Sodium ions (Na\(^+\)) allosterically modulate the binding of orthosteric agonists and antagonists to many class A G protein-coupled receptors, including the dopamine D\(_2\) receptor (D\(_2\)R). Experimental and computational evidences have revealed that this effect is mediated by the binding of Na\(^+\) to a conserved site located beneath the orthosteric binding site (OBS). SB269652 acts as a negative allosteric modulator (NAM) of the D\(_2\)R that adopts an extended bitopic pose, in which the tetrahydroisoquinoline moiety interacts with the OBS and the indole-2-carboxamide moiety occupies a secondary binding pocket (SBP). In this study, we find that the presence of a Na\(^+\) within the conserved Na\(^+\)-binding pocket is required for the action of SB269652. Using fragments of SB269652 and novel full-length analogues, we show that Na\(^+\) is required for the high affinity binding of the tetrahydroisoquinoline moiety within the OBS, and that the interaction of the indole-2-carboxamide moiety with the SBP determines the degree of Na\(^+\)-sensitivity. Thus, we extend our understanding of the mode of action of this novel class of NAM by showing it acts synergistically with Na\(^+\) to modulate the binding of orthosteric ligands at the D\(_2\)R, providing opportunities for fine-tuning of modulatory effects in future allosteric drug design efforts.

Dopamine receptors (DRs) are class A G-protein-coupled receptors (GPCRs) implicated in the biology and treatment of a number of central nervous system disorders\(^1\). Drug development at these receptors has focused upon targeting the orthosteric site where dopamine binds. However, the advantages of targeting allosteric sites have begun to be explored for many GPCRs. This includes the dopamine D\(_2\) receptor (D\(_2\)R), for which drug-like positive (PAM) and negative (NAM) allosteric modulators have recently been identified\(^2\)–\(^7\). SB269652 (1) is the first small molecule NAM of the D\(_2\)R despite having a structure similar to many competitive antagonists of the D\(_2\)-like DRs\(^6\). This structure is comprised of a tetrahydroisoquinoline (THIQ) primary pharmacophore (PP) connected by a cyclohexylene linker to a lipophilic 1H-indole-2 carboxamide secondary pharmacophore (SP). Truncated derivatives of SB269652 containing the THIQ PP, which is protonated at physiological pH, act in a competitive manner with dopamine. Our previous modelling studies predicted that the basic tertiary amine within the THIQ

---

1Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, 399 Royal Parade, Parkville, VIC 3052, Australia. 2Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, 399 Royal Parade, Parkville, VIC 3052, Australia. 3Computational Chemistry and Molecular Biophysics Unit, National Institute on Drug Abuse Intramural Research Program, National Institutes of Health, 333 Cassell Drive, Baltimore, Maryland, 21224, United States. 4Departments of Psychiatry and Pharmacology, New York State Psychiatric Institute, New York, New York, 10032, United States. 5College of Physicians and Surgeons, Columbia University, and Division of Molecular Therapeutics, New York State Psychiatric Institute, New York, New York, 10032, United States. 6Present address: Tri-Institutional Therapeutics Discovery Institute, 413 E 69th St, New York, NY, 10021, United States. Christopher J. Draper-Joyce and Ravi Kumar Verma contributed equally to this work. Correspondence and requests for materials should be addressed to L.S. (email: lei.shi2@nih.gov) or J.R.L. (email: rob.lane@monash.edu)
PP of SB269652 forms a salt bridge with Asp^3.32 (Ballosteros and Weinstein numbering system) in the orthosteric binding site (OBS), while the 1H-indole-2-carboxamide SP of SB269652 extends away from the OBS to interact with residues in a secondary binding pocket (SBP) between the extracellular ends of transmembrane segments (TMs) 2 and 7. To reconcile an orthosteric mode of engagement with an allosteric mechanism of action, we propose that SB269652 acts to engage one protomer of a D₂R dimer in a bitopic binding mode to modulate dopamine binding and function in the adjacent protomer.

The modulatory effects of sodium ions (Na⁺) on orthosteric ligand binding at class A GPCRs has been well characterised. Recently, high resolution structures of the adenosine A₂A receptor (A₂AR) ²⁰ the β₁ adrenergic receptor, protease activated receptor ²⁰, the δ-opioid receptor, and the D₃R ²⁰, revealed a Na⁺-binding site below the orthosteric pocket in which a Na⁺ is coordinated by the side chain oxygen atoms of Asp ³.⁵⁰, Ser ³.⁹⁹, and three water molecules. Molecular dynamics (MD) studies of the A₂AR show that the presence of Na⁺ in this pocket inhibits conformational movement of TM7 toward TM3 at the intracellular side, stabilising an “inactive” conformation. Thus, the absence of Na⁺ is associated with the active conformation of the receptor, which has a greater dynamic flexibility in the movement of Trp ³.⁴₈ and Asn ⁷.⁴₅ ²⁰,²¹.

Physiologically relevant concentrations of Na⁺ have been shown to decrease agonist affinity for the D₃R while enhancing the affinity for some classes of antagonists.²²,²³ Substituted benzamides, including eticlopride and sulpiride, display increased affinity in the presence of Na⁺, whereas sipperone is insensitive and the affinity of zotepine is decreased.²²,²³ Substitution of Asp²⁰⁰ of the D₃R with an uncharged residue reduces the affinity of some antagonists ²¹,²². Our previous docking and MD studies provided insight into the mechanism behind the distinct Na⁺ sensitivities of these antagonists, revealing an allosteric interaction network that propagates cooperative effects from the Na⁺ binding site to the OBS.²² In particular, Na⁺ binding stabilised an interaction between the side chains of Asp²⁰⁰ and Tyr²⁴⁹. In the Na⁺-unbound condition, the Na⁺ sensitive antagonists are correlated with the weakening of this hydrogen bond interaction. Another study revealed that Na⁺ binding to this conserved site was important for the subtype selectivity of D₃R-selective agonists and antagonists through modulation of the interaction of the SP of such ligands to a residue within the SBP.²⁵ Using MD simulations, Selent and coworkers proposed that Na⁺ enters the D₃R via the extracellular side of the receptor, moving along a network of negatively charged residues including Glu²⁰².⁵⁰, before entering the Na⁺-binding site coordinated by Asp⁸⁰².⁵⁰. This Na⁺ binding was proposed to lock the rotamer toggle switch Trp³⁸⁶.⁴₈ in the inactive state ²⁶.

In addition to these effects on orthosteric ligand binding, Na⁺ ions have been shown to modulate the action of BMS986122, a PAM of the µ-opioid receptor, which was shown to exert its effect through disruption of the Na⁺ binding site.²⁷ This study, as well as the critical role of Na⁺ in orthosteric D₃R ligand binding, prompted us to investigate the role of Na⁺ in the action of SB269652. Here, we demonstrate that the action of SB269652 is exquisitely sensitive to the presence of Na⁺, so that in the absence of Na⁺, SB269652 no longer inhibits orthosteric ligand binding at the D₃R. Using mutagenesis, MD simulations and derivatives of SB269652, we demonstrate that a Na⁺ ion, coordinated by Asp²⁸⁰⁻⁵⁰, is required for the high affinity binding of the THIQ PP. Interestingly, the particularly high Na⁺-sensitivity of SB269652, compared to both truncated fragments and structurally similar extended derivatives, is determined by the interaction between the 1H-indole-2-carboxamide moiety and the SBP.

Results

The binding of [³H]spiperone is insensitive to Na⁺. Saturation ligand binding studies were performed with the antagonist [³H]spiperone at wild-type (WT), D₈₀².₅₀Å and E₉₅².₆₅Å c-myc-D₂LRs stably expressed in membranes of Flp-In CHO cells. These assays were performed in the presence or absence of 100 mM NaCl. In the absence of NaCl, we included 100 mM of the organic cation N-methyl-D-glucamine (NMDG) to maintain B_max.

In the presence of 100 mM NaCl, increasing concentrations of SB269652 caused both an incomplete displacement of [³H]spiperone and modulation of dopamine binding (Fig. 2B). These data were fit to an allosteric ternary complex model to obtain a value of affinity for SB269652 (Kₐ = 355 nM) and cooperativity with dopamine (logα = −0.54 ± 0.02, α = 0.29) and [³H]spiperone (logα = −0.45 ± 0.08, α = 0.35, Fig. 2B). In contrast, in the absence of Na⁺, SB269652 failed to modulate dopamine binding and function in the adjacent protomer ²⁶.
The affinity of dopamine is not significantly changed in the absence of Na⁺. Competition binding experiments performed using membranes of Flp-In CHO cells stably expressing a human Myc-hD₂₃R and the antagonist [³H]spiperone reveal that in the presence of 100 mM NaCl (p < 0.05, one-way ANOVA, Tukey’s post hoc test). Statistically different from corresponding affinity estimate of ligand at WT D₂₃R in the presence of 100 mM NaCl (p < 0.05, one-way ANOVA, Tukey’s post hoc test). Statistically different from corresponding affinity estimate of ligand at WT D₂₃R in the presence of 100 mM NaCl (p < 0.05, one-way ANOVA, Tukey’s post hoc test). Statistically different from corresponding affinity estimate of ligand at WT in the presence of 100 mM NMDG (p < 0.05, one-way ANOVA, Tukey’s post hoc test). ND signifies that no inhibition of [³H]spiperone binding was detected. N/A signifies that the parameter was not calculated. '-' signifies that no binding experiments were performed for that condition.

### Table 1. Binding parameters of orthosteric ligand and SB269652 derivatives at N-terminal c-Myc-tagged WT, D80²⁵⁰A, and E95²⁶⁵A D₂₃R constructs stably expressed in Flp-In CHO cell membranes. Data is the mean ± SEM of three individual experiments. *Estimate of the negative logarithm of the equilibrium dissociation constant. †Estimate of the logarithm of the cooperativity factor between the modulator and [³H]spiperone. ‘experiments performed using 0.5 nM [³H]spiperone. ‡Statistically different from corresponding affinity estimate of ligand at WT D₂₃R in the presence of 100 mM NaCl (p < 0.05, one-way ANOVA, Tukey’s post hoc test). §Statistically different from corresponding affinity estimate of ligand at WT D₂₃R in the presence of 100 mM NaCl (p < 0.05, one-way ANOVA, Tukey’s post hoc test). ¶Statistically different from corresponding affinity estimate of ligand at WT D₂₃R in the presence of 100 mM NaCl (p < 0.05, one-way ANOVA, Tukey’s post hoc test). ND signifies that no inhibition of [³H]spiperone binding was detected. N/A signifies that the parameter was not calculated. '-' signifies that no binding experiments were performed for that condition.

| Ligand | Kᵢ (µM) | Bₘₐₓ (fmol/mg) | pKᵢ | logBᵢ | α | Kᵢ(µM) | pKᵢ(µM) | logBᵢ(µM) | α |
|--------|---------|-----------------|------|-------|---|---------|---------|-----------|---|
| WT     |         |                 |      |       |   |         |         |           |   |
| 100 mM NaCl | 10.45 ± 0.06 (0.04) | 237.0 ± 6.7 | 10.31 ± 0.12 (0.05) | 216.1 ± 10.8 | 9.94 ± 0.11 (0.1) | 215.4 ± 7.0 | 10.24 ± 0.14 (0.06) | 192.4 ± 10.7 | 10.21 ± 0.09 (0.06) | 225.3 ± 27.2 | 10.10 ± 0.03 (0.08) | 196.6 ± 8.8 |
| WT     |         |                 |      |       |   |         |         |           |   |
| 100 mM NMDG | 10.45 ± 0.06 (0.04) | 237.0 ± 6.7 | 10.31 ± 0.12 (0.05) | 216.1 ± 10.8 | 9.94 ± 0.11 (0.1) | 215.4 ± 7.0 | 10.24 ± 0.14 (0.06) | 192.4 ± 10.7 | 10.21 ± 0.09 (0.06) | 225.3 ± 27.2 | 10.10 ± 0.03 (0.08) | 196.6 ± 8.8 |

**Figure 1.** The affinity of dopamine is not significantly changed in the absence of Na⁺. Competition binding experiments performed using membranes of Flp-In CHO cells stably expressing a human Myc-hD₂₃R and the antagonist [³H]spiperone reveal that in the presence of 100 mM NaCl (closed symbols) the mutations D80²⁵⁰A and E95²⁶⁵A do not significantly change the affinity of dopamine relative to that at the wild type (WT) receptor. In the absence of Na⁺ (open symbols), whereby 100 mM of the organic cation N-methyl-D-glucamine (NMDG) is included in the buffer to maintain ionic strength, no change in dopamine affinity is observed at the WT, D80²⁵⁰A or E95²⁶⁵A hD₂₃R relative to the affinity determined in the presence of Na⁺.

[^H]spiperone or displace [³H]spiperone binding (Fig. 2C, Table 1). Thus, we were unable to detect any evidence of SB269652 binding in the absence of Na⁺. We extended this study to determine whether this exquisite Na⁺ sensitivity reflects a need for a Na⁺ ion bound within the conserved Na⁺-binding pocket by measuring the ability of SB269652 to displace [³H]spiperone binding to the D80²⁵⁰A mutant (Fig. 2D, Table 1), in which the conserved Na⁺ site is disrupted. Similar to our observations in the absence of Na⁺, SB269652 no longer inhibited the binding of [³H]spiperone at this mutant even in the presence of 100 mM NaCl. Thus, SB269652 binding is dependent on the presence of a Na⁺ ion in the conserved Na⁺-binding pocket coordinated by Asp80²⁵⁰.

A Na⁺ ion coordinated by Asp80²⁵⁰ is important for high affinity binding of the THIQ moiety within the OBS. Our previous studies have shown that the presence of a Na⁺ in the conserved Na⁺-binding pocket can modulate the affinity of some but not all D₂R antagonists. With the hypothesis that dopamine affinity or displace [³H]spiperone binding (Fig. 2C, Table 1). Thus, we were unable to detect any evidence of SB269652 binding in the absence of Na⁺. We extended this study to determine whether this exquisite Na⁺ sensitivity reflects a need for a Na⁺ ion bound within the conserved Na⁺-binding pocket by measuring the ability of SB269652 to displace [³H]spiperone binding to the D80²⁵⁰A mutant (Fig. 2D, Table 1), in which the conserved Na⁺ site is disrupted. Similar to our observations in the absence of Na⁺, SB269652 no longer inhibited the binding of [³H]spiperone at this mutant even in the presence of 100 mM NaCl. Thus, SB269652 binding is dependent on the presence of a Na⁺ ion in the conserved Na⁺-binding pocket coordinated by Asp80²⁵⁰.

[^H]spiperone or displace [³H]spiperone binding (Fig. 2C, Table 1). Thus, we were unable to detect any evidence of SB269652 binding in the absence of Na⁺. We extended this study to determine whether this exquisite Na⁺ sensitivity reflects a need for a Na⁺ ion bound within the conserved Na⁺-binding pocket by measuring the ability of SB269652 to displace [³H]spiperone binding to the D80²⁵⁰A mutant (Fig. 2D, Table 1), in which the conserved Na⁺ site is disrupted. Similar to our observations in the absence of Na⁺, SB269652 no longer inhibited the binding of [³H]spiperone at this mutant even in the presence of 100 mM NaCl. Thus, SB269652 binding is dependent on the presence of a Na⁺ ion in the conserved Na⁺-binding pocket coordinated by Asp80²⁵⁰.

[^H]spiperone or displace [³H]spiperone binding (Fig. 2C, Table 1). Thus, we were unable to detect any evidence of SB269652 binding in the absence of Na⁺. We extended this study to determine whether this exquisite Na⁺ sensitivity reflects a need for a Na⁺ ion bound within the conserved Na⁺-binding pocket by measuring the ability of SB269652 to displace [³H]spiperone binding to the D80²⁵⁰A mutant (Fig. 2D, Table 1), in which the conserved Na⁺ site is disrupted. Similar to our observations in the absence of Na⁺, SB269652 no longer inhibited the binding of [³H]spiperone at this mutant even in the presence of 100 mM NaCl. Thus, SB269652 binding is dependent on the presence of a Na⁺ ion in the conserved Na⁺-binding pocket coordinated by Asp80²⁵⁰.

[^H]spiperone or displace [³H]spiperone binding (Fig. 2C, Table 1). Thus, we were unable to detect any evidence of SB269652 binding in the absence of Na⁺. We extended this study to determine whether this exquisite Na⁺ sensitivity reflects a need for a Na⁺ ion bound within the conserved Na⁺-binding pocket by measuring the ability of SB269652 to displace [³H]spiperone binding to the D80²⁵⁰A mutant (Fig. 2D, Table 1), in which the conserved Na⁺ site is disrupted. Similar to our observations in the absence of Na⁺, SB269652 no longer inhibited the binding of [³H]spiperone at this mutant even in the presence of 100 mM NaCl. Thus, SB269652 binding is dependent on the presence of a Na⁺ ion in the conserved Na⁺-binding pocket coordinated by Asp80²⁵⁰.

[^H]spiperone or displace [³H]spiperone binding (Fig. 2C, Table 1). Thus, we were unable to detect any evidence of SB269652 binding in the absence of Na⁺. We extended this study to determine whether this exquisite Na⁺ sensitivity reflects a need for a Na⁺ ion bound within the conserved Na⁺-binding pocket by measuring the ability of SB269652 to displace [³H]spiperone binding to the D80²⁵⁰A mutant (Fig. 2D, Table 1), in which the conserved Na⁺ site is disrupted. Similar to our observations in the absence of Na⁺, SB269652 no longer inhibited the binding of [³H]spiperone at this mutant even in the presence of 100 mM NaCl. Thus, SB269652 binding is dependent on the presence of a Na⁺ ion in the conserved Na⁺-binding pocket coordinated by Asp80²⁵⁰.
of SB269652 upon dopamine or [3H]spiperone affinity at the hD2LR is observed. (e) Evidence that a Na\(^+\) antagonist [3H]spiperone reveal that in the presence of 100 mM sodium (closed symbols) SB269652 acts to partially displace the radioligand consistent with the action of a negative allosteric modulator (NAM). SB269652 has no effect in the absence of Na\(^+\) (open symbols) or at the D80A and E95A-R receptor mutants.

However, SB269652 displays relatively modest affinity for the D2R and weak negative cooperativity, resulting in the absence of Na\(^+\) bound in the conserved pocket to bind with high affinity to the OBS, we performed competition binding experiments with two orthosteric fragments of SB269652: 2-propyl-1,2,3,4-tetrahydroisoquinoline-7-carbonitrile (2, MIPS1071) and a more extended fragment N-((trans)-4-(2-(7-cyano-3,4-dihydroisoquinolin-2(1H)yl)ethyl)cyclohexyl)acetamide (3, MIPS1059). Both MIPS1071 and MIPS1059 have previously been shown to behave as competitive antagonists of dopamine in functional assays and acted to completely displace the binding of [3H]spiperone (Kᵢ = 1.3 μM and 275 nM respectively, Fig. 3). MIPS1071 displayed a significant loss of affinity both in the absence of Na\(^+\) and at the D80A-R mutant receptor (Fig. 3A, Table 1). In both cases, MIPS1071 was unable to completely displace the binding of [3H]spiperone at a concentration of 100 nM. However, by assuming complete displacement, we could estimate values of affinity in the Na\(^+\)-free condition (pKᵢ = 4.92 ± 0.07, Kᵢ = 12 μM) and at the D80A-R mutant receptor (pKᵢ = 5.03 ± 0.07, Kᵢ = 9.3 μM) that correspond to a 9-fold and 7-fold loss in affinity respectively (one-way ANOVA, Tukey’s post-hoc test, P_\(\text{Na}^+\) = 0.0001, P_\(\text{DHA}^+\)  0.0001). Similarly, MIPS1059 displayed a 6-fold or 5-fold decrease in affinity in the absence of Na\(^+\) (Kᵢ = 1.7 μM) or at the D80A-R mutant (Kᵢ = 1.5 μM) respectively (Fig. 3B, Table 1, one-way ANOVA, Tukey’s post-hoc test, P_\(\text{Na}^+\) < 0.0001, P_\(\text{DHA}^+\)  0.0001). In both cases, no additional effect of the Na\(^+\)-free condition was observed at the D80A-R mutant. Thus, the Na\(^+\) sensitivity of both orthosteric fragments provides evidence that a Na\(^+\) ion, coordinated by Asp80\(^{2,50}\), is required for the high affinity binding of the THIQ PP of SB269652 within the OBS. However, given that in the absence of Na\(^+\), or at the D80A-R mutant these orthosteric fragments display a maximal 10-fold decrease in affinity, if SB269652 displayed a similar loss of affinity then we would still be readily able to detect the activity of SB269652 in our binding studies. Thus, the absence of Na\(^+\) must either have a greater effect upon SB269652 affinity and/or an additional effect upon SB269652 cooperativity. The lack of effect of SB269652 in the absence of Na\(^+\) means that it is not possible to distinguish these two possibilities. However, SB269652 displays relatively modest affinity for the D2R and weak negative cooperativity, resulting in only a small window to detect changes in pharmacology. To address this, we extended our study to extended derivatives of SB269652 that display higher affinity and negative cooperativity with dopamine.

A Na\(^+\) ion coordinated by Asp80\(^{2,50}\) modulates the affinity and cooperativity of high affinity derivatives of SB269652. To understand the effect of Na\(^+\) on the affinity and/or cooperativity of an
The absence of Na\(^+\) disrupts the binding modes of SB269652 and its derivatives. Our previous modelling studies predicted that SB269652 binds to the D\(_2\)R in an extended pose in which the THIQ PP occupies the OBS while the 1H-indole-2-carboxamide SP of SB269652 extends away from the OBS to interact...
with residues within the SBP. In particular, these studies suggested that a hydrogen bond interaction between Glu952.65 and the indolic NH is important for the affinity and cooperativity of SB269652. To test how the absence of the Na\(^+\) bound at the canonical Na\(^+\)-binding site near Asp802.50 would alter the binding poses of SB269652 and its aforementioned derivatives, we carried out comparative MD simulations of SB269652, MIPS1059, MIPS1726, and MIPS1868 bound to the D\(_2\)R. For all these complexes, we simulated both the Na\(^+\)-bound and Na\(^+\)-unbound conditions (See Methods and Table 2).

For all these compounds, whereas in the Na\(^+\)-bound conditions the THIQ moiety consistently faces the TM4-TM5 interface in the OBS, in the Na\(^-\)-unbound conditions this moiety has a strong tendency to move towards the TM5-TM6 interface and deeper into the OBS (Fig. 5A–D, Supplementary Figures 1 and 2). In addition, the salt-bridge interaction between the Asp1143.32 and the protonated amine nitrogen (N1, see Supplementary Figure 1) of the ligands is significantly weakened compared to that in the Na\(^+\)-bound conditions (Supplementary Figures 1 and 2). We hypothesised that these changes are responsible for the observed differences in the binding affinities of these Na\(^-\)-sensitive ligands. Indeed, the results of our MM/GBSA calculations of the

| Ligand      | Na\(^+\) | Number of trajectories | Total Length (µs) |
|-------------|----------|------------------------|-------------------|
| SB269652    | +        | 37                     | 18.0              |
|             | -        | 4                      | 9.0               |
| MIPS1059    | +        | 2                      | 4.8               |
|             | -        | 2                      | 4.8               |
| MIPS1726    | +        | 2                      | 4.2               |
|             | -        | 4                      | 6.6               |
| MIPS1868    | +        | 4                      | 6.0               |
|             | -        | 2                      | 4.8               |
| Total       |          | 57                     | 58.2              |

Table 2. Summary of MD simulations of D\(_2\)R in complex with SB269652 and its derivatives.

Figure 4. High affinity Derivatives of SB269652 display lower affinity and cooperativity in the absence of a sodium ion coordinated by D802.50. (A) In the presence of 100 mM NaCl (closed symbols) the azaindole derivative of SB269652, (4, MIPS1726) acted to partially displace 0.5 nM \([^3H]\)spiperone in a competition binding assay using membranes from FlpIN CHO cells stably expressing the WT hD\(_2\)R. These data could be fit to an allosteric ternary complex model to derive a value of affinity and cooperativity with \([^3H]\)spiperone (Table 1). In the presence of Na\(^+\), MIPS1726 displayed lower affinity at the E952.65A and D802.50 receptor mutants (Table 1). In the absence of Na\(^+\) (100 mM NMDG, open symbols) MIPS1726 displayed lower affinity at the WT and E952.65A D\(_2\)Rs but no change in affinity at the D802.50A mutant (Table 1). (B) In the presence of 100 mM NaCl (closed symbols) the 7-fluoro-indole-2-carboxamide derivative of SB269652 (5, MIPS1868) acted to partially displace 0.15 nM \([^3H]\)spiperone in a competition binding assay using membranes from FlpIN CHO cells stably expressing the WT hD\(_2\)R. These data could be fit to an allosteric ternary complex model to derive a value of affinity for SB269652 and cooperativity with \([^3H]\)spiperone (Table 1). In the presence of Na\(^+\), MIPS1868 displayed lower affinity at the E952.65A receptor mutant but lower affinity and cooperativity at the D802.50 receptor mutant (Table 1). In the absence of Na\(^+\) (100 mM NMDG, open symbols) MIPS1868 displayed lower affinity at the WT D\(_2\)R but no change in affinity at the E952.65A or D802.50A mutants.
ligand binding energies indicate that SB269652 and its derivatives bind more favourably in the Na\(^+\)-bound conditions than their corresponding Na\(^+\)-unbound conditions (Supplementary Figure 3). We were also interested in investigating how the Na\(^+\)-unbound condition would affect the orientation of the SP of SB269652 and derivatives within the SBP. We found that the interactions between Glu952.65 and the amide nitrogen (N3) or the nitrogen of the aryl moiety (N4) of MIPS1868 and MIPS1726 were consistently maintained in the Na\(^+\)-unbound condition; in comparison, the corresponding interactions were predicted to be much weaker for SB269652 (Supplementary Figure 2). This suggests that the modifications to the SP of MIPS1726 and MIPS1868 contribute additional interactions within the SBP that confer the higher affinity of these ligands. This in turn may explain why the Na\(^+\)-unbound condition ablates the action of SB269652, whereas the NAM activities of MIPS1726 and MIPS1868 are still detectable in the absence of Na\(^+\).

Na\(^+\)-binding modulates the interaction of SB269652 derivatives with the SBP. We extended our study to further explore how Na\(^+\) binding might influence the interaction between the indolc NH of SB269652 and the SBP residue Glu952.65. Consistent with our previous findings, the affinity of SB269652 (6-fold, Student's two-tailed unpaired t-test, \(P = 0.004\)) was decreased at the E952.65A mutant\(^\text{+}\). As observed at the WT D\(_2\)R, in the absence of Na\(^+\), SB269652 was unable to inhibit the binding of [\(^3\)H]spiperone at the E952.65A mutant (Fig. 2C) - thus we could not measure an additional effect of the Na\(^+\)-free condition. The affinity of the orthosteric fragment MIPS1071 was not changed, as compared to WT, by the mutation E952.65A in the presence or absence of Na\(^+\) (Table 1, one-way ANOVA with Tukey’s post-hoc test, \(P_{\text{Na}^+} > 0.99, P_{\text{Na}^-} = 0.55\)). However, the extended fragment MIPS1059 displayed a significant 5-fold affinity reduction at the E952.65A mutant compared to the WT in the presence of Na\(^+\) (Table 1, one-way ANOVA with Tukey’s post-hoc test, \(P = 0.0002\)). This loss of affinity may reflect the loss of a hydrogen bond interaction between Glu952.65 and the carboxamide NH of MIPS1059. A significant 3-fold loss of affinity at the E952.65A mutant was also observed in the absence of Na\(^+\) (one-way ANOVA with Tukey’s post-hoc test, \(P = 0.01\)). Similar to observations at the WT D\(_2\)R, MIPS1059 displays 5-fold lower affinity at the E952.65A mutant in the presence of Na\(^+\) compared to the E952.65A mutant in the presence of Na\(^+\) (one-way ANOVA with Tukey’s post-hoc test, \(P = 0.0008\)).

The extended SB269652 derivative, MIPS1726, displayed a significant 4-fold loss in affinity at the E952.65A (\(K_a = 123\) nM) mutant compared to WT D\(_2\)R (one-way ANOVA with Tukey’s post-hoc test, \(P = 0.0003\)). The absence of Na\(^+\) caused a further significant 7-fold decrease in affinity at the E952.65A mutant consistent with these
effects being additive, similar to our observations with the fragment MIPS1059 (one-way ANOVA with Tukey’s post-hoc test, P < 0.0001). The loss of affinity for MIPS1726 at the E95^2.65A mutant as compared to the WT D2R in both the presence and absence of Na^+ is consistent with presence of a hydrogen bond interaction between the azaindole moiety of MIPS1726 and Glu95^2.65 in both conditions (Table 1, Fig. 4B). The mutation E95^2.65A caused a modest 2-fold decrease in affinity of the extended SB269652-derivative (one-way ANOVA, Tukey’s post-hoc test, P = 0.049), MIPS1868, in the presence of Na^+. The Na^+-free condition caused a further 3-fold decrease in the affinity of MIPS1868 at this mutant (one-way ANOVA, Tukey’s post-hoc test, P = 0.02). However, in contrast to the 2-fold difference observed for MIPS1726, comparison of the affinity of MIPS1868 at the WT D2R and the E95^2.65A mutant in the absence of Na^+ revealed no significant difference (one-way ANOVA with Tukey’s post-hoc test, P > 0.99). Interestingly, results from our MD simulations revealed that the extra nitrogen (N5 atom) or the fluoro atom on the SP of MIPS1726 and MIPS1868, respectively, form interactions with the hydroxyl group of Tyr34^1.32 in Na^+-bound condition (Supplementary Figure 4). In the absence of Na^+, the interaction between the fluoro atom of MIPS1868 and Tyr34^1.32 remained stable, whereas the interaction between the N5 atom of MIPS1726 and Tyr34^1.32 weakens, which suggests that interaction between MIPS1868 and Tyr34^1.32 is relatively strong and may mask the presence and absence of the interactions with Glu95^2.65.

Finally, it is interesting to note that the observed difference in affinity of MIPS1059, MIPS1726 and MIPS1868 between the WT and E95^2.65A mutant was significantly and consistently (2-fold) greater in the presence of Na^+ as compared to the difference observed in the absence of Na^+ (Supplementary Table 1). This suggests that in all cases the presence of Na^+ has a subtle but significant effect upon modulating the interaction between these SB269652 derivatives and the SBP.

**Discussion**

The potentially conserved functional role of Na^+ at GPCRs^{28} prompted us to explore the impact of Na^+ binding on the pharmacology of SB269652, the first small-molecule NAM identified at the D2R. We found that SB269652 displayed exquisite sensitivity to Na^+, losing its modulatory effect in the absence of this ion. Recent crystallographic, computational modelling and biochemical efforts have revealed a Na^+ binding site beneath the OBS that includes a highly conserved aspartate (Asp^2.50)\(^{15,16}\), which allowed us to carry out a mechanistic study using both mutagenesis and computational approaches.

A recent study demonstrated that a μ opioid receptor PAM, BMS986122, decreased the ability of Na^+ ions to inhibit agonist binding. This observation can be accommodated within a two-state model, whereby BMS986122 favours an active receptor conformation with a collapsed Na^+-binding pocket\(^27\). The action of SB269652 cannot be reconciled within such a model because it exerts negative allosteric modulation with respect to both agonist and antagonist binding. Previous studies of the D2R revealed that in the absence of Na^+, the competitive antagonist raclopride exerted non-competitive effects upon both its own binding and that of the antagonist [\( ^3\)H]spiperone\(^{30,31}\). These observations were rationalised within a model of a D2R dimer whereby the binding of one molecule of raclopride to one protomer of this dimer, exerted negative cooperativity in the binding of another molecule to the orthosteric site of the other protomer\(^39\). We have previously proposed a novel mechanism of action for SB269652, where it binds as a bitopic ligand at one protomer and exerts negative cooperativity for the binding and action of dopamine at an adjacent protomer\(^2\). However, SB269652 and its derivatives MIPS1726 and MIPS1868 all display negative allosteric pharmacology with [\( ^3\)H]spiperone in the presence of Na^+ ions. Thus, the non-competitive action of these ligands cannot be explained by the mechanism proposed to explain the behaviour of [\( ^3\)H]raclopride in the absence of Na^+\(^28\).

Some classes of D2R antagonists such as substituted benzamides show a significant loss of affinity in the Na^+-unbound state\(^12\). We previously demonstrated that in the Na^+-unbound state the weakening of a critical hydrogen bond interaction between the Asp^3.32 and Tyr^7.43 residues, positioned above the Na^+-binding site, led to non-optimal binding modes for these Na^+-sensitive antagonists\(^34\). Results from these MD simulations were consistent with results from a previous study that demonstrated that mutation of Tyr^7.43 to cysteine dramatically decreased the affinity of sulphide (185-fold), while only a subtle loss of affinity was observed for the Na^+-insensitive N-methyl-spiperone (3-fold)\(^32\). Ligands that displayed Na^+-insensitivity, such as spiperone, make additional contacts within the ligand binding pocket that mask the impact of the absence of bound Na^+\(^24\).

The results of mutagenesis and MD simulations in this study reveal that the Na^+ sensitivity of SB269652 can be reconciled with a mechanism whereby the occupancy of the OBS by the THIQ moiety is dependent upon Na^+. Indeed, the two fragments of SB269652 (MIPS1071 and MIPS1059) that contain the THIQ moiety and act as competitive antagonists displayed pronounced Na^+ sensitivity, akin to that of the substituted benzamides\(^2,23,24\). This Na^+ sensitivity is mediated by the interaction of Na^+ with Asp80^2.50, as mutation of this residue to alanine produced an identical effect in either the presence or absence of Na^+. Consistent with this hypothesis, the predominant effect of the Na^+-free condition was a decrease in affinity of the high affinity allosteric SB269652 analogues MIPS1726 and MIPS1868.

However, while the lack of effect of SB269652 in the absence of Na^+ may reflect a significant decrease in affinity for the OBS, the absence of a Na^+ coordinated by Asp80^2.50 caused a relatively modest decrease in affinity of the THIQ-containing fragments MIPS1071 and MIPS1059 (10-fold and 6-fold, respectively). Similarly, the higher affinity SB269652 derivatives MIPS1726 and MIPS1868 show 12-fold and 5-fold lower affinities respectively in the absence of Na^+. In comparison, the lack of activity of SB269652 in the absence of Na^+ up to a concentration of 30 μM requires a much larger (greater than 80-fold) decrease in affinity. This suggests that the absence of Na^+ exerts effects of greater magnitude upon SB269652 affinity and/or additional effects such as a decrease in negative cooperativity. In agreement with a larger impact upon SB269652 affinity, our MD simulations showed that the interactions of Glu95^2.65 in the SBP with the protonated amide nitrogen and the protonated nitrogen of the aryl moiety of MIPS1868 and MIPS1726 were maintained even in the Na^+-free condition, whereas the corresponding interactions were predicted to be much weaker for SB269652. This suggests that the modifications to the SP of
MIPS1726 and MIPS1868 relative to the 1H-indole-2-carboxamide SP of SB269652 contribute additional interactions within the SBF that confer the higher affinities of these two derivatives and may explain why the Na"+-free condition abolishes the action of SB269652, while retaining detectable NAM activities of MIPS1726 and MIPS1868. The orthosteric fragments MIPS1071 and MIPS1059 lack this SP. Interestingly, the addition of the indole moiety of SB269652 does not confer a gain in affinity relative to MIPS1059. Therefore, one possible explanation for the greater effect of the Na"+-free condition upon SB269652 as compared to these orthosteric fragments, is that the addition of the SP of SB269652 not only confers a relatively weak interaction with Glu952.65 but decreases the strength of the interaction with residues within the OBS, together conferring a greater sensitivity to Na"+. It is interesting to note that the observed difference in affinity of MIPS1059, MIPS1726 and MIPS1868 between the WT and E952.65A mutant was significantly and consistently (2-fold) greater in the presence of Na"+ as compared to the difference observed in the absence of Na"+ (Supplementary Table 1). Such a difference may reflect a change in orientation of these ligands such that the interaction with Glu952.65 has less impact upon ligand affinity, or that Na"+ within the conserved Na"+-binding pocket may modulate the shape of the SBF. Indeed, the presence of Na"+ within this conserved binding pocket was proposed to modulate the affinity of 1,4-disubstituted piperidines/piperazines for the D₃R by increasing the accessibility of the aryl substituents with V912.61, a residue within the SBF26. However, these data may also be consistent with a transient Na"+-binding interaction with E952.65 proposed by Selent and coworkers that may in turn modulate the interaction of extended compounds such as SB269652 with the SBF26.

Finally, the D802.50A mutation caused a similar decrease in the affinity of MIPS1726 as observed in the Na"+-free condition and no change in negative cooperativity was observed. In contrast this mutation caused an additional 6-fold decrease in the negative cooperativity of MIPS1868, an effect not observed at the WT D₂R in the Na"+-free condition. This suggests that this residue plays a role in determining the cooperativity of MIPS1868 that cannot be reconciled simply through its role in coordinating an Na"+ ion. The differential effects appear to depend on the structure of the aryl amide tail moiety, which differs between MIPS1726 and MIPS1868. Thus, the interaction between the aryl amide tail moiety of SB269652 or its derivatives and the SBF is important in determining not only affinity and the magnitude of negative cooperativity but also the effect of Na"+-binding and the D802.50A mutation3. We speculate that the absence of the Na"+-coordinated by Asp802.50 would alter the position of the orthosteric THIQ moiety bound in the OBS, leading to a change in position of the aryl amide moiety within the SBS. It is interesting to note that in the absence of Na"+, the mutation E952.65A has no additional effect upon the affinity of MIPS1868 but caused a 2-fold decrease in the affinity of MIPS1726, an effect that may result from a distinct orientation of these two ligands within the SBS in the Na"+-free condition. Indeed the resulting poses of these two ligands from our MD simulations show noticeable differences in the SBS, with the indole ring of MIPS1868 being positioned more extracellularly than that of MIPS1726 in the Na"+-free condition, when they lose the interaction with Asp3.32 (Supplementary Figure 4). Furthermore, the interaction between the N5 atom of MIPS1726 and Tyr341.32 was weakened in the absence of Na"+ whereas the interaction of the same residue with fluoro atom of MIPS1868 and Tyr341.32 remained stable and may mask the effect of the E952.65A mutation.

To date, investigations into the influence of Na"+ upon dopamine receptor function have been limited to effects on the affinity and efficacy of competitive orthosteric ligands. Here we reveal profound Na"+-sensitivity for the action of the NAM, SB269652. This effect is predominantly mediated by a Na"+ ion located within the conserved Na"+-binding site and coordinated by Asp802.50. Our previous studies have proposed a mechanism of action for SB269652, whereby it adopts a dual orthosteric/allosteric (bitopic) mode of action at the D₂R. This study reveals that the bound Na"+ is required for the high affinity binding of the THIQ moiety of SB269652 and its analogues within the OBS. These observations are consistent with a role for the Na"+-free condition of the OBS. It is interesting to note that the orthosteric fragments MIPS1726 and MIPS1868 lack this SP. Interestingly, the addition of the indole moiety of SB269652 does not confer a gain in affinity relative to MIPS1059. Therefore, one possible explanation for the greater effect of the Na"+-free condition upon SB269652 as compared to these orthosteric fragments, is that the addition of the SP of SB269652 not only confers a relatively weak interaction with Glu952.65 but decreases the strength of the interaction with residues within the OBS, together conferring a greater sensitivity to Na"+.

Methods

Materials. Dulbecco’s modified Eagle’s medium, Flp-In CHO cells, and hygromycin B were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from ThermoTrace (Melbourne, VIC, Australia). [3H]spiperone and Ultima Gold scintillation cocktail were from PerkinElmer (Boston, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). All compounds were synthesized as described previously2,3 with the exception of MIPS1868 and MIPS1726, which were synthesised as described in supplementary information.

Molecular biology. cDNA in pcDNA3.1+ encoding the long isoform of the wild-type human D₂ dopamine receptor (D₂R) was obtained from Missouri University of Science and Technology (http://www.cdna.org/). Oligonucleotides were purchased from GeneWorks (Hindmarsh, Australia). An N-terminal c-Myc epitope tag (EQKLISEEDL) was introduced to the sequence of the D₂R, and flanking ATT sites were introduced to the WT D₂R by overlap extension PCR to allow subcloning into the pDONR201TM vector. The c-Myc–tagged WT D₂R receptor construct in pDONR201TM was transferred into the pEF5/frt/V5/dest vector using the LR clonase enzyme mix (Invitrogen). Desired mutations were introduced using the Quikchange site-directed mutagenesis kit (Agilent). Mutations were confirmed by DNA sequencing (Australian Genome Research Facility, Melbourne, Australia). Receptor constructs in pEF5/frt/V5/dest were used to transfect Flp-In CHO cells.

Cell culture and membrane preparation. Flp-In CHO cells (Invitrogen) were transfected with the pOG44 vector encoding Flp recombinase and the pDEST vector encoding the wild-type or mutant c-Myc-D₂R.
at a ratio of 9:1 using polyethyleneimine (Polysciences, Warrington, PA) as transfection reagent. 24 hours after transfection the cells were subcultured and the medium was supplemented with 700µg/ml hygromycin B as selection agent to obtain cells stably expressing the c-Myc-D₃R. FpIn CHO cells stably expressing the wild-type or mutant c-Myc-D₃R were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 200µg/ml of Hygromycin-B, and maintained at 37 °C in a humidified incubator containing 5% CO₂. When cells were approximately 90% confluent, they were harvested and centrifuged (300 g, 3 min, 4 °C). The resulting pellet was resuspended in assay buffer either in the presence (20 mM HEPES, 100 mM NaCl, 6 mM MgCl₂, 1 mM EGTA, and 1 mM EDTA, pH 7.4) or without (20 mM HEPES, 100 mM NMDG, 6 mM MgCl₂, 1 mM EGTA, and 1 mM EDTA, pH 7.4) sodium ions. The centrifugation procedure was repeated. The intact cell pellet was then resuspended in assay buffer and homogenized using a Polytron homogenizer for three 10 s intervals on the maximum setting, with 30 s periods on ice between each burst. The homogenate volume was brought up to 30 mL, the sample was centrifuged (350 g, 5 min, 4 °C), the pellet was discarded, and the supernatant was centrifuged at 30 000 g for 1 hr at 4 °C. The resulting pellet was resuspended in 5 mL of assay buffer, and the protein content was determined using the bicinchoninic acid assay (BCA assay). The homogenate was then separated into 500µl aliquots and stored at −80 °C until it was required for binding assays.

**[^3H]spiperone binding assay.** For saturating binding assays cell membranes (c-Myc-D₂L-Flp-In CHO, 20µg) were incubated with varying concentrations of [^3H]spiperone and 10µM haloperidol as a non-specific control, in binding buffer with (20 mM HEPES, 100 mM NaCl, 6 mM MgCl₂, 1 mM EGTA, and 1 mM EDTA, pH 7.4) or without (20 mM HEPES, 100 mM NMDG, 6 mM MgCl₂, 1 mM EGTA, and 1 mM EDTA, pH 7.4) sodium ions to a final volume of 1 mL and were incubated at 37 °C for 3 h. For competition and interaction binding assays cell membranes (c-Myc-D₂L-Flp-In CHO, 20µg) were incubated with varying concentrations of test compound in binding buffer with (20 mM HEPES, 100 mM NaCl, 6 mM MgCl₂, 1 mM EGTA, and 1 mM EDTA, pH 7.4) or without (20 mM HEPES, 100 mM NMDG, 6 mM MgCl₂, 1 mM EGTA, and 1 mM EDTA, pH 7.4) sodium ions, containing 0.15nM of [^3H]spiperone and 100µM GppNHp to a final volume of 1 mL and were incubated at 37 °C for 3h. Binding was terminated by fast-flow filtration over GF/B membranes using a Brandel harvester followed by three washes with ice-cold 0.9% NaCl. Bound radioactivity was measured in a Tri-Carb 2900TR liquid scintillation counter (PerkinElmer).

**Data analysis.** Competition binding curves between [^3H]spiperone and dopamine in the absence and presence of SB269652 were best fit to a one-site model:

\[
Y = \frac{B_{\text{max}} [A]}{[A] + \left( \frac{K_{\text{A}} K_{\text{B}}}{K_{\text{A}} + K_{\text{B}}} \right) \left( 1 + \left( \frac{[B]}{K_{\text{B}}} \right) + \frac{[A][B]}{K_{\text{A}} K_{\text{B}}} + \frac{\alpha [A][B]}{K_{\text{A}} K_{\text{B}}} \right)}
\]

(1)

where \(K_i\) is the equilibrium dissociation constant of dopamine.

Competition binding curves between [^3H]spiperone and SB269652, MIPS1726, MIPS1217 and MIPS1500 could be fit to the allosteric ternary complex model using the following equation:

\[
Y = \frac{[A]}{K_{\text{A}} + \left( \frac{[B]}{K_{\text{B}}} \right) + \left( \frac{[A][B]}{K_{\text{A}} K_{\text{B}}} \right) + \frac{\alpha [A][B]}{K_{\text{A}} K_{\text{B}}} \right)}
\]

(2)

Where \(Y\) is percentage (vehicle control) binding; \([A]\) and \([B]\) are the concentrations of [^3H]spiperone and ligand of interest, respectively; \(K_{\text{A}}\) and \(K_{\text{B}}\) are the equilibrium dissociation constants of [^3H]spiperone and ligand of interest, respectively; and \(\alpha\) is the cooperativity between the ligand of interest and [^3H]spiperone. Values of \(\alpha > 1\) denote positive cooperativity, values \(< 1\) (but \(> 0\)) denote negative cooperativity and values \(= 1\) denote neutral cooperativity.

The concentration of ligand that inhibited half of the [^3H]spiperone binding (IC₅₀) was determined using the following equation:

\[
Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{X-\log IC_{50}/n_Y}}
\]

(3)

Where \(Y\) denotes the percentage specific binding, Top and Bottom denote the maximal and minimal asymptotes, respectively, IC₅₀ denotes the X-value when the response is midway between Bottom and Top, and \(n_Y\) denotes the Hill slope factor. IC₅₀ values obtained from the inhibition curves were converted to \(K_i\) values using the Cheng and Prusoff equation.

**Molecular modelling and simulations.** The binding mode of SB269652 and its derivatives in the D₂R were investigated as described in our previous study. Briefly, to acquire a reference binding mode for the tetrahydroisoquinoline (THIQ) core of SB269652 in the high-resolution structure of the D₃R (Protein Data Bank code 3PBL), THIQ core in the protonated form was first docked into the D₂R structure with the induced-fit docking (IFD) protocol implemented in Schrödinger software (release 2016–1, Schrödinger, LLC: New York, NY), and the minimum MM/GBSA energy pose from the largest pose cluster was selected as the reference pose. Assuming similar binding modes of the THIQ moiety in the near-identical OBS of the D₂R and D₃R, the pose from the IFD trial with our previous equilibrated D₂R model that is closest to the reference pose in the D₃R structure was
selected. The full-length SB269652 was then docked into the D2R model by a core-constrained IFD protocol\(^{38}\) with restraints on the heavy atoms (heavy-atom RMSD deviation <2.0 Å) of the selected pose of the THIQ core. Representative poses for the SB269652 derivatives, were acquired similarly by using the core-restrain IFD protocol, where restraints were applied on the THIQ core of the full-length SB269652, assuming that the THIQ core adopts a similar pose in SB269652 derivatives as well.

MD simulations of the D2R-ligand complexes were performed in the explicit water and 1-palmitoyl-2-

-oleoylphosphatidylcholine lipid bilayer solvent environment using Desmond MD system (version 4.5; D. E. Shaw Research, New York, NY) with the CHARMM36 protein force field\(^{39–41}\), CHARMM36 lipid force field\(^{42}\), and TIP3P water model. The ligand parameters were obtained from the GAAMP server\(^{43}\), with the initial force field based on CGenFF assigned by ParamChem\(^{44}\). The system charges were neutralized, and a solvent concentration of 150 mM NaCl was added. The average system size is ~111,300 atoms. Each system was first minimized and then equilibrated with restraints on the ligand heavy atoms and protein backbone atoms, followed by an isothermal-isobaric simulation at 310 K with all atoms unrestrained, as described previously\(^{45–47}\). For each complex, we ran multiple trajectories (Table 2).

MD simulation systems for Na\(^{+}\)-unbound conditions were generated by removing Na\(^{+}\) ions from WT trajectories and then re-neutralizing the overall charge of the system by removing appropriate number of Cl\(^{-}\) ions in the water milieu.

**MM/GBSA Energy Calculations.** MM/GBSA energy calculations on the D2R WT Na\(^{+}\)-bound and -unbound conditions were performed using protocol implemented in CHARMM (version 40b2)\(^ {48}\) using the GBSW implicit solvent model\(^{49}\). For each condition, we extracted frames at 1.2 ns interval from the last 300 ns of the MD trajectories.

**Data Availability.** The datasets generated during the current study are available from the corresponding author on reasonable request.

**References**

1. Beaulieu, J.-M. & Gainetdinov, R. R. The Physiology, Signaling, and Pharmacology of Dopamine Receptors. *Pharmacol Rev* **63**, 182–217 (2011).

2. Lane, J. R. et al. A new mechanism of allosteroy in a G protein–coupled receptor dimer. *Nat Chem Biol* **10**, 745–752 (2014).

3. Shonberg, J. et al. Structure–Activity Study of N-(trans)-4-(2-(7-Cyano-3,4-dihydroisoquinolin-2(1 H)-yl)ethyl)cyclohexyl)-1 H-indole-2-carboxamide (SB269652), a Bitopic Ligand That Acts as a Negative Allosteric Modulator of the Dopamine D2 Receptor. *J Med Chem* **58**, 5287–5307 (2015).

4. Wood, M. et al. In Vitro and In Vivo Identification of Novel Positive Allosteric Modulators of the Human Dopamine D2 and D3 Receptor. *Mol Pharmacol* **89**, 303–312 (2016).

5. Silvano, E. et al. The tetrahydroisoquinoline derivative SB269,652 is an allosteric antagonist at dopamine D3 and D2 receptors. *Mol Pharmacol* **78**, 925–934 (2010).

6. Kumar, V. et al. Synthesis and Pharmacological Characterization of Novel trans-Cyclopropylmethyl-Linked Bivalent Ligands That Exhibit Selectivity and Allosteric Pharmacology at the Dopamine D3 Receptor (D3R). *J Med Chem* **60**, 1478–1494 (2017).

7. Furnman, C. A. et al. Investigation of the binding and functional properties of extended length D3 dopamine receptor-selective antagonists. *Eur Neuropsychopharmacol* **25**, 1448–1461 (2015).

8. Ballesteros, J. A. & Weinstein, H. Methods in Neurosciences. **25**, 366–428 (Elsevier, 1995).

9. Pert, C. B., Pasternak, G. & Snyder, S. H. Opiate agonists and antagonists discriminated by receptor binding in brain. *Science* **182**, 1359–1361 (1973).

10. Limbird, L. E., Speck, J. L. & Smith, S. K. Sodium ion modulates agonist and antagonist interactions with the human platelet alpha 2-adrenergic receptor in membrane and solubilized preparations. *Mol Pharmacol* **21**, 609–617 (1982).

11. Neve, K. A. et al. Modeling and mutational analysis of a putative sodium-binding pocket on the dopamine D2 receptor. *Mol Pharmacol* **60**, 373–381 (2001).

12. Neve, K. A., Cox, B. A., Hemningsen, R. A., Spanoyannis, A. & Neve, R. L. Pivotal role for aspartate-80 in the regulation of dopamine D2 receptor affinity for drugs and inhibition of adenyl cyclase. *Mol Pharmacol* **39**, 733–739 (1991).

13. Martin, S., Botto, J. M., Vincent, J. P. & Mazella, J. Pivotal role of an aspartate residue in sodium sensitivity and coupling to G proteins of neurotransin receptors. *Mol Pharmacol* **55**, 210–219 (1999).

14. Gao, Z.-G. et al. Identification of essential residues involved in the allosteric modulation of the human A3 adenosine receptor. *Mol Pharmacol* **63**, 1021–1031 (2003).

15. Liu, W. et al. Structural basis for allosteric regulation of GPCRs by sodium ions. *Science* **337**, 232–236 (2012).

16. Miller-Gallacher, J. L. et al. The 2.1 Å resolution structure of cyanoindolol-bound β3-adrenoceptor identifies an intramembrane Na+ ion that stabilises the ligand-free receptor. *PloS One* **9**, e92727 (2014).

17. Zhang, C. et al. High-resolution crystal structure of a human protease-activated receptor 1. *Nature* **492**, 387–392 (2012).

18. Fenalti, G. et al. Molecular control of β-opioid receptor signalling. *Nature* **506**, 191–196 (2014).

19. Wang, S. et al. D4 dopamine receptor high-resolution structures enable the discovery of selective agonists. *Science* **358**, 381–386 (2017).

20. Rodriguez, D., Piteiro, A. & Gutierrez-de-Teran, H. Molecular dynamics simulations reveal insights into key structural elements of adenosine receptors. *Biochemistry* **50**, 4194–4208 (2011).

21. Gutierrez-de-Teran, H. et al. The role of a sodium ion binding site in the allosteric modulation of the A2A adenosine G protein-coupled receptor. *Structure* **21**, 2175–2185 (2013).

22. Neve, K. A. Regulation of dopamine D2 receptors by sodium and pH. *Mol Pharmacol* **39**, 570–578 (1991).

23. Neve, K. A. et al. Sodium-dependent isomerization of dopamine D-2 receptors characterized using [125I]epidepride, a high-affinity substituted benzamide ligand. *J Pharmacol Exp Ther* **252**, 1108–1116 (1990).

24. Michino, M., Free, R. B., Boyle, T. B., Sibley, D. R. & Shi, L. Structural basis for Na(+) sensitivity in dopamine D2 and D3 receptors. *Chembiochem* (Camb.) **51**, 8618–8621 (2015).

25. Erickson, S. S., Cummings, D. F., Weinstein, H. & Schetz, J. A. Ligand Selectivity of D2 Dopamine Receptors Is Modulated by Changes in Local Dynamics Produced by Sodium Binding. *J Pharmacol Exp Ther* **328**, 40–54 (2008).

26. Selent, I., Sanz, F., Pastor, M. & De Fabritiis, G. PLOS Computational Biology: Induced Effects of Sodium Ions on Dopaminergic G-Protein Coupled Receptors. *PloS Comp Biol* (2010).

27. 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. *Proceedings of the National Academy of Sciences* **111**, 18369–18374 (2014).
28. Armstrong, D. & Strange, P. G. Dopamine D2 receptor dimer formation: evidence from ligand binding. \textit{J Biol Chem} \textbf{276}, 22621–22629 (2001).
29. Newton, C. L., Wood, M. D. & Strange, P. G. Examining the Effects of Sodium Ions on the Binding of Antagonists to Dopamine D2 and D3 Receptors. \textit{PLoS One} \textbf{11}, e0158808 (2016).
30. Katritch, V. et al. Allosteric sodium in class A GPCR signaling. \textit{Trends Biochem Sci} \textbf{39}, 233–244 (2014).
31. Vives, J., Lin, H. & Strange, P. G. Investigation of cooperativity in the binding of ligands to the D(2) dopamine receptor. \textit{Mol Pharmacol} \textbf{69}, 226–235 (2006).
32. Fu, D., Ballesteros, J. A., Weinstein, H., Chen, J. & Javitch, J. A. Residues in the seventh membrane-spanning segment of the dopamine D2 receptor accessible in the binding-site crevice. \textit{Biochemistry} \textbf{35}, 11278–11285 (1996).
33. Nawaratne, V., Leach, K., Felder, C. C., Sexton, P. M. & Christopoulos, A. Structural determinants of allosteric agonism and modulation at the M4 muscarinic acetylcholine receptor: identification of ligand-specific and global activation mechanisms. \textit{J Biol Chem} \textbf{285}, 19012–19021 (2010).
34. Christopoulos, A. & Kennan, T. G protein-coupled receptor allosterism and complexing. \textit{Pharmacol Rev} \textbf{54}, 323–374 (2002).
35. Cheng, Y. & Prusoff, W. H. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. \textit{Biochem Pharmacol} \textbf{22}, 3099–3108 (1973).
36. Chien, E. Y. T. et al. Structure of the Human Dopamine D3 Receptor in Complex with a D2/D3 Selective Antagonist. \textit{Science} \textbf{330}, 1091–1095 (2010).
37. Sherman, W., Day, T., Jacobson, M. P., Friesen, R. A. & Farid, R. Novel Procedure for Modeling Ligand/Receptor Induced Fit Effects. \textit{J Med Chem} \textbf{49}, 534–553 (2006).
38. Newman, A. H. et al. Molecular determinants of selectivity and efficacy at the dopamine D3 receptor. \textit{J Med Chem} \textbf{55}, 6689–6699 (2012).
39. MacKerell, D. A. et al. CHARMM: The Energy Function and Its Parameterization. (John Wiley & Sons, Inc.).
40. MacKerell, A. D., Feig, M. & Brooks, C. L. Extending the treatment of backbone energetics in protein force fields: limitations of gas-phase quantum mechanics in reproducing protein conformational distributions in molecular dynamics simulations. \textit{J Comput Chem} \textbf{25}, 1400–1415 (2004).
41. Best, R. B. et al. Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone φ, ψ and side-chain χ(1) and χ(2) dihedral angles. \textit{J Chem Theory Comput} \textbf{8}, 3257–3273 (2012).
42. Klauda, J. B. et al. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. \textit{J Phys Chem B} \textbf{114}, 7830–7843 (2010).
43. Huang, L. & Roux, B. Automated field force parameterization for non-polarizable and polarizable atomic models based on ab initio target data. \textit{J Chem Theory Comput} \textbf{9}, (2013).
44. Vanommeslaeghe, K. et al. CHARMM general force field: A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. \textit{J Comput Chem} \textbf{31}, 671–690 (2010).
45. Michino, M. et al. A single glycine in extracellular loop 1 is the critical determinant for pharmacological specificity of dopamine D2 and D3 receptors. \textit{Mol Pharmacol} \textbf{84}, 854–864 (2013).
46. Michino, M. et al. What can crystal structures of amniogenic receptors tell us about designing subtype-selective ligands? \textit{Pharmacol Rev} \textbf{67}, 198–213 (2015).
47. Michino, M. et al. Toward Understanding the Structural Basis of Partial Agonism at the Dopamine D3 Receptor. \textit{J Med Chem} \textbf{60}, 580–593 (2017).
48. Brooks, B. R. et al. CHARMM: the biomolecular simulation program. \textit{J Comput Chem} \textbf{30}, 1545–1614 (2009).
49. Im, W., Feig, M. & Brooks, C. L. An implicit membrane generalized bond theory for the study of structure, stability, and interactions of membrane proteins. \textit{Biophys J} \textbf{85}, 2900–2918 (2003).

Acknowledgements
This research was supported by Project Grant 1049564 of the National Health and Medical Research Council (NHMRC) and Program Grant 1055134 (NHMRC) and the Intramural Research Program of the National Institutes of Health, National Institute on Drug Abuse (R.K.V., M.M., A.M.A. and L.S.). J.R.L. is a Larkin’s Fellow (Monash University, Australia). C.D.-J. acknowledges an Australian Postgraduate Awards.

Author Contributions
C.J.D.J. performed radiolgand-binding experiments. R.K.V., M.M., A.M.A. performed molecular modelling and simulations. J.S., A.K., P.J.S., B.C. synthesised compounds. C.K.H. generated cell lines. C.J.D.J., R.K.V., M.M., A.M.A., D.M.T., J.A.J., A.C., L.S., J.R.L. analysed data. C.J.D.J., R.K.V., M.M., A.M.A., D.M.T., J.A.J., A.C., L.S., J.R.L. & J.R.L. wrote the manuscript with contributions from all the authors. L.S. & J.R.L. designed the studies. All authors reviewed the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-19642-1.

Competing Interests: A.C. has a research contract and consultancy with Servier, France.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.