3-iodothyronamine differentially modulates α -2A-adrenergic receptor-mediated signaling

Juliane Dinter^{1,*}, Jessica Mühlhaus^{1,*}, Simon Friedrich Jacobi^{1,2}, Carolin Leonie Wienchol¹, Maxi Cöster³, Jaroslawna Meister³, Carolin Stephanie Hoefig², Anne Müller¹, Josef Köhrle⁴, Annette Grüters¹, Heiko Krude¹, Jens Mittag², Torsten Schöneberg³, Gunnar Kleinau¹ and Heike Biebermann¹

¹Institut für Experimentelle Pädiatrische Endokrinologie, Charité-Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany

²Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden

³Institut für Biochemie, Molekulare Biochemie, Medizinische Fakultät, University of Leipzig, Leipzig, Germany

⁴Institut für Experimentelle Endokrinologie, Charité-Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany

*J Dinter and J Mühlhaus contributed equally to this work

Correspondence
should be addressed
to H Biebermann

Email
heike.biebermann@charite.de

Abstract

Most *in vivo* effects of 3-iodothyronamine (3-T1AM) have been thus far thought to be mediated by binding at the trace amine-associated receptor 1 (TAAR1). Inconsistently, the 3-T1AM-induced hypothermic effect still persists in *Taar1* knockout mice, which suggests additional receptor targets. In support of this general assumption, it has previously been reported that 3-T1AM also binds to the α -2A-adrenergic receptor (ADRA2A), which modulates insulin secretion. However, the mechanism of this effect remains unclear.

We tested two different scenarios that may explain the effect: the sole action of 3-T1AM at ADRA2A and a combined action of 3-T1AM at ADRA2A and TAAR1, which is also expressed in pancreatic islets. We first investigated a potential general signaling modification using the label-free EPIC technology and then specified changes in signaling by cAMP inhibition and MAPKs (ERK1/2) determination. We found that 3-T1AM induced $G_{i/o}$ activation at ADRA2A and reduced the norepinephrine (NorEpi)-induced MAPK activation. Interestingly, in ADRA2A/TAAR1 hetero-oligomers, application of NorEpi resulted in uncoupling of the $G_{i/o}$ signaling pathway, but it did not affect MAPK activation. However, 3-T1AM application in mice over a period of 6 days at a daily dose of 5 mg/kg had no significant effects on glucose homeostasis. In summary, we report an agonistic effect of 3-T1AM on the ADRA2A-mediated $G_{i/o}$ pathway but an antagonistic effect on MAPK induced by NorEpi. Moreover, in ADRA2A/TAAR1 hetero-oligomers, the capacity of NorEpi to stimulate $G_{i/o}$ signaling is reduced by co-stimulation with 3-T1AM. The present study therefore points to a complex spectrum of signaling modification mediated by 3-T1AM at different G protein-coupled receptors.

Key Words

- ▶ G protein-coupled receptor
- ▶ adrenergic receptor
- ▶ thyronamine
- ▶ 3-T1AM

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Introduction

3-iodothyronamine (3-T1AM) is a decarboxylated and deiodinated derivative of the classical thyroid hormone levothyroxine (Scanlan 2009), and it was originally isolated from rat brain homogenates, but it is also detectable in peripheral blood and various peripheral organs, such as the liver and heart (Scanlan *et al.* 2004, Saba *et al.* 2010, Hoefig *et al.* 2011, Zucchi *et al.* 2014). Application of 3-T1AM in rodents, for example, results in a decreased body temperature (Scanlan *et al.* 2004), mediates a switch between lipid and glucose metabolism for energy consumption (Braulke *et al.* 2008), and shows dose-dependent effects on feeding behavior and body weight (Dhillon *et al.* 2009, Manni *et al.* 2012, Haviland *et al.* 2013). *In vitro*, the receptor target of 3-T1AM is assigned to the trace amine-associated receptor 1 (TAAR1). 3-T1AM activates TAAR1 via the G_s /adenylyl cyclase system (Scanlan *et al.* 2004). Inconsistently, the previously reported 3-T1AM effects on thermoregulation (Scanlan *et al.* 2004), which are hypothermic, still persist in *mTaar1* knockout mice (Panas *et al.* 2010), which suggests that there are additional receptor targets *in vivo*. It has been previously reported that 3-T1AM also binds to the α -2A-adrenergic receptor (ADRA2A), a receptor that influences glucose homeostasis (Regard *et al.* 2007), but the underlying mechanism is still unclear. We consequently hypothesized that signaling of ADRA2A is induced or modulated in the presence of 3-T1AM. In the present study, we assumed two possibilities for modulation: an influence of 3-T1AM either on ADRA2A alone or on a putative ADRA2A/TAAR1 hetero-oligomer. Interaction of ADRA2A with other G protein-coupled receptors (GPCRs) has been demonstrated to lead to uncoupling of the ADRA2A signal transduction pathway (Vilardaga *et al.* 2008). To test both possibilities, we characterized the signal transduction of ADRA2A and ADRA2A/TAAR1 hetero-oligomers in response to 3-T1AM respectively.

We first used the label-free EPIC technology (measurement of dynamic mass redistribution) and detected direct or indirect signaling modulation by 3-T1AM at ADRA2A. Classically, ADRA2A signals via $G_{i/o}$ (Bylund & Ray-Prenger 1989). Therefore, we expressed ADRA2A in HEK293 cells and tested receptor signaling related to $G_{i/o}$ and MAPKs directly and in combination with norepinephrine (NorEpi), the endogenous agonist of ADRA2A. Complementary to this, we characterized the capacity of TAAR1 and ADRA2A to form hetero-oligomers. Moreover, 3-T1AM has been shown to demonstrate high affinities to pancreatic islets, to increase blood glucose concentration

2 h following 3-T1AM application, and, in turn, to reduce insulin concentration (Regard *et al.* 2007). Therefore, we additionally examined the long-term effect of 3-T1AM application *in vivo* in order to examine whether insulin secretion remains blocked after 3-T1AM stimulation of Adra2a. Finally, our analysis revealed important functional aspects of 3-T1AM-mediated signaling by interaction with ADRA2A and provided detailed insights into a diverse and extended set of ligand–receptor interplays.

Material and methods

Cloning of ADRA2A and TAAR1

All human full-length constructs were amplified from genomic DNA or purchased (ADRA2A, Missouri S&T cDNA Resource Center, Rolla, MO, USA) and cloned into the eukaryotic expression vector pcDps. Constructs were N-terminally tagged with a hemagglutinin (5'-YPYDVP-DYA-3') epitope (NHA) or C-terminally tagged with a Flag epitope (C-Flag, 5'-DYKDDDDK-3'). Plasmids were sequenced and verified by BigDye-terminator sequencing (PerkinElmer, Inc., Waltham, MA, USA) using an automatic sequencer (ABI 3710xl; Applied Biosystems).

Cell culture and transient transfection

For cAMP and serum response element-luciferase (SRE-luc) measurements, HEK293 cells were seeded in poly-L-lysine-coated (Biochrom AG, Berlin, Germany) 96-well plates (15 000 cells/well). Transient transfection of HEK293 cells was performed 24 h after seeding in supplement-free advanced MEM (Life Technologies) using Metafectene (Biontex, Munich, Germany) according to the manufacturer's protocol.

Determination of Adra2a and Taar1 expression in pancreatic islets by RNA sequencing analysis

To determine GPCR expression levels in pancreatic islets, data from a recently performed RNA sequencing analysis were utilized (Meister *et al.* 2014). In brief, total RNA from isolated pancreatic islets (ten WT male 129S6 mice) was extracted and cDNA libraries were generated using TruSeq RNA Sample Preparation Kits v2 (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Indexed islet libraries of good quality were pooled and used for sequencing on two flow cell lanes on an Illumina

HiScanSQ System. Trimmed paired-end reads were mapped to the reference mouse genome (July 2007 NCBI37/mm9) with Ensembl v66 annotations using Tophat 1.3.3. (Trapnell *et al.* 2012), which aligns reads using Bowtie (version 0.9.9). Fragments per kilobase of transcript per million mapped reads (FPKM) values were calculated using Cufflinks version 1.3.0 (Roberts *et al.* 2011, Trapnell *et al.* 2012).

Measurement of cAMP accumulation

G_s and G_{i/o} signaling were determined by measuring cAMP accumulation. Forty-eight hours following transfection, stimulation was conducted with compounds diluted in a HEPES-buffered solution containing 1 mM 3-isobutyl-1-methylxanthine (Sigma–Aldrich) to inhibit cAMP degradation by phosphodiesterases. Cells were incubated for 40 min with 3-T1AM (Santa Cruz Biotechnology, Inc.) and/or NorEpi (Sigma–Aldrich). For G_{i/o} pathway examination, cells were co-stimulated with 50 μM forskolin (FSK, AppliChem GmbH, Darmstadt, Germany) in order to stimulate overall adenylyl cyclases. Substance incubation was performed in triplicate and at 37 °C with 5% CO₂ and was stopped by aspirating the medium. Cells were then lysed at 4 °C on a shaking platform. Intracellular cAMP accumulation was determined by a competitive immunoassay based on the AlphaScreen technology (PerkinElmer Life Science, Boston, MA, USA) as previously described (Kleinau *et al.* 2011).

Measurement of MAPK by luc reporter gene assay

MAPK activation was determined by luc activity in a luc reporter gene assay (SRE-luc; Promega). Cells were co-transfected with a reporter construct containing a SRE and the firefly luc reporter gene (SRE-luc, pGL4.33), together with either receptor (of equimolar concentrations in case of co-transfection) or empty vector plasmid DNA (mock transfection). Two days post-transfection, cells were incubated for 6 h with 3-T1AM and/or NorEpi in supplement-free MEM at 37 °C with 5% CO₂. Reaction was terminated by aspirating the media. Cells were lysed for 15 min on a shaking platform at room temperature using 1× passive lysis buffer (Promega). Measurement was conducted with automatic luc substrate injection of 40 μl in a black 96-well plate using a Berthold Microplate Reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The thyrotropin (TSH) receptor served as a positive assay control and was stimulated with 100 mU/ml bovine TSH (Sigma–Aldrich).

Determination of hetero-oligomerization by sandwich-ELISA

Receptor–receptor oligomerization was determined by a sandwich-ELISA as described previously (Piechowski *et al.* 2013). In brief, N-terminally HA-tagged ADRA2A and C-terminally Flag-tagged TAAR1 (or *vice versa*) were co-expressed in COS-7 cells. These cells were selected because they are more robust than HEK293 cells, which is beneficial in light of the numerous washing steps of the ELISA. The growth hormone secretagogue receptor (GHSR) homo-oligomer served as a positive control (Rediger *et al.* 2011), whereas N-HA-tagged/C-Flag-tagged rat muscarinic 3 receptor (rM3R) acted as a ‘non-interaction’ partner. N-HA-tagged ADRA2A and TAAR1 served as a negative control (mock). Cells were harvested and lysed. Proteins were then added to an anti-Flag-MAB (Sigma–Aldrich) coated plate. HA-tagged protein was determined using a biotin-labeled anti-HA-MAB (Roche) with the addition of a streptavidin-labeled HRP that converted *o*-phenyldiamine. Absorption was measured at 450 nm with correction at 620 nm using an Anthos Reader 2001 (AnthosLabtech Instruments, Salzburg, Austria). Protein concentrations were analyzed by the Biuret method using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in accordance with the manufacturer’s protocol.

Determination of cell surface expression

Cell surface expression of ADRA2A, TAAR1, and the hetero-oligomer was determined by a surface ELISA in COS-7 cells transiently co-transfected with equimolar concentrations of N-HA-tagged ADRA2A (N-HA-ADRA2A) or TAAR1 (N-HA-TAAR1) and C-Flag-tagged rM3R. COS-7 cells were used because they are more adherent after numerous washing steps than HEK293 cells are. To investigate whether ADRA2A surface expression is influenced by TAAR1 or *vice versa*, N-HA-tagged receptors were co-expressed with C-Flag-tagged receptors. Cells co-transfected with mock and rM3R-C-Flag served as a negative control. N-HA-ADRA2A and rM3R-C-Flag represented the positive control, because ADRA2A expression at the cell surface is not altered by the non-interaction partner rM3R and does not form hetero-oligomers. Forty-eight hours post-transfection, cells were fixed, blocked, and incubated with biotin-labeled anti-HA antibody (Roche; 1:200). Bound antibodies were detected with HRP-labeled streptavidin (BioLegend, London, UK; 1:2500). Color reaction was performed as described

previously (Schoneberg *et al.* 1996), and absorption was measured at 492/620 nm using an Anthos Reader 2001.

i.p. glucose tolerance test and liver glycogen determination

C57BL/6J male mice that were 3–4 months old were housed in single cages at 21–22 °C on a 12 h light:12 h darkness cycle with *ad libitum* food and water. 3-T1AM was dissolved in 60% DMSO and 40% physiological saline (pH 7.4). Either daily doses of 5 mg/kg 3-T1AM (5 μ l/g) or the same volume of vehicle (identical DMSO concentration as compared to 3-T1AM) were administered via i.p. injection for 7 days. Daily food intake was measured before and during the sham/treatment period.

Six days following the injection, animals were moved to a clean cage and fasted overnight (~16 h) with continued access to water. Basal blood glucose (tail tip cut) was measured by Accu Check Aviva (Roche Diagnostics). Two grams per kilogram glucose were given via i.p. injection using a 20% glucose solution, and blood glucose levels were taken after 0, 15, 30, 60, and 120 min for a glucose tolerance test (GTT). After the final measurement, animals were replaced in cages with *ad libitum* food.

On day 8, the mice were killed and their livers were snap-frozen in liquid nitrogen. All animal care procedures were in accordance with the guidelines set forth by the European Community Council Directives (86/609/EEC) and were approved by Stockholm's Norra Djurförsöksetiska Nämnd.

Glycogen levels were determined in the liver samples of both groups using an extraction protocol as previously described (Vujovic *et al.* 2009).

Quantitative real-time PCR

RNA was extracted from liver using the RNeasy Mini Kit (Qiagen) followed by cDNA synthesis with the transcriptor first-strand cDNA synthesis kit (Roche). Quantitative real-time RT-PCR was carried out with SYBR Green PCR Master Mix (Roche) using the 7300 Real-Time PCR System (Applied Biosystems). Primer sequences of phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate kinase (PYRK) have been previously published (Sjogren *et al.* 2007). To ensure PCR quality and efficiency, melting curves were recorded for every measurement and a standard curve was used. Results were normalized by comparison with the reference gene hypoxanthine phosphoribosyltransferase.

Results

Modulation of ADRA2A signaling by 3-T1AM

To test a general 3-T1AM signaling effect on ADRA2A, we took advantage of the label-free EPIC technology (dynamic mass redistribution assay, Corning Epic Biosensor Measurements, Corning Inc., New York, NY, USA) to prescreen signaling events (detailed methodology described in the [Supplementary Material and methods](#), see section on [supplementary data](#) given at the end of this article). In short, the Corning Epic system detects potential cell signaling mechanisms by biosensorial measurement of cellular mass redistributions during the signaling process in living cells. The wavelength shift of reflected light therefore indicates the relocation of the intracellular components that are triggered in response to potential receptor activation. HEK293 cells transiently transfected with ADRA2A and stimulated with NorEpi (10^{-5} M) served as a positive control and demonstrated robust positive signaling as compared to non-stimulated cells ([Supplementary Figure 1A](#)). HEK293 cells that express ADRA2A were then incubated with 3-T1AM (10^{-6} M). This resulted in a decreased trend in EPIC signaling as compared to non-stimulated (basal) ADRA2A ([Supplementary Figure 1B](#)). To investigate whether 3-T1AM influences NorEpi-induced signaling, we co-stimulated ADRA2A with both ligand types NorEpi (10^{-5} M) and 3-T1AM (10^{-6} M). Interestingly, signaling activity was significantly increased as compared to NorEpi alone ([Supplementary Figure 1A](#)).

3-T1AM activates $G_{i/o}$ via ADRA2A

To clarify whether 3-T1AM induces signaling at ADRA2A, we investigated the effect of 3-T1AM on the activation of $G_{i/o}$, the primary signaling pathway of ADRA2A. HEK293 cells that express ADRA2A were incubated with 50 μ M FSK in the presence of NorEpi (10^{-5} M) as well as 3-T1AM (10^{-5} M). 3-T1AM caused a significant 26% reduction of the FSK signal as compared to a 63% reduction with NorEpi ([Fig. 1A](#)), which confirms that the marginal signal observed by the EPIC technology has a signal transduction counterpart.

We also performed ligand co-stimulation in HEK293 cells that express ADRA2A treated with constant concentrations of 10^{-5} M NorEpi ([Fig. 1B](#), dotted line indicates the $\pm$ S.E.M. area of NorEpi (10^{-5} M) alone) and with increasing amounts of 3-T1AM (10^{-9} – 10^{-5} M). Co-stimulation of NorEpi with 3-T1AM decreased the NorEpi-induced $G_{i/o}$ activation and thereby increased cAMP concentrations (graph

Figure 1

3-T1AM stimulates and modulates $G_{i/o}$ and MAPK activation at ADRA2A. cAMP accumulation was measured in HEK293 cells that express ADRA2A or empty vector (mock). Data are displayed as mean $\pm$ s.e.m. fold over mock, stimulated with the respective ligand and ligand at indicated concentrations. Data are based on four independent experiments, each performed in triplicate (A and B) MAPK activity was determined using a serum response element-luciferase (SRE-luc) reporter assay. SRE-luc levels are displayed as original values in mean $\pm$ s.e.m. relative light units (RLU). Data are based on three independent experiments performed in triplicate (C and D). (A) Cells were co-incubated with 50 μ M forskolin (FSK) and 10^{-5} M NorEpi or 10^{-5} M 3-T1AM. Statistical analysis was performed with an unpaired two-tailed *t*-test with Welch correction in comparison to stimulated mock; * $P \leq 0.05$, and *** $P \leq 0.001$. (B) Cells were co-incubated

with a constant concentration of 10^{-5} M NorEpi and varying concentrations of 3-T1AM (10^{-9} – 10^{-5} M) in the presence of 50 μ M FSK. Dotted lines indicate $\pm$ s.e.m. of NorEpi-induced signaling (10^{-5} M). Statistical significance was determined by one-way ANOVA; * $P \leq 0.05$. (C) HEK293 cells that express ADRA2A were incubated with 10^{-5} M NorEpi or 10^{-5} M 3-T1AM, and SRE-luc levels were measured. A one-way ANOVA with a Kruskal–Wallis test was carried out for statistical analysis. Significances are calculated in comparison to basal (untreated) levels; ** $P \leq 0.01$. (D) Cells were co-incubated with constant concentrations of 10^{-5} M NorEpi and varying concentrations of 3-T1AM (10^{-10} – 10^{-5} M). The dotted line indicates $\pm$ s.e.m. of NorEpi-induced signaling. Statistical significance was determined by one-way ANOVA; * $P \leq 0.05$ and ** $P \leq 0.01$.

above the dotted line represents the $\pm$ s.e.m. range of NorEpi stimulation).

3-T1AM antagonizes NorEpi-induced MAPKs signaling at ADRA2A

ADRA2A is known to signal additionally via β -arrestin activation (Shenoy *et al.* 2006). We determined MAPK activation as a read-out system for β -arrestin activation. HEK293 cells that express ADRA2A strongly responded to NorEpi stimulation with MAPK activation (Fig. 1C). However, 3-T1AM did not induce MAPK signaling at ADRA2A (Fig. 1C), which indicates that 10^{-5} M 3-T1AM is a biased ligand at ADRA2A and only stimulates $G_{i/o}$

(Fig. 1A). 3-T1AM was not observed to influence MAPK signaling at TAAR1 (Supplementary Figure 2A, see section on supplementary data given at the end of this article).

To investigate whether 3-T1AM modulates NorEpi-induced MAPK activation, we performed co-stimulation studies of a constant concentration of NorEpi (10^{-5} M) with increasing concentrations of 3-T1AM (10^{-10} – 10^{-5} M) (Fig. 1D). The highest concentration of 3-T1AM (10^{-5} M) reduced NorEpi-induced MAPK activation by about 67%, whereas the lowest concentration (10^{-10} M) did not significantly modify MAPK signaling (Fig. 1D). In addition to a reduction of NorEpi-induced ERK signaling in the presence of 10^{-5} M 3-T1AM, we also observed significant reduction in the presence of 10^{-7} and 10^{-9} M.

Figure 2

RNA sequencing demonstrates significant expression of *Adra2a* and *Taar1* in mouse pancreatic islets. Receptor expression in the pancreatic islets of ten male mice was determined by RNA sequencing analysis. The glucagon-like peptide 1 receptor (*Glp1r*) served as a positive control. Data are represented as mean $\pm$ s.d. fragments per kilobase of transcript per million mapped reads (FPKM): *Adra2a* (15.1 ± 2.0), *Taar1* (3.40 ± 0.7), and *Glp1r* (96.5 ± 15.1) are highlighted in red.

Adra2a and Taar1 are both expressed in mouse pancreatic islets

To investigate the *in vivo* relevance of 3-T1AM action on TAAR1 and ADRA2A, both GPCR transcripts were first quantified by RNA sequencing analysis. *Adra2a* (15.1 ± 2.0 FPKM) and *Taar1* (3.40 ± 0.7 FPKM) were found to be significantly expressed in mouse pancreatic islets (upper 5% of 851 annotated mouse GPCRs, Fig. 2).

The formation of ADRA2A/TAAR1 hetero-oligomers impairs the capacity for ADRA2A-mediated $G_{i/o}$ signaling

In order to test the hypothesis of whether 3-T1AM acts at the receptors ADRA2A and TAAR1, we characterized both receptors for their hetero-oligomerization capacity. Via a sandwich-ELISA approach (detailed methodology described in the [Supplementary Material and methods](#)), we demonstrated that TAAR1 homo-oligomerized and, furthermore, that TAAR1 and ADRA2A have the capacity to form hetero-oligomers (Fig. 3).

Next, we questioned whether the interaction of ADRA2A and TAAR1 influences signaling properties.

Similarly to experiments performed with ADRA2A alone, HEK293 cells were co-expressed with ADRA2A and TAAR1 and incubated with a concentration of 10^{-5} M NorEpi alone or in combination with increasing concentrations of 3-T1AM (10^{-9} – 10^{-5} M). Co-transfection of ADRA2A with TAAR1 completely abolished the NorEpi-induced

Figure 3

TAAR1 and ADRA2A are able to form hetero-oligomers and do not influence each other in cell surface expression. (A) For dimerization studies, a sandwich-ELISA was performed in COS-7 cells that co-express N-HA-tagged ADRA2A and C-Flag-tagged TAAR1 constructs (or *vice versa*) in equal amounts (detailed methodology described in the [Supplementary Material and methods](#)). The GHSR homo-oligomer served as a positive control (28 ± 4 -fold over negative control per g/l protein, data not shown). Cells that express N-HA-tagged ADRA2A and C-Flag-tagged rM3R (or *vice versa*) served as a negative control. Data are indicated as mean $\pm$ s.e.m. of fold over negative control and were assessed from three to four independent experiments with six replicates each. A *t*-test with Welch correction was performed for statistical analysis in relation to mock transfection (cells that express N-HA-tagged TAAR1 and ADRA2A); *** $P \leq 0.001$. (B) Cell surface expression of ADRA2A, TAAR1, and ADRA2A/TAAR1 was determined via ELISA. The dotted line represents a base-line from the control for comparison. COS-7 cells were co-transfected with N-HA-tagged ADRA2A or TAAR1 receptor constructs and C-Flag-tagged rM3R for analysis of the homo-oligomeric state and co-transfected with N-HA-ADRA2A and TAAR1-C-Flag (and *vice versa*) for analysis of the hetero-oligomeric states. Mock/rM3R-C-Flag served as a negative control. Results are depicted as means $\pm$ s.e.m. based on five independent experiments measured in six replicates. Data are represented as relative cell surface expression (OD492 nm/620 nm). Significance was calculated using a *t*-test.

$G_{i/o}$ activation of ADRA2A (Fig. 4A), which indicates the uncoupling of ADRA2A from its signaling pathway. Moreover, increasing concentrations of 3-T1AM (10^{-10} – 10^{-5} M) only insignificantly modulated signaling properties of co-expressed ADRA2A/TAAR1 at a constant concentration of NorEpi (Fig. 4B, graph above the dotted line indicates the $\pm$ s.e.m. of 10^{-5} M constant NorEpi concentration).

To explore whether the abolished $G_{i/o}$ signaling of the ADRA2A/TAAR1 hetero-oligomers is the result of suppressed cell surface expression (detailed methodology described in the [Supplementary Material and methods](#)), we investigated the cell surface expression of co-expressed ADRA2A/TAAR1. The N-HA-ADRA2A/TAAR1-Flag hetero-oligomer is expressed at the cell surface in the same

quantity as N-HA-ADRA2A/rM3R-C-Flag (which represents the ADRA2A homo-oligomer) is; therefore, TAAR1 does not modify ADRA2A surface expression. Furthermore, TAAR1 surface expression is not influenced by ADRA2A for N-HA-TAAR1/ADRA2A-C-Flag co-expression (Fig. 3B) as compared to the N-HA-TAAR1/rM3R-C-Flag (which represents the TAAR1 homo-oligomer).

Attenuation of the 3-T1AM-induced effect on NorEpi MAPK signaling as a result of the co-expression of ADRA2A/TAAR1

In contrast to the lack of $G_{i/o}$ signaling, we observed a strong MAPK signaling with the co-expression of ADRA2A and TAAR1 by stimulation with 10^{-5} M NorEpi (Fig. 4C) as

Figure 4

The formation of ADRA2A/TAAR1 heterodimers impairs the capacity for ADRA2A-mediated $G_{i/o}$ but not MAPK signaling. HEK293 cells were co-expressed with ADRA2A and TAAR1 or empty vector (mock). Data are displayed as mean $\pm$ s.e.m. fold over stimulated mock (A and B) or of original values (C and D). (A) Cells were co-incubated with 50 μ M forskolin (FSK) and with 10^{-5} M NorEpi, and cAMP levels were measured. Data are based on five independent experiments performed in triplicate. Statistical analysis was performed with an unpaired two-tailed *t*-test with Welch correction in comparison to stimulated mock (empty vector co-transfected with MC3R). (B) Cells were co-incubated with a NorEpi concentration of 10^{-5} M and varying concentrations of 3-T1AM (10^{-9} – 10^{-5} M). cAMP accumulation was measured. Dotted lines indicate $\pm$ s.e.m. of ADRA2A and TAAR1 stimulated with 10^{-5} M NorEpi. Statistical significance was determined by a one-way

ANOVA. (C) Cells that express empty vector (mock) or co-express ADRA2A and TAAR1 were incubated with 10^{-5} M NorEpi. MAPK activity was determined via the measurement of ERK1/2 activation by a serum response element-luciferase (SRE-luc) reporter assay. Statistical analysis was performed by a one-way ANOVA with a Kruskal–Wallis test. Data are based on three to four independent experiments performed in triplicate. Significances are calculated in comparison to basal (untreated) levels; *** $P \leq 0.001$. (D) Cells were co-incubated with a concentration of 10^{-5} M NorEpi and varying concentrations of 3-T1AM (10^{-10} – 10^{-5} M). MAPK kinase activity was measured by an SRE-luc reporter gene assay. Dotted lines indicate $\pm$ s.e.m. of NorEpi 10^{-5} M. Original data are shown from three to four independent experiments.

compared to stimulation with 3-T1AM (Supplementary Figure 2B). In order to proof for a potential modulatory effect of 3-T1AM on NorEpi-induced MAPK signaling at co-expressed ADRA2A/TAAR1, we co-stimulated with a constant NorEpi concentration of 10^{-5} M and increasing concentrations of 3-T1AM (10^{-10} – 10^{-5} M). Interestingly, we did not observe a modulatory effect of 3-T1AM on NorEpi-induced MAPK activation (Fig. 4D).

3-T1AM application *in vivo* does not influence glucose metabolism

To determine the physiological relevance of the observed *in vitro* effects of 3-T1AM, the *in vivo* effects of 3-T1AM were investigated. 3-T1AM-injected animals displayed no differences as compared to the sham-injected control group in body weight before (24.85 ± 0.35 g vs 25.48 ± 0.66 g) and after (21.97 ± 0.40 g vs 22.67 ± 0.56 g) overnight fasting ($n=6$ for each group). Furthermore, food intake was also not found to be altered following 3-T1AM treatment as compared to controls (Fig. 5A).

Single doses of 3-T1AM in mice have been previously shown to cause hyperglycemia after either i.p. (Regard *et al.* 2007) or i.c.v. (Manni *et al.* 2012) administration. The former effect was attributed to strong $G_{i/o}$ activation of ADRA2A on pancreatic β cells, which is known to result in a reduction in insulin secretion (Regard *et al.* 2007). In order to determine the influence of a long-term application, we treated C57BL/6J male mice over a period of 7 days with a concentration of 5 mg/kg 3-T1AM (i.p. injected), and glucose tolerance was subsequently measured following

16 h of fasting. Basal fasting glucose measurement demonstrated slightly but not significantly decreased blood glucose levels in the 3-T1AM group vs controls (4.98 ± 0.13 mmol/l vs 5.33 ± 0.14 mmol/l, $P=0.095$; Fig. 5B). At 15, 30, and 60 min, blood glucose levels of the 3-T1AM-injected group were moderately increased, which shows a trend toward impaired glucose tolerance in 3-T1AM animals as compared to controls, but this was also not significantly significant. This is in line with the area under the curve, which was not altered between the 3-T1AM-treated as compared to the sham-treated group (1971 ± 87 min $\times$ mmol/l in the treated group vs 1850 ± 87 min $\times$ mmol/l in the untreated group, $P=0.35$). After 120 min, glucose returned to similar levels in both groups (3-T1AM-treated 7.40 ± 0.36 mmol/l vs control 7.63 ± 0.29 mmol/l; Fig. 5B).

Because it is a good indicator of glucose turnover, hepatic glycogen content was measured in both groups. Consistent with the i.p. GTT data, liver glycogen levels were not significantly altered in the 3-T1AM treated group as compared to the sham control group ($P=0.39$; Fig. 4C). Relative expression levels of PEPCK and PYRK, which are regulators for glucose neo-genesis and glycolysis–glucose homeostasis respectively also remained unaltered as determined by RT-PCR (Fig. 5D).

Discussion

3-T1AM is an agonist for ADRA2A

The present study provides experimental evidence that 3-T1AM acts directly on ADRA2A and induces $G_{i/o}$

Figure 5

Long-term 3-T1AM treatment does not show significant differences regarding glucose metabolism. C57BL/6J male mice were treated with daily doses of either 5 mg/kg 3-T1AM for 7 days (i.p. injected, $n=6$ /group). Data are shown as means $\pm$ s.e.m. An unpaired *t*-test was performed for statistical analysis. (A) Food intake remained unaltered following chronic 3-T1AM application as compared to sham injection. (B) Sixteen hours after the last injection and fasting, basal blood glucose was measured and a glucose tolerance test (GTT) was performed (2 g/kg glucose, i.p. injected). Glucose levels were measured after 0, 15, 30, 60, and 120 min (continuous line).

3-T1AM-injected mice did not show a significant difference regarding fasting glucose levels and during i.p. GTT as compared to sham-treated animals (scattered line). Glucose area under the curve was calculated and did not differ between the treated (1971 ± 87 min $\times$ mmol/l) and sham-treated group (1850 ± 87 min $\times$ mmol/l, $P=0.35$). (C) Significant changes were not observed for hepatic glycogen levels following 3-T1AM treatment in comparison to sham-injected animals (set to 1). (D) Quantitative RT-PCR of PEPCK and PYRK revealed no significant change in relative gene expression levels in liver between 3-T1AM and sham treatments.

signaling (Fig. 1A) similarly to the classical adrenergic receptor agonist NorEpi. The agonistic effect of 3-T1AM is in accordance with the previously reported physiological and postulated functional effects of ADRA2A. Regard *et al.* (2007) suggested, based on their data with pancreatic islets, that the inhibitory effect of a single dose of 3-T1AM (at a concentration of 50 mg/kg) on insulin secretion is related to $G_{i/o}$ activation and might be mediated by ADRA2A. In the present study, a synergistic effect in $G_{i/o}$ activation by simultaneous treatment with the ligands NorEpi and 3-T1AM was not observed. However, 3-T1AM treatment resulted in a decrease in NorEpi-mediated $G_{i/o}$ signaling (Fig. 1B), which suggests that 3-T1AM is a partial agonist at ADRA2A but possibly has higher binding affinity as compared to NorEpi, as was already speculated by Regard *et al.* (2007). The ligands utilized in the present study demonstrate chemical similarities, and the adrenergic receptors and TAAR are evolutionary closely related (Borowsky *et al.* 2001, Roeder 2005). As a consequence, their ligand binding pockets show significant overlapping properties and specificities, including, for instance, a conserved aspartate residue at transmembrane helix 3 (Huang 2003, Kratochwil *et al.* 2005, Kleinau *et al.* 2011). Therefore, it can be speculated that 3-T1AM and NorEpi both bind at ADRA2A, but slight differences in the receptor–ligand interaction pattern should exist because of the differences in ligand structures, which altogether should cause three general findings: i) the observed non-induction of MAPK by 3-T1AM; ii) the consequential antagonistic effect of 3-T1AM on NorEpi-induced MAPK (by ligand competition at the overlapping ligand binding region); and iii) the induction of G_i signaling also by 3-T1AM.

3-T1AM is an antagonist for MAPKs activation by NorEpi at ADRA2A

The present data provide the first evidence that 3-T1AM inhibits the MAPK signaling pathway induced by NorEpi at ADRA2A. As presented in Fig. 1C and D, MAPK activation is diminished by 3-T1AM in a concentration-dependent manner. The fluctuation of NorEpi-induced ERK pathway modulation in the presence of varying 3-T1AM concentrations shows a biphasic characteristic that has also been reported for other GPCRs, such as the PTH1R (Eishengdrel & Kongsamut 2013). Such a biphasic effect has also been shown for the PAR2 and activation of inositol phosphate (Covic *et al.* 2002). The fact that high and low concentrations of 3-T1AM influence NorEpi-induced ERK activation might hint at possibly different physiological

functions. On the one hand, 3-T1AM acts as an activator, but on the other hand, 3-T1AM is a low potency inhibitor that requires high concentration for its action.

However, 3-T1AM alone does not activate MAPK at ADRA2A or TAAR1 (Fig. 1C and Supplementary Figure 2A), which leads to the overall conclusion that NorEpi and 3-T1AM share functional similarities ($G_{i/o}$ activation) at ADRA2A, but they differ in respect to MAPK activation. This indicates that 3-T1AM is a biased ligand at ADRA2A, which is particularly interesting considering the fact that MAPK activation in β cells has been described as having effects on apoptosis, cell growth, and differentiation (Verga Falzacappa *et al.* 2006).

ADRA2A/TAAR1 co-expression results in impaired $G_{i/o}$ activation by NorEpi

One possible explanation for the previously observed effects of 3-T1AM application in mice (reviewed in Piehl *et al.* (2011)) might be provided by the formation of hetero-oligomers between TAAR1 and ADRA2A (Fig. 3A), seeing as both receptors are expressed *in vivo* in β cells (Fig. 2), and in addition, both receptors can interact with this ligand.

We assume that the shift in signaling properties (the decrease in $G_{i/o}$ signaling) might be caused by an interaction between the two receptors. 3-T1AM at TAAR1 only activates G_s but not $G_{i/o}$ or MAPK signaling, whereas 3-T1AM at ADRA2A stimulates $G_{i/o}$ but not MAPK signaling (Fig. 1A and C). In cells that co-express TAAR1/ADRA2A, NorEpi-induced $G_{i/o}$ signaling is abolished (Fig. 4A), which indicates potential signaling pathway modulation by hetero-oligomerization, a concept already known for GPCRs (reviewed in Rozenfeld & Devi (2011)). This finding is in accordance with the previously reported general mechanism of the uncoupling of the ADRA2A signaling pathway that occurs as a result of hetero-oligomerization with the μ -opioid receptor (Vilardaga *et al.* 2008), and it has also been reported for ADRB2 interacting with the prostaglandin receptor EP1R, which is of therapeutic relevance (Barnes 2006, McGraw *et al.* 2006). The decreased $G_{i/o}$ -mediated signaling in ADRA2A heterodimers may have two explanations, which are still speculative: i) there is a so-called ‘lateral off-target allostery’ between the receptors in a heterodimeric constellation (the suppression of the G_i signaling capacity of ADRA2A by interaction with TAAR1) and ii) the number of $G_{i/o}$ -sensitive ADRA2A receptors is reduced by the interaction of a certain receptor fraction with TAAR1. On the one hand, steric exclusion of G protein binding

could occur, but on the other hand, the probability that the receptors themselves will couple $G_{i/o}$ could be impaired by the direct protomer–protomer interaction via mutual structural constraints. However, for each of these models, further experimental evidence would be needed; it was outside the scope of the present study.

However, the hetero-oligomer constellation causes a modified signaling profile that is a further example of a phenomenon known for a few GPCRs (reviewed in Lambert (2010)) and particularly for activation switching between non- and G protein-mediated signaling (Rozenfeld & Devi 2007). In addition, it cannot be excluded that the application of 3-T1AM in the hetero-oligomers might stimulate G_s at TAAR1, which produces a contradictory and silencing effect on the simultaneous activation of $G_{i/o}$ at ADRA2A by 3-T1AM. It should be noted that ADRA2A and TAAR1 cell surface expression is not influenced by the formation of hetero-oligomers (Fig. 3B).

Long-term application of 3-T1AM *in vivo* has no significant effect on glucose tolerance

The general link between 3-T1AM and glucose homeostasis is strengthened by the finding that 3-T1AM is a modulator of the hypothalamus–pancreas–thyroid axis, because the administration of single doses in mice resulted in decreased insulin sensitivity, which can be prevented in the presence of a glucagon release blocker (Galli *et al.* 2012, Manni *et al.* 2012). In addition, a previous study suggested that ADRA2A might play a role in type 2 diabetes, seeing as ADRA2A knockout mice showed lower blood glucose levels and altered glucose homeostasis as compared to WT mice (Fagerholm *et al.* 2004). Moreover, a single-nucleotide polymorphism at ADRA2A was identified to be a risk factor for reduced insulin secretion and type 2 diabetes (Rosengren *et al.* 2010, Tang *et al.* 2014).

3-T1AM effects following application are acutely dependent on the dose and mode of application. For instance, chronic i.p. 3-T1AM treatment (31 mg/kg every day) for 14 days showed a reduced food intake (Hettinger *et al.* 2010), whereas a daily i.p. injection of 10 mg/kg for 8 days resulted in a body weight loss in obese mice even with no changes in food intake (Haviland *et al.* 2013). Most interestingly, in the present study, chronic i.p. 3-T1AM treatment (5 mg/kg per day) for 6 days did not profoundly alter glucose homeostasis, as demonstrated by an unaltered i.p. GTT and, moreover, by no change in the glycogen liver content of normal-weight animals. The comparison to other studies is difficult because the mode of application (i.p. (Regard *et al.* 2007) vs i.c.v.

(Manni *et al.* 2012)) is different and because the dosage of applied 3-T1AM differs in each study. In these other studies, blood glucose (Manni *et al.* 2012) or glucose and insulin levels (Manni *et al.* 2012) were determined. Interestingly, the high dose of 50 mg/kg (Regard *et al.* 2007) and the low dose of 1.3 µg/kg (Manni *et al.* 2012) both influenced glucose homeostasis. In contrast, in the present study, no significant changes were observed in food intake or in the liver expression of enzymes that control glucose metabolism.

Regard *et al.* (2007) suggested that 3-T1AM stimulates insulin secretion via G_s activation at TAAR1 and inhibits insulin secretion via $G_{i/o}$ activation at ADRA2A, depending on the relative expression of both receptors. The present experimental data (concurrent $G_{i/o}$ activation by 3-T1AM at ADRA2A and the known G_s activation at TAAR1), implies that in the case of the co-expression of both receptors, assuming similar protein expression and independent action, the signaling effects related to $G_{i/o}$ and G_s at both receptors should neutralize one another. The present *in vivo* experiments further support this hypothesis. Moreover, our data from an RNA sequencing analysis showed that *Adra2a* and *Taar1* are significantly expressed in the pancreatic islets of mice. These findings are in parallel with previous data in human islets that demonstrated significant RNA expression levels for ADRA2A and TAAR1 (Eizirik *et al.* 2012). However, the concrete regulation of the expression and actual presence of either TAAR1 or ADRA2A and their influence on, for example, β cells is still under debate and has not yet been clarified (Regard *et al.* 2007). The simultaneous and condition-dependent occurrence of both receptors in the same tissue or cell type might determine the exact physiological reaction.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-15-0003>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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