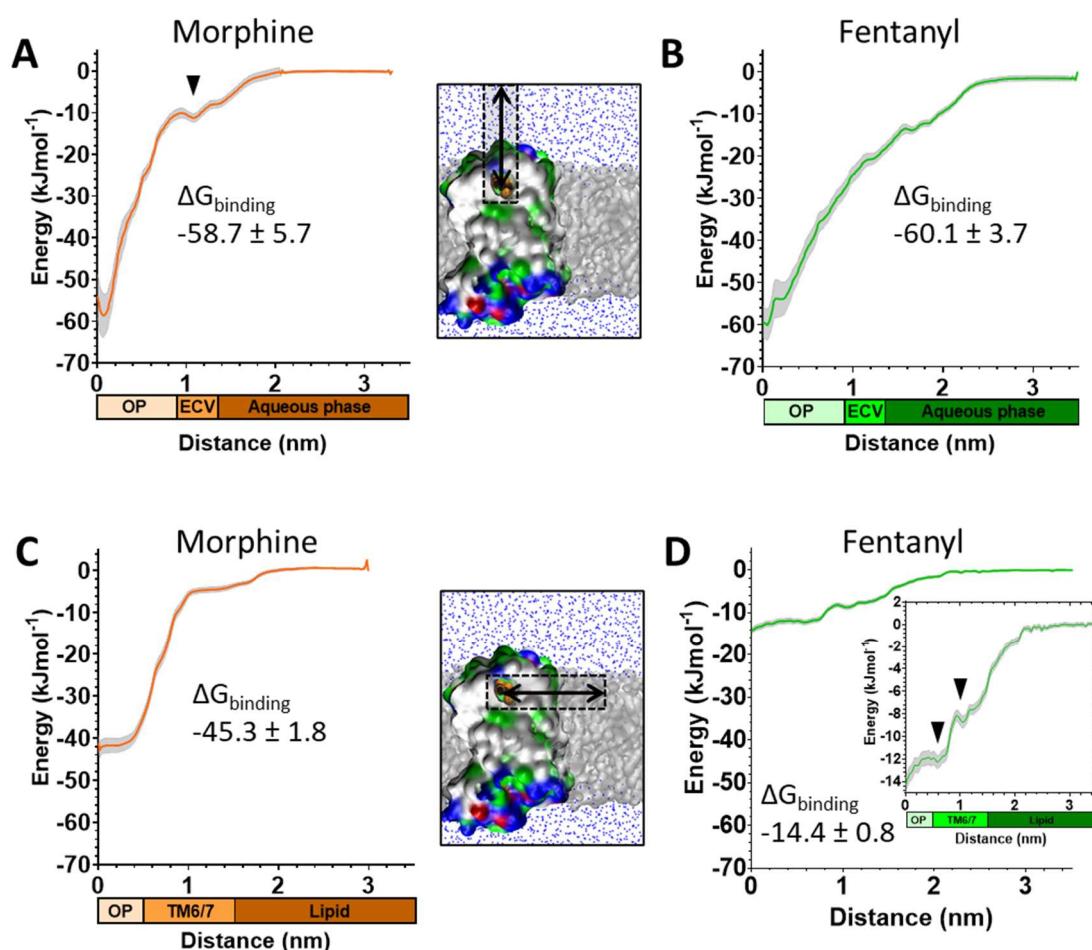


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588 **Figure. 3. Morphine binds to the MOPr from the aqueous phase, via an extracellular**
589 **vestibule site**

590 **A.** Ligand density maps averaged over the 5 μ s simulation, show morphine densities above
591 and within the orthosteric pocket (orange). The protein is coloured according to residue
592 properties (hydrophobic; grey, polar; green, acidic; red, basic; blue). **B.** Distance between the
593 Qd bead of morphine and the SC1 bead of D147^{3,32} over the entire 5 μ s and in the first 200 ns
594 (inset). Data are presented as the raw values (grey) and moving average over 10 frames
595 (orange). **C.** Snapshots from the unbiased simulation of morphine binding to MOPr. Morphine
596 moves from the aqueous solvent to an extracellular vestibule and finally the orthosteric site.
597 **D.** Morphine in the extracellular vestibule site. Morphine is depicted as orange beads, and the
598 residues comprising the vestibule site as coloured beads.

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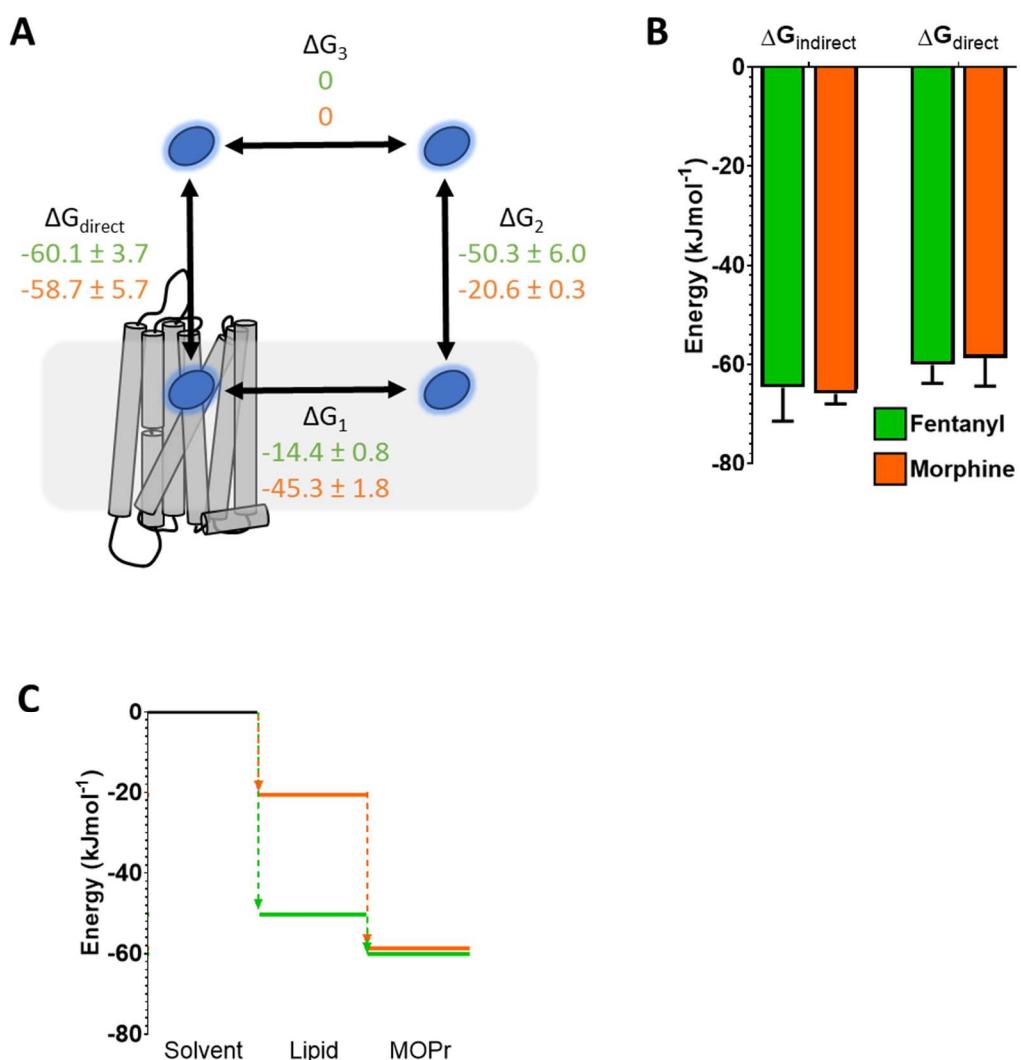
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601 **Figure 4. Free energy calculations for ligand binding pathways**

602 Steered MD was used to recreate the spontaneous binding events reported in Figs 2 and 3.
603 Umbrella sampling and the weighted histogram analysis method were then employed to
604 determine the free energy of binding for each ligand in each pathway. In all plots the distance
605 along the reaction coordinate is defined as the distance between the centre of mass of the
606 ligand and receptor. Coloured bars beneath the x-axes indicate the orthosteric pocket (OP),
607 extracellular vestibule (ECV), TM6/7 interface, lipid and aqueous phases. Data are plotted as
608 an average (coloured line) and statistical error (grey), calculated from bootstrap analysis.
609 $\Delta G_{\text{binding}}$ is expressed as mean \pm statistical error. **A.** PMF profile for morphine binding via the
610 aqueous pathway. **B.** PMF profile for fentanyl binding via the aqueous pathway. **C.** PMF profile
611 for morphine binding via the lipid pathway. **D.** PMF profile for fentanyl binding via the lipid
612 pathway. Inset shows the same data with expanded y axis.

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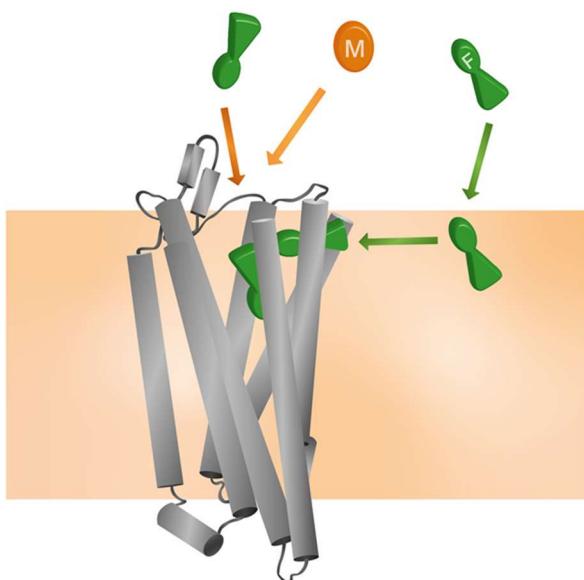
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616 **Figure. 5. Comparison of free energy landscapes for fentanyl and morphine binding to the**
617 **MOPr**

618 **A.** Thermodynamic cycle for opioid ligand binding to MOPr; either by the direct, aqueous
619 pathway (ΔG_{direct}) or via the lipid membrane ($\Delta G_1 + \Delta G_2$). Values for protonated fentanyl
620 (green) and protonated morphine (orange) are taken from the PMF calculations in Fig 1D and
621 4. Diffusion through the solvent (ΔG_3) is assumed to be 0. **B.** Comparison of the free energy of
622 binding to MOPr directly via the aqueous solvent, or indirectly via the membrane, where
623 $\Delta G_{\text{indirect}} = \Delta G_1 + \Delta G_2 + \Delta G_3$. **C.** 2D representation of the indirect, lipid binding route, using the
624 same values as Fig 5A. Fentanyl (green) has a greater propensity to move into the lipid from
625 the solvent, than morphine (orange).

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629 **Figure. 6. Model for the unique pharmacology of fentanyl at the MOPr**

630 In competition with a morphinan ligand (such as morphine or naloxone), fentanyl (green) can
631 access the orthosteric pocket via two binding routes; the canonical aqueous pathway and by
632 the novel lipid pathway. In contrast, the morphinan ligand (orange) only has access to one
633 binding route.

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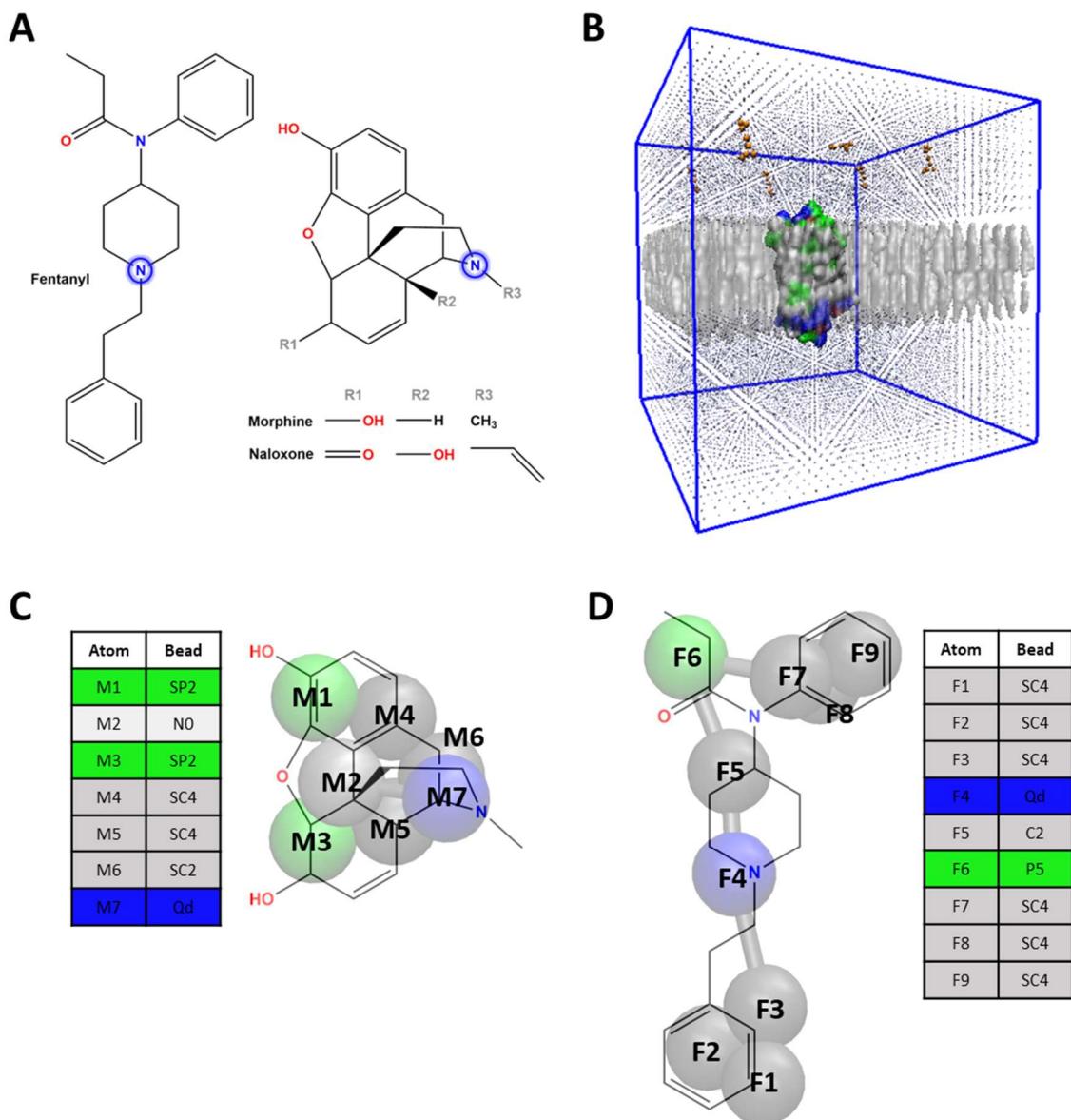
635 **Supplementary Information**

Ligand (6 copies)	n	Simulation time (μs)	Ligand at the TM6/7 interface?	Orthosteric site binding?
Fentanyl (protonated)	6	5; 5; 5; 5; 5; 5	Yes; Yes; Yes; No; Yes; Yes	No; No; Yes; No; No; No
Fentanyl (neutral)	6	5; 5; 5; 5; 5; 5	Yes; Yes; No; Yes; No; Yes	No; No; No; No; No; No
Morphine (protonated)	3	1; 5; 1	No; No; No	No; Yes; No
Morphine (neutral)	3	1; 1; 1	No; No; No	No; No; No

636

637 **Supplementary Table 1. Long-timescale, independent CG simulations for each opioid ligand**

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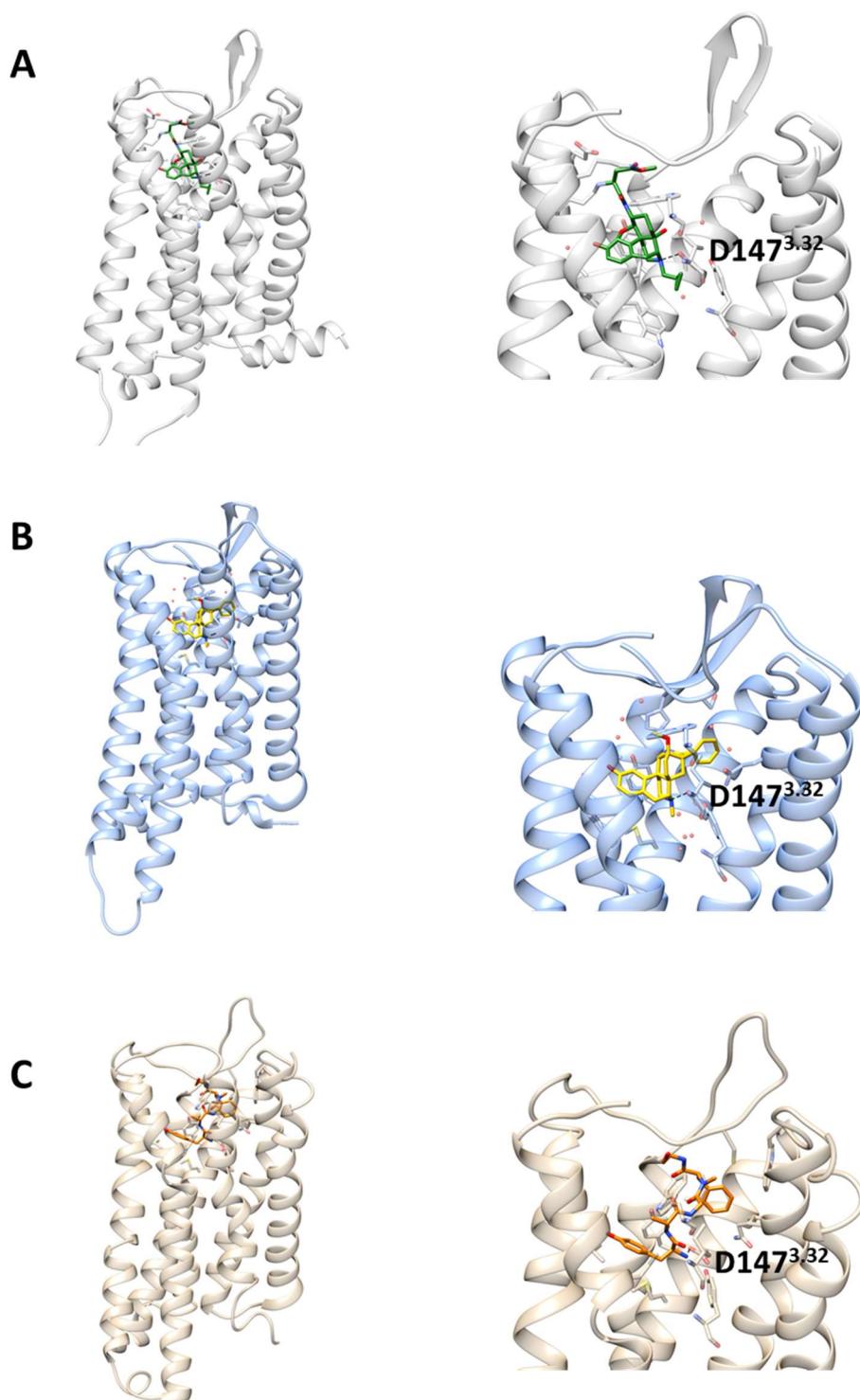


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641 **Supplementary Figure 1. Ligand parameterization and system set up**

642 **A.** Elongated structure of fentanyl compared to the rigid ring structures of morphine and
643 naloxone. The protonatable amine in both molecules is highlighted. **B.** The systems were set
644 up with CG MOPr embedded in a POPE, POPC, cholesterol membrane (grey), solvated in water
645 and ion beads (blue) and 6 molecules of either fentanyl or morphine (orange) randomly
646 placed in the solvent. **C.** Morphine was parameterized for the Martini forcefield using 7
647 Martini beads. **D.** Fentanyl was parameterized for the Martini forcefield using 9 Martini beads.
648 For simulations of neutral (unprotonated) ligands the Qd beads were replaced with Nd beads.
649 For explanation of MARTINI bead types, see *Marrink et al 2007*.

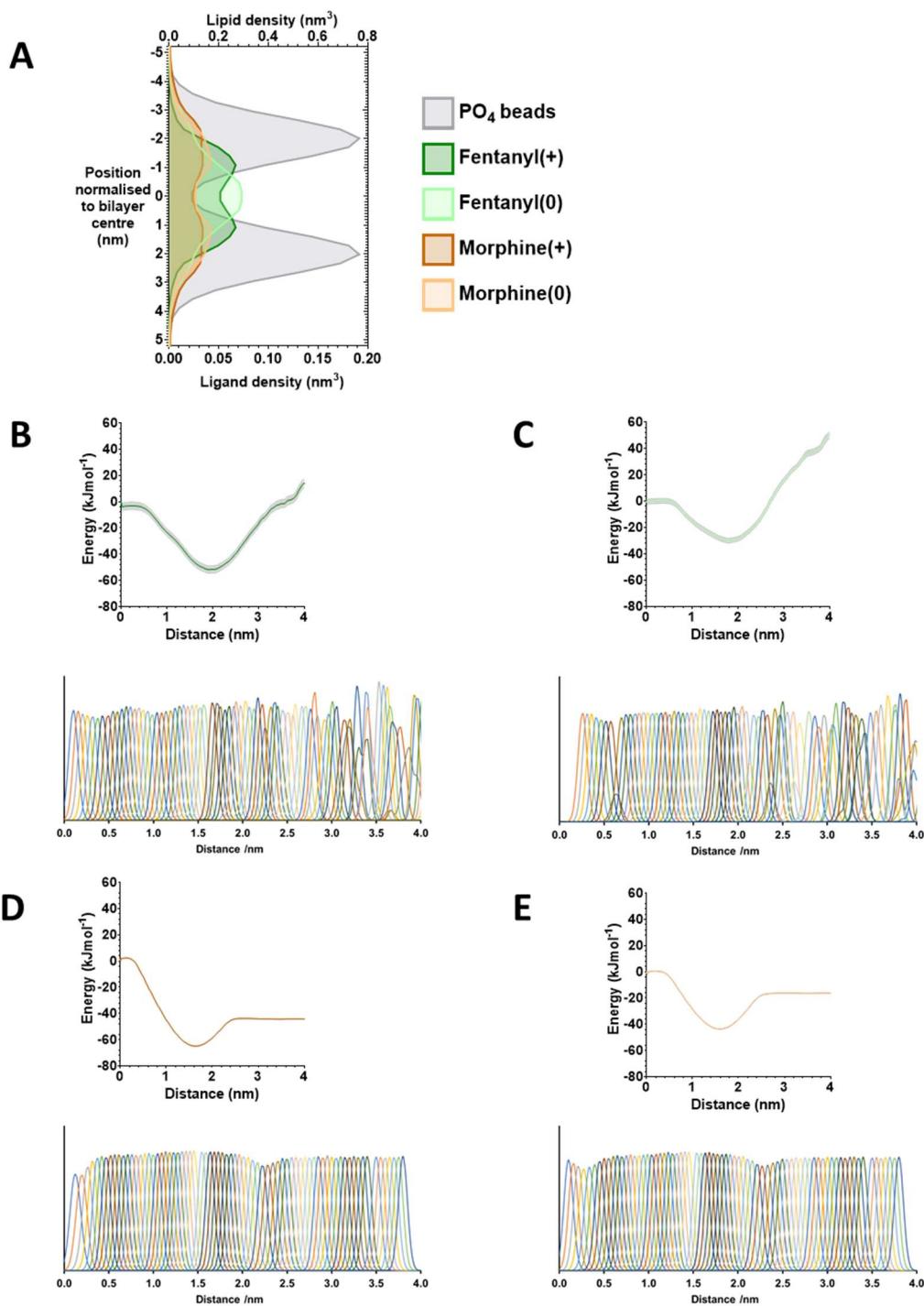
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Supplementary Figure 2. Structures of the MOPr

A. X-ray crystal structure of MOPr (grey) bound to the antagonist β -FNA (green). PDB 4DKL (*Manglik et al. 2012*). **B.** X-ray crystal structure of MOPr (blue) bound to the agonist BU72 (yellow). PDB 5C1M (*Huang et al. 2015*). **C.** Cryo-EM structure of MOPr (tan) bound to the peptide agonist DAMGO (orange). PDB 6DDF (*Koehl et al 2018*). In each case, residues forming the binding pocket are displayed as sticks and the key amino-D147^{3.32} interaction is indicated with a dashed line.



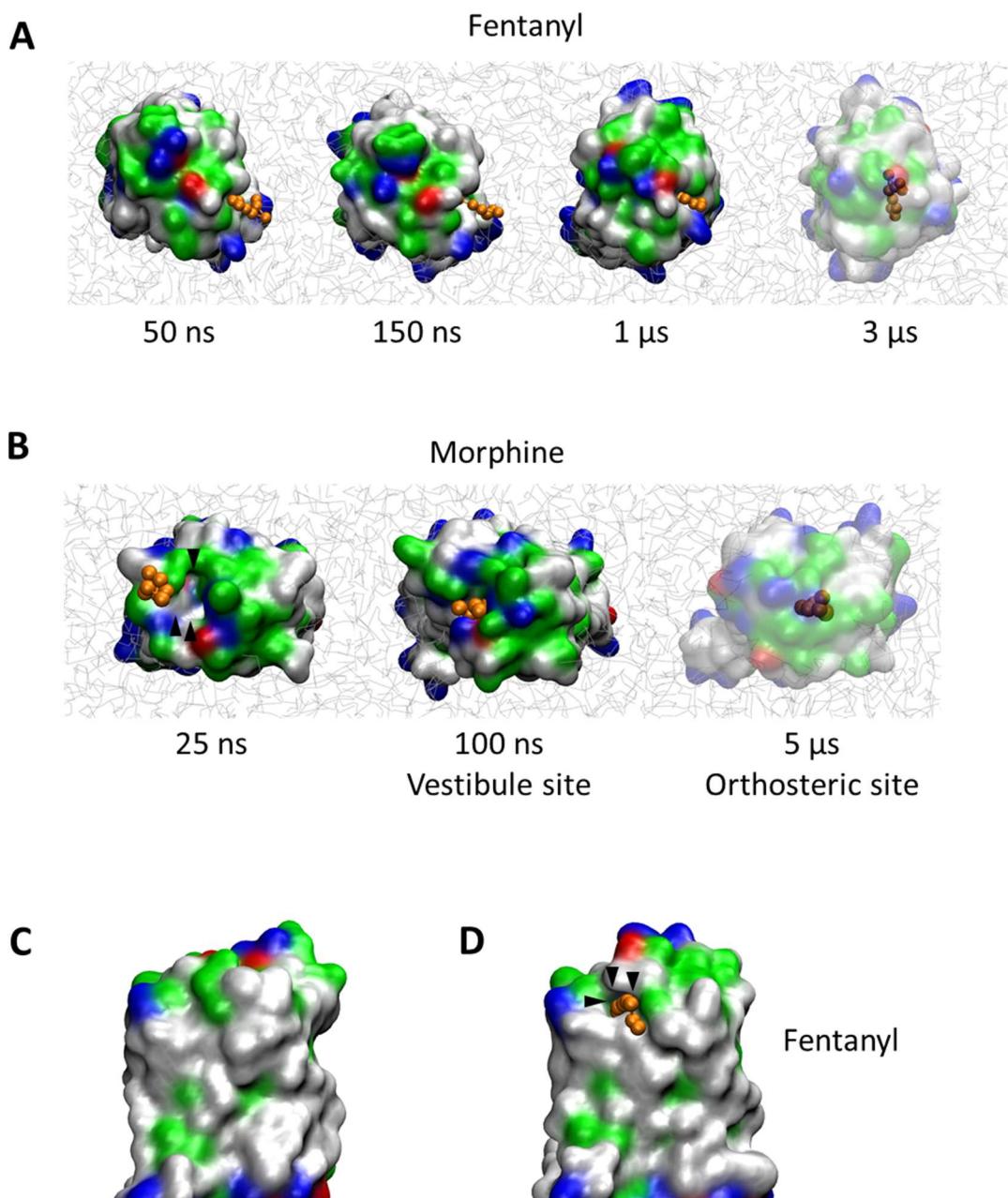
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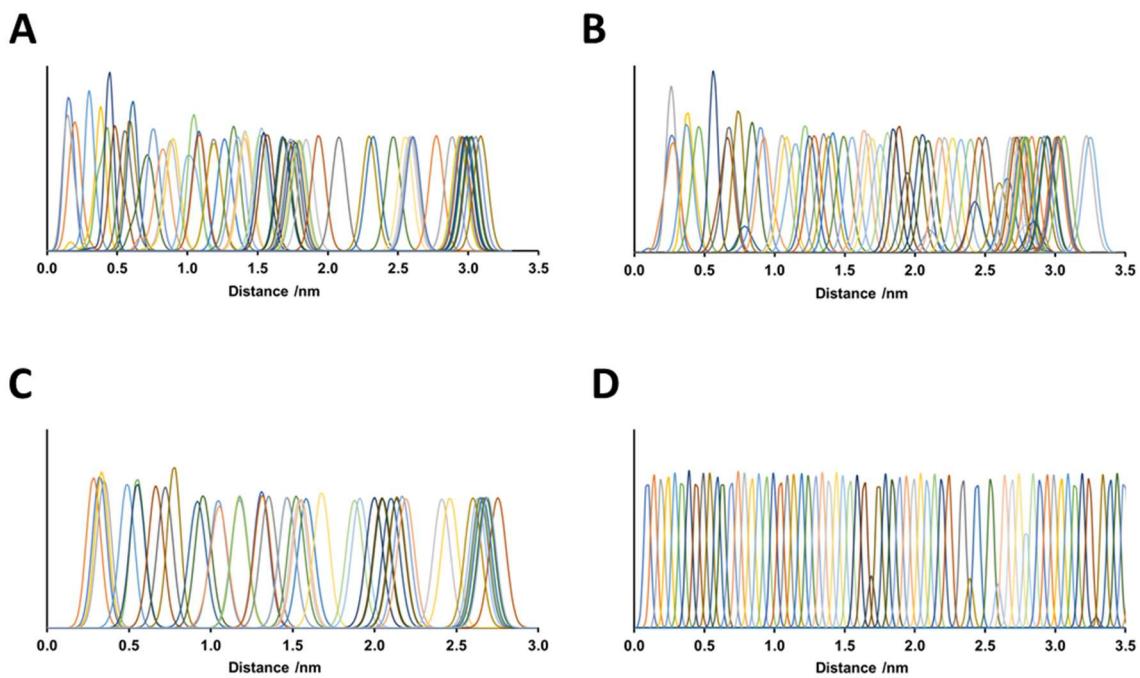
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661 **Supplementary Figure 3. Free energy calculations for ligand solvent/membrane partitioning**

662 **A.** Density plots showing the average position of protonated fentanyl (dark green), neutral fentanyl
 663 (light green), protonated morphine (dark orange) and neutral morphine (light orange), in relation to
 664 the phosphate beads of the lipid membrane (grey). **B-D.** Full PMF profiles along the reaction
 665 coordinate for the umbrella sampling simulations of ligands partitioning between the centre of the
 666 lipid bilayer (0 nm) and the bulk aqueous solvent (4 nm): **B.** protonated fentanyl, **C.** neutral fentanyl,
 667 **D.** protonated morphine and **E.** neutral morphine. The histograms alongside each PMF profile indicate
 668 the umbrella sampling windows fully captured the entire reaction coordinate.

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Supplementary Figure 5. Free energy calculations for ligand binding pathways

A. Histograms along the reaction coordinate for the umbrella sampling simulations of morphine binding via the aqueous pathway. **B.** Histograms along the reaction coordinate for the umbrella sampling simulations of fentanyl binding via the aqueous pathway. **C.** Histograms along the reaction coordinate for the umbrella sampling simulations of morphine binding via the lipid pathway. **D.** Histograms along the reaction coordinate for the umbrella sampling simulations of fentanyl binding via the lipid pathway.

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692 **Supplementary Movie 1. Fentanyl binding via the lipid bilayer and transmembrane domains**
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695 **Supplementary Movie 2. Morphine binding via the aqueous solvent and extracellular vestibule**
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