Selective activation of Gαob by an adenosine A₁ receptor agonist elicits analgesia without cardiorespiratory depression

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The development of therapeutic agonists for G protein-coupled receptors (GPCRs) is hampered by the propensity of GPCRs to couple to multiple intracellular signalling pathways. This promiscuous coupling leads to numerous downstream cellular effects, some of which are therapeutically undesirable. This is especially the case for adenosine A₁ receptors (A₁Rs) whose clinical potential is undermined by the sedation and cardiorespiratory depression caused by conventional agonists. We have discovered that the A₁R-selective agonist, benzoyloxy-cyclopentyladenosine (BnOCPA), is a potent and powerful analgesic but does not cause sedation, bradycardia, hypotension or respiratory depression. This unprecedented discrimination between native A₁Rs arises from BnOCPA’s unique and exquisitely selective activation of Gob among the six Gαi/o subtypes, and in the absence of β-arrestin recruitment. BnOCPA thus demonstrates a highly-specific Gα-selective activation of the native A₁R, sheds new light on GPCR signalling, and reveals new possibilities for the development of novel therapeutics based on the far-reaching concept of selective Gα agonism.
protein-coupled receptors (GPCRs) are the targets of many FDA-approved drugs. However, the promiscuity with which they couple to multiple G protein- and β-arrestin-activated intracellular signalling cascades leads to unwanted side effects. These side effects limit both the range of GPCRs suitable for drug-targeting, and the number of conditions for which treatments could be developed. One family of GPCRs that have particularly suffered as drug targets from their promiscuous coupling and wide-ranging cellular actions are the four GPCRs for the purine nucleoside adenosine, despite the potential for using adenosine receptor agonists to treat many pathological conditions including cancer, and various cardiovascular, neurological and inflammatory diseases. For example, activation of the widely-distributed adenosine A1 receptor (A1R) with currently available agonists elicits multiple actions in both the central nervous system (CNS) and the cardiorespiratory system. In the CNS A1Rs inhibit synaptic transmission, induce neuronal cell death, and contribute to reducing blood pressure (hypotension) and heart rate (bradycardia), and contribute to reducing blood pressure (hypotension), cardiac contractility, and depress respiration (dyspnoea). These multiple effects severely limit the prospects of A1R agonists as life-saving therapies. These side effects limit both the range of GPCRs suitable for drug-targeting, and the number of conditions for which treatments could be developed.

The therapeutic limitations of promiscuous GPCR coupling might be overcome through the development of biased agonists—compounds that preferentially recruit one intracellular signalling cascade over another. This signalling bias has most frequently been expressed in terms of Ga vs β-arrestin signalling and has been pursued at a variety of receptors, for example, at the angiotensin II type 1 receptor (AT1R) and at neurotensin receptors in the treatment of drug addiction. Bias agonist has been sought in the context of opioid receptors, but with some controversy, for compounds producing analgesia with reduced respiratory depression, gastrointestinal disturbance and tolerance.

However, while other forms of bias exist, including between individual Ga subunits, the challenge remains in translating GPCR signalling bias observed in vitro to tangible, and physiologically- and clinically-relevant, selectivity at native receptors in vivo. According to the potential to preferentially drive the G protein-coupling of A1Rs has been described in several in vitro studies, and to date no A1R-specific agonist has been reported that can elicit biased Ga agonism at native A1Rs in intact physiological systems, let alone the selective activation of one Ga subunit. To achieve such selectivity among Ga subunits would introduce novel therapeutic opportunities across a wide range of debilitating clinical conditions.

Here we show, utilising molecular dynamics (MD) simulations, and Ga/o subunit- and β-arrestin-specific cellular signalling assays, how one A1R-selective agonist, BnOCPA, fulfills the criteria for a selective Ga agonist in exclusively activating Gob among the six members of the Ga/o family of G protein subunits, and in the absence of β-arrestin recruitment. In addition, through a combination of CNS electrophysiology, physiological recordings of cardiorespiratory parameters, a sensitive assay of attention and locomotor function, and the use of a clinically-relevant model of chronic neuropathic pain, we demonstrate selective activation of native A1Rs and the delivery of potent analgesia without sedation, motor impairment or cardiorespiratory depression. Our data thus demonstrate the translation of agonist Ga selectivity in vitro to therapeutically tangible clinically-relevant observations in vivo. Such observations reveal the possibility of achieving Ga selectivity at native receptors, highlight the physiological benefits of such selectivity, and specifically speak to the possibility of unlocking the widespread clinical potential of A1R agonists.

**Results**

The novel A1R agonist BnOCPA exquisitely discriminates between native pre- and postsynaptic A1Rs in the intact mammalian CNS. BnOCPA (Fig. 1a), a molecule first described in a patent as a potential treatment for glaucoma or ocular hypertension, is a cyclopentyl derivative of adenosine and a highly selective and potent, full agonist at human adenosine A1Rs (hA1Rs; Fig. 1b; Supplementary Table 1). Our characterisation of BnOCPA, synthesised independently as part of a screen for suitable scaffolds for the generation of fluorescent ligands for the A1R, began with an exploration of the binding characteristics of BnOCPA at the hA1R using classical radioligand binding (where the antagonist [3H]DPCPX was used as a tracer), and a NanoBRET agonist binding assay (using a novel NECA-TAMRA compound, which acts as a full agonist (NECA - 7.23 ± 0.13; See Methods). Using both assays we observed that BnOCPA was able to bind to the hA1R with an affinity equal to that of the prototypical A1R agonist CPA and NECA, and higher than that of the endogenous agonist adenosine (Fig. 1b; Supplementary Table 1).

Significantly, using NECA-TAMRA as the fluorescent agonist tracer, the high-affinity state of the biphasic binding profile observed in the NanoBRET assay was equivalent to that reported previously for BnOCPA (3.8 nM compared to 1.7 nM).

These initial pharmacological studies at recombinant hA1Rs in cell lines did not reveal anything extraordinary about BnOCPA. However, when we investigated BnOCPA at native A1Rs in rat hippocampal slices, against which BnOCPA is also a potent agonist, with ~8000- and >150-fold greater efficacy at rat A1Rs (rA1Rs) than at rat A2ARs (rA2ARs) and A3Rs (rA3Rs), respectively (Supplementary Table 2), we discovered properties of BnOCPA that were not consistent with those of typical A1R agonists such as adenosine, CPA and NECA. In accordance with the effects of standard A1R agonists, BnOCPA potently inhibited excitatory synaptic transmission in rat hippocampal slices (IC50 ~65 nM; Fig. 1c–g and Supplementary Fig. 1a–d). This effect was attributable to the activation of native presynaptic A1Rs on glutamatergic terminals (Fig. 1c; Supplementary Fig. 1e, f), and cannot be attributed to any action of BnOCPA at A1Rs since even a high concentration (1 µM) of the potent and selective A1R agonist 2-Cl-IB-MECA had no effect on synaptic transmission (Supplementary Fig. 1g, h). However, in stark contrast to adenosine and CPA, BnOCPA did not activate postsynaptic A1Rs (Fig. 1c) to induce membrane hyperpolarisation, even at concentrations 15 times the IC50 for the inhibition of synaptic transmission (Fig. 1b, i).

This peculiar and unique discrimination between pre- and postsynaptic A1Rs might possibly be explained in terms of either some hindrance in the binding of BnOCPA to A1Rs on postsynaptic neurones, or, and unprecedented for an A1R agonist, binding to the postsynaptic A1R, but without the ability to activate the receptor. To test the latter hypothesis—that BnOCPA actually bound to postsynaptic A1Rs, but without efficacy—we reasoned that BnOCPA might behave in a manner analogous to a receptor antagonist in preventing or reversing activation by other A1R agonists, a property that has been predicted and observed for biased agonists at other receptors.

To test this, we pre-applied BnOCPA and then applied CPA (in the continued presence of BnOCPA). Remarkably, the co-application of CPA and BnOCPA resulted in a significant reduction of the effects of CPA on membrane potential (Fig. 1i; Supplementary Fig. 2a, b). In addition, membrane hyperpolarisation induced by the endogenous...
agonist adenosine was reversed by BnOCPA (Supplementary Fig. 2c). In contrast, the A3R agonist 2-Cl-IB-MECA had no effect on membrane potential and did not interfere with the membrane hyperpolarisation caused by adenosine (Supplementary Fig. 2d, e), further reaffirming the actions of BnOCPA as being selectively mediated by A1Rs.

To test whether the inability of BnOCPA to affect membrane potential was a trivial action due to BnOCPA blocking K⁺ channels mediating the postsynaptic hyperpolarisation, or in some other way non-specifically interfering with G protein signalling, we applied the GABAB receptor agonist baclofen to CA1 pyramidal neurons. BnOCPA had no effect on membrane potential.
Fig. 1 BnOCPA is an A1R agonist that discriminates between pre- and postsynaptic A1Rs in the CNS. a Chemical structures of adenosine, CPA and BnOCPA. Schematic representing assays used to characterise the affinity and efficacy of agonists (green arrows) to the human (h) A1R. bii agonist displacement of [3H]DPCPX, a selective antagonist for the hA1R (n = 5–10 individual repeats). bili CAMPS levels measured in CHO-K1-hA1R cells following co-stimulation with 1 μM forskolin and each agonist (n = 4–10 individual repeats). biv Both CPA and BnOCPA displace the fluorescent AR agonist NECA-TAMRA in a biphasic manner indicating that both agonists display high affinity and low affinity binding (n = 4 individual repeats). Diagram illustrating: left, hippocampal slice preparation showing positioning of stimulating, patch-clamp and extracellular recording electrodes together with representative electrophysiological recordings; membrane potential (Vm), a fEPSP (field excitatory postsynaptic potential) and seizure activity; right, pre- and postsynaptic A1Rs at hippocampal synapses and their physiological effects upon activation. d, e Increasing concentrations of CPA or BnOCPA reduced the fEPSP, an effect reversed by the A1R antagonist 8-CPT (2 μM). Inset, superimposed fEPSP averages in control (largest fEPSP) and BnOCPA, respectively. f, g Concentration-response curves for the inhibition of synaptic transmission by CPA (IC50 = 11.8 ± 2.7 nM; n = 17 slices) or BnOCPA (IC50 = 65 ± 0.3 nM; n = 11 slices). h CPA hyperpolarised the membrane potential while BnOCPA had little effect. Scale bars measure 4 mV and 30 s. i Summary data for membrane potential changes. The mean hyperpolarisation produced by CPA (300 nM; 7.26 ± 0.86 mV, n = 7 cells) was significantly different (one-way ANOVA, F(2,23) = 70.46; P = 1.55 × 10−10) from that produced by BnOCPA (300 nM or 1 μM; 0.33 ± 0.14 mV, n = 10 and 5 cells, respectively; P = 8.26 × 10−3) and for CPA (300 nM) applied in the presence of BnOCPA (300 nM; 2.75 ± 0.48 mV, n = 4 cells, P = 2.89 × 10−5). See Supplementary Fig. 2a for an example trace. In an in vitro model of seizure activity, represented frequent spontaneous spiking from baseline, CPA (300 nM) reversibly blocked activity while BnOCPA (300 nM) had little effect. Scale bars measure 0.5 mV and 200 s. k Summary data for seizure activity expressed in terms of the frequency of spontaneous spiking before, during and after CPA or BnOCPA. CPA abolished seizure activity (n = 4) whereas BnOCPA did not significantly reduce seizure frequency (n = 6). Data represented as mean ± SEM; Two-way RM ANOVA (BnOCPA vs CPA slices): F(1, 3) = 186.1, P = 8.52 × 10−4 with the following Bonferroni post hoc comparisons: BnOCPA vs Control; P = 1; CPA vs control; P = 0.010; BnOCPA vs CPA; P = 0.027. Averaged data are presented as mean ± SEM. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001. Source data are provided as a Source Data file.

BnOCPA demonstrates unique Ga signalling in the selective activation of Gob. The observation that BnOCPA discriminated between pre- and postsynaptic A1Rs might be explained if these receptors were to activate different intracellular pathways to mediate their effects, and that BnOCPA was not able to activate the pathway responsible for postsynaptic membrane hyperpolarisation. To test whether the actions of BnOCPA and the prototypical A1R agonists were mediated via β-arrestins (β-arrestin1 and β-arrestin2), we used a BRET assay36–40 for β-arrestin recruitment (Supplementary Fig. 3). We observed no β-arrestin recruitment at the A1R using either BnOCPA, CPA or adenosine, regardless of whether β-arrestin1 or β-arrestin2 was expressed (Supplementary Fig. 3). This was in contrast to β-arrestin2 recruitment by the A1R in response to adenosine and NECA, but not BnOCPA (Supplementary Fig. 3). Moreover, the lack of recruitment of β-arrestin1 and β-arrestin2 by the A1R was independent of any of the six G protein receptor kinase (GRK) isoforms co-expressed with β-arrestin1 and β-arrestin2; only low levels of recruitment were observed even when GRKs were highly (five-fold) overexpressed compared to the levels in the A1R assays (Supplementary Fig. 4). These observations of a lack of β-arrestin recruitment by A1Rs are consistent with those previously reported for recombinant A1Rs expressing native sequences41–45, and are likely due to the absence of serine and threonine residues in the A1R cytoplasmic tail, which makes the A1R intrinsically biased against β-arrestin signalling39,46. Accordingly, the differential actions of BnOCPA at pre- and postsynaptic A1Rs are more likely to reside in selective activation of one Ga-mediated pathway over another.

To investigate whether BnOCPA has the ability to discriminate between the various Gai/o subunits activated by adenosine, we generated a recombinant cell system (CHO-K1 cells) expressing both the hA1R and individual pertussis toxin (PTX)-insensitive variants of individual Gai/o subunits. Against these individual Gai/o subunits we tested adenosine, CPA, NECA, BnOCPA, and the agonist HOCPA33,47, a stereoisomer of GR7923648,49, which behaved similarly to adenosine and CPA in both inhibiting synaptic transmission and causing membrane hyperpolarisation (Supplementary Fig. 5). In cells treated with PTX to inhibit endogenous Gai/o30,33 we observed that adenosine, CPA, NECA and HOCPA activated a range of Gai/o subunits. Common to all of these agonists was the activation of both Gao isoforms, Goa and Gob, with differential activation of Gi1 (HOCPA), Gi2 (NECA, CPA) and Gz (adenosine; Fig. 2a–e; Supplementary Figs. 5 and 6). Such promiscuous and biased Ga coupling has been described previously for adenosine, CPA, and NECA at recombinant A1Rs in cell lines39,50, including using novel BRET-based assays for adenosine at some Gai/o51. These previous observations are in keeping with ours, confirming the validity of the PTX-based approach. In stark contrast, BnOCPA displayed a unique and highly distinctive Gai/o subunit activation profile: BnOCPA was not able to activate Gi1, Gi2, Gi3 or Gz, and was furthermore capable of discriminating between the two Gao isoforms via the selective activation of Gob, and not of Goa (Fig. 2a–e; Supplementary Fig. 6).

The selective and unique activation of Gob among the six Gai/o subunits by BnOCPA could be observed in a comparison of the activation of Goa and Gob by the native and selective A1R agonists in their ability to inhibit the forskolin-stimulated accumulation of cAMP (Fig. 2f). Whereas adenosine, CPA and HOCPA activated both Goa and Gob to inhibit cAMP accumulation, BnOCPA selectively activated Gob, with no discernible activation of Goa. Further quantification of this Gob selectivity, through the application of the operational model of receptor agonism42–44 to remove potential issues of system bias, confirmed selective activation of Gob by BnOCPA, with no detectable response at Goa (Fig. 2g). As further validation of the ability of BnOCPA to discriminate between the activation of Goa and Gob, we took advantage of BRET assays of GPCR activation55,56, which utilise a reduction in a Ga-Gβγ BRET signal to infer agonist-induced G protein activation, including for Goa and Gob (Fig. 2h; Supplementary Fig. 7a). Using the TRUPATH GPCR BRET assay55, adenosine, CPA, and HOCPA
elicited equipotent activation of both Goa and Gob. In stark contrast to these agonists, BnOCPA was >10-fold more efficacious in activating Gob than Goa, and, of all the agonists tested, BnOCPA displayed the weakest potency at Goa. While subtle differences between the Goa and Gob response exist across the two very different in vitro assays, these data nonetheless confirm that BnOCPA demonstrates a previously unprecedented ability for an A1R agonist to discriminate between Ga subtypes, and in particular between Goa and Gob.

To establish the functional implications of BnOCPA’s profound selectivity for Gob over Goa, we hypothesised that BnOCPA should reduce the actions of adenosine on the
Fig. 2 BnOCPA selectively activates Gob. a cAMP accumulation in PTX-pre-treated CHO-K1-hA1R cells expressing PTX-insensitive Goat following co-stimulation with 1 μM forskolin and each agonist (1 nM–1 μM; n = 6 individual repeats). b as for a but cells were transfected with PTX-insensitive Goat (n = 6 individual repeats). Stimulation of BnOCPA production in a reflects BnOCPA’s activation of endogenous, PTX-resistant Gs by the A1R (see Supplementary Figs. 5 and 6 and29,153,154). c, d Heatmaps summarising E_max (c %) and potency (d pEC_{SO} = –log (agonist concentration) required for 50% inhibition of cAMP accumulation) for individual Goat subunit and β-arrestin1 and 2 activation by selective A1R agonists for the inhibition of forskolin-stimulated CAMP production. Data taken from: adenosine, CPA, BnOCPA Fig. 1, Supplementary Figs. 3, 6; NECA, Supplementary Fig. 3, 6; HOCPA, Supplementary Fig. 5. e Venn diagram of agonist interactions with individual Gob/o subunits. f The inhibition of cAMP accumulation via A1R-Goa or A1R-Gob by adenosine, CPA, HOCPA and BnOCPA. Each data point represents a determination relative to the natural agonist adenosine using the change in (Δτ/Kr) ratio. The values were calculated for all compounds at each individual G protein and the data was fitted globally to determine single values for τ and K for and then normalised to a reference agonist (adenosine). This approach, used by others155, precludes the provision of individual data points. Compared to adenosine, BnOCPA elicits no measureable response (NR) at Goa. h The TRUPATH assay for direct G protein activation reveals no preference between Goa and Gob by adenosine, CPA or HOCPA, but a significant >10-fold greater activation of Gob vs Goa by BnOCPA (two-tailed unpaired Student’s t-test; P = 0.0009; see also Supplementary Fig. 7a; n = 8 individual repeats for each agonist). i Adenosine/Goa-mediated inhibition of cAMP accumulation was antagonised by BnOCPA in a concentration-dependent manner (n = 3–4 individual repeats). j Example current traces produced by adenosine (10 μM) in control conditions or in the presence of intracellular Goa interfering peptide, scrambled Goa peptide or Gob interfering peptide (all at 100 μM). Scale bars measure 25 pA and 100 s. k Summary data of adenosine-induced outward current experiments. The mean amplitude of the outward current induced by adenosine (40.6 ± 2.2 pA, n = 16 cells) was significantly reduced (one-way ANOVA; F(3,37) = 12.40, P = 9.22 × 10^{-6}) to 20.9 ± 3.6 pA (n = 10 cells, P = 2.65 × 10^{-5}) in 100 μM Goa interfering peptide. Neither the scrambled Goa peptide (0 μA, n = 7 cells, P = 1) nor the Gob interfering peptide (39.2 ± 2.7 pA, n = 8 cells, P = 1) reduced the amplitude of the adenosine-induced outward current compared to control, but each were significantly different from the Goa interfering peptide (P = 8.20 × 10^{-5}; P = 8.86 × 10^{-4}, respectively). Averaged data are presented as mean ± SEM. ****, P < 0.0001 relative to other groups. Source data are provided as a Source Data file.

inhibition of cAMP accumulation via Goa. This was indeed the case (Fig. 2i): BnOCPA antagonised the Goa-mediated inhibition of cAMP production by adenosine in a concentration-dependent manner. This classic attribute of an antagonist enabled a Schild analysis estimate of BnOCPA’s affinity (Kd) to be 113 nM, with a pKd ~6.958, a value that was quantitatively similar to BnOCPA’s ability to bind to the hA1R (pKd ~6.6; Fig. 1b). Importantly, this observation, of the ability of BnOCPA to antagonise the actions of adenosine on cAMP inhibition (Fig. 2i), revealed no agonist action of BnOCPA at Goa at concentrations up to 100 μM (>10^5 greater than the IC_{50} against cAMP accumulation; Fig. 1b and ~10^4 greater than the EC_{50} in the TRUPATH assay; Fig. 2h), and, moreover, had parallels with the antagonistic effects of BnOCPA on membrane potential in the CNS (Fig. 1h, i; Supplementary Fig. 2a, c). These data suggest that BnOCPA has the unique ability of displaying both agonist and antagonist-like properties at both recombinant and native A1Rs: properties that are expected of a truly G protein-selective agonist.

The data from whole-cell patch-clamp recordings showed that BnOCPA did not influence neuronal membrane potential at native A1Rs (Fig. 1h, i), while experiments in recombinant hA1Rs showed that BnOCPA did not activate Goa (Fig. 2a, c–f), and indeed prevented the activation of Goa by adenosine (Fig. 2i). We thus predicted that A1Rs in the hippocampus, where Goa is found at levels 10–15 times higher than Gob59, should act via Goa to induce membrane hyperpolarisation, and thereby providing a potential explanation for the lack of effect of BnOCPA on membrane potential. To test this prediction, we injected a series of previously-validated interfering peptides against Goa and Gob into CA1 pyramidal cells during whole-cell voltage-clamp recordings. Introduction of the Goa interfering peptide caused a significant attenuation of the adenosine-induced outward current (Fig. 2j, k). In contrast, neither the scrambled Goa peptide, nor the Gob peptide, which reduced the modulation of Ca^{2+} channels by muscarinic M4 receptors in striatal cholinergic interneurons63, had any effect on outward current amplitude (Fig. 2j, k). To confirm the specificity and potency of the interfering peptides used in hippocampal neurons, we transfected plasmids coding for the last 11 C-terminal amino acids of either Goa, Gob and the scrambled version of Goa, into the Goa and Gob vectors in the TRUPATH assay used in Fig. 2h (Supplementary Fig. 7b). The interfering peptides reduced the activation of their cognate G protein in a dose-dependent manner, but had no effect on the alternate Go isoform. The scrambled peptide sequence had no effect on Goa or Gob activation.

Thus, adenosine-mediated membrane potential hyperpolarisation occurs mainly through A1R activation of Goa, in keeping with the high levels of expression of Goa vs Gob in the hippocampus59, and with the observation that the Goa-activating agonists adenosine, CPA and HOCPA (Fig. 2c–e, Supplementary Figs. 5 and 6) all induced membrane hyperpolarisation (Fig. 1h, i; Supplementary Figs. 2 and 5). Moreover, the absence of an effect of adenosine on membrane potential in Gz knockout mice70 argues against the possibility that the selective activation of Gz by adenosine observed in our PTX assays (Fig. 2c, d; Supplementary Fig. 6) contributes to membrane hyperpolarisation. The data from recombinant receptors demonstrating the inability of BnOCPA to activate Goa (Fig. 2a, c–g) thus explains why BnOCPA did not cause membrane hyperpolarisation, and indeed prevented or reversed the hyperpolarisation induced by CPA or adenosine, respectively (Fig. 1h, i; Supplementary Fig. 2a, c).

The Ga selectivity displayed by BnOCPA is reflected in non-canonical binding modes and selective interaction with Gai/o subunits. To better understand the unusual signalling properties of BnOCPA and the highly specific Ga coupling to Gob, we carried out dynamic docking simulations to study the basic orthosteric binding mode of BnOCPA in an explicit, fully flexible environment using the active cryo-EM structure of the A1R (PDB code 6D9H; Supplementary Movie 1). We previously reported that modifications at position N^6 of the adenine scaffold modulated the agonist binding path to A1R71. More precisely, N^6- cyclopentyl analogues (CPA and HOCPA) markedly interact with the extracellular loop 2 (ECL2) compared to adenosine, while BnOCPA (which bears the N^6-cyclopentyl-2-benzoxlyloxy group) is most prone to engage residues of the A1R located at the top of transmembrane helix 1 (TM1) and TM7. In the present study, we compared the bound-state BnOCPA to the non-Ga selective
agonists adenosine and HOCPA, and an antagonist (PSB36) of the A1R (Fig. 3a–c). BnOCPA engaged the receptor with the same fingerprint as adenosine72 (Fig. 3a) and HOCPA (Fig. 3b, Supplementary Movie 2). Further explorations of the BnOCPA docked state using metadynamics (MetaD) simulations73 revealed interchangeable variations on this fingerprint (namely Modes A, B, and C; Fig. 3d–f; Supplementary Fig. 8) that could be distinguished by the orientation of the BnOCPA-unique benzyl group. Having established the possible BnOCPA binding modes, we examined the respective contribution of the orthosteric agonists, the G protein α subunit α5 (C-terminal) helix (GαCT), and the G protein subunit74,75 to the empirically-observed G protein selectivity displayed by BnOCPA (Fig. 2a–h, Supplementary Fig. 6).

Firstly, following Dror et al.76, we compared the dynamics of the BnOCPA-bound A1R with the corresponding dynamics of the receptor77,78 bound to either HOCPA (Fig. 3b), the A1R antagonist PSB36 (Fig. 3c), or the apo receptor in the absence of G protein, our hypothesis being that there may be ligand-dependent differences in the way that the intracellular region of the receptor responds in the absence of the G protein. In these simulations the G protein was omitted so that inactivation was possible and so that the results were not G protein-dependent. The BnOCPA binding Modes A–C were interchangeable during MD simulations (Table 1) but were associated with distinctly different dynamics, as monitored by changes in a structural hallmark of GPCR activation, the N7.49PXXY7.53 motif79 (Supplementary Fig. 9). Given the high flexibility shown by the
Fig. 3 Molecular dynamics simulations reveal that BnOCPA binding modes can uniquely drive both agonist- and antagonist-like intracellular conformations of the A₁R. a Adenosine binding pose: N2546.55 (Ballesteros-Weinstein superscript enumeration) is engaged in key hydrogen bonds, while important hydrophobic contacts are shown as cyan transparent surfaces (F177ECL2 and I2747.39). b On the basis of structural similarities and the dynamic docking (Supplementary Movie 2), HOCPA was predicted to bind with a geometry analogous to adenosine; the cyclopropyl group makes further hydrophobic contacts with L2536.54, as shown by simulation. c The xanthine scaffold of the antagonist PSB36 makes hydrogen bonds with N2546.55 side chains and hydrophobic contacts with F177ECL2 and I2747.39. d BnOCPA agonist-like binding Mode A (Supplementary Movie 1); the benzyl group orients towards the ECL2 and makes hydrophobic contacts with I175ECL2 (and M177ECL2) side chains. e BnOCPA antagonist-like binding Mode B: the benzyl group orients towards the top of TM5/TM6 and makes hydrophobic contacts with L2586.59 side chain. f BnOCPA agonist-like binding Mode C: the benzyl group orients towards the top of TM7 and makes hydrophobic contacts with Y2727.36 side chain. g Binding orientation of BnOCPA in antagonist-like Mode D: the benzyl group orients under ECL3 and occupies the hydrophobic pocket defined by L2536.54, T2757.55, T2707.35, and L2697.34. Key hydrogen bonds with N2546.55 and T2777.42 are shown as dotted lines; main hydrophobic contacts are highlighted as cyan transparent surfaces. h Extracellular view of the A₁R showing the four BnOCPA binding Modes A (cyan), B (magenta), C (green), and D (red) as randomly extracted from the MD simulations. i, j Root-mean-square deviation (RMSD) distributions considering the inactive N7.49PXXY7.53 motif on the distal part of TM7 as reference. j HOCPA (blue broken line), BnOCPA Mode A (cyan curve), BnOCPA Mode C (green curve) and the apo receptor (dark green broken line) have a common distribution centring around the active conformation of the A₁R (orange broken line; Supplementary Fig. 9) leading to A₁R signalling. In contrast, j PSB36 (black broken line), BnOCPA Mode B (magenta curve) and BnOCPA Mode D (red curve) RMSD values have the tendency to move closer to the inactive N7.49PXXY7.53 geometry (leftward shift of the curves towards broken grey line at x = 0) preventing A₁R signalling.

BnOCPA benzyl group during the simulations and its lipophilic character, we hypothesised and simulated a further binding mode (namely Mode D) not explored during MD or MetaD simulations. This conformation involves a hydrophobic pocket underneath ECL3 (Fig. 3g) which is responsible for the A₁/A₂A selectivity72. Superimposition of the four BnOCPA binding Modes A–D reveals the highly motile nature of the benzyl group of BnOCPA (Fig. 3h) under the simulated conditions.

Quantification of the N7.49PXXY7.53 dynamics revealed that HOCPA, BnOCPA Mode A, BnOCPA Mode C and the apo receptor show a similar distribution of the RMSD of the conserved N7.49PXXY7.53 motif (Fig. 3i; Supplementary Fig. 9). In contrast, the non-canonical BnOCPA binding Modes B and D were responsible for a partial transition of the N7.49PXXY7.53 backbone from the active conformation to the inactive conformation (Supplementary Fig. 9) in a manner analogous with the antagonist PSB36 (Fig. 3i). Overall, the simulations revealed Mode D as the most stable BnOCPA pose (6.8 µs out of 9 µs simulated starting from this configuration – Table 1), while Mode B accounted for 3.6 µs out of 30 µs.

Next, to simulate the agonist-driven interaction between the A₁R and the G protein, the a5 (C-terminal) helix (GaCT) of the G protein (Gi2, Goa, Gob) was dynamically docked to the HOCPA- and BnOCPA-bound active A₁R structure (again lacking G protein; Supplementary Movie 3). This allowed us to evaluate the effect of different GaCT on the formation of the complex with A₁R to test the hypothesis that, of Goa, Gob and Gi2, only the GaCT of Goa would fully engage with the BnOCPA-bound active A₁R, in line with the empirical observations of G protein selectivity summarised in Fig. 2c, d. Figure 4a shows that the GaCT of Goa docked to the A₁R via a metastable state (MS1) relative to the canonical state (CS1; Supplementary Movie 3), regardless of whether HOCPA or BnOCPA was bound. Figure 4b, c show that the CS1 geometry corresponds to the canonical arrangement as found in the cryo-EM A₁R/G protein complex, whereas state MS1 resembles the recently reported non-canonical state observed in the neurotensin receptor:Gi protein complex80. Overall, Goa interacted more with TM3 and ICL2 residues (Fig. 4g, h), while TM5 and TM6, along with ICL1, were more engaged by Goa (Fig. 4g, h). Interestingly, R2917.56 and I2928.47, which are located under the N7.49PXXY7.53 motif, showed a different propensity to interact with Goa or Gob. In this scenario, it is plausible that a particular A₁R conformation stabilised by BnOCPA (as suggested by the simulations in the absence of G protein, Fig. 3i, j) may favour different intermediate states during the activation process of Goa and Gob.

To test the prediction from the MD simulations that R2917.56 and I2928.47 were involved in A₁R/Ga coupling, we performed a series of site-directed mutagenesis (to alanine) against R2917.56, I2928.47 and the adjacent hydrophilic residues Q2938.48 and K2948.49 (Fig. 4i) and compared the inhibition of forskolin-stimulated cAMP production in response to adenosine, CPA, NECA, HOCPA and BnOCPA in Flp-In-CHO cells against the wild-type (WT) hA₁R (Fig. 4j). Of these residues, none of which are reported to affect binding83, 84, 85, 86, 87, 88, 89, 90 had the least impact on potency; of the agonists, the mutations had minimal effects on HOCPA. In contrast A₁R/Ga coupling induced by adenosine, CPA, NECA and BnOCPA was affected, but differentially so. These effects on potency (IC₅₀ values) can be readily observed when individual mutant IC₅₀ values are normalised to their respective WT controls (Fig. 4k), and revealed that R2917.56, I2928.47 and Q2938.48 are especially important for CPA and NECA coupling, R2917.56 for adenosine potency, and Q2938.48 for BnOCPA. These observations reinforce the MD simulations predictions related to H8 residues involved in G protein coupling of the agonist-stimulated A₁R, and in particular suggest that R2917.56, I2928.47 and Q2938.48 are especially required for selective agonist coupling to Gao/i, and may thus contribute to the Ga bias observed among these agonists (Fig. 2c, d). A more detailed analysis, involving saturation mutagenesis of these residues, is required to provide a full characterisation of their actions to direct agonist bias but is beyond the scope of this current study.
BnOCPA does not depress heart rate, blood pressure or respiration: evidence for in vivo physiological selectivity at native A1Rs. Given BnOCPA’s clear differential effects in a native physiological system (Fig. 1), strong Gob selectivity (Fig. 2), unique binding characteristics (Fig. 3) and selective Gob interaction (Fig. 4), we hypothesised that these properties might circumvent a key obstacle to the development of A1R agonists for therapeutic use—their powerful effects in the cardiovascular system (CVS) where their activation markedly reduces both heart rate and blood pressure\textsuperscript{12}. These cardiovascular effects are likely through Goa, which is expressed at high levels in the heart\textsuperscript{84,85}, particularly in the atria\textsuperscript{86}, and which plays an important role in regulating cardiac function\textsuperscript{87}. In contrast, and with parallels of differential Goa vs Gob expression in the hippocampus\textsuperscript{59}, Gob may be absent or expressed at very low levels in the heart\textsuperscript{86,88}. Given this differential expression of Goa and Gob, and the lack of functional effect of BnOCPA on Goa (Fig. 2a–g), we predicted that BnOCPA would have minimal effects on the CVS. Moreover,
given the antagonism of Goα-mediated actions by BnOCPA at native and recombinant A1Rs (Fig. 1h, i, Supplementary Fig. 2a, c, Fig. 2i), we further predicted that the actions of adenosine on the CVS may be attenuated by BnOCPA.

In initial experiments, we screened BnOCPA for its effects on heart rate using an isolated frog heart preparation. In contrast to adenosine and CPA, which depress heart rate through hyperpolarisation caused by activation of cardiac sinoatrial K⁺ channels⁸⁹, BnOCPA had no effect on heart rate, but markedly reduced the bradycardia evoked by adenosine (Supplementary Fig. 10a). Thus, BnOCPA appears not to activate A1Rs in the heart, but instead behaves like an antagonist in preventing the actions of the endogenous agonist. These observations have parallels with BnOCPA's inability to activate A1Rs to hyperpolarise neurones, and indeed inhibiting or reversing the post-synaptic hyperpolarisation induced by typical A1R agonists (Fig. 1h, i, Supplementary Fig. 2a, c), and in preventing the A1R/Goα-mediated inhibition of cAMP accumulation by adenosine (Fig. 2i). Such antagonist-like behaviour may be explained by BnOCPA causing unique A1R conformations unlike those of conventional agonists (Fig. 3i, j), and driving non-canonical and ultimately non-productive interactions with Goα (Fig. 4a).

To investigate the effects of BnOCPA in an intact mammalian system, we measured the influence of BnOCPA on heart rate and blood pressure in urethane-anaesthetised, spontaneously breathing adult rats. As expected, both resting heart rate and arterial blood pressure were significantly reduced by adenosine and CPA (Fig. 5a–d). In complete contrast, BnOCPA had no effect on either heart rate (Fig. 5a, c) or blood pressure (Fig. 5b, d), even when applied at two or three times the initial dose (Supplementary Fig. 11; Fig. 6e, f). These negative observations could not be explained by metabolism of BnOCPA to an inactive substance since BnOCPA is a very stable compound (half-life (~115.5 μL/min/mg). This was in contrast to the reference compounds verapamil and tenormin (1.0 μL/mg, 37 °C), and the intrinsic clearance (Clint) calculated as <1.0 L/min/mg. Further evidence that BnOCPA was present and active during these experiments was obtained from studies analogous to those in frog heart when BnOCPA was applied together with adenosine. In the intact anaesthetised rat, when co-applied with adenosine or CPA, BnOCPA abolished the bradycardia induced by both agonists, indicating its ability to bind to the A1R at the dose applied (Fig. 5a, c; Fig. 6g, Supplementary Figs. 10b and 11). Volumes of saline equivalent to the drug injections had no effect on either heart rate or blood pressure and there was no waning in the effects of adenosine responses with repeated doses (Supplementary Fig. 10c, d). Thus, BnOCPA does not appear to act as an agonist at CVS A1Rs, but instead antagonises the bradycardic effects of A1R activation on the heart.

Since adverse effects on respiration (dyspnœa) limit the use of systemic A1R agonists⁷, we additionally examined the effects of BnOCPA on respiration. In urethane-anaesthetised, spontaneously breathing adult rats, intravenous injection of BnOCPA had no appreciable effect on respiration (Fig. 6a–d), even if the dose of BnOCPA was doubled or trebled (Fig. 6e, f). In stark contrast the selective A1R agonist CPA caused significant respiratory depression (Fig. 6a–d). Parallelising BnOCPA's antagonism of adenosine- and CPA-induced depressions of heart rate (Fig. 5a, c; Supplementary Figs. 10b and 11), BnOCPA reduced the depression of respiratory frequency and minute ventilation caused by CPA (Fig. 6g, Supplementary Fig. 11). These data suggest that while BnOCPA targets and clearly engages the A1R responsible for adenosine and CPA's cardiorespiratory depression, BnOCPA has no agonist action at these A1Rs.

**BnOCPA is a potent analgesic.** Our observations of a lack of effect of BnOCPA on the CVS and respiration prompted an investigation into a potential application of A1R agonists that had previously been severely curtailed by adverse cardiorespiratory events⁷,¹⁶, namely the use of A1R agonists as analgesics. Since sedation or motor impairment can be mistaken for analgesia, we tested BnOCPA in a sensitive assay for balance and motor coordination, the rotarod, in which the ability of a rodent to remain upon a slowly accelerating rotating cylinder is a measure of alertness and motor function. As a positive control for the sensitivity of the test, we showed that the ability of animals treated with morphine to remain on the rotating cylinder was strongly impaired (Fig. 7a). In contrast, the performance of animals treated with BnOCPA, delivered either intravenously or intraperitoneally, was indistinguishable from vehicle-treated mice (Fig. 7a). This was true even if BnOCPA was injected intravenously at three times the dose (Fig. 7a), which, while having no cardiorespiratory actions on its own, prevented cardiorespiratory depression caused by adenosine and CPA (Figs. 5 and 6; Supplementary Figs. 10 and 11). Thus, BnOCPA does not induce
The population91,92, and which carries a major global burden of chronic pain, which affects between 20 and 50% of the limb is rendered sensitive to previously innocuous tactile stimuli, a feature of which is mechanical allodynia whereby the affected disability93. To test if this analgesia was several orders of magnitude lower than the non-opioid analgesics doses devoid of sedative or cardiorespiratory effects, and at manner. Thus, BnOCPA exhibits powerful analgesic properties at potently reversed mechanical allodynia in a dose-dependent pretoty. 

**Fig. 5 BnOCPA does not affect heart rate or blood pressure.** a Examples of heart rate (HR) and b arterial blood pressure traces from a urethane-anaesthetised, spontaneously breathing rat showing the effects of adenosine (1 mg kg⁻¹), BnOCPA (8 µg kg⁻¹) and CPA (6 µg kg⁻¹). Grey diamonds reflect saline flushing of the femoral vein catheter. Insets are expanded HR and blood pressure responses to adenosine and BnOCPA (black and blue traces, respectively; boxed regions in a and b). Scale bars measure: HR, 200 BPM and 6 s; blood pressure, 40 mm Hg and 6 s. c, d Summary data for 4 rats where each rat is shown as a different symbol with the means (± SEM, light grey bars) connected. One-way RM ANOVA for: c HR, Greenhouse-Geisser corrected F(2.33, 7.00) = 68.27, P = 2.07 × 10⁻⁵; d mean arterial blood pressure (MAP), Greenhouse-Geisser corrected F(1.84, 5.52) = 10.51, P = 0.014; with the following Bonferroni post hoc comparisons: The resting HR of 432 ± 21 BPM was significantly reduced to 147 ± 12 BPM (~66%, P = 2.76 × 10⁻¹¹) by adenosine. BnOCPA had no effect on HR (~6%, 442 ± 20 vs 416 ± 21 BPM; P = 1) but prevented the bradycardic effects of adenosine (P = 2.71 × 10⁻⁹ vs adenosine) when co-injected (mean change 51 ± 4 BPM: -12%; P = 0.67). CPA significantly decreased HR (from 408 ± 17 to 207 ± 29 BPM; -50%, P = 1.85 × 10⁻⁸), a decrease that was not different to the effect of adenosine (P = 0.12), but was significantly different to the effect of both BnOCPA (P = 9.00 × 10⁻⁹) and adenosine in the presence of BnOCPA (P = 6.69 × 10⁻⁷). The resting MAP (86 ± 9 mm Hg) was significantly reduced by adenosine (~47%, 46 ± 4 mm Hg; P = 0.001). BnOCPA had no effect on its own on MAP (88 ± 11 vs 85 ± 13 mm Hg; P = 1) and did not prevent adenosine in lowering MAP to a value similar to adenosine on its own (51 ± 4 mm Hg; P = 1 vs adenosine; P = 0.012 vs BnOCPA alone), CPA significantly decreased MAP (from 83 ± 8 to 51 ± 5 mm Hg; P = 0.017), a decrease that was not different to the effect of adenosine in the absence or presence of BnOCPA (P = 1 for both), ns, not significant; **, P < 0.02; ****, P < 0.0001. Source data are provided as a Source Data file.

Discussion

Biased agonists at GPCRs offer great potential for the preferential activation of desirable intracellular signalling pathways, while avoiding, or indeed blocking those pathways that lead to adverse or unwanted effects3,27. While this, and the potential to exploit previously unattractive drug targets such as the A1R, have been appreciated, translation of in vitro observations, particularly of Gα bias, to native receptors in vivo has been problematic3,4,27. Here we have shown that translation of in vitro selectivity among Gα subunits, identified using two separate assays, to an intact physiological system is possible through a benzyloxy derivative (BnOCPA) of the selective A1R agonist CPA. Moreover, this Gα selectivity has occurred in the context of the A1R, an attractive, but notoriously intractable drug target by virtue of the profound cardiorespiratory consequences of its activation by conventional A1R agonists.

BnOCPA was first reported as a final compound in a patent where it was described to be selective for the A1R with respect to its binding affinity, and effective in reducing elevated intraocular pressure for the potential treatment of glaucoma or ocular hypertension34. We have previously prepared BnOCPA (and HOCPA)33 for assessment as part of our synthetic campaign to develop potent and A1R-selective fluorescent ligands. The N⁶-substituent (1R,2 R)-2-amino cyclo pentan-1-ol) present in BnOCPA and HOCPA is also found in the experimental and later discontinued103 drug CVT-3619 (later named GS 9667), which elevated adenosine tone100 and activation of A1Rs101 in the neuropathic spinal cord, which may have resulted in the desensitisation of A1R-mediated responses102. These observations confirm that the analgesia provoked by BnOCPA is mediated via the selective activation of A1Rs.
has been described as a partial, selective agonist of the A1R and shown to reduce cAMP content and lipolysis in rat adipocytes\textsuperscript{104}.

Having identified BnOCPA as a selective Gob agonist at recombinant A1Rs in vitro, we established that this unusual property can be translated into the selective activation of native A1Rs in both the in vitro CNS and in vivo cardiorespiratory and peripheral nervous systems. Moreover, these properties of BnOCPA were observed at A1Rs expressed by three different species: amphibian, rat, and human. While BnOCPA bound to and induced A1R coupling to G\textsubscript{ai/o} subunits recruited by prototypical A1R agonists such as adenosine and CPA, BnOCPA selectively activated Gob among the six G\textsubscript{ai/o} subunits. This likely reflected BnOCPA’s non-canonical binding profile at the A1R, which had profound implications for the interaction with the
GaCT in terms of different binding pathways and intermediate states, and in the different intra- and intermolecular hydrogen bond patterns and contacts observed in the simulations of the A1R in complex with either Goa (inactive) or Gob (active). Predictions from the MD simulations suggested four hitherto uncharacterised residues as being important for the interaction between the A1R and Ga/o. Individual mutations in three of these contacts, R291<sup>17</sup>-<sup>56</sup>, I292<sup>47</sup>, Q293<sup>38</sup>-<sup>48</sup>, differentially impacted agonist efficacy, with adenosine and HOCPA being relatively unaffected compared to the stronger effects on the efficacy of CPA, NECA and BnOCPA. These and other molecular differences in the coupling of the A1R to Ga/o are likely to underlie the ability of the BnOCPA-bound A1R to selectively trigger Gob activation among the six Ga/o subtypes.

The unique and unprecedented Ga selectivity displayed by BnOCPA has physiological importance since it is able to inhibit excitatory synaptic transmission without causing neuronal membrane hyperpolarisation, sedation, bradycardia, hypotension or dyspnoea. BnOCPA thus overcomes cardiovascular and respiratory obstacles to the development of adenosine-based therapeutics that have plagued the field since their first description nine decades ago<sup>105</sup>. As a first, but significant, step towards this, we demonstrate that BnOCPA has powerful analgesic properties via A1Rs in an in vivo model of chronic neuropathic pain, potentially through a mechanism that may involve a combination of inhibition of synaptic transmission in peripheral and spinal pain pathways, and the hyperpolarisation of Gob-containing nociceptive neurons. Chronic pain, a condition that affects a large proportion of the population suffers on a constant or frequent basis<sup>91</sup>,<sup>92</sup> and associated with a major global burden of disability<sup>93</sup> is, however, a disorder for which the current treatments are either severely lacking in efficacy<sup>106</sup> or, in the case of opioids, come with unacceptable harms such as adverse gastrointestinal effects, respiratory depression, tolerance, dependence and abuse potential<sup>107</sup>. Accordingly, novel treatments for chronic pain are urgently required.

We have shown that highly selective Ga agonism in vitro can be translated into selective activation of native A1Rs to mediate differential physiological effects, and have identified a novel molecule capable of doing so. We have also explored molecular mechanisms by which this could occur, and demonstrated pain as one potential and wide-reaching therapeutic application. Such discoveries are of importance in both understanding GPCR-mediated signalling, and in the generation of both new research tools and therapeutics based on the untapped potential of biased, and indeed Ga-selective, agonists such as BnOCPA.

### Methods

#### Approvals

All experiments involving animals were conducted with the knowledge and approval of the University of Warwick Animal Welfare and Ethical Review Board, and in accordance with the U.K. Animals (Scientific Procedures) Act (1986) and the EU Directive 2010/63/EU. In vivo cardiorespiratory studies were conducted under the auspices of UK PPL 70/8936 and chronic neuropathic pain studies under the auspices of P9D9428A9. Rotarod studies were approved by the Monash University Animal Ethics Committee in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (2013) under Monash AEC protocol number 13333.

#### Preparation of hippocampal slices

Sagittal slices of hippocampus (300–400 μm) were prepared from male Sprague-Dawley rats, at postnatal days 12–20<sup>108</sup>. Rats were kept on a 12-hour light-dark cycle with slices made 90 min after entering the light cycle. In accordance with the U.K. Animals (Scientific Procedures) Act (1986), rats were killed by cervical dislocation and then decapitated. The brain was removed, cut down the midline and the two sides of the brain stuck down to a metal base plate using cyanoacrylate glue. Slices were cut along the midline with a Microm HM 650 V microlicer in cold (2–4 °C), high Mg<sup>2+</sup>, low Ca<sup>2+</sup> artificial cerebrospinal fluid (aCSF), composed of (mM): 127 NaCl, 1.9 KCl, 8 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 1.2 K<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 n-glucose (pH 7.4 when bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, 300 mM). Slices were stored at 34 °C for 1–6 h in aCSF (1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>) before use.

#### Extracellular recording

A slice was transferred to the recording chamber, submerged in aCSF and perfused at 4–6 ml min<sup>−1</sup> (32 ± 0.5 °C). The slice was placed on a grid allowing perfusion above and below the tissue and all tubing was gastight (to prevent leakage of oxygen). An aCSF-filled glass microelectrode was placed within stratum radiatum in area CA1 and recordings were made using either a differential model 3000 amplifier (AM systems, WA USA) or a DP-301 differential amplifier (Warner Instruments, Hampden, CT USA). Field excitatory postsynaptic potentials (fEPSPs) were evoked with either an isolated pulse stimulator model 2100 (AM Systems, WA) or ISO-Flex (AMPI, Jerusalem, Israel). For fEPSPs a 10–20 min baseline was recorded at a stimulus intensity that gave 40–50% of the maximal response. Signals were acquired at 10 kHz, filtered at 3 kHz, and digitised on line (10 kHz with a Micro CED (Mark 2) interface controlled by Spike software (Vs 6.1, Cambridge Electronic Design, Cambridge UK) or with WinLTP<sup>109</sup>. For fEPSP slope, a 1 ms linear region after the fibre volley was measured. Extracellular recordings were made independently on two electrophysiology rigs. As the data obtained from each rig was comparable, both sets of data have been pooled.
Seizure model. Seizure activity was induced in hippocampal slices using nominally Mg\(^{2+}\)-free aCSF that contained no added Mg\(^{2+}\) and with the total K\(^{+}\) concentration increased to 6 mM with KCl. Removal of extracellular Mg\(^{2+}\) facilitates depolarisation via glutamate N-methyl-D-aspartate (NMDA) receptor activation. Increasing the extracellular concentration of K\(^{+}\) depolarises neurons leading to firing and release of glutamate to sustain activity. Both the increase in K\(^{+}\) concentration and removal of Mg\(^{2+}\) are required to produce spontaneous activity in hippocampal slices\(^{110}\). Spontaneous activity was measured with an aCSF-filled microelectrode placed within stratum radiatum in area CA1.

Whole-cell patch-clamp recording from hippocampal pyramidal cells. A slice was transferred to the recording chamber and perfused at 3 ml min\(^{-1}\) with aCSF at 32 ± 0.5 °C. Slices were visualised using IR-DIC optics with an Olympus BX51WI microscope (Scientifica) and a CCD camera (Hitachi). Whole-cell current- and voltage-clamp recordings were made from pyramidal cells in area CA1 of the hippocampus using patch pipettes (5–10 MΩ) manufactured from thick walled glass (Harvard Apparatus, Edenbridge UK) and containing (mM): potassium gluconate 135, NaCl 7, HEPES 10, EGTA 0.5, phosphocreatine 10, MgATP 2, NaGTP 0.3 and biocytin 1 mg ml\(^{-1}\) (290 mOSM, pH 7.2). Voltage and current recordings were obtained using an Axon Multiclamp 700B amplifier (Molecular Devices, USA) and digitised at 20 KHz. Data acquisition and analysis was performed using pClamp 10 (Molecular Devices, USA). For voltage-clamp experiments, CA1 pyramidal cells were held at −60 mV. Peptides to interfere with G protein signalling were introduced via the patch pipette into the recorded cell. The cell was held for at least 10 min before adenosine (10 µM) was added to induce an outward current.

Frog heart preparation. Young adult male Xenopus leavis frogs were obtained from Portsmouth Xenopus Resource Centre. Frogs were euthanized with MS222 (0.2% at a pH of 7), decapitated and pithed. The animals were dissected to reveal the heart and the pericardium was carefully removed. Heart contractions were measured with a force transducer (AD instruments). Heart rate was acquired via a PowerLab 26 T (AD instruments) controlled by LabChart 7 (AD instruments). The heart was regularly washed with Ringer solution and drugs were applied directly to the heart.

In vivo anaesthetised rat preparation for cardiorespiratory recordings. Anaesthesia was induced in adult male Sprague–Dawley rats (230–330 g) with isofluorane (2–4%; Piramal Healthcare). The femoral vein was catheterised for drug delivery. Anaesthesia was maintained with urethane (1.2–1.7 g kg\(^{-1}\); Sigma) in sterile saline delivered via the femoral vein catheter. Body temperature was
maintained at 36.7 °C via a thermocoupled heating pad (TCAT 2-LV; Physitemp). The trachea was cannulated and the femoral artery catheterised, and both were connected to pressure transducers (Digitimer) to record respiratory airflow and arterial blood pressure, respectively. Blood pressure and airflow signals were amplified using the Neurolog signal processing system (Digitimer) connected to the \'H still\' interface and acquired on a computer using Spike2 software (v7.08a; Cambridge Electronic Design). Arterial blood pressure recordings were used to derive heart rate (HR: beats.min⁻¹; BPM), and to calculate mean arterial blood pressure (MAP: Diastolic pressure + 1/3[Systolic Pressure–Diastolic pressure]). Airflow measurements were used to calculate: tidal volume (Vₜ; mL; pressure sensors were calibrated with a 3 mL syringe), and respiratory frequency (f; breaths min⁻¹; BrPM). Minute ventilation (Vₑ; mL min⁻¹) was calculated as f x Vₑ. Cardiovascular and respiratory parameters were allowed to stabilise before experiments began. A₁Rs agonists were administered by intravenous (IV) injection and the changes in HR, MAP, f, Vₑ, and Vₑ were measured. In pilot studies, the optimal dose of adenosine was determined by increasing the dose until robust and reliable changes in HR and MAP were produced (1 mg kg⁻¹). The dose of CPA was adjusted until equivalent effects to adenosine were produced on HR and MAP (6 µg kg⁻¹). For BnOCPA we initially used 1 µg kg⁻¹, but saw no agonist effect on HR and MAP. To ensure this was not a false negative we increased the dose of BnOCPA (6 µg kg⁻¹). For CPA (6 µg kg⁻¹) IV were prevented by the A₁ receptor antagonist DPCPX (1 mg kg⁻¹ IP), but not the A₁-selective antagonist MR5123 (2 mg kg⁻¹ IP). Fisher LSD post hoc comparisons showed significant differences at: IT 1 nmol at 1 and 2 hrs, P = 0.001 and 4.16 x 10⁻⁵, respectively, and 3 nmol at 1, 2 and 4 hrs, P = 9.52 x 10⁻¹¹, 1.42 x 10⁻¹¹ and 1.41 x 10⁻⁹, respectively; IV 3 µg kg⁻¹ at 1, 2 and 4 hrs, P = 0.044, 0.008 and 0.019, respectively, and 10 µg kg⁻¹ at 1, 2 and 4 hrs, P = 1.42 x 10⁻⁹, 6.81 x 10⁻¹⁴ and 3.23 x 10⁻⁴, respectively, b, c n = 6 per treatment, except for 1 nmol BnOCPA, n = 5. The agonistic effects of BnOCPA (6 µg kg⁻¹ IV) were prevented by the A₁ receptor antagonist DPCPX (1 mg kg⁻¹ IP), but not the A₁-selective antagonist MR5123 (2 mg kg⁻¹ IP). Post hoc LSD comparisons across all four groups and four-time points (pre-dose, 1, 2 and 4 hrs; F(3,116) = 26.8, P = 0) revealed that BnOCPA at 6 µg kg⁻¹ IV elicited significant analgesia compared to vehicle-treated animals at 1, 2, and 4 h post-dosing (P = 4.69 x 10⁻⁹, 3.50 x 10⁻⁶, 4.69 x 10⁻⁹, respectively), which persisted in the presence of the selective A₁ receptor antagonist MR5123 over the same time period (P = 4.42 x 10⁻¹³, 3.38 x 10⁻¹⁴, 1.81 x 10⁻¹⁰, respectively). In contrast, the PWT in DPCPX-treated animals did not differ from those in the vehicle group (P = 0.872, 0.748, 0.453 at 1, 2, and 4 h, respectively). n = 11 for BnOCPA and vehicle groups; n = 6 for the DPCPX group and n = 5 for the MR5123 group. Averaged data are presented as mean ± SEM, ns, not significant; *, P < 0.05; **, P < 0.02; ***, P < 0.001; ****, P < 0.0001. Source data are provided as a Source Data file.

Spinal nerve ligation (Chung model) (30). Adult male Sprague–Dawley rats, 7-8 weeks old, weighing around 250 g at the time of Chung model surgery, were purchased from Charles River UK Ltd. The animals were housed in groups of 4 in an air-conditioned room on a 12-hour light/dark cycle. Food and water were available ad libitum. They were allowed to acclimatisate to the experimental environment for 3 days before leaving them on a raised mesh for at least 40 min. The baseline paw withdrawal threshold (PWT) was examined using a series of graduated von Frey hairs (see below) for 3 consecutive days before surgery and re-assessed on the 6th to 8th day after surgery and on the 13th to 17th day after surgery before drug dosing.

Prior to surgery each rat was anaesthetised with 3% isoflurane in oxygen (1 L min⁻¹) followed by an intramuscular injection of ketamine (60 mg kg⁻¹) plus xylazine (10 mg kg⁻¹). The back was shaved and sterilised with povidone-iodine. The animal was placed in a prone position and a para-medial incision was made on the skin covering the L4-L6 level. The L5 spinal nerve was carefully isolated and tightly ligated with 600 silk suture. The wound was then closed in two layers after a complete haemostasis. A single-dose of antibiotics (Amoxicillin, 15 mg/rat, intraperitoneally, IP) was routinely given for prevention of infection after surgery. The animals were placed in a temperature-controlled recovery chamber until fully awake before being returned to their home cages. The vehicle (normal saline or DMSO) was administered via the IV route at 1 ml/kg⁻¹ and via the intrathecal (IT) route at 10 µl for each injection. The A₁-selective antagonist DPCPX (1 mg kg⁻¹) and the A₁,α₂-selective antagonist MR5123 (2 mg kg⁻¹) were administered IP 30 mins before vehicle or BnOCPA treatment. The rats with validated neuropathic pain state were randomly divided into 11 groups: vehicle IV, BnOCPA at 1, 3, 6, 10 µg kg⁻¹ IV; vehicle IT, BnOCPA 0.3, 1, 3 nmol IT; 6 µg kg⁻¹ BnOCPA IV plus 1 mg kg⁻¹ DPCPX IP; 6 µg kg⁻¹ BnOCPA IV plus 2 mg kg⁻¹ MR5123 IP groups and tested blind to treatment.

To test for mechanical allodynia the animals were placed in individual Perspex boxes on a raised metal mesh for at least 40 min before the test. Starting from the filaments of lower force, each filament was applied perpendicularly to the centre of the ventral surface of the paw until slightly bent for 6 seconds. If the animal withdrew or lifted the paw upon stimulation, then a hair with force immediately lower than that tested was used. If no response was observed, then a hair with force immediately higher was tested. The highest value was set at 15. The lowest amount of force required to induce tolerable responses (positive in 3 out of 5 trials) was recorded as the value of PWT. On the testing day, PWT were assessed before and 1, 2 and 4 h following BnOCPA or vehicle administration. The animals were returned to their home cages to rest (about 30 min) before two neighbouring testing time points. At the end of each experiment, the animals were deeply anaesthetised with isoflurane and killed by decapitation.

Fig. 7 BnOCPA is a potent analgesic without causing sedation or motor impairment. a BnOCPA did not induce sedation or affect motor function when injected intraperitoneally (IP; 10 µg kg⁻¹) or intravenously (IV; 10 or 25 µg kg⁻¹). In contrast, morphine caused sedation and motor impairment (15 mg kg⁻¹ subcutaneously, SC). Saline (VeH, SC) did not affect rotoroad performance. Data points (mean ± SEM; n = 6 for each compound) are normalised to pre-dose performance and are offset for clarity. b, c BnOCPA alleviates mechanical allodynia in neuropathic pain when administered via an intrathecal (IT) or IV route. Pre-surgery (pre-surg) animals had similar sensitivity to tactile stimulation as assessed by von Frey hair stimulation. Spinal nerve ligation caused hypersensitivity to touch (mechanical allodynia) at 1 week after surgery as evidenced by the reduction in the tactile pressure necessary to elicit paw withdrawal (paw withdrawal threshold, PWT). PWT reaches a similar nadir across all groups prior to the vehicle or BnOCPA infusion (pre-dose). Administration of BnOCPA significantly increased PWT in the limb dependent to the site of injury in a dose-dependent manner (one-way ANOVA (pre-dose, 1, 2 and 4 hrs) for IT BnOCPA F(3, 88) = 21.9, P = 1.10 x 10⁻¹⁰; for IV BnOCPA F(3, 92) = 18.1, P = 2.70 x 10⁻⁹). Fisher LSD post hoc comparisons showed significant differences at: IT 1 nmol at 1 and 2 hrs, P = 0.001 and 4.16 x 10⁻⁵, respectively, and 3 nmol at 1, 2 and 4 hrs, P = 9.52 x 10⁻¹¹, 1.42 x 10⁻¹¹ and 1.41 x 10⁻⁹, respectively; IV 3 µg kg⁻¹ at 1, 2 and 4 hrs, P = 0.044, 0.008 and 0.019, respectively, and 10 µg kg⁻¹ at 1, 2 and 4 hrs, P = 1.37 x 10⁻⁹, 6.81 x 10⁻¹⁴ and 3.23 x 10⁻⁴, respectively, b, c n = 6 per treatment, except for 1 nmol BnOCPA, n = 5. The agonistic effects of BnOCPA (6 µg kg⁻¹ IV) were prevented by the A₁ receptor antagonist DPCPX (1 mg kg⁻¹ IP), but not the A₁-selective antagonist MR5123 (2 mg kg⁻¹ IP). For BnOCPA, or CPA on respiration. Adult male Sprague–Dawley rats (400-500 g) were anaesthetised with urethane and instrumented as described above, with the exception that the arterial cannulation was not performed.

After allowing the animal to stabilise following surgery, BnOCPA (8 µg kg⁻¹) was administered. After a 20 min recovery period CPA (6 µg kg⁻¹) was administered. All injections were administered IV as a 350 µl kg⁻¹ bolus. Changes in f, Vₑ, and Vₑ were measured. If the dosing occurred close to a respiratory event such as a sigh or a second IV dose was administered, with 20 min recovery periods either side of the injection. Measurements for the effect of BnOCPA were time-matched to when CPA induced a change in respiration in the same preparation. As no difference was observed between the respiratory responses to BnOCPA in these rats (n = 4) and those instrumented for both cardiovascular and respiratory recordings (n = 4), the data were pooled (n = 8; Fig. 6a-d).

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Rotarod test for motor function. A rotarod test was used to assess motor coordination following intravenous and intraperitoneal administration of BnOCPA. An accelerated rotarod (Ugo Basile) was set to speed increased from 6 to 80 rpm every 170 seconds. Male Sprague–Dawley rats (n = 24), 7 weeks of age (212-258 g) were trained on the rotarod twice daily for two days (≥2 trials per session) until performance times were stable. On the day of the experiment, three baseline trials were recorded. The compound was administered IP (10 μg kg⁻¹, n = 6) or IV via tail vein injection (10 μg kg⁻¹, further 16-18 h). Latency to fall (seconds) was measured in triplicate at 1, 2, 3 and 5 h post drug administration.

Constructs, transfections and generation of stable cell lines. Cell lines were maintained using standard subculturing routines as guided by the European Collection of Cell Culture (ECACC) and checked annually for mycoplasma infection using the MycoAlert® mycoplasma detection kit from Lonza. CHO cells (ThermoFisher - R75807) and CHO-K1-hA1R cells in ice-cold buffer (2 mM MgCl₂, 20 mM HEPES, pH 7.4). The ability to induce neuronal cell death following intravenous and intraperitoneal administration of BnOCPA. An accelerated rotarod (Ugo Basile) was set to speed increased from 6 to 80 rpm every 170 seconds. Male Sprague–Dawley rats (n = 24), 7 weeks of age (212-258 g) were trained on the rotarod twice daily for two days (≥2 trials per session) until performance times were stable. On the day of the experiment, three baseline trials were recorded. The compound was administered IP (10 μg kg⁻¹, n = 6) or IV via tail vein injection (10 μg kg⁻¹, further 16-18 h). Latency to fall (seconds) was measured in triplicate at 1, 2, 3 and 5 h post drug administration.

Cell signalling assays. CHO cell lines expressing ARs of interest (including mutants of the hA₁R, hA₂AR, hA₃R, hA₂BR-H) were transiently transfected with 1 μg plasmid using Fugene HD (Promega) at a ratio of 3:1 (v/w) (Fugene:DNA). Transfected cells were harvested and resuspended in growth media containing 10% fetal bovine serum (FBS), at 37 °C with 5% CO₂, in a humidified atmosphere. For cAMP accumulation experiments, cells were seeded at a density of 2000 cells per well of a white 384-well optiplate and stimulated, for 30 min, with a range of agonist concentrations (100 pM – 100 μM) in the presence of 25 μM rolipram (Cayman Chemicals). For cAMP inhibition experiments, cells were co-stimulated with 1 μM forskolin and a range of antagonist concentrations (1 μM–100 μM), in the presence or absence of 1 μM antagonist. cAMP levels were then determined using a LANCE® cAMP kit as described previously.111

β-arrestin recruitment assays. HEK 293 T cells (ATCC CRL-3216) were routinely grown in DMEM/F-12 GlutaMAXmedia (Thermo Fisher Scientific, UK) supplemented with 10% FBS (Sigma-Aldrich) and 1% AA (Sigma, UK). After being grown overnight, cells in each well were transfected using polyethyleneimine 25 kDa (PEI, Polysciences Inc., Germany) at a 1:6 ratio of PEI to DNA, diluted in 150 mM NaCl. Cells were transfected with hA₁R, Gαq-RLuc8 or Gob-RLuc8, Gαq-CPX, Gαq-CPX and pgCYP2C and pcDNA3.1 containing Kozak motifs for enhanced translation. The peptide sequences used were: peptide MGLNRGNAYLCIGMG was used. Constructs were sequenced to confirm identity and low affinity values for the unlabelled agonists. For all ARs, filtered light emission at 450 nm and >610 nm (640-685 nm band pass filter) was measured using a Mithras LB 940 (Berthold technology) using MicroWin 2000 software. Non-specific binding was determined by adding 1 nM of reference compound (i.e., agonist): 100 nM. β-arrestin:hGRK ratio of 1:5:4) using polyethyleneimine (PEI, 1 mg/ml, MW 25,000 g/mol; Polysciences Inc., Germany) for 60 min at room temperature. Free radioligand was separated from bound radioligand by incubating for 10 min prior to adding the combined mix dropwise to the filter. Latency to fall (seconds) was measured in triplicate at 1, 2, 3 and 5 h post drug administration.

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media was discarded and replaced with 80 µl assay buffer (1× Hank’s balanced salt solution (HBSS) with calcium, supplemented with 20 mM HEPES and 0.1% BSA at pH 7.4). The assay was started by adding 10 µl of coelenterazine 400A (NanoLight technology, USA) to a final concentration of 5 µM. The plates were then incubated in the dark for 5 min, prior to the addition of 10 µl compounds (in a range of 0.01 nM – 1 µM). In order to investigate the effect of interfering peptides on Goa and Gob activation, cells were transferred with the TRUPATH constructs for Goa and Gob with the A1R as described above. However, the vector was replaced by either interfering or scrambled peptides, as appropriate, with increasing concentration: 0, 100, and 400 ng and was complemented by pCDN3.1(-) up to 400 ng. CPA 10 µl was used as the ligand in a range of concentration: 0, 1 nM – 1 µM. BRET signal was measured for 30 min on a Mithras LB940 plate reader allowing sequential integration of signal detected from GFP2 and RluCh. The BRET ratio corresponds to the ratio of light emission from GFP2 (515 nm) over RluCh (400 nm). Net BRET ratio was used to generate the concentration-response curve by taking 11-minute time-point after baseline correction. Data was analysed as change in the presence of the interfering peptides relative to control alone at 1 µM CPA.

Statistics and reproducibility

Data and statistical analysis. Concentration-response curves for the effects of A1R agonists on synaptic transmission were constructed in OriginPro 2018 (OriginLab; Northampton, MA, USA) and fitted with a logistic curve using the Levenberg Marquardt iteration algorithm. OriginPro 2018 was also used for statistical analysis. Statistical significance was tested as indicated in the text using paired or unpaired two-tailed t-tests or one-way or two-way ANOVAs with repeated measures (RM) as appropriate. Bonferroni corrections for multiple comparisons were performed. All in vitro cell signalling assay data was analysed using Prism 8.4 (Graphpad software, San Diego, CA), with all concentration-response curves being fitted using a 3 parameter logistic equation to calculate EC50 and IC50. All cAMP data was normalised to a forskolin concentration-response curve ran in parallel to each assay. Where appropriate the operational model of receptor agonism was used to obtain efficacy (log τ) and equilibrium dissociation constant (log Kd) values. Calculation of bias factors (Δlog(τa/Ka), τa relative to adenosine was performed as described in West et al. 2016)11. Error for this composite measure was propagated by applying the following Eq. (1):

\[
Pooled \ SE = \sqrt{SE_m^2 + SE_b^2} \quad (1)
\]

Where, σa and σb are the standard deviations of measurement A and B and x and n is the same as the number of repeats.

Single-dose Schild analysis was performed on data using BnOCPA as an antagonist to adenosine in the cAMP assays so enabling determination of BnOCPA's affinity constant (Kd) using the following Eq. (2):

\[
\frac{D}{D_0} = 1 + [AK] \quad (2)
\]

where D' and D = EC50 values of adenosine with and without BnOCPA present, respectively, [A] = BnOCPA concentration. BnOCPA, and Kd = the affinity constant (Kd) of the BnOCPA.18

Statistical significance relative to adenosine was calculated using a one-way ANOVA with a Dunnett's post-test for multiple comparisons. Radioligand displacement curves were fitted to the one-site competition binding equation yielding IC50 values. One-way ANOVA (Dunnett’s post-test) was used to determine significance by comparing the log(Ki) value for each compound when compared to adenosine. To determine the extent of ligand-induced recruitment of β-arrestin-2 YFP to either the A1R or A2B, the BRET signal was calculated by subtracting the 530 nm/450 nm emission for vehicle-treated cells from ligand-treated cells (ligand-induced ∆BRET). ∆BRET for each concentration at 5 min (maximum response) was used to produce concentration-response curves.

All in vivo cardiovascular and respiratory data were analysed using OriginPro 2018. One-way ANOVAs, with repeated measures as appropriate, and with Bonferroni correction for multiple comparisons were used. Statistical significance for the effects of IV saline and the antagonist effect of BnOCPA on CPA were tested using two-tailed paired t-tests. Data are reported throughout as mean ± SEM and n values are reported for each experiment. For the neuropathic pain studies, one-way ANOVAs with Fisher’s Least Significant Difference (LSD) post hoc test was used to compare drug treatment groups to the vehicle group (OriginPro 2018).

The significance level was set at P < 0.05, with actual P values reported in the figure legends and summaries, by way of abbreviations and asterisks, on the graphs: ns, not significant; * P < 0.05; ** P < 0.02; *** P < 0.001; **** P < 0.0001.

Reproducibility. Samples sizes for in vitro pharmacological or in vivo cardio- respiratory experiments were based on prior experience and were routinely in excess of 4 independent biological replicates. In many cases, the pool of animals was divided to allow time over interlaced experiments with other compounds and often by other investigators, ensuring the consistency and reproducibility of experimental observations. Data was only excluded when technical failures occurred. Randomisation and blinding was not performed for in vitro or cardiorespiratory studies. In the in vivo studies, the sample sizes for which (typically 6 animals) were based on prior experience, the experimenters were blinded to the reagents used. For the pain studies, all animals were pre-screened for hypersensitivity (von Frey hair) when they arrived, and those that showed this, i.e., a paw withdrawal threshold (PWT) less than 8 g, were eliminated from the study. Those rats successive with moderate mechanical allodynia (with PWT lower than 4 g) were randomly assigned into different groups according to their PWT values of pre-surgery, 1 week post-surgery and pre-dosing to ensure a balanced distribution across groups. For the rotarod studies, rats were randomly assigned to treatment groups by animal care technicians not involved in the experimental studies. Experiments occurred over a period of time with various types of manipulations interleaved to avoid bias effects. Key observations were replicated with different batches of animals and reagents and by different experimenters. Results across different independent labs working in different experimental systems were consistent with the main theories and conclusions of the study.

Drugs and substances. Drugs were made up as stock solutions (10-10 Mm) and then diluted in aCSF or saline on the day of use. BnOCPA (2R,3R) R,S,S-{{2–6}[(1R,2S)-2-benzoyloxycyclopentylamino]–9H–pyrin-9-yli}–5(4-hydroxymethyl)oxo–lane-3,4-diol) and HOCPA (2R,3R) R,S,R–I–2–6–{[(1R,2S)–2–hydroxyxycyclopentylamino]–9H–pyrin-9-yli}–5(4-hydroxymethyl)oxolane-3,4-diol), the (1R,2S)–2–hydroxyxycyclopentylamino buta-epimer of known AR agonist GR797269, were synthesised as described previously19 and dissolved in dimethyl-sulphoxide (DMSO, 0.01% final concentration). Adenosine, 8-CPT (8-cyclopentyltheophylline), NECA (5'-N-Ethylcarboxamido) adenosine), DPCPX, ZM241385, MRS1220 and CPA (N'-Cyclopentyladenosine) were purchased from Sigma-Aldrich (Poole, Dorset, UK). MRS1529 was purchased from Cayman Chemicals (Cambridge Bioscience Ltd, Cambridge UK). [H-]DPCPX was purchased from PerkinElmer (Life and Protein Sciences, Waltham, MA). CA200645 and peptides for interfering with G protein signalling were obtained from Helio Bio (Bristol, UK) and were based on published sequences60. NECA-TAMARA was synthesised in-house (Noel et al., in preparation), while AV039, a highly potent and selective fluorescent antagonist of the human A1R based on the 1,2,4-Triazolo[4,3-a]quinazolin-1-one linked to BY630 was kindly gifted to us by Stephen Hill and Stephen Birdson (University of Nottingham). For Gna, the peptide had a sequence of MGIANLRRGCGG. The scrambled version was LNRAGNLCIGMG. For Gna, the peptide had a sequence of MGIANLRRGCGG. Peptides were made up as stock solutions (2 mM) and stored at –20 °C. The stock solutions were dissolved in filtered intracellular solution just before use.

BnOCPA Pharmacokinetics. The stability in solution and metabolism of BnOCPA (0.1 µM or 1 µM) was assessed by Eurofins Panlabs (Supplementary Data 1). The parameter examined were: half-life (t1/2) in PBS (1 µM BnOCPA, 37 °C; pH 7.4; Assay #6060); t1/2 in human plasma (0.1 µM BnOCPA, 37 °C; pH 7.4; Assay #6087) and intrinsic clearance by human liver microsomes (0.1 µM BnOCPA, 0.1 mg/ml, 37 °C; Assay #607).

Half-life determination in PBS. At the end of the incubation at each of the time points (0, 1, 2, 3, 4 h), an equal volume of an organic mixture (acetone/toluene/methanol, 50/50 v/v) was added to the incubation mixture. Samples were analysed by HPLC-MS/MS and corresponding peak areas were recorded for each analyte. The ratio of precursor compound remaining after each time-point relative to the amount present at time 0, expressed as a percentage, is reported as chemical stability. The t1/2 was estimated from the slope of the linear initial range of the logarithmic curve of compound remaining (%) versus time, assuming first-order kinetics.

Half-life determination in human plasma. At the end of incubation at each of the time points (0, 0.5, 1, 1.5, 2 h), acetonic was added to the incubation mixture followed by centrifugation. Samples were analysed by HPLC-MS/MS and peak areas were recorded for each analyte. The area of precursor compound remaining after each of the time points relative to the amount remaining at time 0, expressed as a percentage, was calculated. Subsequently, the t1/2 was estimated from the slope of the initial linear range of the logarithmic curve of compound remaining (%) versus time, assuming first-order kinetics.

Intrinsic clearance by human liver microsomes. Metabolic stability, expressed as a percentage of the parent compound remaining, was calculated by comparing the peak area of the compound at the time-point (0, 15, 30, 45, 60 min) relative to that at time 0. The t1/2 was estimated from the slope of the initial linear range of the logarithmic curve of compound remaining (%) versus time, assuming first-order kinetics. The apparent intrinsic clearance (Clint, in µl/min/mg) was calculated according to the following Eq. (3):

\[
Cl_{int} = \frac{t_1/2}{(0.001\text{mg}\text{ protein/µL})} \quad (3)
\]

The behaviour of BnOCPA was compared to appropriate standards. Data is available in Supplemental Data File 1.

Molecular dynamics simulations

Ligand parameterisation. The CHARMM36111,117 CgFRP1118-120 force field combination was employed in all MD simulations performed. Initial topology and parameter files of BnOCPA, HOCPA, and PSB36 were obtained from the
Paramchem webserver\textsuperscript{118}. Higher penalties were associated with a few BnOCPA dihedral terms, which were therefore optimised at the HF/6-31 G* level of theory using both the high throughput molecular dynamics (HTMD)\textsuperscript{123} parameterise functionality and the visual molecular dynamics (VMD)\textsuperscript{122} Force Field Toolkit (ffTK)\textsuperscript{122}, after fragmentation of the molecule. Short MD simulations of BrnOCPA in water were performed to visually inspect the behaviour of the optimised rotatable bonds.

Systems preparation for fully dynamic docking of BnOCPA and HOCPA. Coordinates of the A1R in the active, adenosine- and G protein-bound state were retrieved from the Protein Data Bank\textsuperscript{124,125} database (PDB ID 6D9H\textsuperscript{137}). Intracellular loop 3 (ICL3) which is missing from PDB ID 6D9H was rebuilt using Modeller 9.19\textsuperscript{126,127}. The G protein, with the exception of the C-terminal helix (helix 5) of the protein alpha subunit (the key region responsible for the receptor TM6 active-like conformation) was removed from the system as in previous work\textsuperscript{128,129}. BnOCPA and HOCPA were placed in the extracellular bulk, in two different systems, at least 20 Å from the receptor vestibule. The resulting systems were prepared for simulations using in-house scripts able to exploit both python HTMD\textsuperscript{133,134} and Tool Command Language (TCL) scripts. Briefly, this multistep procedure performs the preliminary hydrogen atoms addition by means of the pdb2pqr\textsuperscript{130} and propka3\textsuperscript{131} software, considering a simulated pH of 7.0 (the proposed protonation of titratable side chains was checked by visual inspection). Receptors were then embedded in a square 80 Å × 80 Å 1-palmitoyl-2-oleyl-snglycero-3-phosphocholine (POPC) bilayer (previously built by using the VMD Membrane Builder plugin 1.1, Membrane Plugin, Version 1.1.; http://www.ks.uiuc.edu/Research/vmd/plugins/membrane/) through an insertion method\textsuperscript{132}, considering the A1R coordinates retrieved from the OPM database\textsuperscript{133} to gain the correct orientation within the membrane. Lipids overlapping the receptor transmembrane bundle were removed and TIP3P water molecules\textsuperscript{134} were added to the simulation box (membrane Plugin, Version 1.1.; http://www.ks.uiuc.edu/Research/vmd/plugins/solvate/). Finally, overall charge neutralization was performed constraining only the protein atoms. Lastly, positional constraints were applied only to the protein backbone alpha carbons for a further 5 ns.

### Dynamic docking of BnOCPA and HOCPA.

The supervised MD (SuMD) approach is an adaptive sampling method\textsuperscript{135} for simulating binding events in a timescale one or two orders of magnitudes faster than the corresponding classical (unsupervised) MD simulations\textsuperscript{136}. SuMD has been successfully applied to small molecules and peptides\textsuperscript{40-146}. In the present work, the distances between the centres of mass of the adenine scaffold of the A1R agonist and N254\textsuperscript{SS}, F171ECL2, T277\textsuperscript{42} and H278\textsuperscript{43} of the receptor were considered for the supervision during the MD simulations. The dynamic docking of BnOCPA was hindered by the ionic bridge formed between the E172ECL2 and K265ECL3 side chains. A metadynamics\textsuperscript{147-149} energetic bias was therefore introduced in order to facilitate the rupture of this ionic interaction, thus favoring the formation of a bound complex. More precisely, Gaussian terms (height $\mu$ kcal mol\textsuperscript{-1} and widths $\pm 0.1$ Å) were deposited every 1 ps along the distance between the E172ECL2 carboxyl carbon and the positively charged K265ECL3 nitrogen atom using PLUMED 2.3\textsuperscript{130}. A similar SuMD-metadynamics hybrid approach was previously employed to study binding/unbinding kinetics\textsuperscript{144} on the A1R subtype. For each replica (Table 1), when the ligands reached a bound pose (i.e. a distance between the adenine and the receptor residues centres of mass <3 Å), a classic (unsupervised and without energetic bias) MD simulation was performed for at least a further 100 ns.

### BrnOCPA bound state metadynamics.

We decided to perform a detailed analysis of the role played by the E172ECL2–K265ECL3 ionic interaction in the dynamic docking of BnOCPA. Three 250 ns long well-tempered\textsuperscript{135} metadynamics simulations were performed using the bound state obtained from a previous dynamic docking simulation, which resulted in binding mode A, as a starting point. The collective variables (CVs) considered were: i) the distance between the E172ECL2 carboxyl carbon and the positively charged K265ECL3 nitrogen atom and ii) the dihedral angle formed by the 4 atoms linking the cyclopentyl ring to the phenyl moiety (which was the most flexible ligand torsion during the previous SuMD simulations). Gaussian widths were set at 0.1 Å and 0.01 radians respectively, heights at 0.01 kcal mol\textsuperscript{-1}, and the deposition was performed every 1 ps (bias-factor 5). Although complete convergence was probably not reached, three replicas (Table 1) allowed sampling of three main energetic minima on the energy surface (Supplementary Fig. 8); these correspond to the representative binding poses shown in Fig. 3d to f. To test the hypothesis that BnOCPA and HOCPA may differently affect TM6 and/or TM7 mobility when bound to A1R (and to further sample the stability of each BrnOCPA binding mode), putative binding conformations A, B and C (Fig. 3) were superposed to the experimental A1R active state coordinates with the modelled ICL3. This should have removed any A1R structural artefacts, possibly introduced by metadynamics. As reference and control, two further systems were considered: i) the pseudo-apo A1R and ii) the selective A1R antagonist PSB36\textsuperscript{72} superposed in the same receptor active conformation (Table 1). The BnOCPA binding mode D was modelled from mode B by rotating the dihedral angle connecting the cyclopentyl ring and the N6 nitrogen atom in order to point the benzyl of the agonist toward the hydrophobic pocket underneath ECL3 (Fig. 3g) delimited by L253\textsuperscript{58}, T257\textsuperscript{52}, K265ECL3, T270\textsuperscript{35}, and L269\textsuperscript{34}. The G protein atoms were removed, and the resulting systems prepared for MD as reported above. A similar comparison was performed in a milestone study on the β2 adrenergic receptor\textsuperscript{76} which sought to describe the putative deactivation mechanism of the receptor.

### Table 1 Summary of the simulations performed.

| Ligand        | MD approach                          | # Replicas | Total simulated time |
|---------------|--------------------------------------|------------|----------------------|
| BnOCPA        | SuMD                                 | 6          | 1.9 μs               |
| BnOCPA        | SuMD-Metadynamics                    | 5          | 4.3 μs               |
| HOCPA         | SuMD                                 | 5          | 3.4 μs               |
| BnOCPA (bound state after dynamic docking) | Metadynamics                  | 3          | 0.75 μs              |
| BnOCPA(A)     | Classic MD                           | 6          | 9.0 μs               |
| BnOCPA(B)     | Classic MD                           | 6          | 9.0 μs               |
| BnOCPA(C)     | Classic MD                           | 3          | 3.0 μs               |
| BnOCPA(D)     | Classic MD                           | 6          | 9.0 μs               |
| HOCPA         | Classic MD                           | 4          | 8.0 μs               |
| PSB36         | Classic MD                           | 4          | 6.0 μs               |
| Apo A1        | Classic MD                           | 4          | 8.0 μs               |
| GuCt Goa (BnOCPA) | SuMD + Classic MD         | 10         | 0.36 μs ± 3.0 μs     |
| GuCt Goa (Gob) | SuMD + Classic MD                    | 10         | 0.33 μs ± 3.0 μs     |
| GuCt Goa (HOCPA) | SuMD + Classic MD                  | 10         | 0.29 μs ± 3.0 μs     |
| BnOCPA(D):Gob | Classic MD                           | 4          | 4.0 μs               |
| BnOCPA(B):Gob | Classic MD                           | 3          | 3.0 μs               |
| HOCPA:Gob     | Classic MD                           | 4          | 4.0 μs               |
| BnOCPA(D):Goa | Classic MD                           | 5          | 5.0 μs               |
| BnOCPA(B):Goa | Classic MD                           | 4          | 4.0 μs               |

*For SuMD and SuMD-metadynamics simulations the time is the sum of productive SuMD time windows. (A), (B), (C) and (D) indicate the respective BrnOCPA binding modes.
Received: 21 July 2020; Accepted: 23 June 2022; Published online: 18 July 2022

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Acknowledgements

We gratefully acknowledge the support of the University of Warwick (URSS Awards to S.H. and J.O.; Warwick Ventures Proof of Concept Fund awards to M.J.W. & B.G.F.), the Leverhulme Trust (RPG-2017-255, CAR and G.L. to fund K.B. and G.D.), the BBBSRC (BB/M010015/1, G.L. and BB/M01116X/1, Ph.D. studenthip to E.H.), the MRC (MR/J003964/1; I.W. and 2270402, iCASE PhD Studentship with NeuroSolutions to C.L.M.) and The Swiss National Science Foundation (PP00P2_123536 and PP00P2_146321, M.Lo). A.S. is supported by a European Scholarship from the Cambridge Trust, S.C. is funded by an AstraZeneca Ph.D. studentship and X.H. is funded by a China Scholarship Council Cambridge International Scholarship. RH is supported by an MRC Discovery Award (MC_PC_15070). C.A.R. is a Royal Society Industry Fellow. We would like to thank: Stephen Hill, Stephen Briddon, and Mark Soave (University of Nottingham) for gifing the Nluc-tagged adenosine receptor cell lines, the fluorescent antagonist AV039, and technical advice; Kathleen Caron and Duncan Mackie (University of North Carolina) for the β-arrestin1/2-YFP constructs, and Annette Gilchrist (Midwestern University) and Heidi Hamm (Vanderbilt University) for assistance with the Gao interfering peptide plasmids. We are grateful to Kevin Moffat and the Biochemistry students of the School of Life Sciences at the University of Warwick for access to their frog heart preparations; Nick Dale, Mark Wigglesworth, Jens Kleinjung for discussions and comments on draft manuscripts, and Arthur Christopoulos for a pre-publication copy of the adenosine A1R cryo-EM structure. In vivo studies on neuropathic pain were funded and undertaken by NeuroSolutions Ltd. Illustrative figures in Figs. 1bi, c; 2a–d, i; 3i, j; 5a; 6a; 7a, b were created with BioRender.com. Venn diagram in Fig. 2e was made at http://bioinformatics.psb.ugent.be/webtools/Venn/.

Author contributions

Experiments were designed by M.J.W., R.H., G.D., C.A.R., G.L., F.Y.Z., W.L., D.Sp., B.G.F., and were performed by M.J.W., E.H., R.H., K.B., I.W., S.C., A.S., H.F.W., D.Sa., X.H., W.L., C.L.M., E.D., C.H., S.H., J.O. Compounds were synthesised by M.L.e., B.P., M.L.o. Molecular dynamics and docking simulations were designed and carried out by G.D. and C.A.R. Work was originally conceived by M.J.W. and B.G.F. The manuscript was written by M.J.W., G.D., C.A.R., G.L., B.G.F., with valuable input from E.H., R.H., K.B., M.Lo., I.W., W.L. and D.Sp.

Competing interests

The University of Warwick has filed a patent application for uses of BnOCPA in which M.W. and B.G.F. are named as the inventors and M.Lo., R.H., G.L., D.Sp., C.A.R. and G.D. are named as contributors. F.Y.Z., H.F.W. and D.Sp. are employees and/or hold shares in NeuroSolutions, which holds a licence to this patent. The remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-31652-2.

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Peer review information Nature Communications thanks Kenneth Jacobson, Daniela Salvemini and the anonymous reviewer(s) for their contribution to the peer review of this work.

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