



Article–Supporting Information

Role of Extracellular Loops and Membrane Lipids for Ligand Recognition in the Neuronal Adenosine Receptor Type 2A: An Enhanced Sampling Simulation Study

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SUPPORTING INFORMATION

Section S1: Well-tempered Metadynamics

Well-tempered metadynamics (WTM) [1,2] works by introducing of a history-dependent potential V acting on a selected number of slow degrees of freedom, the collective variables (CVs). This forces the dynamics to explore conformations that were not previously visited and discourages the system from returning to these regions. Therefore, it allows the system escaping minima along low free energy paths and exploring other minima in the free energy landscape. This occurs independently of the minima one starts from. From the potential V, one can calculate the free energy.

Let us consider only one CV (*s* hereafter) for simplicity. The free energy surface along *s*, F(s), can be then calculated from the equation:

$$V(s,t \to \infty) = -\frac{T + \Delta T}{\Delta T}F(s)$$

In the well-tempered formulation[1] (the ones implemented here), the potential used to bias the dynamics in order to accelerate the sampling is:

$$V(s,t) = \Delta T ln \left(1 + \frac{\omega N(s,t)}{\Delta T}\right)$$

where ω has the dimension of an energy rate, ΔT is a temperature and N(s, t) comes from the biased simulation. As pointed out by the authors [1], an important property of this formulation is that, it insures the bias eventually to converge, yet "slow enough for the final result not to depend on the initial condition V (s, 0)."

Section S2: H264^{7.29}- E169^{ECL2} salt bridge: intramolecular interactions.

H264^{7.29} is predicted to be doubly protonated by PROPKA[3] at neutral pH. Hence, it is expected to form a salt-bridge with E169^{ECL2}. 18 out of 19 X-ray hA₂AR/ligand structures at pH<7.6 feature this putative salt bridge (see Table S1). The only exception occurs with the agonist UK43907 (PDBid: 3QAK). Here, because of steric reasons, the bulky ring of UK43907 causes a displacement of H264^{7.29} and E169^{ECL2}, with an increase of 3~4 Å of the C α -C α distance).¹ The salt bridge might behave as a 'gate' closing the ligand in the inner binding site and separating it from the vestibular binding site[4-6].

Section S3: H264^{7.29} and E169^{ECL2}salt bridge: conservation across A2ARs.

The following analysis shows that the two groups are fairly conserved across all A2ARs, while this is not the case for all the other human adenosine receptors.

Methods. By using the CLUSTAL Omega (1.2.2) web server[7], we investigated all A_{2A}Rs, along with the other three adenosine receptors (A₁Rs, A₃Rs and A_{2B}Rs) deposited in the UniProt **[8]** UniProtKB database (<u>http://www.uniprot.org/</u>). The conservation of E169^{ECL2} and H264^{7.29} across all A_{2A}Rs are 61 % and 39 %, respectively.

Results. H264^{7.29} is replaced by R and V for the 33% and 17% of the cases, respectively (Figure S7). In the R264 variants, the residues in position 169 are always E. Hence, the putative salt bridge (either formed by E169 and H264, or by E169 and R264) is conserved for slightly more than 60% across all A_{2A}Rs.

The pairwise sequence identities of the other human adenosine receptors (hA1R, hA2BR, and hA3R) with A2AR ranges from 58% to 39% (Figure S8). Position 169 is E hA1R and hA2BR while it is V in hA3R (see Figure S7). Position 264 is H in hA1R, E in hA3R, N in hA2BR. Here, N is embedded between two K's in a "KNK" motif.

The conservation of the E169^{ECL2}-H264^{7.29} positions across all A₁Rs is 58% (Figure S9). E169 and H264^{7.29} are conserved for 92% and 33.3% of the cases, respectively. The position 264 features also both positively (26%) and negatively (33%) charged residues.

The E169^{ECL2}-H264^{7.29} positions are not conserved across all A_{2B}Rs and A₃Rs (Figure S10-S11). It may be replaced by residues which could form H-bond interactions (as opposed to a salt bridge) or a salt bridge other than that formed here: indeed, in the case ofA₃Rs, the E169/K264 pair is present in 30% of the sequences.

Section S4: Sodium allosteric binding site.

This site has a fundamental importance in allosteric modulation of GPCRs [9,10]. The residues that mostly contribute to sodium binding along the GPCRs family, i.e S^{3,39}, N^{7,45} and D^{2,50} are described in literature as highly conserved. Position 2.50, in particular is an aspartic acid in 90% of the eukaryotic GPCRs, accordingly to our alignments taken from a curated multiple sequence alignment from GPCRdb. This residue can highly modulate the function of GPCRs. Indeed, the role of sodium modulation is well known for several GPCRs [10]. Mutagenesis studies on residues involved in the sodium ion coordination, and in particular D^{2,50}, highlighted the different effects that allosteric sodium may have in various class A GPCRs signaling [9]. Indeed, D^{2,50} replacement with uncharged amino acids can drastically reduce the agonist-induced G protein activation [11,12] [13] [14,15] or modulate the allosteric cavity can also exert different effects on the constitutive signaling of GPCRs [17,18]. In many cases, the presence of bound sodium seems to stabilize the inactive conformation of the receptor reducing the constitutive G-protein [13,15], whereas in other receptors

¹ In high pH environment, H264^{7.29} is instead predicted as deprotonated, and the salt bridge with E169^{ECL2} is expected to be broken. This is indeed the case for the remaining 5 structures crystalized at pH > 7.6 (see Table S1).

the substitution of sodium coordinating D^{2.50} abolishes the constitutive G-protein coupling and activation without affecting the agonist-stimulated activity [19]. Exhaustive studies have also revealed that the sodium pocket collapses due to the activation-related movements of the transmembrane helixes [18]. Recently, we have shown that the disruption of the sodium binding site of GPR3 strongly biased the receptor to the inactive state [20]. Thus, most of the studies agree with the fact that the constitutive activity can be dramatically affected by mutations in this cavity. Indeed, a constitutive active mutant (CAM) on human mu-opioid receptors, has been observed to disrupt the allosteric sodium binding cavity, favoring the exploration of active-like conformations even in the apo state [21]. In particular, for A2A receptors, very recently, White and collaborators [22] obtained the crystal structures of agonist complexes for two variants sodium binding site, D 52 ^{2.50}N and S91^{3.39}A. In both cases the structures are active-like but, the variants induce important changes in the activation motif NPxxY. The authors, combining several experimental techniques provide a basis for understanding the role of the sodium-coordinating residues on stability and G-protein signaling.

4. Tables

Table S1. H264^{7.29} **protonation state across hA**_{2A}**R X-ray structures**. The table reports: the resolution of the X-ray structures, the type of ligand (antagonists and agonists are colored in red or blue respectively), the pH of crystallization, the protonation state of H264^{7.29} at corresponding crystallization pH value (H264^{7.29} protonation), as predicted using PROPKA[3], the presence of H264^{7.29} and E169^{ECL2} salt-bridge/HB interactions and H264^{7.29} CA - E169^{ECL2} CA distances (Å).

PDBid	Resolution	ligand	рН	H264 ^{7.29} Protonation	H264 ^{7.29} -E169 ^{ECL2} salt bridge—/HB interaction	CA-CA distance	Reference
3EML	2.6	ZMA	6.5	Yes	Yes	11.5	[23]
4EIY	1.8	ZMA	5.0	Yes	Yes	11.4	[24]
3VG9	2.7	ZMA	6.5	No	Yes	11.3	[25]
3VGA	3.1	ZMA	6.5	Yes	Yes	11.0	[25]
2YDO	3.0	adenosine	7.6	Yes	Yes	11.5	[26]
2YDV	2.6	NECA	6.4	Yes	Yes	11.5	[26]
3QAK	2.7	UK-432097	5.0	Yes	No	15.3	[27]
4UHR	2.6	CGS21680	4.8	Yes	Yes	12.1	[28]
4UG2	2.6	CGS21680	4.8	Yes	Yes	11.2	[28]
3PWH	3.3	ZMA	8.1	No	No	11.9	[29]

3RFM	3.6	caffeine	8.2	No	No	11.8	[29]
3REY	3.3	xanthine	8.2	No	No	11.9	[29]
3UZC	3.3	T4E	8.0	No	No	11.7	[30]
3UZA	3.3	T4G	8.0	No	No	11.8	[30]
5G53	3.4	NECA	5.5	Yes	Yes	11.1	[31]
5IU4	1.7	ZMA	5.5	Yes	Yes	11.4	[5]
5IU7	1.9	6DY	5.4	Yes	Yes	11.4	[5]
5IU8	2.0	18F	5.5	Yes	Yes	11.4	[5]
5IUA	2.2	6DX	5.4	Yes	Yes	11.4	[5]
5IUB	2.1	6DV	5.5	Yes	Yes	11.4	[5]
5K2A	2.5	ZMA	5.0	Yes	Yes	11.4	[32]
5K2B	2.5	ZMA	5.0	Yes	Yes	11.4	[32]
5K2C	1.9	ZMA	5.0	Yes	Yes	11.4	[32]
5K2D	1.9	ZMA	5.0	Yes	Yes	11.4	[32]
5UIG	3.5	8D1	6.5	Yes	Yes	10.5	[33]
5UVI	3.2	ZMA	5.0	Yes	Yes	11.3	[34]
5JTB	2.8	ZMA	5.2	Yes	No	11.5	[35]
5N2R	2.8	8JN	5.5	Yes	No	12.2	[36]
5MZP	2.1	Caffeine	5.5	Yes	Yes	11.5	[36]
5MZJ	2.0	TEP	5.5	Yes	Yes	11.3	[36]
5NM4	1.7	ZMA	5.0	Yes	Yes	11.3	[37]
5NM2	1.9	ZMA	5.0	Yes	No	12.9	[37]
5NLX	2.1	ZMA	5.0	Yes	Yes	11.4	[37]
5VRA	2.4	ZMA	5.0	Yes	Yes	11.5	[38]

6AQF	2.5	ZMA	5.0	Yes	Yes	11.5	[39]
50M4	2.0	T4E	5.3	Yes	Yes	11.4	[40]
50M1	2.1	T4E	5.3	Yes	Yes	11.5	[40]
50LZ	1.9	T4E	5.3	Yes	Yes	10.3	[40]
50LV	2.0	LUAA47070	5.3	Yes	Yes	11.5	[40]
50LO	3.1	Tozadenant	5.3	Yes	No	12.4	[40]
50LK	2.6	Vipadenant	5.3	Yes	Yes	11.4	[40]
50LG	1.9	ZMA	5.3	Yes	Yes	11.4	[40]
5WF6	2.9	UKA	5.0	Yes	No	14.6	[22]

Table S2. Ligand hydration (defined here as the number of water molecules within 4 Å of ZMA) and OBS volume for free energy minima **A**, **B**, **C**, **D**, **E** and **F**.

State	OBS Volume (nm ³)	Ligand hydration
А	0.38±0.07	12±3
В	0.42±0.07	10±3
С	0.45±0.08	13±3
D	0.34±0.06	21±4
Е	0.39±0.06	16±4
F	0.33±0.06	33±6

Table S3. Conservation of residues forming the VBS of $h_{2A}R$, as emerging from our calculations. Sequences of four human adenosine subtypes and 18 sequences of adenosine receptor type 2A across species were used for multiple sequence alignment on the web server of CLUSTAL O (1.2.2) [7].

Residue	Conservation in human ARs	Conservation in A2ARs
M1 ^{1.27}	0%	33%
P2 ^{1.28}	0%	33%
Y91.35	100%	50%
E13 ^{1.39}	100%	50%

S67 ^{2.65}	75%	50%
T68 ^{2.66}	100%	50%
G69 ^{2.67}	100%	50%
N154 ^{ECL2}	0%	39%
H155 ^{ECL2}	0%	11%
A 165 ^{ECL2}	0%	17%
M2707.35	50%	72%
	50%	12/0
Y271 ^{7.36}	75%	44%

Table S4. Amino acid coevolution profile computed using the Coeviz tool within the web server polyview-2d [41]. The chi-squared covariance, weighted by phylogeny derived from alignments of hA2AR sequence (as defined in PDBid 3PWH [29]) against NCBI NR database with 90% identity [41]. For each amino acid, the genetic number and binding site location are annotated.

	VBS	VBS	VBS	VBS	VBS	OBS	OBS	VBS'	VBS'	OBS	OBS	OBS/VBS	OBS/VBS	OBS	OBS
	Ү91.35	E13 ^{1.39}	I64 ^{2.61}	S672.64	G69ecl1	$V84^{3.32}$	L85 ^{3.33}	G142 ^{ECL2}	W143 ^{ECL2}	M1775.40	N181 ^{5.43}	M270 ^{7.34}	Y271 ^{7.35}	I274 ^{7.38}	H2787.42
Y9 ^{1.35}	0.1 33	0.5 51	0.3 49	0.3 23	0.3 54	0.3 29	0.4 56	0.4 13	0.4 19	0.4 45	0.4 44	0.3 89	0.2 72	0.4 62	0.5 22
E13 ^{1.39}		0.0 62	0.4 31	0.3 14	0.4 26	0.4 71	0.5 12	0.5 22	0.4 99	0.5 62	0.5 32	0.4 55	0.3 28	0.5 73	0.6 52
I64 ^{2.61}			0.0 97	0.1 84	0.2 06	0.2 19	0.2 95	0.2 62	0.2 79	0.3 06	0.2 79	0.2 67	0.2 06	0.3 50	0.4 04
S67 ^{2.64}				0.1 42	0.1 04	0.2 07	0.3 46	0.1 75	0.1 87	0.2 49	0.1 98	0.2 38	0.1 26	0.3 20	0.3 04
G69 ^{EC} L1					0.1 72	0.3 02	0.2 93	0.2 82	0.2 87	0.3 58	0.3 29	0.2 72	0.2 03	0.3 51	0.4 34

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V84 ^{3.32}			0.0 47	0.3 99	0.3 29	0.3 36	0.4 17	0.4 62	0.4 38	0.3 11	0.3 68	0.4 60
L85 ^{3.33}				0.0 69	0.3 56	0.3 63	0.4 63	0.3 91	0.4 31	0.3 45	0.4 01	0.4 95
G142 ^E CL2					0.1 32	0.6 39	0.4 36	0.3 64	0.3 12	0.2 14	0.4 10	0.4 98
W143 ECL2						0.1 35	0.4 42	0.3 70	0.3 19	0.2 23	0.4 05	0.5 02
M177 ⁵ .40							0.1 07	0.4 67	0.3 85	0.3 21	0.4 86	0.5 49
N181 ^{5.} 43								0.0 73	0.4 18	0.2 73	0.4 20	0.5 10
M270 ⁷ .34									0.0 47	0.3 07	0.3 72	0.4 41
Y271 ^{7.} 35										0.0 57	0.2 86	0.3 22
I274 ^{7.3} 8											0.1 22	0.5 57
H278 ^{7.} 42												0.0 62

Table S5. Presence of residue coevolution between orthosteric binding site (OBS) and extracellular loops (ECLs) of human receptors in class A, B, C and F. X-ray structures of 27 human GPCRs with OBS-bound ligand were used for evolutionary correlation analysis with Coeviz tool in polyview-2d webserver **[41]**. The threshold of PCS score was chosen as >0.3 to identify evolutionarily correlated residue pairs. Among them, 22 GPCRs have residue-based ECL-OBS coevolution relation. **'0"** ECL is missing in X-ray structure; "Yes" presence of residue-based evolutionary correlation; "No" absence of residue-based evolutionary correlation.

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Receptor	PDBid	Class	ECL1-OBS Correlation	ECL2-OBS Correlation	ECL3-OBS Correlation
β2-adrenergic receptor	3D4S	А	No	Yes	Yes
CXCR4	30DU	А	Yes	Yes	Yes
D3 receptor	3PBL	А	Yes	No	Yes
A2A receptor	3PWH	А	Yes	Yes	Yes
H1 receptor	3RZE	А	Yes	Yes	Yes
M1 receptor	5CXV	А	Yes	Yes	Yes
S1P1 receptor	3V2Y	А	Yes	Yes	Yes
к receptor	4DJH	А	Yes	Yes	0
NOP receptor	4EA3	А	No	No	No
PAR1 receptor	3VW7	А	Yes	Yes	Yes
5-HT1B receptor	4IAR	А	Yes	No	0
5-HT2B receptor	4IB4	А	Yes	No	No
SMO receptor	4JKV	F	Yes	Yes	Yes
CRF1 receptor	4K5Y	В	Yes	Yes	0
glucagon receptor	4L6R	В	0	No	No
CCR5 receptor	4MBS	А	No	No	No
M2 receptor	3UON	А	Yes	Yes	Yes
δ receptor	4N6H	А	No	No	No
mGlu1 receptor	40R2	С	Yes	Yes	No
mGlu5 receptor	5CGD	С	Yes	Yes	Yes
P2Y12 receptor	4NTJ	А	No	No	Yes

FFA1 receptor	4PHU	А	Yes	Yes	Yes
OX2 receptor	4S0V	А	Yes	Yes	Yes
AT1 receptor	4YAY	А	No	No	No
LPA1 receptor	4Z35	А	Yes	Yes	Yes
OX1 receptor	4ZJC	А	Yes	Yes	No
M4 receptor	5DSG	А	Yes	Yes	Yes

5. Figures



Figure S1-part A. Receptor ligand interaction 2D scheme obtained by MOE (Molecular Operating Environment) [42].



Figure S1-part B. Receptor ligand interactions 2D scheme obtained by MOE (Molecular Operating Environment) [42].



Figure S2. Pairwise Root Main Square Deviation (RMSD) matrix across twelve of the deposited structures of ZMA-bound hA₂AR receptor and the minima **A**, **B**, **C** calculated in Å considering the C α atoms of the overall receptor (upper-right triangle) or the residues belonging to the binding site (lower-left triangle).



Figure S3. ZMA binding poses in the orthosteric site corresponding to minima **B** and **C** in Figure 1 are shown in A and B panels, respectively, as 3D representation. The protein backbone is rendered as cartoon, ZMA is shown as a green licorice, residues interacting with ZMA are shown as grey lines. The E169^{ECL2} and H264^{7.29} residues are shown in cyan licorice. Hydrogen, oxygen and nitrogen atoms are specifically colored in white, red and light blue, respectively. C-D) 2D scheme of binding poses in A) and B), respectively.



Figure S4 Superimposition of hA_2AR representative structure in the minima B (yellow tube) and C (cyan tube). ZMA is shown in yellow and cyan licorice representation for B and C minima, respectively.

sp PODMS8 AA3R_HUMAN	VTTHRRIWLALGLCWLVSFLVGLTPMFGWNMKLTSEYHF	FLSC
sp P30542 AA1R_HUMAN	VVTPRRAAVAIAGCWILSFVVGLTPMFGWNNLSAVERAWAANGSMGE	VIKC
sp P29274 AA2AR HUMAN	LVTGTRAKGIIAICWVLSFAIGLTPMLGWNNCGQPKEGKNHSQGCGEG	QVACI
sp P29275 AA2BR HUMAN	LVTGTRARGVIAVLWVLAFGIGLTPFLGWNSKDSATNNCTEPWDGTTNESCC	LVKCI
	:.* * :. *:::* :****::***	: *

Figure S5. Conservation of solvent-exposed motif of ECL2 in human Adenosine receptor subfamily. This multiple sequence alignment was generated using the web server of CLUSTAL O (1.2.2) [7]. Amino acid residues are colored according to this scheme: small and hydrophobic residues including aromatic residues are colored in red, acidic residues are colored in blue, basic residues are colored in magenta, and hydroxyl, sulfhydryl, amine residues and glycine are colored in green. Same alignment method and coloring schemes are applied in the following Figures S6-11.

tr	A0A0S7EPN9	A0A0S7EPN9_9TELE	GWHNLSASGYYSTNTSSTLFSSSQCTFLSVISLPFMVYFNFLGCIMAPLLVMTFLYVIIF
tr	A0A0S7I6S9	A0A0S716S9_9TELE	
tr	A0A0S715U9	A0A0S715U9_9TELE	
tr	A0A0S71265	A0A0S7I265_9TELE	KGCPNGLTQCLFETVVPMDYMTYFNFFGFVLTPLLVMLVIYIKIF
tr	A0A0S71736	A0A0S71736_9TELE	KGCPKGLTQCLFETVVPMDYMTYFNFFGFVLTPLLVMLVIYIKIF
tr	A0A0S7I5T3	A0A0S7I5T3_9TELE	KGCPKGLTQCLFETVVPMDYMTYFNFFGFVLTPLLVMLVIYIKIF
tr	A0A0S7EMS2	A0A0S7EMS2_9TELE	GWHKTPPDSGYCFFVLVVDMTYMVYFNFFACVLAPLVIMFLIYAQIF
tr	W8C370 W8C	370_CERCA	GWHVKPENEAEMSCHFVKVMDYNYLVFL-YFATIITPALLMLAFYTHIY
tr	A0A034WCJ8	A0A034WCJ8_BACDO	GWHVKPENEADMTCHFVKVMDYNYLVFL-YFATIITPALLMLAFYTHIY
tr	A0A0S7ELH4	A0A0S7ELH4_9TELE	GWHKESNSTETNNNTCQFGQMKCLFEAVVNMEYMVYFNFFACVLIPLLLMLAIYLCIF
tr	A0A0S7I6R8	A0A0S7I6R8_9TELE	GWNTGRNLITLHSSEKKGCPGGET
sp	P30543 AA2	AR_RAT	GWNNCSQRDGNSTRTCGEGRVTCLFEDVVPMNYMVYYNFFAFVLLPLLLMLAIYLRIF
sp	Q60613 AA2	AR_MOUSE	GWNNCSQKDENSTKTCGEGRVTCLFEDVVPMNYMVYYNFFAFVLLPLLLMLAIYLRIF
sp	P46616 AA2	AR_CAVPO	GWNNCSQFKGDKNHSESCDEGQVTCLFEDVVPMNYMVYYNFFAFVLVPLLLMLGIYLRIF
sp	Q6TLI7 AA2	AR_HORSE	GWNNCHHWGEGENQSQGCGEGQVACLFEDVVPMNYMVYYNFFACVLVPLLLMLGVYLRIF
sp	P29274 AA2	AR_HUMAN	GWNNCGQBKEGKNHSQGCGEGQVACLFEDVVPMNYMVYFNFFACVLVPLLLMLGVYLRIF
tr	U6CZN7 U6C	ZN7_NEOVI	GWNNCGQPKAGQNQSEVCGEGQVTCLFEDVVPMNYMVYYNFFACVLVPLLLMLGVYLRIF
sp	P11617 AA2	AR_CANLF	GWNNCSQRKEGRNYSOGCGEGQVACLFEDVVPMNYMVYYNFFAFVLVPLLLMLGVYLRIF

Figure S6. Conservation of solvent-exposed motif of ECL2 in Adenosine receptor A₂R across different species. Color-code and alignment method as in Figure S5.

tr A0A0S7EPN9 A0A0S7EE	N9_9TELE GW	HNLSASGYYSTNTSSTLPSSSQCTF	ISVISLPFMVYFNFLGCIMAPLLVMTFLYVIIF
tr A0A0S716S9 A0A0S716	S9_9TELE		
tr A0A0S715U9 A0A0S715	U9_9TELE		
tr A0A0S71265 A0A0S712	265_9TELE	KGCPKGLTQCLF	JIVVPMDYMTYFNFFGFVLTPLLVMLVIYIKIF
tr A0A0S71736 A0A0S717	736_9TELE	KGCPKGLTQCLF	TVVPMDYMTYFNFFGFVLTPLLVMLVIYIKIF
tr A0A0S715T3 A0A0S715	T3_9TELE	KGCPKGLTQCLF	ETVVPMDYMTYFNFFGFVLTPLLVMLVIYIKIF
tr A0A0S7EMS2 A0A0S7EM	IS2_9TELE GW	HKTPPDSGYCFF	LVVDMTYMVYFNFFACVLAPLVIMFLIYAQIF
tr W8C370 W8C370_CERCA	GW	HVKPENEAEMSCHF	XXVMDYNYLVFL-YFATIITPALLMLAFYTHIY
tr A0A034WCJ8 A0A034WC	CJ8_BACDO GW	HVKPENEADMTCHF	WKVMDYNYLVFL-YFATIITPALLMLAFYTHIY
tr A0A0S7ELH4 A0A0S7EL	GW GW	HKESNSTETNNNTCQPGQMKCLF	EAVVNMEYMVYFNFFACVLIPLLLMLAIYLCIF
tr A0A0S716R8 A0A0S716	R8_9TELE GW	NTGRNLTLHSSEKKGCPGGET	T
sp P30543 AA2AR_RAT	GW	NNCSQKDGNSTKTCGEGRVTCLF	DVVPMNYMVYYNFFAFVLLPLLLMLAIYLRIF
sp Q60613 AA2AR_MOUSE	GW	NNCSQKDENSTKTCGEGRVTCLF	DVVPMNYMVYYNFFAFVLLPLLLMLAIYLRIF
sp P46616 AA2AR_CAVPO	GW	NNCSQPKGDKNHSESCDEGQVTCLF	DVVPMNYMVYYNFFAFVLVPLLLMLGIYLRIF
sp Q6TLI7 AA2AR_HORSE	GW	NNCHHWGEGENQSQGCGEGQVACLF	DVVPMNYMVYYNFFACVLVPLLLMLGVYLRIF
sp P29274 AA2AR_HUMAN	GW	NNCGQPKEGKNHSQGCGEGQVACLF	EDVVPMNYMVYFNFFACVLVPLLLMLGVYLRIF
tr U6CZN7 U6CZN7_NEOVI	GW	NNCGQPKAGQNQSEVCGEGQVTCLF	DVVPMNYMVYYNFFACVLVPLLLMLGVYLRIF
sp P11617 AA2AR_CANLF	GW	NNCSQPREGRNYSQGCGEGQVACLF	DVVPMNYMVYYNFFAFVLVPLLLMLGVYLRIF
tr A0A0S7EPN9 A0A0S7EE	N9_9TELE LV	LTLFAFCWIPLHLMNCLLLLWGPQA	TQGTLYTGRSELFSYSLHRC-
tr A0A0S716S9 A0A0S716	SS9_9TELE	TDTGKHGGIINCLNHLCKGCK	RPTIWVMNIAIILSHANSVVNPFIYAYRIREFR
tr A0A0S7I5U9 A0A0S7I5	5U9_9TELE	TDTGKHGGIINCLNHLCKGCK	RPTIWVMNIAIILSHANSVVNPFIYAYRIREFR
tr A0A0S71265 A0A0S712	265_9TELE II	VGLFAICWLPVHIINCLNHLCKGCK	RPTIWVMNIAIILSHANSVVNPFIYAYRIREFR
tr A0A0S71736 A0A0S717	736_9TELE II	VGLFAICWLPVHIINCLNHLCKGCK	RPTIWVMNIAIILSHANSVVNPFIYAYRIREFR
tr A0A0S715T3 A0A0S715	ST3_9TELE II	VGLFAICWLPVHIINCLNHLCKGCK	PTIWVMNIAIILSHANKTNLMF
tr A0A0S7EMS2 A0A0S7EM	4S2_9TELE LV	LFLFTVCWIPLHIINCFLLLCP	
tr W8C370 W8C370_CERCF	II II	VLFFMICWFPLYTINCIKAFCPDCQ	S-GKLTLFSIILSHLNSAGNPVLYAYHLKDFR
tr A0A034WCJ8 A0A034WC	IJ8_BACDO II	VLFFMICWFPLYTINCIKAFCPQCQ	MP-GKLTLFCIILSHLNSAGNPVLYAYHLKDFR
tr A0A0S7ELH4 A0A0S7EI	LH4_9TELE II	VGLFAVCWLPLHIINCFTLFCPECP	RPPGWIMYVAIILSH
tr A0A0S7I6R8 A0A0S7I6	SR8_9TELE		T
sp P30543 AA2AR_RAT	II	VGLFALCWLPLHIINCFTFFCSTCR	HAPPWLMYLAIILSHSNSVVNPFIYAYRIREFR
sp Q60613 AA2AR_MOUSE	II	VGLFALCWLPLHIINCFTFFCSTCQ	HAPPWLMYLAIILSHSNSVVNPFIYAYRIREFR
sp P46616 AA2AR_CAVPO	II	VGLFALCCLPLNIINCFTFFCPECD	APPWLMYLTIILSHGNSVVNPLIYAYRIREFR
sp Q6TLI7 AA2AR_HORSE	II	VGLFALCWLPLHIINCFTFFCPECP	APLWLMYPAIILSHFNSVVNPFIYAYRIREFR
sp P29274 AA2AR_HUMAN	II	VGLFALCWLPLHIINCFTFFCPDCS	HAPLWLMYLAIVLSHTNSVVNPFIYAYRIREFR
tr U6CZN7 U6CZN7_NEOVI	I II	VGLFALCWLPLHIINCFTFFCPECS	HAPPELMYLTIILSHTNSVVNPFIYAYRIREFR
SD P11617 AA2AR CANLF	II	VGLFALCWLPLHIINCFTFFCPECS	HAPLWLMYLTIVLSHTNSVVNPFIYAYRIREFR

Figure S7. Conservation of H264^{7.29} and E169^{ECL2} in Adenosine receptor A_{2A}R across different species. Color-code and alignment method as in Figure S5.

sp PODMS8 AA3R HUMAN	FVSVMRMDYMVYFSFLTWIFIPLVVMCAIYLDIFYIIRNKLSLNLSNSKETGAFYGR
sp P30542 AA1R_HUMAN	FEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFYLIRKQLNKKVSASSGDPQKYYGK
sp P29274 AA2AR_HUMAN	FEDVVPMNYMVYFNFFACVLVPLLLMLGVYLRIFLAARRQLKQMESQPLPGERARSTLQK
sp P29275 AA2BR_HUMAN	FENVVPMSYMVYFNFFGCVLPPLLIMLVIYIKIFLVACRQLQRTELMDHSRTTLQR
	* .*: *.****.*: :: **::* :*: :* .:*. :
	E169
sp PODMS8 AA3R_HUMAN	EFKTAKSLFLVLFLFALSWLPLSIINCIIYFNGEVPQLVLYMGILLSHANSMMNPI
sp P30542 AA1R_HUMAN	ELKIAKSLALILFLFALSWLPLHILNCITLFCPSCHKPSILTYIAIFLTHGNSAMNPI
sp P29274 AA2AR_HUMAN	EVHAAKSLAIIVGLFALCWLPLHIINCFTFFCPDC-SHAPLWLMYLAIVLSHTNSVVNPF
sp P29275 AA2BR_HUMAN	EIHAAKSLAMIVGIFALCWLPVHAVNCVTLFQPAQGKNKPKWAMNMAILLSHANSVVNPI
	H264

Figure S8. Conservation of H264^{7.29} and E169^{ECL2} in human Adenosine receptor subtypes hA1R, hA2AR, hA2BR, hA3R. Color-code and alignment method as in Figure S5.

NNLQFLLKNGTVTTEELVVTCEFETVISMDYMVYFNFFGWVLPPLLLMLAIYVEIFY
NNLNKVLGTRDLNVSHSEFVIKCQFETVISMEYMVYFNFFVWVLPPLLLMLLIYLEVFN
NNLREVQRAWAANGSVGEPVIKCEFEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFY
NNLSAVERDWLANGSVGEPVIECOFEKVISMEYMVYFNFFVWVLPPLLLMVLIYMEVFY
NRLGGG
NRLGEAQRAWAANGSGGEPVIKCEFEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFY
NNLSVVEQDWRANGSVGEPVIKCEFEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFY
NNLSEVEQAWIANGSVGEPVIKCEFEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFY
NNLSKIEMAWAANGSVGEPVIKCEFEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFY
NNLSAVERAWAANGSMGEPVIKCEFEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFY
white a supervise a supervise and the supervise supervis
NNLSAVERAWAANGSMGEPVIRCEPDRVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFY
NNLSAVERAWAANGSMGEPVIRCEPORVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFY ISWLPLHIINCITFFCPQODK
NNLSAVEKAWAARGSNGEFVIRCEPDRVISMEINVIFNFFWVDPPLLLAVLIVLEVF - ISWLPLHIINCITFFCPQODK IHRQLNKKVTASHMDPRRYFGKELKLAKSLALVLFLFAVSNLPHIINCITFFLFQCM
NNLSAVERAWAANGSNGEPVIKCEPDAVISHEYRVYFNFYWVLPPLLAWLIILSVU
NNLSAVEKAWAANSSHGEPVIRCEERAVISHEIMVIT REVWULEPLLAVLIILEVIT
NNLSAVEKAWAANSSIGEFVIRCETERVISHEINVISHEINVISHEINVISHEILAVLIILEVUI
NILSAVERAWAAANSINGEPUIRCEENVISHEIMVITRETWULEPULLAVUITLEVIT
INDERVEKAWAANSSIGGEVIRCEERVISHEINVISHEINVISHEINVISHEINUSTEEVIR
NILSAVERAWAAASSIGGEVIRCEERIN ISBETAVITRETWALEPELLAVLITLEVIT
NILSAVERAWAANSSHGEPVIRCEPRVISHERVYTRFFVWLEPPLLAVLITLEVT
NILSAVERAWAAASSHGEPVIRCEERIN ISHEIMVITRETWULEPLLIAVLIILLUUTLEUFT
NILSAVERAWAAASSGREEVIRCEERVISE

Figure S9. Conservation of H264^{7.29} and E169^{ECL2} in Adenosine receptor A1R across different species. Color-code and alignment method as in Figure S5.

tr A0A0S7ID29 A0A0S7ID29 9TELE	TARKIIAILWILSFVIGLIPFFGWNLKNSSWENSSSVNNTKCKECYFDSVV
sp Q1LZD0 AA2BR BOVIN	RARGVIAALWVLAFGIGLTPFLGWNDRKIAT-NCTEPGDAATNVSCCLIRCLFENVV
sp Q60614 AA2BR MOUSE	RARGIIAVLWVLAFGIGLTPFLGWNSKDSATSNCTELGDGIANKSCCPVTCLFENVV
SP P29276 AA2BR RAT	RARGIIAVLWVLAFGIGLTPFLGWNSKDRATSNCTEPGDGITNKSCCPVKCLFENVV
tr U6D4J8 U6D4J8 NEOVI	RARRVIAVLWVLAFGIGLTPFLGWNSKNTAA-NCTEPWDGATNVSCCLVRCLFENVV
SP Q6W3F4 AA2BR CANLF	RARGVIAVLWVLAFGIGLTPFLGWNSKDSAT-NCTEPWDGTTNESCCLVKCLFENVV
sp P29275 AA2BR HUMAN	RARGVIAVLWVLAFGIGLTPFLGWNSKDSATNNCTEPWDGTTNESCCLVKCLFENVV
SP Q32ZE2 AA2BR RABIT	RARGVIAVLWVLAFGIGLTPFLGWNSKDSATANCTEPRDGTTNESCCLVKCLFENVV
tr A0A0S7GS81 A0A0S7GS81 9TELE	GVLFYPDKHADRESS
tr C1BIS9 C1BIS9 OSMMO	
sp 013076 AA2BR_CHICK	RARGLIAVLWLLSFVIGLTPLMGWNKAMSGCPNSTNETGADHGAGHHGCFISCLFENVV
tr A0A0S7ID29 A0A0S7ID29 9TELE	AKSLSIIVGLFTLCWLPVHILNCLTLFYGDLKKPVFVMYVAIILSHANSAINPIIYA
sp Q1LZD0 AA2BR_BOVIN	AKSLALIVGIFALCWLPVHTINCASLFQPTWAKVKPKWAINTAILLSHANSAVNPIVYA
sp Q60614 AA2BR MOUSE	AKSLAMIVGIFALCWLPVHAINCITLFHPALAKDKPKWVMNVAILLSHANSVVNPIVYA
sp P29276 AA2BR RAT	AKSLAMIVGIFALCWLPVHAINCITLFHPALAKOKPKWVMNVAILLSHANSVVNPIVYA
tr U6D4J8 U6D4J8 NEOVI	AKSLAMIVGIFALCWLPVHAINCVTFFQPAKSKAKPKWVMNTAILLSHANSVVNPIVYA
sp Q6W3F4 AA2BR CANLF	AKSLAMIVGIFALCWLPVHAINCVTLFQPARAKDKPKWAMNMAILLSHASSVVNPIVYA
sp P29275 AA2BR HUMAN	AKSLAMIVGIFALCWLPVHAVNCVTLFQPAQGKNKPKWAMNMAILLSHANSVVNPIVYA
sp Q32ZE2 AA2BR RABIT	AKSLAMIVGIFALCWLPVHAINCVTLFQPAQAKDKPKWAMNTAILLSHANSVVNPIVYA
tr A0A0S7GS81 A0A0S7GS81 9TELE	
tr C1BIS9 C1BIS9_OSMMO	
sp 013076 AA2BR CHICK	AKSLAIIVGLFAFCWLPLHILNCITHFHEEFSKSKPEWVMYVAIILSHANSVINPIIYA

Figure S10. Conservation of H264^{7.29} and E169^{ECL2} in Adenosine receptor A_{2B}R across different species. Color-code and alignment method as in Figure S5.

sp	Q61618 AA3R_MOUSE	RTVTTQRRIWLFLGLCWLVSFLVGLTPMFGWNRKATLASSQNSSTLLCHFFSVVSLDYMV
sp	P28647 AA3R RAT	RTVTTQRRIWLFLGLCWLVSFLVGLTPMFGWNRKVTLELSQNSSTLSCHFRSVVGLDYMV
sp	002667 AA3R RABIT	RRVTTQRRIWLALGLCWVVSLLVGFTPMFGWNMKPTLESARNYSDFQCKFDSVIPMEYMV
sp	P35342 AA3R SHEEP	RRVTTQRRIWLALGLCWLVSFLVGLTPMFGWNMRLSSA-DENLTFLPCRFRSVMRMDYMV
sp	QOVC81 AA3R BOVIN	RRVTTORRIWLALGLCWLVSFLVGLTPMFGWNMKLSSA-DKNLTFLPCOFFSVMRMDYMV
sp	PODMS8 AA3R HUMAN	KRVTTHRRIWLALGLCWLVSFLVGLTPMFGWNMKLTSEYHRNVTFLSCOFVSVMRMDYMV
tr	U6CRM7 U6CRM7_NEOVI	RRVTTORRIWLALGLCWLLSFLVGLTPMFGWNMKLTSEYDRNVTFLSCOFFSVMRMDYMV
sp	Q28309 AA3R_CANLF	RRVTTORRIWLALGLCWLVSFLVGLTPMFGWNMKLTSEHORNVTFLSCOFOSVMRMDYMV
tr	A0A0S7MIV4 A0A0S7MIV4_9TELE	KFNSSEKCMFINVITLNYLV
tr	A0A0S7KYU4 A0A0S7KYU4 9TELE	MSYLV
		:.*:*
sp	Q61618 AA3R MOUSE	FLFALCWLPLSIINFVSYFDVKIPDVAMCLGILLSHANSMMNPIVYACKIKKFKETYF
sp	P28647 AA3R_RAT	FLFALCWLPLSIINFVSYFNVKIPEIAMCLGILLSHANSMMNPIVYACKIKKFKETYF
sp	002667 AA3R RABIT	ALFAGCWLPLSIINCVTYFKCKVPDVVLLVGILLSHANSMMNPIVYACKIQKFKETYI
sp	P35342 AA3R_SHEEP	FLFALCWLPLSIINCILYFDGOVPQTVLYLGILLSHANSMMNPIVYAYKIKKFKETYL
sp	Q0VC81 AA3R_BOVIN	FLFALSWLPLSIINCIIYFNGEVPQIVLYLGILLSHANSMMNPIVYAYKIKKFKETYI
sp	PODMS8 AA3R_HUMAN	FLFALSWLPLSIINCIIYFNGEVPQLVLYMGILLSHANSMMNPIVYAYKIKKFKETYI
tr	U6CRM7 U6CRM7 NEOVI	FLFALSWLPLSIINCITYFHGEVPQIFLYLGILLSHANSMMNPIVYAYKIKKFKETYI
вр	Q28309 AA3R CANLF	FLFAFSWLPLSIINCITYFHGEVPQIILYLGILLSHANSMMNPIVYAYKIKKFKETYI
tr	A0A0S7MIV4 A0A0S7MIV4_9TELE	ALFALSWLPLHIMNCILLFTNHAIKIAFHIGISLSHANSAVNPVVYAFKIKR
tr	A0A0S7KYU4 A0A0S7KYU4_9TELE	TLFVVSWLPLHIMNIVVYFHGPDTVPYIAFYVGILLSHANSAVNPVVYAFKIKKIKTAYV

Figure S11. Conservation of H264^{7.29} and E169^{ECL2} in Adenosine receptor A₃R across different species. Color-code and alignment method as in Figure S5.

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