Angiotensin II type 1/adenosine A2A receptor oligomers: a novel target for tardive dyskinesia

Paulo A. de Oliveira1, James A. R. Dalton2, Marc López-Cano3,4, Adrià Ricarte2, Xavier Morató5,6, Filipe C. Matheus1, Andréia S. Cunha1, Christa E. Müller1, Reinaldo N. Takahashi1, Víctor Fernández-Dueñas3,4, Jesús Giraldo2, Rui D. Prediger1,6 & Francisco Ciruela3,4

Tardive dyskinesia (TD) is a serious motor side effect that may appear after long-term treatment with neuroleptics and mostly mediated by dopamine D2 receptors (D2Rs). Striatal D2R functioning may be finely regulated by either adenosine A2A receptor (A2AR) or angiotensin receptor type 1 (AT1R) through putative receptor heteromers. Here, we examined whether A2AR and AT1R may oligomerize in the striatum to synergistically modulate dopaminergic transmission. First, by using bioluminescence resonance energy transfer, we demonstrated a physical AT1R-A2AR interaction in cultured cells. Interestingly, by protein-protein docking and molecular dynamics simulations, we described that a stable heterotetrameric interaction may exist between AT1R and A2AR bound to antagonists (i.e. losartan and istradefylline, respectively). Accordingly, we subsequently ascertained the existence of AT1R/A2AR heteromers in the striatum by proximity ligation in situ assay. Finally, we took advantage of a TD animal model, namely the reserpine-induced vacuous chewing movement (VCM), to evaluate a novel multimodal pharmacological TD treatment approach based on targeting the AT1R/A2AR complex. Thus, reserpinized mice were co-treated with sub-effective losartan and istradefylline doses, which prompted a synergistic reduction in VCM. Overall, our results demonstrated the existence of striatal AT1R/A2AR oligomers with potential usefulness for the therapeutic management of TD.

Angiotensin II (AII) is a peptidic hormone that causes vasoconstriction through activation of angiotensin receptor type 1 (AT1R). Indeed, it is a key component of the renin-angiotensin system (RAS), which regulates blood pressure1. Accordingly, blocking AT1Rs with selective antagonists (i.e. losartan) constitutes the first-line therapy to deal with hypertensive patients2. Interestingly, AII is also synthesized in the brain, where its levels are much higher than those observed in plasma3. In addition, AT1Rs are expressed both in neurons and glial cells4. Thus, the existence of an endogenous brain angiotensin system has been postulated, which may respond to AII synthesized in and/or transported into the brain (for review see ref. 5). The function of AII in the brain has still not been fully elucidated. However, a role in the control of stress reaction and cerebral circulation, and in the mechanisms leading to brain ischemia, neuronal injury and inflammation has been demonstrated6. In addition, AT1R blockade reduced brain inflammation responses7 and had beneficial effects in processes involving microglial activation and neuroinflammation (such as animal models of Alzheimer's disease, brain ischemia and multiple sclerosis) (for review see ref. 7). Similarly, in animal models of parkinsonism induced by neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an increase in AII levels, with concomitant AT1R overactivation, has been observed8–10. On the other hand, the presence of RAS components in the basal ganglia in general and in the nigrostriatal system in particular has also been reported. Altogether, it has been

1Departamento de Farmacologia, Universidade Federal de Santa Catarina, Trindade, 88049-900, Florianópolis, SC, Brazil. 2Institut de Neurociències y Unitat de Bioestadística, Universitat Autònoma de Barcelona, Network Biomedical Research Center on Mental Health (CIBERSAM), Bellaterra, Spain. 3Unitat de Farmacologia, Departament de Patologia i Terapèutica Experimental, Facultat de Medicina, IDIBELL-Universitat de Barcelona, L’Hospitalet de Llobregat, Spain. 4Institut de Neurociències, Universitat de Barcelona, Barcelona, Spain. 5PharmaCenter Bonn, Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Bonn, Germany. 6Programa de Pós-graduação em Neurociências, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Trindade, 88049-900, Florianópolis, SC, Brazil. Paulo A. de Oliveira and James A. R. Dalton contributed equally to this work. Correspondence and requests for materials should be addressed to J.G. (email: Jesus.Giraldo@uab.es) or R.D.P. (email: rui.prediger@ufsc.br) or F.C. (email: fciruela@ub.edu)
postulated that brain RAS may be involved in dopaminergic degeneration, especially when the dopaminergic system is impaired, thus contributing to the pathogenesis and progression of dopaminergic-related pathologies such as Parkinson’s disease (PD).

The concept that cell surface receptors may physically interact forming oligomers appeared early in the eighties, while characterizing G protein-coupled receptors (GPCRs) for neurotransmitters. Notably, striatal dopaminergic receptors in general, and the dopamine D2 receptor (D2R) in particular, constitute the archetypal GPCR capable of forming receptor-receptor complexes. Indeed, the potential impact of these oligomers in pathophysiological conditions involving dopaminergic dysfunction has been extensively studied. Interestingly, the D2R has been shown to oligomerize with several GPCRs, including the adenosine A3R receptor (A3R) receptor interaction is defined as its “biochemical fingerprint.” Noteworthy, the D2R-A3R heteromer has been defined as a potential pharmacological target for pathologies associated with dysfunctional dopaminergic signaling, such as PD and schizophrenia. Indeed, A3R antagonists (i.e. istradefylline) are currently used for PD treatment in Japan. On the other hand, the D2R has also been shown to oligomerize with the AT1R in the striatum, thus the potential use of AT1R ligands to modulate dopaminergic signaling has been postulated. Interestingly, early studies also indicated interactions between the adenosinergic and the angiotensinergic systems, for instance the antinociceptive effect of AII was related to that produced by adenosine A1 agonists. In addition, an A3R- and AT1R-mediated synergistic interaction in the peripheral RAS was described. Thus, while adenosine was able to reverse the stimulatory effect of AII on Na+ transport via A3R activation, AT1R blockers reduced AII-mediated ROS formation via Nox2 in endothelial cells.

Conversely, AII potentiated the adenosine-induced contraction of afferent arterioles, while losartan-mediated AT1R blockade abolished the adenosine-mediated vasorelaxation in the renal cortex. Altogether, the aforementioned evidence highlights the need for a better understanding of the adenosinergic system-RAS interaction. Furthermore, this interaction may be relevant not only in the periphery but also in the brain, where a functional interplay with the dopaminergic system may occur.

Here, we study the possible existence, both in cultured cells and in mouse striatum, of a physical AT1R-A3R interaction, which may be a potential target for managing dopaminergic-related disorders (i.e. tardive dyskinesia, TD). Also, we seek to characterize the most likely heteromeric receptor arrangement through protein-protein docking and long-timescale molecular dynamics (MD) simulations. Finally, we propose a novel multimodal treatment for TD based on the use of AT1R and A3R antagonists at sub-effective doses, and test it in a mouse TD model, namely the reserpine-induced vacuous chewing movement (VCM).

**Results**

**AT1R and A2AR form heteromers in cultured cells.** Based on the existence of AT1R/D2R heteromers, we aimed to elucidate whether AT1R is also able to oligomerize with the A2AR, a well-known D2R partner. To this end, we first assessed the co-distribution of AT1R and A2AR in cultured cells through the fluorescence detection of CFP/YFP tagged receptors. Thus, by means of confocal microscopy analysis of HEK-293T cells transiently expressing AT1RCFP and A2ARYFP, a high overlapping in the distribution of the former receptors was observed. Next, we examined the possible physical interaction of AT1R and A2AR in living cells by means of the BRET approach. Thus, cells were transiently transfected with receptor constructs carrying the appropriate fluorophore pair (A2ARFluc and AT1RTPP). A positive and saturable BRET signal was observed in cells co-transfected with a constant concentration of A2ARFluc and increasing concentrations of AT1RTPP (Fig. 1b). Of note, as the control pair GABAARFluc and AT1RTPP led to a low and linear distribution, the specificity of the saturation (hysteretic) assay for the A2ARFluc and AT1RTPP pair could be established (Fig. 1b). Overall, these results demonstrate that AT1R and A2AR form heteromers in living HEK-293T cells.

**Structure of AT1R/A3R heteromer.** Computational modeling, protein-protein docking, and MD simulations were used to probe the interaction between AT1R and A3R, and determine their most likely heteromeric arrangement. Initially, AT1R and A3R antagonists (losartan and istradefylline, respectively), were docked into their respective inactive-state receptor crystal structure using Autodock4.2. The corresponding best docked AT1R-losartan and A3R-ivardefylline complexes were then embedded in lipid bilayer membranes and subjected to MD simulations of 250 ns and 500 ns, respectively, where both bound antagonists were observed to stabilize. In particular, in AT1R, ARG167, located on extracellular loop 2 (ECL2) above the orthosteric pocket, was observed to make H-bonds with losartan at both ends of the ligand (see SI Fig. 1) in a similar manner to that observed in the AT1R crystal structure containing alosinexartan. Likewise, in A3R, ASN253 (ASN285 in Ballesteros-Weinstein numbering) made an H-bond with istradefylline in a similar manner to other co-crystallized A3R xanthine antagonists (Fig. S1).

As both AT1R and A3R are thought to form functional homodimers at the cell surface, we investigated the likely structure and behavior of these respective homodimers with bound antagonists, prior to investigating heteromeric interactions. In order to do this we utilized the A3R homodimer crystal structure with co-crystallized antagonist as a structural guide for initializing AT1R-losartan and A3R-ivardefylline homodimer models. This dimeric crystal structure is observed to contain an interface between TM4 and TM5 helices of each monomer, with TM4 of one monomer interacting with TM5 of the other, and vice versa. Initial AT1R and A3R homodimer models were refined with protein-protein docking using the ROSSIE server, each consisting of two antagonist-bound receptors in the same MD-generated conformation (see above). Following protein-protein docking, the A3R and AT1R homodimers were subjected to further MD simulations of 1.5 μs and 750 ns, respectively. During these simulations, both AT1R and A3R homodimers were seen to form significant interactions via their TM4 and TM5 helices, respectively, with considerable contact between monomers, indicative of energetically stable dimers (Fig. S2). In addition, the respective bound antagonists remained stably bound in...
each participating monomer, with all receptor subunits maintaining an inactive state. From these results, it was inferred that the antagonist-bound homodimeric states of AT1R and A2AR are stable in silico, and likely form the minimum constituents that participate in cross-receptor heteromeric interaction.

As other described heteromeric interactions involving A2AR fit a heterotetramer model, and as MD simulations of AT1R and A2AR homodimers suggest their respective stability, we investigated heterotetrameric interactions between the two receptor homodimers. As there is no crystal structure for GPCRs in tetrameric formation, we performed extensive protein-protein docking with ROSIE to identify the highest possible scoring interaction of AT1R and A2AR homodimers. The “best” conformation involved a tetramer with cross-receptor interfaces involving TM5 and TM6 of one receptor with TM1 and TM2 of the other, and vice versa (Fig. 2). In order to assess the stability of the proposed interaction, the heterotetramer complex was subjected to an MD simulation in a membrane for 2µs. Results show the receptors progressively stabilized (RMSD curve in Fig. S3) and enhanced their interaction, whilst maintaining the original tetrameric configuration (Fig. 2). Furthermore, the respective AT1R and A2AR homodimers remained stable and unperturbed within the tetramer, maintaining their respective inactive states. In conclusion, stable heterotetrameric interaction between AT1R and A2AR is plausible at a molecular level and compatible with bound antagonists, losartan and istradefylline.

Functional consequences of the AT1R and A2AR oligomerization. The formation of AT1R-A2AR complexes in transfected cells suggests that there might exist a functional coupling between these two receptors. Thus, we assessed the impact of A2AR expression on AT1R-mediated intracellular Ca2+ mobilization from internal stores by means of Fluor4 determinations. Thus, in Fluor4 loaded cells expressing AT1R alone, the activation with angiotensin II increased intracellular Ca2+ (Fig. 3a, red trace), as expected. Interestingly, in cells co-expressing AT1R and A2AR, the angiotensin II-mediated intracellular Ca2+ mobilization was boosted (Fig. 3a, blue trace). Indeed, in cells expressing only A2AR, a residual and not significant effect of angiotensin II was observed, probably because of the endogenous expression of AT1R in HEK-293T cells (Fig. 3a, black trace). Quantification of the results (Fig. 3c) demonstrated a significant [F(2,6) = 8.40 (P < 0.05)] difference between the experimental groups assessed, thus a significant (P < 0.05) increase in the AT1R-mediated intracellular calcium accumulation.
in AT1R-A2AR cells was observed (Fig. 3c). These results suggest that a functional interplay between AT1R and A2AR might exist upon expression in heterologous cells.

**AT1R and A2AR heteromers are expressed in mouse striatum.** Once demonstrated that AT1R and A2AR assemble into functionally interacting complexes in living cells, we aimed to determine the existence of AT1R/A2AR heteromers in native tissue, namely the striatum. To this end, we first conducted immunofluorescence experiments to assess the expression levels and distribution of both AT1R and A2AR in mouse striatum. Interestingly, both receptors showed a high degree of co-distribution throughout the striatal neuropil (Fig. 4a, upper panels) and eventually within the medium spiny neurons (MSN) cell bodies (Fig. 4a, lower panels). Importantly, the myelinated fiber bundles that penetrate the striatum were visible as dark (not stained) structures within the stained neuropil (Fig. 4a, upper panel). These results give rise to the possibility that these two receptors might be forming heteromers under native conditions. Subsequently, to confirm the existence of AT1R/A2AR heteromers in the striatum we implemented the P-LISA approach, a well described technique providing enough...
sensitivity to evaluate receptor's close proximity within a named GPCR oligomer in native conditions. Thus, by using proper antibody combinations, the AT₁R/A₂A R heteromer expression in mouse striatum was addressed by P-LISA assays. Indeed, red dots reflecting a positive P-LISA signal was observed in the striatum of wild-type mice (Fig. 4b), thus allowing the visualization of the AT₁R/A₂A R receptor-receptor interaction. Interestingly, in striatal slices from the A₂A R-KO mice the P-LISA signal was negligible (Fig. 4b), thus reinforcing the specificity of our P-LISA assay. Indeed, when the P-LISA signal was quantified the wild-type animal showed $4 \pm 0.5$ dots/nuclei under the same experimental conditions. Thus, a marked and significant ($P < 0.005$) reduction in the P-LISA signal was observed in the A₂A R-KO striatal slices. Taken together, data gathered from our P-LISA experiments strongly support the existence of AT₁R/A₂A R heteromers in the mouse striatum.

**Functional interplay between AT₁R and A₂A R in an animal model of TD.** A₂A R-containing oligomers, including $A₂A R/D₂ R$, are thought to be involved in the control of locomotor function both in normal...
and pathological conditions. However, although A2AR has been linked to neuroleptic-induced TD, its impact on this syndrome is still ambiguous. Consequently, we sought to investigate whether the AT1R/A2AR heteromer might play a role in TD. We took advantage of the vacuous chewing movement (VCM) model of TD in mice. Interestingly, administration of the AT1R antagonist losartan dose-dependently reduced reserpine-induced VCM (Fig. 5a). Similarly, administration of the A2AR antagonist istradefylline dose-dependently reduced reserpine-induced VCM (Fig. 5b). Subsequently, we investigated whether co-treatment at sub-effective low doses of AT1R and A2AR antagonists would elicit a significant reduction of VCM in our reserpine-induced TD animal model. Therefore, for combination treatment, 0.05 mg/kg of losartan and 0.03 mg/kg of istradefylline were selected as they were not effective in reducing VCM. Noteworthy, the combined treatment produced a significant (*P < 0.05) reduction in VCM (Fig. 5c), thus demonstrating a synergistic interaction between both drugs. Overall, these results suggest that co-treatment with AT1R and A2AR antagonists at sub-effective low doses is a useful therapeutic approach for TD management.

Finally, in an attempt to ascertain the role of AT1R/A2AR oligomers in the synergistic effect observed upon receptor antagonist co-treatment, we assessed the efficacy of the VCM sub-effective losartan dose in mice lacking the A2AR (i.e. A2AR-KO mice). Interestingly, the low dose of losartan (0.05 mg/kg) was able to significantly (P < 0.05) reduce the number of VCM in the A2AR-KO mice (Fig. 5d). Hence, in the absence of A2AR the efficacy of losartan was higher, thus indicating that AT1R/A2AR heteromers are crucial for finely modulating TD. Collectively, these results suggest that AT1R and A2AR functionally interact in vivo and that this functional interplay may be provided by the existence of AT1R/A2AR oligomers.

**Discussion**

TD is a serious motor side effect associated to long-term treatment with neuroleptics. Notably, D2R occupancy and its transience to occupation have been identified as a potential mechanistic substrate to develop antipsychotic-induced TD. Indeed, D2R-mediated control of motor function has been related to the ability of this receptor to oligomerize with otherGPCRs in general and with the A2AR in particular. Also, in the brain, dopaminergic neurotransmission can be modulated by AII through AT1R. Thus, AT1R blocking precludes AII-mediated dopamine release. Furthermore, a functional interaction between angiotensin and dopamine...
and AT1R-mediated anti-LID effects are related to their ability to heteromerize with D2R, and revealed for the first time the existence of AT1R/D2R/A2AR oligomers in the striatum and its implications in TD.

Our experimental data shows that AT1R and A2AR form heteromers both in co-transfected cells and in mouse striatum. This feature is especially strengthened by our in-silico analysis, which has predicted a heterotetrameric receptor arrangement that was stable during 2μs of MD simulation. The “best” receptor-receptor interface identified for the AT1R/A2AR heterotetramer involves TM5 and TM6 of one receptor with TM1 and TM2 of the other, and vice versa, while in the respective homodimers the TM4 of one monomer interactac with TM5 of the other, and vice versa. Interestingly, the D2R/A2AR heterodimeric interface has been postulated to be formed by the TM4 and TM5 of D2R interacting with TM4 and TM5 of the A2AR. Therefore, when considering a putative AT1R/D2R/A2AR oligomer new in-silico analysis will be needed to accurately determine TM-TM contacts and receptor rearrangement defining AT1R/D2R/A2AR oligomer stoichiometry. Overall, this information will be extremely valuable when assessing potential multimodal TD pharmacotherapeutic interventions based on drugs targeting these receptors.

The AT1R/A2AR oligomerization was shown to elicit functional consequences, since co-expression with A2AR boosted AT1R signaling. This AT1R gain of function may most likely result from an A2AR-mediated AT1R increased cell surface targeting, as was previously reported. Alternatively, an A2AR-mediated direct trans-activation of AT1R could not be excluded, as has been described for other A2AR-containing oligomers. Thus, further work is needed to elucidate the precise molecular mechanism behind this AT1R/A2AR oligomer-dependent AT1R gain of function. Nevertheless, our main purpose consisted of ascertaining the in vivo implications of the AT1R/A2AR oligomer formation, which is the cornerstone when describing a new GPCR oligomer. Indeed, our P-LISA data strongly supported the existence of AT1R/A2AR heteromers in the mouse striatum, thus warranting the need to assess the impact of this oligomer in behaving animals. Accordingly, we demonstrated an unprecedented synergism of AT1R and A2AR antagonists on the control of involuntary mandibular movements induced by reserpine in an animal model of TD. Thus, co-treatment with AT1R and A2AR antagonists at sub-effective low doses robustly (>60%) reduced reserpine-mediated VCM. Certainly, this makes this multimodal pharmacological approach an attractive solution for TD management.

The striatum is considered a pivotal brain region, since it receives projections from other basal ganglia areas and from many other brain regions involved in motor and non-motor functions, such as the motor cortex, the prefrontal cortex and the hippocampus. Indeed, both the renin-angiotensin and the adenosinergic systems play an important role in controlling the striatal function. Thus, the ability of AT1R and A2AR to heteromerize in the striatum might constitute a way of fine-tuning multiple receptor-signaling pathways harmonizing dopaminergic neurotransmission. Therefore, the AT1R/A2AR oligomer could be envisaged as a potential drug target for striatum-related adverse motor dysfunctions associated to therapy, including TD and L-DOPA induced dyskinesia (LID). Indeed, A2AR antagonists have been postulated and licensed as antiparkinsonian drugs and eventually studied in the management of LID. Furthermore, A2AR has been linked to neuroleptic-induced TD, although with some debate. Similarly, preclinical studies have demonstrated that blockade of AT1R reduces LID. It is assumed that these A2AR- and AT1R-mediated anti-LID effects are related to their ability to heteromerize with D2R, and thus controlling dopaminergic neurotransmission. However, it could be speculated that AT1R and A2AR might control D2R function through functional AT1R/D2R/A2AR-containing complexes in GABAergic striatopallidal neurons. A number of facts support this last statement: i) the high and selective co-expression of AT1R, D2R and A2AR in these particular cells; ii) the demonstration of A2AR/D2R, AT1R/D2R and AT1R/A2AR heteromers; and iii) the existence of strong multiple interactions between the three receptors. In conclusion, the demonstration of their simultaneous physical interaction may constitute a novel and very attractive target for developing new drugs in the management of pathologies in which these receptors play a key role, such as TD.

Methods
Reagents. The primary antibodies used were: rabbit anti-AT1R polyclonal antibody (Abcam, Cambridge, UK), and mouse anti-A2AR monoclonal antibody (Millipore, Billerica, MA, USA). The secondary antibodies were: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pierce Biotechnology, Rockford, IL, USA) and Cy3-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The ligands used were: losartan (Abcam); angiotensin II, istradefylline (KW-6002), reserpine and ibomycin from Sigma-Aldrich (St. Louis, MO, USA).

Plasmid constructs. To perform co-localization and BRET experiments, the A2AR constructs containing a cyan fluorescent protein (CFP; A2ARCFP), or the Renilla luciferase (RhLuc; A2ARRhLuc) were used. The AT1R and GABAa2 receptor constructs containing a yellow fluorescent protein (YFP; AT1RYFP, GABAa2YFP) were cloned, as previously described.

Animals. CD-1 mice (Charles River Laboratories and from the central animal facility of Federal University of Santa Catarina) and A2AR-KO mice developed in a CD-1 genetic background (animal facility of University of Barcelona) weighing 20–25 g were used. The University of Barcelona and Federal University of Santa Catarina Committee on Animal Use and Care approved the protocol. Animals were housed and tested in compliance with the guidelines described in the Guide for the Care and Use of Laboratory Animals and following the European Union directives. All efforts were made to minimize animal suffering and the number of animals used. All animals were housed in groups of five in standard cages with ad-libitum access to food and water and maintained under 12 h dark/light cycle (starting at 7:30 AM), 22 °C temperature, and 66% humidity.
Cell culture. Human embryonic kidney (HEK)-293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL streptomycin, 100 mg/mL penicillin and 5% (v/v) fetal bovine serum at 37°C and in an atmosphere of 5% CO2. HEK-293T cells growing in 25 cm2 flasks or six-well plates containing 18 mm coverslips were used for western blot and fluorescence imaging, respectively. They were transiently transfected with the cDNA encoding the specified proteins using Polyethylenimine (Polysciences, Inc. Warrington, PA, USA).

Fixed brain tissue preparation. Mice were anesthetized and perfused intracardially with 100–200 ml ice-cold 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 8.07 mM NaH2PO4, 1.47 mM KH2PO4, 137 mM NaCl, 0.27 mM KCl, pH 7.2). Brains were post-fixed in the same solution of PFA at 4°C during 12 h. Coronal sections (25 μm) were processed using a vibratome (Leica Lasertecnik GmbH, Heidelberg, Germany). Slices were collected in Walker’s Antifreezing solution (30% glycerol, 30% ethylene glycol in PBS, pH 7.2) and kept at −20°C until processing.

Bioluminescence resonance energy transfer measurements. Bioluminescence resonance energy transfer (BRET) experiments in HEK-293T cells were performed as previously described. In brief, HEK-293T expressing the indicated constructs were rapidly washed, detached, and resuspended in HBSS buffer (137 mM NaCl, 5 mM KCl, 0.34 mM Na2HPO4, 0.44 mM KH2PO4, 1.26 mM CaCl2, 0.4 mM MgSO4, 0.5 mM MgCl2, 10 mM HEPES, pH 7.4) containing 10 mM glucose. Cell suspensions (20 μg of protein) were distributed in 96-well microplate plates, 5 μM h-coelenterazine (Nanolight Technology, Pinetop, AZ, USA) was added and BRET determined in a POLARstar Optima plate-reader (BMG Labtech, Durham, NC, USA) as previously described.

Intracellular calcium determination. The AT1R-mediated intracellular Ca2+ accumulation was assessed by Fluo4-NW Calcium Assay Kit (Invitrogen, Carlsbad, CA, USA). Thus, transiently transfected HEK-293T cells were lifted and plated in 96-well black plates with transparent bottoms. Cells were incubated with the Fluor4-NW following the instructions of the manufacturer and washed with HBSS. Fluorescence signals were measured at 530 nm during 60 s while injecting Angiotensin II (50 nM) and ionomycin (5 μM) at seconds 5 and 40 respectively, using a POLARstar Optima plate-reader (BMG Labtech). The specific Angiotensin II-induced Fluo4 signal (F) was expressed as percentage of the signal elicited by ionomycin (Fi) in each set of experimental conditions.

Immunohistochemistry. Previously collected slices were washed three times in PBS, permeabilized with 0.3% Triton X-100 in PBS for 2 hours and rinsed back three times more with wash solution (0.05% Triton X-100 in PBS). The slices were then incubated with blocking solution (10% NDS in wash solution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h at R.T. and subsequently incubated with the primary antibodies overnight at 4°C. After two rinses (10 min each) with 1% NDS in wash solution, sections were incubated for 2 h at R.T. with the appropriate secondary antibodies conjugated with Alexa dyes (Invitrogen, Carlsbad, CA, USA), then washed (10 min each) two times with 1% NDS in wash solution and two more times with PBS, and mounted on slides. Fluorescence striatal images were obtained using a Leica TCS 4D confocal scanning laser microscope (Leica Lasertecnik GmbH).

Proximity ligation in situ assay. Duolink in situ PLA detection Kit (Olink Bioscience, Uppsala, Sweden) was performed in a similar manner as immunohistochemistry explained above until the secondary antibody incubation step. The following steps were performed following the manufacturer’s protocol, as previously described. Fluorescence images were acquired on a Leica TCS 4D confocal scanning laser microscope (Leica Lasertecnik GmbH) using a 60x N.A. = 1.42 oil objective from the selected area. High-resolution images were acquired as a z-stack with a 0.2 μm z-interval with a total thickness of 5 μm. Nonspecific nuclear signal was eliminated from PLA images by subtracting DAPI labeling. Analyze particle function from Image J (NIH) was used to calculate protein-nuclei and neuron nuclei was obtained and ratio among them was calculated.

Computational modeling. Ligand docking. Crystal structures of AT1R (PDB id: 4ZUD) and A2AR (PDB id: 4E1Y) were converted into apo w t forms by removing co-crystallized ligands and non-native fusion proteins i.e. cytochrome b562, building missing intracellular and extracellular loop sections with MODELLER v9.14, and energy minimizing in the AMBER14SB force-field with CHIMERA and docked into respective receptor structures with Autodock4.2 using default parameters. Grid points were generated to cover total orthosteric pocket volumes. The final docking conformations of losartan and istradefylline represented top hits identified by best predicted affinity (nM). These were checked to be consistent with previously reported binding modes of relevant co-crystallized antagonists. In particular, losartan was docked to interact with Arg167 and istradefylline was docked to interact with Asn253. Subsequent energy minimization of docked structures was performed with CHIMERA in the AMBER-14SB force-field.

Protein-protein docking. For generating homodimers of respective receptors: AT1R and A2AR, with bound antagonists, two molecular dynamics (MD)-generated receptor-ligand monomers (see MD methods) of either AT1R or A2AR, in each case, were superimposed onto the A3R homodimer crystal structure (PDB id: 4E1Y), yielding an initial homodimer model, which was then submitted to the ROSIE webserver for protein-protein docking. For both AT1R and A2AR, the best docked homodimer was identified by three factors: best possible ROSETTA interface score (I_sc), lowest possible RMSD in relation to initial model, and acceptable membrane-compatible
orientation. For construction of an AT₁R-A₂AR heterotetramer, two initial tetrameric arrangements were manually generated by combining respective MD-generated AT₁R and A₂AR homodimers (see MD section) in alternative ways: (i) where homodimers are arranged side-to-side in a rectangular-like configuration, where each homodimer subunit interacts with a subunit of the other homodimer (by respective TM1/2–5/6 helices), (ii) where homodimers are partially displaced with respect to one another creating a parallelogram-like configuration, where both subunits of one homodimer interact with a single subunit of the other homodimer (by respective TM4/5 helices). Both these alternative configurations were submitted to the ROSIE webserver for identification of the best possible tetrameric arrangement according to the same criteria implemented previously. For all protein–protein docking runs executed on the ROSIE webserver, default local parameters were used, i.e. perturbation of 3 Å between proteins, 8° of tilt, and 360° rotation around protein centers, with generation of 1000 docking solutions per case.

**Molecular dynamics system setup.** Five different systems were generated using the CHARMM-GUI web-based interface 73, each in a POPC membrane and solvated with TIP3P water molecules: AT₁R monomer with bound losartan, A₂AR monomer with bound istradefylline, AT₁R homodimer with bound losartan, A₂AR homodimer with bound istradefylline, and AT₁R-A₂AR heterotetramer with bound antagonists. All receptor structures were orientated according to the OPM database 68 entry: aei4. Charge neutralizing ions (0.15 M KCl) were introduced to each system. Parameters of membrane, water and protein were automatically generated by CHARMM-GUI 73 according to CHARMM36 force-field 75 with ACEMD 79 on specialized GPU-computer hardware, totaling 5 μs across systems. In detail, monomer AT₁R-A₂AR systems were equilibrated for 20 ns at 300 K and 1 atm, while AT₁R-A₂AR homodimers and heterotetramer systems were equilibrated for 50 ns under same conditions. During equilibration, positional harmonic restraints on protein and antagonist heavy atoms were progressively released over the first 8 ns and then continued without constraints. After equilibration, AT₁R and A₂AR monomers were subjected to unbiased production runs of 250 ns and 500 ns under same conditions, respectively. Likewise, AT₁R and A₂AR homodimers were subjected to unbiased production runs of 750 ns and 1.5 μs, respectively. The AT₁R-A₂AR heterotetramer was subjected to an unbiased production run of 2 μs. Simulation trajectories were analyzed using VMD software v1.9.2 80.

**Reserpine-induced vacuous chewing movements.** The VCM model of TD 48 was induced in mice through two subcutaneous (s.c.) reserpine injections (1 mg/kg) administered with an interval of 48 h. Twenty-four hours after the last reserpine administration, mice were treated by intraperitoneal (i.p.) route with losartan (0.05–50 mg/kg) and/or istradefylline (0.03–0.06 mg/kg). VCM parameters were evaluated as previously described 81 but with some modifications. Thus, the evaluation of VCM frequency consists of a manual counting of continuous single mouth openings in a vertical plane, not directed to a physical material. Mirrors were placed on the table and behind the glass cylinder (Ø 19 cm and 22 cm height) to allow observation of the orofacial movements when mice were not facing the observer. The evaluation of this parameter during 10 min was performed by a blind observer, 30 min after the pharmacological treatments administered 24 h after the second reserpine injection 81.

**Statistics.** The number of samples (n) in each set of experimental conditions is indicated in figure legends. Statistical analysis was performed by one-way ANOVA followed by Newman-Keuls post-hoc test or Student’s t-test when appropriate. Statistical significance was considered at P < 0.05.

**References**

1. Brunnert, H. R., Chang, P., Wallach, R., Sealey, J. E. & Laragh, J. H. Angiotensin II vascular receptors: their avidity in relationship to sodium balance, the autonomic nervous system, and hypertension. J. Clin. Invest. 51, 58–67, doi:10.1172/JCI106797 (1972).
2. Geppetti, P. & Wagstaff, A. J. Losartan potassium: a review of its pharmacology, clinical efficacy and tolerability in the management of hypertension. Drugs 51, 820–45, doi:10.2165/00003495-19965010-00008 (1996).
3. Hermann, K., McDonald, W., Unger, T., Lang, R. E. & Ganten, D. Angiotensin biosynthesis and concentrations in brain of normotensive and hypertensive rats. J. Physiol. (Paris). 79, 471–80 (1984).
4. Garrido-Gil, P., Valenzuela, R., Villar-Cheda, B., Lanciego, J. L. & Labandeira-Garcia, J. L. Expression of angiotensinogen and receptors for angiotensin and prorenin in the monkey and human substantia nigra: an intracellular renin-angiotensin system in the nigra. Brain Struct. Funct. 218, 373–88, doi:10.1007/s00429-012-0402-9 (2013).
5. Saavedra, J. M. Brain Angiotensin II: New Developments, Unanswered Questions and Therapeutic Opportunities. Cell. Mol. Neurobiol. 25, 485–512, doi:10.1023/A:10097105-001-5 (2005).
6. Saavedra, J. M. Angiotensin II AT(1) receptor blockers ameliorate inflammatory stress: a beneficial effect for the treatment of brain disorders. Cell. Mol. Neurobiol. 32, 667–81, doi:10.1007/s10571-011-9754-6 (2012).
7. Labandeira-Garcia, J. L. et al. Brain renin-angiotensin system and dopaminergic cell vulnerability. Front. Neuroanat. 8, 67, doi:10.3389/fnana.2014.00067 (2014).
8. Grammatopoulos, T. N. et al. Angiotensin type 1 receptor antagonist losartan, reduces MPTP-induced degeneration of dopaminergic neurons in substantia nigra. Mol. Neurodegener. 2, 1, doi:10.1186/1750-1236-2-1 (2007).
9. Zawada, W. M. et al. Generation of reactive oxygen species in 1-methyl-4-phenylpyridinium (MPP+) treated dopaminergic neurons occurs as an NADPH oxidase-dependent two-wave cascade. J. Neuroinflammation 8, 129, doi:10.1186/1742-2094-8-129 (2011).
10. Sonsalla, P. K. et al. The angiotensin converting enzyme inhibitor captopril protects nigrostriatal dopamine neurons in animal models of parkinsonism. Exp. Neurol. 250, 376–83, doi:10.1016/j.expneurol.2013.10.014 (2013).
11. Agnati, L. F., Fuxe, K., Zini, I., Lenzi, P. & Hokfelt, T. Aspects on receptor regulation and isoreceptor identification. Med. Biol. 58, 182–187 (1980).
12. Fuxe, K. et al. Evidence for the existence of receptor–receptor interactions in the central nervous system. Studies on the regulation of monoamine receptors by neurosteroids. J. neural Transm. 18, 165–179 (1983).
13. Gomes, I. et al. G Protein–Coupled Receptor Heteromers. *Ann. Rev. Pharmacol. Toxicol.* **56**, 403–425, doi:10.1146/annurev-pharmaco-061616-155952 (2016).

14. Ciruela, F. et al. Combining Mass Spectrometry and Pull-Down Techniques for the Study of Receptor Heteromerization. Direct Epitope–Epitope Electrostatic Interactions between Adenosine A2A and Dopamine D2 Receptors. *Anal. Chem.* **76**, 5354–5363, doi:10.1021/ac049295s (2004).

15. Ferre, S. et al. An Update on G Protein–Coupled Receptors. *Curr. Pharm. Des.* **14**, 1468–1474, doi:10.2174/138945008784840108 (2008).

16. Müller, T. The safety of irstradefylline for the treatment of Parkinson's disease. *Expert Opin. Drug Saf.* **14**, 769–75, doi:10.1517/14743383.2015.104798 (2015).

17. Martinez-Pinilla, E. et al. Dopamine D2 and angiotensin II type 1 receptors form functional heteromers in rat striatum. *Biochem. Pharmacol.* **96**, 131–142, doi:10.1016/j.bcp.2015.05.006 (2015).

18. Pechova, D. M. & Georgiev, V. P. Interaction of angiotensin II and adenosine A1 and A2A receptor ligands on the writhing test in mice. *Pharmacol. Biochem. Behav.* **72**, 23–8, doi:10.1016/S0091-3057(01)00707-9 (2002).

19. Tchekalarova, J., Kambourova, T. & Georgiev, V. P. Interaction of angiotensin II and adenosine A1 and A2A receptor ligands on the writhing test in mice. *Pharmacol. Biochem. Behav.* **72**, 23–8, doi:10.1016/S0091-3057(01)00707-9 (2002).

20. Thévenin, D., Lazarova, T., Roberts, M. F. & Robinson, C. R. Oligomerization of the fifth transmembrane domain from the adenosine A2A receptor. *Structure of the Adenosine A2A Receptor in Complex with ZM241385 and the Xanthines XAC and Caffeine*. *J. Clin. Invest.* **101**, 769–776, doi:10.1122/jc1480 (1998).

21. Fernández-Dueñas, V. et al. Untangling dopamine-adenosine receptor-receptor assembly in experimental parkinsonism in rats. *Dis. Model. Mech* **8**, 57–63, doi:10.1242/dmm.0118143 (2015).

22. Morris, G. M. et al. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. Comput. Chem.* **30**, 2785–91, doi:10.1002/jcc.x30.16 (2009).

23. Gracia, E. et al. A2A adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase. *Biochim. Biophys. Acta - Biomembr.* **1840**, 26, doi:10.1016/j.bbamem.2011.02.007 (2011).

24. Doré, A. S. S. Dimerization and ligand binding affect the structure network of A2A adenosine receptor. *Structure of the Adenosine A2A Receptor in Complex with ZM241385 and the Xanthines XAC and Caffeine*. *J. Neurochem.* **105**, 338–40, doi:10.1111/j.1471-4159.2005.02200.x (2004).

25. Karip, E., Turu, G., Supeki, K., Szidonya, L. & Hunyady, L. Cross-inhibition of angiotensin AT1 receptors supports the concept of receptor oligomerization. *Neurochem. Int.* **51**, 261–267, doi:10.1016/j.neuint.2007.05.018 (2007).

26. Barnard, D. L., Lutter, G., Langer, A., el Faramawy, Y. & Quitterer, U. Factor XIIIa Transglutaminase Crosslinks AT1 Receptor Dimers of Monocytes at the onset of Atherosclerosis. *Cell 119*, 345–354, doi:10.1016/j.cell.2004.10.006 (2004).

27. Hansen, J. L., Theilade, J., Haunso, S. & Sheikh, S. P. Oligomerization of Wild Type and Nonfunctional Mutant Angiotensin II Type 1 Receptors Inhibits Gq Protein Signaling but Not ERK Activation. *J. Biol. Chem.* **279**, 24108–24115, doi:10.1074/jbc.M400922200 (2004).

28. Gracia, E. et al. A2A adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase. *Biochim. Biophys. Acta - Biomembr.* **1840**, 26, doi:10.1016/j.bbamem.2011.02.007 (2011).

29. Fanelli, F. & Felline, A. Dimerization and ligand binding affect the structure network of A2A adenosine receptor. *Biochem. Biophys. Acta - Biomembr.* **1808**, 1256–1266, doi:10.1016/j.bbamem.2010.08.006 (2011).

30. Tchekalarova, J., Kambourova, T. & Georgiev, V. P. interaction of angiotensin II and adenosine A1 and A2A receptor ligands on the writhing test in mice. *Pharmacol. Biochem. Behav.* **29127–29139, doi:10.1016/j.pss.2011.06.014 (2011)."
81. Cunha, A. S. 
76. Vanommeslaeghe, K. 
75. Huang, J. & MacKerell, A. D. CHARMM36 all-atom additive protein force field: Validation based on comparison to NMR data. 
74. Lomize, M. A., Lomize, A. L., Pogozheva, I. D. & Mosberg, H. I. OPM: Orientations of Proteins in Membranes database. 
77. Vanommeslaeghe, K., Raman, E. P. & MacKerell, A. D. Automation of the CHARMM General Force Field (CGenFF) II: Assignment of Bonded Parameters and Partial Atomic Charges. J. Chem. Inf. Model. 52, 3144–3154, doi:10.1021/ci100364x (2012). 
78. Vanommeslaeghe, K. & MacKerell, A. D. Automation of the CHARMM General Force Field (CGenFF) II: Assignment of Bonded Parameters and Partial Atomic Charges. J. Chem. Inf. Model. 52, 3155–3168, doi:10.1021/ci1003649 (2012). 
69. Sali, A. Comparative protein modeling by satisfaction of spatial restraints. Mol. Med. Today 1, 270–7, doi:10.1036/S1357-4310/95/11170-7 (1995). 
70. Case, D. A. et al. The Amber biomolecular simulation programs. J. Comput. Chem. 26, 1668–1688, doi:10.1002/jcc.20290 (2005). 
71. Kim, S. et al. PubChem Substance and Compound databases. Nucleic Acids Res. 44, D1202–D1213, doi:10.1093/nar/gkv951 (2016). 
72. Beinert, H. Protein motions: The importance of side-chains. Curr. Opin. Struct. Biol. 19, 485–90, doi:10.1016/j.sbi.2009.01.006 (2009). 
73. Jo, S., Kim, T., Iyer, V. G. & Im, W. CHARMM-GUI: a web-based graphical user interface for CHARMM. J. Comput. Chem. 29, 1859–65, doi:10.1002/jcc.20495 (2008). 
64. Ledent, C. et al. Aggression, hypolaesthesia and high blood pressure in mice lacking the adenosine A2a receptor. Nature 388, 674–8, doi:10.1038/41771 (1997). 
65. Clark, J. D., Gebhart, G. F., Gonder, J. C., Keeling, M. E. & Kohn, D. F. Special Report: The 1996 Guide for the Care and Use of Laboratory Animals. ILAR J. 38, 41–48, doi:10.1093/ilar/38.1.41 (1997). 
66. Ciruela, F. & Fernández-Duñach, V. GPCR oligomerization analysis by means of BRET and dFRAP. Methods Mol. Biol. 1272, 133–144, doi:10.1007/978-1-4939-2336-6_10 (2015). 
67. Garcia-Negredo, G. et al. Coassembly and coupling of SK2 channels and mGlu5 receptors. J. Neurosci. 34, 14793–802, doi:10.1523/JNEUROSCI.0383-14.2014 (2014). 
68. Matamales, M. et al. Stratal medium-sized spiny neurons: identification by nuclear staining and study of neuronal subpopulations in RAC transgenic mice. PLoS One 4, e4770, doi:10.1371/journal.pone.0004770 (2009). 
69. Sali, A. Comparative protein modeling by satisfaction of spatial restraints. Mol. Med. Today 1, 270–7, doi:10.1036/S1357-4310/95/11170-7 (1995). 
70. Case, D. A. et al. The Amber biomolecular simulation programs. J. Comput. Chem. 26, 1668–1688, doi:10.1002/jcc.20290 (2005). 
71. Pettersen, E. F. et al. UCSF Chimera–a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–12, doi:10.1021/ct049770a (2004). 
72. Kim, S. et al. PubChem Substance and Compound databases. Nucleic Acids Res. 44, D1202–D1213, doi:10.1093/nar/gkv951 (2016). 
73. Jo, S., Kim, T., Iyer, V. G. & Im, W. CHARMM-GUI: a web-based graphical user interface for CHARMM. J. Comput. Chem. 29, 1859–65, doi:10.1002/jcc.20495 (2008). 
74. Lomize, M. A., Lomize, A. L., Pogozheva, I. D. & Mosberg, H. I. OPM: Orientations of Proteins in Membranes database. Bioinformatics 22, 623–625, doi:10.1093/bioinformatics/btk233 (2006). 
75. Huang, J. & MacKerell, A. D. CHARMM36 all-atom additive protein force field: Validation based on comparison to NMR data. J. Comput. Chem. 34, 2135–2145, doi:10.1002/jcc.23354 (2013). 
76. Vanommeslaeghe, K. et al. CHARMM general force field: A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. J. Comput. Chem. 31, 871–90, doi:10.1002/jcc.21367 (2010). 
77. Vanommeslaeghe, K., Raman, E. P. & MacKerell, A. D. Automation of the CHARMM General Force Field (CGenFF) II: Assignment of Bonded Parameters and Partial Atomic Charges. J. Chem. Inf. Model. 52, 3155–3168, doi:10.1021/ci3003649 (2012). 
78. Vanommeslaeghe, K. & MacKerell, A. D. Automation of the CHARMM General Force Field (CGenFF) II: Bond Perception and Atom Typing. J. Chem. Inf. Model. 52, 3144–3154, doi:10.1021/ci300363x (2012). 
79. Harvey, M. J., Giupponi, G. & Fabritius, G. de ACEMD: Accelerating Biomolecular Dynamics in the Microsecond Time Scale. J. Chem. Theory Comput. 5, 1632–9, doi:10.1021/ct01090088 (2009). 
80. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. J. Mol. Graph. 14, 33–8, 27–8 (1996). 
81. Cunha, A. S. et al. Agmatine attenuates reserpine-induced oral dyskinesia in mice: Role of oxidative stress, nitric oxide and glutamate NMDA receptors. Behav. Brain Res. 312, 64–76, doi:10.1016/j.bbr.2016.06.014 (2016).
Competing Interests: The authors declare that they have no competing interests.

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

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017