Supporting Information

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

Thomas T. Joseph, MD, PhD[1]
Weiming Bu, PhD[1]
Wenzhen Lin, PhD[1,2]
Lioudmila Zoubak[3]
Alexei Yeliseev, PhD[3]
Renyu Liu, MD, PhD[1]
Roderic G. Eckenhoff, MD[1]
Grace Brannigan, PhD[4]

1 Department of Anesthesiology and Critical Care, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA USA

2 Department of Biochemistry and Molecular Biology, Guangxi Medical University, Nanning, Guangxi, China

3 National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD USA

4 Center for Computational and Integrative Biology and Department of Physics, Rutgers University, Camden, NJ USA

Corresponding author:

Thomas T. Joseph, MD, PhD
Room 319A, John Morgan Building
Department of Anesthesiology and Critical Care
Perelman School of Medicine, University of Pennsylvania
3620 Hamilton Walk
Philadelphia, PA 19104 USA
Tel: +1 917 512 1664 Fax: +1 215 349 5078
Email: thomas.joseph@pennmedicine.upenn.edu

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

$$P([L], K_D) = \frac{[L]}{K_D + [L]} \tag{S1}$$

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 if $[L] = K_D$ then $P([L], K_D) = \frac{1}{2}$. Next, let us determine the probability of no ligand occupying the site, given the total ligand concentration $[L]$ which includes both protonation states.

$$\begin{aligned} P_{\text{unoccupied}} &= (1 - P(f_0[L], K_0))(1 - P(f_1[L], K_1)) \\ &= \frac{K_0 K_1}{(K_0 + f_0[L])(K_1 + f_1[L])} \end{aligned} \tag{S2}$$

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

$$P_{\text{either}} = 1 - \frac{K_0 K_1}{(K_0 + f_0[L])(K_1 + f_1[L])} \tag{S3}$$

$$= P(f_0[L], K_0) + P(f_1[L], K_1) - P(f_0[L], K_0)P(f_1[L], K_1). \tag{S4}$$

(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_{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:

$$\begin{aligned} P_{\text{either}} &= 1 - \frac{\lambda_0 f_0 \lambda_1 f_1}{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])} \\ &= \frac{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])}{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])} - \frac{\lambda_0 f_0 \lambda_1 f_1}{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])} \\ &= \frac{\lambda_0 f_0 \lambda_1 f_1 + \lambda_0 f_0 f_1[L] + \lambda_1 f_1 f_0[L] + f_0 f_1[L]^2 - \lambda_0 f_0 \lambda_1 f_1}{(\lambda_0 f_0 + f_0[L])(\lambda_1 f_1 + f_1[L])} \\ &= \frac{\lambda_0 f_0 \lambda_1 f_1 + \lambda_0 f_0 f_1[L] + \lambda_1 f_1 f_0[L] + f_0 f_1[L]^2 - \lambda_0 f_0 \lambda_1 f_1}{\lambda_0 \lambda_1 f_0 f_1 + \lambda_0 f_0 f_1[L] + \lambda_1 f_0 f_1[L] + f_0 f_1[L]^2} \\ &= \frac{\lambda_0 f_0 f_1[L] + \lambda_1 f_0 f_1[L] + f_0 f_1[L]^2}{\lambda_0 \lambda_1 f_0 f_1 + \lambda_0 f_0 f_1[L] + \lambda_1 f_0 f_1[L] + f_0 f_1[L]^2} \\ &= \frac{f_0 f_1[L](\lambda_0 + \lambda_1 + [L])}{f_0 f_1(\lambda_0 \lambda_1 + \lambda_0[L] + \lambda_1[L] + [L]^2)} \\ &= \frac{(\lambda_0 + \lambda_1)[L] + [L]^2}{\lambda_0 \lambda_1 + (\lambda_0 + \lambda_1)[L] + [L]^2} \end{aligned} \tag{S5}$$

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:

$$K'_D = [L]_{0.5} = \begin{cases} \frac{1}{2}\left(-\lambda_0 - \lambda_1 - \sqrt{\lambda_0^2 + 6\lambda_0\lambda_1 + \lambda_1^2}\right) \\ \frac{1}{2}\left(-\lambda_0 - \lambda_1 + \sqrt{\lambda_0^2 + 6\lambda_0\lambda_1 + \lambda_1^2}\right) \end{cases} \tag{S6}$$

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
$\Delta G_{solvation}$ for neutral species	6.4 kcal/mol	6.3 kcal/mol	7.7 kcal/mol
$\Delta G_{solvation}$ 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^{1+}				(R)-Ketamine^{1+}				(R)-Norketamine^{1+}				(S)-Norketamine^{1+}				(2R,6R)-HNK^{1+}			
B-W	**M**	**K**	**M**	**M H+**	**K**	**K H+**	**M**	**M H+**	**K**	**K H+**	**M**	**M H+**	**K**	**K H+**	**M**	**M H+**	**K**	**K H+**	**M**	**M H+**	**K**	**K H+**
				*								*								*		
2.46	L110	L101																				×
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	I144	I135	×					×								×		×	×			
3.31	I146	I137								×												
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_a
HSAF	S-ketamine	$K_0 = 5.6\times10^{-7}$	K_D = 42 μM	7.0	8.87
MOR	S-ketamine	$K_1 = 9.6\times10^{-11}$ $K_1 = 2.6\times10^{-8}$ (H297+)	K_i = 11 μM	7.4	*, * (H297+)
	R-ketamine	$K_1 = 2.1\times10^{-9}$ $K_1 = 1.6\times10^{-8}$ (H297+)	K_i = 28 μM	7.4	*, 4.21 (H297+)
	HNK	$K_1 = 5.5\times10^{-9}$ = 5.5 nM $K_1 = 1.0\times10^{-6}$ (H297+)	IC_{50} = 0.56 nM	7.5	*, * (H297+)
KOR	S-ketamine	$K_1 = 7.2\times10^{-11}$ $K_1 = 9.1\times10^{-7}$ (H291+)	K_i = 24 μM	7.4	1.97, 6.03 (H291+)
	R-ketamine	$K_1 = 3.2\times10^{-8}$ $K_1 = 8.8\times10^{-8}$ (H291+)	K_i = 100 μM	7.4	*, 4.43 (H291+)
	HNK	$K_1 = 5.7\times10^{-10}$ = 0.57 nM $K_1 = 5.2\times10^{-12}$ (H291+)	$IC_{50} = 2.1\times10^{-14}$ M	7.5	*, * (H291+)

Supporting Table 3. Ligand pK_a 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_a 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 [^{35}S]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.

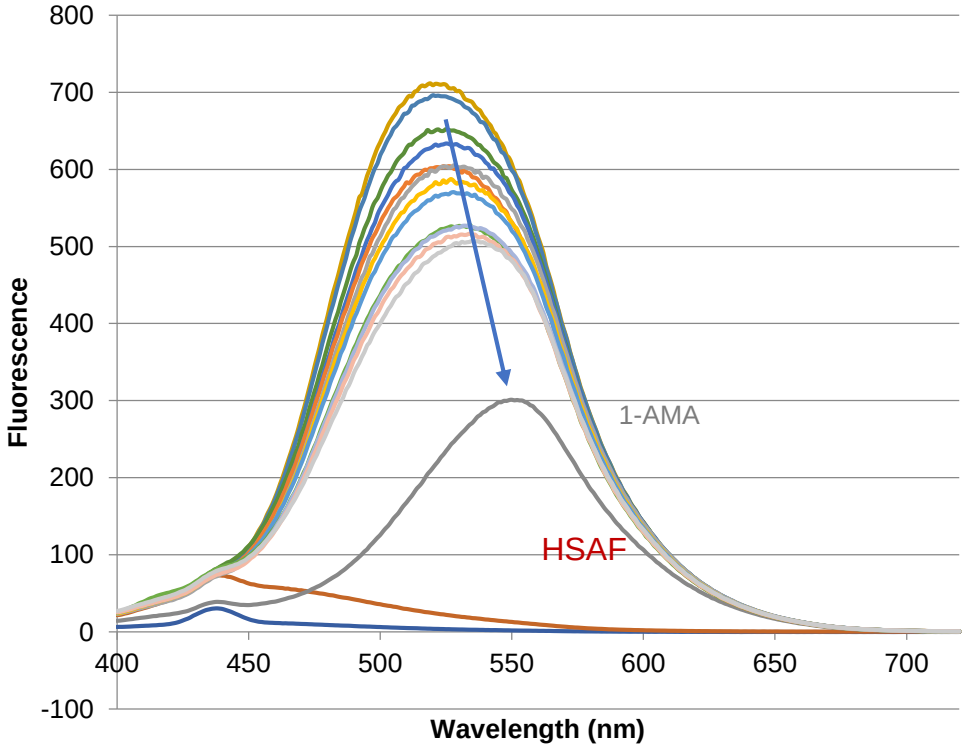
Coordinates of systems and ligands used for FEP MD calculations are available at:

https://osf.io/r5j2p/

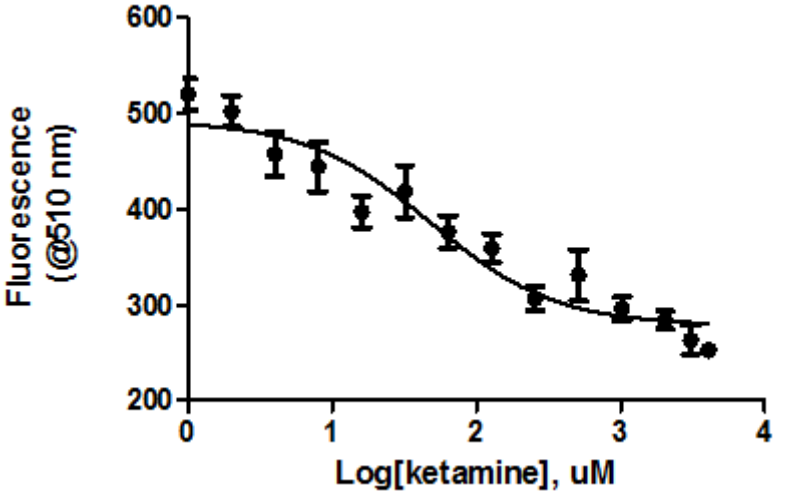
This data is hosted by the Center for Open Science.

Supp Figure 1 (p. S10)

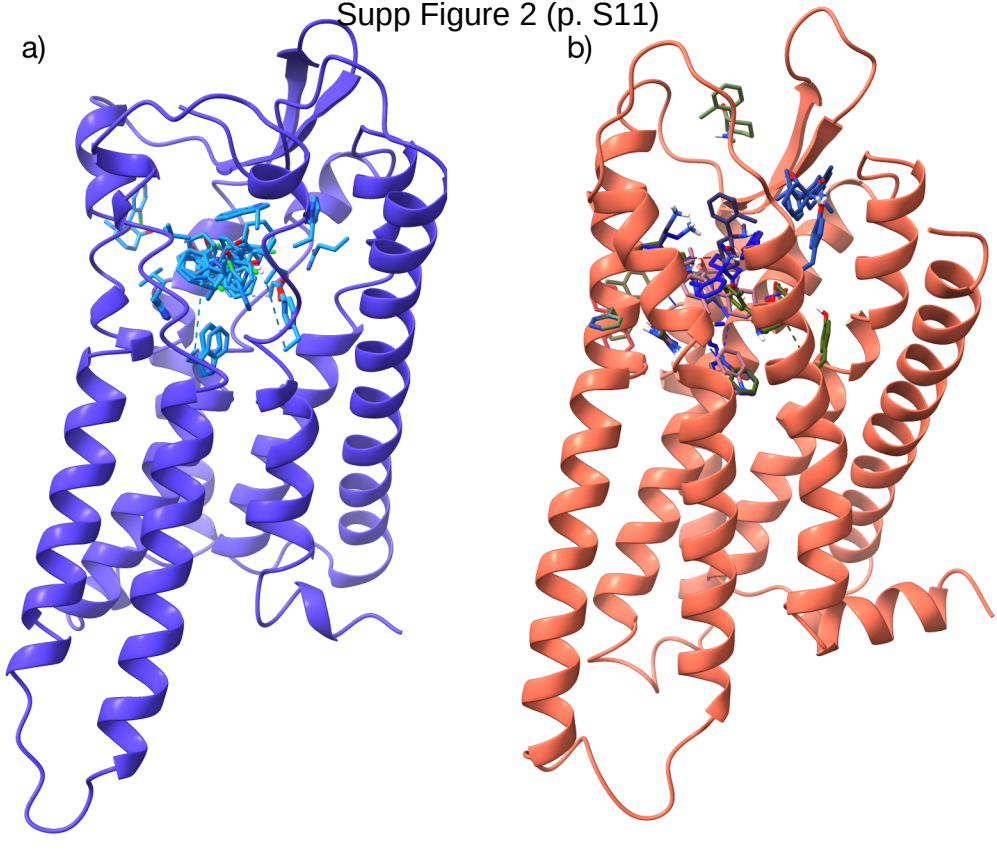
a)



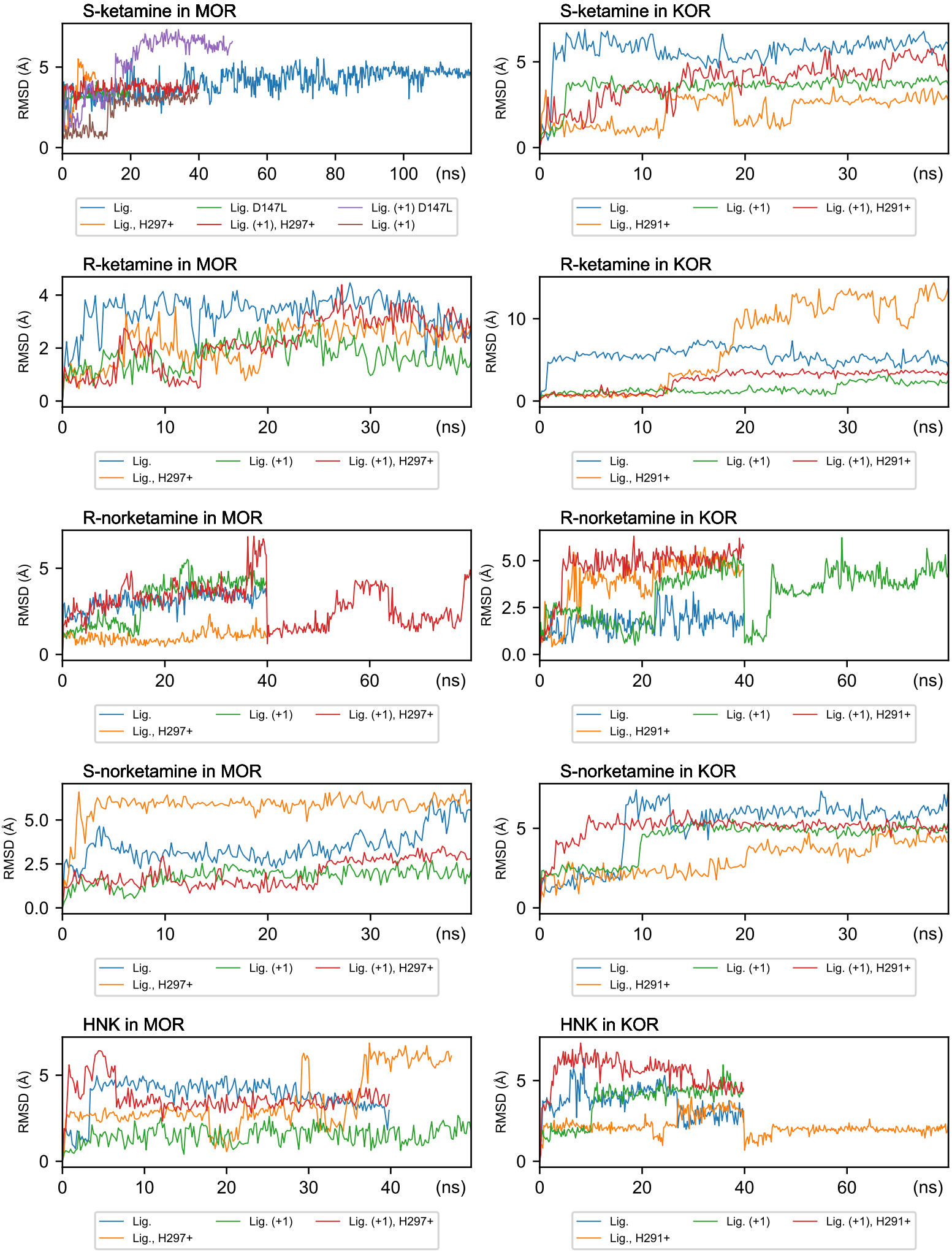
b)



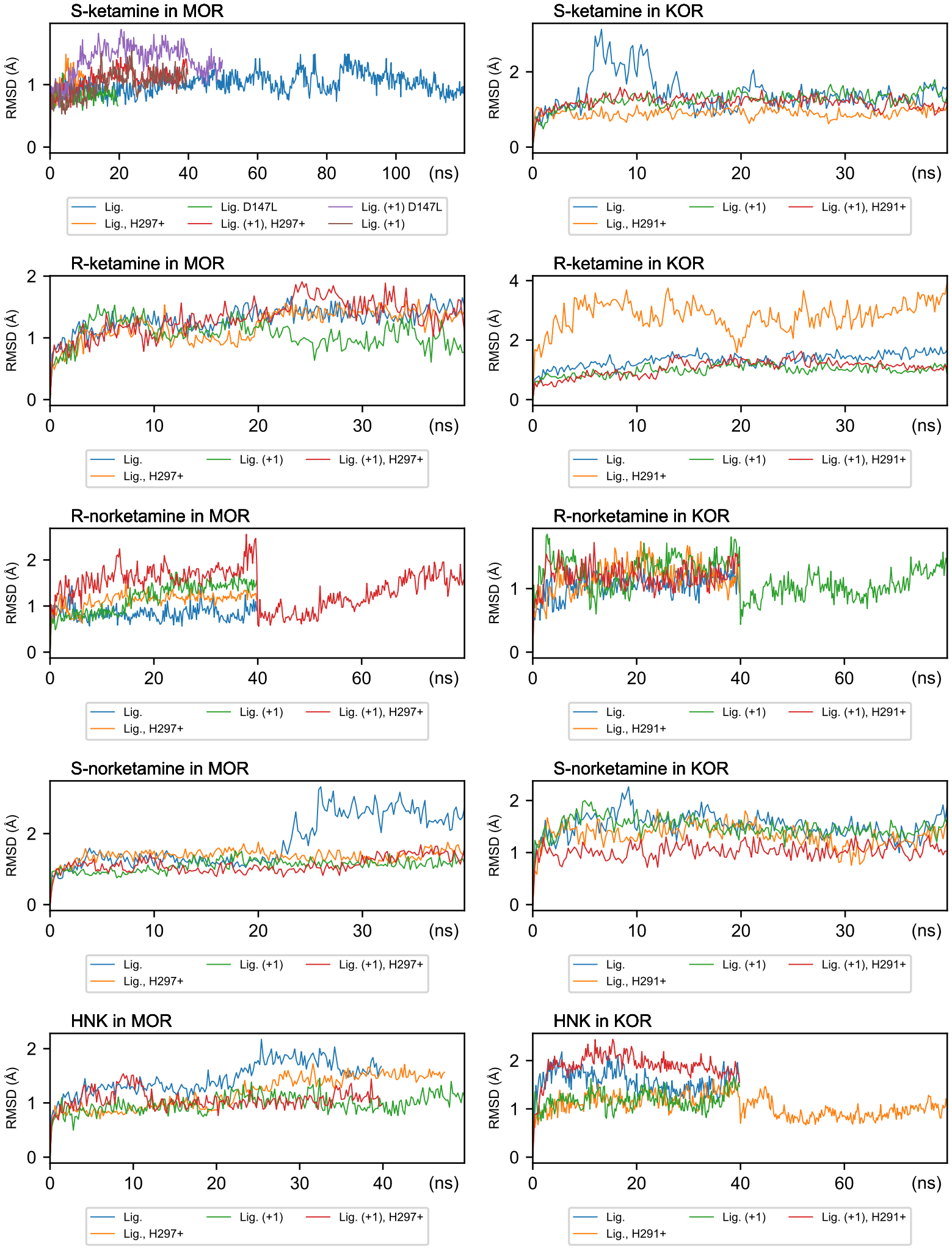
Supp Figure 2 (p. S11)



a) Ligand RMSD during equilibrium MD

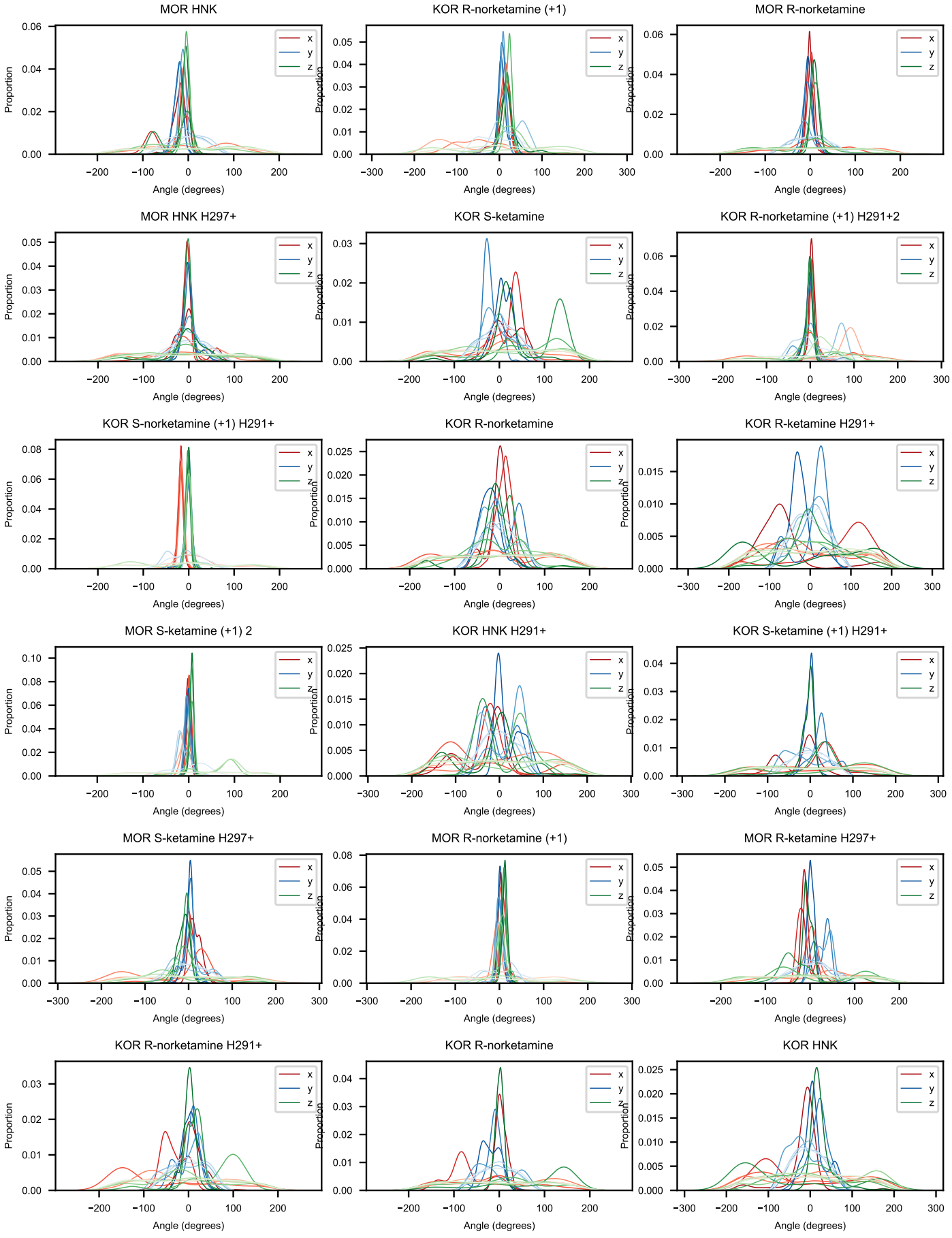


b) Receptor binding pocket RMSD during equilibrium MD



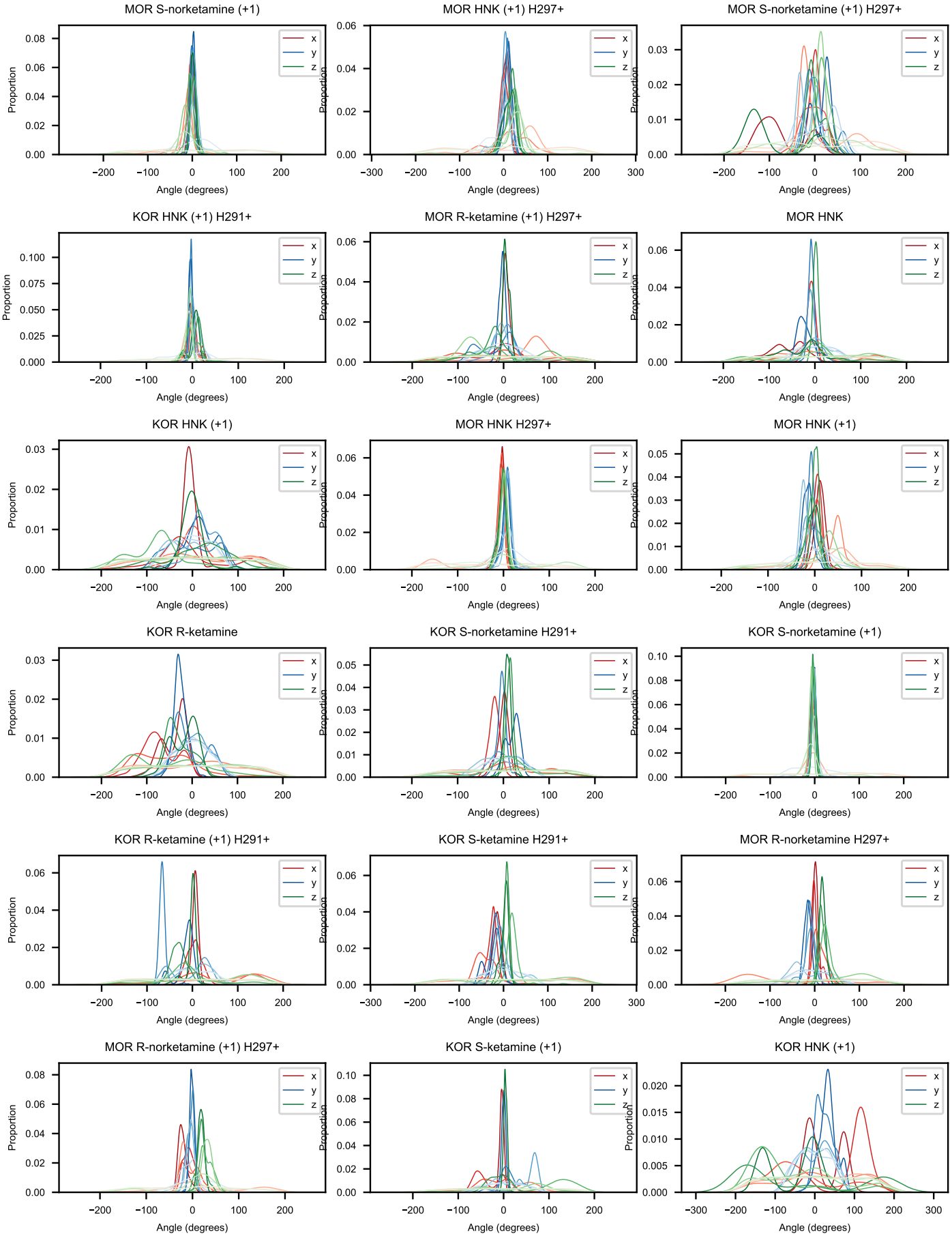
Supp Figure 3 (p. S14)

c) Ligand rotational sampling during FEP MD

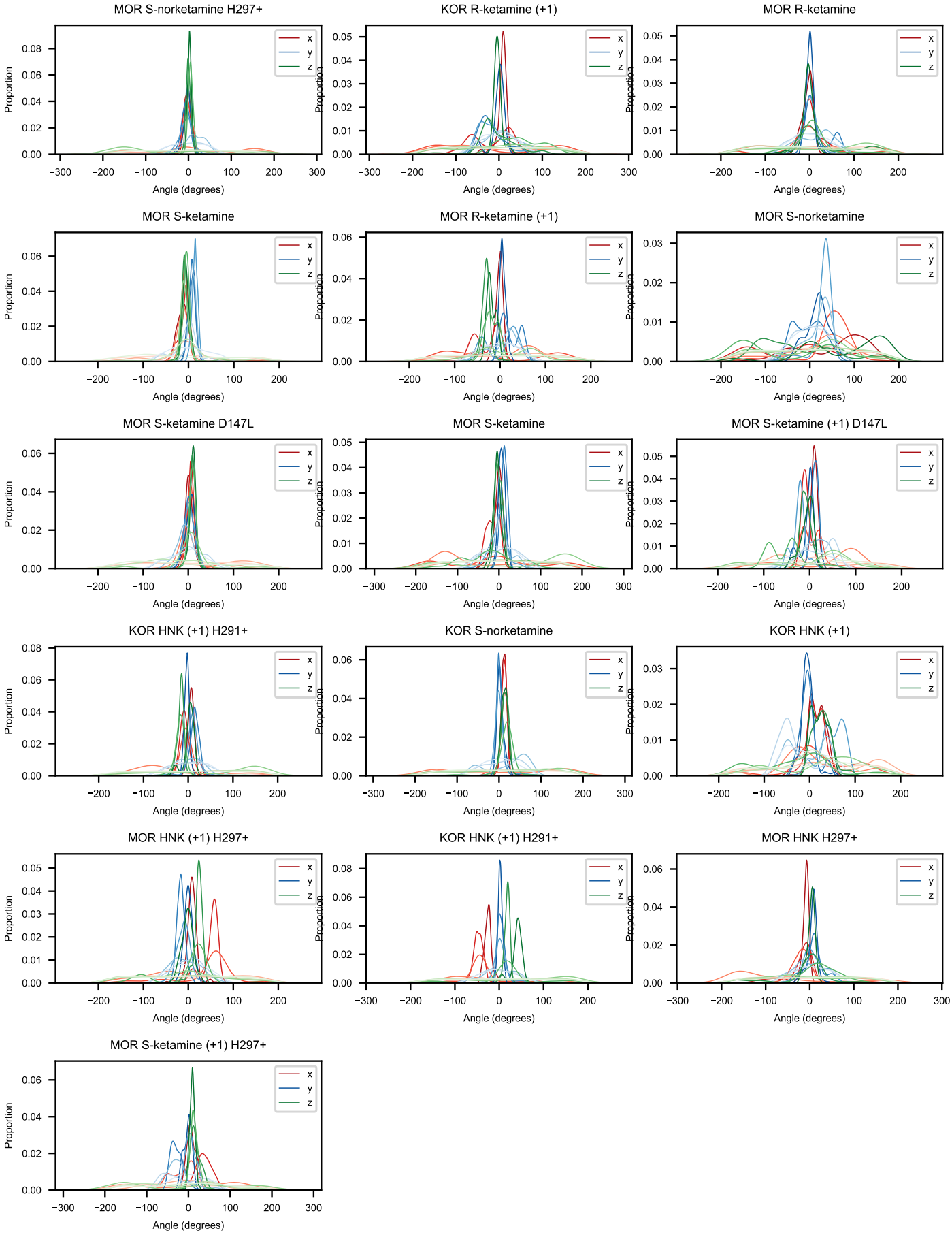


Supp Figure 3 (p. S15)

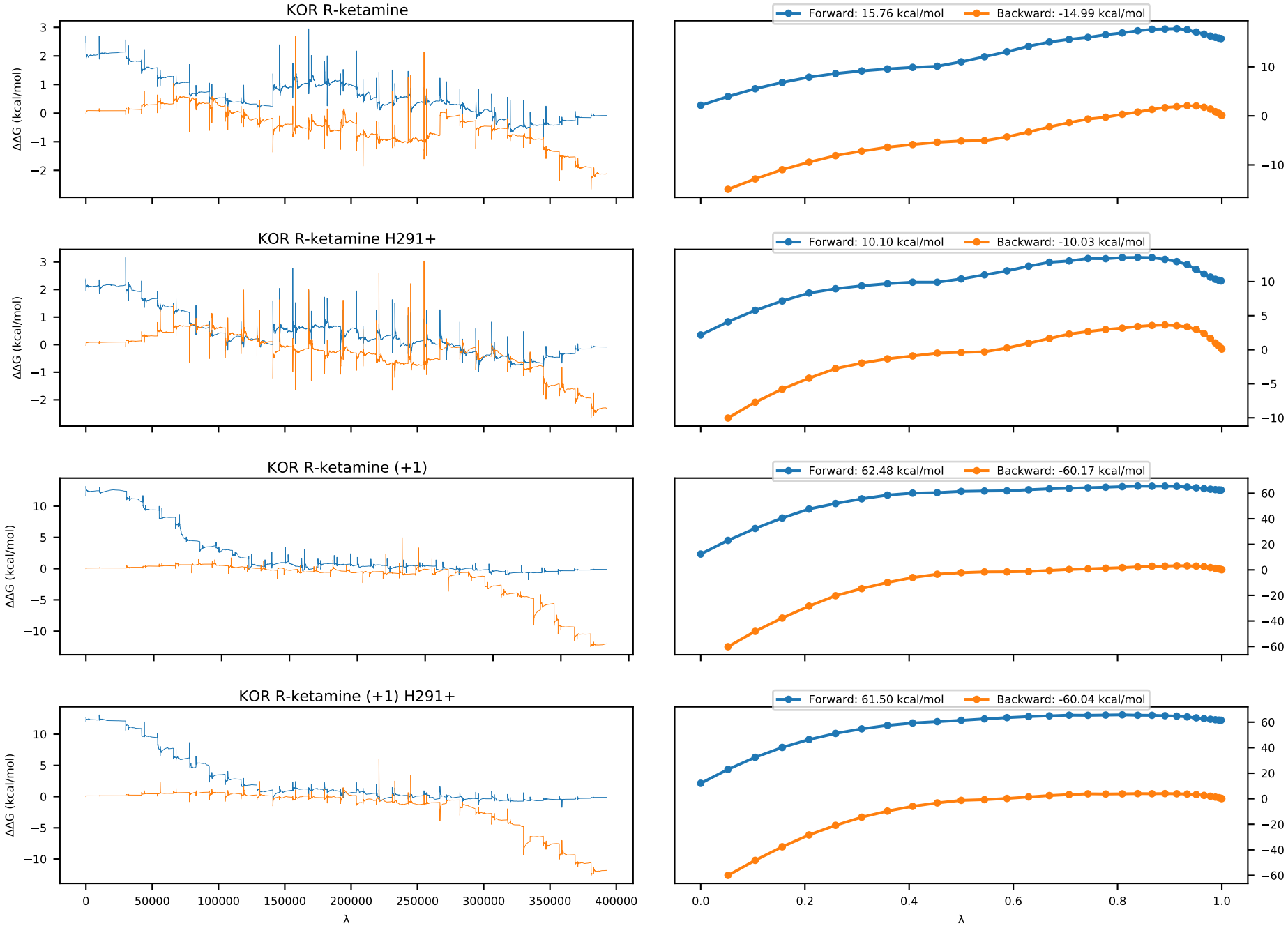
c) Ligand rotational sampling during FEP MD



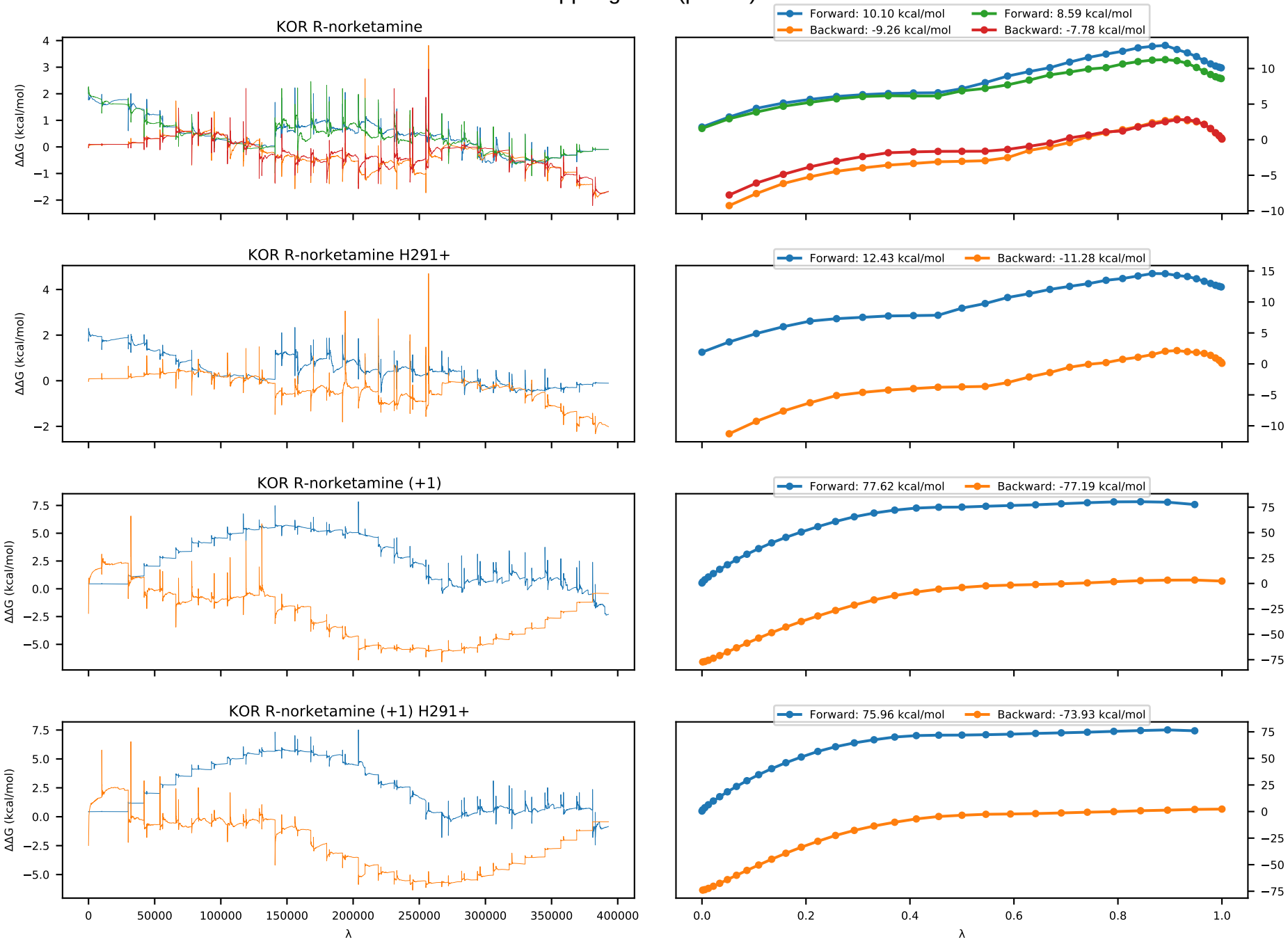
c) Ligand rotational sampling during FEP MD



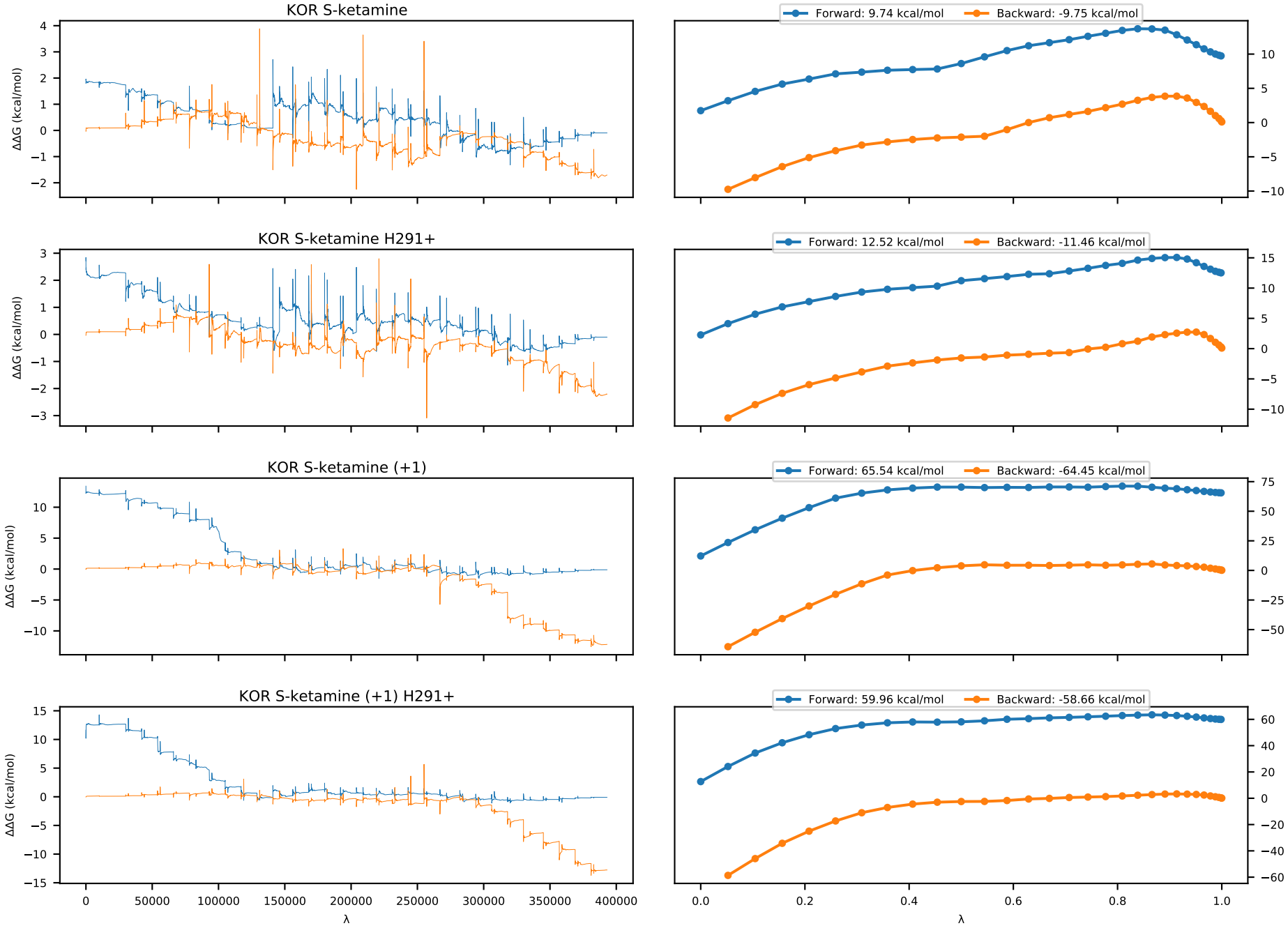
Supp Figure 4 (p. S17)



Supp Figure 4 (p. S18)



Supp Figure 4 (p. S19)



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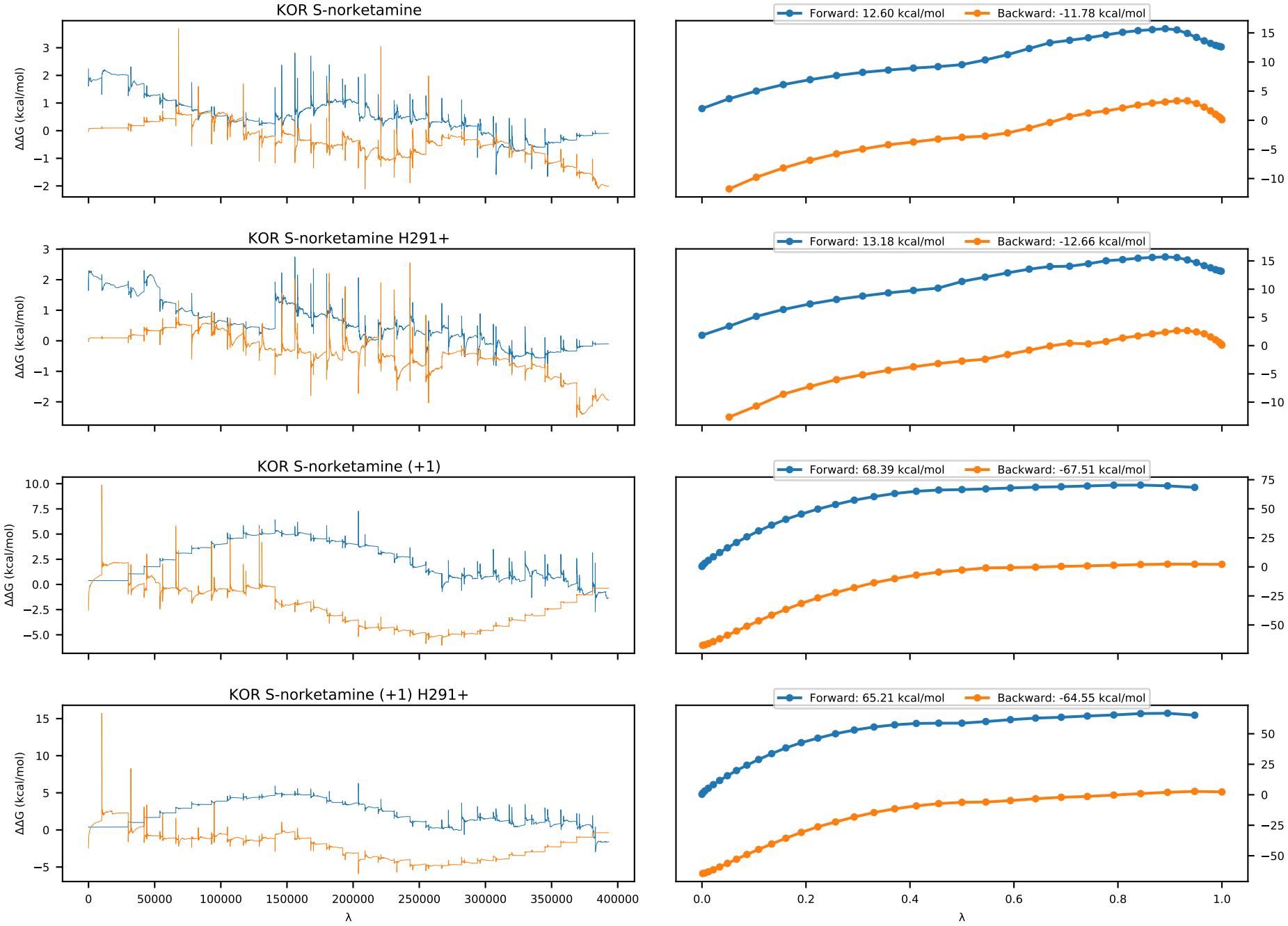

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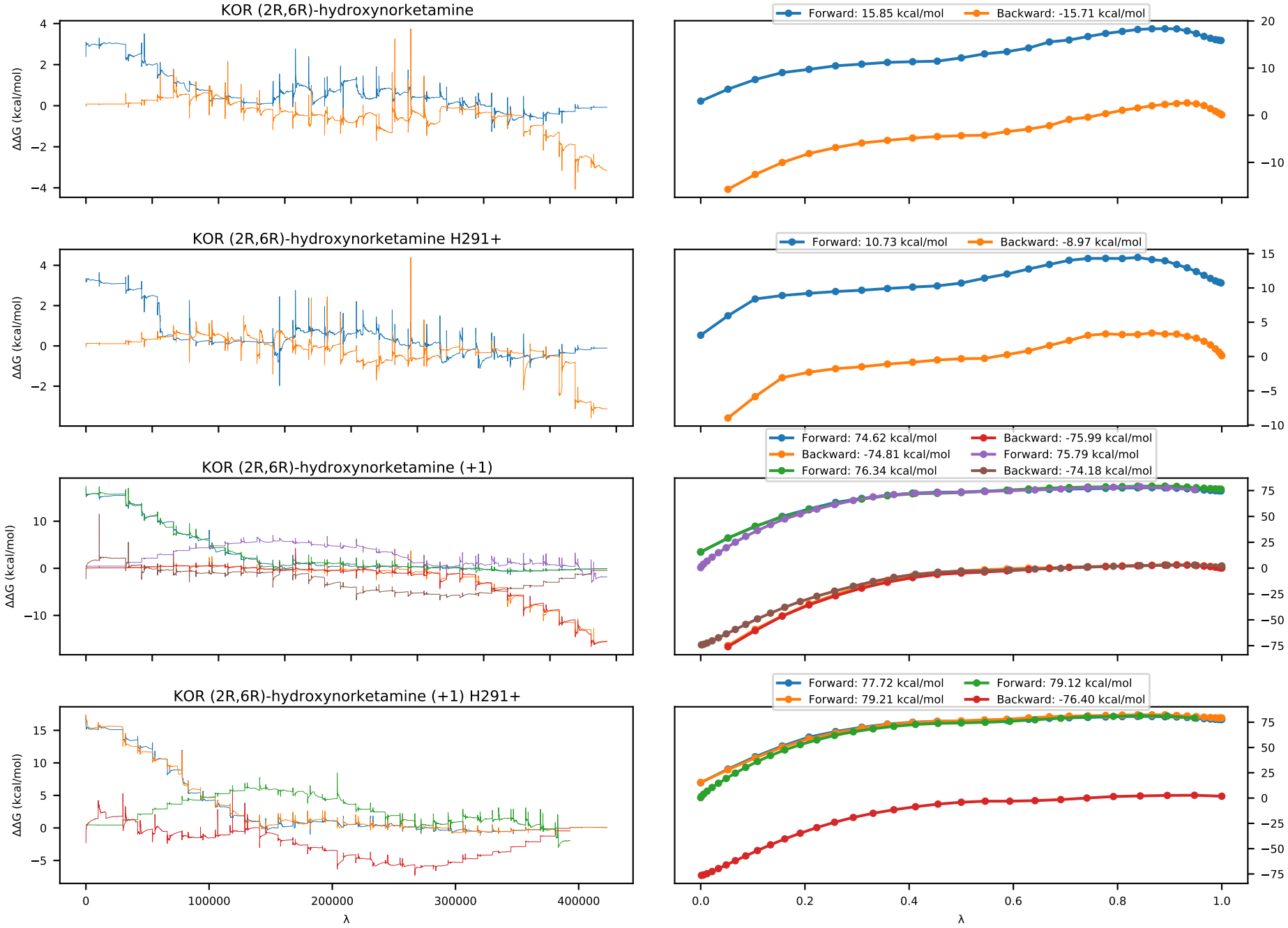

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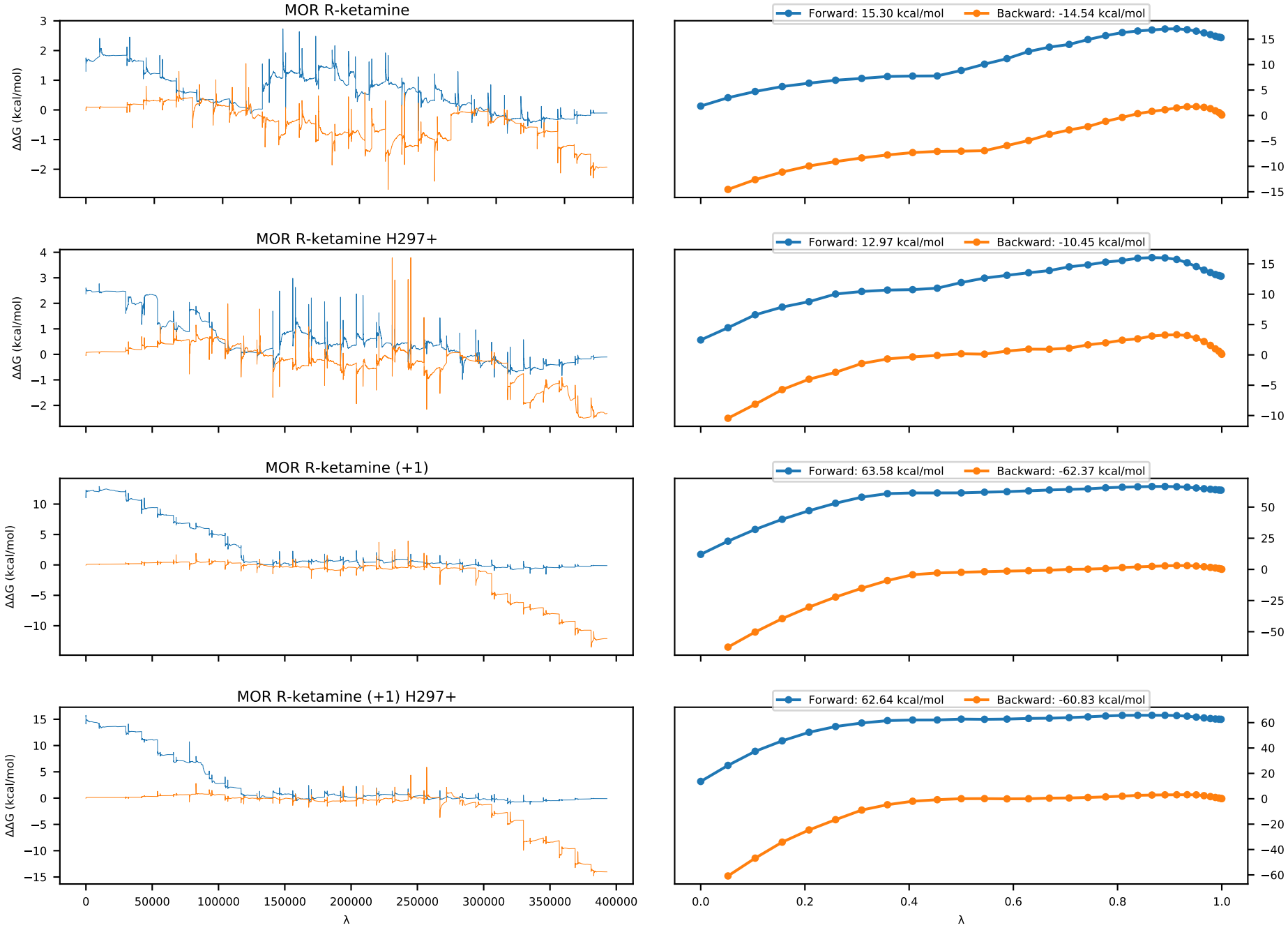

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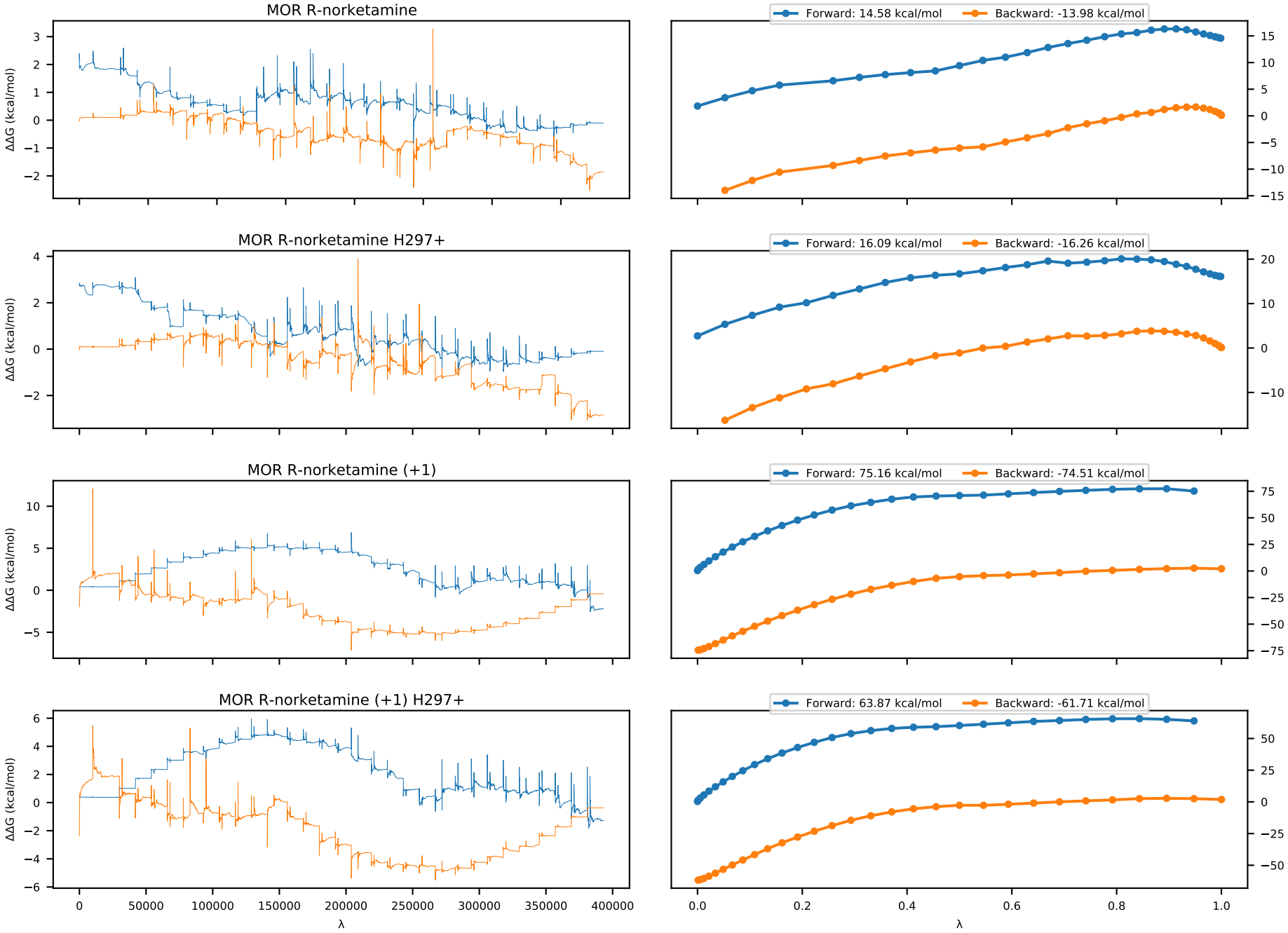

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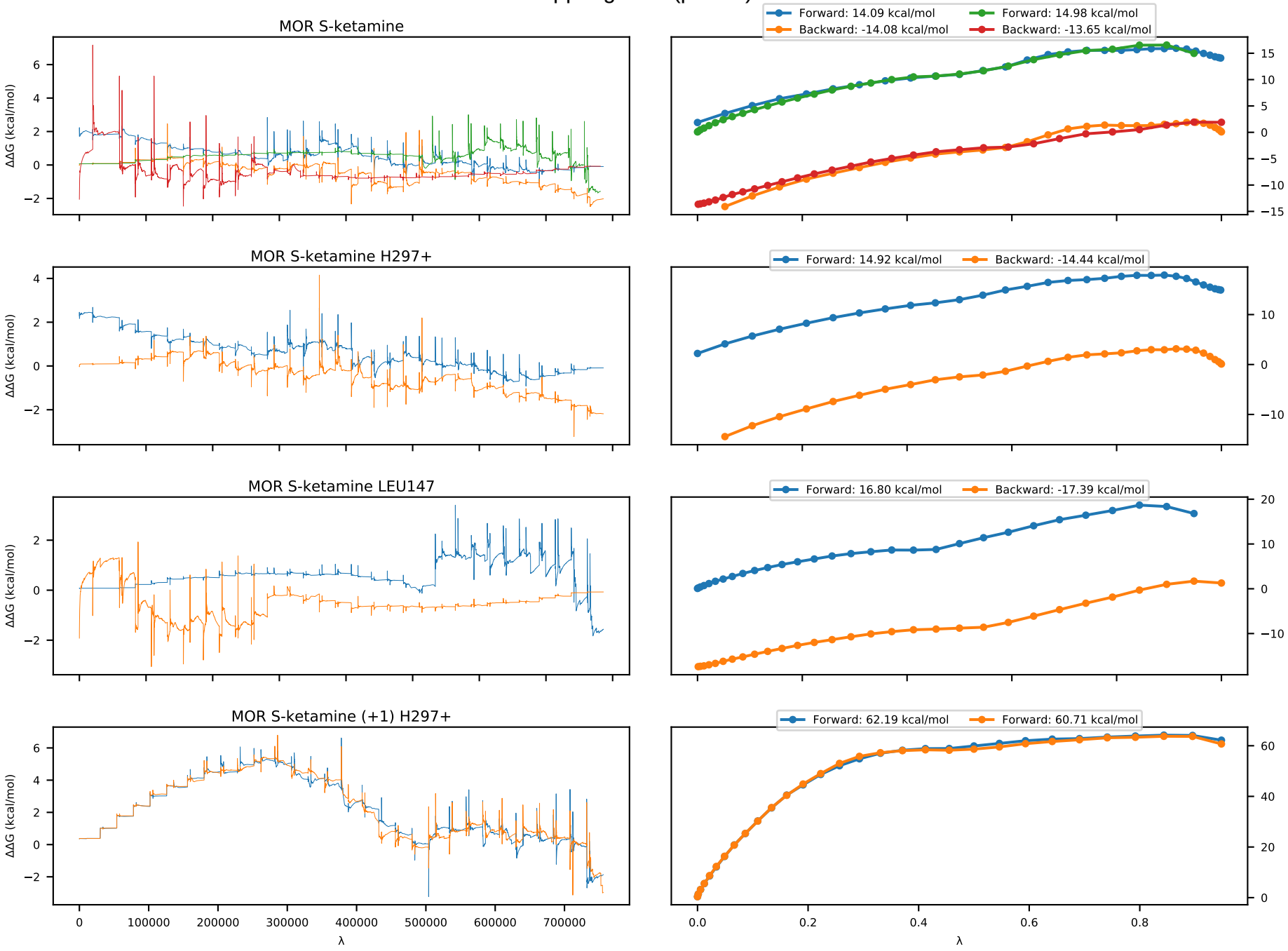

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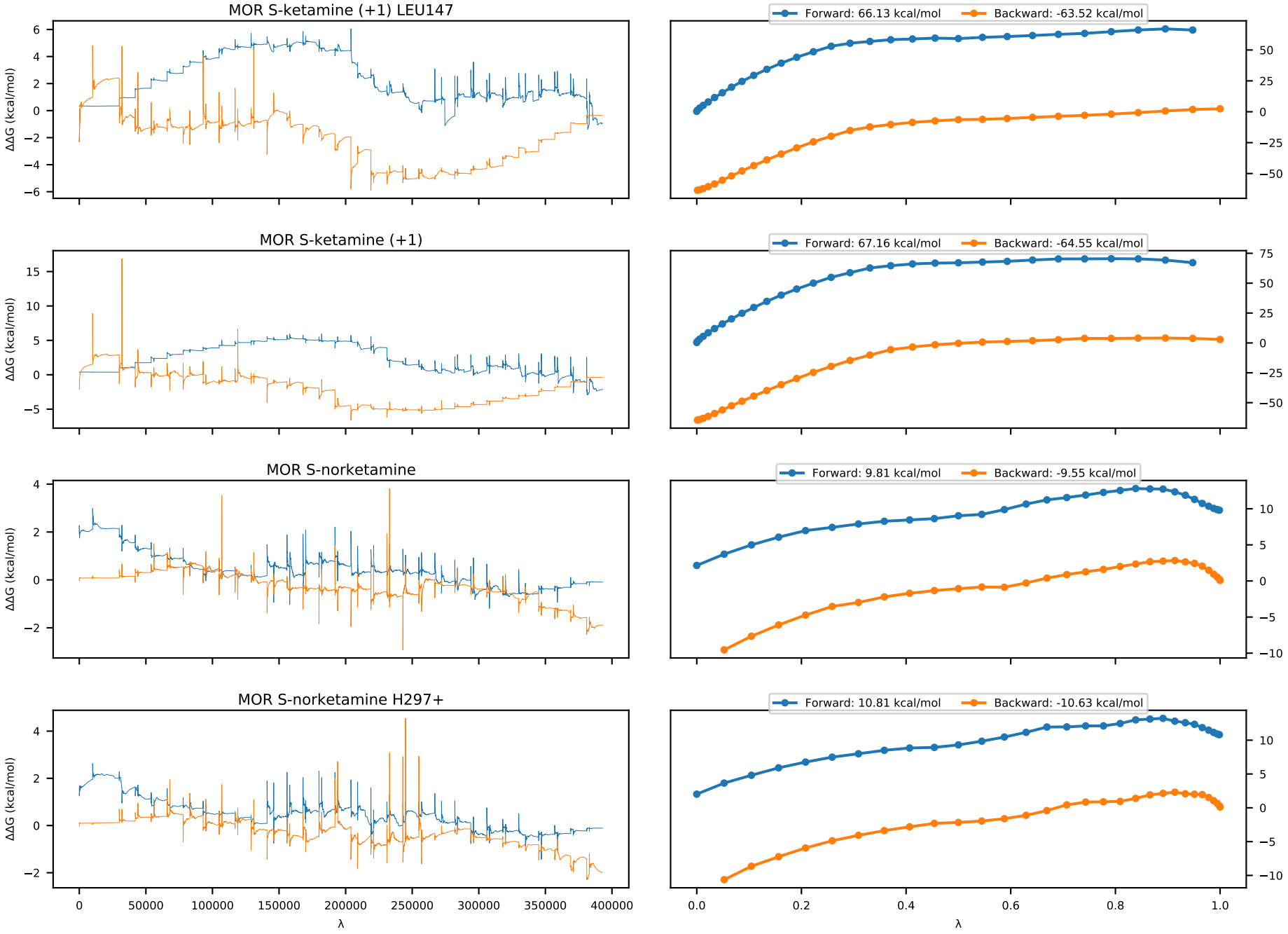

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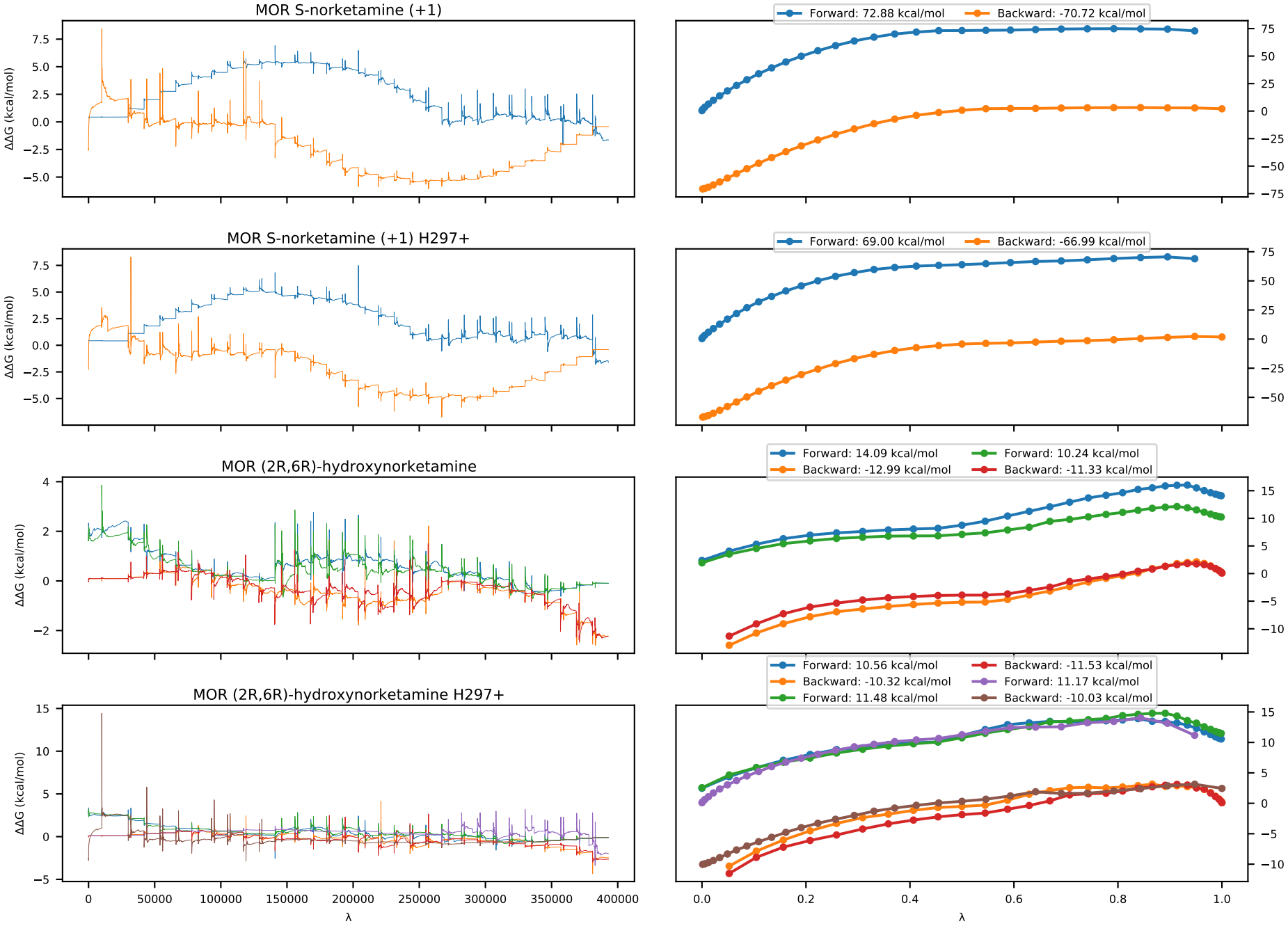
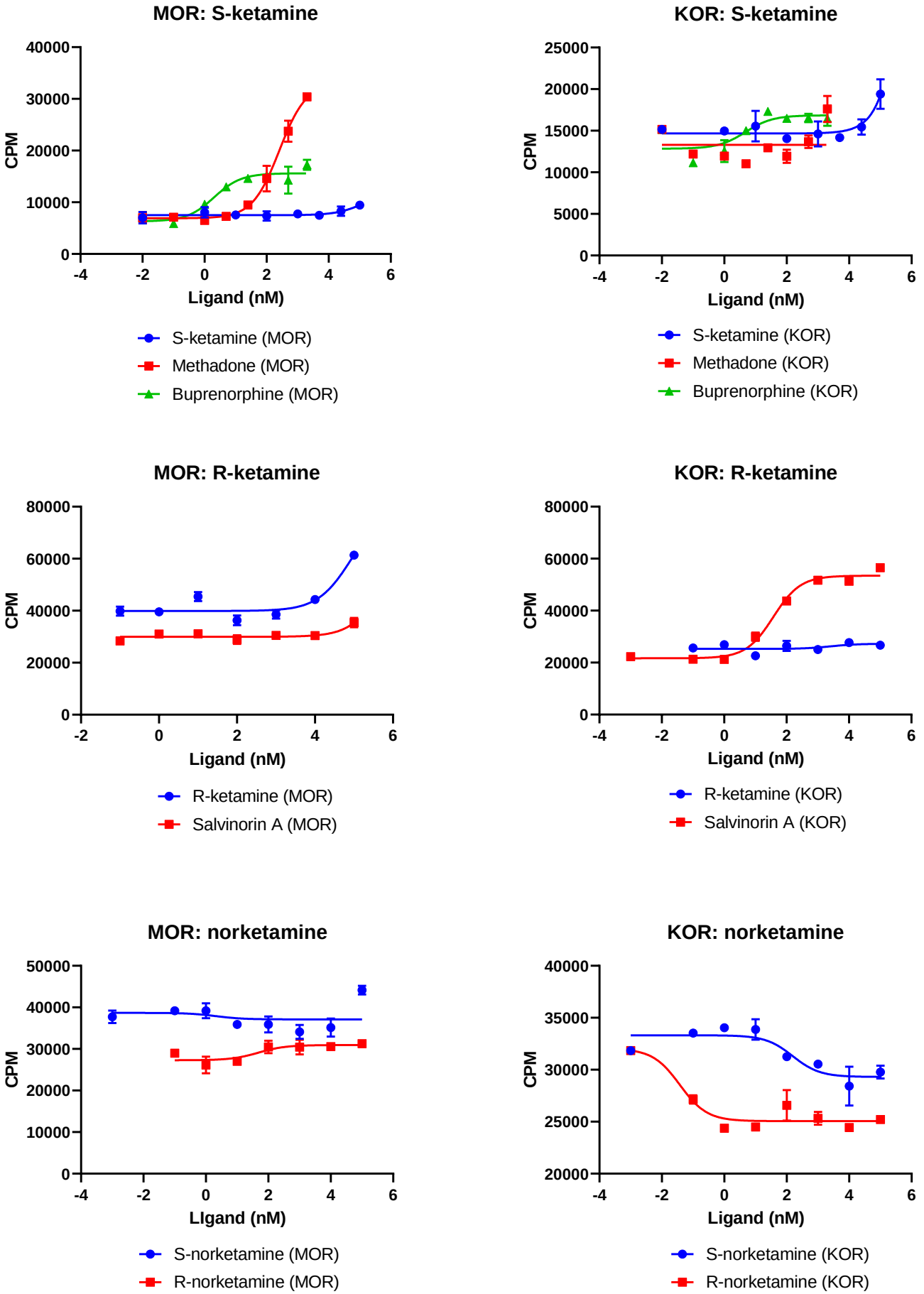
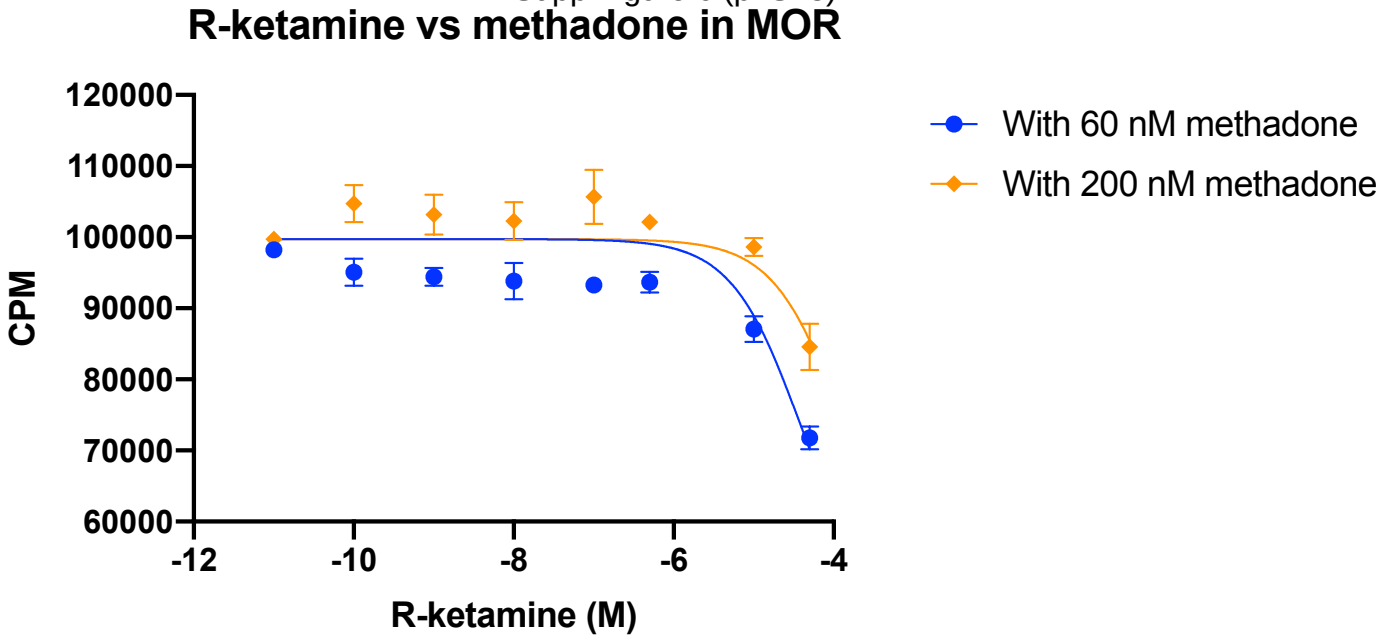
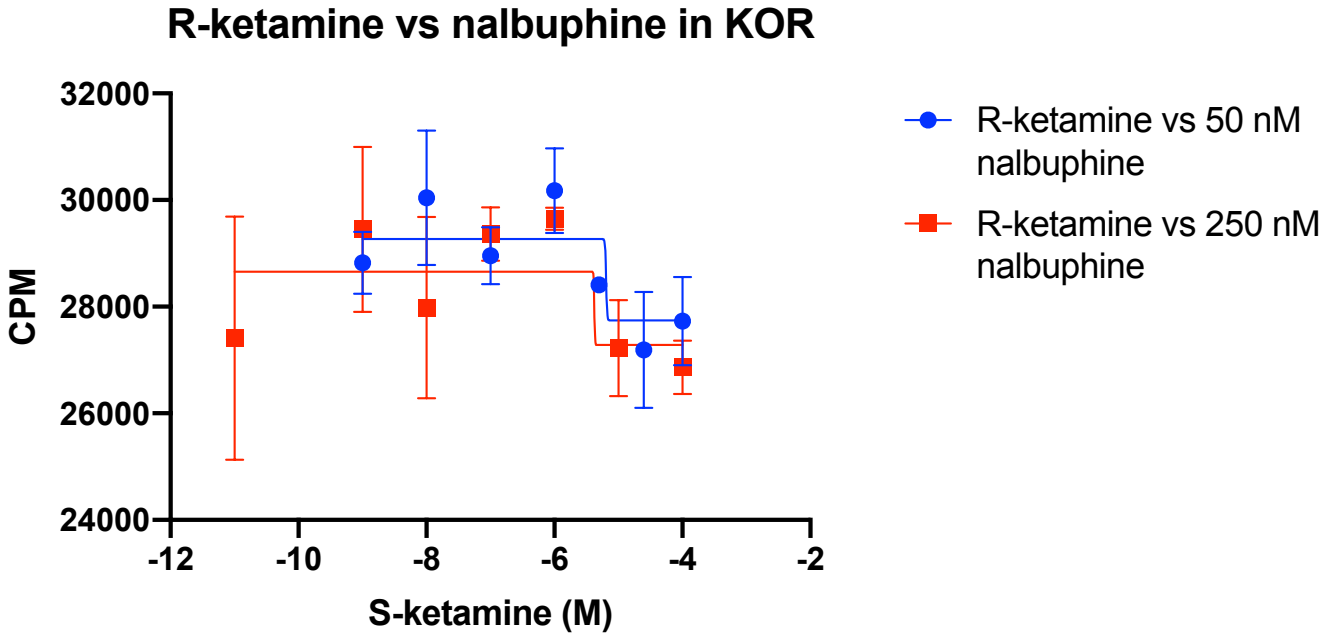
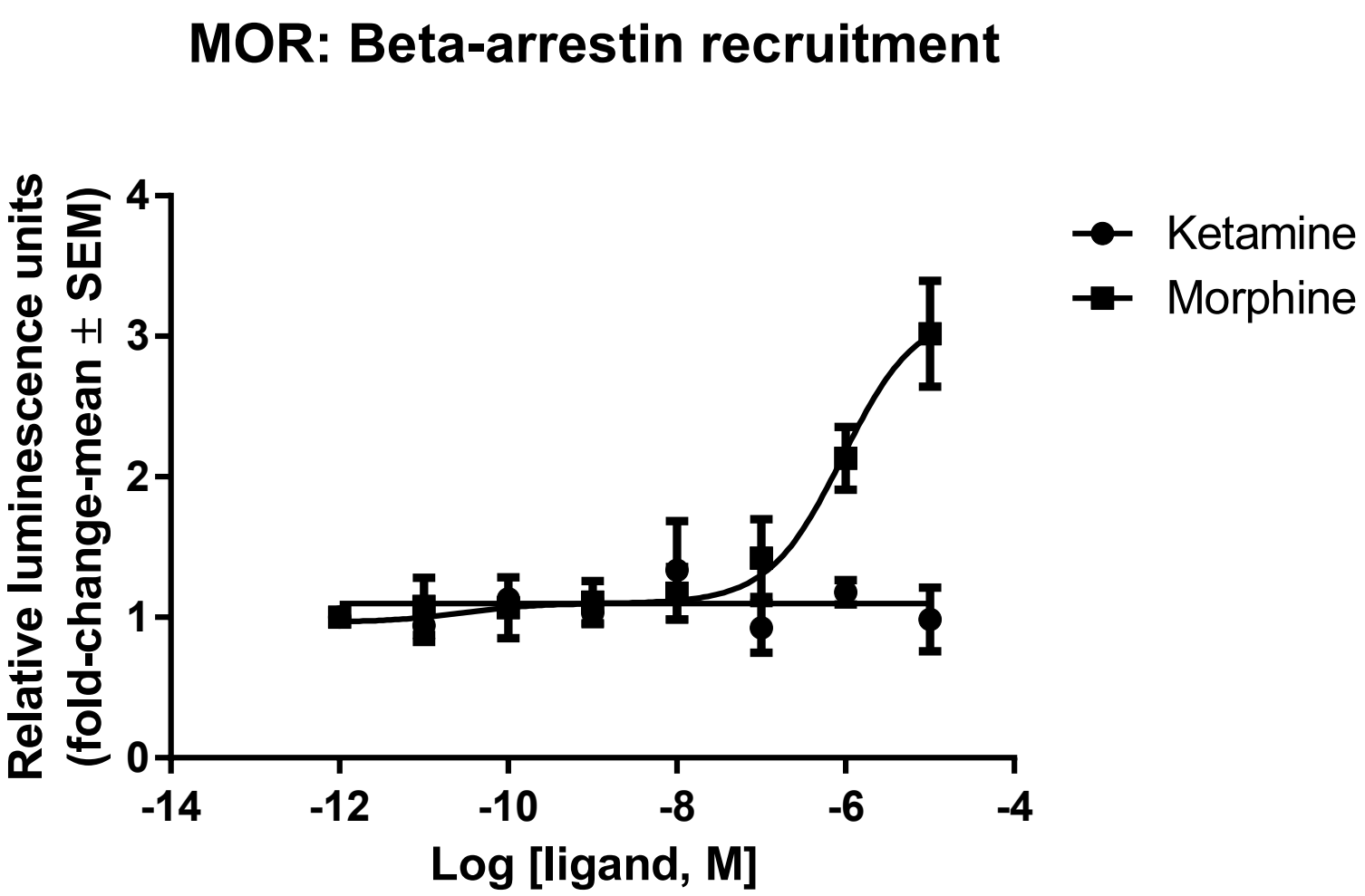
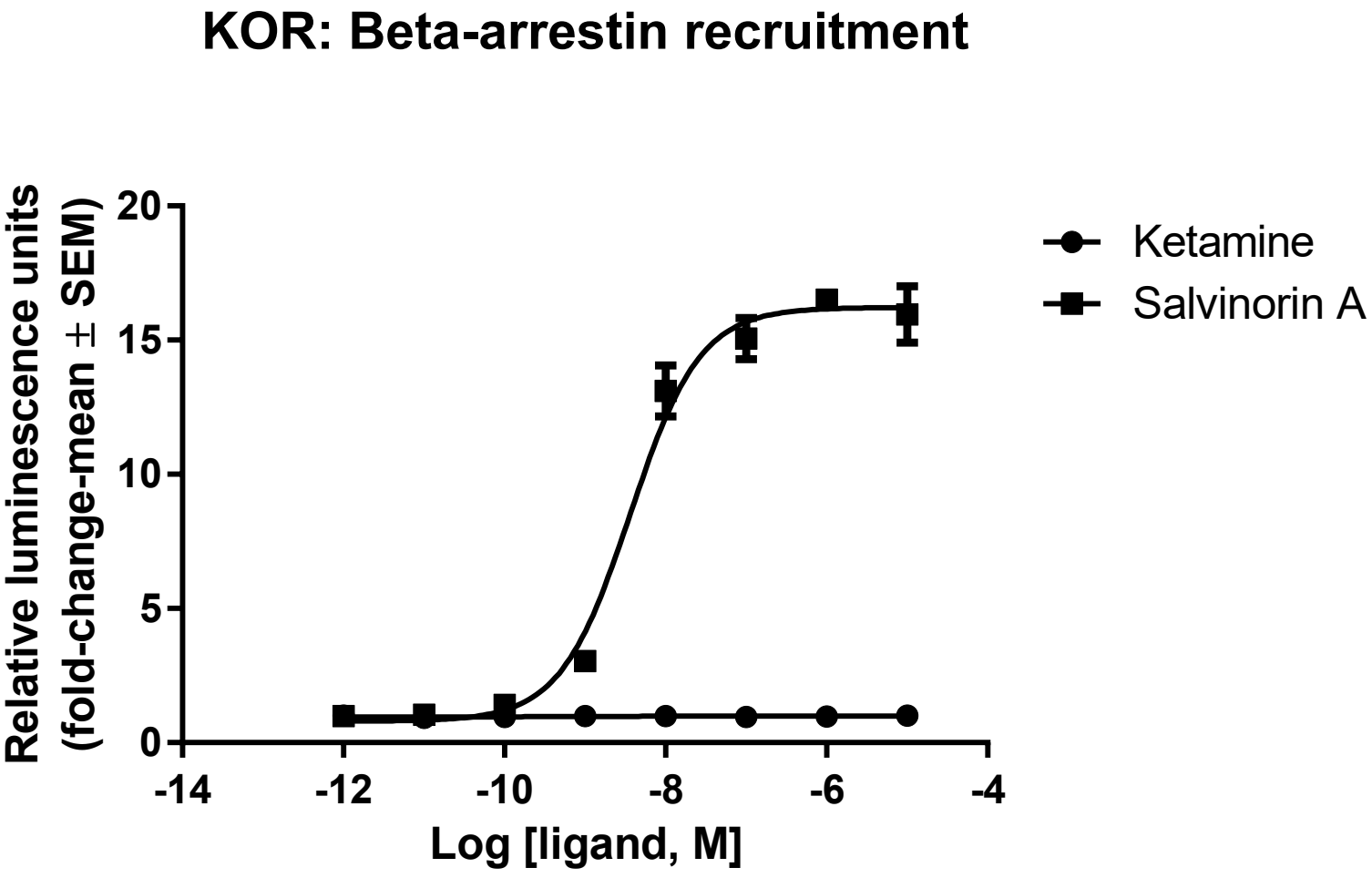



MOR: S-ketamine
CPM
Ligand (nM)
S-ketamine (MOR)
Methadone (MOR)
Buprenorphine (MOR)
KOR: S-ketamine
CPM
Ligand (nM)
S-ketamine (KOR)
Methadone (KOR)
Buprenorphine (KOR)
MOR: R-ketamine
CPM
Ligand (nM)
R-ketamine (MOR)
Salvinorin A (MOR)
KOR: R-ketamine
CPM
Ligand (nM)
R-ketamine (KOR)
Salvinorin A (KOR)
MOR: norketamine
CPM
LIgand (nM)
S-norketamine (MOR)
R-norketamine (MOR)
KOR: norketamine
CPM
Ligand (nM)
S-norketamine (KOR)
R-norketamine (KOR)


R-ketamine vs methadone in MOR
120000
110000
100000
90000
80000
70000
60000
CPM
-12
-10
-8
-6
-4
R-ketamine (M)
With 60 nM methadone
With 200 nM methadone


R-ketamine vs nalbuphine in KOR
32000
30000
28000
26000
24000
CPM
-12
-10
-8
-6
-4
-2
S-ketamine (M)
R-ketamine vs 50 nM nalbuphine
R-ketamine vs 250 nM nalbuphine


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


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