Functional Characterization of Sodium Channel Inhibitors at the Delta-Opioid Receptor

Abdulhamid O. Mohamud,§ Manel Zeghal,§ Shivani Patel, Geneviève Laroche, Nuria Blgacim, and Patrick M. Giguère*

ABSTRACT: Existing pharmacotherapies acting on the opioid receptor system have been extensively used to treat chronic pain and addictive disorders. Nevertheless, the adverse side effects associated with opioid therapy underscore the need for concerted measures to develop safer analgesics. A promising avenue of research stems from the characterization of a sodium-dependent allosteric regulation site housed within the delta-opioid receptor and several other G protein-coupled receptors (GPCRs), thereby revealing the presence of a cluster of sodium and water molecules lodged in a cavity thought to be present only in the inactive conformation of the receptor. Studies into the structure-function relationship of said pocket demonstrated its critical involvement in the functional control of GPCR signaling. While the sodium pocket has been proposed to be present in the majority of class A GPCRs, the shape of this allosteric cavity appears to have significant structural variation among crystallographically solved GPCRs, making this site optimal for the design of new allosteric modulators that will be selective for opioid receptors. The sodium pocket supports the accommodation of small molecules, and it has been speculated that promiscuous amiloride and 5′-substituted amiloride-related derivatives could target this cavity within many GPCRs, including opioid receptors. Using pharmacological approaches, we have described the selectivities of 5′-substituted amiloride-related derivatives, as well as the hitherto undescribed activity of the NHE1 inhibitor zoniporide toward class A GPCRs. Our investigations into the structural features of the delta-opioid receptor and its ensuing signaling activities suggest a bitopic mode of overlapping interactions involving the orthosteric site and the juxtaposed Na⁺ pocket, but only at the active or partially active opioid receptor.

INTRODUCTION

Delta-Opioid Receptor. Delta-opioid receptors (DOR) belong to the class A of G protein-coupled receptors (GPCRs), a superfamily of seven-transmembrane proteins of which more than 30% of prescribed drugs target. Besides their critical role in pain management, DOR agonists have also been shown to exhibit antidepressant activity and have the potential to treat spasms associated with Parkinson’s disease. However, the clinical use of DOR agonists is limited due to the possible occurrence of potentially life-threatening side effects such as tolerance, convulsions, and seizures.³ GPCRs transduce extracellular stimuli into intracellular outcomes through two main mechanisms, the G-protein-dependent pathway, which facilitates a change in the concentration of an intracellular second messenger via the activation of the heterotrimetric G-protein, as well as a G-protein-independent mechanism, whereby receptor signaling is attenuated by phosphorylation and internalization, processes both dependent on the initial recruitment of the protein adaptor β-arrestin.

GPCR activation is tightly controlled through intramolecular determinants serving as intrinsic locks or switches. These motifs are highly conserved within GPCRs, especially within the class A family. Recent studies have revealed the presence of a highly conserved cavity serving as an allosteric binding site for sodium ion and water molecules, forming a cluster in the middle of the 7TM bundle of the majority of class A GPCRs, including the delta-opioid receptor (DOR).² Although most drugs target the orthosteric site of GPCRs, exploiting allosteric binding sites provides several advantages. Among others, this strategy would allow for more precise control of subtype selectivity, would preserve the spatiotemporal activity of endogenous ligand, and could be used to control functional selectivity of the natural ligand, all of which hold great potential for developing efficacious novel compounds and candidate drugs.³,⁴ This sodium cavity is formed by the side chains of 16 amino acid residues, of which 15 residues are conserved in all class A branches. At the same time, structural
studies revealed an important disparity in the structure of the sodium cavity, which seems to affect the selectivity and function of ligands. In the human DOR, the oxygen atom of side-chain Asn131.35 directly coordinates with the sodium ion, while the nitrogen atom forms a hydrogen bond to Asp128.32 and a salt bridge with the nitrogen group of naltindroide bound to the orthosteric site. Thus, these interactions between Asn131.35, Asp128.32, and the sodium ion serve not only as an ionic lock to stabilize the inactive state of the receptor but also as a propagation link between the two sites during the activation of the receptor.2,6

Therefore, this allosteric pocket could be critical for the modulation of signaling and ligand binding. Importantly, some unique features of DOR’s sodium cavity distinguish it from other sodium sites in Class A GPCRs. The sodium ion interacts with conserved residues, which are arranged into two shells; the first coordination shell of the sodium ion in the allosteric site is formed by five oxygen atoms, three of them from Asp95.50, Ser135.39, and Asn131.35 side chains and the other two oxygen atoms of molecules of water. The second coordination shell consists of side chains of three amino acid residues, Trp274.48, Asn310.45, and Asn314.49, with two other water molecules in contact with the first shell. Additionally, the aspartic acid residue in position 2.50 (Asp.50) is essential for binding sodium in this site, as it forms a strong salt bridge with the positively charged sodium ion. Remarkably, these conserved residues of the sodium cavity are among the most conserved motifs in class A GPCRs and have critical roles in the activation of the receptor.2,5 Despite this emergent information on the allosteric behavior of sodium ions in GPCRs are still poorly understood. Interestingly, the effect of sodium ions on ligand binding at certain GPCRs and especially at opioid receptors was observed more than forty years ago. It has been reported that the presence of a high concentration of sodium increases the binding affinity of antagonists to the opioid receptors, while the decrease in Na⁺ has no significant effect on the affinity of the agonist. Therefore, researchers drew upon the presence or absence of Na⁺ to discriminate whether a ligand was an agonist or an antagonist.10 This finding suggested that the sodium ion stabilized the inactive state of opioid receptors and was described to likely act as an allosteric modulator. Later, the same allosteric qualities of sodium ion were observed at six other different GPCRs, including Neurotensin NTSR1, Dopamine D2R, Adrenergic α2-AR, Adenosine A₂A-AR, and Protease Activated PAR-1.6

Amiloride and Its Derivatives as GPCR Allosteric Modulators. The suggested functional importance of the allosteric pocket in receptor activation and its exceptionally high conservation in class A GPCRs makes it an attractive target for the discovery of small molecules with unique functional and pharmacological properties, insights of which can be applied and served as novel starting points for drug discovery.

Some reports have proposed that the diuretic amiloride could exert allosteric modulation on different GPCRs, albeit with modest affinity (>10 μM).13 Extending on these findings, a few amiloride-related derivatives have shown comparable or stronger affinities at several receptors.12–18 As a potassium-sparing diuretic, amiloride works by directly blocking the epithelial sodium channels (ENaCs) and acid-sensing ion channel 3 (ASIC3), as its mechanism of action entails the reduction of potassium excretion and inhibition of sodium reabsorption in the distal tubule, resulting in the loss of sodium and water from the body.19 It is also used to alleviate edema associated with hepatic cirrhosis and in the treatment of heart failure by blocking Na⁺/H⁺ exchangers-1 (NHE1), resulting in decreased reperfusion injury in ischemic attacks.19

Interestingly, mutation of the conserved Asp.50 in selected GPCRs abolishes the effect of amiloride and its derivatives on orthosteric ligands, with Na⁺ modulating this effect.20–26 It has thus been proposed that the guanidinium group, which is found in all amiloride-related derivatives, can bind with the carbonyl group of Asp.50 residue, which is now known to coordinate the Na⁺ ion.22 However, the low affinity and selectivity of amiloride toward GPCRs, and the lack of molecular and structural information have hampered the systematic study of these allosteric ligands (reviewed in ref 22).

Most studies using amiloride-related derivatives have used biochemical characterization, such as binding experiments, due to said compounds’ low affinities and low allosteric constants.27 At the concentration required to have an effect in vitro (>10 μM), these Na⁺ channel inhibitors are toxic to cells (data not shown). Moreover, few studies have addressed the functional effect. One such study revealed a modest effect with 10 μM of S-(N,N-hexamethylene)amiloride (HMA) at the gonadotropin-releasing hormone receptor (GnRHR) using an NFAT-reporter assay15 whole another using isolated rat tracheal rings showed a reduction of the Emax of acetylcholine toward muscarinic receptors using 100 μM − 1 mM of amiloride.27

From this premise, we tested nontoxic concentrations of S’ substituted amiloride-related derivatives in a functional cellular-based assay at most class A GPCRs. Herein, we aimed to identify receptors with a greater affinity toward these molecules, which would enable the execution of better-informed pharmacological studies toward potentially targeting the Na⁺ pocket.

**RESULTS**

The PRESTO-Tango GPCR assay possesses unique and advantageous features that enable the simultaneous testing of a set of amiloride derivatives at all class A GPCRs.28,29 The difficulty in screening the entire druggable GPCRome in parallel is mainly due to the inherent diversity of G protein signal-transduction cascades. The measurement of G protein-independent β-arrestin recruitment provides a universal assay platform, as nearly all tested GPCRs can induce β-arrestin translocation.28,30,31 We selected to test 5-(N,N-dimethyl)-amiloride (DMA), 5-(N-methyl-N-isobutyl)amiloride (MIA), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), 5-(N,N-hexamethylene)amiloride (HMA), and the sodium-hydrogen exchanger isoform 1 (NHE1) inhibitor zoniporide. We also opted to include zoniporide, which is the ligand with the lowest degree of structural similarity to amiloride, as it contains a guanidinium group and showed no toxicity at 10 μM. This set of Na⁺ channel inhibitors was tested at 10 μM for their capacity to modulate the basal activity of class A GPCRs. Importantly, the ligands were tested in ‘agonist mode’ and not in ‘allosteric mode,’ meaning that the stimulation of receptors with their corresponding agonists was not required for this screen. This reasoning stems from previous reports of several
allosteric modulators exhibiting partial agonist activity at high concentrations. As such, we assumed that modulation of the Na+ pocket, which locks the receptor in an inactive conformation, would modulate basal activity and would therefore be detectable in agonist mode. As shown in Figure 1 and Table S1, only six GPCRs were found to be modulated by at least one of the ligands tested at 10 μM, given the set threshold of >3-fold increase. Interestingly, even with high sequence homology, the kappa-OR (KOR) was not detected in this primary screening. As shown in the inset of Figure 1B, the secondary screening at the DOR receptor and stimulated with increasing concentrations of the indicated agonist or antagonist, in the presence or absence of 10 μM EIPA or amiloride. Data were normalized toward BW373U86, which represents the agonist with the highest efficacy (n = 3 in quadruplicate).

**Figure 1.** Parallel interrogation of the class A GPCR-ome by four sodium channel inhibitors. (A) HTLA cells were plated in 384-well plates, transfected with 350 GPCR Tango constructs, and either stimulated with the indicated compounds at 10 μM or with vehicle buffer (-compound). The vector pcDNA3.1+ was used as a negative control, and the DRD2 receptor stimulated with quinpirole was used as a positive control (excluded from the heatmap). A heatmap was generated following extraction of the fold-over basal (treated/nontreated) increase for each receptor assayed in quadruplicate. The adjacent table highlights the six receptors showing >3-fold increase (treated/nontreated), and KOR was added to highlight its insensitivity toward the modulators. The complete list of GPCR tested is shown in Table S1. (B) Dose–response curves for compound profiling and demonstration of β-arrestin2 recruitment to DOR in secondary screening. HTLA cells were transiently transfected with the DOR-Tango receptor and stimulated with increasing concentrations of the indicated agonist or antagonist, in the presence or absence of 10 μM EIPA or amiloride. Data were normalized toward BW373U86, which represents the agonist with the highest efficacy (n = 3 in quadruplicate).

**Figure 2.** Parallel interrogation of class A GPCR-ome at external sodium concentrations of 30 and 200 mM. (A) HTLA cells were plated in 384-well plates, transfected with 350 GPCR Tango constructs, and media was replaced for modified DMEM containing 30 or 200 mM NaCl. Data are represented as the ratio between the relative light unit (RLU) at 200 mM divided by RLU at 30 mM. Data = 0 suggests no difference, >1 suggests an increase in activity with 200 mM Na+, and <1 suggests an increase in activity with 30 mM Na+. Only three receptors showed a strong effect: M2, M3, and M4 muscarinic receptors. (B) Dose–response curves and demonstration of β-arrestin2 recruitment to M1–M5 receptors in secondary screening. HTLA cells were transiently transfected with M1–M5-Tango receptors and stimulated with increasing concentrations of carbachol in media containing either 140 mM (normal media), 30 mM, or 200 mM NaCl. Osmolarity was adjusted with choline chloride for all media (n = 3 in quadruplicate).
PAM mode of action on the receptor. At a nontoxic concentration (10 μM), the prototypic inhibitor amiloride was found to have no activity. This is not surprising as most biophysical studies using amiloride found activity only at >100 μM, concentrations which cannot be used in a cell-based assay.

Using a similar approach, we tested the effect of different concentrations of extracellular Na⁺ on the basal activity of the class A GPCRome. Using Na⁺-free DMEM media, we adjusted the osmotic strength using choline chloride and tested three different concentrations of Na⁺, specifically 30, 140, and 200 mM. The physiologic Na⁺ concentration is approximately 140–150 mM Na⁺ in the extracellular space and 5–15 mM in the intracellular environment; moreover, Na⁺ concentration is extremely variable and dynamic throughout the body. Using ²³Na-MR imaging, it has been shown to be modulated in various pathophysiological states ranging from 5 to 300 mM in certain regions, with a median concentration of approximately 40 mM in brain white matter.²²,³³ Although the intracellular Na⁺ concentration is usually around 5–15 mM, we observed some level of cell death at 15–20 mM extracellular Na⁺, and as such, we chose to work with 30 mM, which is the lowest concentration with no effect on cell viability. As shown in Figure 2, comparisons at 200 vs 30 mM Na⁺ revealed little impact on the context of basal activity across the interrogated GPCRome. As the Na⁺ pocket is highly conserved within class A GPCRs, we were expecting to have a broad effect on the basal activity of GPCRs. As this was not observed, these findings indicate that Na⁺ concentration is probably not a significant mode of regulation of GPCR activity and determinant for interaction, but rather Na⁺ is ligand interaction that alters the conformation of the Na⁺ pocket and hence the release of the Na⁺ ion from the pocket. The only outliers detected from our screen were the muscarinic acetylcholine receptor subtypes M2, M3, and M4; these were confirmed in secondary Tango experiments by performing dose−response validation using the agonist carbachol at the five muscarinic acetylcholine receptors. A significant increase in basal activity (>10-fold) was observed by reducing extracellular Na⁺ to 30 mM at M2, M3, and M4. Although modest at the M1 and M5, we found that these two receptors had much higher basal activity than M2, M3, and M4, and thus the detected difference in basal activity is limited to <2-fold. We also observed a loss of agonist efficacy and potency for all five subtypes. Conversely, increasing external Na⁺ to 200 mM significantly increases carbachol efficacy by more than 3-fold, without affecting potency for M2, M3, and M4 and weakly increasing efficacy at M1 and M5. Although the physiological significance of these findings is unknown, it is yet tempting to speculate a link to the other acetylcholine receptor, the ligand-gated nicotinic acetylcholine receptor (nAChR), which is an important Na⁺ channel. Spatiotemporal control of Na⁺ concentration at the synaptic cleft could potentially be a control mechanism of the muscarinic receptor. Seeing as M1, M3 and M5 are Gq-coupled receptors, whereas M2 and M4 are Gi-coupled, it appears that the coupling mechanism is not relevant to the observed effect of Na⁺, although we do not exclude its influence on G-protein signaling itself.

Similar selectivities of amiloride-related derivatives have been previously described for the human A2A adenosine receptor by the group of Ijzerman.³⁴ However, most of the experiments were performed using in vitro biochemical and biophysical experiments. This likely explains why the A2A receptor was not observed as a potential hit from our primary screening and the null effect observed at A2A in the cell-based Tango assay; moreover, a similar conclusion can also be drawn for all GPCRs previously reported to be modulated by amiloride. Various S′-substituted amiloride-related derivatives have been previously synthesized to perform structure−activity-relationship (SAR) at the A2A receptor.³⁴ The same derivatives, generously donated by the Ijzerman group, and others were tested at the DOR, as shown in Figure 3 (structures are shown in Figure S1). Some derivatives were found to be toxic at >5 μM, which accounts for the flat curves for derivatives 7363, 7355, 7439, and 7440. With the exception of 7327, 7403, DMA, KR-32568, and phenamil, all other compounds demonstrated some level of allosteric activity. MIA remains the best ago-PAM, while zoniporide remains the best PAM without intrinsic agonist activity. The lack of DMA activity also highlights the importance of having a large hydrophobic moiety at the S′-substitution. The single addition of the chloride group on the phenyl in 7327 abolishes its activity compared to 7332. Altogether, we found that the S′-amiloride substitution was relatively permissive but required an extensive hydrophobic substitution, and that none of the compounds tested outperformed the original MIA, EIPA, HMA, and zoniporide used in the primary screening.

It has been proposed that S′-substituted amiloride-related derivatives bind to the sodium ion site and influence orthosteric ligand binding, implicating the possible interference of sodium ion with the interaction of the ligands by direct competition. Thus, a Schild analysis of HMA, MIA, zoniporide, and the inactive phenamil was performed at different permissive extracellular sodium concentrations. As shown in Figure S2, reducing extracellular Na⁺ to 30 mM slightly increases the agonistic activities of HMA and MIA, while 200 mM does the opposite. Although this result does not exclude interaction within the Na⁺ pocket, the effect of the Na⁺ ion is
weak, indicating a more complex mode of binding or main interaction outside this pocket.

Relevant to this idea, we conducted experiments probing the structure–activity relationship at the receptor level, specifically by creating several mutants and chimeras to increase our understanding of the residues contributing to the actions of amiloride-related derivatives and zoniporide (Figures 4 and S3). A chimera between the delta and the kappa-opioid receptor was generated because the latter is unresponsive to amiloride-related derivatives. A DOR chimera comprising a KOR fragment spanning the N-terminus to the end of TM1 (KOR-TM1) gives a functional receptor as demonstrated by the dose–response curve with DADLE (Figure 4), as well as the antagonist response toward naltrindole in the presence of 100 nM DADLE (Figure S3). We observed a loss of allosteric modulation (PAM) by MIA, HMA, and zoniporide, but the agonistic effect is still present. The loss of allosteric regulation is likely caused by the chimera’s increased efficacy observed solely with DADLE compared to the wild type (WT) receptor, which perhaps reaches the maximum efficacy of the receptor with DADLE alone. Yet, this result strongly supports that TM1 is not an essential determinant of the resultant findings. The ECL2 chimera or any chimeras involving TM2-TM6 results in inactive receptors. The chimeras, including the KOR ECL3, showed no efficacy toward DADLE but are still sensitive to MIA, and HMA, as corroborated by agonist activity. Naltrindole still reverses the effect with the ECL3 chimera but with a relatively low affinity, supporting the idea of a bitopic mode of interaction involving the orthosteric site and a juxtaposed allosteric site, probably at the apex of the Na⁺ pocket. A chimera comprising the DOR ECL2 of the leopard frog (Rana pipiens) has previously been shown to be functional; indeed, this DORrpECL2 was found to be nearly identical to the WT receptor. Given the low homology between the human and R. pipiens ECL2, we believe that ECL2 does not play a substantial role in the interactions and functional effects of amiloride-related derivatives and zoniporide.

Thereafter, we examined specific mutations of the exposed residues that might be involved in the binding and functional effect of our modulators (Figures 4 and S3), commencing with mutants in the Na⁺ pocket. We previously demonstrated that
mutants within this pocket act as an efficacy switch and reverse the antagonist naltrindole to an agonist by disrupting an important ionic lock that stabilizes the inactive conformation. The ΔG required for the transition to the active conformation is probably lowered, and naltrindole has the minimal requirement to stabilize a partially active state. Therefore, it is not surprising to observe this effect with all mutants performed except D95N.2,50 Mutations of the key Na⁺-coordinating residue D95 to alanine (D95A) results in an almost complete loss of original properties observed for MIA, HMA, and zoniporide. Similarly, the D95N mutant also had nearly full nullification of activity. However, the

![Figure 5. Assessment of the functional impact of three modulators on the binding of DOR ligands.](https://example.com/figure5)

(A) 3H-DADLE saturation assays were performed to determine the Kd at each mutant receptor. Each mutant presenting a confident Kd was evaluated in a competition experiment with increasing concentrations of the indicated ligands. Results are presented as average ± s.e.m. from two or more separate experiments, each assayed in triplicate. Binding curves were fit to a one-site model. (B) 3H-DADLE and 3H-naltrindole saturation assays were performed to determine the Kd at the WT receptor. (C) Subsequent allosteric competition experiments were performed with increasing concentrations of the indicated ligand, in the presence or absence of the indicated modulator. A representative result is shown from three independent experiments performed in triplicate.
remaining ago-PAM properties of MIA are reversed with naltrindole, indicating that this residue is not critical for interaction but rather for function. Other mutations were considered, chiefly N310A7.45 and N314A7.49, which lead to reduced effect but not a total abrogation of detected activity. As for mutating residues N131V3.35 and S135A3.39, which are less conserved in class A GPCR but present in all three opioid receptors, we noted an increase in the ago-PAM properties of zoniporide (which is absent with WT), and interestingly, for S135A, the efficacy of DADLE is restored in the presence of amiloride-related derivatives and zoniporide. These latter results strongly support an allosteric mechanism underlying the observed effects. Another interesting observation is that when the S135A mutant is stimulated with naltrindole, it abolishes the agonist activity of our allosteric modulators, but the modulators do not abrogate the agonist activity of naltrindole. This result suggests a state-dependent effect of the amiloride-related derivatives and zoniporide. We thus propose that our modulators bind and stabilize a partially active receptor, hence attributing to their partial agonist activities and low potency. A similar change in the conformational ensemble has also been described for Fg754, a recently described bitopic modulator targeting the Na⁺-cavity at the A2A receptor.36

Interestingly, a similar effect was observed with the juxtaposed mutant residue S311A7.46, which is also present in the Na⁺ pocket but not involved in the coordination of sodium or water molecules (Figure 4). Mutation of other residues in helix VII showed that only G307A7.41 completely suppressed the effects of our tested modulators (Figure 4). The Y318F3.53 of the NPxxY microswitch motif does not inhibit the modulators’ response, indicating that this layer is not of high importance to this phenomenon. Mutation within the DRY or PIF motif did not abolish the effects of the allosteric modulators, as shown for R146A3.50 and Y147A3.51. The mutation of F270A6.44 of the PIF motif results in complete loss of DADLE efficacy, which can be rescued using all three modulators in a manner similar to what was observed for S135A3.39 and S311A7.46 mutants described above (Figure 4).

Other scattering mutations were performed, including T84A2.39, which revealed increased agonist activity of our PAM, including the zoniporide, a result similar to that noted with K108A2.63 (Figure 4). The latter also shows heightened efficacy of DADLE, very similar to that observed with the KOR TM1 chimera described previously. The K1082.63 side-chain points directly within the top of the orthosteric pocket and is potentially involved in peptide-ligand interaction, but not with morphinan ligands which situate deeper in the pocket. The last TM2 mutation of Y109A2.64 did not lead to significant disturbance of our modulators. On the other hand, the Y129A3.33 mutation completely abrogated the efficacy of DADLE, but all three modulators retained their activity. It also cancels the binding of naltrindole, which is not surprising since this residue forms a hydrogen bond with water molecules, as well as the critical hydroxy group of the tyrosine in opioid peptides or corresponding phenol group in morphinan. Therefore, this residue is critical for opiate binding but not for receptor activation, as our allosteric modulators can still activate the receptor when mutated. L129M3.45 in TM3, another distinct residue in KOR, was also found to mediate none of the actions of the three allosteric modulators. Finally, a series of mutations were performed at the top of TM6 and TM7, which was found to be important for controlling DADLE efficacy. To elaborate, these mutants increased the ago-PAM behaviors produced, excluding their role in interacting or affecting our modulators. Nonfunctional mutations are shown in Figure S4.

Our mutational studies suggest that amiloride-related derivatives and zoniporide bind deep into the orthosteric pocket, with possible overlapping interactions with the top of the Na⁺ pocket. Residues G307A41 and D955.00 are the two mutants that remained functional and showed the greatest reduction in allosteric modulation by all three compounds (MIA, HMA, and zoniporide). The residues S1353.39, S3117.46, and F270A6.44, all devoid of DADLE response, regained efficacy in the presence of the modulators, likely to compensate for the lack of the propagation between the orthosteric site and the TM7, which is bounded by the Na⁺ pocket. Given that naltrindole blocked the allosteric agonism of MIA and HMA, but that MIA, HMA, and zoniporide did not block the agonist effect of naltrindole, it is likely that both types of compounds do not bind simultaneously to the receptor and probably interact with a different receptor intermediary state. It is important to note that naltrindole, a morphinan antagonist at the WT receptor, is an agonist at the Na⁺-pocket mutants, as previously reported.2

Next, we sought to estimate the binding affinities of the three selected modulators, namely, MIA, HMA, and zoniporide, toward the DOR. As shown in Figure 5A, all three modulators can displace the radioligand agonist 3H-DADLE with an estimated Kᵢ between 0.5 and 1 μM. It is thus questionable whether both molecules can simultaneously bind the receptor, as discussed below. Performing radioligand binding at mutant receptors is always challenging, in part due to the low affinities or low expression levels (low Bₘ₉₉) of many mutants, making it difficult to quantify the Kᵢ. Figure 5A highlights some of the mutants that consistently perform well in that experiment. In agreement with our functional data, the G307A mutant lost almost all modulator affinity. Surprisingly, the D95A mutant still binds to all modulators with similar affinity, indicating that D₉₅ is not critical for interaction yet still important for receptor regulation by the modulators, thus being a functionally disrupted mutant.

Certain mutants that demonstrated increased activities also had boosted affinities, such as W284E/K and S135A mutants. In the case of S135A, the affinity of naltrindole and DADLE are also shifted leftward, indicating that the loss of DADLE efficacy observed in the β-arrestin recruitment assay is not related to a decrease in affinity, but rather to a breakdown in signal propagation between the orthosteric site and the TM7 due to Na⁺-pocket disturbance. As shown in Figure 5B, all three modulators failed to efficiently displace the radioligand antagonist 3H-naltrindole, exhibiting a low displacement at 10 μM. Given the poor ability of DADLE to displace naltrindole, we added the superagonist BW373U86 (BW) as a control. BW373U86 (like most SNC series of compounds) is a superagonist in arrestin recruitment and is unaffected by Na⁺ because it directly modulates the TM-VII at the top of the orthosteric site7.17 (and unpublished data). A binding experiment performed in allosteric mode showed that MIA has no effect on the Kᵢ of naltrindole toward 3H-DADLE, suggesting that the two molecules do not interact simultaneously with the receptor. However, this functional outcome is brought about by noncompetitive allosteric antagonists, as MIA cannot displace naltrindole and has no effect on DADLE and BW373U86 affinity (Figure 5C).
DISCUSSION

Commonly defined as ligands that bind to topologically distinct sites on receptors and hence, do not occupy the natural ligand binding sites, allosteric modulators are ubiquitous among GPCRs, including those of endogenous nature such as the heterotrimeric G proteins and sodium ions. Allosteric ligands convey several advantages over their orthosteric counterparts, including spatial and temporal fine-tuning of the response of endogenous ligands, which could reduce side effects generated by chronic activation of receptors throughout the body. Moreover, other allosteric sites unassociated with endogenous molecules also harbor exploitable features given that they are generally less conserved, offering thus another level of selectivity. The virtues of allosteric regulation are particularly pertinent for the modulation of the opioid system, as chronic and overstimulation of opioid receptors are well-known to trigger side effects such as tolerance and addiction, as well as constipation and respiratory depression. Modulating only those receptors where the natural opioid peptides are present could efficiently relieve pain and reduce undesired opioid actions. Relevant to this application, the continued discoveries of novel class A GPCR allosteric modulators and the increased availability of structure-based computational methods and GPCR crystallography are advancing the development and optimization of suitable allosteric agents, bypassing the difficulties associated with chemical screening using functional assays. Different allosteric modulators have been described for the delta and mu-opioid receptors (MORs), the most active being the BMS-986122 at the MOR. Interestingly, the activity of this modulator was found to be correlated with the action of sodium and was able to disrupt the occupation of Na⁺ ion, facilitating agonist binding and hence the positive allosteric modulation observed. Although the binding site is unknown, it was shown to not compete with the orthosteric ligand and showed probe dependency, which is dependent on agonist efficacy. This is quite interesting as Na⁺ ion was found to not only modulate receptor state and thus agonist versus antagonist affinity, but also control efficacy by facilitating the transition toward the active state, as seen with antagonists that are reverted to agonists as reported before and herein.

5'-Substituted amiloride-related derivatives are weak and promiscuous allosteric inhibitors at many GPCRs. The proposed binding mode, based on docking and mutational studies at the adenosine 2A receptor, suggests the interaction of the guanidium group with the carboxy moiety of the D2. Certain limitations render pharmacological evaluation difficult with a cell-based assay, including the fact that the affinity of most amiloride-related derivatives at GPCRs is >5 µM and that most of these channel inhibitors are toxic to cells at >10 µM. Amiloride is a potassium-sparing diuretic that blocks the epithelial sodium channel (ENaC) and has also been shown to be a weak Na⁺/H⁺ exchanger (NHE) inhibitor, resulting in the generation of more potent NHE inhibitors from 5-alkylamino substituted derivatives of amiloride such as, among others, 5-(N,N-hexamethylene)-amiloride (HMA), 5-(N,N-dimethyl)-amiloride (DMA), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), and 5-(N-methyl-N-isobutyl)amiloride (MIA). Although useful in illustrating the potential clinical benefit of NHE1 inhibition in cardiac pathology, these derivatives were found to be nonselective NHE inhibitors, ultimately leading to the generation of zoniporide, a more selective NHE1 inhibitor derived from the lead NHE1 inhibitor CP-545,470. Although distinct from amiloride, most NHE inhibitors contain an acylguanidine group essential for their activity. Similar to amiloride-related derivatives, zoniporide was found to have a weak affinity for a few GPCRs. However, it is interesting to note that, as reported by Tracey et al. in 2003, zoniporide has a fairly good affinity for the rat mu-opioid (60 nM) and mouse delta-opioid (238 nM), but no detectable interaction with the kappa-opioid receptor. To obtain better pharmacological profiles of these drugs at GPCRs, we decided to perform reverse pharmacology by screening a nontoxic concentration of the selected inhibitors. The Presto-Tango is a unique open resource that allows simultaneous testing of most class A GPCRs using β-arrestin2 recruitment to measure receptor activity. This signal amplification platform is extremely sensitive and allows the detection of weak partial agonists, which usually run undetected by most assays. As a reporter assay, stimulation is performed over 16 h, conducive to increased sensitivity and minimization of the temporal aspect of drug action and interaction. While allosteric modulators are normally screened in the presence of an agonist, the simultaneous interrogation of over 350 GPCRs would make it difficult to test them in the presence of their respective agonists. Therefore, we opted for screening in “agonist” mode, stimulating the receptors with a single concentration of said modulators, to detect any activity at the class A GPCR-ome level. Although it is not optimal for studies of pure allosteric modulators, considering that amilorides were found to be quite promiscuous with mixed pharmacological properties in radioligand binding experiments, and given the proposed mode of action toward the Na⁺-binding site, which is an important efficacy switch, we were confident in detecting a potential modulating effect. Indeed, as this publication demonstrates, we found that the mu- (MOR) and delta- (DOR) opioid receptors were strongly modulated by some of the derivatives tested. We chose to further characterize the interaction at the delta-opioid receptor, given our previous structural studies of the Na⁺-cavity on said receptor. Our work provides a clear illustration of the direct activity of the derivatives at DOR, with MIA and HMA demonstrating the best agonist allosteric modulator activity (Ago-PAM). In contrast, zoniporide was found to have very weak agonist activity but still retains a similar PAM in the presence of the prototypic agonist DADLE. Considering the proposed mode of interaction with the Na⁺-coordinating residue D2.50, present in more than 95% of class A GPCRs, we first examined the effect of different Na⁺ concentrations. We reasoned that if the D2.50 is critical for the interaction, changes in Na⁺ concentration should have a significant impact, which was not observed. A weak effect was detected, but not robust enough to implicate such an important role to D2.50 in the matter of coordinating ionic interaction with the guanidium group of our derivatives.

Toward increasing our understanding of the structure-activity relationship, we used site-directed mutagenesis at DOR. Although many mutants were inactive or not expressed, we found interesting effects using our tested amiloride-related derivatives (MIA, HMA, and zoniporide). The Na⁺ pocket is crafted by 15 of the 34 most conserved residues in the majority of nonolfactory class A GPCRs. The pocket integrates three important well-known molecular switches, FxxCW, FxxP, NPXXY, and a cluster of residues making a hydrogen-bonding network; it should be noted that only the DRY motif is excluded from the pocket. Depending on the receptor, five or
six of the 15 residues lining the pocket are involved in the direct interaction with the Na\(^+\) ion, or through coordination between water and the Na\(^+\) ion.\(^6,47\) While the D95A\(^2.50\) mutant abrogated the observed functional effect, it did not strongly reduce binding, thus excluding it as a direct interacting residue with our modulators. Some of the residues forming the pocket have been found to increase the activity of our modulators, such as N131V\(^3.35\), S135A\(^3.39\), and S311A\(^7.46\), and may even rescue the loss of DADLE efficacy. As a result, we propose that these important residues lining the Na\(^+\) pocket are not important for the interaction with any of the modulators, but rather control a favorable receptor state for their interaction. This is supported by the result obtained using the antagonist naltrindole, which is reversed to partial agonist at all mutants of the Na\(^+\)-H\(_2\)O coordinating residues. In the presence of our modulators, naltrindole acts slightly as an antagonist until DOR reaches an intermediate active state, wherein naltrindole and our modulator have similar efficacy. While naltrindole did not displace our modulator in the binding experiment, the most plausible explanation is that transient states are favorable for both molecules; while both act as partial agonists, naltrindole displaces our modulator by a noncompetitive inhibitory mechanism since it has a higher affinity. Some mutants gave results that were very difficult to interpret; for example, the N67A\(^1.50\) mutant completely abolished the effects of DADLE and our modulators but gained efficacy with the antagonist naltrindole. This mutant is therefore still functional to a certain level, but we cannot conclude its role in the drug interaction. The only active mutant abrogating the modulators’ effect is the G307A\(^7.41\)(\(^7.42\)). This residue and the adjacent

---

**Figure 6.** S′-Substituted amiloride-related derivatives and the sodium channel inhibitor zoniporide stabilize a partially active conformation. (A) HTLA cells were transiently transfected with the DOR-Tango S135A mutant receptor and stimulated with increasing concentrations of naltrindole or DADLE, in the presence or absence of the indicated modulator at 10 \(\mu\)M. Elbow connector lines are used to designate a common partially active state stabilized by naltrindole and all three modulators, compared to the fully active state stimulated by MIA and DADLE (green), indicated by the arrow line. (B) Proposed model for binding of MIA and zoniporide to DOR. Docking poses of MIA (blue) and zoniporide (pink) were generated using the inactive structure (PDB:4N6H) bound to naltrindole (gray). The TM VII (green) and TM V (pink) are indicated as reference. Some important residues of the docked complex as well as the water network of the inactive structure are represented and labeled. The polar interaction network is concentrated on the left side of the binding pocket, while the right side is mainly hydrophobic. The residue G\(^7.41\), found to be critical for the interaction, is highlighted in green, and its hydrophobic surface is shown in the form of a mesh. The Na\(^+\) ion is shown as a purple sphere for reference (from the inactive structure) but is not present in the docked complex.
Y3087.42 are believed to play an important role in β-arrestin recruitment in the mu-opioid receptor (MOR). Interaction of the ligand with this residue on TM7 and TM2-TM3 stabilizes a balanced signaling state, whereas ligands that do not interact with TM7 G7.41-Y7.42 are G protein biased, such as shown for TRV130.48 It is thus possible that the weak engagement of this region by the S'-substituted amiloride stabilizes a partial agonist state. Interestingly, partial agonists such as morphine do not engage G7.41-Y7.42, and the full agonist DAMGO interacts with this TM7 region based on computational modeling.48 None of the mutations within the Na' pocket of the DOR receptor completely abolishes the functional activity nor the binding of the tested modulators, clearly excluding a major role for this pocket during their interaction. Many mutations have been shown to increase the agonist or PAM activity of all modulators, as shown for T84A3.39 and K1088.63 where zoniporide gains agonist activity, behavior which is absent when zoniporide is tested with the WT receptor. The two most exciting mutants are the S311A7.46 and S135A3.39, two residues that are exposed at the top of the sodium pocket and coordinate Na' through water molecules. In both cases, naltrindole is converted to a partial agonist that stabilizes the same receptor state as all three modulators (Figure 6A). We cannot exclude that both molecules can bind to the receptor simultaneously, seeing as none of the modulators displace naltrindole in the binding experiment. Hence, the reversal effect of naltrindole at the WT receptor could be a consequence of stabilizing the inactive conformation, which is not permissive to interaction with the modulators. Our docking and modeling studies also support our hypothesis that the modulators bind deep in the orthosteric pocket, with the guanidium moiety sitting on the apex of the Na' pocket. Some key residues important for receptor activation, such as D1283.32, W2744.48, and Y3087.42, are likely to be directly modulated by hydrophobic interactions. Given the lack of a side chain for the G3077.41, we believe that the loss of interaction and effect upon mutation to alanine is caused by steric hindrance of the methyl side chain in alanine, as well as the destabilization of the Y3088.42 (Figure 6B). Altogether, the partial agonist effect of MIA is caused by disruption of the D3.32-Y7.42 TM3-TM7 lock, which in turn disrupts the Na' ionic lock. This strongly supports that some sodium channel inhibitors harboring a guanidium group can stabilize a partially active receptor similar to the Na'-free receptor stabilized with naltrindole. Finally, we were unable to effectively quantify G protein signaling initiated by these compounds at the opioid receptors. Although the aforementioned actions were also detected using cAMP as the reading output of G protein signaling, we observed a receptor-independent modulation of cAMP, making results difficult to interpret.

CONCLUSIONS
In conclusion, we aimed to delineate the interactions of S'-substituted amiloride-related derivatives and the NHE1 inhibitor zoniporide at the delta-opioid receptor. Although most in vitro studies using those derivatives have been performed using radioligand binding or in silico modeling, our characterization approach employed a β-arrestin recruitment assay. Since the affinity of these derivatives toward the delta- and mu-opioid receptors is higher than other receptors, this allowed us to use nontoxic concentrations to study their functional impact on delta-opioid receptor signaling. Our results suggest binding of our modulators deep in the orthosteric site, which does not allow co-hosting with an orthosteric ligand such as DADLE or naltrindole. S'-substituted amiloride-related derivatives such as MIA, HMA, and EIPA have agonist activity and positive allosteric modulation, which increases efficacy when co-incubated with the full agonist DADLE. However, zoniporide, which is chemically distinct from amiloride, has a PAM activity devoid of intrinsic agonist activity. We proposed a bitopic binding mode deep in the orthosteric site overlapping the apex of the Na' pocket, wherein G3077.41 is a critical residue for the interaction, and S3117.46, S1353.39, and D952.50 are important for the transmission of the signal toward the TM7 and consequently β-arrestin recruitment. Recently, the group of Peterson reported that NHE1 inhibitors could reduce opioid self-administration in a zebrafish model.49 Although preliminary, it could be very interesting to test whether this result is mediated by NHE1, or a direct allosteric effect on opioid receptors, or both simultaneously. Many GPCRs interact with the Na(+)/H(+) exchange regulatory cofactor (NHERF-1/2), which was originally characterized as a cAMP-dependent regulator of Na(+)/H(+) exchange (NHE). Additionally, some GPCRs have been shown to regulate proton efflux through NHE1 and NHE3. Therefore, there is a clear link between GPCRs, cAMP, and Na(+)/H(+) exchangers.0,31 Since these modulators have a greater affinity toward the delta- and mu-opioid receptors, this polypharmacological effect at NHE1 and opioid receptors could be a promising avenue to explore, with the overall aim to reduce the side effects associated with opioid analogs, including dependence and tolerance.

METHODS
Cell Culture. Human Embryonic Kidney cells (HEK293T) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 5% bovine calf serum (BCS), and 100 μg/mL of penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO2. HTLA cells (kindly provided by Dr. Richard Axel), which are HEK293T stably expressing human β-arrestin fused to Tobacco Etch Virus (TEV) protease and luciferase reporter gene, were maintained in DMEM supplemented with 5% FBS, 5% BCS, 100 μg/mL penicillin and streptomycin, 2.5 μg/mL puromycin, and 50 μg/mL hygromycin. 

Tango β-Arrestin Recruitment Assay. Assays were performed using modifications of the original Tango assay52 as described and detailed previously.29,53,24 HTLA cells were transfected by the PEI precipitation method. The next day, the cells were plated in DMEM supplemented with 1% dialyzed FBS into Poly-1-Lys (PLL) coated 384-well white clear-bottom cell culture plates, at a density of 15,000 cells per well and in a total volume of 40 μL. The following day, ligand solutions were prepared in filtered assay buffer (20 mM HEPES, 1X Hanks’ balanced salt solution (HBSS), pH 7.40) at 3X and added to cells (20 μL per well) for overnight incubation (16–20 h). On the next day, media and drug solutions were removed, and 20 μL per well of homemade Glo reagent (108 mM Tris–HCl; 42 mM Tris-Base, 75 mM NaCl, 3 mM MgCl2, 5 mM dithiothreitol (DTT), 0.2 mM coenzyme A, 0.14 mg/mL D-luciferin, 1.1 mM adenosine triphosphate (ATP), 0.25% v/v Triton X-100, 2 mM sodium hydrosulfite) was added. The plates were incubated for 10 min at room temperature in the dark before counting using a Hidex Sense Beta Plus (Gamble

16948 https://doi.org/10.1021/acsof.1c07226 ACS Omega 2022, 7, 16939–16951
Technologies, ON). Data were subjected to nonlinear least-squares regression analysis using the sigmoidal dose–response function provided in GraphPad Prism 9.0. Data of three independent experiments \((n = 3)\) performed in quadruplicate are presented as Relative Light Unit (RLU) or normalized as indicated in figure legends. Parallel interrogation was performed as previously published by us, with the exception that custom-made DMEM (Wisent, Inc., QC, Canada) was used when different sodium concentrations were tested. Na+-Free DMEM was adjusted with the desired NaCl concentration and compensated with choline chloride for a final concentration of 140 mM \(\text{Na}^+\) (final osmolarity of 337 mOsm/kg). Trypan blue exclusion was used to measure cell viability for the different conditions tested; all conditions selected did not affect cell viability.

**Radioligand Binding Assays.** \(^3\)H-DADLE or \(^3\)H-naltrindole binding assays were performed using HEK293T membrane preparations transiently expressing WT or mutant DOR receptors. HEK293T cells were transfected to make membranes, and binding assays were set up in 96-well plates as previously described. All binding assays were conducted in the DOR binding buffer \((50 \text{ mM Tris HCl, } 2 \text{ mM EDTA, pH 7.40})\) in the absence of external NaCl, using 25 to 40 μg of membrane per well. Saturation binding assays with 0.2–30 nM \(^3\)H-DADLE or \(^3\)H-naltrindole in DOR binding buffer were performed to determine equilibrium dissociation constant \((K_d)\), while 10 μM naltrindole was used to define nonspecific binding. To quantify the allosteric potential of each modulator, a series of concentrations of tested ligands \((\text{e.g., DADLE})\) were incubated with a fixed concentration of \(^3\)H-DADLE or \(^3\)H-naltrindole, in the absence and presence of increasing concentrations of the indicated modulator. Reactions \((\text{either saturation or competition binding})\) were incubated for 2 h at room temperature in the dark and terminated by rapid vacuum filtration onto chilled 0.3% PEl-soaked GF/A filters, followed by three quick washes with cold washing buffer \((50 \text{ mM Tris HCl, pH 7.40})\) and quantified as previously described. Results \((\text{with or without normalization})\) were analyzed using GraphPad Prism 9.0 using one-site models.

**Molecular Biology.** Codon optimized DOR-Tango construct \((\text{Addgene #66461})\) was used in all experiments, including as a template for mutagenesis and chimera construction. Single-site mutagenesis was performed using QuikChange mutagenesis kit \((\text{Agilent, ON})\), and chimeras were created using Gibson Assembly Cloning Kit \((\text{NEB, ON})\), with OPRK1-Tango \((\text{Addgene #66462})\) serving as the source for the KOR chimera, and rpECL2 fragment synthesized by IDT \((\text{Iowa})\). All plasmids and/or more information are available upon request.

**Molecular Docking.** Compound structures of \(5-(\text{N-methyl-N-isobutyl})\)amiloride \((\text{MIA})\) and zoniporide were obtained from the PubChem database, and were subjected to molecular docking against the target crystal structures of the inactive \((\text{PDB: 4N6H})\) and active delta-opioid receptor, solved in complex with the peptide agonist KGCHM07 \((\text{PDB: 6PT2})\). Ligand and protein target preparations, and subsequent docking simulations, were performed using ICM-Pro software \((\text{Molsoft L.L.C., version 3.9.2a})\). Receptor preparation included removing unnecessary fusions and preserving water molecules known to play a role in Na–ion binding. Ligands were also preprocessed, with charges assigned to the compounds using the Merck Molecular Force Field \((\text{MMFF})\). Following the generation of receptor grid maps, five independent docking simulations were performed with a sampling thoroughness of 1. Obtained docking poses were rescoring at the end of each run using the default ICM scoring functions, individually loaded, and subsequently sorted by their predicted binding scores. They were also clustered with RMSD cutoff values to remove redundancy within the conformations generated during each simulation.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at [https://pubs.acs.org/doi/10.1021/acsomega.1c07226](https://pubs.acs.org/doi/10.1021/acsomega.1c07226).

Structures of the 5′-substituted amiloride-related derivatives and drug compounds used in this study (Figure S1); measurement of the sodium effect at the three modulators of interest \((\text{HMA, MIA, and zoniporide})\) (Figure S2); measurement of the pharmacological properties of the three modulators of interest \((\text{HMA, MIA, and zoniporide})\) at different DOR mutants (Figure S3); measurement of the pharmacological properties of the three modulators of interest \((\text{HMA, MIA, and zoniporide})\) at different nonfunctional DOR mutants (Figure S4); and parallel interrogation of the class A GPCR-ome by four sodium channel inhibitors \((\text{Table S1})\) \((\text{PDF})\).

### AUTHOR INFORMATION

#### Corresponding Author

Patrick M. Giguère — Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H8M5, Canada; Brain and Mind Research Institute, University of Ottawa, Ottawa, ON K1H8M5, Canada; orcid.org/0000-0002-0695-4855; Email: patrick.giguere@uottawa.ca

### Authors

Abdulhamid O. Mohamud — Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H8M5, Canada

Manel Zeghal — Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H8M5, Canada

Shivani Patel — Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H8M5, Canada

Geneviève Laroche — Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H8M5, Canada

Nuria Blgacim — Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H8M5, Canada

Complete contact information is available at: [https://pubs.acs.org/10.1021/acsomega.1c07226](https://pubs.acs.org/10.1021/acsomega.1c07226)

### Author Contributions

A.O.M. and M.Z. contributed equally to this work.

### Notes

The authors declare no competing financial interest.
ACKNOWLEDGMENTS

This work was supported by the Canadian Institutes of Health Research (CIHR grant #MOP142219) and the Natural Sciences and Engineering Research Council of Canada (NSERC RGPIN-2017-06151). The authors thank Dr. Adriaan P IJzerman for generously sharing his S'-substitute amiloride-related derivatives.

ABBREVIATIONS

Ago-PAM: agonist-positive allosteric modulator
ASIC3: acid-sensing ion channel 3
BW:Superagonist BW373U86
DADLE:[d-Ala2, d-Leu5]-Enkephalin
DMA: S-(N,N-dimethyl)amiloride
DOR: Delta-opioid receptor
EIPA: S-((N-ethyl-N-isopropyl)amiloride
ENaC: epithelial sodium channel
GPCR: G protein-coupled receptor
HMA: S-(N,N-hexamethylene)amiloride
KOR: Kappa-opioid receptor
MIA: S-(N-methyl-N-isobutyl)amiloride
MOR: Mu-opioid receptor
nAChR: nicotinic acetylcholine receptor
NHE1/3: sodium-hydrogen exchanger isoform 1/3
NHERF-1/2: Na(+)/H(+) exchange regulatory cofactor 1/2
OPRL1/NOP: nociceptorin receptor
OR: Opioid receptor
PAM: positive allosteric modulator
RLU: relative light unit
SAR: structure–activity relationship
TM: transmembrane domain

REFERENCES

1. Waldhoer, M.; Bartlett, S. E.; Whistler, J. L. Opioid receptors. Annu. Rev. Biochem. 2004, 73, 953–990.
2. Fenalti, G.; Giguere, P. M.; Katritch, V.; Huang, X. P.; Thompson, A. A.; Cherezov, V.; Roth, B. L.; Stevens, R. C. Molecular control of delta-opioid receptor signalling. Nature 2004, 406, 191–196.
3. van der Westhuizen, E. T.; Valant, C.; Sexton, P. M.; Christopoulos, A. Endogenous allosteric modulators of G protein-coupled receptors. J. Pharmacol. Exp. Ther. 2015, 353, 246–260.
4. Alavi, T.; Christopoulos, A. Measurements of ligand bias and functional affinity. Nat. Rev. Drug Discovery 2013, 12, 483. Comment Letter.
5. Sun, X.; Laroche, G.; Wang, X.; Agren, H.; Bowman, G. R.; Giguere, P. M.; Tu, Y. Propagation of the Allosteric Modulation Induced by Sodium in the Delta-Opioid Receptor. Chemistry 2017, 23, 4615–4624.
6. Katritch, V.; Fenalti, G.; Abola, E. E.; Roth, B. L.; Cherezov, V.; Stevens, R. C. Allosteric sodium in class A GPCR signaling. Trends Biochem. Sci. 2014, 39, 233–244.
7. Liu, W.; Chun, E.; Thompson, A. A.; Subburaju, P.; Xu, F.; Katritch, V.; Han, G. W.; Roth, C. B.; Heitman, L. H.; IJzerman, A. P.; et al. Structural basis for allosteric regulation of GPCRs by sodium ions. Science 2012, 337, 232–236. Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov’t.
8. Livingston, K. E.; Traynor, J. R. Disruption of the Na+ ion binding site as a mechanism for positive allosteric modulation of the mu-opioid receptor. Proc. Natl. Acad. Sci. U.S.A. 2014, 111, 18369–18374.
9. White, K. L.; Eddy, M. T.; Gao, Z. G.; Han, G. W.; Lian, T.; Deary, A.; Patel, N.; Jacobson, K. A.; Katritch, V.; Stevens, R. C. Structural Connection between Activation Microswitch and Allosteric Sodium Site in GPCR Signaling. Structure 2018, 26, 259–269 e255.
10. Pert, C. B.; Pasternak, G.; Snyder, S. H. Opiate agonists and antagonists discriminated by receptor binding in brain. Science 1973, 182, 1359–1361. In Vitro
11. Garritsen, A.; IJzerman, A. P.; Tulp, M. T.; Crone, E. J.; Jr.; Soudijn, W. Receptor binding profiles of amiloride analogues provide no evidence for a link between receptors and the Na+/H+ exchanger, but indicate a common structure on receptor proteins. J. Recept. Res. 1991, 11, 891–907.
12. Chun, E.; Thompson, A. A.; Liu, W.; Roth, C. B.; Griffith, M. T.; Katritch, V.; Kunken, J.; Xu, F.; Cherezov, V.; Hanson, M. A.; Stevens, R. Fusion partner toolchest for the stabilization and crystallization of g protein-coupled receptors. Structure 2012, 20, 967–976.
13. Gao, Z. G.; Melman, N.; Erdmann, A.; Kim, S. G.; Muller, C. E.; AP, I. J.; Jacobson, K. A. Differential allosteric modulation by amiloride analogues of agonist and antagonist binding at A(1) and A(3) adenosine receptors. Biochim. Pharmacol. 2003, 65, 525–534. Research Support, Non-U.S. Gov’t.
14. Horstmann, D. A.; Brandon, S.; Wilson, A. L.; Gayer, C. A.; Crone, E. J.; Jr.; Limbird, L. E. An aspartate conserved among G-protein receptors confers allosteric regulation of alpha 2-adrenergic receptors by sodium. J. Biol. Chem. 1990, 265, 21590–21595. Comparative Study In Vitro Research Support, Non-U.S. Gov’t Research Support, U.S. Gov’t, P.H.S.
15. Hooie, S. R. J.; Coldwell, M. C.; Armstrong, D.; Strange, P. G. Regulation of human D(1), d(2(long)), and d(2(short)), D(3) and D(4) dopamine receptors by amiloride and amiloride analogues. Br. J. Pharmacol. 2000, 130, 1045–1059.
16. Pauwels, P. J. Competitive and silent antagonism of recombinant 5-HT1B receptors by amiloride. Gen. Pharmacol. 1997, 29, 749–751. Comparative Study.
17. Heitman, L. H.; Ye, K.; Oosterom, J.; IJzerman, A. P. Allosteric derivatives and a nonpeptidic antagonist bind at two distinct allosteric sites in the human gonadotropin-releasing hormone receptor. Mol. Pharmacol. 2008, 73, 1806–1815. Comparative Study.
18. Barbhaiya, H.; McClain, R.; IJzerman, A.; Riskees, S. A. Site-directed mutagenesis of the human A1 adenosine receptor: influences of acidic and hydroxy residues in the first four transmembrane domains on ligand binding. Mol. Pharmacol. 1996, 50, 1635–1642. Research Support, Non-U.S. Gov’t Research Support, U.S. Gov’t, P.H.S.
19. Warnock, D. G.; Kusche-Vihrog, K.; Tarjus, A.; Sheng, S.; Oberleithner, H.; Kleyman, T. R.; Jaisser, F. Blood pressure and amiloride-sensitive sodium channels in vascular and renal cells. Nat. Rev. Nephrol. 2014, 10, 146–157.
20. Gao, Z. G.; Kim, S. K.; Gross, A. S.; Chen, A.; Blaustein, J. B.; Jacobson, K. A. Identification of essential residues involved in the allosteric modulation of the human A(3) adenosine receptor. Mol. Pharmacol. 2003, 63, 1021–1031.
21. Hori, T.; Okuno, T.; Hirata, K.; Yamashita, K.; Kawano, Y.; Yamamoto, M.; Hato, M.; Nakamura, Shimizu, T.; Yokomizo, T.; et al. Na(+)–mimicking ligands stabilize the inactive state of leukotriene B4 receptor BLT1. Nat. Chem. Biol. 2018, 14, 262–269.
22. Massink, A.; Amelia, T.; Karamychev, A.; AP, I. J. Allosteric modulation of G protein-coupled receptors by amiloride and its derivatives. Perspectives for drug discovery? Med. Res. Rev. 2020, 40, 683–708.
23. Massink, A.; Gutierrez-de-Teran, H.; Lenselink, E. B.; Ortiz Zacarias, N. V.; Xia, L.; Heitman, L. H.; Katritch, V.; Stevens, R. C.; AP, I. J. Sodium ion binding pocket mutations and adenosine A2A receptor function. Mol. Pharmacol. 2015, 87, 305–313.
24. Neve, K. A.; Cumbay, M. G.; Thompson, K. R.; Yang, R.; Buck, D. C.; Watts, V. J.; DuRand, C. J.; Teeter, M. M. Modeling and mutational analysis of a putative sodium-binding pocket on the dopamine D2 receptor. Mol. Pharmacol. 2001, 60, 373–381.
25. Parker, M. S.; Wong, Y. Y.; Parker, S. L. An ion-responsive motif in the second transmembrane segment of rhodopsin-like receptors. Amino Acids 2008, 35, 1–15.
(26) Schetz, J. A.; Sibley, D. R. The binding-site crevice of the D4 dopamine receptor is coupled to three distinct sites of allosteric modulation. J. Pharmacol. Exp. Ther. 2001, 296, 359−363.

(27) Santacana, G. E.; Silva, W. I. Differential antagonism by amiloride and pirenzepine of the muscarinic receptors of rat tracheal smooth muscle. Biol. Asoc. Med. P. R. 1990, 82, 403−406.

(28) Kroeze, W. K.; Sassano, M. F.; Huang, X. P.; Lansu, K.; McCorvy, J. D.; Giguere, P. M.; Scialy, N.; Roth, B. L. PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. Nat. Struct. Mol. Biol. 2015, 22, 362−369.

(29) Zeghal, M.; Laroche, G.; Giguere, P. M. Parallel Interrogation of beta-Arrestin2 Recruitment for Ligand Screening on a GPCR-Wide Scale using PRESTO-Tango Assay. J. Visualized Exp. 2020, No. e60823.

(30) Ledkowitz, R. J.; Shenoy, S. K. Transduction of receptor signals by beta-arrestins. Science 2005, 308, 512−517.

(31) Roth, B. I.; Marshall, F. H. NOBEL 2012 Chemistry: Studies of a ubiquitous receptor family. Nature 2012, 492, No. 57.

(32) Huhn, K.; Engelhorn, T.; Linker, R. A.; Nagel, A. M. Potential of Sodium MRI as a Biomarker for Neurodegeneration and Neuroinflammation in Multiple Sclerosis. Front. Neural. 2019, 10, No. 84.

(33) Malzacher, M.; Kalacyayan, R.; Konstandin, S.; Haneder, S.; Schad, L. R. Sodium-23 MRI of whole spine at 3 Tesla using a 3-channel receive-only phased-array and a whole-body transmit resonator. Z. Med. Phys. 2016, 26, 95−100.

(34) Massink, A.; Louvel, J.; Adlere, I.; Van Cen, C.; Huismans, B. J.; Dijksteel, G. S.; Guo, D.; Lenselink, E. B.; Buckley, B. J.; Matthews, H.; et al. S’-Substituted Amlor-Idone Derivatives as Allosteric Modulators Binding in the Sodium Ion Pocket of the Adenosine A2A Receptor. J. Med. Chem. 2016, 59, 4769−4777.

(35) Vardy, E.; Sassano, M. F.; Rennekamp, A. J.; Kroeze, W. K.; Mosier, P. D.; Westkaemper, R. B.; Stevens, C. W.; Katritch, V.; Stevens, R. C.; Peterson, R. T.; Roth, B. Single-Amino Acid Variation Underlies Species-Specific Sensitivity to Amphibian Skin-Derived Opioid-like Peptides. Chem. Biol. 2015, 22, 764−775.

(36) Lu, Y.; Liu, H.; Yang, D.; Zhong, L.; Xin, Y.; Zhao, S.; Wang, M. W.; Zhou, Q.; Shui, W. Affinity Mass Spectrometry-Based Fragment Screening Identified a New Negative Allosteric Modulator of the Adenosine A2A Receptor Targeting the Sodium Ion Pocket. ACS Chem. Biol. 2021, 16, 991−1002.

(37) DiCello, J. J.; Carbone, S. E.; Saito, A.; Pham, V.; Szemaszkiewicz, A.; Gondin, A. B.; Alvi, S.; Marique, K.; Shenoy, P.; Veldhuis, N. A.; et al. Positive allosteric modulation of endogenous delta opioid receptor signaling in the enteric nervous system is a potential treatment for gastrointestinal motility disorders. Am. J. Physiol.: Gastrointest. Liver Physiol. 2022, 322, G66−G78.

(38) Kandasamy, R.; Hillhouse, T. M.; Livingston, K. E.; Kochan, K. E.; Meurice, C.; Eans, S. O.; Li, M. H.; White, A. D.; Roques, B. P.; McLaughlin, J. P.; et al. Positive allosteric modulation of the mu-opioid receptor produces analgesia with reduced side effects. Proc. Natl. Acad. Sci. U.S.A. 2021, 118, No. e2000017118.

(39) Schetz, J. A.; Sibley, D. R. The binding-site crevice of the D4 dopamine receptor is coupled to three distinct sites of allosteric modulation. J. Pharmacol. Exp. Ther. 2001, 296, 359−363.

(40) Guzman-Perez, A.; Wester, R. T.; Allen, M. C.; Brown, J. A.; Buchholz, A. R.; Cook, E. R.; Day, W. W.; Homanaka, E. S.; Kennedy, S. P.; Knight, D. R.; et al. Discovery of zoniporide: a potent and selective sodium-hydrogen exchanger type 1 (NHE-1) inhibitor with high aqueous solubility. Bioorg. Med. Chem. Lett. 2001, 11, 803−807.

(41) Tracey, W. R.; Allen, M. C.; Frazier, D. E.; Fossa, A. A.; Johnson, C. G.; Marala, R. B.; Knight, D. R.; Guzman-Perez, A. Zoniporide: a potent and selective inhibitor of the human sodium-hydrogen exchanger isoform 1 (NHE-1). Cardiovasc. Drug Rev. 2006, 21, 17−32.

(42) Schetz, J. A.; Sibley, D. R. The binding-site crevice of the D4 dopamine receptor is coupled to three distinct sites of allosteric modulation. J. Pharmacol. Exp. Ther. 2001, 296, 359−363.

(43) Mosier, P. D.; Westkaemper, R. B.; Stevens, C. W.; Katritch, V.; Schlessinger, A.; Sali, A.; Roth, B. L.; Shoichet, B. K. Ligand discovery to record receptor activation. Proc. Natl. Acad. Sci. U.S.A. 2020, 117, 16546−16555.

(44) Bosse, G. D.; Cadeddu, R.; Floris, G.; Farero, R. D.; Vigato, E.; Lee, S. J.; Zhang, T.; Gaikwad, N. W.; Keefe, K. A.; Phillips, P. E.; et al. The GPCR-ductase inhibitor finasteride reduces opioid self-administration in animal models of opioid use disorder. J. Clin. Invest. 2021, 131, No. 143990.

(45) Barondet, D.; Ahmadzai, M. M.; Kammala, A. K.; Yang, C.; Occhiuto, C.; Das, R.; Subramanian, H. Roles of NHERF Family of PDZ-Binding Proteins in Regulating GPCR Functions. Adv. Immunol. 2017, 136, 353−385.

(46) Lefkowitz, R. J.; Shenoy, S. K. The genetic design of signaling cascades to record receptor activation. Proc. Natl. Acad. Sci. U.S.A. 2008, 105, 64−69.

(47) Allen, J. A.; Roth, B. L. Strategies to discover unexpected targets for drugs active at G protein-coupled receptors. Annu. Rev. Pharmacol. Toxicol. 2011, 51, 117−144. Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review.

(48) Carlson, J.; Coleman, R. G.; Setola, V.; Irwin, J. J.; Fan, H.; Schlessinger, A.; Sali, A.; Roth, B. L.; Shoichet, B. K. Ligand discovery from a dopamine D3 receptor homology model and crystal structure. Nat. Chem. Biol. 2011, 7, 769−778. Research Support, N.I.H., Extramural Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't.

(49) Besnard, J.; Ruda, G. F.; Setola, V.; Aebcassis, K.; Rodriguz, R. M.; Huang, X. P.; Norval, S.; Sassano, M. F.; Shin, A. I.; Webster, L. A.; et al. Automated design of ligands to polypharmacological profiles. Nature 2012, 492, 215−220. Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't.

(50) Kim, S.; Thiessen, P. A.; Shao, B.; Bolten, E. E.; Chen, J.; Fu, G.; Gindulute, A.; Han, L.; He, J.; He, S.; Shoemaker, B. A.; et al. PubChem Substance and Compound databases. Nucleic Acids Res. 2016, 44, D1202−1213.

(51) Vijay, N. O.; Carvalheda, C. A.; Zaidi, S. A.; Pisslakov, A. V.; Katritch, V.; Zacharias, U. Intracellular Transfer of Na(+)+ in an Active-State G-Protein-Coupled Receptor. Structure 2018, 26, 171−180.e172.

(52) Halgren, T. A. Merck molecular force field. I. Basis, scope, parameterization, and performance of MMFF94. J. Comput. Chem. 1996, 17, 490−519.