Evidence for Heterodimerization and Functional Interaction of the Angiotensin Type 2 Receptor and the Receptor MAS

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Abstract—The angiotensin type 2 receptor (AT2R) and the receptor MAS are receptors of the protective arm of the renin–angiotensin system. They mediate strikingly similar actions. Moreover, in various studies, AT2R antagonists blocked the effects of MAS agonists and vice versa. Such cross-inhibition may indicate heterodimerization of these receptors. Therefore, this study investigated the molecular and functional interplay between MAS and the AT2R. Molecular interactions were assessed by fluorescence resonance energy transfer and by cross correlation spectroscopy in human embryonic kidney-293 cells transfected with vectors encoding fluorophore-tagged MAS or AT2R. Functional interaction of AT2R and MAS was studied in astrocytes with CX3C chemokine receptor-1 messenger RNA expression as readout. Coexpression of fluorophore-tagged AT2R and MAS resulted in a fluorescence resonance energy transfer efficiency of 10.8 ± 0.8%, indicating that AT2R and MAS are capable to form heterodimers. Heterodimerization was verified by competition experiments using untagged AT2R and MAS. Specificity of dimerization of AT2R and MAS was supported by lack of dimerization with the transient receptor potential cation channel, subfamily C-member 6. Dimerization of the AT2R was abolished when it was mutated at cysteine residue 35. AT2R and MAS stimulation with the respective agonists, Compound 21 or angiotensin-(1–7), significantly induced CX3C chemokine receptor-1 messenger RNA expression. Effects of each agonist were blocked by an AT2R antagonist (PD123319) and also by a MAS antagonist (A-779). Knockout of a single of these receptors significantly induced CX3C chemokine receptor-1 messenger RNA expression. Effects of each agonist were blocked by an AT2R antagonist (PD123319) and also by a MAS antagonist (A-779). Knockout of a single of these receptors made astrocytes unresponsive for both agonists. Our results suggest that MAS and the AT2R form heterodimers and that—at least in astrocytes—both receptors functionally depend on each other. (Hypertension. 2017;69:1128-1135. DOI: 10.1161/HYPERTENSIONAHA.116.08814.) ● Online Data Supplement

Key Words: AT2 receptor ▪ heterodimerization ▪ homodimerization ▪ MAS ▪ renin–angiotensin system

The renin–angiotensin system (RAS) is an essential regulator of blood pressure and fluid volume.1 The main effector hormone, angiotensin II, binds to the angiotensin type 1 receptor (AT1R) and the angiotensin type 2 receptor (AT2R). While AT1R mediates vasoconstriction, water and sodium retention, fibrosis, and inflammation, AT2R has opposing actions mediating vasodilation, natriuresis, antifibrosis, and anti-inflammation.2

Angiotensin-(1–7) [Ang-(1–7)] is another active hormone within the RAS. Ang-(1–7) is cleaved from angiotensin II by angiotensin-converting enzyme 2 and binds to the receptor MAS.3 AT2R and MAS both mediate tissue-protective and -regenerative effects, which are of striking similarity.4 Likewise, intracellular signaling coupled to these receptors, such as activation of phosphatases, seems widely identical.

Interestingly, in several studies, the effects of Ang-(1–7) could be blocked by the AT2R antagonist PD123319.4–7 Other recent publications showed the reverse: a blockade of effects of the AT2R agonist, Compound 21 (C21), by the MAS antagonist, A-779.4–7 As recently reviewed by us in detail, this cross-inhibition may be because of insufficient selectivity of AT2R and MAS agonists and antagonists.4 However, because...
cross-inhibition is a common feature of dimerizing receptors, it may indicate dimerization of AT2R and MAS.

This study was designed to test by 2 independent methods, fluorescence resonance energy transfer (FRET) and fluorescence cross correlation spectroscopy (FCCS), whether AT2R and MAS form homodimers or heterodimers. Furthermore, we studied a potential functional dependence of both receptors using astrocytes in primary culture naturally expressing both AT2R and MAS.

Material and Methods
Detailed methods are provided in the online-only Data Supplement.

Fluorescence Resonance Energy Transfer
Receptor cDNA was subcloned into vectors encoding various fluorophores. A point mutation of cysteine residue 35 (C35) of AT2R was generated using site-directed mutagenesis and the mutated receptor tagged with YFP (yellow fluorescent protein; AT2R-C35A-YFP). Plasmids were transiently transfected into human embryonic kidney 293 cells. FRET was measured by gradual acceptor photobleaching in cells from at least 3 independent transfections each involving at least 12 single cells. Sensitized emission FRET measurements were performed to evaluate whether stimulation of MAS or AT2R with respective agonists (C21 or Ang-(1–7); 1 μmol/L) has any impact on MAS/AT2R dimerization.

Fluorescence Cross Correlation Spectroscopy
We used FCCS in transiently transfected human embryonic kidney 293 cells coexpressing the GFP (green fluorescent protein)- or mCherry-tagged receptor constructs to monitor fluorescent fluctuations of the fluorescently tagged receptors AT2R and MAS using a LSM710-ConfoCor3 system.

Astrocyte Cell Culture Experiments
Astrocyte cultures (>98% purity) were prepared from brains of 2-day-old C57BL/6J, AT2R-knockout (AT2R-KO), or MAS-knockout mice on postnatal day 2. Cells were incubated for 6 hours with Ang-(1–7) (1 μmol/L), C21 (1 μmol/L), A-779 (10 μmol/L), or PD123319 (10 μmol/L) or a combination of agonists/antagonists. Tumor necrosis factor-α (TNFα; 10 ng/mL; Gibco, Carlsbad, CA) served as an internal control to assure technical accuracy. Gene expression of CX3C chemokine receptor-1 (CX3CR1) was quantified by real-time polymerase chain reaction. Data were normalized to hypoxanthine-guanine phosphoribosyltransferase and analyzed according to the ΔΔCt method.

Statistical Analysis
Statistical analyses were performed using Prism 5.0 (GraphPad, San Diego, CA). Data are presented as the mean±SEM. The statistical significances of differences in mean values were assessed by 1-way analysis of variance with Bonferroni post hoc test. Differences were considered significant at a P value <0.05.

Results
Molecular Interaction of Receptor MAS and the AT2R
Fluorescence Resonance Energy Transfer
Successful transfection of human embryonic kidney 293 cells and expression of CFP (cyan fluorescent protein)-tagged MAS and AT2R-YFP on cell membranes was verified by fluorescence microscopy (Figure 1A). Applying FRET with gradual acceptor photobleaching, MAS-AT2R-heterodimerization was strongly indicated by a FRET efficiency of 10.8±0.8% (Figure 1B, first column). AT2R and MAS also formed homodimers as indicated by FRET efficiencies of 9.2±0.7% (for AT2R; Figure 1C) or 7.4±0.8% (for MAS; Figure 1D).

To verify specificity of dimerization, a series of controls was performed. To allow binding competition between tagged and untagged receptors, an equal amount of fluorophore-tagged and -untagged vectors encoding MAS or AT2R were transfected. Co-transfection with 50% untagged receptors resulted in a significant, >50% reduction of FRET efficiency, strongly suggesting that the dimerization really occurred at the receptor level and not between fluorophores (Figure 1B through 1D, second columns). In another control experiment, plasmid-cDNA encoding the fluorophore-tagged receptors was cotransfected with an equal amount of empty vectors. Because this did not affect FRET efficiency, it can be assumed that it was not the reduction of the plasmid concentration that influenced the lowering of the FRET efficiency on the competition test (Figure 1B through 1D, third columns).

YFP-tagged transient receptor potential cation channel, subfamily C, member 6, an ion channel with 6 transmembrane domains, served to prove that AT2R and MAS would not dimerize with every other receptor displaying similar subcellular distribution. Neither coexpression of AT2R-CFP nor coexpression of MAS-CFP with transient receptor potential cation channel, subfamily C, member 6-YFP resulted in significant FRET efficiency, indicating that the FRET signal for AT2R and MAS homo- and heterodimerization was specific (Figure 1C and 1D, fourth columns).

Based on the observation by Miura et al that homodimerization of AT2R relies on disulfide bonding between C35 in one and C290 in the other receptor of the dimer, we tested whether a point mutation of C35 of AT2R would affect the efficiency of MAS/AT2R heterodimerization. Cells transfected with vectors encoding mutant AT2R-C35A-YFP and nonmutant MAS-CFP showed a significantly decreased FRET efficiency of 3.9±1.2%, suggesting that C35 is indeed essential for the formation of AT2R-MAS dimers (Figure 1B, fourth column). Furthermore, we confirmed that C35 is essential for AT2R homodimerization (Figure 1C, fifth column).

To determine whether stimulation of MAS (with 1 μmol/L Ang-(1–7)) or AT2R (with 1 μmol/L C21) has an impact on MAS/AT2R dimerization, we performed dynamic FRET. None of the agonists induced a significant change in FRET efficiency on the competition test (Figure 2).

Fluorescence Cross Correlation Spectroscopy
To provide evidence for AT2R/MAS dimerization by a second method, human embryonic kidney 293 cells were cotransfected with MAS-GFP and AT2R-mCherry for FCCS, which requires low expression of fluorescently tagged receptors in the cells. The confocal volume was focused to the plasma membrane of the cells. Normalized autocorrelation and cross-correlation curves were analyzed. Cross-correlation amounted to ≈11%, which was significantly more (P<0.001) than the autocorrelation (8%) obtained from the respective fluorophores (GFP and mCherry), thus, strongly suggesting heterodimerization of the 2 receptors (Figure 3).
Experiments investigating a potential functional interaction between the AT2R and MAS were performed in astrocytes isolated from C57BL/6J mice. Expression of AT2R and MAS was confirmed in these cells by reverse transcription polymerase chain reaction (Figure 4). Importantly, knockout of 1 receptor did not affect expression of the other.

CX3CR1, which was identified in a prestudy to be significantly increased by AT2R or MAS stimulation in mouse astrocytes in primary culture, served as read-out to investigate potential functional interactions between AT2R and MAS.

**Effect of AT2R/MAS Agonists on CX3CR1 Expression**

Initially, we confirmed that stimulation of MAS with Ang-(1–7) (1 μmol/L) for 6 hours significantly increased CX3CR1 mRNA expression (Figure 5A). This effect was blocked by the MAS antagonist A-779 (10 μmol/L) and was absent in astrocytes isolated from MAS-deficient mice. The effect of TNFα (10 ng/μL) on CX3CR1 expression served as an internal control to assure technical accuracy especially in experiments with an otherwise negative response. TNFα indeed significantly reduced CX3CR1 expression (Figure 5A through 5D).

Similarly, AT2R stimulation with C21 (1 μmol/L) resulted in increased CX3CR1 mRNA expression, an effect that was blocked with the AT2R antagonist PD123319 (10 μmol/L) and absent in cells isolated from AT2R-KO mice (Figure 5B). Again, the assay was responsive to TNFα stimulation.

**Cross-Inhibition of MAS and AT2R Effects**

The phenomenon of cross-inhibition between AT2R and MAS was confirmed in our experimental setting by inhibition of C21-induced CX3CR1 expression by the MAS antagonist A-779 (Figure 5C) and by inhibition of Ang-(1–7)-induced CX3CR1 expression by the AT2R antagonist PD123319 (agonists, 1 μmol/L; antagonists, 10 μmol/L; Figure 5D).

Because the observed cross-inhibition could also be a result of unselectivity of the agonists or antagonists, we tested whether genetic deletion of one receptor would have an impact...
on the function of the other receptor. Knockout of MAS prevented the C21-induced increase in CX3CR1 expression (Figure 5C), despite the fact that—as shown in Figure 4—AT2R was still present. The same was true in reverse: knockout of AT2R prevented the increase in CX3CR1 expression by Ang-(1–7), although the receptor MAS was still present (Figure 5D). Co-stimulation of cells by Ang-(1–7) plus C21 did not result in an additive or synergistic effect (Figure 5E).

**Discussion**

This study was performed to clarify whether the phenomenon of cross-inhibition, which has been repeatedly reported for AT2R and MAS,\(^4,4\) may be because of heterodimerization of these receptors and, if so, whether dimerization of these receptors is of functional relevance.

The main findings of this study were, first, strong evidence for heterodimerization of AT2R and MAS derived by 2 distinct methods, FRET and FCCS, and, second, evidence for functional dependence of AT2R and MAS on each other, at least in mouse astrocytes in primary culture.

G-protein–coupled receptors, including those of the RAS, have a high tendency to form oligomers, thus, influencing each other’s function.\(^5,12,20–23\) Regarding receptors of the RAS, homodimerization has been described for AT1R\(^3\) and AT2R\(^19,20\) and heterodimerization for AT1R/AT2R,\(^29\) AT1R/MAS,\(^22,24,25\) AT1R/B2-bradykinin receptor (although this has also been questioned),\(^26–29\) AT2R/B2-bradykinin receptor,\(^30\) MAS/B2-bradykinin receptor,\(^31\) AT1R/β-adrenergic receptor,\(^12\) AT1R/dopamine D1, D3, and D5 receptors,\(^32–34\) and AT1R/endothelin B receptor.\(^35\) In our study, we could confirm AT2R homodimerization and in addition found evidence for MAS homodimerization and AT2R/MAS heterodimerization, the latter using 2 independent methods, FRET and FCCS.

To exclude random colocalization and to support specificity of AT2R/MAS heterodimerization, we performed a series of control experiments. In competition assays, equal amounts of fluorophore-tagged and -untagged AT2R and MAS were coexpressed, resulting in a ±50% reduction in FRET efficiency, thus, strongly indicating specific AT2R/MAS heterodimerization. In contrast, the lack of reduction of FRET efficiency after coexpression of equal amounts of empty vectors with tagged AT2R or MAS excluded that reduced FRET efficiency in the competition tests was only because of the reduction of the total number of tagged plasmids and also that unspecific protein aggregation accounted for the measured FRET efficiency. Specificity of dimerization was further substantiated by FRET experiments with either CFP-tagged AT2R or CFP-tagged MAS and an unrelated YFP-tagged receptor, for which transient receptor potential cation channel, subfamily C, member 6, an ion channel with 6 transmembrane domains, was chosen.\(^36\) FRET efficiency of <1 clearly indicated that neither AT2R nor MAS formed dimers with transient receptor potential cation channel, subfamily C, member 6, thus, supporting specificity of AT2R/MAS heterodimerization.

AT2R homodimerization was reported to depend on disulfide bonds between the extracellular C35 of one receptor and of C290 of the other dimerization partner.\(^39\) In our hands, mutation at C35 of AT2R reduced FRET efficiency of AT2R/MAS heterodimerization by >50%, thus, strongly indicating that C35 is also critical for AT2R/MAS heterodimerization most likely by being the anchoring point for a disulfide bridge.

Dimer formation can occur either constitutively/ligand independently or it can be induced/enhanced on ligand binding.\(^37\) With respect to dimers that include receptors of the RAS, ligand-independent dimerization seems by far prevalent as shown for homodimerization of AT1R and AT2R or heterodimerization of AT1R/AT2R, AT1R/MAS, AT1R/B2, and AT1R/β-adrenergic receptors.\(^19,21,26\) In our study, dynamic

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**Figure 3.** Fluorescence cross correlation spectroscopy (FCCS). Human embryonic kidney 293 (HEK293) cells transfected with AT2R-mCherry/MAS-GFP (green fluorescent protein) or fluorophore-tagged, empty plasmids as control. AT2R/MAS cross-correlation was significantly higher (∼11%) than autocorrelation of empty plasmids (8%). Values are means±SEM from 3 independent experiments. n=100. **P<0.001. AT2R indicates angiotensin type 2 receptor.**
A V0991, could be blocked by PD123319.39 Because 2 stud-

FRET experiments revealed that AT2R/MAS heterodimeriza-
tion was ligand independent, too, because addition of C21 or 

Although cross-inhibition strongly speaks in favor of 
dimerization and functional dependence of 2 receptors, our 

B AVE0991 has no (or extremely low) affinity for AT2R38,40 and because PD123319 has only very low affinity for MAS,1 the inhibition of AVE0991-

Another explanation for the inhibition of Ang-(1–7) 
effects by PD123319 may be that PD123319 is also an antag-

Because according to the above considerations, pharma-
cological studies cannot provide final proof whether the phe-

Although our data provide strong evidence for AT2R/MAS 
heterodimerization and functional dependence, data can be 

Evidence for functionality of MAS in the absence of AT2R can be found in 2 studies, one of 
them showing Ang-(1–7)-induced lowering of blood pres-
sure,6 the other Ang-(1–7)-induced prevention of neointima 
formation in AT2R-KO.44 However, in the latter study, the 
effect of Ang-(1–7) was less strong in AT2R-KO compared 
with wild-type mice, which may again point to dependence 
of MAS on AT2R expression for full functionality at least 
in some tissues or under certain conditions. Dimerization of 
G-protein–coupled receptors can in fact be tissue/cell-specific 
or depend on environmental conditions, such as density of 
receptor expression, pathological conditions, or receptor 
maturaton.45-48
It is a limitation of this study that although we observed changes in CX3CR1 expression by AT2R/MAS stimulation, we did not further examine the physiological relevance of this observation, which would have required confirmation of this effect on protein level. According to the literature, binding of the chemokine fractalkine to its receptor CX3CR1 acts anti-inflammatory in the central nervous system by controlling microglia activation, which results in a neuroprotective effect, for example, in brain injury. Therefore, it can be speculated that an AT2R-mediated increase in CX3CR1 expression could be part of the mechanism underlying the neuroprotective effect of AT2R stimulation.

**Perspectives**

The present study provides evidence that MAS and AT2R heterodimerize and are functionally dependent on each other.
biological relevance of oligomerization can be multifaceted and impact receptor expression and function, including receptor trafficking, agonist binding, G-protein coupling, potency, efficacy, and G-protein selectivity. For example, heterodimerization of AT1R/AT2R or AT1R/MAS leads to suppression of AT1R signaling in a ligand-independent manner, while heterodimerization of AT1R or MAS with the bradykinin B2 receptor enhances their affinity or activity, respectively. Furthermore, AT1R homodimers may be of pathophysiologic relevance because these dimers are increased in hypertensive patients and can be reduced by antihypertensive treatment with angiotensin-converting enzyme inhibitors.

Cross-inhibition as result of oligomerization can be of major pharmacological relevance. Generally, cross-inhibition probably occurs much more often than currently known and may be causative for side effects of respective drugs because of inhibition of dimerization partners in addition to inhibition of the actually targeted receptor. Furthermore, receptor oligomerization may become of relevance in drug development, for example, by future drugs, which specifically target oligomers, but not monomers, or vice versa, thus, inducing/blocking distinct signaling pathways.

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Disclosures
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References
7. Theilade J, Haunsø S, Sheikh SP. Oligomerization of wild-type and nonfunctional mutant angiotensin II type I receptors inhibits ghrelin protein signaling but not ERK activation.
12. Hansen JL, Theilade J, Haunsø S, Sheikh SP. Oligomerization of wild-type and nonfunctional mutant angiotensin II type I receptors inhibits ghrelin protein signaling but not ERK activation.
27. AbdAlla S, Lother H, Abi-Abib R, Fernandes MS, Santos DP, Caruso-Neves C. Angiotensin II and angiotensin-(1-7) inhibit the inner cortex Na+ -ATPase activity through AT2 receptor.


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Novelty and Significance

What Is New?

- This study is the first to provide evidence that the angiotensin type 2 receptor (AT2R) and the receptor MAS form heterodimers and that they functionally depend on each other.

What Is Relevant?

- These findings are of major relevance for the understanding of 2 receptors of the RAS, which is a prime system for blood pressure control. Apart from an advanced understanding of AT2R and MAS function, our findings are also important for the interpretation of research data, in particular, when using AT2R/MAS antagonists and for the prediction and understanding of side effects of future drugs targeting AT2R and MAS.

Summary

AT2R and MAS form constitutive heterodimers. Heterodimerization is essential for the receptors to be functional—at least in certain cell types.
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EVIDENCE FOR HETERODIMERIZATION AND FUNCTIONAL INTERACTION OF THE ANGIOTENSIN AT2-RECEPTOR AND THE RECEPTOR MAS

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MATERIAL AND METHODS

HEK293 Cell Culture
Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, Virginia, USA) were cultured in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F12 (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a 5% CO2 atmosphere at 37° C.

Construction of fluorescent or non-fluorescent protein expression vectors

MAS-CFP; MAS-YFP; MAS-GFP, AT2R-CFP, AT2R-YFP and AT2-mCherry:
cDNA of human MAS or AT2R was amplified by PCR using MAS or AT2R expression vectors as templates (Imagenes GmbH, Berlin, Germany). cDNA was subcloned into vectors containing yellow (YFP), cyan (CFP), green (GFP) or red (mCherry) fluorescent proteins. Vectors used for this purpose being tagged or untagged with fluorescent protein cDNA (pcDNA3-CFP, pcDNA3-YFP or pcDNA3.1) were a kind gift by Dr. Michael Schäfer, University of Leipzig, Germany. pmCherry-N1 and pEGFP-N1 were obtained from Clontech (Mountain View, CA). 5’-end cleaving site (EcoRI) and the translation stop codon of MAS or AT2R were added with the insertion of the cleavage site (XbaI). All subcloned plasmids were verified by DNA sequencing (GATC Biotech AG, Konstanz, Germany).
**AT2R-C35A-YFP:**
Site-Directed Mutagenesis (QuikChange™ Site-Directed Mutagenesis Kit) was performed, using the AT2R-CFP expression vector (described above) as template and high fidelity polymerase (PfuUltra high-fidelity DNA Polymerase, Agilent Technologies) to replace the codon that will transduce the Cysteine to an Alanine at the 35 position of the AT2R (AT2R-C35A).

Fw- AT2-C35A : 5'-AGTCTACCTTGAACGCTCACAGAAACCAT-3'
Rv-AT2-C35A: 5’-ATGGTTTCTGTGAGGCCTCAAGGTGACT-3'

**TRPC6-CFP:**
This vector was kindly donated by Dr. Michael Schaefer, University of Leipzig, Germany.

**HEK293 Cell Transfection**
For FRET experiments, HEK293 cells were transiently transfected with 3 µg of total plasmid cDNA using 6 µg polyethylenimine (Sigma-Aldrich, St. Louis, Missouri, USA). For every set of experiments relative CFP and YFP fluorescence intensities were determined for several ratios of plasmids, not exceeding 3 µg of total plasmid. Only cells with a plasmid ratio emitting a fluorescence intensity within the ratio (YFP:CFP) of approximately 3 were used for all FRET experiments to minimize CFP-only multimers. Furthermore, only cells with a certain brightness (between 100 and 500 units) were used to control for similar expression levels between experiments.

**Acceptor Photobleaching FRET for Detection of Heterodimers**
To test whether there is a physical interaction between MAS and AT2R, we measured Fluorescence Resonance Energy Transfer (FRET) by gradual acceptor photobleaching. HEK293 cells were transiently transfected with vectors encoding the respective receptor of interest tagged with a donor fluorophore and the other receptor of interest with an acceptor fluorophore. The concept of FRET is that if both receptors are expressed in close spacial proximity (less than 10 nm), energy will be transferred from the donor to the acceptor in a non-radiative way, resulting in a reduced donor energy emission.

Cells were seeded on cover slips and covered with HEPES-buffered solution containing 128 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L MgCl₂, 5.5 mmol/L glucose, 10 mmol/L HEPES, 1 mmol/L CaCl₂, 2 mg/ml BSA at a pH of 7.4 for FRET measurements performed 24 hours after transfection.

FRET was evaluated using an inverted epifluorescent microscope (DMI6000B, Leica Microsystems) with a 63x/1.4 objective using the Leica Application Suite Advanced Fluorescence Software. The FRET efficiencies were determined by the acceptor photobleaching protocol, which monitors the increase in the CFP (FRET-donor) fluorescence emission during selective YFP (FRET-acceptor) photobleaching similar to the protocol of Hellwig et al. ¹. Briefly, the protocol started with 15 cycles for the baseline detection of the fluorophores (each cycle consisting of 12 ms excitation at 436/12 nm for CFP-detection and 20 ms at 500/20 nm for YFP detection using Leica filter cube N2.1). The next 43 cycles applied illumination between 515-560 nm for 1800 ms/cycle (additionally to the detection measurements) to the cells to quench the YFP fluorophore. Fluorophore brightness, fluorophore ratio, and FRET efficiency
were determined for each individual cell. FRET efficiencies were determined from at least three independent transfections each involving at least 12 single cells.

**Dynamic FRET after Application of Receptor Agonists**

To evaluate whether stimulation of MAS or the AT2R with respective agonists has any impact on MAS/AT2R dimerization, we recorded FRET efficiency after treatment of cells with C21 (1µmol/L) or Ang-(1-7) (1µmol/L), respectively. Sensitized emission FRET measurements [25] were performed, which allow real time FRET recordings in living cells to study the influence of substances on the FRET signal in the same cell with a high temporal resolution. HEK293 cells grown on a coverslip were co-transfected with vectors encoding MAS-YFP and AT2R-CFP, placed in a perfusion chamber and rinsed with HEPES-buffered solution (HBS) as previously described ². Briefly, FRET sensitized emission (SE) recordings were performed with 4 X 4 binning using an inverted epifluorescence microscope (DMI6000B, Leica Microsystems) with the manufacturer’s FRET SE wizard within the LAS AF software suite. SE recordings were performed for 50 seconds under basal conditions followed by stimulation of the cells with Ang-(1-7) (1µmol/L) or C21 (1µmol/L) and another 400 seconds of recording. FRET efficiency was calculated by correction for donor cross-talk and acceptor cross-excitation ³.

**Fluorescence cross correlation spectroscopy (FCCS)**

Fluorescence cross correlation spectroscopy (FCCS) allows the detection of fluorescence signals in living cells at a single molecule level. Principles of FCCS have been described previously ⁴,⁵. If two different fluorescent molecules are measured in a confocal volume, cross-correlation analyses with mathematical procedures can be performed. A significant cross-correlation then indicates parallel (non-random) movements and consequently interaction of the molecules. In contrast a lack of cross-correlation indicates independent diffusion and monomeric molecules. The confocal volume is defined by the focused laser beam of the laser scanning microscope (LSM), which was focused to the plasma membrane of the cells.

We used FCCS in order to monitor fluorescent fluctuations of the fluorescently tagged receptors AT2R and MAS. Transiently transfected HEK 293 cells co-expressing the GFP- or mCherry-tagged receptor constructs were grown as described above. FCCS measurements were performed at room temperature on a LSM710-Confocor3 system ⁶. GFP and mCherry fluorescence signals were recorded using a x40/1.2 numerical aperture water objective (GFP: argon laser, λex=488 nm, 505–540-nm band pass filter; mCherry: diode-pumped solid state laser λex=561 nm, 580-nm long pass filter), and the spectral parts were split using an MBS 488/561 and an NFT 565 beam splitter, respectively. Membranes were located by z-scans. Intensity fluctuations were recorded for 4s and 25 repetitions. Average autocorrelation and cross-correlation curves were derived from the fluctuations using the LSM710 software ZEN 2010 (Carl Zeiss MicroImaging GmbH). For average calculations, only convergent curves were used. The curves were normalized to the correlation amplitude for display purposes. The normalized form of the autocorrelation function is defined as follows:

$$ G(\tau) = \langle \delta F(t) \delta F(t + \tau) \rangle / F(t)^2 $$

where angle brackets indicate average times, and $\delta F(t) = F(t) - \langle F(t) \rangle$, the fluctuations around the mean intensity.
Correlation curves were derived using a two-component model of free diffusion in two dimensions with triplet fraction and offset for membrane-associated proteins (equation below) using the ZEN2010 software. A two-component model was used for the two-dimensional fits to obtain satisfactory fits. The first component was too fast to reflect membrane diffusion, and thus the diffusion time of the second component was considered to be significant. The analytical function of the model is described by the equation,

\[ G(\tau) = 1 + G_{\infty} + \frac{1}{N} \left( 1 + \frac{\tau e^{-\tau/\tau_{F}}}{1-T} \right) \left( \frac{1}{1+\tau/\tau_{D1}} + \frac{1-f}{1+\tau/\tau_{D2}} \right) \]

where \( G_{\infty} \) is the offset from 1, and \( N \) and \( T \) represent the total number of particles and the triplet fraction, respectively. \( \tau_{D1} \) and \( \tau_{D2} \) represent free diffusion times (the subscripts indicate the different molecule species) \( \tau_{F} \) is the triplet time, \( f \) and \( 1-f \) are the fractions of species 1 and 2, and \( \tau \) is the correlation time.

**Astrocyte Cell Culture Experiments**

Astrocytes were prepared from brains of C57BL/6J, AT2R-knockout (AT2R-KO) or MAS-knockout (MAS-KO) mice (knockouts were on a C57BL/6J background) on postnatal day 2 according to the protocol by McCarthy and de Vellis. All mice were obtained from the Department of Experimental Medicine (FEM), Charité, University Medicine Berlin. AT2R-KO mice were originally obtained from Prof. M. Horiuchi, Ehime University Graduate School of Medicine, Japan, and backcrossed 8 generations to C57BL/6J. MAS-KO mice were originally obtained from one of the authors, MB, Max-Delbrück-Center for Molecular Medicine, Berlin. Animal housing, breeding and care complied with the Guide for the Care and Use of Laboratory Animals of the State Government of Berlin, Germany. Sacrifice of animals for the purpose of organ sampling was approved by the State Government of Berlin (approval number: T0059/05). Briefly, brain tissue was triturated and dissociated with a Pasteur Pipette and transferred into cell culture flasks. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, PAA Laboratories GmbH, Pasching, Austria) supplemented with 10 % fetal bovine serum (FBS, PAA Laboratories GmbH, Pasching, Austria), 100 µg/ml streptomycin and 100 U/ml penicillin in a 5 % CO\(_2\) atmosphere at 37 °C. Purity of astrocytes was >98 % as estimated by GFAP and GLAST staining. Cells were used at passage 5. After reaching confluence, the medium was replaced by serum-deprived medium containing 0.5 % FBS, 100 µg/ml streptomycin and 100 U/ml penicillin for 24 h. For the experiments, cells were incubated with either the MAS agonist Ang-(1-7) (1 µmol/L, Bachem, Torrance, USA), the AT2R agonist Compound 21 (1 µmol/L, Vicore Pharma, Gothenburg, Sweden), the MAS antagonist A-779 (10 µmol/L, Bachem, Torrance, USA), the AT2R antagonist PD123319 (10 µmol/L, Sigma-Aldrich, St. Louis, USA) or a combination of agonists and/or antagonists. TNFα (10 ng/µl, Gibco, Carlsbad, USA), which has been shown to significantly reduce CX3CR1 levels in murine astrocytes, served as an internal control to assure technical accuracy. Incubation time was 6 hours. Antagonists were added 1 hour prior to the respective agonists. The number of independent experiments for every treatment group was at least three.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA from each sample was extracted using NucleoSpin RNA II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to manufacturers’ instructions.
The eluted RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI). After inactivation of DNase by the addition of the provided stop reagent, 1000 ng of mRNA were transcribed into cDNA using SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, USA). The gene expression of CX3CR1 was quantified by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA). Data were normalized to stably expressed hypoxanthine-guanine phosphoribosyltransferase (HPRT) and data analysis was performed according to the ΔΔCT method.

Primer sequences used for PCR were:
- CX3CR1, forward 5´-TGTCACCTCCTCCCTGAAGCTGAACCTG-3´
- reverse 5´-CGAACGTGAAGACGAGGGCGT-3´
- HPRT, forward 5´-TGAGCCATTGTGAGCGGCGGC-3´
- reverse 5´-CGCTAATCACGACGCTGGGACTG-3´.

All samples were measured in triplicate and non-template controls were used to confirm specificity.

Validation of MAS and AT2R Expression in Wild-type and Knockout Astrocytes
For receptor detection, real-time PCR was performed as described above. Gene primer sequences used for PCR were:
- MAS, forward 5´-CTCTGGTTCCTCTGCTTCCG-3´
- reverse 5´-CCTCTCCACACTGATGGCTG-3´
- AT2R, forward 5´-CTCACTGTTTTGTTGTC-3´
- reverse 5´-CAATGGTTCTGACATCC-3´.

Amplification products were confirmed by agarose gel electrophoresis. 2% agarose gels were stained with ethidium bromide, loaded with the PCR amplicons and DNA was visualized under ultra-violet light.

Statistical Analysis
Statistical analyses were performed using Prism 5.0 (GraphPad, San Diego, California, USA). Data are presented as the mean ± SEM. The statistical significances of differences in mean values were assessed by one-way ANOVA with Bonferroni post-hoc test. Differences were considered significant at a P value <0.05.

REFERENCES


