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Dual agonist occupancy of AT₁-R- α_{2C} -AR heterodimers results in atypical G_s-PKA signaling

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Author contributions

M.B. designed and performed most of the experiments, analyzed and interpreted data and helped to write the manuscript. S.G. and C.B. helped in the anisotropy experiments and performed β -arrestin and cAMP experiments, analyzed data and helped to write the manuscript. H.J.M. performed NE-release experiments in primary cultures of sympathetic neurons and analyzed the data. F.D. designed, performed and analyzed mouse microneurography experiments. C.D. helped in the binding studies, the design of most experiments and the interpretation of the data. J.J. helped with construction of the Venus and Luciferase fusion BRET probes, the interpretation of the data and the editing of the manuscript. S.M. performed anisotropy experiments and analyzed the data. V.P. and M.-H.S. established some plasmid constructs. S.J.S. and J.L.H. performed some binding studies and analyzed and interpreted data. A.P. assisted in data processing and analysis and with manuscript preparation. A.G. supervised the neuron experiments and wrote the manuscript. J.-M.S. supervised some aspects of the project, analyzed the data and wrote the manuscript. C.G. conceived and supervised the project, performed data analysis and wrote the manuscript.

Competing financial interests

The authors declare competing financial interests: details accompany the online version of the paper.

Additional information

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Abstract

Hypersecretion of norepinephrine (NE) and angiotensin II (AngII) is a hallmark of major prevalent cardiovascular diseases that contribute to cardiac pathophysiology and morbidity. Herein, we explore whether heterodimerization of presynaptic AngII AT₁ receptor (AT₁-R) and NE α_{2C}-adrenergic receptor (α_{2C}-AR) could underlie their functional cross-talk to control NE secretion. Multiple bioluminescence resonance energy transfer and protein complementation assays allowed us to accurately probe the structures and functions of the α_{2C}-AR–AT₁-R dimer promoted by ligand binding to individual protomers. We found that dual agonist occupancy resulted in a conformation of the heterodimer different from that induced by active individual protomers and triggered atypical G_s–cAMP–PKA signaling. This specific pharmacological signaling unit was identified *in vivo* to promote not only NE hypersecretion in sympathetic neurons but also sympathetic hyperactivity in mice. Thus, we uncovered a new process by which GPCR heterodimerization creates an original functional pharmacological entity and that could constitute a promising new target in cardiovascular therapeutics.

The pathogenesis of arterial hypertension (HT) and heart failure (HF) is multifactorial, but hyperactivity of both the sympathetic nervous system (SNS) and the renin-angiotensin-aldosterone system is fundamental in their development^{1,2}. Both systems are functionally interconnected as NE secretion through the SNS stimulates AngII production and vice versa. Consequently, HT and HF management is currently based on the use of molecules from similar pharmacological classes. However, the efficacy of drugs reducing renin-angiotensin-aldosterone activity, such as AngII AT₁-R antagonists, on morbidity and mortality remains uncertain. Moreover, although centrally acting drugs such as α₂-adrenergic agonists efficiently reduce SNS activity, they have never proven to effectively reduce morbidity and mortality³. Therefore, a better understanding of the molecular mechanisms regulating NE secretion could help to develop new efficient therapeutics.

NE secretion by SNS endings is controlled by a balance between inhibitory and stimulatory presynaptic G protein–coupled receptors (GPCRs). The α₂-adrenergic receptors (α_{2A}-AR and α_{2C}-AR), comprising the main NE inhibitory secretion system, have a prominent role in controlling sympathetic neural output, and their dysfunction constitutes a central mechanism for sympathetic hyperactivity in HT and HF^{4,5}. In contrast, AT₁-R facilitates NE secretion⁶.

Several *ex vivo* studies have highlighted the existence of functional crosstalk between the α_2 and AT₁ receptors in the modulation of NE secretion but led to inconsistent results^{7–9}. It has been suggested that α_2 -AT₁ cross-talk relies on intracellular communication involving G $\beta\gamma$ from G_i or G_o proteins coupled to the α_2 -AR⁹. However, it is now well established that GPCRs can exist both as monomeric and oligomeric entities and that GPCR dimerization, specifically hetero-dimerization, has impacts on different functions of each receptor protomer including traffic, ligand binding and signaling¹⁰. Recently, GPCR heterodimers were suggested to have a role in physiology and pathophysiology^{11–15}. Numerous GPCRs have been shown to dimerize, and α_2 -AR or AT₁-R are no exception^{15–18}. However, AT₁-R- α_2 -AR heterodimerization is not reported. In this context, we hypothesized that the cross-talk between AT₁-R and α_2 -AR for the regulation of NE release could rely on the existence of AT₁-R- α_2 -AR heterodimers. The fact that AT₁-R stimulates NE secretion and that α_{2C} -AR subtype does not influence NE secretion *per se* but acts as a regulator of α_{2A} -AR-promoted NE secretion⁴ prompted us to examine the heterodimerization of AT₁-R with α_{2C} -AR in the context of neurohumoral activation.

In this study, we used different biophysical and newly developed technical approaches, combined with *in vitro* and *in vivo* functional assays, to provide an exhaustive characterization of the structure and function of the α_{2C} -AR-AT₁-R heterodimer. We demonstrated that NE-AngII costimulation creates the α_{2C} -AR-AT₁-R heterodimer, a new pharmacological entity, that signals through a unique G_s-cAMP-PKA signaling pathway and is associated with both NE hypersecretion and sympathetic nerve hyperactivity *in vivo*. These results present the α_{2C} -AR-AT₁-R heterodimer as a promising potential new pharmacological target for the management of HT- and HF-associated sympathetic hyperactivity that will need to be explored in the future.

RESULTS

α_{2C} -AR and AT₁-R form constitutive heterodimers

The interaction between α_{2C} -AR and AT₁-R was assessed in human embryonic kidney (HEK293T) cells by bioluminescence resonance energy transfer (BRET). Saturating BRET signals between AT₁-R and α_{2C} -AR (Fig. 1a) highlighted constitutive and specific association between both receptors. The specificity of interaction was confirmed by weak BRET signals not linearly increasing with the amount of interacting probes when measured between AT₁-R-RLuc8 or α_{2C} -AR-RLuc8 and CD8-GFP2, an unrelated transmembrane protein (Fig. 1a). Subcellular localization of α_{2C} -AR-AT₁-R heterodimers was investigated using bimolecular fluorescence complementation (BiFC). Each receptor was fused to complementary nonfluorescent halves of a Venus fluorophore (V1 and V2) at its C terminus as previously described¹⁹, resulting in receptor fusion constructs preserving their cell surface trafficking (**Supplementary Results, Supplementary Fig. 1a**) and their cognate G-protein activation (**Supplementary Fig. 1b**). Hemagglutinin (HA)-AT₁-R-V1 coexpression with HA- α_{2C} -AR-V2 generated a functional fluorescent Venus (Fig. 1b) colocalizing with cell surface HA staining, indicating that the α_{2C} -AR-AT₁-R heterodimer was expressed at the plasma membrane. In contrast, no fluorescence was detected when coexpressing HA-AT₁-R-V1 or HA- α_{2C} -AR-V2 with CD8-V2 or CD8-V1, whereas Venus fluorescence was

observed at the cell surface and in intracellular compartments following coexpression of CD8-V1 and CD8-V2 (Fig. 1b). The expression of α_2C -AR-AT₁-R heterodimers using BiFC was also observed at the cell surface of sympathetic-like neurons from nerve growth factor (NGF)-differentiated PC12 neurites (**Supplementary Fig. 2**).

Distinct active conformations of the α_2C -AR-AT₁-R dimer

Few studies have reported how ligand binding affects protomer association in a dimer, and this has led to conflicting interpretations^{20,21}, most likely due to the use of poor-resolution RET techniques²¹. Also, to assess ligand modulation of the α_2C -AR-AT₁-R heterodimer, we used BRET² with high spectral resolution and sensitivity^{21,22}. The natural α_2C -AR agonists NE and epinephrine (E) decreased the BRET between HA- α_2C -AR-Rluc8 and HA-AT₁-R-GFP₂, whereas the AT₁-R-specific agonists AngII and SII increased it (Fig. 2a). NE- and AngII-dependent BRET modulation correlated with G-protein activation promoted by wild-type HA-AT₁-R or Myc- α_2C -AR when expressed alone (NE half-maximum effective concentration (EC₅₀) = 1.2 ± 0.4 nM; AngII EC₅₀ = 3.4 ± 2.2 nM (s.e.m.); Fig. 2a and **Supplementary Fig. 1b**). The specificity of ligand-promoted BRET changes was confirmed by pretreatment with 10 μ M of specific α_2C -AR (yohimbine) or AT₁-R (candesartan) antagonists, which completely blocked the NE-mediated decrease or AngII-mediated increase in BRET (Fig. 2b). The agonist-induced changes in BRET could indicate enhanced or decreased physical association or dissociation between α_2C -AR and AT₁-R but could also reflect conformational rearrangements within protein complexes as this measure relies on the distance and dipole orientation of the energy donor and acceptor^{21,23,24}. To distinguish between these two possibilities, we manipulated the distance and dipole orientation by exchanging the GFP₂ energy acceptor fused to AT₁-R for Venus and measured BRET1 between HA- α_2C -AR-Rluc8 and HA-AT₁-R-Venus. In this configuration, NE and E still decreased the BRET signal, whereas AngII but not SII promoted an increase in BRET (Fig. 2c). As the BRET changes are consistent with conformational changes within the dimer, it seems that α_2C -AR and AT₁-R agonists stabilize two different conformations of the HA- α_2C -AR-HA-AT₁-R heterodimer. Moreover, the AT₁-R agonists AngII and SII also induce different dimer conformations, in agreement with our previous demonstration that SII stabilizes a different AT₁-R entity to that stabilized by AngII²⁴. Further evidence for NE and AngII promoting different conformations of the HA- α_2C -AR-HA-AT₁-R heterodimer was provided by real-time BRET2 kinetics, which revealed a faster NE-induced decrease in BRET than the AngII-mediated increase in BRET (AngII $t_{1/2}$ = 517 ± 6 ms (s.e.m.) versus NE $t_{1/2}$ < 200 ms; Fig. 2d).

NE and AngII hypersecretion observed in some cardiac diseases results in high concentrations of both agonists at sympathetic nerve endings, where they can bind simultaneously on putative α_2C -AR-AT₁-R heterodimers. Thus, we tested whether the simultaneous presence of the natural agonists of each receptor protomer would further modify the conformation of the α_2C -AR-AT₁-R heterodimer. When measuring BRET2 between HA- α_2C -R-Rluc8 and HA-AT₁-R-GFP₂, NE and AngII respectively increased and decreased BRET, and costimulation with both agonists reduced the BRET to a level similar to that resulting from stimulation with NE alone (Fig. 2e). When the experiment was repeated following energy donor-acceptor exchange between the two receptors, NE- and

AngII-promoted BRET modulation remained unchanged, but costimulation had no effect (Fig. 2f). These experiments not only suggest that ligand-modulated BRET results from conformational changes in the α_2C -AR-AT₁-R heterodimer but also show that stimulation with AngII, NE and AngII plus NE stabilize three different conformational states of the heterodimer. This was further supported by fluorescence anisotropy measurements of complemented Venus fluorophore. In HEK cells coexpressing HA-AT₁-R-V1 and HA- α_2C -AR-V2, the mean Venus anisotropy was different in AngII-treated cells compared to that in cells treated with NE and NE plus AngII (**Supplementary Fig. 3**), indicating that AngII promoted a conformation of the AT₁-R- α_2C -AR dimer different than that stabilized by NE or AngII plus NE. Sequential addition of AngII and NE (**Supplementary Fig. 4a-d**) did not modify BRET modulation between AT₁-R and α_2C -AR (Fig. 2e,f) for each energy donor-acceptor combination. Thus, contrary to previous reports¹⁰, these results are consistent with a model in which the ligand occupancy of one protomer does not necessarily influence the binding properties of the other. In line with this assumption, neither the binding affinity nor the maximal binding capacity of AngII or NE were modified in the presence of the other agonist in HEK cells coexpressing α_2C -AR and AT₁-R (**Supplementary Table 1**), suggesting that the ligand binding pocket was not affected, as previously reported for allosteric communication between protomers²⁵.

Distinct heterodimer-effector complex conformations

We next explored the consequences of the different α_2C -AR-AT₁-R dimer conformations by analyzing the interactions of the heterodimer with G proteins and β -arrestin. To specifically isolate ligand effects arising from the heterodimer, we used complemented donor-acceptor resonance energy transfer (CODA-RET)²⁶ combining BiFC and BRET. Thus, HA- α_2C -AR and HA-AT₁-R were fused at their C termini to an N-terminal (L1) or C-terminal fragment (L2) of Rluc8 and to an N-terminal (V1) or C-terminal fragment (V2) of Venus, resulting in fusion receptors with preserved cell surface expression and function (**Supplementary Fig. 1**). Analysis of fluorescence or luciferase complementation for α_2C - α_2C , AT₁-AT₁ and α_2C -AT₁ receptors showed that all of the combinations demonstrated fluorescence or luminescence reconstitution (**Supplementary Fig. 5**), suggesting that α_2C -AR and AT₁-R have a similar propensity for forming homo- or heterodimers.

When HA-AT₁-R-V1 was coexpressed with HA-AT₁-R-V2 in the presence of Rluc- β -arrestin 2, real-time BRET1 measurements showed β -arrestin 2 recruitment to the AT₁-R-AT₁-R homodimer only in the presence of AngII (**Supplementary Fig. 6a**). Similarly, with α_2C - α_2C homodimers, no β -arrestin recruitment was measured in the presence of NE or AngII (**Supplementary Fig. 6b**), despite cell surface expression of the homodimer (**Supplementary Fig. 5**). These results agree with previous reports for β -arrestin 2 recruitment to AT₁-R24 but not to α_2C -AR18 (**Supplementary Fig. 7**). Notably, individual stimulation of the α_2C -AR-AT₁-R heterodimer with AngII or NE reproduced the same β -arrestin 2 recruitment profile as that obtained with the cognate receptor homodimers or monomers (Fig. 3a), suggesting that heterodimerization does not affect the functionality of each receptor protomer in the β -arrestin 2 pathway. However, costimulation with AngII and NE significantly ($P = 0.0002$) potentiated maximal AngII-induced β -arrestin 2 recruitment (AngII plus NE BRET_{max} = 121.9% $\pm$ 2.3; AngII BRET_{max} = 100.0% $\pm$ 2.0 (s.e.m.)). When

similar experiments were performed using β -arrestin 2 fused to the Rluc probe at its C-terminal (β -arrestin 2-Rluc), NE was still unable to recruit β -arrestin 2, whereas stimulation with AngII or AngII plus NE led to significant but similar maximal β -arrestin 2 recruitment (AngII plus NE $\text{BRET}_{\text{max}} = 86.1\% \pm 10.9$; AngII $\text{BRET}_{\text{max}} = 98.6\% \pm 14.4$ (s.e.m.); Fig. 3a). This suggests that different $\alpha_2\text{C}$ -AT₁ heterodimer conformations stabilized by AngII, NE or AngII plus NE translate into three distinct heterodimer- β -arrestin 2 conformational changes rather than modifications of β -arrestin 2 levels associated to the receptor.

We applied the same strategy to G-protein activity by measuring the BRET between complemented split luciferase receptor fusions and the G α -Venus subunit of the G $\alpha\beta\gamma$ heterotrimer, a BRET assay accurately sensing R-G complex conformations^{23,24}. Consistent with the cognate G-protein coupling of each receptor, AngII increased the BRET signal between the HA-AT₁-R-L1/HA-AT₁-R-L2 homodimer and G α_q -Venus or G α_{i1} -Venus but not G α_s -Venus (**Supplementary Fig. 8a**), whereas a significant ($P < 0.01$) BRET increase was detected in the presence of NE but only between the HA- $\alpha_2\text{C}$ -AR-L1/HA- $\alpha_2\text{C}$ -AR-L2 homodimer and G α_{i1} -Venus (**Supplementary Fig. 8b**). When the HA-AT₁-R-L1/HA- $\alpha_2\text{C}$ -AR-L2 heterodimer was coexpressed with the different G α -Venus isoforms, the R-G complex exhibited distinct conformations depending on the G α isoform and the nature of the ligand, as shown by the different BRET modulations (Fig. 3b). It is noteworthy that although NE was unable to promote $\alpha_2\text{C}$ -AR coupling to G α_q , it significantly ($P < 0.05$) decreased the BRET between the AT₁-R- $\alpha_2\text{C}$ -AR heterodimer and G α_q . Remarkably, although AT₁-R and $\alpha_2\text{C}$ -AR do not usually couple to G α_s , and as corroborated by the absence of conformational rearrangements between AT₁-R or $\alpha_2\text{C}$ -AR homodimer receptors and G α_s (**Supplementary Fig. 8a,b**), costimulation with AngII and NE significantly ($P < 0.05$) increased the BRET between the heterodimer and G α_s (Fig. 3b), suggesting an original G α_s engagement to the heterodimer. Swapping the luciferase splits between AT₁-R and $\alpha_2\text{C}$ -AR led to similar results for all G proteins except that heterodimer stimulation with AngII promoted a BRET increase instead of a decrease in the $\alpha_2\text{C}$ -AT₁-G α_q complex (Fig. 3b). This demonstrates that the AT₁-R- $\alpha_2\text{C}$ -AR-G α_q complex adopts different conformations in the presence of AngII, NE or AngII plus NE, in agreement with the notion that specific ligand combinations can stabilize different heterodimer conformations.

$\alpha_2\text{C}$ -AR-AT₁-R costimulation activates G α_s -cAMP-PKA signaling

We next questioned the functional consequences of the different conformations of complexes formed between the AT₁-R- $\alpha_2\text{C}$ -AR dimer and β -arrestin 2 or the G α subunit and stabilized by the three agonist combinations.

Given the central role of β -arrestin in GPCR internalization, we examined the internalization profile of the AT₁-R- $\alpha_2\text{C}$ -AR dimer in HEK 293T cells coexpressing β -arrestin 2-mCherry together with HA-AT₁-R-V1 and HA- $\alpha_2\text{C}$ -AR-V2. In agreement with the BRET assay (Fig. 3a), 120 min NE stimulation did not promote β -arrestin 2 recruitment (Fig. 4 and **Supplementary Fig. 9**), whereas internalization of the Venus dimer could be observed. In contrast, cell treatments with 10 μM AngII or AngII plus NE for 10 min recruited β -arrestin 2 to the AT₁-R- $\alpha_2\text{C}$ -AR dimer. However, after 30-min treatments, stimulation with AngII plus NE sustained β -arrestin 2 colocalization with the heterodimer in internalized vesicles,

whereas colocalization markedly decreased with AngII (Fig. 4 and **Supplementary Fig. 9**) and persisted over 120 min stimulation. These results indicate that the three heterodimer conformations stabilized by the three ligand combinations traffic via distinct intracellular routes, suggesting diverse signaling outcomes.

We next explored the G-protein activation pattern associated with the heterodimer. Indeed, α_{2C} -AR and AT₁-R coexpression in HEK293T cells will lead to the formation of monomer, dimers and higher-order oligomers, making it impossible to detect only heterodimer activity. To bypass this problem, we designed an assay based on the ligand-forcing dimerization of FKBP12 rapamycin-binding domain (FRB) (DmrC) and FK506 binding protein (FKBP) (DmrA), which has previously been used to explore protein dimerization^{27–29}. Thus, we engineered a chimeric α_{2C} -AR fused to DmrA at its C terminus (Myc- α_{2C} -AR–DmrA), whereas AT₁-R was fused to DmrC (HA–AT₁-R–DmrC). These fusions neither altered cell surface trafficking nor the receptor activity (**Supplementary Fig. 10**). We directly evaluated the activation of G α_{i1} , G α_q and G α_s in living cells using a BRET2 probe, which measured the increased distance between the G α helical domain (G α -Rluc8) and the G γ_2 N terminus (GFP10-G γ_2) during the GDP-GTP exchange and sensed a decrease in the BRET signal following receptor activation^{23,24}. Myc- α_{2C} -AR–DmrA and HA–AT₁-R–DmrC were coexpressed in HEK293T cells with the different G α BRET probes in the presence of NE, AngII or both. No G-protein activation was detected following 1 min of ligand stimulation in the absence of fusion receptor expression (**Supplementary Fig. 11**). In the presence of the G α_{i1} BRET probe, NE strongly decreased the BRET signal in the absence of the A-C dimerizing agent, indicating potent G α_{i1} activation consistent with action through the G $_i$ -coupled α_{2C} -AR (Fig. 5a). Further addition of AngII did not modify the G α_{i1} activation profile, whereas AngII alone activated the G α_{i1} , but to a much lower extent (Fig. 5a), in agreement with lower efficiency of AngII on AT₁-R-mediated G $_i$ versus G $_q$ coupling²⁴. Notably, for similar receptor expression, forced dimerization of α_{2C} -AR–AT₁-R with the A-C dimerizer significantly ($P < 0.05$) reduced G α_{i1} activation by stimulation with NE or NE plus AngII, whereas the AngII response was unchanged (Fig. 5a). This result highly suggests that in the absence of the dimerizer, G α_{i1} activation most probably arises from a mixed population of active monomeric, homodimeric and heterodimeric α_{2C} and AT₁ receptors that the dimerizer disrupts to favor the heterodimeric population, resulting in a lower overall level of G α_{i1} activation. In contrast, all of the ligand combinations activated G α_q , and no difference was observed when α_{2C} -AR–AT₁-R dimerization was forced (Fig. 5a). NE-induced G α_q activation was unexpected because α_{2C} -AR is thought to exclusively couple to G $_i$ and G $_o$ proteins²⁴, and NE is unable to bind AT₁-R. This could indicate that NE-mediated G α_q activation is triggered by the spontaneous formation of the heterodimer with expression level sufficient for G α_q activation as supplementation with the A-C dimerizer did not change the activation profile. This hypothesis agrees with our earlier results showing that NE induced conformational changes of the complex formed between α_{2C} -AR–L1/AT₁-R–L2 and Venus-G α_q (Fig. 3b). Finally, we found that only stimulation with NE plus AngII promoted G α_s activation in forced α_{2C} -AR–AT₁-R heterodimer conditions (Fig. 5a). This correlates with conformational rearrangements observed within the α_{2C} -AR–AT₁-R–G α_s complex (Fig. 3b) and demonstrates that rearrangement stabilized by dual occupancy of the heterodimer-G α_s complex by AngII and NE efficiently translated

into G_s activation. The lack of detectable G_s activation in the absence of the dimerizing agent could argue for production of a peculiar α_{2C} -AR-AT₁-R heterodimer entity coupled to G_s that did not spontaneously form by itself. However, BRET experiments showed that free mobile α_{2C} and AT₁ receptors can constitutively interact (Fig. 1b) and adopt a specific conformation alone (Fig. 2e,f) or in complex with G_s (Fig. 3b) in the presence of both AngII and NE. This most likely supports a model in which dual AngII and NE binding promotes G_s coupling to the α_{2C} -AR-AT₁-R heterodimer in the absence of the dimerizer, but the G_s BRET probe is not sensitive enough for detection.

Validation of G_s activation by dual occupancy of the α_{2C} -AR-AT₁-R heterodimer by AngII and NE was determined at the level of the cAMP-dependent protein kinase (PKA) effector. HEK293T cells were cotransfected with the BRET-based PKA biosensor, which measures the separation between RLuc8-tagged regulatory subunits and GFP2-tagged catalytic subunits of PKA as a reflection of activation²⁴. In the absence of fusion receptor expression, NE significantly ($P < 0.001$) activated the PKA biosensor, and activation was completely blocked by propranolol (**Supplementary Fig. 12a**), suggesting the presence of an endogenous G_s -coupled β -adrenergic receptor. Although AngII stimulation alone did not promote any PKA response (**Supplementary Fig. 12b**), the same results were obtained in the presence of both NE and AngII (**Supplementary Fig. 12c**). That endogenous β -adrenergic receptor activated PKA was quite puzzling as no G_s activation could be measured after 1 min of NE stimulation in the absence of transient receptor expression (**Supplementary Fig. 11c**). However, when activation time was extended to 5 min, treatment with NE or NE plus AngII induced G_s activation, consistent with PKA activation in similar conditions (**Supplementary Fig. 11c**). In this context, the ability of the heterodimer to activate PKA was studied in the presence of propranolol when using NE. When the PKA biosensor was coexpressed with Myc- α_{2C} -AR-DmrA and HA-AT₁-R-DmrC, no PKA activation was found downstream of NE or AngII treatment, regardless of the presence of a dimerizing agent (Fig. 5b). Significant ($P < 0.05$) PKA activation was measured only in the presence of both NE and AngII with forced α_{2C} -AR-AT₁-R dimerization (Fig. 5b). This supports the idea that G_s is specifically activated downstream of the α_{2C} -AR-AT₁-R heterodimer.

To confirm the atypical G_s activation stimulated by the heterodimer, we directly quantified cAMP production in HEK293T cells coexpressing α_{2C} -AR and AT₁-R. Although no cAMP inhibition or production was detected in the absence of receptor expression (**Supplementary Fig. 13a**), NE inhibited cAMP production, consistent with activation of G_i - α_{2C} -AR (**Supplementary Fig. 13b**). More unexpectedly, no cAMP production was detected in the presence of NE plus AngII, as expected on the basis of G_s -PKA biosensor activation (Fig. 5a,b). Because the cAMP production could result from the concomitant activation of both activator and inhibitory signals whose detection is highly dependent on the activation level of each pathway, we repeated the experiments in the presence of pertussis toxin (PTX) to inhibit the G_i -cAMP pathway. The inhibition of NE-mediated cAMP production was totally prevented by PTX pretreatment, but, remarkably, AngII promoted significant ($P < 0.01$) cAMP production, which was significantly ($P < 0.001$) potentiated by NE (**Supplementary Fig. 13b**). The potentiating effect of stimulation with AngII plus NE on cAMP production

was reinforced by fluorescence resonance energy transfer (FRET)-based imaging of cAMP levels in living sympathetic-like neurons from NGF-differentiated PC12 cells coexpressing α_{2C} -AR, AT₁-R and a cAMP FRET biosensor and cocultured with H9C2 cardiomyoblasts (to establish neurocardiac synapses); in these neurons, stimulation with AngII plus NE promoted a decrease of the FRET signal, indicating cAMP production that significantly differed from FRET signals obtained with individual treatments with NE ($P < 0.001$) or AngII ($P < 0.01$) (**Supplementary Fig. 14**).

AngII plus NE promotes PKA-dependent SNS hyperactivity

Given the G_s coupling of the α_{2C} -AR–AT₁-R heterodimer with dual NE plus AngII occupancy and the implication of both receptors in NE secretion, we wondered whether a similar pharmacological unit could affect NE secretion. As expected, NE or AngII stimulation decreased or increased NE release in primary cultures of sympathetic neurons, respectively (Fig. 6). However, NE potentiated AngII-induced NE secretion (Fig. 6), which was completely prevented by blocking the G_s -PKA with Rp-8-cAMPS, a selective PKA inhibitor (Fig. 6a). Notably, pretreatment with candesartan selectively inhibited AngII-mediated NE release but had no effect on the potentiation of NE release induced by costimulation with NE and AngII (Fig. 6a). This is in agreement with results showing that candesartan or yohimbine, alone or in combination, are unable to block G_s stimulation promoted by treatment with NE plus AngII on the α_{2C} -AR–AT₁-R dimer (**Supplementary Fig. 15**) and thus suggests that an endogenous α_{2C} -AR–AT₁-R dimer could be involved in NE secretion in sympathetic neurons.

We next assessed the *in vivo* relevance of costimulation with NE and AngII on the firing rate of sympathetic nerves, which correlates with NE release³⁰. We measured renal sympathetic nerve activity (RSNA) in mice following intravenous injections of AngII, NE or both in the presence of hexamethonium to prevent baroreflex-induced changes in RSNA subsequent to changes in arterial pressure³¹. Hexamethonium significantly lowered mean arterial pressure (MAP; 60 ± 8 Hg versus 88 ± 6 mm (s.e.m.) Hg in controls; $P < 0.001$), did not modify heart rate (553 ± 19 beats min⁻¹ versus 537 ± 19 (s.e.m.) beats min⁻¹ in untreated controls) and decreased RSNA (14.4 ± 5.0 bursts s⁻¹ versus 35.7 ± 7.7 (s.e.m.) bursts s⁻¹ in controls; $P < 0.001$). RSNA significantly ($P < 0.001$) increased following AngII administration (Fig. 6b and **Supplementary Table 2**). In contrast, NE administration tended to decrease RSNA (Fig. 6b and **Supplementary Table 2**). Notably, administration of both AngII and NE markedly increased RSNA by $125.7 \pm 17.8\%$ versus $65.9 \pm 6.3\%$ (s.e.m.) with AngII alone (Fig. 6b and **Supplementary Table 2**). This RSNA hyperactivity induced by costimulation with AngII and NE was not related to differences in drug-induced vasoconstriction (**Supplementary Table 2**). Blocking the G_s -PKA pathway using the H89 PKA inhibitor completely prevented the RSNA potentiation induced by AngII plus NE (Fig. 6b and **Supplementary Table 2**), indicating involvement of a G_s -coupled receptor and correlating with the original G_s coupling of the α_{2C} -AR–AT₁-R dimer we identified *in vitro*.

DISCUSSION

Numerous studies show that GPCR dimerization modulates the pharmacological properties of individual receptor protomers through alterations in ligand binding or signaling properties³². In most cases, signaling modulation of the dimer refers to increased or decreased activation of the G protein coupled to each protomer, although in several studies, a G protein–coupling switch between promoters was reported³². Only one study suggested the creation of a new form of G_q protein coupling in the D1-D2 dopamine receptor heterodimer³³. However, given the existence of multiple receptor signaling units (monomers, homodimers and heterodimers) with variable cellular expression levels in all of these studies, it is difficult to infer that the effects result from altered coupling of the heterodimer *per se* or from downstream signaling cross-talk.

In this context, a major finding of this study is that, using a strategy of ligand-forcing GPCR dimerization, we provide clear evidence that dual occupancy of the α_2C -AT₁ receptor heterodimer by two natural selective agonists generates a new G_s coupling that arises from the heterodimer only. This new active pharmacological entity, the AngII- and NE-occupied α_2C -AR–AT₁-R heterodimer, was confirmed by the conformational rearrangement of the receptor-receptor dimeric interface observed by BRET to the receptor heterodimer–effector complexes observed by CODA-RET. Although all homodimeric, heterodimeric and monomeric receptor species are likely to coexist simultaneously when two different receptors are coexpressed^{34–37}, our strategy allowed us to shift this equilibrium toward the heterodimeric state. When compared to the bicomplementation approach, which provides a static snapshot of the receptor dimer immediately after its biosynthesis, FRB-FKBP–driven dimerization only occurs following addition of the dimerizing agent once all receptor species have already been expressed, thereby offering the possibility of modulating the equilibrium of different receptor species. Accordingly, we found changes in G-protein coupling following addition of the dimerizing agent, strongly supporting the notion that different receptor populations do coexist and that the net signaling output following the coexpression of different receptors most likely reflects the average signaling arising from these different receptor populations. This notion is further reinforced by the observation that cAMP production, which is generally associated with G_s coupling and arises from costimulation with AngII and NE of the α_2C -AT₁ dimer, could not be detected without inactivating G_i proteins (**Supplementary Fig. 13b**), indicating activation of dual G_s - G_i signals. It thus follows that a specific GPCR dimer function can be easily missed.

The exclusive G_s -cAMP-PKA signaling identified for the NE- and AngII-occupied α_2C -AR–AT₁-R heterodimer could be involved in the regulation of arterial blood pressure, but it could also be particularly relevant to the pathophysiological context of HT and HF with sustained production of both agonists. Indeed, the unusual and unexpected sympathetic hyperactivity and NE hypersecretion in sympathetic neurons measured following coadministration of AngII and NE, which are both dependent on a PKA pathway, strongly supports a specific molecular mechanism unrelated to the individual activities of presynaptic α_2C -AR or AT₁-R. Although this study is far from proving this, the implication of the α_2C -AR–AT₁-R heterodimer in the regulation of sympathetic nerve activity deserves further investigations. Permanent costimulation of the α_2C -AR–AT₁-R heterodimer, similar to

conditions in HT and HF, could promote continuous G_s -PKA-dependent NE release by enhancing the exocytotic machinery, as previously described³⁸. In this context, the α_{2C} -AR-AT₁-R heterodimer could constitute a potential new target for the treatment of NE hypersecretion-associated diseases. Specific pharmacological blocking of the α_{2C} -AR-AT₁-R heterodimer using bivalent ligands targeting the two protomers of the dimer or monovalent ligands specifically targeting the heterodimer, as previously shown³⁹, could be particularly relevant.

A second major finding of this study is that the different ligand occupancy states of the α_{2C} -AT₁ receptor heterodimer stabilize distinct conformations of the dimer, which then translate into specific signaling outputs. Whereas NE bound to the α_{2C} -AR protomer or AngII bound to the AT₁-R protomer stabilized different conformations of the dimer, the concomitant presence of both agonists promoted a third conformation triggering a new form of G_s signaling. The activation of G_s by the α_{2C} -AR-AT₁-R heterodimer we describe herein differs from the asymmetric model, in which numerous class A or C GPCRs are proposed to reach their active dimeric state^{40–46} with one protomer controlling the activation process of the dimer and the other fine-tuning it. Contrarily, our data highlight a new model whereby the conformational change of the two protomers act in concert to create a unique structural conformation of the heterodimer, which translates into a specific G-protein response. Until now, the functional asymmetric modes described have relied essentially on receptor homodimer models. In the future, it will be interesting to see whether the new complementary mode demonstrated here is specific to the α_{2C} -AR-AT₁-R dimer or is a general feature of heterodimers, thus giving rise to a new receptor functional entity.

In summary, our finding that a HT- and HF-like neurohumoral active state of the AT₁-R- α_{2C} -AR heterodimer provides a new structurally and functionally unique pharmacological entity indicates that, in the future, GPCR heterodimer function itself should be screened without *a priori* knowledge of the function of the individual protomers. Thus, a larger set of effectors should be included in the evaluation. The examination of structural rearrangements within GPCR dimers and their associated signaling cascades based on the ligand-forcing FRB-FKBP dimerization approach could be applied generally to identify new pharmacological signatures. In this study, we have found a novel RSNA activity under HT- and HF-like neurohumoral conditions. It remains to be fully confirmed whether the AT₁-R- α_{2C} -AR heterodimer is specifically involved and thus contributes to HT and HF pathophysiology, yet results presented here provide evidence that the HT- and HF-like neuro-humoral active state of AT₁-R- α_{2C} -AR could constitute a promising target for future HT and HF treatment.

ONLINE METHODS

Live animals

Two-month-old male or 1- to 3-d-old C57BL/6 mice were used in accordance with the US National Institutes of Health (NIH) guidelines. *In vivo* experiments were approved by the Comité d'éthique d'expérimentation animale 122.

Materials

Angiotensin II (AngII), L-(–)-norepinephrine (NE), (–)-epinephrine (E), 3-isobutyl-1-methylxanthine (IBMX) and yohimbine were purchased from Sigma-Aldrich. Candesartan was kindly provided by J.L. Hansen. Coelenterazine 400a and h were purchased from Biotum. Levo-[7-³H]norepinephrine and [¹²⁵I]Tyr4-angiotensin II were purchased from Perkin-Elmer.

cDNA expression vectors

Plasmids encoding rat HA-AT₁-R and HA-AT₁-R-GFP2 were a generous gift from J.L. Hansen. All receptors were fused in-frame at their C terminus to either Rluc8, GFP2 or Rluc8 or Venus splits. Human HA-α_{2C}-AR-GFP2 was obtained by inserting the receptor coding sequence lacking its stop codon into the humanized pGFP2-N1 vector (Perkin Elmer, Life Science). HA-α_{2C}-AR-Rluc8 was obtained from pGFP2-MCS-Rluc8 (customized) vector by replacing the GFP2-MCS sequence with the receptor coding sequence of α_{2C}-AR. HA-AT₁-R-V1, HA-AT₁-R-V2, HA-α_{2C}-AR-V1 and HA-α_{2C}-AR-V2 as well as HA-AT₁-R-L1, HA-AT₁-R-L2, HA-α_{2C}-AR-L1 and HA-α_{2C}-AR-L2 were constructed from HA-AT₁-R-GFP2 and HA-α_{2C}-AR-GFP2 by replacing GFP2 with the coding sequence of the different Venus fragments (from CD8-V1 and CD8-V2 (ref. 19), kindly provided by J.A. Javitch) or Rluc8 fragments (from D2-L1 and D2-L2, kindly provided by J.A. Javitch). Plasmids encoding HA-AT₁-R-Venus, α_{i1}-91Rluc8, α_q-97Rluc8, α_s-113Rluc8, GFP10-Gγ₂, Gβ₁, Rluc-βarr₂, RIα-Rluc8 and GFP2-Cα were previously described²⁴. α_{i1}-Venus and α_s-Venus were a generous gift from J.A. Javitch. α_q-Venus was obtained from α_q-97Rluc8 by replacing Rluc8 with the coding sequence of Venus. HA-AT₁-R-DmrC and Myc-α_{2C}-AR-DmrA were generated from HA-AT₁-R-V1 and Myc-α_{2C}-AR-V1, respectively, by replacing V1 with the encoding sequence of FRB (DmrC) or FKBP12 (DmrA), respectively (obtained by PCR from pHet-1 and pHet-Nuc1 vectors using iDimerize Inducible Heterodimer System, Clontech). All generated constructs were confirmed by DNA sequencing. Rluc-Barr2 and β-arrestin 2-cherry vectors were kindly provided by S. Marullo and were already described^{47,48}. Pm-Epac2-camps was a generous gift from V. Nikolaev.

Cell culture and transfection

Human embryonic kidney 293 cells (HEK293T) cells were cultured in DMEM Glutamax supplemented with 10% (v/v) FBS and 100 U/ml penicillin/streptomycin at 37 °C in a humidified atmosphere at 5% CO₂. Transient transfections were performed 24 h after cell seeding using polyethylenimine (PEI, Polysciences Inc.) or Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Mouse superior cervical ganglion neurons were cultured as previously described⁴⁹. Briefly, superior cervical ganglions were dissected from 1- to 3-d-old C57BL/6J mice and dissociated for 20 min in 3 mg/ml collagenase with 0.5 mg/ml trypsin, followed by washing (10% FBS in DMEM). Cells were plated and incubated with 3% FBS in UltraCulture medium containing NGF for 2 h at 37 °C to allow fibroblasts to adhere. The supernatant medium (containing SCG cells) was centrifuged, resuspended in medium supplemented with FBS and NGF and cultured on poly-D-lysine- and collagen-coated glass-bottom plates for 14 d before experiments (treating with 1 μM 5-fluoro-5-deoxyuridine).

Immunofluorescence microscopy

HEK293T cells were seeded and transfected using Lipofectamine 2000 in six-well plates containing glass cover slips precoated with poly-L-lysine (1 mg/ml; Sigma). Forty-eight hours after transfection, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, blocked in PBS-0.2% BSA and incubated for 2 h with a mouse anti-HA antibody (16B12, Covance) or a mouse anti-Myc tag (9E10, Santa Cruz). Immunoreactivity was revealed using an Oregon Green- or Texas Red-conjugated secondary goat anti-mouse antibody (Molecular Probes). Cell membranes and nuclei were respectively stained with Texas Red-conjugated wheat germ agglutinin (WGA; Molecular Probes) and DAPI (Sigma). Fluorescent images were acquired with an Axio Observer Z.1m inverted microscope associated with ApoTome (Carl Zeiss Jena, Germany) and a 63× objective lens (Plan-Apochromat, 1.4 Oil DIC). Colocalization was assessed by overlay using Zeiss microscopy software AxioVision Rel. 4.8. Confocal imaging was performed using a LSM 780 microscope, piloted by manufacturer software, with a 63× Plan-Neofluar objective (Carl Zeiss), and the colocalization parameter (correlation coefficient) was calculated for each cell using ZEN software.

FRET imaging

PC12 cells were electroporated with the Epac2-pm cAMP FRET sensor together with HA-AT₁-AR and Myc- α_2C -AR according the Neon Transfection kit (Invitrogen) recommendation. Twenty four hours after transfection, cells were placed in a differentiation medium (RPMI 1% HS, 70 ng/ml NGF, Pen/Strep), and H9C2 cells were added into the culture 8h after. Cells were imaged 4 to 6 d after differentiation using a confocal LSM 780 microscope piloted by ZEN software, with a 63× Plan-Neofluar objective (Carl Zeiss). Contact between PC12 and H9C2 was imaged with the following parameters: electronic zoom 2, confocal pinhole 1.5 Airy units no frame averaging, 6 slices Z-stack. CFP and EYFP were excited using 452-nm and 51-nm laser lines (from the 488 nm Argon laser), and emitted light was collected at 475–510 nm and 520–555 nm (CFP and EYFP or FRET imaging, respectively). A first image with bright field, CFP and YFP excitation was acquired to choose the cell; then, to study agonist-induced changes in FRET, we acquired the data in the CFP channel and in the FRET channel only with CFP excitation. Ten images before stimulation and 20 images after stimulation were acquired, with 10-s intervals. At least 25 cells were analyzed per condition over two experiments. After background subtraction (bleed through correction), net FRET was calculated as the ratio of the FRET channel over the CFP channel using ImageJ. The difference in net FRET between stimulated and unstimulated for 1–5 ROIs (regions of interest) was then expressed as a percentage and plotted over time.

BRET

Receptors, G-protein subunits or β -arrestin-encoding vectors were transiently transfected into HEK293T cells. Forty-eight hours after transfection, cells were washed with PBS, detached in PBS/5 mM EDTA and resuspended in PBS/0.1% (w/v) glucose at room temperature. Cells were then distributed (80 μ g of protein per well) into a 96-well microplate (Wallac, PerkinElmer Life and Analytical Sciences) and incubated in the presence of the

different ligands for 1 min or 5 min, except for the kinetics study (Fig. 2d) and PKA activation (Fig. 5b and **Supplementary Fig. 10**). BRET2 between native or complemented RLuc8 and GFP10 or complemented GFP2 was measured after the addition of the RLuc substrate coelenterazine 400a (5 μ M, Interchim). For BRET1 experiments, BRET between RLuc and native or reconstituted Venus was measured following the addition of the RLuc substrate coelenterazine h (5 μ M, Interchim). Where indicated in Figure 2d, BRET signal values were corrected by subtracting the background signal detected when an RLuc8/RLuc-tagged construct was expressed alone from the BRET signal detected in cells coexpressing both RLuc8/RLuc-tagged and GFP2/Venus constructs (Net BRET).

For titration experiments (Fig. 1a), the expression level of each tagged protein was determined by direct measurement of total fluorescence and luminescence in aliquots of the transfected cells. Total fluorescence was first measured with an excitation filter at 400 nm and an emission filter at 510 nm for GFP2. Then, the same sample was incubated for 8 min with 5 μ M coelenterazine h, and the total luminescence was measured using a modified Infinite F500 (Tecan Group Ltd).

For kinetics analysis of α_2C -AR-AT₁-R interactions (Fig. 2d), coelenterazine 400a was added before injection of the ligand. Readings were then collected at 200-ms intervals for 12 s. Ligands were injected 2 s after the beginning of the reading to allow baseline recording followed by real-time recording of the BRET changes. Net BRET signals were determined for each time point by calculating the ratio of the light emitted by GFP10 over that emitted by RLuc8.

BRET readings were collected using a modified Infinite F500 (Tecan Group Ltd). The BRET signal was calculated by the ratio of GFP10/GFP2 (510–540 nm) to RLuc/RLuc8 (370–450 nm) for BRET2 readings or Venus (520–570 nm) to RLuc/RLuc8 (370–480 nm) for BRET1 readings.

Anisotropy measurements

Anisotropy measurements were performed on a LSM 710 confocal microscope (Zeiss, Germany) implemented for anisotropy imaging. Two consecutive images were recorded, corresponding to the orientation of polarizers in the emission path of the microscope set first to parallel and then to perpendicular, with respect to the excitation polarization. Images were recorded through a 10 $\times$ /0.30 objective to prevent mixing of different polarization components. Venus dye was excited with a 488-nm argon laser line, and fluorescence emission was recorded from 500 nm to 600 nm. Anisotropy images were calculated from raw data with ImageJ (NIH) using equation (1) and a custom-built macro. G-factor was calculated with equation (2) from the measurement of an isotropic solution of fluorescein.

$$\langle r \rangle = \frac{I_{\parallel} - G \cdot I_{\perp}}{I_{\parallel} + 2 \cdot G \cdot I_{\perp}} \quad (1)$$

$$G = \frac{I_{\parallel}}{I_{\perp}} \quad (2)$$

Histograms of frequency versus anisotropy values were calculated for each individual anisotropy image and fitted with a Gaussian model to accurately estimate the mean anisotropy value.

Radioligand binding assay

For AT₁-R, whole-cell radioligand binding studies were performed as previously described⁵⁰. Competition studies were done with HEK293T cells transiently transfected with either 1 µg AT₁-R-Rluc8 or 1 µg AT₁-R-Rluc8 plus 6 µg HA-α_{2C}-AR-GFP2, in the presence of 10⁻¹⁰ M [¹²⁵I]Tyr4 AngII ([¹²⁵I]AngII, Perkin-Elmer) and increasing concentrations (from 10⁻¹² M to 10⁻⁶ M) of unlabeled AngII in the presence or absence of 10 µM NE.

For α_{2C}-AR, competition experiments were done as previously described⁵¹ on crude membrane from HEK293T cells transiently transfected with either 10 µg HA-α_{2C}-AR-Rluc8 or 10 µg HA-α_{2C}-AR-Rluc8 plus 10 µg HA-AT₁-R-GFP2, using 10⁻⁸ M [³H](-)norepinephrine ([³H]NE, Perkin-Elmer) as a tracer and increasing concentrations of unlabeled NE (from 10⁻¹² to 10⁻⁶ M) as a competitor agent, in the presence or absence of 10 µM AngII.

Each experiment was realized in triplicate, and competition curves were fitted using GraphPad Prism (version 5.04, GraphPad Software, USA) using a one-site competition model, followed by pK_i (-log K_i) calculation.

cAMP quantification

Quantification of cAMP levels was performed using the homogeneous time-resolved fluorescence (HTRF) assay (femto cAMP kit (Cisbio, Bedford, USA), based on a competitive immunoassay using the Lumi4-TbTM cryptate-labeled antibodies anti-cAMP and d2-labeled cAMP). For that purpose, HEK293 cells were transiently cotransfected with HA-AT₁R and Myc-α_{2C}R using X-tremeGENE 9 DNA transfection reagent (Roche). Twenty-four hours after transfection, cells were pretreated overnight with PTX (100 ng/ml) or vehicle. The day of the experiment, cells were washed and resuspended in PBS/5 mM glucose/2 mM IBMX. Then, 35,000 cells/well (384 wells per plate) were plated and stimulated for 1 h at room temperature with 10 µM NE, AngII or NE plus AngII in the presence of 10 µM propranolol in a final volume of 10 µl. Cells were then lysed using 5 µl of lysis buffer containing d2-labeled cAMP and 5 µl of Lumi4-TbTM cryptate-labeled anti-cAMP. The signal was measured after 1 h room temperature incubation using a modified Infinite F500 (Tecan Group Ltd). The RET signal was calculated by the ratio of d2-cAMP/Lumi4-TbTM (665 nm/620 nm), the specific signal being inversely proportional to the concentration of cAMP in the sample. For each experiment, a calibration curve was established with cAMP standards allowing the quantification of cAMP levels by regression.

Culture of mouse superior cervical ganglion neurons

Mouse superior cervical ganglion neurons were cultured as previously described⁴⁹. Briefly, superior cervical ganglions were dissected from 1- to 3-d-old C57BL/6J mice and dissociated for 20 min in 3 mg/ml collagenase and 0.5 mg/ml trypsin, followed by washing (10% FBS in DMEM). Cells were plated and incubated with 3% FBS in UltraCulture medium containing NGF for 2 h at 37 °C to allow fibroblasts to adhere. Supernatant medium (containing SCG cells) was centrifuged, resuspended in medium supplemented with FBS and NGF and cultured on poly-D-lysine- and collagen-coated glass-bottom plates for 14 d before experiments (treating with 1 μ M 5-fluoro-5-deoxyuridine).

Determination of [³H]noradrenaline release

Neurons cultures were washed, incubated in culture medium with 0.2 μ M [³H]NE (2 h) and rinsed with fresh culture medium. Subsequently, cultures were washed (1 h) in a buffer containing NaCl (120 mM), KCl (6.0 mM), CaCl₂ (2.0 mM), MgCl₂ (2.0 mM), glucose (10 mM), HEPES (10 mM), ascorbic acid (0.50 mM) and bovine serum albumin (1%) and adjusted to pH 7.4 with NaOH. Cultures were treated with 10 μ M propranolol and 10 μ M cocaine (10 min) in above buffer. Inhibitors (candesartan or Rp-cAMP) or vehicle were added during this preincubation period. Cultures were treated with drugs in above buffer (10 min (37 °C), including inhibitors) and tritium content was measured by scintillation counting. Cultures were extracted using 0.1% SDS, and remaining radioactivity was evaluated by scintillation counting.

In vivo measurement of RSNA

Experiments were performed on adult C57BL/6J (12–17 weeks old, 25–35 g) male mice housed in a temperature-controlled room with a 12 h/12 h light/dark cycle with free access to standard laboratory chow and tap water.

Mice were anesthetized with isoflurane gas administration (induction: 3%, maintenance: 1.5%). Body temperature was continuously monitored using rectal probe and maintained within a normal range using a heating pad. The trachea was cannulated with PE-90 polyethylene tubing and, until the end of the surgical procedure, the mice spontaneously breathed isoflurane gas using a mask connected to the cannula. The right jugular vein and left carotid artery were cannulated with PE-10 polyethylene catheters for drug administration and measurement of arterial pressure, respectively. The arterial pressure catheter was connected to a pressure transducer allowing signal amplification (hydraulic pressure; Millar Instruments, Houston, Texas, USA). The left kidney was exposed retroperitoneally through a left flank incision. Under the dissecting microscope, the left renal sympathetic nerve was isolated from the surrounding connective tissue and mounted on a bipolar 36-gauge platinum-iridium electrode (Cooner Wire Co, Chadsforth, CA). Once optimal recording parameters were established, the nerve was fixed to the electrode with silicone gel (Kwik-Sil; World Precision Instruments Inc., Sarasota, FL). The nerve electrode was attached to a high-impedance probe (HIP-511; Grass Instruments Co, Quincy, MA). The signal was amplified 105 times with a Grass P5 AC preamplifier and filtered at both low- (100 Hz) and high-frequency (1,000 Hz) cut-off. To prevent baroreflex-induced decrease in RSNA subsequent to the increase in arterial pressure induced by NE or AngII, a

ganglionic blocker (hexamethonium, 30 $\mu\text{g}\cdot\text{g}^{-1}$, intravenous) was administered, and mice were artificially ventilated (MiniVent ventilator, Hugo Sachs Elektronik, Harvard Apparatus, USA). Then, mice received in a randomized order intravenous injections of AngII (4 ng g^{-1}) or NE (NE, 40 ng g^{-1}) alone or in combination. Arterial pressure and RSNA were sampled at 2,000 Hz and sent to a PowerLab analog digital converter (Ad Instruments, Castle Hill, New South Wales, Australia) for recording on a PC computer and off-line data analysis (LabChart 7 Pro, Ad Instruments, Castle Hill, New South Wales, Australia). Heart rate (HR) was extrapolated from phasic arterial pressure. Mean arterial pressure (MAP; mm Hg), HR (beats per min, b.p.m.) and RSNA (bursts/s) were averaged over a 1-min period before drug injection (baseline) and over a 5-s period 20–30 s after injections, i.e., at the time of the maximal pressure effect of injected drugs. The mean time interval between each drug injection (5 min) was defined in preliminary experiments as the time allowed for MAP to return to baseline values. RSNA was analyzed as previously described subtracting the integrated voltage after death (background noise) from the total integrated voltage.

Data and statistical analysis

Results are expressed as mean values $\pm$ s.e.m. of at least three independent experiments. Statistical analysis was carried out using GraphPad Prism 4 software (GraphPad Software Inc.). Statistical tests used are indicated in the figure legends.

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Figure 1. Characterization of the heterodimerization between AT_1 -R and α_{2C} -AR in HEK293T cells

(a) BRET was measured in HEK293T cells coexpressing a fixed amount of the indicated Rluc8-tagged receptor and increasing amounts of the indicated GFP2-tagged receptors. Results were analyzed by nonlinear regression on a pooled data set from three independent experiments, assuming a model with one-site binding (GraphPad Prism 4.0). (b) HEK293T cells were transiently cotransfected with HA- AT_1 -R-V1 and HA- α_{2C} -AR-V2 (upper panels). Specificity was controlled in cells expressing the HA- AT_1 -R-V1 and CD8-V2 receptor (middle panel), the HA- α_{2C} -AR-V2 and CD8-V1 receptor or CD8-V1 and CD8-V2, as indicated. The green channel illustrates the expression of Venus complementation (V1 + V2), whereas the red channel shows cell surface anti-HA staining, highlighting only HA- AT_1 -R and/or HA- α_{2C} -AR present at the cell surface. Cell surface membranes and nuclei were visualized by Texas Red-wheat germ agglutinin staining (WGA; red) or DAPI staining (blue) respectively. Scale bars, 10 μ m.

Figure 2. Characterization of individual agonist effects on the AT₁-R- α_2C -AR heterodimer (a,b,d,e) BRET in HEK293T cells coexpressing HA- α_2C -Rluc8 and HA-AT₁-R-GFP2. **(a)** Cells were stimulated or not for 1 min with 10 μ M or increasing concentrations of the indicated compounds. SII, [¹Sar⁴Ile⁸Ile]angiotensin IIS. **(b)** Cells were first pretreated with 10 μ M specific antagonist (yohimbine (Yo) or candesartan (Cande)) for 10 min before agonist incubation. Cells were stimulated or not for 1 min with 10 μ M of the indicated compounds. **(d)** BRET kinetics were measured every 200 ms for 12 s before and after 1 μ M injection of AngII (top arrow) or NE (bottom arrow). Agonists were injected 2 s after the beginning of the reading. Inset, kinetics of net agonist-promoted BRET using data from **d**. Results were analyzed by nonlinear regression assuming a model with one-phase exponential association (GraphPad Prism 4.0). The figure is representative of one experiment performed twice in triplicate. **(e)** Cells were stimulated or not for 1 min with 10 μ M of the indicated compounds alone or in combination. **(c)** BRET in HEK293T cells coexpressing HA- α_2C -AR-Rluc8 and HA-AT₁-R-Venus, stimulated or not for 1 min with 10 μ M of the indicated compounds. **(f)** BRET in HEK293T cells coexpressing HA- α_2C -GFP2 and HA-AT₁-R-Rluc8, stimulated or not for 1 min with 10 μ M of the indicated compounds. In all panels, results are expressed as the difference in BRET signals measured in the presence and absence of agonist. Data represent the mean $\pm$ s.e.m. of at least four independent experiments. The statistical significance between stimulated and unstimulated cells was assessed using a paired Student's *t*-test (**P* < 0.05, ***P* < 0.01, ****P* < 0.001), and

statistical difference between ligand treatments was assessed using one-way analysis of variance followed by a Tukey's test ($^{\#}P < 0.05$, $^{###}P < 0.001$). NS, not statistically significant.

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Figure 3. CoDA-RET characterization of β -arrestin 2 and G-protein recruitment to heteromeric α_{2C} and AT₁ receptors

(a) Real-time BRET1 measurements in HEK293T cells coexpressing HA-AT₁-R-V1 and HA-α_{2C}-AR-V2 (illustrated by the diagrams) with Rluc-β-arrestin 2 (β-Arr2; top) or β-Arr2-Rluc (bottom) with or without the presence of 10 μM AngII (black), NE (gray) or both (dark gray). Data represent the BRET ratio expressed as the percentage of AngII-induced maximal β-arrestin 2 recruitment. Curves were fitted according to a one-phase hyperbolic association model and compared using two-way analysis of variance, with time and agonist as interaction factors. BRET_{max} and $t_{1/2}$ are indicated in the table. Data represent the mean ± s.e.m. of three to seven independent experiments, as indicated in the table. *** $P < 0.001$; NS, not statistically significant. **(b)** BRET1 was measured in HEK293T cells coexpressing the indicated Gα-Venus subunits (Gα₁₁-Venus, Gα_q-Venus or Gα_s-Venus) with β₁γ₂ G-protein untagged subunits in the presence of HA-AT₁-R-L1 and HA-α_{2C}-AR-L2 (top) or HA-α_{2C}-AR-L1 and HA-AT₁-R-L2 (bottom), with or without stimulation by 10 μM NE (light gray), AngII (black) or both (dark gray) for 1 min. Results are expressed as the difference in the BRET signal measured in the presence and absence of agonist. Data represent the mean ± s.e.m. of three to five independent experiments. The statistical significance between stimulated and unstimulated cells was assessed using a paired Student's t -test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 4. The nature of ligands dictates different β -arrestin 2 trafficking pathways activated by the AT_1 -R- α_{2C} -AR heterodimer

β -Arrestin 2 (β -Arr2) recruitment to AT_1 -R- α_{2C} -AR heterodimer was visualized by confocal microscopy on HEK293T cells coexpressing β -arrestin 2-mCherry (red), HA- AT_1 -R-V1 + HA- α_{2C} -AR-V2 (green upon BiFC), with or without stimulation (basal) for 10 min, 30 min or 120 min with 10 μ M NE, 10 μ M AngII or 10 μ M of both (AngII plus NE). Quadrants show 5 $\times$ electronic magnification, and arrows outline two dimer-positive spots. Nuclei labeled with DAPI (blue). Scale bars, 10 μ m. Quantification of β -arrestin 2-mCherry and Venus (AT_1 -R- α_{2C} -AR) fluorescence colocalization is shown in **Supplementary Figure 9**.

Figure 5. AngII-NE costimulation of the AT₁-R-α_{2C}-AR heterodimer generates new G_s-cAMP-PKA signaling

(a,b) For G-protein coupling measurement (a), BRET was measured in HEK293T cells coexpressing HA-AT₁-R-DmrC and Myc-α_{2C}-AR-DmrA, with GFP10-Gγ₂, Gβ₁ and Gα_{i1}-Rluc8 (left); Gα_q-Rluc8 (middle); or Gα_s-Rluc8 (right) and with or without stimulation by 10 μM NE, AngII or both (AngII plus NE) for 1 min, whereas for PKA activation evaluation (b), BRET was measured in HEK293T cells coexpressing Rluc8 and GFP2-Cα in the presence of HA-AT₁-R-DmrC and Myc-α_{2C}-AR-DmrA, with or without stimulation for 30 min with 10 μM NE, AngII or both (AngII plus NE) with or without the presence of 10 μM propranolol, as indicated. Attached cells were pretreated (+) or not (-) with 400 nM A-C for 1 h to force AT₁-R-α_{2C}-AR before the BRET experiment. Results are expressed as the difference in the BRET signal measured in the presence and absence of agonist (upper panels in a and b) or as the difference in the G-protein activation induced by ligands in the presence and absence of A-C (lower panels in a). Data represent the mean ± s.e.m. of four to six independent experiments. The statistical significance was assessed using a paired Student's *t*-test comparing ligand-stimulated and unstimulated cells (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) or comparing A-C-treated and untreated cells (#*P* < 0.05). NS, not statistically significant.

Figure 6. NE potentiates AngII-induced increase in NE release in neurons or RSNA in mice through a PKA pathway

(a) NE release was assayed in superior cervical ganglion neurons (SCGN) primary cultures with or without stimulation by NE (1 μ M), AngII (10 μ M) or both and with or without 10-min pretreatment with the PKA antagonist Rp-cAMP (left; 100 μ M, Rp) or the AT₁-R antagonist candesartan (right; 10 μ M, Cande). Ligand-modulated NE release is depicted as the percentage of release in the presence of drugs versus basal release (in the presence of vehicle). Data represent the mean $\pm$ s.e.m. of 10 (left panel) to 12 (right panel) independent experiments. (b) RSNA was measured after randomized intravenous injection of AngII (4 ng g^{-1}), NE (40 ng g^{-1}) or a combination of both (AngII + NE) without (vehicle, white bars, $n = 14$) or with H89 pretreatment (5 mg per kg body weight, intraperitoneal, black bars, $n = 7$) in hexamethonium-treated (30 μ g g^{-1} , intravenous) mice. Results are depicted as the percentage of the corresponding basal value normalized to 100% (detailed results are in **Supplementary Table 2**). Data represent the mean $\pm$ s.e.m. of 7–14 independent experiments. The statistical significance was assessed using one-way analysis of variance followed by the Newman-Keuls test (a) or ANOVA for repeated measures followed by Dunnett's test (b). $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$ \$ \$}P < 0.001$ for the indicated pairs; $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus basal value using paired Student's t -test; NS, not significant.