Table A: Position conservations (top conserved residue identities and their percentages), for residues discussed in the paper, across eucariotic class A GPCRs, from the curated GPCRdb server alignment (Isberg, 2014)

Generic residue numbering (class A)	Residue Conservation			
3.36	M=19%	G,L=12%	V,C=10%	F,S=8%
3.40	I=40%	V=24,3%	L=11,5%	A=5%
3.43	L=73%	I=10%	V=5%	F,G,H,T=1% each
3.44	T=24%	C=16%	A,V,L=12%	
6.40	V=35%	I=28%	L=15%	M=6%
6.43	V=30%	A=22%	L,I=11%	
6.44	F=71%	Y=6%	V=5%	L=3%
7.42	A=38%	G=22%	I=7%	S=10%
7.45	N=59%	S=15%	H=10%	
7.52	I=41%	L=32%	V=13%	Y,C=4% each
7.53	Y=85%	F=4%	L=3%	

To allow comparison of residues in equivalent position in both inactive and active receptors state of GPCRs, we used the GPCRdb numbering scheme (Isberg, 2016). To calculate the conservation of the residue analyzed in this article we obtained the multiple alignment from the GPCRdb and defined the percentage of conservation as (X/P)*100 where X is the residue and P the length of the multiple alignment. Plants are not included since GPCR in plants are not well characterized (Taddese, 2014). The alignment of 1618 non-olfactory (the inclusion of curated sequences of about 3000 olfactory receptors does not change the conservation values) class A GPCRs were obtained from the GPCRdb database. A total of 276 positions, spanning all the seven transmembrane segments were aligned.

Table B: Effects of mutations in residues involved in this article within class A GPCRs

Generic residue numbering (class A)	Receptor	Mutants	Effect	Reference
3.40	M1 muscarinic	V3.40A	Increased agonist affinity	Hulme (2013)
	M5 muscarinic	V3.40A	Increased agonist affinity	Hulme (2013)
	MOR136-1	S3.40A	Abolished agoinist binding	Ho (2015)
	Histamine H1R	I3.40A	Lower basal activity and lower agonist response	Sansuk (2011)
	Histamine H1R	I3.40G	Lower basal activity and lower agonist response	Sansuk (2011)
	TSHR	V3.40A	Constitutive activity	Duprez (1994)
3.43	S1P1	L3.43E/G	Abolished activation	Fujiwara (2007)
	B2AR	L3.43R/K/A	Increased basal activity	Tao (2000)
	M1 muscarinic	L3.43A	Constitutive activity	Lu (1999)
	TSHR	L3.43Q/N/R	Constitutive activity	Kosugi (2000), Nishihihara (2006), Trulzsch (2001)
	C5A	L3.43A	Constitutive activity	Baranski (1999)
	CB1	L3.43A	Constitutive activity ; elevated basal cAMP accumulation ; enhanced affinity for agonists and diminished affinity for inverse agonists.	D'Antona (2006)
6.40	C5A	V6.40A	Increased basal activity	Baranski (1999)
	M5 muscarinic	I6.40S	Constitutive activity	Spalding (1998)
	Histamine H1R	I6.40E/G/A/R/K/S	Constitutive activity	Bakker (2008), Sansuk (2011)
	Rhodopsin	M6.40Y	Constitutive activity	Deupi (2012)
	AT1R	I6.40T	Increased basal activity	Parnot (2000)
	Melanocortin-4 receptor	L6.40Q	Increased basal activity	Vaisse (2000)
	TSHR	L6.40F	Increased basal activity	Tonacchera (1998)
	Lutropin-choriogonadotropic receptor	L6.40A	Constitutive activity	Fanelli (2000)
	S1P1	V6.40A/T	Reduced activation	Fujiwara (2007)
	S1P1	V6.40L	Abolished activation	Fujiwara (2007)
6.44	M5 muscarinic	F6.44S/T/L	Constitutive activity	Spalding (1998)
	Rhodopsin	F6.44T/V	Increased basal activity	Han (2007)
	Rhodopsin	F6.44A	No significant change in basal activity	Han (2007)
	Rhodopsin	F6.44W	Decreased basal activity	Han (2007)

	A1B adrenergic	F6.44L	Increased basal activity	Greasley(2002)
	A1B adrenergic	F6.44Y	No significant change in basal activity	Greasley (2002)
	A1B adrenergic	F6.44A/G	Decreased basal activity	Greasley (2002)
	Ghrelin receptor	F6.44Y/L	Decreased basal activity	Valenti-Hansen (2012)
	Ghrelin receptor	F6.44A	Increased basal activity	Valenti-Hansen (2012)
	GPR119	F6.44A	Decreased basal activity; loss of agonist-induced response	Valenti-Hansen (2012)
	B2AR	F6.44A	Decreased basal activity; loss of agonist-induced response	Valenti-Hansen (2012)
	NK1	F6.44A	Decreased basal activity; loss of agonist-induced response	Valenti-Hansen (2012)
	GPR39	L6.44A/Y	Decreased basal activity	Valenti-Hansen (2012)
	GPR39	L6.44F	Decreased basal activity; loss of agonist-induced response	Valenti-Hansen (2012)
	TSHR	D6.44A/E/H/Y	Constitutive activity	Porcellini (1994), Parma (1997), Russo (1996)
7.45	adenosine	N7.45A	Eliminated the allosteric effects of modulators but had little effect on agonist binding.	Gao (2003)
7.52	TSHR	L7.52V	Increased basal activity	Liu (2015)
	A2bAR	V7.52A	Reduced agonist-induced activation	Russo (1999)
	Melanocortin-1	I7.52T	Reduced basal activity	Lubrano-Berthelier (2006)
7.53	V2 vasopressin	Y7.53F/A	Reduced ability to bind the G-protein	Venkatakrishnan (2016)

Table C: Effects of mutations in 7.52 corresponding, for non-class A GPCRs

Generic class A residue numbering	Class	Equivalent residue numbering	Receptor	Mutants	Effect	Reference
7.52	B1 TASTE2	7.56	PTHR-1 TAS2R38	I7.56R V296F	natural mutation generating Jansen's chrondrodysplasia; constitutive activity; change in activation of the receptor	Gensure (2001), Biarnes (2010)

MD simulation of M_2 muscarinic receptor: Setup of the system

mAchRs are expressed in the Pre-Frontal Cortex (Thiele 2013). Neuronal cells belonging to this region of the brain have a specific membrane composition (Soubias and Gawrisch 2012). Computational and experimental studies, also from our lab, showed that the composition of the membrane may influence the receptor behavior (Cao, Rossetti et al. 2015). Therefore, we built a model membrane of a neuronal cell located in the Pre-Frontal Cortex, consistent with the experimental measured composition (Table S5) (Soubias and Gawrisch 2012). All the simulations are performed with the M_2 receptor embedded in such a membrane. Notice that lipids with of concentration below 2% were not included. Their influence was considered negligible.

Lipids	Percentage
PhosphatidylCholine (PC)	16
PhosphatidylEthanolamine (PE)	16
PhosphatidylSerine (PS)	4
PhosphatidylInositol + Phosphatidic acid lipids (PI+PA)	2
Sphingomyelin	14
Cholesterol	48

Table S5: Membrane composition of a neuronal cell in the pre-frontal cortex from Ref. 19.

The CHARMM-GUI web server was employed in order to obtain a pre-equilibrated membrane (Jo, Kim et al. 2008). This is composed by 192 lipids embedded in a 110 x 110 x 30 $Å^3$ long box.

All the available structures of mAchRs lack the so-called ICL3 loop, a large cytoplasmic loop (about 130 residues). The latter might play a role in the activation mechanism of the receptor (Bernstein, Ramineni et al. 2004). ICL3 structural ensemble was predicted here by ab-initio modeling (Rodriguez, Chinea et al. 1998) because of the unavailability of reliable structural templates. Specifically, we used I-Tasser web server suite (Roy, Kucukural et al. 2010).

The AMBER99SB-ILDN force fields (Best and Hummer 2009), the Slipids (Jämbeck and Lyubartsev 2012, Jämbeck and Lyubartsev 2012), the TIP3P (Jorgensen, Chandrasekhar et al. 1983) force fields were used for the protein and ions, the lipids, and the water molecules respectively. The General Amber force field (GAFF) parameters (Wang, Wolf et al. 2004) were used for the ligands, along with the RESP atomic charge using Gaussian 09 (Frisch, Trucks et al. 2009) with the HF-6-31G* basis set (Wang, Cieplak et al. 2000, Case, Cheatham et al. 2005). MD simulations were performed using Gromacs v4.6.7 package (Van Der Spoel, Lindahl et al. 2005). MD simulation was conducted in the NPT ensemble (constant pressure and temperature) under periodic boundary conditions. A rectangular box

of 90 x 90 x 152 $Å^3$ along the x, y and z direction respectively has been used for M_2 in complex with antagonist and a 91 x 91 x 147 $Å^3$ box for M_2 in complex with agonist. The number of atoms for the latter is 128,867 and 127,733 for the M_2 in complex with the antagonist. Constant temperature and pressure conditions were achieved via independently coupling protein, lipids, solvent and ions to Nosè-Hoover thermostat (Hünenberger 2005) at 300 K and Andersen-Parrinello-Rahman Barostat (Parrinello and Rahman 1981) at 1 atm. The Particle Mesh Ewald method (Darden, York et al. 1993) was used to treat the long-range electrostatic interaction with a real space cutoff of 1.2 nm. A 1.2 nm cutoff was used for the short-range non-bonded interaction. A time step of 2 fs was set. The LINCS algorithm (Hess, Bekker et al. 1997) was applied to constrain all bonds involving hydrogen atoms. The final systems, antagonist and agonist, were then simulated for 190ns and 200ns, respectively.

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