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Harnessing Ion-Binding Sites for GPCR Pharmacology

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Abstract—Endogenous ions play important roles in the function and pharmacology of G-protein coupled receptors (GPCRs). Historically the evidence for ionic modulation of GPCR function dates to 1973 with studies of opioid receptors, where it was demonstrated that physiologic concentrations of sodium allosterically attenuated agonist binding. This Na⁺-selective effect was distinct from effects of other monovalent and divalent cations, with the latter usually counteracting sodium's negative allosteric modulation of binding. Since then, numerous studies documenting the effects of mono- and divalent ions on GPCR function have been published. While ions can act selectively and nonselectively at many sites in different receptors, the discovery of the conserved sodium ion site in class A GPCR structures in 2012 revealed the unique nature of Na⁺ site, which has emerged as a near-universal site for allosteric modulation of class A GPCR structure and function. In this review, we synthesize and highlight

recent advances in the functional, biophysical, and structural characterization of ions bound to GPCRs. Taken together, these findings provide a molecular understanding of the unique roles of Na⁺ and other ions as GPCR allosteric modulators. We will also discuss how this knowledge can be applied to the redesign of receptors and ligand probes for desired functional and pharmacological profiles.

Significance Statement—The function and pharmacology of GPCRs strongly depend on the presence of mono and divalent ions in experimental assays and in living organisms. Recent insights into the molecular mechanism of this ion-dependent allostereism from structural, biophysical, biochemical, and computational studies provide quantitative understandings of the pharmacological effects of drugs in vitro and in vivo and open new avenues for the rational design of chemical probes and drug candidates with improved properties.

I. Historical Overview

Endogenous ions are involved in all aspects of human biology, including their key roles in the function and pharmacology of GPCRs, which comprise the largest family of clinically relevant protein targets (Lagerström and Schiöth, 2008; Katritch et al., 2013; Hauser et al., 2017). GPCRs signal both at the plasma membrane and

in intracellular membranes, including endosomes and golgi (Calebiro et al., 2010; Irannejad et al., 2013; Vilardaga et al., 2014; Godbole et al., 2017; Eichel and von Zastrow, 2018), and are likely exposed to large spatiotemporal variations in ionic and pH conditions that may affect their function. Thus, for instance, extracellular Na⁺ is normally maintained in 135–145 mM range, while its intracellular levels are about 10 times

ABBREVIATIONS: A_{2A}R, A_{2A} adenosine receptor; BLT1, leukotriene B4 receptor 1; cAMP, cyclic adenosine monophosphate; CaSR, calcium sensing receptor; CB1, cannabinoid receptor type 1; CCR2, C-C chemokine receptor type 2; CLTR1, cysteinyl leukotriene receptor 1; CPMG, Carr Purcell Meiboom and Gill NMR; DOR, delta opioid receptor; DRD2, dopamine receptor D2; DRD3, dopamine receptor D3; DRD4, dopamine receptor D4; EC, extracellular; ECL, extracellular loop; ECL2, extracellular loop 2; EM, electron microscopy; EP3, prostaglandin E receptor subtype EP3; ET_A, endothelin ET_A receptor; ETB1, endothelin ETB1 receptor; GPCR, G-protein-coupled receptors; HMA, 5-(N,N-hexamethylene)amiloride; MD, molecular dynamics; M2R, muscarinic acetylcholine receptor 2; MOR, μ -opioid receptor; Na⁺, sodium ion; NAM, negative allosteric modulation; NMR, nuclear magnetic resonance; NTS, neurotensin receptor; NTSR1, neurotensin receptor type 1; NTSR2, neurotensin receptor type 2; PAM, positive allosteric modulation; PAR1, protease-activated receptor 1; PAR2, protease-activated receptor 2; μ -OR, μ -opioid receptor; 5HT_{2B}, 5-hydroxytryptamine receptor 2B; 7TM, 7 transmembrane.

lower in most cells (Lodish et al., 2000); intracellular sodium levels rapidly increase during depolarization in neurons. Also, some GPCRs are directly (Wingler et al., 2019) and selectively modulated by inorganic ions as a part of their physiologic function, e.g., CaSR by Ca^{2+} (Silve et al., 2005) and GPR39 by Zn^{2+} (Sato et al., 2016). Other GPCRs are proton sensing, including GPR68, GPR4, TDAG8, and G2A (Ludwig et al., 2003; Radu et al., 2005; Yang et al., 2007; Liu et al., 2010; Huang et al., 2015b). In this review though, we will mostly focus on the function of endogenous ligands, and therapeutic drugs, being allosterically modulated by ions interacting with GPCRs.

Historically, the first evidence for ionic modulation of GPCRs dates well before they were recognized as a large family of receptors sharing a common seven-transmembrane (7TM) architecture. In 1973, studies of opioid receptors showed that agonist binding is negatively modulated by monovalent cations like Na^+ (Pert et al., 1973; Pert and Snyder, 1974), while being positively modulated by divalent cations (Pasternak et al., 1975). Several subsequent studies provided biochemical data suggesting that these effects were mediated by an allosteric mechanism (Simon and Groth, 1975; Horstman et al., 1990). A similar negative allosteric modulation of agonist binding affinity was soon discovered for many other class A GPCRs including adrenergic (Tsai and Lefkowitz, 1978), dopaminergic (Neve, 1991;

Neve et al., 1991) and somatostatin (Kong et al., 1993) receptors. Since then, hundreds of papers have appeared documenting the actions of sodium, as well as other cations and anions on the function of many GPCRs [see Katritch et al. (2014) and (Strasser et al., 2015) for review].

Moreover, high-resolution structural information for GPCRs and their complexes, which has emerged in the past few years (Liu et al., 2012b; Fenalti et al., 2014; Miller-Gallacher et al., 2014; Wang et al., 2017) has made it possible to identify a variety of ion binding sites in GPCRs (Fig. 1; Table 1). While some of the ions, like the multiple Zn^{2+} and Hg^{2+} ions in rhodopsin structures were introduced to assist crystallization and/or anomalous diffraction phasing (Teller et al., 2001), many other ion binding sites may be relevant for endogenous ligand binding at specific receptors. For example, the crystallographically observed PO_4^{3-} site in H1 histamine receptor (Shimamura et al., 2011) or Na^+ binding in the extracellular loop in the $\beta_1\text{AR}$ adrenergic receptor (Miller-Gallacher et al., 2014).

Only the sodium site, however, stands out as highly conserved among most class A GPCRs (Katritch et al., 2014) and potentially tracing its origin to other distant 7TM relatives like prokaryotic channel rhodopsins (Shalaeva et al., 2015). This site binds allosteric sodium in the middle of the 7TM helical bundle of class A GPCRs (Fig. 2, A and B), anchored at the most

Fig. 1. Ions identified in GPCR crystal structures. (A) Monovalent ions: Na^+ (blue) and Cl^- (green). (B) Polyvalent ions Zn^{2+} (magenta), Hg^{2+} (cyan), PO_4^{3-} (phosphate colored green), SO_4^{2-} (sulfur colored yellow). GPCR structures shown as gray cartoons.

TABLE 1

Receptor	PDB code	Resolution Range, Å	Ion	Site
A _{2A} AR	4EIY, 5IU4, 5IU7, 5IU8, 5IUA, 5IUB, 5K2A, 5K2B, 5K2C, 5K2D, 5MZJ, 5MZIP, 5N2R, 5NLX, 5NM2, 5NM4, 5OLG, 5OLH, 5OLV, 5OLZ, 5OM1, 5OM4, 5VRA	1.7–2.8	Na ⁺	D2.50, conserved, tight binding ^d
DRD4	5WIV	2.1	Na ⁺	D2.50, conserved, tight binding
δ-OR	4N6H, 4RWD	1.8, 2.7	Na ⁺	D2.50, conserved, tight binding
PAR1	3VW7	2.2	Na ⁺	D2.50, conserved, tight binding
PAR2	5NDD	2.8	Na ⁺	D2.50, conserved, tight binding
CLTR1	6RZ4, 6RZ5	2.5–2.7	Na ⁺	D2.50, conserved, tight binding
β1AR	3ZPR, 4BVN, 5A8E	2.1–2.7	Na ⁺	D2.50, conserved, tight binding
β1AR	2VT4, 2Y00, 2Y02, 2Y03, 2Y04, 2YCW, 3ZPQ, 3ZPR, 4AMJ, 4BVN, 5A8E, 6H7J, 6H7K, 6H7L, 6H7M, 6H7N, 6H7O	2.1–3.1	Na ⁺	ECL2, backbone, tight binding
β2AR	4LDE, 4LDL	2.8, 3.1	Na ⁺	ECL2, backbone, tight binding
SMO, class F	5L7D	3.2	Na ⁺	Loose binding
A _{2A} AR	5OM1	2.1	Cl [−]	Helices VI and VIII (H230, R293), loose binding
PAR1	3VW7	2.2	Cl [−]	Helices II, III, VI, (K135, R200, K307), loose binding
Rhodopsin	1GZM, 1F88, 1U19, 1L9H, 1HZX, 2HPY, 2G87, 2PED, 3C9L, 3OAX	2.2–3.0	Zn ²⁺	Additive in crystallization protocols. Loose binding in multiple sites.
PAF	5ZKQ	2.9	Zn ²⁺	Helices I, VI, VII (H4, H8, E259, H268), tight binding
SMO, class F	4QIM, 4N4W	2.6, 2.8	Zn ²⁺	Loose binding
M2R	5YC8	2.5	Hg ²⁺	Used as anomalous scatterers for phasing. Loose binding in multiple sites.
Rhodopsin	1U19, 1L9H, 1HZX, 1F88, 2PED, 2G87, 2HPY, 3OAX	2.2–3.0	Hg ²⁺	Loose binding
5HT _{2B}	6DRY, 6DRZ	2.9, 3.1	PO ₃ [−]	ICL2 and Helix III, tight binding
DRD4	5WIU, 5WIV	2, 2.1	PO ₃ [−]	7TM bundle, ECL, (K179, K191, Y431, H450), tight binding
H1R	3RZE	3.1	PO ₄ ^{3−}	Loose binding
μ-OR	5C1M	2.1	PO ₄ ^{3−}	Loose binding
A _{2A} AR	3EML	2.6	SO ₄ ^{2−}	Loose binding
CB1	5U09	2.6	SO ₄ ^{2−}	Loose binding
CCR2	5T1A	2.8	SO ₄ ^{2−}	Helix VIII, ICL1, tight binding
ETB1	5X93, 5GLI	2.2, 2.5	SO ₄ ^{2−}	Inside 7TM, intracellular (R199, R208, K210), tight binding, common ^b
PD2	6D27, 6D26	2.7, 2.8	SO ₄ ^{2−}	Inside 7TM, intracellular (R143, R2243), tight binding, common ^b
EP3	6M9T	2.5	SO ₄ ^{2−}	+ Other loose binding sites
Rhodopsin	3PQR, 4I4Q, 4PXF, 5DYS, 5EN0, 5TE3	2.3–2.9	SO ₄ ^{2−}	Loose binding
β2AR	2RH1, 5D5A	2.4, 2.5	SO ₄ ^{2−}	Inside 7TM, intracellular (T68, R131), tight binding, common ^b
μ-OR	4DKL	2.8	SO ₄ ^{2−}	VI, VII (K270, K273, R328), tight binding Inside 7TM, intracellular (T103, R179), tight binding, common ^b Other six loose binding sites

^aDirectly coordinated by two or more ionic interactions with charged residues.
^bSO₄²⁻ bound at a common intracellular site in several receptors.

Fig. 2. Conserved Na⁺ sites in GPCRs. (A) GPCR superfamily tree with blue circles highlighting receptor structures with Na⁺ resolved in the conserved pocket and red dots marking GPCR with established allosteric effect of Na⁺. (B) Overview of the Na⁺ position in 7TM helical bundle. (C) Type I Na⁺ site, first discovered in A_{2A}R (green cartoon and sticks) and conserved in majority of Class A GPCRs structures (gray). (D) Distinct coordination of Na⁺ by two acidic residues D^{2.50} and D^{7.49} in Type II sodium site as resolved in PAR1 (cyan), PAR2 and CLTR1 (gray) in δ -branch GPCRs.

conserved aspartate residue D^{2.50} (superscript shows generic numbering of GPCR residues as described in Isberg et al. (2015)). Analysis of the structures and sequences of class A GPCRs revealed the highly conserved nature of the sodium pocket, 15 residues of which are conserved exactly in 45 diverse receptors and with minor variations in a vast majority of class A GPCRs families (Fig. 2, C and D) (Katritch et al., 2014). Moreover, those class A receptors that lack the key residues of the sodium site naturally, or via introduced

mutations, have their ligand-induced signaling dramatically reduced or completely abolished (Katritch et al., 2014; Massink et al., 2015; White et al., 2018). While our understanding of ion binding sites and their biochemical and physiologic effects on GPCR signaling has greatly expanded in the last few years, we are only beginning to understand the new possibilities for harnessing this knowledge for the discovery of safer and more efficient drugs that have improved subtype and/or functional selectivity (Roth, 2019).

II. Structural Data for Conserved and Nonconserved Ion Binding Sites in G-Protein-Coupled Receptors

A. Conserved Sodium Binding Site in Class A G-Protein-Coupled Receptors

1. High-Resolution Structures of Sodium in the Conserved Site. Sodium ion in the conserved sodium pocket was first crystallographically identified in the A_{2A} adenosine receptor (Liu et al., 2012b), quickly followed by PAR1 thrombin (Zhang et al., 2012), β_1 AR adrenergic (Miller-Gallacher et al., 2014), δ -OR opioid (Fenalti et al., 2014), and D4 dopamine receptors (Wang et al., 2017). Remarkably, despite these structures representing receptors in different major branches of class A GPCRs and having low sequence identity between them (20%–35%), the sodium-binding positions in the structures were found to be almost identical (within 0.5–1.5 Å) with all of them anchored at the negatively charged D^{2.50} side chain. Moreover, the sequence of all 16 residues lining the sodium binding pocket and their conformations in receptor structures are remarkably conserved either in the whole class A GPCR or its individual branches (Table 2). The unprecedented level of conservation of the Na⁺ pocket as a structural feature was also emphasized by the fact that the positions of up to 10 water molecules in the pocket comprising Na⁺/water cluster were found conserved between such distant receptors as A_{2A}, β_1 AR, and δ -OR. Such a high level of sequence and structural conservation implied a critical functional role of this Na⁺ site in class A GPCRs (Liu et al., 2012b; Katritch et al., 2014). It is important to note, also, that the sodium pocket lies in close proximity and, in most structures of class A GPCRs, is directly connected to the orthosteric pocket making

it potentially accessible to ligand design, as discussed below in *section IV*.

A number of high-resolution structures have been more recently obtained (see Table 1), shedding light on the Na⁺ pocket and revealing new features of the Na⁺ binding site. Thus, more than 20 additional structures of antagonist-bound complexes for each of the A_{2A} adenosine and β_1 adrenergic receptors show that the Na⁺/water cluster can be reliably resolved in GPCR structures at up to about 2.3 Å resolution. Among them, the X-ray free-electron laser crystal structure of A_{2A}R (1.96 Å resolution) is especially important (Batyuk et al., 2016), because it was determined at room temperature. This structure demonstrated that existence of a well-defined conformation of Na⁺/water in the pocket detected in crystal structures of many GPCRs was not an artifact of cryo-freezing, but rather a result of the unique stability of the cluster itself.

Key insights were obtained from the antagonist-bound dopamine receptor D4 (DRD4) high-resolution structure (Wang et al., 2017), which was determined both with and without sodium. Importantly, an electron density for sodium was observed only when Na⁺ (~200 mM) was added during crystallization, thus providing the most direct structural evidence for Na⁺ in its binding site. Remarkably, even though another, the sodium-free structure of DRD4, was slightly higher resolution, the electron densities for water molecules forming the Na⁺/water cluster disappeared, showing that Na⁺ is critical for the stability of the whole cluster. Indeed, water clusters in polar pockets can usually dynamically form many combinations of their hydrogen bonding network, which compromise detection of individual water molecules. In contrast, the presence of the Na⁺ with a strong ionic bridge to D^{2.50} creates a specific configuration of the whole cluster, characterized

TABLE 2
Residue conservation in the sodium pocket for different branches of Class A GPCRs.

Numbers show % conservation, also highlighted by color – from low (white), to medium (yellow), to green (high).

Position	1.50	1.53	2.46	2.47	2.49	2.50	3.35	3.39	3.43	6.44	6.48	7.45	7.46	7.49	7.50	7.53
α -branch (90)	N	V	L	A	A/S/T	D	F/C/L/S	S	L	F	W	N	S	N/D	P	Y
	100	83	88	79	58	97	26	84	81	89	92	82	77	82	93	93
					17		20							17		
					17		18									
							18									
β -branch (38)	N	V/L	L	A/S	S/A	D	S	S/T	L	F/Y	W	N/S	S/T	N	P	Y/L
	92	47	95	76	45	92	61	71	71	76	89	47	61	87	92	74
		24		16	32			24		21		42	18			18
γ -branch (65)	N	V	L	A	A/S	D	N/S	S/G	L/I	F	W	N/H	S/C	N	P	Y
	97	86	97	88	71	94	61	71	67	86	88	55	64	95	97	97
					23		18	17	21			35	30			
δ -branch (66)	N	A/V	L	A	A/S	D	N	S/G	L	F	F	N	S/C	D/N	P	Y
	98	42	85	57	65	91	52	72	82	62	60	80	49	57	94	94
		38			23			18					23	34		

by well-defined electron densities, as observed previously in A_{2A}, β₁AR, and δ-OR high-resolution structures. It is also worth noting that there was no significant difference in the receptor conformation itself between Na⁺-bound and sodium-free structures of DRD4, even in the sodium-coordinating pocket residues, suggesting that the presence of sodium ion does not “induce” any specific conformational macrostate of the receptor. Instead, the observed stabilizing role of Na⁺ is manifested in shifting equilibrium toward the same inactive state conformation as observed without sodium.

The recently solved structures of the PAR2 proteinase-activated receptor (Cheng et al., 2017a) and of the cysteinyl leukotriene receptor CLTR1 (Luginina et al., 2019) further confirm the presence of sodium in the δ-branch of GPCRs as was identified previously for PAR1 (Zhang et al., 2012). These receptors provide a distinct structure of the Na⁺ pocket (Fig. 2D; Table 2), where sodium is coordinated by two acidic residues, D^{2.50} and D^{7.49}, instead of only D^{2.50} in most other class A receptors, which have N^{7.49}. This double salt bridge coordination shifts the sodium position about 1.5 Å “down” along the polar channel and changes the overall Na⁺ coordination and conservation pattern compared with “classical” sodium pocket in α- and γ-branches of GPCRs.

2. Sodium Ion Detection Criteria. While divalent ions in crystal structures are often detected by their anomalous diffraction, monovalent ions including Na⁺ lack such anomalous diffraction. Reliable detection of monovalent ions like Na⁺ in the protein crystal structures is based on high resolution (usually <2.3 Å), the strong electron density in a potential Na⁺ ion position and the unambiguous detection of at least five oxygen (or potentially nitrogen) atoms that comprise the Na⁺ coordination shell. The sodium ion can be identified then by its 5-atom coordination geometry and short characteristic distances to the coordinating atoms (2.3–2.5 Å), as discussed in Liu et al. (2012b). These criteria help to differentiate Na⁺ from four-atom tetrahedral coordination of water molecules and characteristic water interaction distances (2.8–3.1 Å). At even higher resolution, e.g., <2.0 Å, the accuracy of measurement may also be sufficient (Cheng et al., 2017a) to differentiate Na⁺ coordination distances from those of other monovalent ions, e.g., longer distances for K⁺ (2.6–2.8 Å) and shorter for Li⁺ (1.9–2.1 Å) (Kuppuraj et al., 2009), thus specifically detecting Na⁺.

It is important to note that the high structural stability of the protein and of the Na⁺/water cluster is as important for detection of Na⁺ as the resolution of the structures. Thus, it was possible to reliably resolve Na⁺ in PAR2 even at somewhat lower 2.8 Å resolution, because Na⁺ was coordinated by five oxygen atoms of the protein side chains, including two from the charged carboxy groups of D^{2.50} and D^{7.49}. At the same time, in some higher resolution structures, for example OX₂R

(PDB: 5WQC, resolution 1.96 Å), allosteric sodium was apparently absent from the conserved pocket (Suno et al., 2018a). Though some waters of the sodium pocket were resolved, the density for sodium and a neighboring position interacting with D^{2.50} were not well defined, precluding Na⁺ detection. This is not surprising, as the Na⁺ concentration used for this crystallization was 120 mM, which is in the range close to EC₅₀ of Na⁺ in some receptors (e.g., ~100 mM for DRD4), and thus may not allow the full saturation required for Na⁺/water cluster stability and detection. Therefore, the lack of sodium density in this structure does not necessarily mean that the fully conserved Na⁺ pocket in OX₂R does not bind sodium, but rather that it could not be detected crystallographically under the conditions used. Further studies of Na⁺ in OX₂R, including validation of the classic Na⁺ effect on agonist binding, may be needed to answer this question more definitively (Suno et al., 2018a).

All structures with bound Na⁺ so far were resolved by crystallography, although it is possible that structural information about sodium pocket may also come in the future from cryo-EM studies. The best GPCR structures by cryo-EM have been solved at ~3.0 Å resolution (Zhao et al., 2019), but the rapid progress in the cryo-EM field and detection of soluble protein structures at resolutions as high as 1.8 Å (Merk et al., 2016) suggests that this crystal-free technology may ultimately allow deciphering the sodium cluster and other ion binding details as well.

3. Lower Resolution Inactive State Structures of Class A Compatible with Sodium Ion Binding. In many other crystal structures of diverse class A GPCRs where the modest resolution (2.4–2.9 Å) was insufficient reliably to resolve sodium ion, the conserved pocket is still fully compatible with sodium presence in the structure.

- In the α-branch of class A GPCRs, this includes A₁ adenosine (Cheng et al., 2017b) and most of the aminergic GPCR structures, for example, β₂AR (2RH1), D₃R (3PBL) that have closely related subtypes with Na⁺ explicitly determined crystallographically.
- In the γ-branch, where sodium was only resolved in high-resolution structure of delta opioid receptor (DOR) so far (Fenalti et al., 2014), the pockets are fully conserved and compatible with Na⁺ binding in structures of all other opioid receptors including mu (MOR) (Manglik et al., 2012), kappa (KOR) (Wu et al., 2012) and nociceptin (NOP) (Thompson et al., 2012) opioid receptors.
- In the β-branch, the NTSR1 neurotensin receptor was previously characterized as having a sodium binding site (White et al., 2012; Krumm et al., 2015), although Na⁺ was not crystallographically resolved in the NTSR1-agonist complex as it

represented a partially active-like state. An interesting observation was made recently for another β -branch GPCR, the ET_B endothelin receptor (Shihoya et al., 2017). Though a weak allosteric sodium effect was detected in the ET_B receptor, it was above the physiologic sodium concentration (>1 M), probably owing it to the fact that one of the key residues of the otherwise conserved sodium pocket, $Y^{7.53}$, was replaced by $L^{7.53}$ in the ET_B receptor. Correspondingly, the crystal structure of ET_B solved at relatively high resolution 2.2 Å had the cavity filled with electron densities that were more compatible with water molecules than with sodium. Intriguingly, the closely related ET_A has much stronger Na^+ binding at $EC_{50} = 245$ mM, and a high-resolution ET_A structure might provide new insights for this family.

4. Structures of Active State G-Protein-Coupled Receptors Are Incompatible with Sodium Binding. Comparison of the sodium pocket conformation in inactive- and active-state structures of A_{2A} , β_1AR , muscarinic, 5-HT, and opioid receptors (Fig. 3) reveals that the sodium pocket shape, conformation, and interaction network change dramatically upon activation,

resulting in a partially collapsed pocket that is not compatible with high-affinity binding of sodium (Katritch et al., 2014). In general, active state conformations are characterized by an inward movement of the TM7 backbone, which directly clashes with the sodium site and rearranges sodium-coordinating side chains so that they form direct hydrogen bonds instead of Na^+ mediated (e.g., between $D^{2.50}$ and $S^{3.39}$) that preclude sodium coordination (Fig. 3C). Other major activation related changes, like the outward movement of TM6, also change the shape of the Na^+ pocket and disrupt the so-called hydrophobic layer (Yuan et al., 2014). Disruption of the hydrophobic layer, comprising residues 1.53, 2.46, 3.43, 7.53 at the bottom of the Na^+ pocket, opens the floodgate for water and sodium ion egress toward the intracellular side.

Such conformational changes in active-like states were described recently for NTSR1 (White et al., 2012; Krumm et al., 2015) and AT2R (Zhang et al., 2015b, 2017) in structures of complexes with agonists, as well as the structures of fully active MOR (PDB: 5C1M) (Huang et al., 2015a) and KOR (PDB: 6B73) (Che et al., 2018) bound to both agonists and nanobodies. The MOR structure is especially important in this respect because

Fig. 3. Conformational changes in the sodium pocket upon activation of opioid receptors. (A) Superimposition of high-resolution structures of δ -opioid receptor in inactive state (green, PDB: 4N6H, resolution 1.8 Å) and μ -opioid receptor in active state (orange, PDB: 5C1M, resolution 2.1 Å); conformational changes shown by arrows. (B) Close up of sodium pocket in inactive state. (C) Close up of the pocket in active state. Hydrogen bonds and salt bridges are shown by cyan dotted lines.

it was solved at a high 2.1 Å resolution. A detailed examination of the pocket structure reveals no electron density suitable for Na⁺, and in general, the active state conformation is incompatible with Na⁺ binding (Huang et al., 2015a).

It should be noted, that while the described above general conformational rearrangements are common to active state conformations, the details of newly formed interactions in the pocket can differ between the structures quite dramatically. This contrasts with the very conserved conformation of the residues in the structures of inactive GPCRs. This loss of uniformity can be explained by natural differences in the pocket between receptors, but also by different activation states (intermediate activated to fully active) and a range crystallographic resolution (from 2.1 to 3.5 Å) (Katritch et al., 2014).

5. Allosteric Ligands can Block Sodium Binding. Although the Na⁺ pocket is small, ~200 Å³ as estimated in A_{2A} (Liu et al., 2012b), it can bind small molecules like amiloride and its analogs, which have a common positively charged moiety connected to an aromatic ring (Fig. 4) (Liu et al., 2012b; Katritch et al., 2014). The allosteric binding of amiloride has been shown biochemically for several receptors, revealing direct competition with Na⁺ binding and a strong dependence on mutations in D^{2.50} and other pocket residues (Howard et al., 1987; Gao and Ijzerman, 2000; Gao et al., 2003a,b; Heitman et al., 2008; Gutiérrez-de-Terán et al., 2013; Massink et al., 2015) (Fig. 4B). Crystallographic observation of ligand binding in the Na⁺ pocket has been elusive, until recently the structure of leukotriene B₄ receptor BLT1 was solved in complex with a ligand

reaching into Na⁺ pocket (Hori et al., 2018). The bitopic ligand BIL260, spanning the orthosteric pocket and reaching all the way to the sodium binding anchor D^{2.50}, was characterized as an inverse agonist. This is an expected functional effect, as the ligand blocks the sodium site and precludes conformational rearrangements in the pocket, which are required for activation. The bitopic ligand comprised an orthosteric BTL1 selective moiety and a positively charged benzamidine group that forms a salt bridge to D^{2.50} in a manner similar to amiloride. Interestingly, the study also shows the benzamidine itself has a negative allosteric effect of on BLT1 activation with $K_B \sim 500 \mu\text{M}$, which is much weaker than K_B values reported for amilorides (Fig. 4B). This allosteric effect of benzamidine was also confirmed in the $\beta_2\text{AR}$, suggesting its potential effect in many other class A GPCRs with a similar Na⁺ pocket structure. A combination of orthosteric selectivity with a controlled allosteric sodium pocket functionality in bitopic ligands was suggested as a beneficial path for drug discovery, as discussed in section IV below.

6. Mutations Abolishing Sodium Binding. A central role of the Na⁺ site in activation-related conformational changes suggests that mutation in this site can modulate the stability of specific functional states. Moreover, by removing Na⁺ as a key “gear” in the transmission mechanism, the conformational space sampled by the receptor along the activation path is modified and can improve the thermostability of the receptor (Katritch et al., 2014). Indeed, several structures of GPCRs have been recently obtained with mutations in the sodium pocket that improved receptor thermostability.

Fig. 4. GPCR pockets for binding allosteric ligands that target conserved sodium binding pocket. Semitransparent surface shows orthosteric pocket (orange) and allosteric conserved sodium binding pocket (cyan). (A) Overview of the pockets in 7TMD. (B) Amiloride (magenta) bound to KOR in complex with selective antagonist 4-phenylpiperidine derivative JD1c (green) (PDB: 4DJH). (C) Benzamidine (yellow) bound to MOR in complex with irreversible agonist β -FNA (green) (PDB: 4DKL).

Specifically, a mutation in the Na⁺ anchor residue D^{7.49}N helped to crystallize and solve structures of P2Y1 receptor in complex with antagonists (PDB: 4XNV) (Zhang et al., 2015a), as well as P2Y12 complex with agonist (PDB: 4PXZ) (Zhang et al., 2014). Some of the established sodium-disrupting mutations (D^{2.50}N, S^{3.39}A, and D^{7.49}N) were included as knowledge-based transferrable mutations in the GPCR thermostabilization algorithm (Popov et al., 2018, 2019). Other mutations in the pocket show promise, e.g., introducing Arg in 3.39 position was theoretically predicted as stabilizing mutation (Yasuda et al., 2017) expected to block the Na⁺ pocket. This mutation recently helped to solve new structures of muscarinic acetylcholine receptor 2 (M2R) (Suno et al., 2018b) and EP4 prostaglandin receptor (Toyoda et al., 2019) in the inactive state.

Because stabilization by sodium pocket destruction usually comes at the expense of losing function, i.e., signaling response to agonists, we specifically studied the structural consequences of some of these mutations in a well-established system such as A_{2A} adenosine receptor (A_{2A}AR) (White et al., 2018). Mutations in sodium-coordinating positions D^{2.50}N and S^{3.39}A were introduced in A_{2A}AR and assessed both functionally and structurally. This study demonstrated robust improvement in thermostability for the D^{2.50}N mutation in the apo-, agonist-, and antagonist-bound receptor, supporting its broad importance. Although D^{2.50}N resulted in complete disruption of G-protein signaling mechanism, it retained a full affinity for antagonists, while even improving binding of agonist, as expected for GPCRs with decoupling mutations. Importantly, the crystal structures of A_{2A}AR D^{2.50}N and S^{3.39}A mutants in complex with agonist UK432097 were conformationally undistinguishable from the wild-type receptor in the same complex, with only minor local variations in the two to three residues directly interacting with the mutation site. This structural resilience to stabilizing mutations in the sodium pocket again suggests that such mutants can be used to facilitate GPCR crystallization (or improved cryo-EM resolution) with minimal disturbance to the resulting overall structure of the receptor.

B. G-Protein-Coupled Receptors that Lack the Conserved Sodium Site

1. Some Class A G-Protein-Coupled Receptors Lack Specific Sodium Site. A limited number of class A GPCRs lack the key polar residues in the sodium pocket and are apparently not suitable for the selective high-affinity binding of sodium. We estimated about 10%–30% of class A GPCRs lack specific Na⁺ binding in the conserved pocket, depending on the Na⁺ affinity cutoff. The most obvious 36 exceptions are listed in Supplemental Table S1 of our previous review (Katritch et al., 2014), including 1) visual rhodopsin and other opsins that lack conservation in the polar pocket,

2) GPCRs lacking D^{2.50} anchor that are known to lack ligand-induced signaling, some constitutively activated and some acting via dimerization with signaling sub-type, 3) some orphan and “putative” GPCR lacking D^{2.50} anchor where ligand signaling has not been established, or 4) receptors where lack of D^{2.50} anchor may be compensated by acidic Asp and Glu in positions 7.49 or 3.39 of the sodium pocket.

Several other interesting cases of receptors with rare deviations in the pocket, which result in dramatically reduced or abolished Na⁺ binding affinity have been studied more recently. Thus, in the NK1 neurokinin receptor (NK1R), the rare E^{2.50} carboxylic acid side chain, which is longer than the common D^{2.50}, was predicted to occupy the sodium position in this site and make direct interactions with conserved S^{3.39}, T^{7.46}, and N^{7.49} (Valentin-Hansen et al., 2015). This modeling prediction was recently confirmed by a 2.2 Å resolution structure of NK1R (Schöppe et al., 2019), which also shows that while E^{2.50} replaces Na⁺ in the site, the structure of water molecules in the pocket is remarkably conserved as in Na⁺/water cluster resolved in other GPCRs. It was hypothesized that by replacing mobile Na⁺ with direct and immobile carboxy side chain interactions of E^{2.50}, the receptor more tightly controls its basal signaling; indeed NK1R lacks appreciable basal activity. Intriguingly, while NK1R lacks Na⁺ binding and allosteric effects, both can be restored in the NK1R receptor by “reintroducing” D^{2.50}, as it is in other two NK receptors, NK2 and NK3 (Schöppe et al., 2019). The E^{2.50}D and other mutations in the Na⁺ pocket of NK1R also dramatically change the constitutive and biased signaling profile of NK1R, suggesting that evolution uses deviations from the canonical Na⁺ site as a way to modulate the functional properties of receptors.

Interestingly, another rare substitution of a small to larger side chain in 7.46 position of the pocket, e.g., S(T,A)^{7.46}N, is found in only two class A GPCRs including angiotensin AT1 receptor. Assessment of the sodium pocket structure of AT1R, including inactive (Zhang et al., 2015b, 2017) and active-like state (Wingler et al., 2019) structures, suggests that N^{7.46} side chain and its hydrogen bond interactions with N^{3.35} may interfere with sodium binding, replacing sodium as a conformational stabilizer and making the receptor insensitive to sodium concentration.

Another example of a GPCR structure with deviations in the sodium pocket incompatible with selective Na⁺ binding is the CCR5 chemokine receptor (CCR5R), which lacks two key sodium coordinating side chains in N^{3.35} and S^{3.39} positions, which are replaced by Gly instead. Indeed, while the inactive state structure of CCR5R in complex with an antagonist was solved at relatively high (2.2 Å) resolution, no density for Na⁺ binding was detected (PDB: 5UIW) (Zheng et al., 2017). The structural deviations in the allosteric pocket

that compromise Na^+ binding appear to be common to a group of other inflammatory chemokine receptors, suggesting that the switch in Na^+ pocket played a key evolutionary role in differentiating the chemokine receptor family into homeostatic (CXCR4-like) and inflammatory (CCR5-like) (Taddese et al., 2018) (see more discussion in section V.C). In general, establishing an accurate structure-activity relationship for the Na^+ pockets of all class A GPCRs is far from finished and will require a combination of computational modeling and experimental efforts.

2. Non-Class A G-Protein-Coupled Receptors Lack Conserved Sodium Sites in 7-Transmembrane Domains. Potential ion binding sites have been identified or proposed for non-class A GPCRs, both in their 7TM domains and soluble extracellular domains; however, they are structurally distinct from the conserved class A GPCR Na^+ site and appear to have different functional and evolutionary roles. In the class B 7TM domain, an allosteric Na^+ site was proposed by MD simulations in the glucagon receptor, with the ion coordinated by residues Glu362^{6.53b}, Asn238^{3.43b}, Tyr239^{3.44b}, and Tyr400^{7.53b}, although the predicted ion residence time in the binding site was very short (Selvam et al., 2018). Moreover, the site is conserved only in four class B receptors that have the key acidic Glu residue in 6.53b position, and the biologic significance of ion binding to class B GPCRs remains unclear. None of the class B structures have ions detected in their crystal structures that bind their 7TM or extracellular domains, even though many of the extracellular structures were solved at sub 2.0 Å resolution.

In Class C GPCRs, the internal cavity extends deep in the 7TM bundle reaching approximately the location of the class A Na^+ site. There are polar residues like Y659^{3.40c}, T781^{6.44c}, and S809^{7.45c} in this region that create a hydrophilic subpocket, and indeed a water molecule has been resolved in this position in the mGluR5 metabotropic glutamate receptor structure (mGluR5) solved at 2.2 Å resolution (PDB: 6FFI) (Christopher et al., 2019). This polar triad is conserved in seven of eight mGluR receptors (but not other class C), suggesting some role for a water binding site. However, the subpocket lacks any acidic residues compatible with specific binding of cations like Na^+ . None of the currently available class C structures have an ion resolved crystallographically, even at 2.2 Å resolution.

Similarly, in class F GPCRs, exemplified by the smoothened receptor structures (Wang et al., 2013) and the recently solved structure of apo FZD4 receptor (Yang et al., 2018), there is an extended polar channel in the 7TM domain, with water molecules coordinated by the conserved in class F residues Y262^{2.52a}, S317^{3.40a}, and Y444^{6.41a}. Again, none of the residues in the core of the 7TM has an acidic side chain, precluding specific ion binding in this region.

Some nonspecific binding ions have been detected in smoothened receptor (SMO) structures, though they are not likely to play a substantial functional role in these receptors.

C. Nonconserved Ion Binding Sites in G-Protein-Coupled Receptors

Sodium ions, as well as other single and polyatomic ions have been found in many crystal structures of GPCRs with sufficient resolution, as listed in Table 1. Thus, for the $\beta_1\text{AR}$ and $\beta_2\text{AR}$, in addition to the conserved sodium site, a second Na^+ ion was also identified in the ECL2, coordinated by three carboxy groups of the protein backbone and 2 water molecules. This tightly bound Na^+ , along with a disulfide bond formed by cysteines of the loop, apparently helps to stabilize the α -helix-loop structural motif in the ECL2 of these receptors, and thus apparently serves a structural role. This particular sequence and structural motif, however, can be found in only three receptors of β -AR subfamily and is not conserved in other GPCRs.

Other notable nonconserved sites include phosphate ion PO_4^{3-} in the ECL region of the H1 histamine (H1R) receptor (Shimamura et al., 2011). As described in this H1R structural paper, the PO_4^{3-} ion plays an important role in binding and selectivity of some of the ligands (see more discussion in section IV.C), but this site is unique for H1R.

Specific binding of Zn^{2+} has also been observed in the of PAF1 platelet activating receptor receptor extracellular loops tightly coordinated by three His and one Glu side chains with distances as low as 2.0–2.2 Å (Cao et al., 2018). This can explain Zn^{2+} induced inhibition of platelet activating factor binding to the receptor and physiologic reduction information of platelets (Nunez et al., 1989). Ions have been described as endogenous ligands to some of the GPCRs. Thus, Ca^{2+} ion is an endogenous agonist for eponymous CaSR calcium-sensing receptor (Chang et al., 2008; Hannan et al., 2018). CaSR is critical for many of the functions dependent upon the regulation of Ca^{2+} metabolism, including the parathyroid gland and bone development. The allosteric modulation by extracellular calcium has been studied extensively for another Class C GPCR family, the metabotropic glutamate receptors, where the Ca^{2+} site adjacent to the Glu site was predicted and biochemically characterized (Jiang et al., 2010, 2014). Most recently, Ca^{2+} was also revealed as an important allosteric modulator of Class B parathyroid hormone receptor signaling, and the structural determinants of the ion binding were proposed (White et al., 2019). Also, Zn^{2+} has been described as an endogenous agonist for the GPR39 receptor, for which the activation site and mechanism have been proposed (Storjohann et al., 2008; Sato et al., 2016). It would be very interesting to test these hypotheses to gain a better understanding of the atomistic mechanisms when the high-resolution structures of these receptors are available.

D. Nonspecific Ion Binding in Crystal Structures

Multiple ions have been found crystallographically in the intracellular region of the receptor (Fig. 1; Table 1); however, most of them have loose interactions with the receptor, suggesting nonspecific binding. As the intracellular region is enriched with positively charged Arg and Lys residues, it is not surprising that all of the ions identified in this region are anions, including Cl^- , PO_4^{3-} , and SO_4^{2-} . In most cases, the ions are coordinated by one or two positively charged side chains, although in general, the binding is rather loose and the ions remain highly exposed to solvent. Note also that these intracellular ion binding sites are not reproduced between receptors and, in most cases, not even between different subunits and different structures of the same receptor, so this binding is probably nonspecific and only identified due to the high concentration of these ions in crystallization conditions.

Some divalent cations like Zn^{2+} and Hg^{2+} have been also detected bound at the lipid interface of the 7TM bundle. Interestingly, in almost all these cases these metal cations have been found in structures of rhodopsin, reflecting specific crystallization conditions that used a high concentration of the ions. One other crystal structure with Hg^{2+} is a M2 muscarinic receptor structure (Suno et al., 2018b), where the ions are bound on the lipid interface of the 7TM domain and do not make any ionic or even substantial polar contacts, making their binding apparently nonspecific.

Many other ions present in high concentrations in crystallographic conditions are likely to be loosely and nonspecifically bound in GPCRs, but were not identified in crystal structures because they lack well-defined electron density. Their physiologic role is usually limited to nonspecific ionic strength effects at high concentrations. For example, some studies report a component of the Na^+ allosteric effects that are independent of $\text{D}^{2.50}$ mutation in H1R (Hishinuma et al., 2017). This is not surprising, given numerous charged residues in GPCRs, often in the orthosteric ligand binding pockets, for example, negative anchor residues in all aminergic opioid and positive phosphate binding residues in P2Y purinergic receptors. Nonspecific ion binding in ICL3 region may also directly modulate downstream effector binding and activation. Although most of these effects, including the overall ionic strength of the solvent, can manifest themselves only at high concentration of ions and unlikely involved in GPCR function, they need to be accounted in experiments by using appropriate controls.

III. Functional Role—Why is Sodium So Special for Class A G-Protein-Coupled Receptors?

Sodium is one of the most abundant ions in the human body, essential for cell energetics, homeostasis, neural function, and many other physiologic functions.

However, our understanding of Na^+ and its role in the physiologic processes involving GPCR signaling is only now starting to unfold.

A. Allosteric Effects of Sodium on Agonist Binding

1. “Classical” Allosteric Effect of Sodium Ion on Agonist Binding. As mentioned above, the selective sodium effect was originally discovered in opioid receptors as a negative allosteric modulation of (NAM) of agonist binding upon increasing sodium concentration (Pert et al., 1973; Pert and Snyder, 1974; Simon and Groth, 1975; Roth et al., 1981). This NAM effect in the μ -opioid receptor (μ -OR) correlated well with ligand efficacy, and for some time was the primary method for differentiating agonists from antagonists; for the latter, the effect was usually neutral or reversed (Pert and Snyder, 1974). This allosteric effect was observed for sodium within the physiologic concentration range of ~ 140 mM, and, in some cases, titration of the effect with sodium concentration allowed measurement of K_B or EC_{50} values as a proxy for Na^+ binding affinity. Importantly, the described above NAM effect on agonists was found specific for Na^+ , while showing much less magnitude for Li^+ and lacking for K^+ and larger monovalent cations (Pert and Snyder, 1974). The divalent cations like Mn^{2+} , Mg^{2+} , and Ca^{2+} displayed the opposite effect on agonist binding, although the effect was not specific (Pasternak et al., 1975). Since then, studies for numerous other class A GPCRs have demonstrated similar sodium allosteric effect on ligand binding, many of these studies also showing the specificity of Na^+ binding by mutations in the pocket and corresponding effects on signaling (for historical data see Table 1 in Katritch et al., 2014).

In the years since the structural detection of Na^+ in the highly conserved GPCR site, a resurgence of interest led to further validation and more detailed biochemical characterization of the sodium allosteric effects in these and many other class A GPCRs (Table 3). Thus, for adenosine A_{2A} receptor titration of the NAM effect of Na^+ on agonist NECA allowed estimation of its IC_{50} value at 44 ± 6 mM (Massink et al., 2015; White et al., 2018), while confirming the positive allosteric modulation (PAM) effect on antagonist ZM241385 (4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl)phenol). The NAM effect was drastically reduced by $\text{S}^{3.39}\text{A}$ and $\text{W}^{6.48}\text{A}$ mutations and completely abolished by $\text{D}^{2.50}\text{A}$, $\text{N}^{7.45}\text{A}$, and $\text{N}^{7.49}\text{A}$ mutations in the Na^+ pocket.

For dopamine receptors, NAM modulation of agonist binding by Na^+ was confirmed for the D4, D2, and D3 dopamine receptors (Michino et al., 2015; Wang et al., 2017), and titration of the effect on Na^+ concentration made it possible systematically to compare Na^+ affinities for several receptors. Thus, Na^+ showed lower affinities to dopamine receptors, $K_B \sim 100$ mM (D4), $K_B \sim 123$ mM (D2), and $K_B \sim 76$ mM (D3) compared

TABLE 3
Recent data on allosteric effect of Na⁺

Receptor	Allosteric Effect [Na ⁺]	Estimate of Na ⁺ Affinity	PDB ID (Na ⁺ in Structure)	Reference
DRD4	Agonist NAM, reduce basal activity	$K_B = 98 \text{ mM}$	5WIV (Na ⁺)	Wang et al. (2017)
DRD2	Agonist NAM, control of other allosteric ligands	$K_B = 123.1 \text{ mM}$	6CM4	Wang et al. (2017), Draper-Joyce et al. (2018)
DRD3	Agonist NAM	$K_B = 76 \text{ mM}$	3PBL	Wang et al. (2017)
MOR	Agonist NAM, control of other allosteric ligands	$K_B = 7.3 \text{ mM}$	4DKL	Wang et al. (2017), Livingston et al. (2018)
DOR	Agonist NAM, control of other allosteric ligands	$K_B = 24.3 \text{ mM}$	4N6H (Na ⁺)	Wang et al. (2017)
A _{2A} R	Agonist NAM	$K_B = 32.4 \text{ mM}$	4E1Y (Na ⁺)	Wang et al. (2017)
CLTR1	Receptor thermostability	$K_B = 39 \text{ mM}$	6RZ4, 6RZ5 (Na ⁺)	Luginina et al. (2019)
BLT1	Agonist NAM	$EC_{50} < 200 \text{ mM}^a$	5X33 (Na ⁺ site blocked by ligand)	Hori et al. (2018)
V1b	Agonist NAM, required for IP3 signaling	$EC_{50} < 50 \text{ mM}^a$	n/a	Koshimizu et al. (2016)
OXTR	Agonist NAM	$EC_{50} < 200 \text{ mM}^a$	n/a	Schiffmann and Gimpl (2018)
H1R	Agonist NAM	$EC_{50} < 100 \text{ mM}^a$	3RZE	Hishinuma et al. (2017)
NTSR1	Agonist NAM	$EC_{50} = 43 \text{ mM}$	4BUO	White et al. (2018)
ET _A	Agonist NAM	$EC_{50} = 245 \text{ mM}$	n/a	Shihoya et al. (2017)

^a Approximately estimated from Na⁺ titration curves in the referenced papers.

with K_B as high as 7.3 mM for MOR, 24.3 mM for DOR, and 32.4 mM for A_{2A}. As expected (see functional effect in *Section B.1*), constitutive activity of the D4 receptor significantly increased at low concentrations of Na⁺, the effect of which can be blocked by the addition of antagonists (Wang et al., 2017).

For the BLT1 leukotriene receptor, structural studies (Hori et al., 2018) were complemented by biochemical assays that revealed a pronounced negative allosteric effect of Na⁺ on agonist leukotriene B4 binding. Interestingly, the study also described similar effects for an allosteric small molecule benzamide that competes with Na⁺ for the sodium pocket binding.

For V1b vasopressin receptors in cell-based assays, a recent study shows that reducing the concentration of external Na⁺ to below 50 mM dramatically increased cell surface binding of radiolabeled agonist [³H]arginine vasopressin (Koshimizu et al., 2016). Interestingly, though agonist binding was increased, the receptor signaling and internalization were reduced in low Na⁺ concentrations. This is an important observation, suggesting that the functional effects of Na⁺ are not limited to NAM effect on agonist binding. Again, the biochemical and functional effects were selective for Na⁺ compared with Cs⁺ or NH₄⁺.

For the oxytocin receptor, while the endogenous agonist oxytocin was positively modulated by divalent ions like Mg²⁺, specific NAM effect of Na⁺ on oxytocin binding was detected at physiologic Na⁺ concentration (Schiffmann and Gimpl, 2018). Thus, the increase of Na⁺ concentration from 0 to 300 mM reduced oxytocin affinity ~15-fold, while no significant effect was observed for K⁺ or other monovalent ions.

For the H1 histamine receptor (Hishinuma et al., 2017), all three agonists studied showed expected NAM effect at 100 mM concentration of Na⁺. While a maximal NAM effect of Na⁺ was observed for histamine and other two agonists, a set of diverse antagonists showed a whole range of effects from NAM to PAM, with the most pronounced PAM found for the most efficacious second-generation antagonists (antihistamines). While these effects were largely abolished by D^{2.50}N mutation, residual D^{2.50}-independent effects were observed for some of the antagonists like fexofenadine, depending on their physicochemical properties. This observation emphasizes that the observed Na⁺ effects are often a combination of specific D^{2.50} pocket Na⁺ binding and nonspecific effects due to multiple low-affinity binding sites and charge screening effect in ligands and receptors.

For the muscarinic receptor subfamily, early studies suggested a classic Na⁺ effect in M2 muscarinic receptors (Rosenberger et al., 1980), recently corroborated by mutation studies (Suga and Ehlert, 2013) and molecular dynamics simulations in M3 muscarinic receptors (Miao et al., 2015). However, a strong nonspecific effect of ionic strength (Birdsall et al., 1979) and ionic

interactions in the orthosteric pocket may interfere with an accurate assessment of Na^+ selective binding in this subfamily.

In general, both the magnitude and affinity (e.g., K_B) of the sodium effect can vary dramatically between receptor and between ligands in the same receptor. As the systematic study for six different receptors shows (Wang et al., 2017), the magnitude of agonist potency change by Na^+ can exceed 100-fold for some receptors (e.g., MOR), while in other receptors is barely detectable at less than fivefold (DRD4). This may somewhat correlate with the K_B of the sodium effect, which is shown to be much higher for MOR than for DRD4.

Interestingly, a strong sensitivity to allosteric Na^+ was characterized recently for ligands that are allosteric modulators themselves. At the D2 dopamine receptor, the allosteric ligand SB269652 completely loses its modulatory effect in the absence of Na^+ ion (Draper-Joyce et al., 2018). Similarly, allosteric ligands effect in MOR was found to be controlled by Na^+ presence (Livingston and Traynor, 2014). The effect has been recently observed in other opioid receptors (Livingston et al., 2018), and the authors conclude that disruption of the Na^+ ion binding site may represent a common mechanism for allosteric modulation of class A GPCRs.

2. Binding of Sodium Not Always Detected by a “Classical” Allosteric Effect. While the negative allosteric modulation of agonist binding has been long considered a hallmark effect of Na^+ in some GPCRs, this effect may be much less pronounced and can easily go undetected in some cases, even when sodium is known to bind in their conserved pocket. Thus, sodium ion anchored by D^{2.50} has been resolved in high-resolution $\beta_1\text{AR}$ structure, revealing the same Na^+ /water cluster as in $\text{A}_{2\text{A}}\text{R}$ (Miller-Gallacher et al., 2014). But in contrast to $\text{A}_{2\text{A}}\text{R}$, any attempts to detect this NAM effect in $\beta_1\text{AR}$ have failed, suggesting that the $\beta_1\text{AR}$ adrenergic receptor lacks any observable dependence of agonist binding on Na^+ concentration. In the closely related $\beta_2\text{AR}$ (65% sequence identity), mutations D^{2.50}A or D^{2.50}N disrupting Na^+ site also failed to detect classic Na^+ NAM effect on agonist binding (Strader et al., 1988).

The lack of “classical” NAM sodium effects on GPCRs that actually bind Na^+ can arise from combination of several factors, such as 1) weak coupling between allosteric and orthosteric pocket conformations, which reduce the magnitude of the allosteric effect and 2) presence of nonspecific binding effects that can at least partially mask/compensate the specific NAM effect. The detection of the “classical” sodium effect can be further complicated in those cases where affinity of Na^+ binding in the specific pocket is low ($K_B > 100 \text{ mM}$), which makes it harder to differentiate from nonspecific ionic strength effect. Corroborating the first factor above, both β_1 - and β_2 -adrenergic receptors are well known to have very weak coupling between extracellular agonist binding and intracellular conformational changes,

reflected in their high basal activity ($\sim 20\%$ – 40%) and incomplete activation by endogenous ligands (Yao et al., 2009). This weak coupling is reflected also in structural studies, showing the agonist binding per se does not convert $\beta_1\text{AR}$ and $\beta_2\text{AR}$ to active (R^*) state (Rosenbaum et al., 2011; Warne et al., 2011), which needs G protein or arrestin binding for stabilization (Rasmussen et al., 2011). Importantly, this carefully documented case of absence of NAM effect of Na^+ on agonist binding in some GPCRs suggest that this “classical” effect, though most easily measurable in vitro, is not, in fact, essential to the functional role of sodium. The NAM effect on agonist binding is only part of the story and probably not the most biologically important part.

B. Evidence for the Functional Importance of the Sodium Ion Site

Although the most commonly documented effect of sodium presence in class A GPCRs is NAM, i.e., reduction of agonist binding and reduction in constitutive activity (Quitterer et al., 1996; Seifert and Wenzel-Seifert, 2001; Wang et al., 2017) there is a substantial evidence that physiologic sodium is actually required for efficient stimulation of the receptors in response to agonists. Indeed, as early as 1982, Cooper et al. (1982) noted the amplification effect of Na^+ on agonist-induced cAMP modulation in rat striatal plasma membrane. More recent studies are further corroborating this hypothesis, including both direct dependence of signaling on Na^+ concentrations and its displacement by an agonist, as well as indirect effects of mutations in Na^+ coordinating residues.

1. Direct Functional Effects of Sodium Ion Presence. Because of the potential interference of changing Na^+ concentration with signal transduction downstream from GPCRs, direct measurements of the Na^+ concentration effect on GPCR signaling are challenging. Nevertheless, several studies demonstrate that such dependencies can be detected in well-controlled assays. Thus in 1982, Cooper et al. (1982) were the first to note the amplification effect of Na^+ on agonist signaling via opioid receptors in rat striatal plasma membrane. Opioid receptors generally signal via the G_i pathway, which inhibits the production of cAMP. The authors found that the presence of Na^+ at 80 mM concentrations results in a dramatic increase of the cAMP inhibition effect of the morphine agonist, especially at high GTP concentrations.

Sodium effects on spontaneous opioid receptor GTPase activity and relative agonist efficacy were also studied by Costa et al. (1990, 1992), revealing “paradoxical difference in the way sodium ions affect GTPase activity and ligand binding.” Most importantly, they showed that buffer exchange from K^+ to Na^+ dramatically reduces basal GTPase activity while maintaining the activity of agonist DADLE, thus selectively amplifying the ligand-induced stimulation (Fig. 5A).

Fig. 5. Direct measurement of Na⁺ effect on GPCR signaling. (A) Exchange of K⁺ to Na⁺ leads to reduced basal activity, but maintains agonist-induced signal (Costa et al., 1990). (B) Addition of Na⁺ dramatically enhances differential between agonists and antagonists of G-protein signaling in MOR (Selley et al., 2000). (C) Addition of Na⁺ reduces basal activity of DRD4 (Wang et al., 2017). This figure reproduced with permission.

Selley et al. (2000) studied the Na⁺ effect on [³⁵S]-GTPγS binding in CHO cells stably transfected with MOR (mMOR-CHO cells) and in rat thalamus. In both systems, an increase of sodium concentration to physiologic levels (~140 mM) had a dual effect of 1) reduced basal G-protein signaling and 2) increased receptor stimulation by full agonists, but not partial agonists. In other words, while in the absence of Na⁺, stimulation by full and partial agonists was almost indistinguishable, increasing Na⁺ concentrations “magnified relative efficacy differences among agonists” (Fig. 5B).

In the recent study on DRD4 (Wang et al., 2017), which combines structural, biochemical, and functional assessment of the receptor, the authors showed that basal (constitutive) activity dependence on Na⁺ can be accurately measured. Thus, the study found that constitutive Gα_{i/o} activity at D4 receptor was dramatically (twofold) reduced at physiologic Na⁺ concentrations. The potentiation of DRD4 constitutive activity in low Na⁺ concentration can be abolished by selective DRD4 antagonist nemonapride, showing that the effect is entirely D4 receptor-mediated (Fig. 5C).

The aforementioned study of V1b vasopressin receptors (Koshimizu et al., 2016) also shows that agonist binding at high Na⁺ concentrations was reduced. However, IP₃ production assays showed that Na⁺ in the external buffer was required for signaling. Thus, in the NaCl-containing buffer, the agonist increased the IP₃ level from basal 6.6 ± 1.2 to 13.2 ± 0.7 nM in the stimulated receptor. In contrast, in the buffer without NaCl, the agonist-stimulated IP₃ levels were below the detection level (<1 nM). These biochemical and functional effects were selective for Na⁺ compared with Cs⁺ or NH₄⁺.

In general, all these results suggest that presence of sodium at physiologic concentrations both reduces the basal activity of receptors and enhances the stimulated response to agonist, thus selectively enhancing the overall efficacy of full agonists. The effects were selective to Na⁺, as shown in K⁺ replacement experiments. Importantly, when the studies were able to accurately titrate sodium allosteric effects on signaling, whether basal or ligand induced (Costa et al., 1990; Selley et al., 2000; Wang et al., 2017), the Na⁺ response curves show

K_B or EC₅₀ values in the range of ~10–200 mM, approximately corresponding to the affinity of Na⁺ in the conserved sodium pocket of these receptors.

2. Mutations in Sodium Ion Pocket Reduce or Abolish Receptor Stimulation by Agonists. A substantial body of evidence for the functional role of Na⁺ comes from indirect studies showing the dramatic impact of mutations in the sodium coordinating residue's classic allosteric binding effect and on signaling function of at least 20 class A GPCRs, as presented in Table 1 of our previous review (Katritch et al., 2014). Several recent studies corroborate these observations, suggesting that removal of Na⁺ site via mutations often has similar consequences as removal of Na⁺ itself from solution. Thus Massink et al. (2015) show that while in A_{2A} adenosine receptor mutations in sodium-coordinating residues S^{3.39} and N^{7.45} reduce or abolish classic dependence of agonist binding on Na⁺, they also increased basal activity and reduced the maximal activity (E_{max}) of the agonist-induced signal. These mutations result in about a fivefold reduction of total cAMP response to ligand compared with the wild-type signal. Importantly, the mutations did not reduce but even slightly improved EC₅₀ values of agonists in these assays, which is similar to improved agonist affinities in lieu of Na⁺. In the case of the D^{2.50}N(A) mutations (Massink et al., 2015; White et al., 2018) in adenosine A_{2A}AR, however, any cAMP activity (basal or induced) of the mutants was disrupted, suggesting that in addition to the Na⁺ anchoring role, D^{2.50} has other roles in the receptor activation, which may also be related to dynamic change in its protonation state (Vickery et al., 2018). A similar effect was recently observed for GPR3, where D^{2.50}A mutation in a recently characterized sodium site completely abolished signaling (Capaldi et al., 2018).

Interestingly, constitutively active mutants have been also observed in a number of class A GPCRs in position 3.43 at the bottom of the sodium pocket, where conserved hydrophobic residues Leu (~74% receptors) or Met (~20%) comprise a hydrophobic layer, keeping the gate closed to the waters and Na⁺ ion escaping toward the intracellular side (Yuan et al., 2014). One of the studies showed that replacing Leu or Met with any small or polar group in 3.43 position (Arg, Lys, or Ala)

all resulted in constitutive activation in thyrotropin receptor (TSHR), but also in β_2 AR, luteinizing hormone (LHR), and follitropin (FSHR) receptors (Tao et al., 2000). Apparently, mutations breaking the hydrophobic layer facilitate Na^+ /water cluster disruption and egress into the cytoplasm, activating receptors even without agonist.

3. A Gain of Function by Introducing Acidic Residues in Sodium Ion Pocket. The importance of allosteric Na^+ binding itself is further corroborated by gain-of-function effects, when acidic residues in the pocket other than D^{2.50} were found to restore, at least partially, signaling function of the receptor lacking D^{2.50}. Such gain of function studies performed for the 5-HT_{2A} serotonin receptor and μ -opioid receptor show that whereas the D^{2.50}N mutant abrogated receptor coupling to G-protein, double mutant D^{2.50}N/N^{7.49}D with another Asp in position 7.49 restored Na^+ binding and regained most of the functional activity (Sealfon et al., 1995; Xu et al., 1999). Similarly, some GPCRs, for example the sodium-dependent GnRHR, have these residues naturally reversed as N^{2.50} and D^{7.49} in the wild-type protein (Flanagan et al., 1999). Another Na^+ coordinating position of the pocket, 3.39, also bears Glu in a few olfactory receptors that lack D^{2.50}, which likely helps them to retain their Na^+ binding properties and signaling.

4. Disease-Associated Mutations in the Sodium Pocket. Because GPCRs play a critical role in many biologic and pathologic pathways, missense mutations modifying their signaling response underlie many monogenic disorders in retinal, endocrine, metabolic, developmental, and other systems (Spiegel and Weinstein, 2004; Insel et al., 2007; Vassart and Costagliola, 2011). Some of the critical mutations occur in the sodium pocket residues, impacting their functional profile. Thus, a disease-relevant SNP in CLTR2 cysteinyl leukotriene receptor residue L129^{3.43} has been associated with uveal melanoma and blue nevi (Moore et al., 2016; Moller et al., 2017). Like other mutations in this position described above, the L129^{3.43}N mutant of CLTR2 receptor constitutively activates endogenous $\text{G}\alpha_q$ and is unresponsive to stimulation by leukotriene (Moore et al., 2016).

As predicted recently by Hauser et al. (2018), many more GPCR point mutations documented in the Exome Aggregation Consortium database may be pathologically and therapeutically relevant and many of them are located in the sodium pocket. More than 220 potential disease-associated mutations have been suggested in the sodium pocket of more than 80 different clinical targets of class A GPCRs (Hauser et al., 2018). Of these, mutations at D^{2.50} position were predicted to be deleterious in 24 different class A GPCRs, S^{3.39} in 14, N^{7.45} in 13, S^{7.46} in 15, and Y^{7.53} in 15 GPCRs. Most of these SNPs are exceedingly rare (rate $<10^{-4}$) or unique, making their disease association hard to detect

and statistically validate. Thus, understanding of their functional role can facilitate full biochemical and in vivo characterization of the mutants, leading to new diagnostics tools for rare diseases. Importantly, as the effect of mutations can vary from elevated basal activity to reduced or completely abolished signaling, the same receptor may have several different disease associations.

C. Mechanism of Sodium Ion Functional Involvement

1. Sodium as an Allosteric Cofactor of Class A G-Protein-Coupled Receptor Signaling. The above evidence suggests that along with selective NAM effects on agonist binding, physiologic concentrations of Na^+ can reduce the basal activity of receptors and, overall, enhance the magnitude of their stimulation by full agonists. These opposing effects of Na^+ on agonist binding and signaling response at GPCRs have been characterized with EC₅₀ or K_B values in the same 20–100 mM concentration range, and the effects can be abolished by D^{2.50}N or other mutations in the sodium pocket, suggesting a common functional mechanism that involves Na^+ binding in the conserved pocket. In 2014, we (Katritch et al., 2014) proposed a dynamic mechanism of Na^+ as an allosteric cofactor in class A GPCR ligand induced signal transduction. It involves Na^+ entrance into the conserved pocket from the extracellular side and along the hydrated channel, which is opened in most class A GPCRs. The extracellular entrance of Na^+ , also observed in all MD simulations (see section V.B below) is also corroborated by the fact that the intracellular side of the pocket of class A GPCRs in an inactive state is sealed by the “hydrophobic layer” right beneath the sodium. Moreover, the intracellular entrance of Na^+ is hindered by a major electrostatic barrier due to excess of positive charges (as high as 10–15) found at the cytoplasmic side of receptors. It is well established that the presence of the sodium/water cluster in the conserved pocket stabilizes the receptor in the inactive state, reducing its basal activity and reducing the availability of high-affinity binding sites for agonists (Chung et al., 2011). Unlike agonists, binding of most antagonists is compatible with Na^+ binding and therefore a synergistic stabilization of inactive state by Na^+ can enhance the affinity of antagonists and inverse agonists.

During activation-related rearrangements in the 7TM bundle and the sodium pocket, Na^+ becomes dislodged from its position in the pocket and exits toward cell cytoplasm via the opening formed in the hydrophobic layer upon activation (Yuan et al., 2013). Importantly, the extracellular entrance and intracellular egress of Na^+ comprises a transfer of Na^+ ion across cell membranes. This transfer goes along with the gradient of Na^+ concentration, which is ~10- to 20-fold higher at the extracellular side, as well as

with electrostatic potential on the plasma membrane, and the reverse transfer against the electrochemical gradient is very unlikely. It was estimated that the transmembrane transfer of Na^+ along with gradient would result in ~ 3 kcal gain in energy, and this transfer can be coupled with signal amplification in class A GPCRs observed in presence of Na^+ .

One of the more recent studies also pointed to possible protonation of $\text{D}^{2.50}$ upon activation, where increased mobility of Na^+ in the pocket results in higher pK_a of this acidic side chain (Vickery et al., 2018). Such protonation would result in the total disappearance of the barrier for sodium intracellular egress and thus facilitate activation (Fig. 6).

D. Other Potential Functional Effects of the Conserved Sodium Ion Binding

1. Voltage Sensing. Selective transfer of Na^+ positive charge through the GPCR transmembrane bundle and coupling of this transfer with receptor activation is likely to make GPCRs sensitive to both sodium concentration gradient and the electrostatic potential on the membrane (Ben-Chaim et al., 2006). Several recent studies, indeed, showed that membrane voltage increased the sensitivity of the α_{2A} adrenoreceptor to norepinephrine (Rinne et al., 2013). Activation of another adrenergic receptor, the $\beta_1\text{AR}$, by catecholamine agonists was also shown to be positively modulated by membrane voltage, while depolarization of membrane dramatically reduced signaling (Birk et al., 2015). Similarly, voltage sensitivity of muscarinic acetylcholine receptors to their full agonists was shown for M2, M3, and M5 subtypes (Navarro-Polanco et al., 2011; Rinne et al., 2015). Several studies, including MD-simulations in M2 and the δ -opioid receptor (Vickery et al., 2018), suggested that Na^+ binding in the sodium pocket may explain

such voltage sensitivity. Limited experimental data from live cell assays, however, have not been conclusive so far. While $\text{D}^{2.50}$ mutations to Ala (Navarro-Polanco et al., 2011) or Asn (Barchad-Avitzur et al., 2016) eliminated gating currents in M2R, voltage sensitivities for agonist binding and conformational changes of the receptor were still present in the mutant (Barchad-Avitzur et al., 2016). This suggests the presence of multiple voltage sensors in muscarinic receptors (Hoppe et al., 2018) and calls for similar assessments of voltage sensitivity in other class A GPCRs, where the effect may be more well defined.

2. pH Dependence. Protonation of $\text{D}^{2.50}$ has been proposed as a facilitator of Na^+ egress from class A GPCRs, thus shifting the conformational equilibrium toward their active state and facilitating signaling (Vickery et al., 2018; Hu et al., 2019). This mechanism is consistent with in vitro observations that lower pH increases both basal and ligand-induced activation, for example in the $\beta_2\text{AR}$ (Ghanouni et al., 2000). This pH dependence may have important physiologic consequences because, in addition to classic cell membrane signaling, GPCR have been shown to be signaling for an extended period of time from endosomes, where pH is dramatically shifted toward an acidic environment (Calebiro et al., 2010; Irannejad et al., 2013; Vilardaga et al., 2014; Godbole et al., 2017; Eichel and von Zastrow, 2018). The conserved Na^+ site protonation would establish a common mechanism for pH dependence for the majority of class A GPCRs; however, more data and further details of the proton transfer need first to be established.

IV. Ion Binding Sites as Ligand Targets—New Approaches to Design Functional Properties

Beyond their physiologic importance, can the ion binding sites in GPCRs be directly exploited for the

Fig. 6. Updated version of the Na^+ involvement in GPCR activation mechanism. (A) Inactive receptor conformation has an Na^+ ion bound to $\text{D}^{2.50}$ in a pocket, which is sealed from the cytosol by a hydrophobic layer around $\text{Y}^{7.53}$. (B) G-protein and agonist bind to the receptor, leading to the formation of a continuous water channel across the GPCR. The increased mobility of the Na^+ ion results in a pK_a shift and subsequent protonation of $\text{D}^{2.50}$. (C) Neutralization of $\text{D}^{2.50}$ and the presence of the hydrated pathway facilitate transfer of Na^+ to the intracellular side, driven by the TM Na^+ gradient and the negative cytoplasmic membrane voltage. (D) The expulsion of Na^+ toward the cytosol results in a prolonged active state of the receptor. This figure from Vickery et al. (2018) reproduced with permission.

discovery of new ligands with potentially therapeutically relevant properties? Indeed, structure-based analysis of known GPCRs suggests that ion binding sites can be critical for designing both subtype and functionally selective ligands (Fig. 7).

A. Targeting Nonconserved Ion Binding Sites for Subtype Selectivity

Targeting selective ionic interactions can often serve as a beneficial strategy for creating subtype selectivity within closely related subfamily members, with some of such cases being characterized pharmacologically and structurally. One of the examples is the development of highly selective drugs for the H1 histamine receptor (H1R) (Fig. 7D). While the first generation of antihistamines, including doxepin, were not subtype selective, the crystal structure of the H1R-doxepin complex revealed a phosphate ion tightly bound in the

extracellular loop (ECL) region and coordinated by nonconserved basic side chains K179^{ECL2} and K191^{5.39} (Shimamura et al., 2011). Docking of the second generation antihistamines like acrivastine, levocetirizine, and fexofenadine showed that H1R selectivity and thus improved safety and pharmacological profile of these drugs can be explained by their acidic carboxy groups mimicking the interactions of the PO_4^{3-} ions.

Interactions with ion binding sites must be taken into consideration in other cases of design of GPCR ligands. The GPR39A, recently identified as Zn^{2+} modulated receptor, presents a good example of ligand identification for an ion-binding receptor. Ligands were discovered by medium-throughput screening assays, which detected selective modulation of Zn^{2+} activity on this GPCR39A (Sato et al., 2016). Another interesting example is proton-sensing receptor GPR68, where the proton site has been detected via a combination of molecular modeling and

Fig. 7. GPCR pockets for binding bitopic ligands that target specific ion binding sites. (A) Overview of the pocket positions in 7TM domain. (B) Structure of BTL1 receptor with bitopic ligand, PDB: 5X33 (Hori et al., 2018). (C) Model of bitopic ligands designed for binding to KOR Na^+ allosteric site (Zaidi et al., 2019). (D) Structure of complex with orthosteric ligand and PO_4^{3-} ion, PDB: 3RZE (Shimamura et al., 2011). In all panels, semitransparent surface shows orthosteric pocket (orange), allosteric conserved sodium pocket (cyan), and allosteric site in EC loops (green).

mutagenesis (Huang et al., 2015b). After initial detection of lorazepam as a selective positive allosteric modulator of the proton activation in GPR68, homology modeling and ligand guided optimization approaches were used to develop a model for virtual screening of $\sim 3\text{M}$ available compounds. The screening yielded several new selective PAMs for GPR68, some of them showing *in vivo* activity (Huang et al., 2015b). Rapidly improving availability of receptor structures and more relevant templates for structural homology modeling makes such approaches more and more practical in application to other ion-binding GPCRs.

B. Allosteric Ligand Binding in the Conserved Sodium Ion Site

The importance of the conserved Na^+ site for the function of class A GPCRs suggests that targeting this site with allosteric or bitopic ligands may be a viable general strategy for modulation of these receptor signaling. The volume of the pocket is usually about $150\text{--}250 \text{ \AA}^3$, thus permitting binding of small fragment-like molecules. Indeed, the Na^+ pocket has been characterized as a binding site of an antidiuretic drug, sodium channel blocker amiloride, and its derivatives (Liu et al., 2012b). Amilorides are known as negative allosteric modulators (NAMs) of many class A GPCRs, including adenosine (Howard et al., 1987; Leppik et al., 2000; Gao et al., 2003a,b, 2011; Gutiérrez-de-Terán et al., 2013), dopamine (Neve, 1991; Hoare et al., 2000), muscarinic (Dehay and Verhasselt, 1995), 5-HT (Pauwels, 1997), GnRHR (Heitman et al., 2008), and potentially many more receptors, as summarized in Katritch et al. (2014). Various affinity estimates for amiloride derivatives show K_B ranging from ~ 1 to $50 \text{ }\mu\text{M}$ in their negative allosteric modulation of orthosteric ligand binding. Docking of amiloride and a bulkier derivative HMA (5-(*N,N*-hexamethylene)amiloride) (Gutiérrez-de-Terán et al., 2013) shows that the positively charged guanidine moiety of the ligand forms a salt bridge to the $\text{D}^{2.50}$ carboxyl, while the bulkier $\text{N}5$ substituents point toward the orthosteric site. The induced docking and conformational modeling also suggested that the fitting of amilorides, especially HMA into the pocket, requires substantial expansion of the pocket, which manifested in adjustments in the $\text{N}^{7.45}$, $\text{N}^{7.49}$, and especially $\text{W}^{6.48}$ side chains. Accordingly, mutations of these residues to alanine in this study only improved affinity of amiloride and HMA severalfold, suggesting that amiloride might not be the optimal chemotype for targeting the pocket. On the chemistry side, a number of additional amiloride derivatives with longer 5N substitutes were characterized in a recent study (Massink et al., 2016), showing that extension of the allosteric ligand into the orthosteric pocket is possible without major reduction of the binding affinity.

Another small molecule characterized recently as an allosteric Na^+ pocket binder in the BLT1 receptor is

benzamidine (Hori et al., 2018), though its affinity (K_B) was estimated much lower than amiloride at $\sim 500 \text{ }\mu\text{M}$, making it only ~ 10 times more potent than Na^+ ion itself (based on Fig. 4A in Hori et al., 2018). The study revealed binding of benzamidine and its NAM effect on G-protein activity in two very different receptors, the BLT1 receptor and $\beta 1\text{AR}$, suggesting that it likely binds at the sodium ion binding site in other class A GPCRs as well.

Intriguingly, because Li^+ can compete with Na^+ in the conserved pocket (Pert et al., 1973), some studies hypothesized that effects of Li^+ on functional properties of GPCRs can be implicated in physiologic and psychoactive effects of the Li^+ (Dudev et al., 2018). The Li^+ effect as a competitor to Na^+ binding is especially intriguing because lithium is widely used in treatment of bipolar disorders; however, more evidence is needed to establish the GPCR mode of action of Li^+ , as this ion can also impart a central nervous system effect via ion channel modulation.

C. Targeting Sodium Ion with Bitopic Ligands

1. Concept of Bitopic Ligands. The highly conserved nature and small size of the sodium pocket itself limit its selectivity, and therefore, the practical utility of small ligands like amilorides and benzamidines as allosteric modulators. On the other hand, a combination of high affinity selective orthosteric moieties with the unique functional properties of the Na^+ site allosteric binders could make bitopic ligands an attractive target for ligand design. One recently characterized example of such a bitopic ligand is benzamidine-containing ligand BIIL260 found in the BTL1 receptor structure (Hori et al., 2018). By reaching into the Na^+ site and forming a salt bridge with $\text{D}^{2.50}$ carboxyl, as well as hydrogen bonds to $\text{S}^{3.39}$ and $\text{S}^{7.45}$, the positively charged benzamidine moiety is expected to block activation related changes. In agreement with this prediction, BIIL260 was characterized as an inverse agonist, completely blocking the basal activity of the receptor. There are several other benzamidine-containing compounds for BLT1 predicted to bind in a similar manner (Hori et al., 2018).

2. Structure-Based Design of Bitopic Ligands for the Sodium Ion Site. Structure-based rational design of bitopic ligands targeting Na^+ site of class A GPCRs was proposed as a potentially broadly applicable mechanism for developing selective ligands with beneficial functional properties, including, e.g., inverse agonism (Katritch et al., 2014). Such design in application to opioid receptors been performed recently (S. Zaidi, T. Che, B. Roth, and V. Katritch, manuscript in preparation). The structure-based design of these ligands (see Fig. 7) is based on the orthosteric agonist nalfurafine, extending its morphinan scaffold at $\text{N}5$ toward the sodium pocket. Both bitopic ligands synthesized and tested, BRI-731 and BRI-751, show high affinities at

the three opioid receptors and retain full G_i agonism of nalfurafine. At the same time, the ligands, and especially guanidine-containing BRI-751, effectively block arrestin recruitment, which makes them highly G_i -biased agonists. The ability of bitopic ligands to block arrestin signaling can be explained by the conformational mechanism, where the allosteric moiety (e.g., amiloride) blocks inward movements of TM7 in the Na^+ pocket, while still allowing outward movement of TM6. Indeed, several previous studies, e.g., by NMR (Liu et al., 2012a) and fluorescent spectroscopy (Rahmeh et al., 2012), established a direct connection between dynamical changes in TM6 and G-protein biased signaling, while TM7 was associated with arrestin-biased signaling. Like the amiloride derivatives described above, the improved affinities of BRI-751 in the Ala mutants of the pocket side chains suggest that the Na^+ site is slightly too small for these moieties and that further optimization could improve the ligand affinities to wild-type receptors.

V. Biophysical and Computational Approaches for Studying Allosteric Ions

As ions impact many aspects of GPCR function, often in subtle dynamic ways, a comprehensive multidisciplinary approach is often needed to fully understand the observed effects. In addition to the key evidence from biochemical and pharmacological studies of allosteric ion effects and structural studies revealing ion binding sites, biophysical and computational approaches make an increasingly important contribution to our understanding of ion dynamics and functional role.

A. Nuclear Magnetic Resonance Spectroscopy

1. Study of G-Protein-Coupled Receptor Dynamics with and without Sodium Ion Site. Nuclear magnetic resonance (NMR) approaches have been widely applied to study conformational dynamics of GPCRs, complementing static the picture produced by crystallography, and the recent examples show that it can greatly contribute to the understanding of the role of ions in GPCR function. Thus, in a recent study (Eddy et al., 2018b) characterized the dynamic behavior of the $\text{A}_{2\text{A}}$ adenosine receptor ($\text{A}_{2\text{A}}\text{AR}$) by assessing NMR spectra of Trp and Gly residues in complexes with specific agonists and antagonists. The study showed that the $\text{D}^{2,50}\text{N}$ mutation disrupting the Na^+ site can drastically reduce signaling-related dynamics in the intracellular half of the receptor, while not affecting conformational dynamics in the extracellular ligand binding half. This is in perfect agreement with the “uncoupling” role of $\text{D}^{2,50}\text{N}$ mutation that abolishes G-protein activation in $\text{A}_{2\text{A}}\text{AR}$ (Eddy et al., 2018a) and validates the dynamic nature of the Na^+ allosteric effect.

2. Direct Nuclear Magnetic Resonance Assessment of Allosteric Sodium Ion Binding Kinetics. Recent NMR studies show that the effect of sodium and divalent

cations on GPCR signaling dynamics can be assessed directly by NMR (Eddy et al., 2018b; Ye et al., 2018). These studies, also performed with $\text{A}_{2\text{A}}\text{AR}$, demonstrated that increasing concentrations of Na^+ selectively shift the equilibrium toward an inactive ensemble, while the addition of K^+ does not have such effect. Moreover, the Ye et al. (2018) study used ^{23}Na NMR binding isotherm to achieve the first direct measurement of the dissociation constant of Na^+ in apo $\text{A}_{2\text{A}}\text{AR}$ as $K_d = 61 \pm 27$ mM, which is in good agreement with previous indirect estimations of Na^+ allosteric effects $K_B = 32$ mM (Massink et al., 2015). Furthermore, relaxation dispersion (CPMG) experiments with ^{23}Na provided a first measure of the bound state lifetime. In the apo state, the specific Na^+ bound lifetime was estimated at 480 μs , in agreement with lack of Na^+ dissociation in the shorter time scales of <10 μs in molecular dynamic simulations of the inactive state. As expected, binding of the antagonist ZM241385 increased the bound fraction of Na^+ by 20% and the average bound state lifetime to 630 μs . Another important observation in this study involves amiloride analog HMA, which was shown by NMR to compete with sodium weakening its effect on titration isotherm.

3. Binding and Effect of Divalent Ions in $\text{A}_{2\text{A}}$ Adrenergic Receptor is Potentially Nonspecific. In stark contrast with Na^+ effects, the same NMR study (Ye et al., 2018) shows that the allosteric effects of divalent cations at $\text{A}_{2\text{A}}\text{AR}$ appear to be nonselective, at least between Ca^{2+} and Mg^{2+} , and require non-physiologically high concentrations (100–500 mM) of these ions. The divalent ions shifted the equilibrium toward active states of the receptor, and this shift was synergistic with agonist binding, which is similar to observations for divalent ions found in opioid receptors earlier (Pert et al., 1973). The MD simulations in this study suggested that at high cation concentrations they can bind in the extracellular loops of the receptor, bridging the key acidic residues and contracting the pocket, thus facilitating agonist binding and receptor activation.

B. Molecular Dynamic Simulations

Owing to its ability to look into atomistic details and generate hypotheses for experimental validation, molecular dynamics (MD) simulations have become an important tool for deciphering roles of ions in GPCR function. On the other hand, adequate modeling of ions, especially Na^+ , is becoming a requirement for adequate MD studies of functional effects in most GPCRs, in the same way as ions are absolutely required in the modeling of ion channels.

1. Sodium Access and Universality of Sodium Ion Binding in Class A G-Protein-Coupled Receptors. Starting from the pioneering work on the Na^+ access into the allosteric pocket in dopamine receptors (Selent et al., 2010), a number of studies characterized Na^+ access kinetics in opioid (Shang et al., 2014) and muscarinic (Miao et al., 2015; Vickery et al., 2016)

receptors. Moreover, conventional and accelerated MD simulations were recently performed systematically for 18 different GPCRs with known 3-D structures by Selvam et al. (2018). The studies predict similar sodium entry pathways from the extracellular side into the 2.50 pocket for 15 of these diverse class A receptors. Interestingly, the predicted average mean first passage time for Na^+ ranged from about 0.1 to 0.3 μs in opioid and orexin-2 receptors, which have relatively open access to the sodium pocket, to $\sim 30 \mu\text{s}$ in sphingosine-1-phosphate receptors, which has the extracellular entrance into the receptor obscured by the extracellular (EC) loops. In two of the receptors assessed, the PAR1 protease activated receptor and the P2Y12 purinergic receptor, occlusion of the EC access blocked the extracellular entrance of Na^+ during the modeling time, even though PAR1 and PAR2 protease activated receptor crystal structures have Na^+ resolved in the conserved pocket. These observations suggest that either Na^+ enters PAR1/PAR2 from the intracellular side against the gradients and electrostatic potential (which is unlikely), or thermal and ligand-induced conformational changes in the EC loops can dynamically open the access for Na^+ from the outside of the cell. This study (Selvam et al., 2018) also predicted very long average unbinding times for the $\text{D}^{2.50}$ anchored Na^+ , ranging from 13 μs in the adenosine receptor to $\sim 120 \mu\text{s}$ in the orexin-2 receptor. This estimate is approaching the same order of magnitude (480 μs) as measured by the NMR study (Ye et al., 2018). While the MD was also used to simulate Na^+ binding to a nonconserved site in the class B glucagon receptor, the predicted class B binding was very weak and unstable, with unbinding times much faster (2 μs) than binding times (27 μs), which further contrasts with strong and highly selective Na^+ binding in class A receptors.

2. Molecular Dynamic Simulations Corroborate New Functional Hypotheses. Molecular dynamic simulations also suggest some intriguing details of Na^+ -dependent functions. Thus, Vickery et al. (2016) used the CompEL approach to simulate the Na^+ gradient and the voltage across the membrane in MD simulations with δ -OR and M2R. The study suggested that the sodium movement along the transmembrane domain in the water-filled pocket can create a gating charge of 0.5e when the ion travels between the extracellular entry and the allosteric binding site, which would make the receptor highly sensitive to membrane polarization and depolarizations as occurs in neuron signaling. Moreover, another study (Vickery et al., 2018) used comprehensive MD-based pKa predictions to show that $\text{D}^{2.50}$ is likely to become protonated when active-like conformation of the receptors and increased mobility of Na^+ can lead to $\text{D}^{2.50}$ protonation, followed by fast and barrier-free egress of Na^+ into the cytoplasm.

Another recent MD study on μ -opioid receptor (MOR) used a $>11 \mu\text{s}$ Markov State Model ensemble MD

simulation to identify the continuous egression path for Na^+ in the MOR active state (Hu et al., 2019). This work draws similar conclusions, showing that protonation of $\text{D}^{2.50}$ can greatly facilitate Na^+ egress from the cytosol in an active MOR. The combination of MD with machine learning also provides insight into conformational changes accompanying Na^+ movements, predicts energetics, and time scales of Na^+ translocation in inactive and active MOR, as well as qualitatively predicts impact of Na^+ binding on agonist affinity. Interestingly, the predicted timescales for sodium intracellular relocation on the order of 1 second are comparable to the experimentally derived lifetimes of GPCR/G protein complexes.

Long-time scale accelerated molecular dynamic simulations in the M3 muscarinic receptor in Miao et al. (2015) further corroborated the notion that sodium ion bound to charged $\text{D}^{2.50}$ residue confines the receptor mostly to an inactive state with reduced flexibility. In contrast, the $\text{D}^{2.50}$ -protonated receptor, which does not bind Na^+ could sample larger conformational space, including large-scale structural rearrangements of the transmembrane helices leading to active-like states. MD simulations in MOR receptor (Yuan et al., 2013) also supported the Na^+ trajectory pathway through a receptor entering the $\text{D}^{2.50}$ binding site from the extracellular side and then exiting at the intracellular site upon conformational changes and redistribution of internal water molecules. The simulations suggested that the egress path of Na^+ can also help to disrupt the ionic lock between $\text{D}^{3.49}$ and $\text{R}^{3.50}$ in the conserved “DRY” motif and thus can facilitate G-protein activation.

Another recent study performed for CXCR4 chemokine receptor simulations used replica exchange MD with enhanced sampling (Cong and Golebiowski, 2018), showing the special role of Na^+ coordinating $\text{N}^{3.35}$ side chain in receptor activation and explaining the constitutive activation role of $\text{N}^{3.35}\text{A}$ and $\text{N}^{3.35}\text{S}$ mutations.

For histamine receptors, the MD simulations and the calculation of Gibbs energy of solvation (Wittmann et al., 2014) also show the preference for $\text{D}^{2.50}$ binding of Na^+ in human H3-receptor (hH3R), which has an experimentally documented allosteric sensitivity to sodium ions. In contrast, the study suggests that the $\text{L}^{7.42}\text{Q}$ change between the hH3R and hH4R is responsible for different sodium dynamics in the two closely related receptor subtypes, even though all 16 residues of the sodium pocket are identical. While $\text{Q}^{7.42}$, unique for hH4R in not directly involved in sodium binding, it can disrupt the water-filled channel connecting orthosteric and allosteric sodium pocket in hH4R and kinetically block the sodium access, which may explain hH4R insensitivity to sodium effects.

MD simulations are now routinely used in many GPCR structural studies to decipher the functional effects of the Na^+ ion, e.g., for D4 dopamine receptor (Wang et al., 2017). The MD simulations of the DRD4

inactive state suggest that sodium and the antagonist nemonapride can mutually stabilize each other's binding, in agreement with the increased affinity of nemonapride-like antagonists in the presence of sodium.

C. Phylogenetic Analysis

While the exceptional level of sequence and structure conservation of the sodium pocket in class A GPCRs has been characterized (Katritch et al., 2014), detailed study of variations in the sequence and structure of the pocket can be used to decipher interesting trends in the evolution of GPCR function. Thus, lack of conservation in the residues corresponding to sodium pocket of opsin receptors suggests different functional features of these receptors. Indeed, driven by much higher energies of photochemical switches in the covalent retinal ligand and the requirement for fast on-off switching, opsins have apparently lost their Na^+ binding and functional dependence. For GPCRs with dissolvable ligands, however, loss of Na^+ anchor residues in the receptor pocket of GPCR subtypes leads to loss of ligand-induced signaling, e.g., NTSR2 neurotensin receptor (NTSR2) is nonresponsive to neurotensin and presumably acts via dimerization with its fully functional and sodium-dependent NTSR1 neurotensin receptor subtype (Katritch et al., 2014).

Phylogenetic analysis also has been applied to make new insights into evolution of function in whole GPCR families, such as chemokine receptors (Taddese et al., 2018). The study used principal component analysis of sequence covariations in nested GPCR sequence sets to decipher evolutionary determinants of chemokine receptors. This approach identified residue positions 2.49, 3.35, and 7.45 as the hallmark positions that define the emergence of chemokine receptors and their subsequent divergence into homeostatic (e.g., CXCR4) and inflammatory (e.g., CCR5) receptors. Polar $\text{N}^{3.35}$ and $\text{S}^{3.39}$ in these key residue positions define high stability of the Na^+ binding in homeostatic receptors like CXCR4, while glycines $\text{G}^{3.35}$ and $\text{G}^{3.39}$ in these positions result in loose and weak Na^+ binding in CCR5 and other inflammatory receptors. Further analysis of chemokines in species also suggested that evolution of chemokine receptors might be driven, at least in part, by dramatic changes in the sodium binding mode. Thus, the ancient jawless fishes had a highly conserved sodium binding site, while this conservation was loosened during the divergence of the chemokine family in modern species.

VI. Unanswered Questions and Future Perspectives

Despite dramatic progress in the understanding of the role of ions in GPCR function, allosteric regulation and fine tuning, numerous questions and venues of inquiry remain wide open.

First, it is still unclear how, with so many potential binding sites in GPCRs for various cations and anions (Strasser et al., 2015), the conserved Na^+ site became an almost universal site across the most populous and highly diverse class of class A GPCRs. Some hints were proposed in a recent study (Shalaeva et al., 2015) tracing sodium site evolution to the much more ancient 7TM family of prokaryotic sodium-translocating rhodopsins, though more comprehensive research is needed to clarify the origins and evolution of the Na^+ site in GPCRs, which may be facilitated by the flow of sequencing data from diverse prokaryotic and eukaryotic species.

Second, ascribing a precise functional role of sodium in the activation mechanism remains challenging. While its role as an NAM for agonist binding is most commonly studied, for the super-conserved site it absolutely is also required for effective agonist-induced signal transduction in class A GPCRs. The definitive experimental proof that not just the residues of the pocket, but the presence of Na^+ in the pocket itself, is critical for the function remains to be obtained. Because Na^+ is critical for the function of many cell components and has an obviously major impact on ion channels involved in GPCR signaling, such ultimate experimental proof requires very accurate and well-controlled experiments where removal of Na^+ and abrogation of its transmembrane gradient does not impact the assay itself.

Third, while the bulk of the structural and molecular modeling studies predict an extracellular entrance of Na^+ into the allosteric pocket and subsequent intracellular egress upon receptor activation, a definitive electrochemical or biochemical experiment directly demonstrating the transmembrane transfer of sodium by GPCRs across plasma membrane remains to be done. The key challenge for the electrophysiological assessment is an ultra-low current in this system, where a single charge transfer is occurring only once per GPCR activation event. Such currents are below the sensitivity thresholds of most methods conventionally used to characterize ion channels, pumps, and transporters and require either supersensitive electrophysiology or other approaches, e.g., using $^{22}\text{Na}^+$ radioisotope.

Fourth, new approaches are needed to further investigate physiologic relevance of Na^+ modulation in some GPCRs and cell types. This includes further studies of gating potential effects in GPCR (Vickery et al., 2018), which may play a role in GPCR response to depolarization in neurons. Another interesting aspect is GPCR signaling in endosomes, where acidic environments can contribute to $\text{D}^{2.50}$ protonation and explain prolonged, pH-dependent signaling observed in this system. In general, the allosteric and the functional effects of sodium need to be incorporated as part of allosteric models and signaling pathway analysis, quantitatively explaining often unusual pharmacology of GPCR signaling in different cells and environments.

Finally, while the first few examples of direct targeting Na⁺ site by bitopic ligands have been emerging (Hori et al., 2018; S. Zaidi, T. Che, B. Roth, and V. Katritch, manuscript in preparation), there are myriad possibilities for using the Na⁺ pocket in many GPCR targets to rationally design chemical probes with new properties and pharmacological profiles.

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Authorship Contributions

Participated in research design: Katritch, Roth.

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Wrote or contributed to the writing of the manuscript: Katritch, Roth, Zarzycka, Zaidi.

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