Supporting Information

The ketamine metabolite (2R,6R)-hydroxynorketamine interacts with mu and kappa opioid receptors

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Derivation of two-state ligand binding model

We have a ligand \( L \) that can exist in two protonation states we call 0 and 1, each of which can bind to a single receptor site. Only one ligand molecule can occupy the binding site. Each binds to the receptor with different affinities \( K_0 \) and \( K_1 \), which we have calculated using FEP MD. We wish to determine a combined dissociation constant \( K_D^* \) that can be compared to the experimentally determined dissociation constant \( K_D \) which includes both states. But we do not know in what fractions \( f_0 \) and \( f_1 \) ligand states 0 and 1 exist. Since there are only two states, we know that \( f_0 + f_1 = 1 \) by definition.

We begin by defining the probability of a ligand being bound as

\[
P([L], K_D) = \frac{[L]}{K_D + [L]}
\]  

(S1)

where \([L]\) is the concentration of the ligand and \(K_D\) is the dissociation constant; this is Equation 1 in the main text. Note that Equation S5 is the same as Equation 3 in the main text. Now we may determine the ligand concentration \([L]\) which includes both protonation states.

\[
P_{\text{unoccupied}} = (1 - P(f_0[L], K_0))(1 - P(f_1[L], K_1))
\]

\[
= \frac{K_0 K_1}{(K_0 + f_0[L])(K_1 + f_1[L])}
\]  

(S2)

The probability that the site will be occupied by either ligand is then:

\[
P_{\text{either}} = 1 - \frac{K_0 K_1}{(K_0 + f_0[L])(K_1 + f_1[L])} = P(f_0[L], K_0) + P(f_1[L], K_1) - P(f_0[L], K_0)P(f_1[L], K_1).
\]  

(S3)

(Equation S4 is another way of writing the probability that one but not both ligands occupy the site, which is Equation 2 in the main text.) To write the expression for \( P_{\text{either}} \) in a more convenient manner, we define \( \lambda_0 = \frac{K_0}{f_0} \) and \( \lambda_1 = \frac{K_1}{f_1} \) then substitute in Equation S3 and simplify:

\[
P_{\text{either}} = 1 - \frac{\lambda_0 f_0 \lambda_1 f_1}{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])}
\]

\[
= \frac{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])}{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])} - \frac{\lambda_0 f_0 \lambda_1 f_1}{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])}
\]

\[
= \frac{\lambda_0 f_0 \lambda_1 f_1}{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])}
\]

\[
= \frac{\lambda_0 \lambda_1 f_0 f_1 + \lambda_0 f_0 f_1 + f_0 f_1[L]}{\lambda_0 \lambda_1 f_0 f_1 + \lambda_0 f_0 f_1[L] + \lambda_1 f_0 f_1[L] + f_0 f_1[L]^2}
\]

\[
= \frac{\lambda_0 \lambda_1 f_0 f_1 + \lambda_0 f_0 f_1[L] + f_0 f_1[L]^2}{\lambda_0 \lambda_1 f_0 f_1 + \lambda_0 f_0 f_1[L] + \lambda_1 f_0 f_1[L] + f_0 f_1[L]^2}
\]

\[
= \frac{f_0 f_1[L] + f_0 f_1[L] + f_0 f_1[L]^2}{\lambda_0 \lambda_1 + \lambda_0 f_0 f_1[L] + f_0 f_1[L]^2}
\]

\[
= \frac{f_0 f_1[L] + f_0 f_1[L] + f_0 f_1[L]^2}{\lambda_0 \lambda_1 + \lambda_0 f_0 f_1[L] + f_0 f_1[L]^2}
\]

\[
= \frac{f_0 f_1[L] + f_0 f_1[L] + f_0 f_1[L]^2}{\lambda_0 \lambda_1 + \lambda_0 f_0 f_1[L] + f_0 f_1[L]^2}
\]

\[
= \frac{f_0 f_1[L] + f_0 f_1[L] + f_0 f_1[L]^2}{\lambda_0 \lambda_1 + \lambda_0 f_0 f_1[L] + f_0 f_1[L]^2}
\]

(S5)

Note that Equation S5 is the same as Equation 3 in the main text. Now we may determine the ligand concentration \([L]_{0.5}\) at which the binding site is half occupied; that is, \( P_{\text{either}} = \frac{1}{2} \), so that \([L]_{0.5} = K_D^* \). Using an automated solver, we find two solutions:
\[ K_D' = [L]_{0.5} = \begin{cases} \frac{1}{2} \left( -\lambda_0 - \lambda_1 - \sqrt{\lambda_0^2 + 6\lambda_0\lambda_1 + \lambda_1^2} \right) \\ \frac{1}{2} \left( -\lambda_0 - \lambda_1 + \sqrt{\lambda_0^2 + 6\lambda_0\lambda_1 + \lambda_1^2} \right) \end{cases} \] (S6)

Only the second solution of Equation S6 makes physical sense, so we take this as the expression for \( K_D' \). This is Equation 4 in the main text. We can achieve further simplification in the cases where \( \lambda_0 \gg \lambda_1 \) or \( \lambda_1 \gg \lambda_0 \), as described in the main text.
|                           | Ketamine        | Norketamine    | (2R,6R)-hydroxynorketamine |
|---------------------------|-----------------|----------------|----------------------------|
| **Density from MD simulation** | 1.15 g/mL       | 1.17 g/mL      | 1.23 g/mL                  |
| **ΔH_{vap} from MD simulation** | 13.34 kcal/mol  | 15.74 kcal/mol | 15.15 kcal/mol             |
| **ΔH_{vap} from Joback method** | 14.78 kcal/mol  | 15.25 kcal/mol | 19.16 kcal/mol             |
| **ΔG_{solvation} for neutral species** | 6.4 kcal/mol    | 6.3 kcal/mol   | 7.7 kcal/mol               |
| **ΔG_{solvation} for protonated species** | 43.5 kcal/mol   | 51.0 kcal/mol  | 55.0 kcal/mol              |

**Supporting Table 1.** Densities and enthalpies of vaporization/sublimation calculated from MD simulation and the Joback method, and free energies of solvation. We are unaware of existing experimental values for enthalpies of vaporization. Free energies of solvation for neutral and protonated species were calculated with FEP MD.
### a)  
| (S)-Ketamine** | (R)-Ketamine** | (R)-Norketamine** | (S)-Norketamine** | (2R,6R)-HNK** |
|----------------|----------------|-------------------|-------------------|---------------|
| **B-W** | **M** | **K** | **M** | **K** | **M** | **K** | **M** | **K** | **M** | **K** | **M** | **K** | **M** | **K** |
| 2.46 | L110 | | | | | | | | | | | | | |
| 2.49 | A113 | A104 | | | | | | | | | | | | |
| 2.50 | D114 | D105 | | | | | | | | | | | | |
| 2.53 | A117 | V108 | | | | | | | | | | | | |
| 2.54 | T118 | T109 | | | | | | | | | | | | |
| 2.56 | T120 | T111 | | | | | | | | | | | | |
| 2.57 | L121 | M112 | | | | | | | | | | | | |
| 2.59 | F123 | F114 | | | | | | | | | | | | |
| 2.60 | Q124 | Q115 | | | | | | | | | | | | |
| 2.61 | S125 | S116 | | | | | | | | | | | | |
| 2.63 | N127 | V118 | | | | | | | | | | | | |
| 2.64 | Y128 | Y119 | | | | | | | | | | | | |
| 3.25 | C140 | C131 | | | | | | | | | | | | |
| 3.28 | V143 | V134 | | | | | | | | | | | | |
| 3.29 | T144 | T135 | | | | | | | | | | | | |
| 3.31 | F146 | T137 | | | | | | | | | | | | |
| 3.32 | D147 | D138 | | | | | | | | | | | | |
| 3.33 | Y148 | Y139 | | | | | | | | | | | | |
| 3.35 | N150 | N141 | | | | | | | | | | | | |
| 3.36 | M151 | M142 | | | | | | | | | | | | |
| 3.37 | F152 | F143 | | | | | | | | | | | | |
| 3.39 | S154 | S145 | | | | | | | | | | | | |
| 3.40 | I155 | I146 | | | | | | | | | | | | |
| 5.42 | V236 | V230 | | | | | | | | | | | | |
| 5.43 | F237 | F231 | | | | | | | | | | | | |
| 5.46 | A240 | A234 | | | | | | | | | | | | |
| 6.44 | F289 | F283 | | | | | | | | | | | | |
| 6.47 | C292 | C286 | | | | | | | | | | | | |
| 6.48 | W293 | W287 | | | | | | | | | | | | |
| 6.51 | I296 | I290 | | | | | | | | | | | | |
| 6.52 | H297 | H291 | | | | | | | | | | | | |
| 6.55 | V300 | I294 | | | | | | | | | | | | |
| 7.36 | H319 | Y313 | | | | | | | | | | | | |
| 7.38 | C321 | C315 | | | | | | | | | | | | |
| 7.39 | I322 | I316 | | | | | | | | | | | | |
| 7.40 | A323 | A317 | | | | | | | | | | | | |
| 7.41 | L324 | L318 | | | | | | | | | | | | |
| 7.42 | G325 | G319 | | | | | | | | | | | | |
| 7.43 | Y326 | Y320 | | | | | | | | | | | | |
| 7.45 | N328 | N322 | | | | | | | | | | | | |
| 7.46 | S329 | S323 | | | | | | | | | | | | |

### b)  
| Ligand                  | Receptor   | Loop region residues |
|-------------------------|------------|----------------------|
| S-ketamine              | MOR H297+  | S55                  |
| R-norketamine           | MOR H297+  | S53, H54, S55, L56, C57 |
| S-norketamine           | MOR        | S55                  |
| S-norketamine           | MOR        | H54, S55, C217, T218 |
| S-norketamine           | KOR H291+  | E209, C210, S211     |
| (2R,6R)-hydroxynorketamine | MOR H297+  | H54, S55              |
Supporting Table 2. a) Residues within 6.5 Å of protonated ketamine and metabolites in greater than 50% of trajectory frames, in equilibrium MD simulation. “M”: mu opioid receptor; “K”: kappa opioid receptor. Versions of receptors with orthosteric histidine 6.52 protonated (H297 and H291 in MOR and KOR respectively) are denoted with “H+”. Only protonated versions of ligands shown, since these contribute the majority of binding affinity. Ballesteros-Weinstein (B-W) numbers are reported to facilitate comparison among GPCRs. b) Residues in loop regions within 6.5 Å of ligands in greater than 50% of equilibrium MD trajectory frames, denoted by asterisks in Table 1a.
| Protein | Ligand | Calc. $K_D$ (highest affinity, M) | Experimental $K_D$ or proxy value | Exp. pH | Required effective ligand pK<sub>a</sub> |
|---------|--------|--------------------------------|---------------------------------|---------|----------------------------------|
| HSAF    | S-ketamine | $K_D = 5.6 \times 10^{-7}$ | $K_D = 42 \mu M$ | 7.0 | 8.87 |
| MOR     | S-ketamine | $K_1 = 9.6 \times 10^{-11}$, $K_2 = 2.6 \times 10^{-6}$ (H297+) | $K_1 = 11 \mu M$ | 7.4 | *, *(H297+) |
|         | R-ketamine | $K_1 = 2.1 \times 10^{-9}$, $K_2 = 1.6 \times 10^{-6}$ (H297+) | $K_1 = 28 \mu M$ | 7.4 | *, 4.21 (H297+) |
| HNK     |         | $K_1 = 5.5 \times 10^{-9}$, $K_2 = 5.5$ nM, $K_3 = 1.0 \times 10^{-6}$ (H297+) | $IC_{50} = 0.56$ nM | 7.5 | *, *(H297+) |
| KOR     | S-ketamine | $K_1 = 7.2 \times 10^{-11}$, $K_2 = 9.1 \times 10^{-7}$ (H291+) | $K_1 = 24 \mu M$ | 7.4 | 1.97, 6.03 (H291+) |
|         | R-ketamine | $K_1 = 3.2 \times 10^{-9}$, $K_2 = 8.8 \times 10^{-6}$ (H291+) | $K_1 = 100 \mu M$ | 7.4 | *, 4.43 (H291+) |
| HNK     |         | $K_1 = 5.7 \times 10^{-10}$, $K_2 = 0.57$ nM, $K_3 = 5.2 \times 10^{-12}$ (H291+) | $IC_{50} = 2.1 \times 10^{-14}$ M | 7.5 | *, *(H291+) |

**Supporting Table 3.** Ligand pK<sub>a</sub> required for combined FEP-calculated $K_D$ to equal experimentally-derived proxy $K_D$ value. The higher affinity FEP-calculated $K_D$ component (denoted $K_0$ for neutral ligand and $K_1$ for positively charged ligand) are also listed. Implausibly low or mathematically impossible required ligand pK<sub>a</sub> values denoted by asterisks.
Supporting Figure Legends

Supporting Figure 1. a) Fluorescence curves from S-ketamine titration in a system containing HSAF and 1-AMA. Downward arrow indicates reduction in fluorescence upon the stepwise addition of S-ketamine. Lowest-intensity red and blue line denote HSAF only in solution, and green line labeled 1-AMA indicates 1-AMA alone in solution. b) Fluorescence at 510 nm as a function of S-ketamine concentration. Error bars indicate standard deviation across 3 experiments. These data are consistent with competitive binding for the interfacial HSAF binding site. Hill slope is -0.8 +/- 0.25.

Supporting Figure 2. Docked conformations of neutral S-ketamine in a) MOR and b) KOR, from AutoDock Vina. Side chains in the orthosteric pocket were made flexible and exhaustiveness was set to 12. Docking scores ranged from -5.7 to -7.5 in MOR and -6.0 to -7.5 in KOR. This diagram is representative – other ligands had qualitatively similar results – and intended to show that there was no clear, credible result from docking other than identifying the orthosteric binding pocket.

Supporting Figure 3. a) Root-mean-square deviation (RMSD, Å) over time (ns) for each receptor-bound ligand heavy atoms, during each equilibration MD simulation prior to FEP MD simulations. These plots include only the RMSD of the ligand. b) RMSD over time of protein binding pocket residues for each ligand during equilibration MD prior to FEP MD simulations. The selected residues are those that were within 6.5 Å of the ligand at the end of the simulation. Only the backbone is included in the calculation. c) Ligand rotation distributions as a function of decoupling parameter λ in each FEP MD simulation. X-rotation curves are in shades of red, y-rotation in shades of green, z-rotation in shades of blue. Colors become lighter as λ progresses. This shows stability of ligand binding conformations prior to evaluation of binding affinities.

Supporting Figure 4. Free energy perturbation molecular dynamics energy plots for decoupling ligand from receptor. Left side: Free energy change as a function of timestep. This includes the equilibration portion of each window, which was not included in the production simulation. Right side: Cumulative sums of energies as a function of λ. Each run is shown separately. “Backward” runs are from interleaved double-wide sampling.

Supporting Figure 5. G-protein activation assays for ketamine and norketamine with MOR and KOR as well as controls. In each graph, X-axis is log nM ligand concentration, and y-axis is relative activity.

Supporting Figure 6. Competition [35S]GTPγS assays for MOR and KOR. Each receptor was pretreated with methadone (MOR) or nalbuphine (KOR) and R-ketamine was added at varying concentrations. G-protein recruitment activity, quantified in counts per minute (CPM), as a function of R-ketamine concentration is shown.
**Supporting Figure 7.** β-arrestin recruitment assays for S-ketamine with MOR and KOR as well as controls: MOR agonist morphine and KOR agonist salvinorin A. X-axis is log M ligand concentration, and y-axis is relative activity.

Coordinates of systems and ligands used for FEP MD calculations are available at:

https://osf.io/r5j2p/

This data is hosted by the Center for Open Science.
Supp Figure 1 (p. S10)

(a) Fluorescence spectra for different compounds, showing peaks at various wavelengths. The compounds are labeled as 1-AMA and HSAF.

(b) Graph showing fluorescence intensity at 510 nm (y-axis) as a function of log concentration of ketamine (x-axis). The data points are accompanied by error bars indicating variability.
a) Ligand RMSD during equilibrium MD

Supp Figure 3 (p. S12)
b) Receptor binding pocket RMSD during equilibrium MD

Supp Figure 3 (p. S13)
Supp Figure 3 (p. S14)

c) Ligand rotational sampling during FEP MD
c) Ligand rotational sampling during FEP MD

Supp Figure 3 (p. S15)
c) Ligand rotational sampling during FEP MD

Supp Figure 3 (p. S16)
Supp Figure 4 (p. S18)
Supp Figure 4 (p. S19)
Supp Figure 4 (p. S21)
Supp Figure 4 (p. S23)
Supp Figure 4 (p. S25)

MOR S-ketamine (+1) LEU147

- ΔG (kcal/mol) vs. λ

Forward: 66.13 kcal/mol
Backward: -63.52 kcal/mol

MOR S-ketamine (+1)

- ΔG (kcal/mol) vs. λ

Forward: 67.16 kcal/mol
Backward: -64.55 kcal/mol

MOR S-norketamine

- ΔG (kcal/mol) vs. λ

Forward: 9.81 kcal/mol
Backward: -9.55 kcal/mol

MOR S-norketamine H297+

- ΔG (kcal/mol) vs. λ

Forward: 10.81 kcal/mol
Backward: -10.63 kcal/mol
Supp Figure 5 (p. S27)

MOR: S-ketamine

KOR: S-ketamine

MOR: R-ketamine

KOR: R-ketamine

MOR: norketamine

KOR: norketamine
R-ketamine vs methadone in MOR

Supp Figure 6 (p. S28)

R-ketamine vs nalbuphine in KOR
MOR: Beta-arrestin recruitment

-14 -12 -10 -8 -6 -4
0 1 2 3 4
Ketamine Morphine
Log [ligand, M]
Relative luminescence units (fold-change-mean ± SEM)

KOR: Beta-arrestin recruitment

-14 -12 -10 -8 -6 -4
0 5 10 15 20
Ketamine Salvinorin A
Log [ligand, M]
Relative luminescence units (fold-change-mean ± SEM)