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(Direktor: Prof. K.-A. Nave, Ph.D.)
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Establishment of a novel technique
to study G protein-coupled receptor activation

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vorgelegt von

Minou Susan Djannatian

aus

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Dekan: Prof. Dr. med. C. Frömmel

I. Berichterstatter: Prof. Dr. med. M. Simons

II. Berichterstatter/in: Prof. Dr. med. W.-H. Zimmermann

III. Berichterstatter/in: Prof. Dr. rer. nat. B. Schwappach

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Abbreviations

5'-AMP	5'-adenosine monophosphate
aa	amino acids
ADRB2	beta-2 adrenergic receptor
AKAP	A kinase-anchoring protein
Akt	Akt serine/threonine kinase
Amp ^R	ampicillin resistance
AP-1	activator protein 1
AP-2	adaptor protein complex AP-2
AVPR1a	arginine vasopressin receptor 1a
AVPR1b	arginine vasopressin receptor 1b
AVPR2	arginine vasopressin receptor 2
ATP	adenosine triphosphate
AVP	arginine vasopressin
βArr2	β-arrestin 2
βARR2Δ	β-arrestin 2 truncation mutant (aa 1-382)
Bcr-Abl	breakpoint cluster region-abelson oncogene
BME	Basal Medium Eagle
BRET	Bioluminescence Resonance Energy Transfer
BSA	Bovine Serum Albumin
Ca ²⁺	calcium ions
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
ccdB	controlling cell death B gene
CML	chronic myeloid leukemia
CMV	cytomegalovirus
cre	cAMP-responsive element
CREB	cAMP-responsive element binding protein
C-TEV	C-terminal aa 119-221 of the TEV protease
DAG	diacylglycerol
db-cAMP	dibutyryl cyclic adenosine monophosphate
DMEM	Dulbecco's modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DRD1	dopamine receptor 1
DRD2	dopamine receptor 2
DRD3	dopamine receptor 3

DRD4	dopamine receptor 4
DRD5	dopamine receptor 5
DTT	1,4-dithiothreitol
EC ₅₀	agonist concentration which results in a half-maximal response
E.coli	Escherichia coli
EGFP	enhanced green fluorescent protein
ErbB	family of receptor tyrosine kinases
ERK	extracellular-signal regulated kinase
EYFPnuc	nuclear localized enhanced yellow fluorescent protein
FBS	fetal bovine serum
Fig.	figure
FRET	Fluorescence Resonance Energy Transfer
FSC	Forward Scatter
g	gram
G5	5-fold Gal4-responsive element
Gal4	yeast DNA binding domain
GDP	guanosine diphosphate
Genta ^R	gentamycin resistance
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
GV	artificial transcription factor, composed of the yeast Gal4 DNA-binding domain and the herpes simplex VP16 transactivation domain
h	hour
HBSS	Hank's Buffered Salt Solution
HEK	human embryonic kidney cell line
HS	horse serum
IGF-1	Insulin-like Growth Factor 1
IHF	integration host factor, encoded by E.coli
IL-2	interleukin-2
Int	integrase, encoded by bacteriophage λ
IP ₃	inositol 1,4,5-trisphosphate
JAK	Janus kinase
Kan ^R	kanamycin resistance
l	liter
LB	Luria Bertani
μ	micro
m	milli

M	molar
MAPK	mitogen-activated protein kinase
n	number of samples, nano
NEAA	non-essential amino acids
NFAT	nuclear factor of activated T cells
NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells
N-TEV	N-terminal aa 1-118 of the TEV protease
PBS	phosphate buffered saline
PC12	rat pheochromocytoma cell line
PCR	polymerase chain reaction
PDE	phosphodiesterase
PEG	polyethylene glycol
pH	negative logarithm of the hydrogen ion concentration
P1'	amino acid carboxyterminally of a protease cleavage site
PI3K	phosphatidylinositol 3-kinase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLCβ	phospholipase Cβ
PLL	poly-L-lysine
PLO	poly-L-ornithine
PMA	phorbol myristate acetate
PPI	protein-protein interaction
Ras	Ras family of small GTPases
RLUs	relative light units
RNA	ribonucleic acid
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
[³⁵ S]GTP _γ S	guanosine 5'-[γ-[³⁵ S]thio]triphosphate
Src	Src non-receptor tyrosine kinase
SSC	Side Scatter
STAT	Signal Transducers and Activators of Transcription
SV40	monkey virus Simian Virus 40
TE	Tris/EDTA
TEV	Nla protease of the tobacco etch virus
tevS	TEV protease cleavage site ENLYFQ'G
tevS*	TEV protease cleavage site ENLYFQ'L
TFIID	transcription factor IID
TK	thymidine kinase

TM-GV	GV coupled to the transmembrane domain of the human PDGF α receptor
TNF α	tumor necrosis factor α
TRIS	Tris(hydroxymethyl)aminomethane
TRP	transient receptor potential
U	unit
U2OS	human osteosarcoma cell line
VC	C-terminal aa 343-371 of the AVPR2 receptor
VP16	Herpes simplex transactivation domain
w/v	weight per volume
Xis	excisionase, encoded by bacteriophage λ
Zeo ^R	zeocin resistance

1 Introduction

1.1 G protein-coupled receptors

Mammalian cells integrate extracellular signals into distinct cellular responses via signal transduction pathways. Receptors located at the cell surface transduce the extracellular stimulus to specific signaling cascades in the cytoplasm by activating specific effector proteins. The largest family of these receptors in mammals is represented by G protein-coupled receptors (GPCRs) which include more than 800 members in the human genome (Lagerstrom and Schiöth 2008). GPCRs are an extremely diverse receptor family, reacting to as different signals as hormones, neurotransmitters, chemokines, odorants, calcium, and light. They direct or modulate diverse physiologic processes including cell growth, inflammation, neurotransmission and hormone signaling under normal and pathological conditions. Currently, about 25% of all approved drugs are targeted against GPCRs highlighting the clinical importance of this protein family (Overington et al. 2006).

All GPCRs have a similar structure in that they consist of seven transmembrane α helices which are bundled together and connected by three extracellular and three intracellular loops. The transmembrane 'central core' is preceded by an extracellular N-terminal domain and followed by an intracellular C-terminal domain. Activation of GPCRs leads to a conformational change in the 'central core'.

1.1.1 Activation of G protein-coupled receptors

GPCRs are coupled to guanine nucleotide-binding proteins (G proteins) which serve as intracellular signal transducers. G proteins are heterotrimeric, consisting of an α subunit and a $\beta\gamma$ subunit under physiological conditions. Several G proteins exist which differ in their α subunits (α_s , $\alpha_{i/o}$, α_q , α_{12} , and others) and couple to different downstream signaling pathways. In the resting state, when no ligand is bound to the receptor, the α subunit binds guanosine diphosphate (GDP) and is closely associated with the $\beta\gamma$ -subunit. Receptor conformation changes during activation by an agonist allow binding of the G protein. GPCRs then catalyze the exchange of GDP by guanosine triphosphate (GTP) and thereby promote the dissociation of α and $\beta\gamma$ subunit. The α , and in some cases the $\beta\gamma$, subunit modulates effectors which catalyse the synthesis of second messenger molecules and thus initiate downstream signaling.

Termination of GPCR-activated signaling cascades takes place within tens of seconds through GTP hydrolysis by the $G\alpha$ subunit itself. Desensitization is another mechanism to end GPCR signaling (see 1.1.4).

1.1.2 Signaling of G protein-coupled receptors

Classical GPCR signaling takes place via three main signaling pathways which depend on the G protein family involved. Signaling via G_s involves activation of the effector molecule adenylate cyclase by the $G\alpha$ subunit which leads to the synthesis of cyclic adenosine monophosphate (cAMP). Binding of two cAMP molecules to the regulatory subunits of protein kinase A (PKA) releases the catalytic subunits of PKA which then display catalytic activity. Substrates of serine/threonine-specific PKA are enzymes of the glucose and glycogen metabolism, lipase, calcium channels in skeletal muscle as well as a number of neuronal proteins.

$G_{i/o}$ inhibits adenylate cyclase and therefore cAMP production via both the α and the $\beta\gamma$ subunit. $\beta\gamma$ subunits of these G proteins can furthermore directly activate potassium channels, phospholipase C β (PLC β) and phosphatidylinositol 3-kinase (PI3K) (Neves et al. 2002).

G_q activates PLC- β which cleaves phosphatidylinositol 4,5-bisphosphate (PIP $_2$) into two second messengers: the membrane-bound, lipophilic diacylglycerol (DAG) and the cytosolic inositol 1,4,5-trisphosphate (IP $_3$). DAG activates protein kinase C (PKC) after calcium-dependent translocation to the plasma membrane. PKC in turn phosphorylates and thereby activates a number of proteins involved in cell growth and metabolism, smooth muscle contraction and neuronal excitation. IP $_3$ induces a transient release of calcium ions (Ca $^{2+}$) from the endoplasmic reticulum into the cytoplasm. Influx of extracellular calcium is initiated through binding of the IP $_3$ -gated Ca $^{2+}$ channels to transient receptor potential (TRP) channels once the Ca $^{2+}$ stores of the endoplasmic reticulum are depleted (Boulay et al. 1999). Calcium signaling is important for muscle activity, neurotransmitter release, modulation of Ca $^{2+}$ -dependent ion channels, metabolism, cell proliferation, and apoptosis (Berridge et al. 2000).

Signal amplification on all levels is a hallmark of these signaling pathways (e.g. synthesis of several hundreds of cAMPs follows the activation of a single receptor molecule). However, signaling by second messengers occurs in local microdomains and is thus spatially well controlled. This allows the cell to react to multiple extracellular stimuli and to run a number of different intracellular signaling processes at the same time while

maintaining specificity of these processes. For example, compartmentalization of cAMP signaling is achieved in form of cAMP gradients. These gradients are shaped by phosphodiesterases (PDEs) which degrade cAMP to 5'-adenosine monophosphate (5'-AMP) and are positioned at discrete locations in the cytoplasm, partly in association with organelles. PDEs can even act as 'sinks' in which cAMP is drained while the concentration is maintained at more distant locations, thus creating multiple cAMP gradients at the same time. Since both PDEs and PKA are anchored to defined intracellular sites by A kinase-anchoring proteins (AKAPs), cAMP molecules can be 'guided' towards their effector proteins in a very specific way (Baillie 2009).

GPCR signaling is highly dependent on cellular environment, meaning that the activation of the same receptor can have a certain effect in one cell type but a completely different one in another cell type. The influence of cell 'phenotype' originates from a variety of factors like receptor density, receptor trafficking, receptor dimerization, presence of receptor-modulating proteins, stoichiometry of receptors to G proteins, and availability of effector molecules (Kenakin 2003).

1.1.3 Transcriptional regulation through G protein-coupled receptor signaling

It has been long established that extracellular signals influence gene regulation through common signaling pathways. Gene transcription can be regulated by the action of transcription factors which are subjected to phosphorylation during signaling. Activation of transcription factors can be achieved by 1) direct phosphorylation of the transcription factor at the membrane or in the cytoplasm followed by its translocation into the nucleus (e.g. in Janus kinase-Signal Transducers and Activators of Transcription (JAK-STAT) pathways), 2) nuclear translocation of kinases followed by phosphorylation of a resident transcription factor (e.g. in mitogen-activated protein kinase (MAPK) pathways), or 3) release of the transcription factor from an inhibitory protein by phosphorylation with subsequent nuclear translocation (e.g. nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)) (Edwards 1994; Hill and Treisman 1995). One of the most investigated examples of transcriptional regulation is mediated via cAMP-responsive element (*cre*) binding protein (CREB). CREB recognizes and binds to the *cre* consensus site TGACGTCA (Montminy et al. 1986) which is a cis element positioned in the promoter region of a variety of genes. As described above, cAMP releases the catalytic subunits of PKA from the regulatory subunits and thus enables them to translocate into the nucleus. Phosphorylation of CREB by PKA enhances its ability to recruit the transcriptional

machinery. CREB builds a complex with the general transcription factor TFIID and CREB binding protein (CBP) which confers gene transactivation through association with RNA-polymerase II complexes (Mayr and Montminy 2001; Nakajima et al. 1997).

A well-characterized example for transcriptional regulation by calcium signaling is the nuclear factor of activated T cells (NFAT). An increase of intracellular calcium levels can activate the phosphatase calcineurin which dephosphorylates cytosolic NFAT proteins. This unmasks a nuclear localization signal and allows nuclear translocation of NFAT, binding to the *nfat* cis element and subsequent transactivation of target genes, e.g. tumor necrosis factor α (TNF α). Some target genes of NFAT require the convergence of different signaling pathways, which provides further specificity. Concomitant activation of NFAT by calcium and of activator protein 1 (AP-1) by PKC/Ras pathways is needed for binding of NFAT/AP-1 complexes to *nfat* binding sites and transactivation of e.g. interleukin-2 (IL-2) (Crabtree and Olson 2002; Macian et al. 2001).

1.1.4 Desensitization of G protein-coupled receptors

A remarkable characteristic of GPCRs is their desensitization, resulting in a reduced response to the agonist over time. Desensitization includes different mechanisms varying in onset and duration, namely uncoupling of the receptor from G proteins, receptor internalization, and downregulation of the receptor mRNA and protein content. Uncoupling from G proteins is achieved through phosphorylation of the receptor by second messenger-dependent kinases (PKA and PKC) and G protein-coupled receptor kinases (GRKs). Both second messenger-dependent kinases and GRKs phosphorylate serine and threonine residues within the third intracellular loop and the C-terminal tail. However, in contrast to second messenger-dependent kinases, GRKs selectively phosphorylate agonist-activated receptors. GRKs furthermore promote the binding of arrestins, leading to a sterical uncoupling of G proteins from the receptor (Ferguson 2001).

1.1.5 Roles of arrestins in receptor desensitization and signal transduction

Arrestins are a protein family that consists of four members, namely visual and cone arrestin, and β -arrestin 1 and 2. Visual and cone arrestin are predominantly found in the retina, whereas β -arrestins are ubiquitously expressed outside the retina. The structure of arrestins is characterized by an N and a C domain which are each composed of a seven stranded β -sandwich and linked by a phosphate sensor domain. Upon receptor activation, interaction of the phosphorylated receptor tail with arrestin leads to a reorientation of the

arrestin N and C domains, thereby promoting arrestin binding to the receptor (Hirsch et al. 1999). β -arrestin 2 mutants with a point mutation of aa 169 from arginine to glutamine or a deletion of aa 383-409 result in “constitutively active” variants, which bind to GPCRs in a phosphorylation-independent manner and exhibit even stronger stimulation-dependent receptor desensitization than wild-type β -arrestins (Kovoor et al. 1999). Besides the physical uncoupling of GPCRs from G proteins, β -arrestins promote the degradation of second messengers by recruiting the appropriate enzymes (Nelson et al. 2007; Perry et al. 2002).

Moreover, β -arrestins target GPCRs to clathrin-mediated endocytosis by interacting with both clathrin and the AP-2 adaptor complex. Internalization of GPCRs allows their degradation in lysosomes, but apparently also plays a role in receptor resensitization (Zhang et al. 1997) and coupling of desensitized GPCRs to G protein-independent signaling pathways (Luttrell et al. 1999). The trafficking pattern of internalized GPCRs is strongly associated with their interaction with β -arrestins. Two classes of GPCRs have been identified which differ in their interaction with arrestins and their fate upon internalization (Oakley et al. 2000). ‘Class A’ GPCRs show a stronger affinity for β -arrestin 2 than for β -arrestin 1 and do not interact with visual arrestin. Upon internalization, β -arrestin dissociates from the receptor and recycles to the plasma membrane. This allows receptors to become dephosphorylated by a GPCR-specific phosphatase (Pitcher et al. 1995), which is important for receptor recycling to the cell surface, and thus, for resensitization. ‘Class B’ GPCRs, in contrast, bind to both β -arrestins with equal affinities, do interact with visual arrestin, and form stable complexes with β -arrestin which are targeted to endosomes.

β -arrestins are not only important for GPCR desensitization but have recently been recognized to mediate signaling on their own. This is connected to their role as scaffold proteins, recruiting intracellular signaling molecules like Src tyrosine kinase family members and MAPK to complexes. Interestingly, it has been described that β -arrestin-mediated extracellular signal-regulated kinase (ERK) 1 and 2 activation is a very distinct mechanism from G protein-mediated ERK activation with respect to kinetics, subcellular targeting, and cellular effects (Ahn et al. 2004). These different mechanisms can be targeted by ‘biased’ agonists which promote either one or the other mechanism. Moreover, ERK activation by β -arrestin seems not to be dependent on GPCR stimulation, but rather on the translocation of β -arrestin to the membrane (Terrillon and Bouvier 2004). The universal role of β -arrestin for MAPK signaling can be estimated from the findings that β -arrestin as well mediates signaling from tyrosine kinase receptors (as described for the

Insulin-like Growth Factor 1 (IGF-1) receptor) (Rakhit et al. 2001) and ligand-gated ion channels (as described for the nicotinic cholinergic receptor) (Dasgupta et al. 2006) to MAP kinases. Additionally to their functions at the plasma membrane and in the cytoplasm, β -arrestins translocate to the nucleus where they regulate gene expression, e.g. by inhibiting NF- κ B-dependent nuclear transcription (Gao H et al. 2004) and by facilitating histone deacetylation at specific gene promoters (Kang et al. 2005).

In summary, β -arrestin functions turned out to be by far more diverse than initially expected, since they are involved in the regulation of a variety of physiological functions, like chemotaxis, cytoskeletal reorganization, metastasis, apoptosis, and behaviour (Lefkowitz et al. 2006).

1.1.6 A closer look at GPCR family members: vasopressin, dopamine and beta-adrenergic receptors

1.1.6.1 Vasopressin receptors

The vasopressin receptor family contains three members, AVPR1a, AVPR1b and AVPR2, which are largely diverse in localization and function. AVPR1a is expressed in vascular smooth muscle cells, liver, kidney, heart, adrenal gland and brain and is important for vasoconstriction, aldosterone and corticosterone secretion, glucose metabolism and platelet aggregation. AVPR1b is found in the anterior pituitary and throughout the brain and is mostly known for regulation of the hypothalamic-pituitary-adrenal axis. AVPR2 is mainly localized in the kidney where it induces the insertion of aquaporin-2 water channels into the plasma membrane of collecting duct cells, allowing water reabsorption and thereby urine concentration (Harmar et al. 2009). AVPR2 pathologies lead to nephrogenic diabetes insipidus (Pan et al. 1992). AVPR1a and AVPR1b mainly employ G_q to stimulate phospholipase C whereas AVPR2 causes cAMP accumulation via G_s (Schoneberg et al. 1998). AVPR2 furthermore signals to ERK via a G protein-independent, β -arrestin 2-mediated pathway (Tohgo et al. 2003).

1.1.6.2 Dopamine receptors

Among the dopamine receptors, 5 different receptors have been identified which can be classified into D1-like (DRD1 and DRD5) and D2-like (DRD2, DRD3, DRD4) receptors according to their structural, pharmacological and biochemical features. Dopaminergic neurons are organized in different pathways throughout the brain: the nigrostriatal pathway (from the substantia nigra in the midbrain to the striatum), the mesolimbic and

mesocortical pathway (from the ventral tegmental area to the nucleus accumbens and the frontal cortex) and the tuberoinfundibular pathway (from the hypothalamus to the pituitary gland). Peripheral dopaminergic neurons can be found in kidney, heart and adrenal cortex (Harmar et al. 2009).

Dopamine receptors are typical examples of GPCRs which are linked to multiple effectors and can therefore confer highly complex and context-dependent signaling. While D1-like receptors typically initiate G_s -dependent cAMP signaling (Brown and Makman 1972) and regulate a variety of ion channels via PKA (Neve et al. 2004), G_i -mediated $PLC\beta$ activation is employed in some cases (Mahan et al. 1990). Most D2-like receptors inhibit adenylate cyclase via $G_{\alpha_{i/o}}$ (Jiang et al. 2001), but β/γ subunits can alternatively activate $PLC\beta$, MAPK, phospholipase A_2 or K^+ channels (Neve et al. 2004).

Dopamine receptor pathologies in the central nervous system are linked to a variety of neuropsychiatric disorders such as Parkinson's disease (Fuxe et al. 2006), schizophrenia (Lewis and Lieberman 2000), attention-deficit hyperactivity disorder (Tripp and Wickens 2009), and drug addiction (Di Chiara et al. 2004).

1.1.6.3 Beta-adrenergic receptors

Beta-adrenergic receptors are found in a wide variety of tissues throughout the body. The three subtypes identified so far differ in localization, pharmacological characteristics (such as sensitivity to their natural ligands, noradrenaline and adrenaline, or response to antagonists), and function. The beta-2 adrenergic receptor subtype (ADRB2) is mainly found in lung, kidney, heart, skeletal muscle, liver and brain (Harmar et al. 2009). Major physiological actions are bronchodilation, blood vessel dilation, increase of cardiac output due to positive chronotropy and inotropy, and glycogenolysis. In the brain, neuronal ADRB2 is involved in learning and memory (Gray and Johnston 1987) whereas astrocytic ADRB2 plays an important role in energy supply and regulation of brain inflammatory processes (Laureys et al. 2010). ADRB2 is the classical example of a G_s -coupling GPCR. However, as an example of dual signalling, additional coupling to G_i in cardiac myocytes seems to restrict cAMP accumulations to local microdomains (Kuschel et al. 1999), and signaling through its β/γ subunit promotes cell survival via a PI3K-Akt pathway (Zhu et al. 2001). ADRB2 furthermore evokes G protein-independent signaling to ERK via a β -arrestin 2-mediated complex with Src kinase (Luttrell et al. 1999).

1.2 Signal transduction revised

Until the last two decades, signal transduction has been believed to be the sum of a great number of linear pathways. Pathways were thought to be organized in a strictly hierarchical way with a fixed sequence of molecular interactions once the pathway was initiated. In this paradigm, chemical compounds would initiate the same pathway in every tissue at any time given. In line with this, it was assumed that diseases are caused by single defects within signaling pathways and that compensating the respective defect would cure the disease. There are indeed diseases for which this holds true and in which selective drugs represent a valuable therapy. For example, almost all of the cases of chronic myeloid leukemias (CML) are characterized by formation of the Bcr-Abl fusion protein due to a chromosomal translocation, and unregulated activity of the Abl tyrosine kinase has been identified as the single cause of the disease (Konopka et al. 1985). Selective inhibition of tyrosine kinase activity with imatinib turned out to be an efficient therapy for CML (Druker et al. 1996).

However, it has become apparent that signaling pathways intersect on various levels, building huge signaling networks (Fig. 1). How is it now possible that distinct pathways use the same components but still result in different outputs? Roles as signaling molecules can be assigned to proteins through posttranslational modifications such as phosphorylation, ubiquitination or acetylation (Scott and Pawson 2009). Signaling can be spatially confined by compartmentalization in cellular subspaces (Baillie 2009) or by scaffolds which recruit a specific subset of proteins (Lefkowitz et al. 2006; Weng et al. 1999). Signal duration can decide about cell fate by promoting either proliferation or differentiation, as has been shown for ERK signaling in pheochromocytoma PC12 cells, thus adding complexity in a temporal dimension (Kholodenko et al. 2010; Marshall 1995). Signaling components, e.g. different MAPK, might require different thresholds for activation and therefore depend on signal strength (Gong et al. 2001). Nonetheless, extensive crosstalk takes place among signaling pathways, e.g. by transport between different subcellular compartments (Weng et al. 1999) or by transactivation across receptor families (Daub et al. 1996). Furthermore, cells adjust to ongoing signal processes by constantly modifying the molecular equipment in feedback and feed-forward loops, e.g. by transcriptional regulation (Weng et al. 1999).

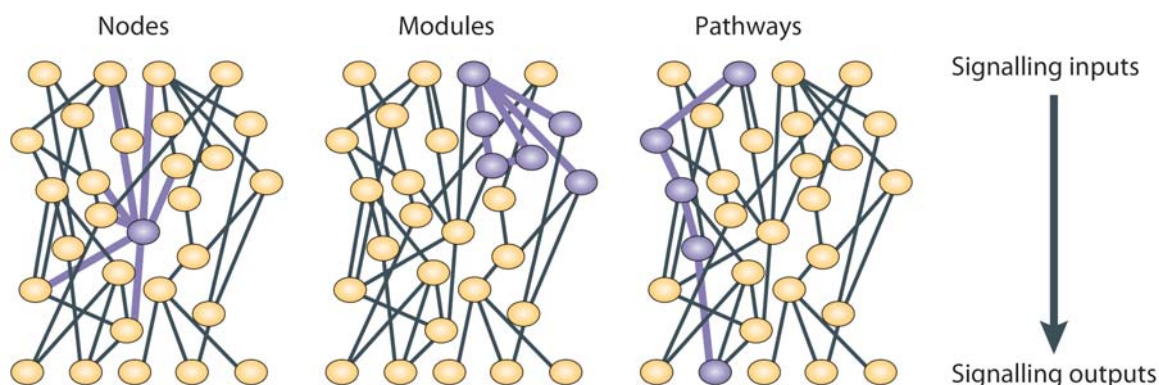


Fig. 1. Signaling network reconstruction.

The diverse connections between components of signaling networks can be described in terms of “nodes” which comprise all interactions of a given component (a), “modules” which group proteins functioning together (b), and “pathways” which describe the connection between a given signaling input and a signaling output (c). Adapted from Papin et al. (2005, p. 104).

Consequently, the function of a given protein results from its interplay with other proteins within a network and can change depending on the network composition, the molecular equipment of cells in different tissues, or time.

1.3 Challenges in drug discovery

Despite the sequencing of the human genome and the implementation of high-throughput screening in drug discovery, the identification of new drug targets did not substantially increase, as was believed to be essential for the development of new drugs. Moreover, the number of drug targets does not necessarily translate into the number of newly developed drugs. Indeed, the number of drug targets of all currently available drugs can be pinned down to about 300, and the majority of new drugs is directed against targets that have been known for quite a while (Overington et al. 2006; Yildirim et al. 2007). On the other hand, many drugs in the pipeline fail in phase II and III trials due to the lack of efficacy or safety (Hopkins 2008). Hence, traditional approaches in drug discovery need to be revised by integrating current knowledge on the systems biology level.

Signaling networks where signaling molecules are interconnected in diverse ways are protected against perturbation by redundancy of functions. Interactions between components are in this sense more vulnerable than the single components themselves (Boran and Iyengar 2010; Kitano 2007). Thorough evaluation of the diverse functions of a drug target within signaling networks in a context-dependent manner is therefore a key for future drug discovery. Moreover, multicomponent drugs or the sequential use of different drugs as known from cancer treatment might be ways to target signaling networks. All this

reinforces the call for the development of theoretical modeling systems as well as assumption-free, complex assay systems (Hopkins 2008; Kenakin 2009; Kitano 2007; Weng et al. 1999).

1.4 GPCR assays

GPCRs have been analyzed for several decades with a continuously growing spectrum of tools. Several levels of GPCR signaling have been addressed: ligand-binding assays which mainly use radioactive labelling of ligands to assess their affinity, assays to measure GPCR/G protein association via binding of the radiolabeled, non-hydrolyzable GTP analog [³⁵S]GTP γ S, assays monitoring GPCR/ β -arrestin interactions, assays which measure intracellular second messenger levels with various readouts or are coupled to second messenger responsive elements, and label-free assays like electrical impedance measurements and optical methods are available (extensive reviews on GPCR screening assays can be found in Eglen et al. (2007), Fang et al. (2008) and Thomsen et al. (2005)).

The stimulation-dependent interaction between GPCRs and β -arrestins has been widely used in cell-based assays to monitor GPCR activation (Eglen et al. 2007; Lefkowitz et al.; Thomsen et al. 2005). Current approaches include indirect measurements based on the translocation of β -arrestin2-green fluorescent protein (GFP) fusion proteins upon stimulation by microscopy (Barak et al. 1997; Ghosh et al. 2005) and direct protein interaction assays based on Bioluminescence Resonance Energy Transfer (BRET) (Hamdan et al. 2005; Vrecl et al. 2004), Fluorescence Resonance Energy Transfer (FRET) (Krasel et al. 2008), or complementation of β -galactosidase fragments (Yan et al. 2002; Zhao et al. 2008). In addition, protease proximity assays termed Tango[®] have been developed using a reporter gene system as readout (Barnea et al. 2008). In the Tango[®] assay, the interaction of GPCR and β -arrestin 2 (β Arr2) fusion proteins brings the TEV protease and its specific cleavage site into close proximity releasing a fused transcription factor by proteolytic cleavage (Barnea et al. 2008). The transcription factor induces the expression of reporter genes, such as firefly luciferase or β -lactamase which can be quantitatively analyzed using appropriate substrates (de Wet et al. 1987; Zlokarnik et al. 1998).

1.5 The split-TEV system

The split-TEV system is a novel technique which allows the monitoring of constitutive and regulated protein-protein interactions (PPIs) at the membrane and in the cytosol of living

mammalian cells (Fig. 2) (Wehr et al. 2006). This system combines the advantages of split enzyme- and reporter gene-mediated assays. A major component of the technique is the Nla protease of the tobacco etch virus (TEV protease), which shows high substrate specificity and lacks endogenous substrates in mammalian cells. In split-TEV, the catalytic triad of the TEV protease (His46, Asp81, and Cys 151) is split onto N- and C-terminal TEV protease fragments (referred to as N-TEV and C-TEV), which on their own do not exhibit any proteolytic activity. Split-TEV can be used to investigate specific PPIs, since TEV protease activity is only reconstituted upon transcomplementation of N- and C-TEV fusion proteins (Fig. 2a).

Previous assays have shown that the N-TEV(1-118)/C-TEV(119-242) pair is particularly suited for transcomplementation, displaying ~30-40% proteolytic activity compared to the full-length TEV protease (Wehr et al, 2006). Owing to the fact that the C-terminus of the TEV protease can back-fold into the catalytic center of the protease (Nunn et al. 2005), C-TEV was truncated deleting amino acids 222-242 and adding a point mutation at amino acid position 219 (S219P). These modifications have increased the proteolytic activity of the transcomplemented split-TEV, compared to the 'native' split-TEV fragments. Thus, N-TEV(1-118)/C-TEV(119-221_S219P) is used as the preferred TEV pair in split-TEV assays.

TEV protease activity is monitored by the release of a reporter, which is coupled to the specific cleavage site of the TEV protease, ENLYFQ'G (tevS). As split-TEV reporter systems, either inactivated reporter proteins ('proteolysis-only' reporters) or inactivated transcription factors ('transcription-coupled' reporters) can be used. 'Proteolysis-only' reporters are directly activated upon TEV-protease cleavage. In contrast, 'transcription-coupled' reporters require two subsequent steps: first, release of the transcription factor by proteolytic cleavage, and second, transcriptional activation of the reporter (Fig. 2b). A transcription factor, termed GV, has been designed for the split-TEV system by fusion of the yeast Gal4 deoxyribonucleic acid (DNA)-binding domain to the herpes-simplex VP16 transactivation domain. GV can be used to activate reporters such as the Gal-4 responsive Firefly luciferase or the Gal4-responsive EGFP. The flexible use of the split-TEV system for different applications is given by the availability of both membrane-bound and cytosolic reporter systems. To increase the sensitivity for membrane-localized PPIs, GV can be fused to the C terminus of the membrane protein-TEV protease fragment. Thereby, the TEV-protease substrate is brought into close proximity of the transcomplemented TEV protease.

An important feature of the split-TEV system is the implemented enzymatic amplification, one for ‘proteolysis-only’ reporters and several for ‘transcription-coupled’ reporters. This way, transient interactions can be converted into a permanent signal, thus enabling the detection of weak PPIs.

The split-TEV system has been successfully implemented to monitor the interaction of native membrane proteins as well as cytosolic proteins. Furthermore, it has been shown to be sensitive to stimulus-dependent interactions. Finally, which was shown for ErbB receptor tyrosine kinase signaling events, the split-TEV system is suited to analyze signaling events at different levels (Wehr et al. 2006; Wehr et al. 2008).

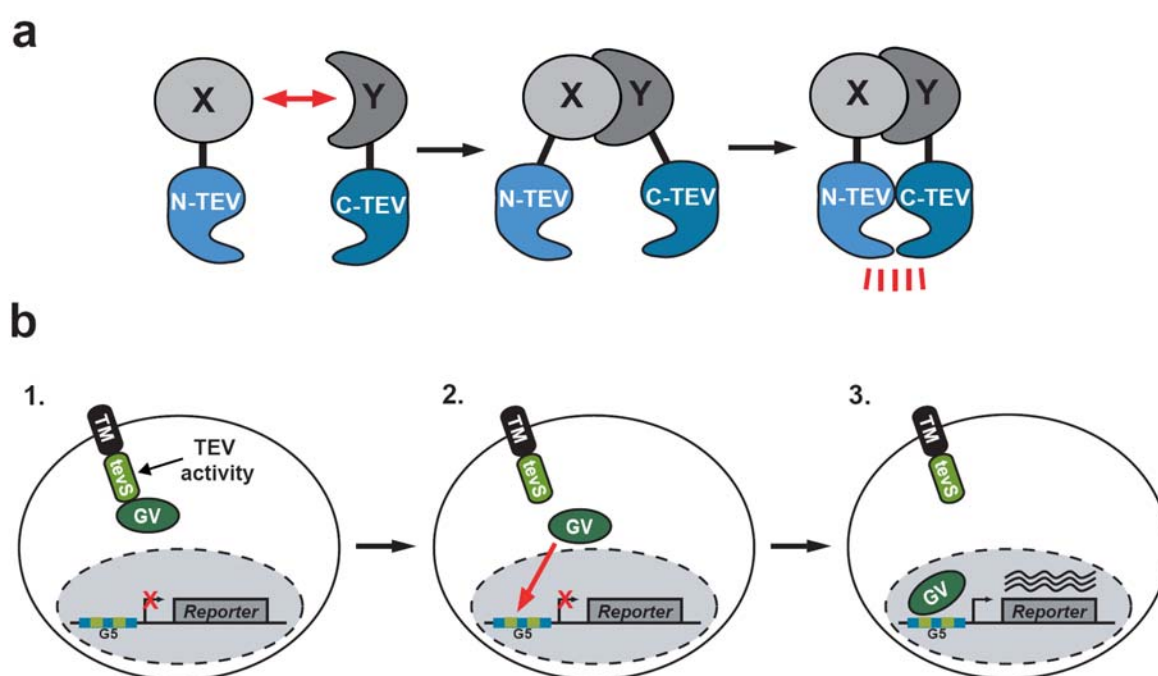


Fig. 2. Principle of split-TEV with the membrane-bound, transcription-coupled reporter system.

(a) The N-TEV and the C-TEV fragment of the TEV protease are coupled to two proteins of interest. Protein-protein interaction leads to the transcomplementation of the TEV protease and thus, to TEV protease activity. (b) (1) TEV protease activity localized to the membrane leads to the cleavage of the Gal4-VP16 transcription factor (GV). (2) GV translocates to the nucleus, (3) where it binds to its specific DNA-binding sequence (G5-promoter) and induces the transcription of the reporter gene (e.g., EGFP or Firefly luciferase). TM-GV: GV coupled to the transmembrane domain of the human PDGF α receptor; tevS: specific cleavage site of the TEV protease, ENLYFQ'G, G5: 5-fold Gal4-responsive element. Adapted from Wehr et al. (2006, p. 986-987).

1.6 Aim of the study: Development of a split-TEV assay for G protein-coupled receptors

The aim of the project was to develop a split-TEV assay to quantitatively measure the activation of GPCRs. The split-TEV system was applied to measure the interaction of β -arrestins with GPCRs, since this interaction is specific for activated GPCRs and can thus be used as a correlate for GPCR activation.

A selection of GPCRs was examined which are coupled to different downstream signaling pathways and show distinct interaction kinetics for β -arrestins. As examples, the vasopressin receptors AVPR1a and AVPR2, the dopamine receptors DRD1 and DRD2 and the beta-adrenergic receptor ADRB2 were chosen. Among the β -arrestins, β -arrestin 2 (β Arr2) was found to be particularly suited for split-TEV assays since its localization is restricted to the cytosol.

β -arrestin-2 and GPCRs were implemented in the split-TEV system through the generation of fusion proteins with the respective TEV fragments. Assays were performed using transcription-coupled reporter systems, which activate the expression of Firefly Luciferase. To monitor GPCR activation, receptors were treated with their natural ligands during the assays.

During development of the assay, several key questions were addressed:

- are GPCR constructs cleavable by TEV protease?
- do GPCR and β Arr2 constructs interact and can this be measured with the reporters?
- do GPCR constructs exhibit physiological behaviour?
- are GPCR split-TEV assays applicable in different cell types, including primary cells?
- how sensitive are GPCR split-TEV assays in comparison to other GPCR assays?

2 Materials

2.1 Chemicals and reagents

6x DNA Loading Dye	Fermentas
Agarose	Bio-Rad, FMC
Ampicillin	Sigma
Arginine vasopressin (AVP)	Sigma
ATP	PJK
Bacto™ Agar	BD Biosciences
Bacto™ Peptone	BD Biosciences
Bacto™ Yeast Extract	BD Biosciences
BME	Invitrogen
BSA	Sigma
Coelenteracin	PJK
Coenzyme A	PJK
db-cAMP	Biolog, Bremen
DMSO	Merck
DNA Ladders (100 bp, 1 kb)	Fermentas
Dopamine hydrochloride	Sigma
Dulbecco's modified eagle medium (DMEM)	Invitrogen
DTT	PJK
EDTA	Sigma
Ethidium bromide	Sigma
Fetal bovine serum (FBS)	Invitrogen
Gentamycin	Sigma
Glacial acetic acid	Merck

L-Glutamine	Invitrogen
GlutaMAX™	Invitrogen
Glycerin	Sigma
Glycogen	Roche
HBSS	Invitrogen
Hoechst dye	Sigma
Horse serum (HS)	Sigma
Ionomycin	Sigma
Isopropanol	Merck
(-)-Isoproterenol hydrochloride	Sigma
Kanamycin	Sigma
KCl	Merck
KH ₂ PO ₄	Merck
K _x PO ₄	Merck
D-Luciferin	PJK
McCoy's A	Invitrogen
MgCl ₂	Merck
4(MgCO ₃)*Mg(OH) ₂ *5H ₂ O	Merck
MgSO ₄	Merck
NaCl	Merck
Na ₂ -EDTA	Merck
Na ₂ HPO ₄ *2H ₂ O	Merck
NaN ₃	Merck
NEAA	Invitrogen
Opti-MEM®	Invitrogen
Papain	Worthington Biochemical Corp.

PEG 8000	Promega
Penicillin/Streptavidin	Sigma
Phorbol myristate acetate (PMA)	Sigma
Poly-L-lysine	Sigma
Poly-L-ornithine	Sigma
RPMI	Invitrogen
Tricine	Merck
Tris Base	Merck
Tris-HCl	Merck
Trypsine	Invitrogen
Zeocin	Invivogen

2.2 Consumables

Cryo tubes	Nunc
1.5 ml and 2 ml tubes	Eppendorf
5 ml, 15 ml and 50 ml tubes	Falcon
10 cm and 15 cm cell culture dishes	Falcon
96-well flat-bottom plates	Falcon

2.3 Equipment

Centrifuges	Sorvall Du Pont, Heraeus, Eppendorf
Digital camera ProgRes C14	Jenoptik
FACSAria flow cytometer	BD Biosciences
GenePulser Xcell	Biorad
Luminometer MicroLumatPlus LB 96 V	Berthold Technologies
Microscopes	Leica DM IRBE (Invers)

	Leica DM RXA
PCR machines	Biometra
Photometer (GenequantII, Ultraspec 3000)	Pharmacia
Sterile hood	Heraeus
Thermocycler T3	Biometra
UV-Illuminator	Intas Systems

2.4 Ready-made reaction systems

GATEWAY Recombination System	Invitrogen
NucleoSpin Plasmid Quick Pure	Macherey-Nagel
NucleoBond PC100 Midiprep	Macherey-Nagel
NucleoSpin Extract II	Macherey-Nagel

2.5 Enzymes

Restriction enzymes	New England Biolabs
HotStarTaq DNA Polymerase	Qiagen
Pfu Ultra Advanced DNA Polymerase	Stratagene
BP Clonase II	Invitrogen
LR Clonase II	Invitrogen

2.6 Solutions and buffers

2.6.1 *Molecular biological solutions*

PBS (Phosphate-buffered Saline)	
NaCl	1.7 M
KCl	34 mM

Na₂HPO₄*2H₂O 40 mM

KH₂PO₄ 18 mM

in H₂O, adjusted to pH 7.2

Tris-EDTA (TE buffer)

Tris-HCl (pH 7.4) 10 mM

EDTA (0.5 M) 1 mM

TAE (50x)

Tris-Base 2M

EDTA 50 mM

adjusted to pH 8 with glacial acetic acid

filled up to 1000 ml with H₂O

Ethidium bromide

Ethidium bromide 1% in H₂O 10 mg/ml

Final concentration in gel 1 µg/ml

PEG/MgCl₂

40 g PEG 8000

in 100 ml of 30 mM MgCl₂

LB medium (Luria and Bertani medium)

Bacto™ Yeast Extract 0.5% (w/v)

Bacto™ Peptone pH 7.5 1% (w/v)

NaCl 1% (w/v)

Low salt 0.5% (w/v)

LB/Amp:	200 µg/ml ampicillin in LB medium
LB/Kan:	50 µg/ml kanamycin in LB medium
LB/Genta:	25 µg/ml gentamycin in LB medium
LB/Zeo:	30 µg/ml zeocin in low-salt LB medium

LB Agar Plates

Bacto™ Yeast Extract	0.5%	(w/v)
Bacto™ Peptone pH 7.5	1%	(w/v)
NaCl	1%	(w/v)
Low salt	0.5%	(w/v)
Bacto™ Agar	1.5%	(w/v)

Antibiotics were added as described for LB medium

2.6.2 Solutions for luciferase assays

Renilla luciferase plasmid mix

pRLuc/SV40	100 µg
phRLuc/TK	20 µg
phRLuc/CMV	10 µg
pEYFPnuc	130 µg

filled up to 1.3 ml with 10 mM Tris, pH 8.5

final concentration: 200 ng/µl (100 ng/µl Renilla luciferase plasmids, 100 ng/µl EYFPnuc)

Passive Lysis Buffer

Promega (5x), dilute in ddH₂O

Firefly Luciferase Substrate

In 500 ml

Tricine	20 mM
(MgCO ₃) ₄ *Mg(OH) ₂ *5H ₂ O	1.07 mM
MgSO ₄	2.67 mM
EDTA	0.1 mM
DTT	33.3 mM
Coenzyme A	270 μM
D-Luciferin, free acid	470 μM
ATP	530 μM

To dissolve magnesium carbonate, pH was titrated until the solutions turns clear. pH was then adjusted to 7.8 using 5 M NaOH. Luciferin and coenzyme A were added in the end. Storage under light protection at -20°C. Thawn at room temperature.

Renilla Luciferase Substrate

NaCl	1.1 M
Na ₂ -EDTA	2.2 mM
K _x PO ₄ (pH 5.1)	0.22 M
BSA	0.44 mg/ml
NaN ₃	1.3 mM
coelenterazine	1.43 mM

adjusted to pH 5.0, then coelenterazine (dissolved in ethanol) was added. Storage under light protection at -20°C. Thawn at room temperature.

2.6.3 Cell culture solutions

PLL 250x

Poly-L-lysine in H ₂ O	5 mg/ml
Final concentration	20 µg/ml

PLO 100x

Poly-L-ornithine in H ₂ O	1.5 mg/ml
Final concentration	15 µg/ml

PC12 tet-off Medium

DMEM (1 g/l glucose)	500ml
HS	10%
FBS	10%
GlutaMAX™	2 mM
Penicillin/Streptomycin	100 U/ml each

U2OS Medium

McCoy's 5A + L-glutamine	500ml
dialyzed FBS	10%
NEAA	0.1 mM
Penicillin/Streptomycin	100 U/ml each

HEK 293 Medium

DMEM (4.5 g/l glucose)	500ml
FBS	10%
GlutaMAX™	2 mM

Penicillin/Streptomycin 100 U/ml each

for stably transfected HEK 293- β Arr2-C-TEV, 200 μ g/ml G418 were added to maintain selection

Medium for primary cultured neurons

RPMI 500 ml
FBS 10%
GlutaMAX™ 2 mM
Penicillin/Streptomycin 100 U/ml each

Medium for primary cultured astrocytes

BME 500 ml
FBS 10%
GlutaMAX™ 2 mM
Penicillin/Streptomycin 100 U/ml each

2x Freezing medium for eucaryotic cell lines

DMEM 40%
DMSO 20%
FBS 40%

2.7 Oligonucleotides

deoxynucleotide trisphosphates (dNTPs) Boehringer
GeneRuler™ 100bp und 1kb marker Fermentas

Oligo name		5'-3' sequence	#
AVPR1a-B1	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTCT ACCATGGACAGCATGCGTCTCTCCGC	11989
AVPR1a-B2	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCA GTTGAAACAGGAATGAATTTGATGGACTTGG AAG	11990
AVPR2-B1	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTCT ACCATGCTCATGGCGTCCACCACTTC	9242
AVPR2-B2	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCC GATGAAGTGTCCCTGGCCAGG	9243
DRD1-B1	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTCT ACCATGAGGACTCTGAACACCTCTGCCATG	12488
DRD1-B2	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTG GTTGGGTGCTGACCGTTTTGTGTG	12489
DRD2-B1	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTCT ACCATGGATCCACTGAATCTGTCCTGGTATG	9236
DRD2-B2	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTG GCAGTGGAGG ATCTTCAGGAAGG	9237
ADRB2-B1	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTCT ACCATGGGGCAACCCGGGAACGG	11987
ADRB2-B2	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCT AGCAGTGAGTCATTTGTACTACAATTCCTCC	11988
bARR2-B1	sense	GGGGACAAGTTTGTACAAAAAAGCAGGCTCT ACCATGGGGGAGAAACCCGGGACC	13930
bARR2-B2	antisense	GGGGACCACTTTGTACAAGAAAGCGGGTCTG CAGAGTTGATCATCATAGTCGTCATCC	13931
bARR2Δ-B1	antisense	GGGGACCACTTTGTACAAGAAAGCTGGGTCT GTGGCATAGTTGGTATCAAATTCATGAGG	17925
VC-N-TEV_s	sense	ATATGGATCCGCCCGGGGACGCACCCC	3023TF
VC-N-TEV_as	antisense	GTTAACAATGCTTTTATATCCTGTAAAGAATC CATTTTCAAATCATGTCAAGGTCTTCTCGAG G	15004

N-TEV-tevS- GV_s	sense	AAAATGGATTCTTTACAGGATATAAAAGCATT GTTAAC	15003
N-TEV-tevS- GV_as	antisense	CATTTAGGTGACACTATAGAATAGGGCCC	3065TF
KpnI_VC-N- TEV-tevS-GV	sense	ATATGGTACCGCCCGGGGACGCACCCCAA	15172
VC-N-TEV- tevS-GV-Sbfl	antisense	ATATCCTGCAGGTTAATAATAAAAATCATAAA TCATAAGACATTCGCCCG	15173
tevS*_s	sense	CGAGAACCTGTACTTCCAGCTTTCTAGAGAA CAGAAGCTG	2978
tevS*_as	antisense	CAGCTTCTGTTCTCTAGAAAGCTGGAAGTAC AGGTTCTCG	2979

2.8 Plasmids

Plasmid name	Resistance
pcDNA3	Amp ^R
3xFlag-TEV	Amp ^R
pG5-FireflyLuciferase	Amp ^R
pG5-EYFPnuc	Kan ^R
pG5-tdTomato	Kan ^R
<i>cre</i> -luciferase	Amp ^R
<i>nfat</i> -luciferase	Amp ^R
pDONR 207	Genta ^R
pENTR_AVPR1a	Genta ^R
pENTR_AVPR2	Genta ^R
pENTR_DRD1	Genta ^R
pENTR_DRD2	Genta ^R
pENTR_ADRB2	Genta ^R

pENTR_βArr2	Genta ^R
pENTR_βArr2Δ383	Zeo ^R
pDEST_EF5/FRT/V5	Amp ^R
pDEST_X-N-TEV-GV	Amp ^R
pDEST_X-N-TEV-tevS*-GV	Amp ^R
pDEST_X-VC-N-TEV-tevS-GV	Amp ^R
pDEST_X-VC-N-TEV-tevS*-GV	Amp ^R
pDEST_X-TEV	Kan ^R
pDEST_X-C-TEV	Kan ^R
pEXPR_βArr2-C-TEV	Kan ^R
pEXPR_βArr2Δ-C-TEV	Kan ^R
pEXPR_βArr2-TEV	Amp ^R
pEXPR_βArr2Δ-TEV	Amp ^R
pEXPR_AVPR1a-N-TEV-tevS-GV	Amp ^R
pEXPR_AVPR1a-N-TEV-tevS*-GV	Amp ^R
pEXPR_AVPR1a-VC-N-TEV-tevS-GV	Amp ^R
pEXPR_AVPR1a-VC-N-TEV-tevS*-GV	Amp ^R
pEXPR_AVPR2-N-TEV-tevS-GV	Amp ^R
pEXPR_AVPR2-N-TEV-tevS*-GV	Amp ^R
pEXPR_DRD1-VC-N-TEV-tevS-GV	Amp ^R
pEXPR_DRD1-VC-N-TEV-tevS*-GV	Amp ^R
pEXPR_DRD2-VC-N-TEV-tevS-GV	Amp ^R
pEXPR_DRD1-VC-N-TEV-tevS*-GV	Amp ^R
pEXPR_DRD2-N-TEV-tevS-GV	Amp ^R
pEXPR_DRD2-VC-N-TEV-tevS-GV	Amp ^R
pEXPR_DRD2-VC-N-TEV-tevS*-GV	Amp ^R

pEXPR_ADRB2-N-TEV-tevS-GV Amp^R

pEXPR_ADRB2-N-TEV-tevS*-GV Amp^R

Amp^R: ampicillin resistance, Genta^R: gentamycin resistance, Kan^R: kanamycin resistance, Zeo^R: zeocin resistance

2.9 Bacteria strains

MegaX DH10B TM (electrocompetent) Invitrogen

One Shot[®] Mach1 TM (chemocompetent) Invitrogen

2.10 Eucaryotic cell lines

PC12 tet-off rat pheochromocytoma cell line carrying the Tet-off transactivator protein

U2OS human osteosarcoma cell line

HEK 293 human embryonic kidney cell line

HEK-βArr2-C-TEV HEK 293 cell line stably transfected with βArr2-C-TEV

3 Methods

3.1 Molecular biological techniques

3.1.1 *The GATEWAY Technology*

Cloning was done with the GATEWAY Cloning Technology (Invitrogen) which uses the bacteriophage λ system for site-specific recombination in *E.coli* (Hartley et al. 2000).

Bacteriophage λ has four recombination sites, attB, attP, attL and attR, which recombine specifically according to the scheme $\text{attB} \times \text{attP} \leftrightarrow \text{attL} \times \text{attR}$. Integration into the *E.coli* genome (attB x attP reaction) is mediated by the proteins Int (integrase, encoded by phage λ) and IHF (integration host factor, encoded by *E.coli*) whereas excision from the genome (attL x attR reaction) is mediated by Int, IHF, and Xis (excisionase, encoded by phage λ). These recombination reactions are conservative, which means they occur without net gain or loss of nucleotides.

The bacteriophage λ recombination system has been modified in the Gateway Cloning Technology to maintain the orientation of the gene of interest throughout the recombinations. Through mutations in the core regions of the recombination sites, specific att sites (attB1, attB2, attP1, attP2, attL1, attL2, attR1, attR2) have been generated which react only in the way attB1 x attP1, attB2 x attP2, attL1 x attR1, and attL2 x attR2. By flanking the gene of interest by a pair of these specific att sites (e.g. attB1 at the N-terminus and attB2 at the C-terminus), the orientation remains unaffected by recombination. Isolation of the recombination product was made possible by positive selection for resistance and negative selection against the lethal ccdB gene which interferes with DNA gyrase.

Under these preconditions, a system with four kinds of vectors was built up (Fig. 3):

The transcriptionally silent Entry Vector (pENTR, Genta^R or Zeo^R) which contains the gene of interest flanked by attL sites allows flexible transfer of this gene into various Expression Vectors (pEXPR, Amp^R or Kan^R) for the expression of native or fusion proteins (carrying attB sites).

The Destination Vector (pDEST, Amp^R or Kan^R) carries the “controlling cell death B” (ccdB) gene flanked by attR sites and is used to recombine an Entry Clones (Vector + gene of interest) to an Expression Clone in a so-called LR reaction.

The Donor Vector (pDONR, Genta^R or Zeo^R) contains the *ccdB* gene flanked by attP sites and mediates the back-recombination of an Expression Clone to an Entry Clone in a “BP reaction”.

Entry Clones can be generated in different ways, including classical cloning and PCR. The latter approach includes amplification of the gene of interest flanked by attB sites and a subsequent BP reaction.

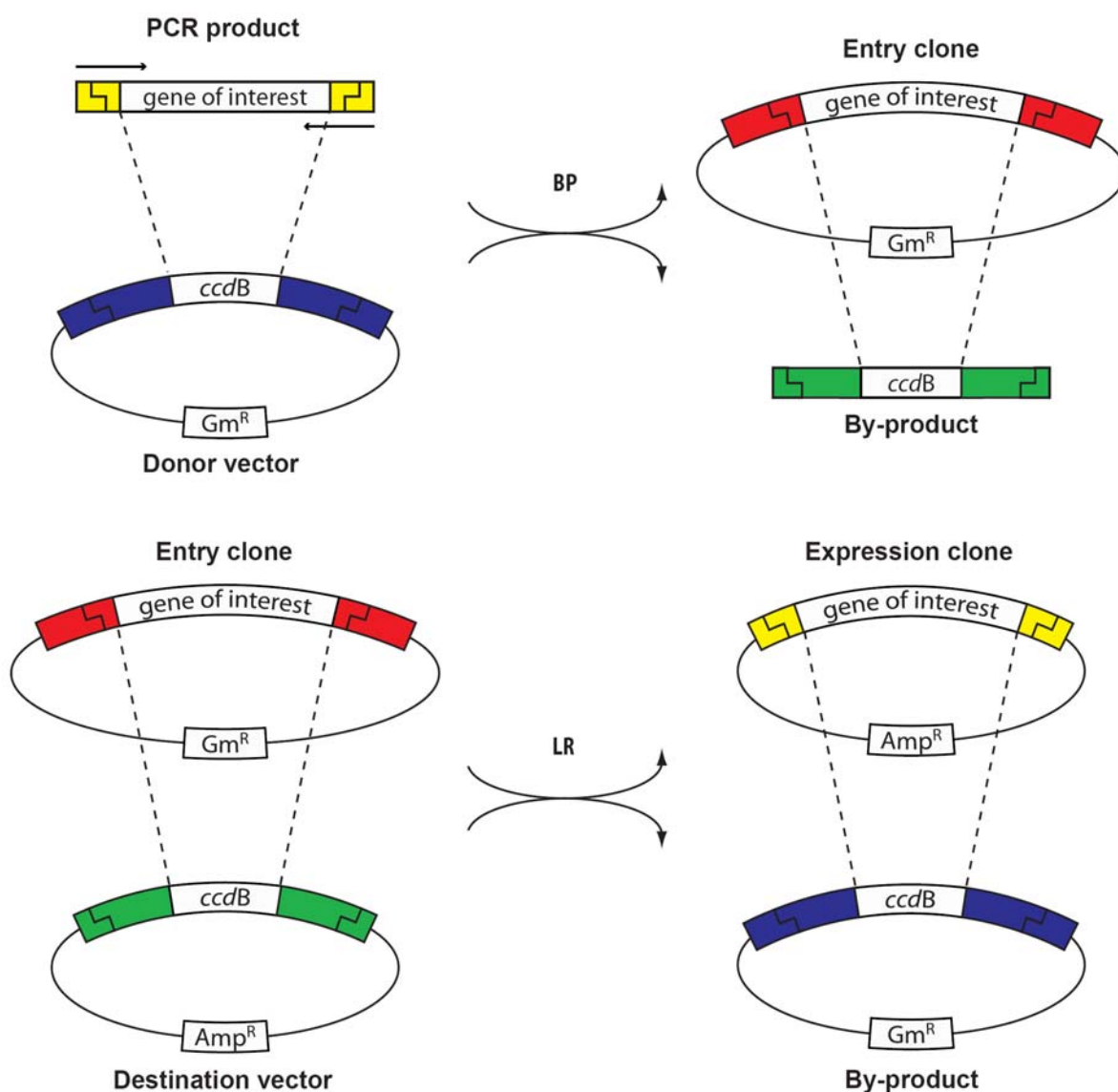


Fig. 3. GATEWAY cloning procedure.

BP reaction (BP). The gene interest (available e.g. as PCR product) can be recombined with a donor vector to give rise to an entry clone. A by-product carrying the lethal *ccdB* gene is generated as well. LR reaction (LR). An entry clone can be recombined with a destination vector to an expression clone. Again, a byproduct carrying the *ccdB* gene is generated.

The basis for the high efficiency of the Gateway technology can be exemplified by the LR reaction: an Entry Clone is recombined with a Destination Vector to an Expression Vector. The Entry Clone is gentamycin- or zeocin-resistant whereas the Destination Vector and the Expression Clone carry an ampicillin- or kanamycin-resistance. The recombination reaction is transformed in *E.coli* and selection for the recombination product is done on Amp- or Kan-positive agar plates, respectively. Selection for resistance favors the Destination Vector, intermediates of the recombination and the Expression Clone, but since the *ccdB* gene lethal to *E.coli* is present in both the Destination Vector and reaction intermediates, the Expression Clone is obtained with high efficiency.

3.1.2 Cloning strategy

All expression constructs were generated by Gateway cloning (Invitrogen). Human *AVPR1a* (NM000706.3), *AVPR2* (NM000054.4), *DRD1* (NM000794), *DRD2* (NM016574), *ADRB2* (NM000024) and β *ARR2* (β -arrestin 2, BC067368) were amplified from a mix of human liver, heart, uterus and fetal and adult brain cDNAs using proofreading polymerases. Gateway-compatible PCR products were generated in a C-open form (N-terminal Kozak sequence, no stop codon) by using the primer pairs 11989/11990, 9242/9243, 12488/12489, 9236/9237, 11987/11988, 13930/13931, respectively (see section 2.7 for oligonucleotide sequences). The truncation mutant β *ARR2* Δ 383 (β *ARR2* Δ) was amplified using the primers 13930 and 17925.

PCR products were recombined with the pDONR 207 vector to obtain pENTR clones. These were further recombined with a pDEST vector to produce pEXPR clones. Customized pDEST vectors generated according to the manufacturer's protocols were used unless stated otherwise. pDEST_N-TEV-tevS-GV comprised aa 1-118 of the TEV protease, the TEV protease cleavage site ENLYFQ'G and a fusion of yeast Gal4 DNA-binding domain and the herpes simplex VP16 transactivation domain, as previously described (Wehr et al. 2006). pDEST_X-C-TEV contained aa 119-221 of the TEV protease including the point mutation S219P. pDEST_TEV contained the full-length TEV protease. pDEST_X-VC-N-TEV-tevS-GV included aa 343-371 of the AVPR2 receptor C-tail (VC) positioned N-terminally of the N-TEV fragment (Barnea et al. 2008). VC-N-TEV was amplified from pEXPR_AVPR2-N-TEV-tevS-GV with the primers 3023 TF and 15004 whereas N-TEV-tevS-GV was amplified from pDEST_N-TEV-tevS-GV with the primers 15003 and 3065TF. A fusion PCR using the primers 3023TF and 3065TF yielded VC-N-TEV-tevS-GV. This product was subjected to another PCR with the primers 15172 and

15173 to attach a 5' terminal KpnI and a 3' SbfI site for subcloning into a pCMV vector. A pDEST vector was generated from this final construct.

pDEST_X-N-TEV-tevS*-GV and pDEST_X-VC-N-TEV-tevS*-GV contained the mutated, low-affinity TEV protease cleavage site ENLYFQ'L (tevS*). These vectors were created by site-directed mutagenesis of pDEST_X-N-TEV-tevS-GV and pDEST_X-VC-N-TEV-tevS-GV, respectively, using the primers 2978 and 2979.

Backbone expression vectors used were pBK-CMV (Stratagene) and pcDNA 3.1 (Invitrogen).

V5 epitope-tagged receptors (C-terminally attaching the amino acids GKPIPPLLGLDST) were obtained by recombination of pENTRs into the pEF5/FRT/V5-DEST vector.

Reporter gene constructs used were G5-luciferase (five clustered Gal4-responsive *cis* elements coupled to the firefly luciferase gene, as described in (Wehr et al. 2006)), *cre*-luciferase and *nfat*-luciferase.

All constructs were verified by sequencing of both strands (AG Benseler, MPI for Experimental Medicine, Göttingen).

3.1.3 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed using the following composition:

x µl DNA template (10 – 100 ng)
1 µl sense primer (10 pmol/µl)
1 µl anti-sense primer (10 pmol/µl)
5 µl 10x buffer (with MgCl₂)
5 µl dNTPs (final concentration: 0.2 mM)
0,2 µl proofreading DNA Polymerase (2.5 U/µl)
ddH₂O up to 50 µl

PCRs were performed using the following standard protocol, with a variable cycle number of 25-35:

95°C, 3 min: initial denaturation

95°C, 30 s: denaturation (cycle)

56°C, 30 s: annealing (cycle)

72°C, 60 s: extension (cycle, 3 min for long fragments)

72°C, 10 min: final extension

hold at 4°C.

PCR products were mixed with 6x Loading Dye and analyzed on 1.5% agarose gels containing 1 µg/ml ethidium bromide. Gel electrophoresis was done at 100-160 V in a gel chamber containing TAE buffer. DNA fragments were visualized with a UV illuminator.

3.1.4 Extraction of DNA fragments from agarose gels

DNA fragments were cut from agarose gels and extracted using the NucleoSpin Extract II Kit (Macherey-Nagel) according to the manufacturer's instructions. In principle, agarose slices were solubilised and adjusted for binding to a silica membrane. Upon adsorption, DNA was washed and eluted under low-salt conditions in 50 µl elution buffer.

3.1.5 DNA purification by precipitation with polyethylene glycol (PEG)

PEG precipitation was performed to clean plasmid DNA from small-size DNA fragments (<300 bp), such as primer dimers or fragments originated from restriction digests. Plasmid DNA was diluted 4-fold with TE buffer, mixed with ½ volume of PEG/MgCl₂ and 1-3 µl glycogen carrier, and centrifuged at room temperature for 15 min at 13000 rpm. After removal of the supernatant, the pellet was resuspended in TE buffer, according to the final concentration wanted.

3.1.6 BP and LR reactions

BP and LR reactions were set up in the following way:

BP reactions:

3 µl PCR product

1 µl pDONR vector (100 ng/µl)

1 µl BP clonase II

LR reactions:

1 μ l pENTR clone

1 μ l pDEST vector (100 ng/ μ l)

0.5 μ l LR clonase II

BP and LR reactions were incubated overnight at room temperature. Reactions were stopped by proteinase K treatment for 10 min at 37°C with subsequent enzyme inactivation for 10 min at 95°C. BP and LR reactions were transformed into DH10B electrocompetent cells or One Shot[®] Mach1[™] chemocompetent cells.

3.1.7 Transformation of DNA into chemocompetent bacteria

2 μ l of recombination reaction were incubated with 25 μ l of One Shot[®] Mach1[™] cells for 30 min on ice. Cells were then heat-shocked for 30 s at 42°C and incubated on ice for another 5 min. After addition of 250 μ l of S.O.C. medium, the transformation reaction was incubated on a shaker for 1 h at 37°C. The transformation reaction was spread on pre-warmed selective agar plates and incubated overnight at 37°C.

3.1.8 Transformation of DNA into electrocompetent bacteria

20 μ l of DH10B cells, diluted 1:4 in 10% glycerol in H₂O, were incubated with 1.5 μ l of recombination reaction for 5 min on ice. Cells were transferred to a 1 mm-electroporation cuvette and electroporated at 1700 kV, 25 μ F, and 200 Ω . After addition of 1000 μ l of cold LB medium, the transformation reaction was incubated on a shaker for 1 h at 37°C. The transformation reaction was spread on selective agar plates and incubated overnight at 37°C.

3.1.9 Preparation of plasmid DNA

Single bacterial colonies from LB agar plates were transferred into 4 ml of selection medium and grown overnight in a shaking incubator at 37°C. Subsequently, bacteria were harvested by centrifugation at 13000 rpm for 1 min. For the preparation of larger amounts of plasmid DNA, 150 ml of bacterial cultures were inoculated, incubated overnight, and harvested by centrifugation at 4000 rpm for 15 min at 4°C.

Plasmid DNA was prepared by use of the NucleoSpin Plasmid Quick Pure (Macherey-Nagel, for low amounts of plasmid) and the NucleoBond PC100 Midiprep kit (Macherey-Nagel, for larger amounts of plasmid) according to the manufacturer's instructions. In principle, bacteria were lysed under alkaline conditions to denature plasmid and chromosomal DNA, and proteins. Chromosomal DNA and proteins were then precipitated under high-salt conditions. Plasmid DNA was adsorbed to a silica membrane (Miniprep Kit) or an anion-exchange resin (Midi Kit), washed, and eluted under low-salt conditions in 100 μ l (mini prep) or 1 ml (midi prep) elution buffer.

3.1.10 Restriction digests

All subclones generated throughout the described cloning procedure were analyzed by digests with type II restriction enzymes. Analytical digests were done in an overall volume of 20 μ l, including 2 μ l of 10x restriction buffer and 0.5 μ l of enzyme. Plasmid DNA was used in a concentration range of 0.5-1 μ g. The respective restriction buffer for each enzyme was chosen according to the manufacturer's instructions. Double digests were carried out with enzymes with matching restriction buffers, using 0.5 μ l of each enzyme. Digests were incubated at 37°C for 1-2 h and analyzed on an agarose gel.

3.2 Cell culture techniques

3.2.1 Basic cell culture techniques

Early passages of mammalian cell lines (PC12-tetoff, U2OS) frozen in liquid nitrogen were quickly thawed at 37°C. The cell suspension was transferred to a Falcon tube filled with 10 ml culture medium and centrifuged for 5 min at 800 rpm to remove DMSO. Cells were resuspended in culture medium and plated on 15 cm-dishes. To allow better adherence, PC12 cells were plated on poly-L-lysine (PLL) coated dishes (0.02 mg/ml PLL in H₂O, incubated for 30 min at room temperature).

PC12-tetoff cells were grown in DMEM (1g/l glucose), supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), 2 mM GlutaMAXTM, and 100 U/ml each of penicillin and streptomycin. U2OS cells were grown in McCoy's A medium, supplemented with 10% dialyzed FBS, 0.1 mM non-essential amino acids (NEAA), and 100 U/ml of penicillin/streptomycin. HEK 293 cells were grown in DMEM (4.5 g/l glucose), supplemented with 10% FBS, 2 mM GlutaMAXTM, and 100 U/ml each of penicillin and

streptomycin. HEK cells stably transfected with β Arr2-C-TEV additionally received 200 μ g/ml G418 to maintain selection. Medium changes were performed every 2-3 days, and cells were passaged at near confluency every 4-7 days.

During passaging, cells were washed with PBS and then treated with 2.5 ml trypsin/EDTA for 1-3 min to break down cell contacts and cell adhesion. Trypsinization was stopped by addition of cell-culture medium, and cells were dissociated to single cells by pipetting up and down. Cells were spread on new dishes with fresh culture medium in a ratio of 1:20 - 1:4, according to the actual requirements.

To plate cells for luciferase assays, cells were dissociated, transferred to a Falcon tube, and centrifuged for 5 min at 800 rpm. Cells were resuspended in a defined volume of culture medium, counted in a Neubauer counting chamber, and plated at the required density.

For conservation in liquid nitrogen, cells were dissociated, centrifuged, and resuspended in 500 μ l culture medium per 5×10^6 cells. Cells were transferred to cryo tubes in 500 μ l aliquots, and 500 μ l of DMSO-supplemented 2x freezing medium were added to each tube. Cryo tubes were frozen at -20°C for 1-2 hrs and subsequently at -80°C overnight, and transferred to liquid nitrogen the next day.

3.2.2 Primary cell culture

For primary neuronal cell culture, brains from E18 Sprague Dawley rat embryos were prepared as described (Wehr et al. 2006). Briefly, after removal of the meninges and the cerebellum, cortex was isolated. Cortices were washed three times with fresh HBSS and then incubated with 5 ml of papain solution for 10 min at 37°C . Cells were subsequently washed three times with fresh HBSS and triturated in 5 ml RPMI medium (supplemented with 10% FBS, 2 mM GlutaMAXTM, and 100 U/ml each of penicillin and streptomycin). After centrifugation for 5 min at 800 rpm, cells were resuspended in RPMI medium and plated on poly-L-ornithine (PLO) coated 96 well-plates (15 μ g/ml PLO in H_2O , incubated overnight at 37°C) at a density of 10^5 cells/well.

Primary astrocytes were prepared from P0 C57BL/6 mice. Whole brains were isolated and cut into pieces after removal of the cerebellum and the meninges. For dissociation, the tissue was incubated with 4ml of trypsin-EDTA for 10 min at room temperature, and gently triturated. The reaction was stopped with 10% serum and tissue pieces were washed twice with HBSS + 10% MgSO_4 . After resuspension in 2 ml BME medium (supplemented with 10% FBS, 2 mM GlutaMAXTM, and 100 U/ml each of penicillin and

streptomycin), the tissue was further mechanically dissociated with a polished Pasteur pipette. The cell suspension was distributed on poly-L-lysine-coated cell culture flasks and cultivated in BME medium for 7-10 days yielding a confluent astrocyte layer with oligodendrocyte precursor cells and microglia on top. During this time, medium was changed every second day. Finally, cellular debris, growing microglia and oligodendrocyte precursor cells were subsequently mobilized by shaking the flask. Supernatant was exchanged by fresh BME medium. Primary astrocytes were then plated on poly-L-lysine-coated 96 well-plates at a density of 5×10^4 cells/well.

3.2.3 Transfection of mammalian cells by lipofection

Cells were transfected by lipofection with Lipofectamine 2000 reagent. Lipofection is based on the formation of complexes between positively charged liposomes and the DNA which interact with the cell membrane and are taken up into the cells by endocytosis.

Lipofectamine 2000 reagent was preincubated with serum- and antibiotic-free Opti-MEM[®] medium for 5 min, mixed with the DNA, and incubated for another 20 min to allow complex formation. Following a medium change, cells were treated with the Lipofectamine-DNA mix. 1-4 hrs after transfection, culture medium with 2x serum concentration was added.

3.3 Reporter gene assays

3.3.1 Luciferase reporter gene assays

The luciferase reporter gene assay is based on the measurement of bioluminescence generated by enzymatic conversion of the luciferase substrate. The amount of emitted light is directly dependent on the amount of luciferase and can therefore be taken as readout for reporter gene expression.

The Dual-Luciferase[®] Reporter Assay System (Promega) allows subsequent measurement of the activities of two luciferases with different substrate specificities, Firefly and Renilla luciferase. Firefly luciferase from the firefly *Photinus pyralis* catalyzes the oxidation of D-luciferin involving the consumption of ATP and oxygen. Light emission occurs at a wavelength of 575-600 nm. Renilla luciferase from the sea pansy *Renilla reniformis* uses coelenterazine as a substrate and emits light at 475 nm during the Ca^{2+} -dependent reaction.

The Dual-Luciferase[®] Reporter Assay is performed on lysed cells expressing Firefly and Renilla luciferases. Addition of Firefly Luciferase Assay Buffer to the test preparation provides the substrate for the Firefly luciferase reaction which can be quantitatively measured with a luminometer. The Firefly luciferase reaction is then quenched by addition of Renilla Luciferase Assay Buffer which simultaneously provides the substrate for the Renilla luciferase reaction. In our assays, Firefly luciferase was used as an “experimental” reporter to monitor expression levels of the reporter gene whereas Renilla luciferase served as an internal control to compensate for variable transfection efficiencies. Normalization of the Dual-Luciferase[®] Reporter Assay was done by calculating the ratios of Firefly to Renilla values (relative light units, RLU).

Luciferase reporter gene assays were performed in rat pheochromocytoma (PC12) and human osteosarcoma (U2OS) cells as well as primary cultured neurons and astrocytes in a 96-well plate format. Cells were seeded at densities of 10^4 cells/well (U2OS), 4×10^4 cells/well (PC12), 5×10^4 cells/well (primary astrocytes) and 10^5 cells/well (primary neurons). Cells were transfected up to 24 h after plating, using Lipofectamine 2000 transfection reagent according to the manufacturer’s protocol. Transfection mixes contained 10 ng each of the plasmids of interest as well as of the reporter plasmid (G5-Firefly luciferase, if not stated otherwise, Fig. 4). A pcDNA backbone vector was used as mock. To standardize for transfection variability, unspecific drug effects and cell death, we additionally transfected a mixture of plasmids coding for renilla luciferase under the three promoters SV40, TK, and CMV (10 SV40 : 2 TK : 1 CMV), along with a CMV-driven nuclear localized enhanced yellow fluorescent protein (EYFPnuc) for optical transfection control (Wehr et al. 2006).

Cells were stimulated 16-18 h after transfection by adding 1 volume of two-fold concentrated test compounds in assay medium (DMEM, McCoy’s A, BME or RPMI, depending on cell type, + 5% dialyzed FBS).

24 h after stimulation, if not specified otherwise, cells were lysed with 30 μ l of 1x Passive Lysis Buffer (Promega) for 20 min at room temperature on a shaker. Lysates were transferred to an opaque 96-well microtiter plate for luciferase assays. Dual-Luciferase[®] Reporter Assays were performed in an automated way in a Mithras LB 940 multimode reader (Berthold Technologies). For each well, the following protocol was applied: injection of 75 μ l Firefly Luciferase Assay Buffer followed by a delay of 2 s, measurement over 10 s and display of an integrated Firefly value, injection of 75 μ l Renilla Luciferase Assay Buffer followed by a delay of 2 s, measurement over 10 s and display of an integrated Renilla value.

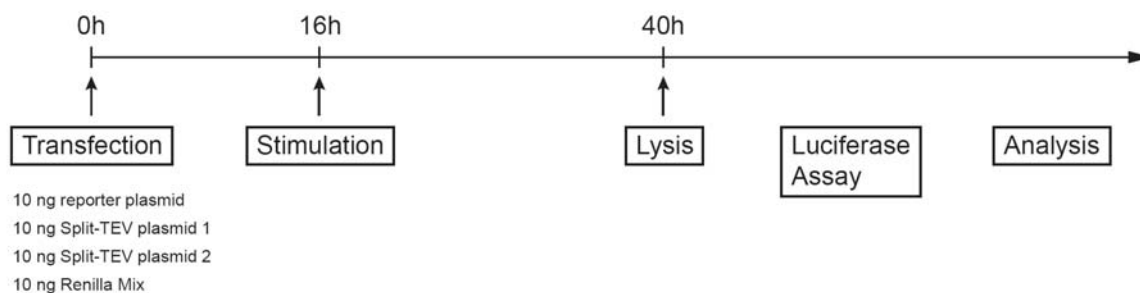


Fig. 4. Time frames for luciferase reporter gene assays.

Cells were transfected with 10 ng of each reporter plasmid, the respective split-TEV plasmids and Renilla Mix. Cells were stimulated 16 h after transfection and lysed after another 24 h. Luciferase assays and data analysis were performed thereafter.

Data were analysed in Excel (Microsoft) and Graphpad Prism® (GraphPad software). Firefly values were divided by Renilla values to obtain Relative Light Units (RLUs). RLUs were averaged over the number of replicates ($n = 6$), and the standard deviation was calculated. Levels of significance (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) were determined with an unpaired, double-sided t-test.

3.3.2 Dose response curves

Dose responses of GPCR/beta-arrestin 2 interactions were carried out using a typical agonist for each given GPCR. Agonists were subsequently diluted in assay medium from a stock concentration and tested within a maximal range of 10^{-15} to 10^{-4} M. Data of 6 technical replicates were acquired per concentration. Cells were stimulated about 16 h after transfection and lysed another 6 h later.

Dose response curves were generated in GraphPad Prism® (GraphPad Software, Inc). Replicates were treated as separate data points. Curves were fitted assuming a sigmoidal dose response according to the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))}$, where X is the logarithm of the concentration. In the curve-fitting process, EC_{50} s (agonist concentration needed to evoke a response halfway between baseline and maximum) and the R^2 Pearson's correlation coefficient (goodness of fit) were determined. Dose responses were normalized by setting the lowest mean of the data array to 0 and the highest mean to 100.

3.3.3 Fluorescence-based reporter gene assays

Fluorescence-based reporter gene assays were carried out on the basis of a fluorescent protein instead of luciferase, allowing an optical readout such as fluorescence microscopy or flow cytometry. Similarly to the G5-luciferase construct, the gene coding for a fluorescent protein such as tdTomato or EYFPnuc was coupled to a cluster of five Gal4-responsive *cis* elements. The resulting constructs were termed G5-tdTomato and G5-EYFPnuc, respectively. Assays were performed in the same manner and with the same time frames as described for luciferase-based assays, but either G5-tdTomato or G5-EYFPnuc were used as reporters. Assays were qualitatively analyzed using a fluorescence microscope, and quantified by flow cytometry. For the latter - instead of lysis - cell medium was exchanged by Hoechst dye, dissolved 1:500 in PBS. Cells were incubated for 20 min at 37°C whereafter nuclear staining was checked under a fluorescence microscope. Cells were then washed with PBS, treated with a 1:1-dilution of trypsin in PBS for 5 min at 37°C and separated mechanically. After further dilution in 3 volumes of PBS, cells of each well were transferred to a separate 5 ml round-bottom tube for flow cytometry.

3.3.4 Flow cytometry

Flow cytometry is suited to analyse cells with respect to their size, shape and inner complexity, and to count and sort them according to distinct parameters. In principle, a laser beam of a unique wavelength is directed onto the fluid stream of a cell suspension which is focused in a way that cells pass the laser one at a time. While passing, cells lead to a scattering of the laser light depending on their morphology. Several detectors in different positions collect the scattered light as Forward Scatter (FSC, reflecting cell size) and Side Scatter (SSC, reflecting granularity which is e.g. defined by the size and structure of the cell nucleus and the presence of vesicles). Fluorescent labelling of cells can be used as an additional parameter for characterization. By using gates with thresholds and maximal cut-offs for FSC, SSC and fluorescent stainings, cell subpopulations with distinct properties can subsequently be confined.

Recordings and analysis were done using a BD FACSAria flow cytometer and the BD FACS Diva™ Software (Becton Dickinson, BD Biosciences). Subpopulations of Hoechst-positive/EYFPnuc-positive or Hoechst-positive/tdTomato-positive cells were analysed with respect to cell numbers and total fluorescence intensity. Data were normalized to the number of cells measured in total.

4 Results

4.1 Design of split-TEV assays for GPCR activation

Split-TEV assays make use of inactive N- and C-terminal fragments of the TEV protease which are coupled to two proteins of interest and are functionally complemented upon their interaction (Wehr et al. 2006). The reconstituted active TEV protease recognizes its specific cleavage site ENLYFQ'G (tevS), thereby releasing a fused transcriptional activator. Here, the artificial transcription factor GV was used, which is composed of the yeast Gal4 DNA-binding domain and the herpes simplex VP16 transactivation domain. Release of GV allows its translocation to the nucleus, followed by the activation of a Gal4-responsive firefly luciferase gene (G5-luciferase) (Wehr et al. 2006). Luciferase activity finally serves as readout. To monitor GPCR activation, we made use of its stimulation-dependent interaction with the adaptor protein β Arr2 which mediates receptor desensitization and internalization in a widely distributed manner throughout the GPCR family (Ferguson 2001; Ferguson et al. 1996; Lohse et al. 1990). The aim was to establish split-TEV based assays monitoring GPCR activation (i) of vasopressin receptors 1a (AVPR1a) and 2 (AVPR2) regulated by the peptide hormone vasopressin (AVP), (ii) of dopamine receptors 1 (DRD1) and 2 (DRD2) and the β -adrenergic receptor 2B (ADRB2) responding to the catecholamines dopamine and (nor)adrenaline, respectively (Harmar et al. 2009).

Receptor constructs were designed in which the N-terminal fragment of TEV (N-TEV) was coupled to GV via the specific TEV cleavage site ENLYFQ'G (tevS), and attached to GPCRs at their respective C-termini (Fig. 5a). Complementary, β Arr2 was coupled to the C-terminal fragment of TEV (C-TEV). Split-TEV assays were compared to the previously described Tango[®]/full-TEV proximity assays (referred to in this thesis as 'full-TEV') in which the full-length TEV protease was coupled to β Arr2 (β Arr2-TEV) and a transcriptional reporter was attached to the GPCR via the TEV protease cleavage site (Fig. 5b). In contrast to split-TEV, these constructs were modified to contain the low-affinity cleavage site ENLYFQ'L (tevS*) which has been shown to be essential for 'full-TEV assays' (Fig. 5b) (Barnea et al. 2008). AVPR1a, DRD1 and DRD2 constructs were further modified by inserting aa 343-371 of the human AVPR2 receptor in between of the respective GPCR C-termini and N-TEV (X-VC-X) thought to enhance β Arr2 coupling as described previously (Fig. 5c) (Barnea et al. 2008; Tohgo et al. 2003). A list of all GPCR constructs used in the assays described below can be found in Table 1..

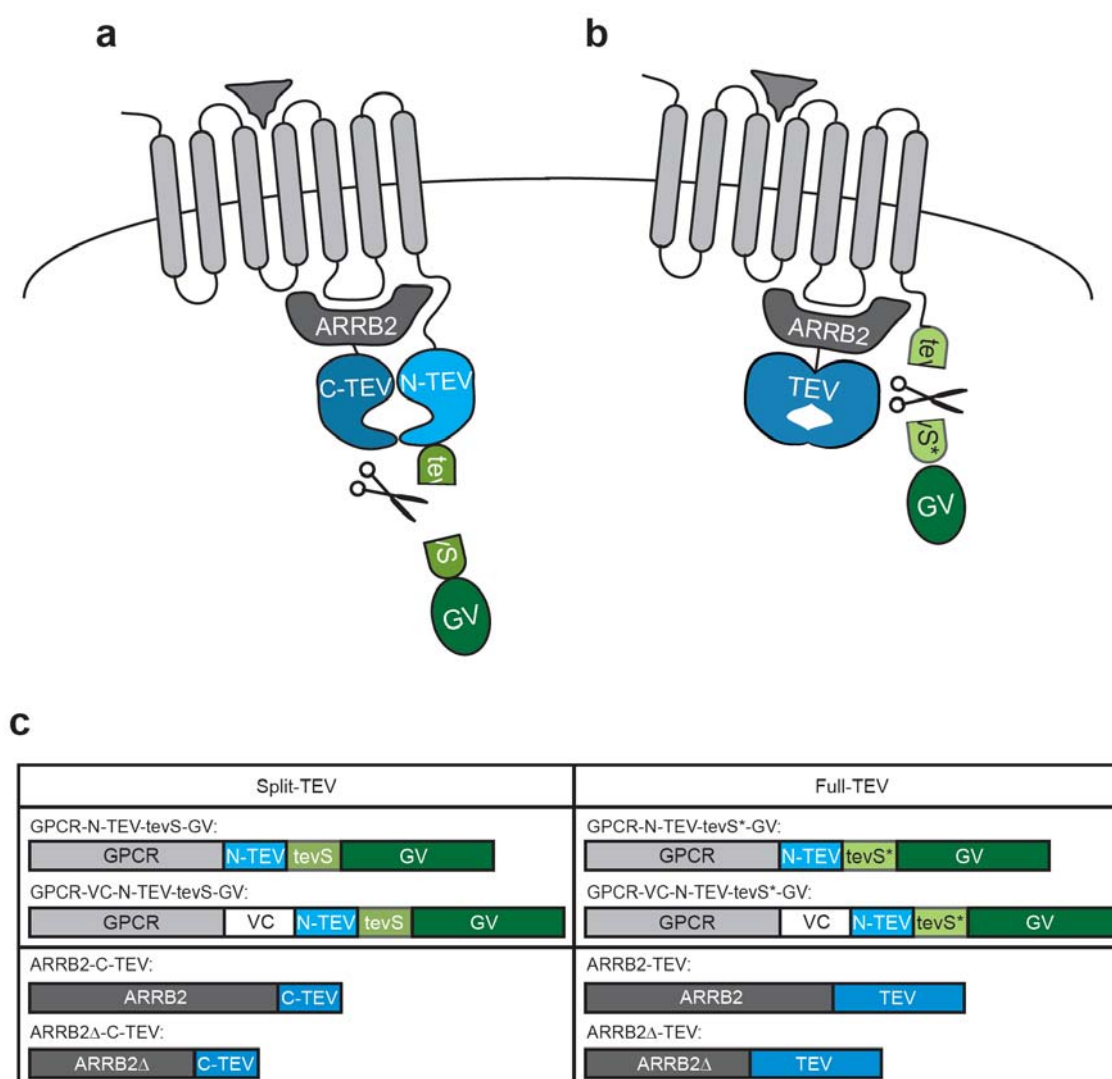


Fig. 5. Design of split-TEV and full-TEV assays to monitor GPCR activation.

a,b) Illustration of split-TEV (a) and full-TEV (b) assays with functionally relevant domains depicted schematically. Split-TEV as well as full-TEV assays rely on the interaction of modified GPCRs with β Arr2 upon GPCR activation. Split-TEV assays are based on the functional reconstitution of inactive N- and C-terminal TEV fragments (N-TEV, C-TEV). N-TEV is fused to the C-terminus of the GPCR followed by a high-affinity TEV cleavage site (tevS) and the artificial transcription factor GV while C-TEV is fused to β Arr2 (a). Interaction of the two fusion proteins leads to the reconstitution of the TEV protease activity releasing GV. Full-TEV (Tango®) assays use a 'proximity approach' where the full-length TEV protease (TEV) is fused to β Arr2 whereas the GPCR is coupled to GV via a low-affinity TEV protease cleavage site (tevS*) (b). Proximity of the two fusion proteins leads to TEV-mediated cleavage and release of the transcription factor. Common for both assays is the TEV-dependent release of GV that finally activates firefly luciferase as reporter gene (not shown). c) Domain structure of constructs used for split-TEV (left panel) and full-TEV assays (right panel). For GPCR-N-TEV-tevS-GV and β Arr2-C-TEV fusion constructs the following additionally modified constructs were tested. GPCR-VC-N-TEV-tevS-GV, harbours a C-terminal tail of the human vasopressin AVPR2 receptor to provide a stronger interaction with β Arr2. β Arr2 Δ -C-TEV, C-terminally truncated form of human β Arr2. N-TEV: N-terminal fragment of TEV protease (aa 1-118), C-TEV: C-terminal fragment of TEV protease (aa 119-221), TEV: full-length TEV protease, VC: C-terminus of the AVPR2 receptor (aa 343-371), tevS: natural TEV cleavage site ENLYFQ'G, tevS*: low-affinity TEV protease cleavage site ENLYFQ'L, GV: Gal4-VP16 transcription factor, β Arr2: β -arrestin 2, β Arr2 Δ : truncated β Arr2 (aa 1-382).

	N-TEV-tevS-GV	N-TEV-tevS*-GV	VC-N-TEV-tevS-GV	VC-N-TEV-tevS*-GV
HEK 293 cleavage assay	AVPR2 (7b,c) DRD2 (7b,c) ADRB2 (7b,c)			
TEV site comparison	AVPR1a (9)	AVPR1a (9)		
HEK 293-βArr2-C-TEV readout comparison	AVPR2 (8a,b,c)			
PC12 tet-off cleavage assay	AVPR2 (6b) ADRB2 (6e)	AVPR2 (6b) ADRB2 (6e)	AVPR1a (6a) DRD1 (6c) DRD2 (6d)	AVPR1a (6a) DRD1 (6c) DRD2 (6d)
split-TEV assay	ADRB2 (10c)		AVPR1a (10a) DRD2 (10b)	
dose response full-TEV assay	AVPR2 (13b)	ADRB2 (10c)	AVPR1a (13a)	AVPR1a (10a) DRD2 (10b)
signaling assay dose response	AVPR1a (16a,b) AVPR2 (16a,b) AVPR1a (16d) AVPR2 (16c)		AVPR1a (16a,b)	
U2OS split-TEV assay	ADRB2 (11c)		AVPR1a (11a) DRD2 (11b)	
dose response	ADRB2 (13e)		DRD1 (13c) DRD2 (13d)	
full-TEV assay		ADRB2 (11c)		AVPR1a (11a) DRD2 (11b)
primary neurons split-TEV assay			AVPR1a (12a) DRD2 (12b)	
full-TEV assay				AVPR1a (12a) DRD2 (12b)
primary astrocytes split-TEV assay dose response full-TEV assay	ADRB2 (12c) ADRB2 (13f)			
	V5	C-TEV		
PC12 tet-off signaling assay	AVPR1a (16a,b) AVPR2 (16a,b)	AVPR1a (16a,b) AVPR2 (16a,b)		

Table 1. Overview of GPCR constructs used in cleavage assays, split-TEV and full-TEV activation assays and signaling assays.

Table shows in which cells each assay was performed and which tagged versions of GPCRs were used in a given assay. Citation of figures is given in brackets.

4.2 Cleavage of GPCR fusion constructs by TEV protease

Cleavage of GPCR fusion proteins by a co-expressed cytoplasmic TEV protease (TEV) was tested to assess construct functionality and to estimate cleavage efficiency. TEV cleavage was highly efficient for all receptor constructs containing the natural TEV protease cleavage site ENLYFQ'G (tevS) (Fig. 6a-e). Cleavage was variable between receptor constructs, ranging from 7-fold signal induction for DRD1-N-TEV-tevS-GV in the presence of TEV to 110-fold induction for AVPR1a-N-TEV-tevS-GV. In contrast, receptor constructs containing the mutated, low-affinity TEV cleavage site ENLYFQ'L (tevS*) were not efficiently cleaved by TEV (Fig. 6a-e) which is in concordance with findings by Barnea et al. (Barnea et al. 2008).

Additionally, a fluorescent readout was used to microscopically assess cleavage. Instead of G5-luciferase, G5-EYFPnuc was used as a reporter. Therefore, release of GV and its subsequent localization to the nucleus drives EYFPnuc expression. Here, cleavage of AVPR1a-N-TEV-tevS-GV, DRD2-N-TEV-tevS-GV and ADRB2-N-TEV-tevS-GV was tested in HEK 293 cells (Fig. 7). All of these receptors showed strong fluorescence in the presence of TEV protease (Fig. 7a) which could clearly be distinguished from controls. However, a considerable fluorescent background was observed in the ADRB2-N-TEV-tevS-GV control. As this might impose problems to qualitatively evaluate less efficient cleavages such as in protein interaction-dependent assays, quantification of fluorescence was done in addition.

Flow cytometry was used to quantify EYFPnuc-positive cells as well as to measure total fluorescence. TEV cleavage was reflected in signal inductions rates of 3 (ADRB2), 5 (DRD2) and 56 (AVPR1a) in the analysis of EYFPnuc-positive cells (Fig. 7b) and of 9 (ADRB2), 28 (DRD2) and 542 (AVPR1a) in the analysis of total fluorescence (Fig. 7c). Ratios were mainly influenced by the amount of background while the percentage of EYFPnuc-positive cells and the total fluorescence in the presence of TEV were about the same among the tested receptor constructs. Analysis of fluorescence seemed to be more sensitive than the analysis of EYFPnuc-positive cells.

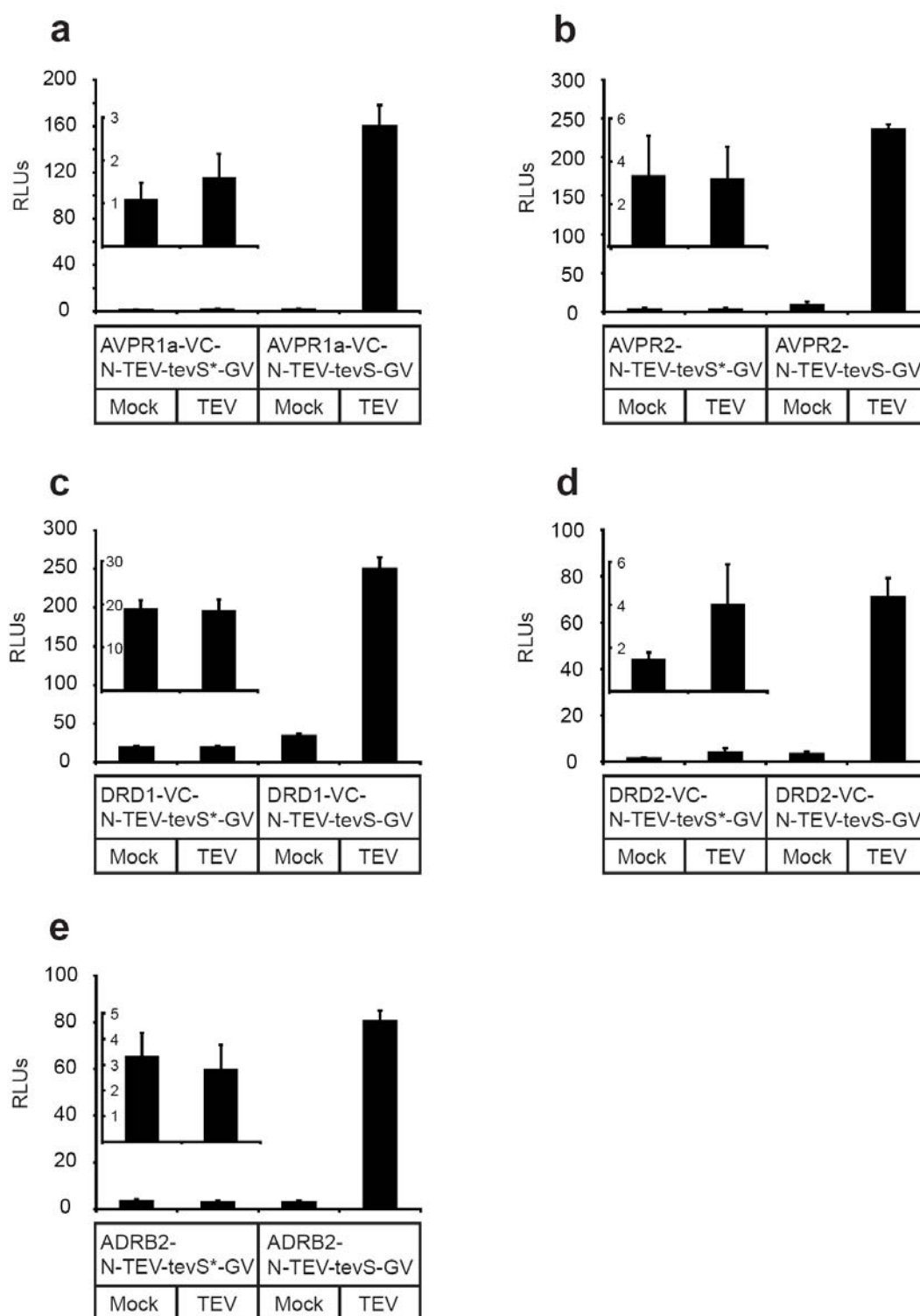


Fig. 6. Cleavage of GPCR constructs by TEV protease.

(a-e) Efficiency of a cytoplasmic TEV protease (TEV) to cleave low- (tevS*, left) and high-affinity (tevS, right) TEV cleavage sites of full-TEV and split-TEV GPCR reporter constructs. a) AVPR1a, vasopressin receptor 1a. b) AVPR2, vasopressin receptor 2. c) DRD1, dopamine receptor 1. d) DRD2, dopamine receptor 2. e) ADRB2, adrenergic receptor 2. Assays were performed in PC12 cells and lysed 24 h after transfection. Insets show data for full-TEV receptor constructs in a separate scale. Mock: pcDNA backbone vector. RLUs, relative light units. Data are given as means of $n=6 \pm$ s.d.

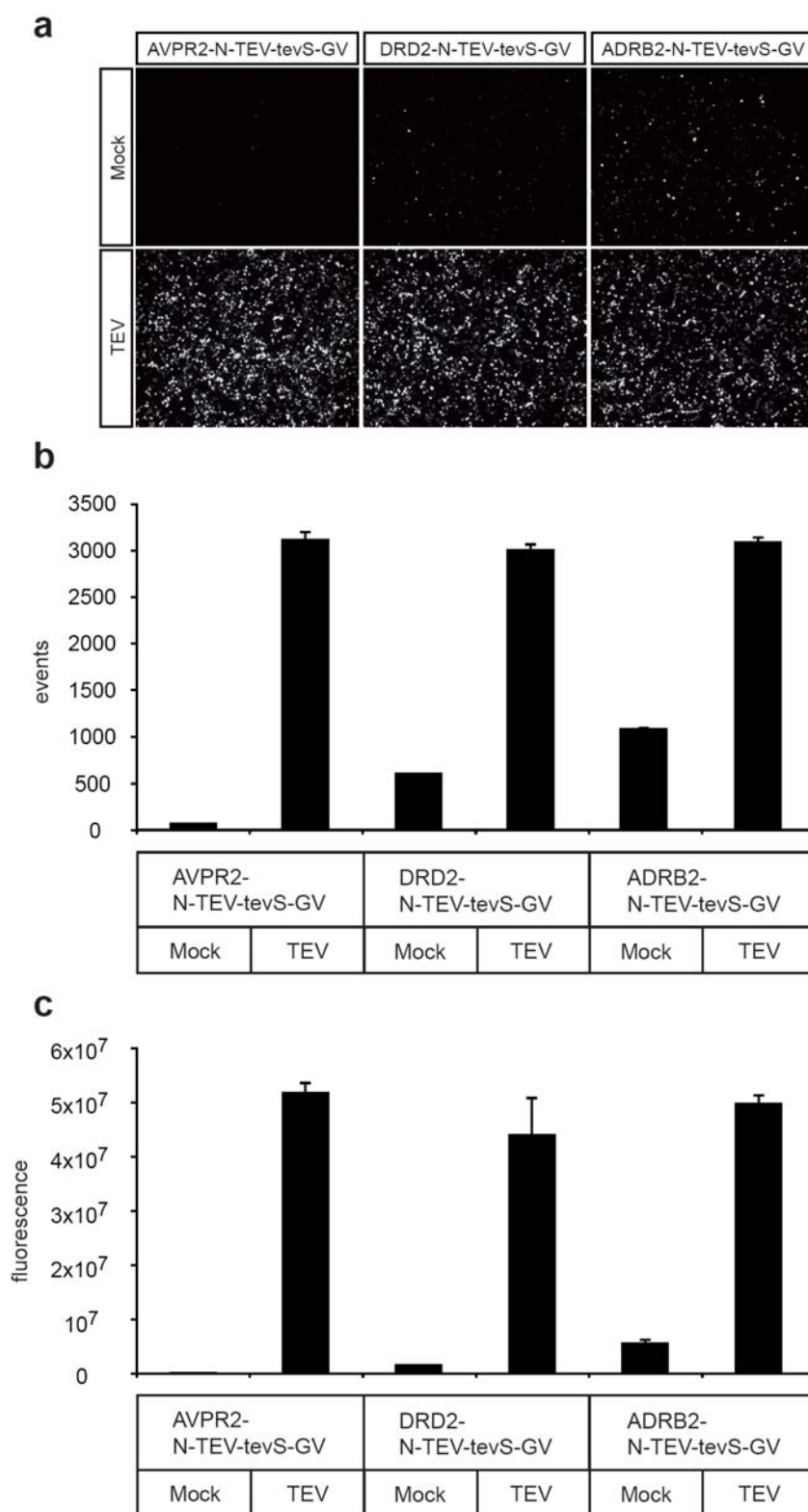


Fig. 7. Cleavage of GPCR constructs monitored by a fluorescent readout.

(a-c) Cleavage of GPCR constructs by TEV protease in HEK 293 cells was qualitatively evaluated by fluorescence microscopy (a) and quantified by flow cytometry (b,c). G5-EYFPnuc was used as reporter. Both the number of EYFPnuc-positive cells (b) and total fluorescence (c) were quantified. As EYFPnuc-positive cells were analyzed as a subset of Hoechst-positive cells in each condition, data were normalized to 10000 cells counted in total. Mock: pcDNA backbone vector. Data are given as means of $n=6 \pm$ s.d.

4.3 Evaluation of different readouts for split-TEV assays

In order to evaluate the use of luciferase and fluorescence readouts for GPCR activation assays, split-TEV assays for AVPR2 were simultaneously conducted with a G5-luciferase and a G5-EYFPnuc reporter (Fig. 8). In this case, split-TEV assays were performed in HEK 293 cells stably transfected with β Arr2-C-TEV. Depending on the readout, assays were analyzed with a luciferase assay or by flow cytometry 64 h after transfection.

Background in the absence of the receptor construct was low in both luciferase assay (Fig. 8a) and flow cytometry (Fig. 8b,c). Transfection of the reporter construct raised the background 16-fold in the luciferase assay (Fig. 8a), 32-fold regarding the amount of tdTomato-positive cells in flow cytometry (Fig. 8b), and 257-fold regarding total fluorescence in flow cytometry (Fig. 8c). Stimulation with the agonist AVP resulted in a 3.3-fold (luciferase assay, Fig. 8a), 3.2-fold (flow cytometry, number of tdTomato-positive cells, Fig. 8b) and 2.7-fold (flow cytometry, total fluorescence, Fig. 8c) signal increase. Thus, in this proof-of-principle split-TEV assay, the stimulation-dependent interaction between β arr2 and AVPR2 was reflected in a very similar way by the luciferase and the fluorescence readout. Nevertheless, the fluorescence readout displayed more background in the absence of stimulation. Mainly based on the easier handling of luciferase assays and therefore a better applicability for larger-scale assays, we decided to use the luciferase readout for all further assays.

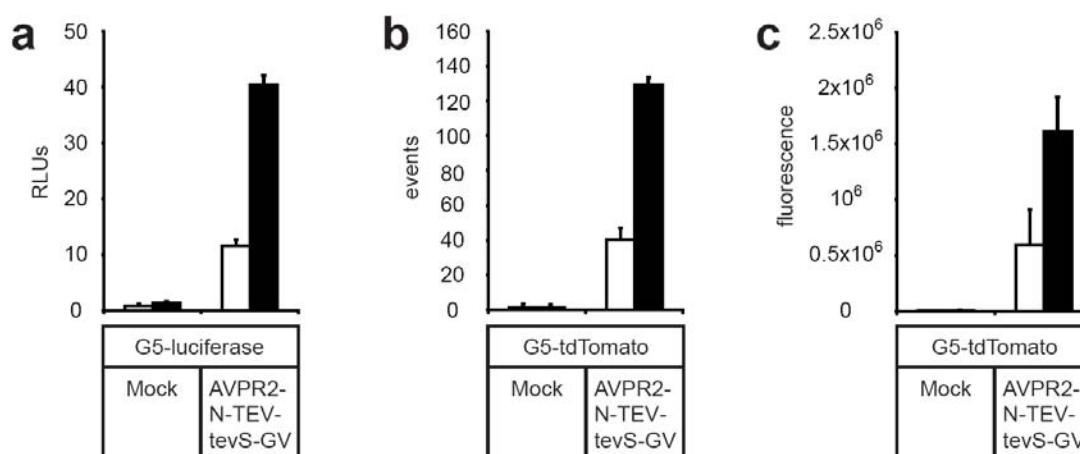


Fig. 8. Comparison of luciferase and fluorescence readouts for split-TEV assays.

(a-c) Luciferase (a) and fluorescence (b,c) readout were compared in parallel split-TEV assays for the AVPR2 receptor in HEK 293 cells stably transfected with β Arr2-C-TEV. G5-luciferase served as a reporter for luciferase assays, whereas G5-tdTomato was used for the fluorescence readout which was quantified by flow cytometry. Cells were stimulated 24h and analyzed 64h after transfection. Both the number of tdTomato-positive cells (b) and total fluorescence are shown for the fluorescence readout. As tdTomato-positive cells were analyzed as a subset of Hoechst-positive cells in each condition, data were normalized to 3000 cells counted in total. Mock: pcDNA backbone vector. Data are given as means of $n=6 \pm$ s.d.

4.4 Evaluation of different cleavage sites for split-TEV and full-TEV assays

In another proof-of-principle experiment, the use of different TEV protease cleavage sites for split-TEV and full-TEV assays was evaluated. The mutated, low-affinity tev site ENLYFQ'L has been described by Barnea et al. to perform better in full-TEV assays than the natural tev site ENLYFQ'G. AVPR1a constructs which either contained ENLYFQ'G (tevS) or ENLYFQ'L (tevS*) were therefore tested in both a split-TEV assay (with β Arr2-C-TEV) and a full-TEV assay (with β Arr2-TEV) in HEK 293 cells (Fig. 9). The split-TEV assay with tevS showed an induction rate of 2.7 ($p < 1.7 \times 10^{-7}$) upon stimulation with the agonist AVP. In the full-TEV assay, a significant induction was seen as well, although the induction rate was lower (1.3-fold, $p < 0.0002$). No significant induction occurred in the split-TEV assay using tevS* ($p < 0.25$). In contrast, the full-TEV assay with tevS* showed a significant induction with a higher ratio than observed for tevS (2-fold, $p < 10^{-5}$). Remarkably, background interaction of AVPR1a and β Arr2 in the absence of stimulation was much higher when using tevS compared to tevS*.

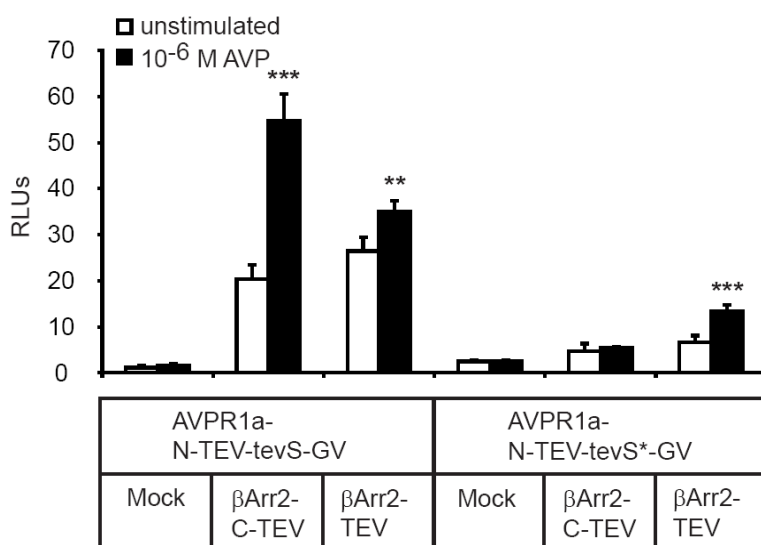


Fig. 9. Performance of different TEV protease cleavage sites in split-TEV and full-TEV assays.

The natural TEV cleavage site ENLYFQ'G and the low-affinity cleavage site ENLYFQ'L were tested in split-TEV and full-TEV assays for the AVPR1a receptor in HEK 293 cells. Split-TEV assays were performed with β Arr2-C-TEV whereas in full-TEV assays, β Arr2-TEV was used. Mock: pcDNA backbone vector. Data are given as means of $n=6 \pm$ s.d.*: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$, two-sided t-test.

4.5 Comparison of split-TEV and full-TEV assays for GPCR activation

Split-TEV and full-TEV assays were performed for the vasopressin receptors AVPR1a, the dopamine receptor DRD2 and the β -adrenergic receptor ADRB2 (Fig. 10). In order to obtain optimal responses, split-TEV assays were performed with GPCR constructs carrying the high-affinity TEV cleavage ENLYFQ'G whereas in full-TEV assays, constructs containing the low-affinity cleavage site ENLYFQ'L were used (Fig. 9, (Barnea et al. 2008)). Assays were performed in the rat pheochromocytoma cell line PC12-tetoff.

The full-TEV assay for AVPR1a showed an over 5-fold signal increase upon agonist stimulation for the interaction with β Arr2-TEV ($p < 8.6 \times 10^{-7}$, Fig. 10a). A ratio of 1.5 ($p < 0.0016$) was obtained in the DRD2 full-TEV assay (Fig. 10b). In the full-TEV assay for ADRB2, an about two-fold agonist-induced increase in baseline reporter activity was observed which was independent of β Arr2 (Fig. 10c).

Split-TEV assays with wild-type β Arr2 allowed to monitor stimulation-dependent interactions between β Arr2 and AVPR1a (4.2-fold induction, $p < 1.2 \times 10^{-9}$, Fig. 10a) and ADRB2 (2.9-fold induction, $p < 4.16 \times 10^{-9}$, Fig. 10c). In contrast, split-TEV assays for DRD2 (Fig. 10b) displayed high backgrounds where β Arr2 did not yield any reproducible stimulation-dependent signal changes.

4.5.1 Use of a β Arr2 truncation mutant for split-TEV and full-TEV assays

In addition to full-length β -arrestin 2 (β Arr2), a deletion mutant lacking the 28 C-terminal amino acids (β Arr2 Δ) was tested. This constitutively active mutant has been found to exhibit a stronger stimulation-dependent receptor desensitization compared to wild-type β Arr2 (Kovoor et al. 1999). It furthermore lacks the AP-2 binding site which is required for clustering of GPCR into clathrin-coated pits and thereby partially inhibits GPCR internalization (Laporte et al. 2000). Using β Arr2 Δ -TEV, an almost 3-fold stimulation-dependent signal increase was obtained in a full-TEV assay for AVPR1a ($p < 0.0007$, Fig. 10a), but no significant signal change was found in the DRD2 full-TEV assay and no remarkably different signal increase compared to the one in the control condition was seen in the ADRB2-full-TEV assay.

In comparison to β Arr2-C-TEV, β Arr2 Δ -C-TEV improved split-TEV assays for all three receptors. In the AVPR1a split-TEV assay, ratios were raised to 16.5 over background ($p < 2.85 \times 10^{-10}$, Fig. 10a) and in the ADRB2 assay, ratios increased to 6.3 ($p < 4 \times 10^{-10}$, Fig. 10c). Ratios of interactions in the presence vs. absence of agonist increased mainly due to

a reduction of background. Importantly, use of β Arr2 Δ -C-TEV provided stable readouts in the DRD2 assay (1.3-fold induction, $p < 0.0004$, Fig. 10b) which could not be obtained with wild-type β Arr2.

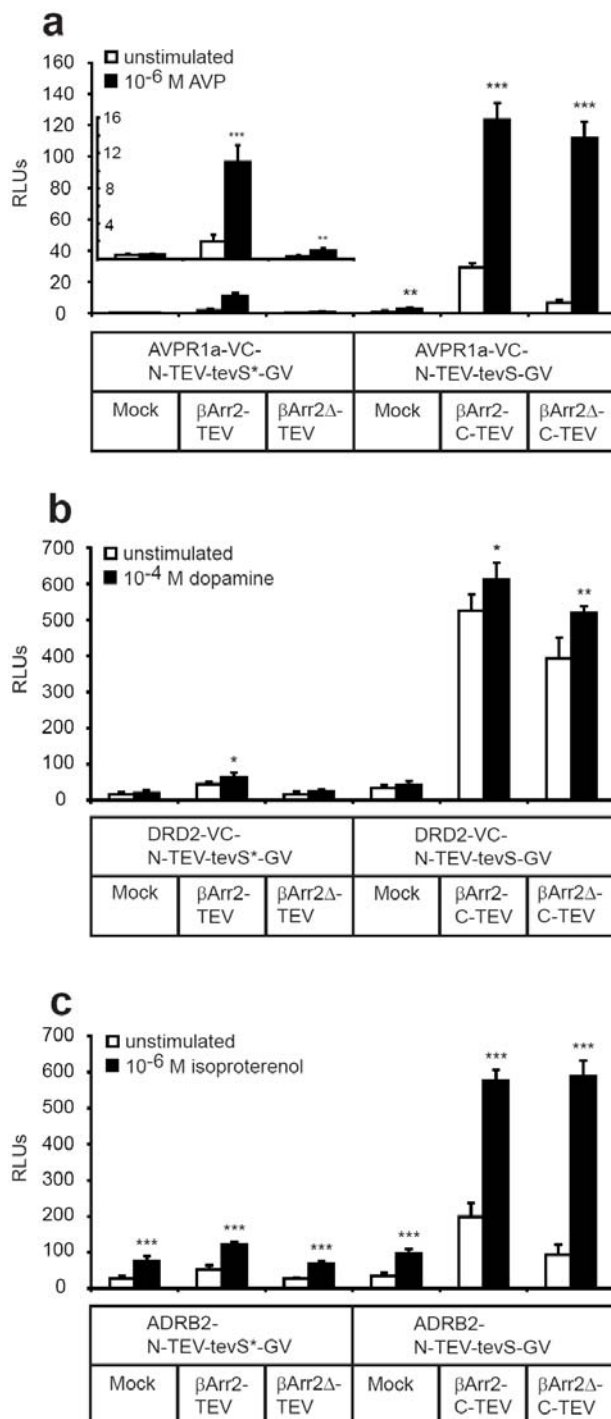


Fig. 10. Comparison of full-TEV and split-TEV assays to monitor GPCR activation in PC12 cells. (a-c) Full-TEV (left) and split-TEV (right) assays were performed for AVPR1a (a), DRD2 (b) and ADRB2 (c) in PC12 cells. β Arr2 was used in its wild-type form (β Arr2) and in a constitutively active mutant form (β Arr2 Δ). Cells were stimulated 16 h after transfection and lysed 24 h later. Mock: pcDNA backbone vector. Data are given as means of $n=6 \pm$ s.d. *: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$, two-sided t-test.

4.5.2 Performance of split-TEV and full-TEV assays in U2OS cells

Split-TEV and full-TEV assays for the same receptors were performed in another heterologous cell line, the human osteosarcoma cell line U2OS, in order to check assay performance in a different cellular background (Fig. 11).

Full-TEV assays for the AVPR1a receptor did not show any robust signal changes upon stimulation compared to controls (Fig. 11a). A 1.6-fold signal increase in the DRD2 full-TEV assay was observed with β Arr2-TEV ($p < 0.005$, Fig. 11b), but none was obtained with β Arr2 Δ -TEV. No stimulation-dependent signal change occurred in any of the ADRB2 full-TEV assays ($p < 0.64$ for β Arr2-TEV and $p < 0.94$ for β Arr2 Δ -TEV, Fig. 11c).

Stimulation-dependent interactions between GPCRs and wild-type β Arr2 could be monitored in split-TEV assays for DRD2 (4.8-fold induction, $p < 2.7 \times 10^{-8}$, Fig. 11b), and ADRB2 (1.4-fold induction, $p < 0.011$, Fig. 11c). In contrast, high backgrounds without reproducible stimulation-dependent signal changes were observed in the AVPR1a split-TEV assay ($p < 0.17$, Fig. 11a).

However, use of the truncation mutant β Arr2 Δ -C-TEV resulted in stable readouts, showing a 2.8-fold induction ($p < 0.0001$, Fig. 11a) upon stimulation. The ADRB2 split-TEV assay was as well improved by β Arr2 Δ (3.7-fold induction, $p < 1.86 \times 10^{-5}$, Fig. 11c) whereas no major difference in split-TEV assay performance between wild-type and mutant β Arr2 was seen for DRD2 (5.2-fold induction, $p < 4 \times 10^{-9}$, Fig. 11b). As already described for PC12 cells, a reduction of background was the main reason for better assay performance with β Arr2 Δ .

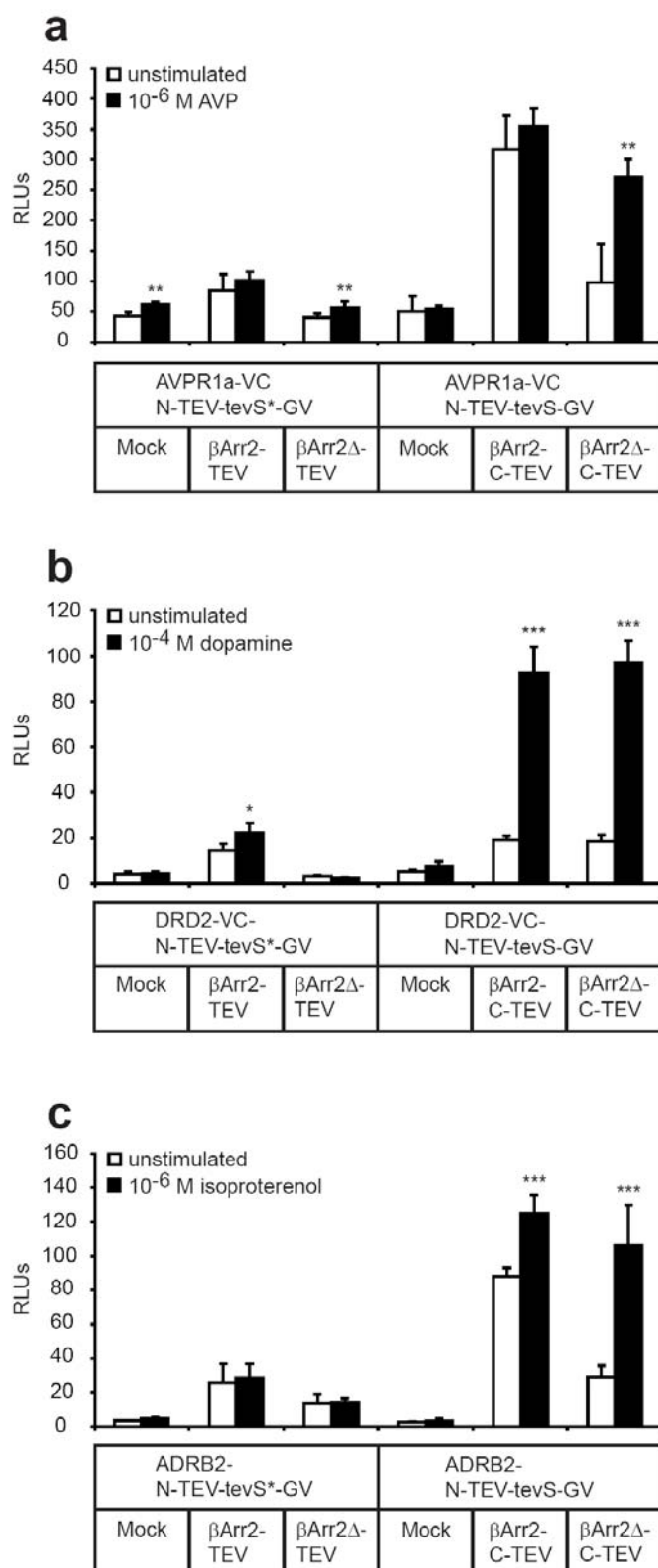


Fig. 11. Comparison of full-TEV and split-TEV assays to monitor GPCR activation in U2OS cells.

(a-c) Full-TEV (left) and split-TEV (right) assays were performed for AVPR1a (a), DRD2 (b) and ADRB2 (c) in U2OS cells. β Arr2 was used in its wild-type form (β Arr2) and in a constitutively active mutant form (β Arr2 Δ). Cells were stimulated 16 h after transfection and lysed 24 h later. Mock: pcDNA backbone vector. Data are given as means of $n=6 \pm$ s.d. *: $p<0.01$, **: $p<0.001$, ***: $p<0.0001$, two-sided t-test.

In summary, the tested split-TEV GPCR activation assays are reliably applicable in the heterologous PC12 and U2OS cell lines using transient transfection protocols, whereas full-TEV assays may require further optimization and/or selected stable cell clones (Hanson et al. 2009). By comparing only two cell lines, the pheochromocytoma 'neuronal-like' PC12 cells and the osteosarcoma-derived U2OS cells, cell type-specific effects with an impact on assay performance were observed.

4.5.3 Split-TEV and full-TEV assays in primary cultured cells

To assess the applicability of transient TEV-based GPCR activation assays in primary cultured cells, primary cultured neurons and astrocytes were used as model systems (Fig. 12). Because of the known importance of dopamine- and vasopressin-mediated signaling in neurons (Caldwell et al. 2008), DRD2 and AVPR1a assays were performed in primary cultured neurons (Fig. 12a,b). Full-TEV assays did not show any significant stimulation-dependent signal changes. Although split-TEV assays for AVPR1a and DRD2 in neurons showed a generally higher variability and lower induction rates compared to heterologous cell lines, clear agonist-dependent effects could be detected. Induction rates of 2 ($p < 0.0038$) and 1.5 ($p < 0.00015$) were obtained for AVPR1a and DRD2, respectively (Fig. 12a,b). Because astrocytes express adrenergic receptors and respond to adrenergic signaling (Laureys et al. 2010), ADRB2 activation was monitored in astrocytes. The full-TEV approach was not functional whereas robust ligand-dependent activation in astrocytes could be detected with the split-TEV assay (1.6-fold induction, $p < 1.34 \times 10^{-6}$, Fig. 12c).

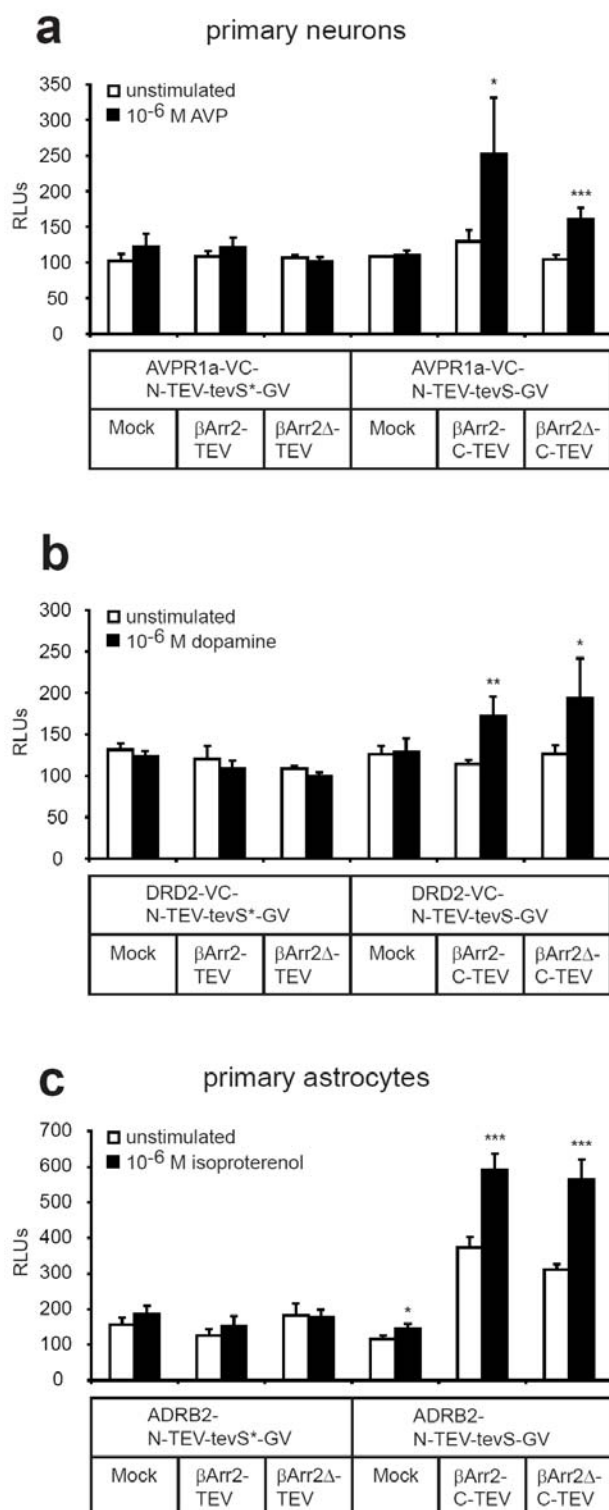


Fig. 12. Comparison of full-TEV and split-TEV assays to monitor GPCR activation in primary cultured neurons and astrocytes.

(a-c) Full-TEV (left) and split-TEV (right) assays were performed for AVPR1a (a) and DRD2 (b) in primary neurons and for ADRB2 (c) in primary astrocytes. Cells were stimulated 16 h after transfection and lysed 24 h later. β Arr2 was used in its wild-type form (β Arr2) and in a constitutively active mutant form (β Arr2 Δ). Mock: pcDNA backbone vector. Data are given as means of $n=6 \pm$ s.d. *: $p<0.01$, **: $p<0.001$, ***: $p<0.0001$, two-sided t-test.

4.6 Dose-dependence of split-TEV GPCR activation assays

When describing drug effects, an important parameter to consider is drug concentration. Most drug effects are elicited in a certain concentration range in which the response appears almost linear. Below a certain threshold concentration, virtually no drug effect can be monitored whereas, at the other end of the concentration range, the maximal possible response cannot be further increased by higher drug concentrations. Dose-response relationships have been widely used to characterize and compare drugs with respect to their pharmacological properties. Conversely, dose-response curves of well-characterized drugs are an excellent tool to evaluate assay systems which monitor concentration-dependent biological processes.

Therefore, dose-response relationships for the GPCR/ β Arr2-interaction were analyzed using split-TEV assays. As a remarkable cell type-dependent variation of split-TEV assay performance has been observed, the best performing cell line/construct combination for a given receptor was chosen to carry out selected dose-response experiments (Fig. 13). Particular interest laid in the determination of the agonist concentration which evokes a half-maximal response (EC_{50}), a measure of potency of the respective drug.

AVPR1a-VC-N-TEV-tevS-GV and AVPR2-N-TEV-tevS-GV showed a dose-dependent interaction with β Arr2 Δ -C-TEV in PC12 cells with an EC_{50} of 0.4 nM and 3.6 nM, respectively (Fig. 13a,b). Dose responses for the dopamine receptors DRD1 and DRD2 were performed in U2OS cells (Fig. 13c,d). The interaction of β Arr2 Δ -C-TEV with either DRD1-VC-N-TEV-tevS-GV or DRD2-VC-N-TEV-tevS-GV showed dose-response characteristics with an EC_{50} of 1 μ M and 2.8 μ M, respectively. Dose-response analysis for the interaction of β Arr2 Δ -C-TEV with ADRB2-N-TEV-GV resulted in an EC_{50} of 33.8 nM in U2OS cells (Fig. 13e). Because of the function of ADRB2 in astrocytes, this receptor was chosen to further assess dose-dependence of the split-TEV assay in primary cultured astrocytes. An EC_{50} of 18 nM was observed (Fig. 13f). Both dopamine and isoproterenol elicited cytotoxic effects at concentrations above 10^{-4} M, imposing difficulties in determining maximal responses.

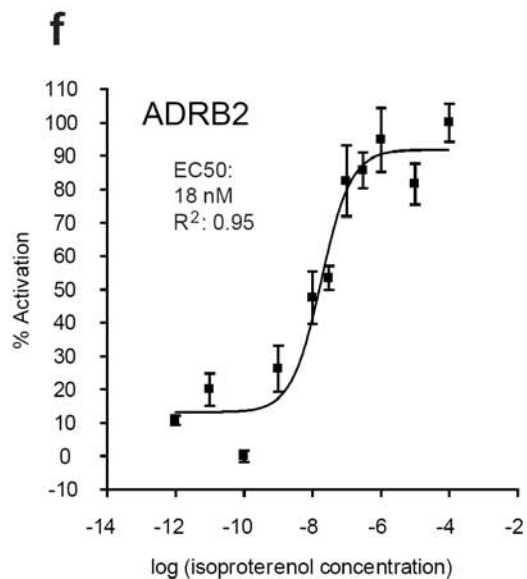
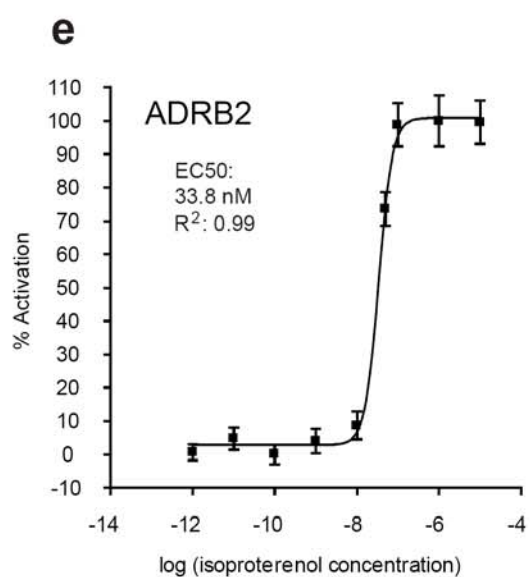
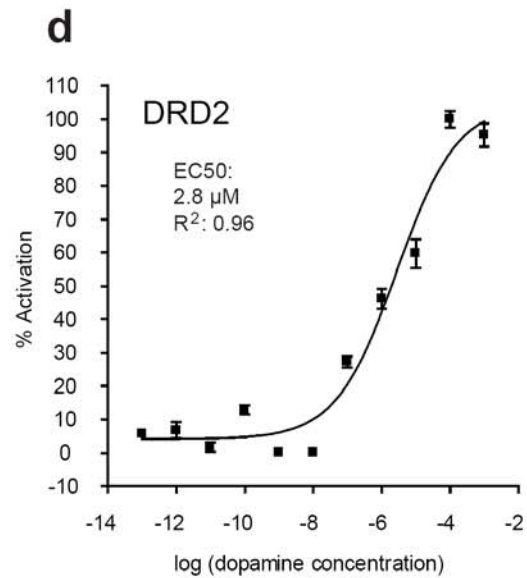
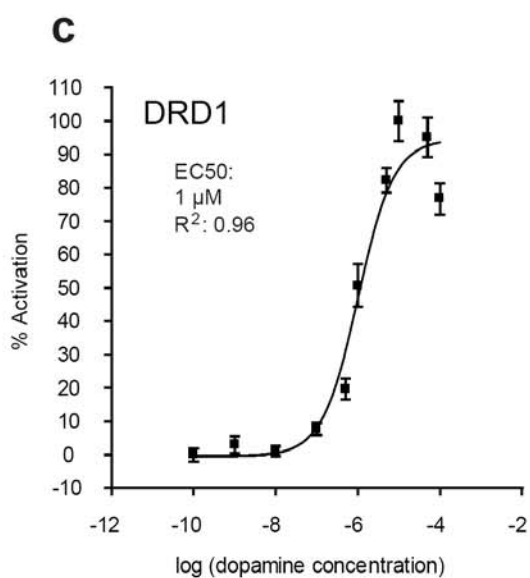
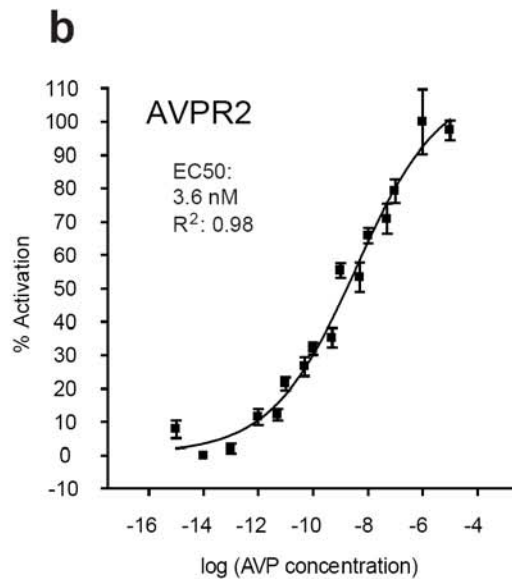
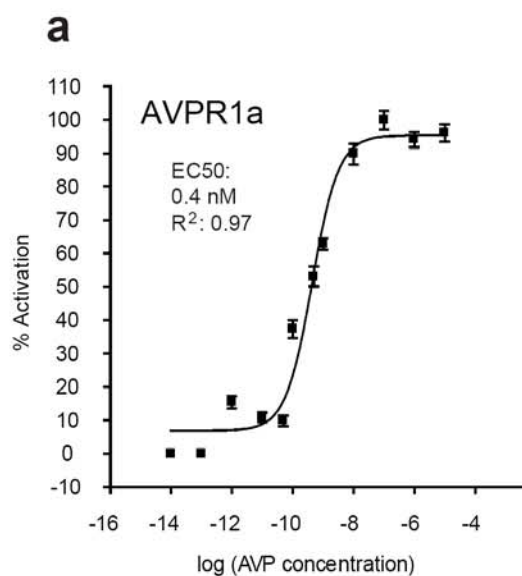


Fig. 13. Dose-response analysis with split-TEV GPCR activation assays in heterologous and primary cells.

a-f). Dose dependence of the interaction between β Arr2 and GPCRs was tested using β Arr2 Δ -C-TEV and either of the receptor constructs AVPR1a-VC-N-TEV-tevS-GV (a), AVPR2-N-TEV-tevS-GV (b), DRD1-VC-N-TEV-tevS-GV (c), DRD2-VC-N-TEV-tevS-GV (d) and ADRB2-N-TEV-tevS-GV (e,f). Assays with vasopressin receptors were carried out in PC12 cells (a,b), assays with dopamine receptors were performed in U2OS cells (c,d) and the ADRB2 receptor in U2OS (e) and primary astrocytes (f). Cells were treated with agonists 24 h after transfection and lysed after 6 h. Indicated EC_{50} values and R^2 Pearson's correlation coefficients were determined by sigmoidal curve fitting algorithm using GraphPad Prism[®]. Data are given as means of $n=6 \pm$ s.d.

4.7 Cellular Signaling by tagged AVPR1a and AVPR2 receptors

Split-TEV as well as the other β Arr2-based recruitment assays rely on tagged constructs and it may be possible that modified receptors display altered signaling properties (Thibonnier et al. 2001). In order to analyze signaling events in response to stimulation by the modified GPCRs, reporter gene assays monitoring cAMP and calcium levels were used. Vasopressin receptor constructs were used in this assay because the AVPR1a receptor preferentially couples to G_q while AVPR2 mainly recruits G_s family members (Fig. 14) (Harmar et al. 2009; Thibonnier et al. 1998).

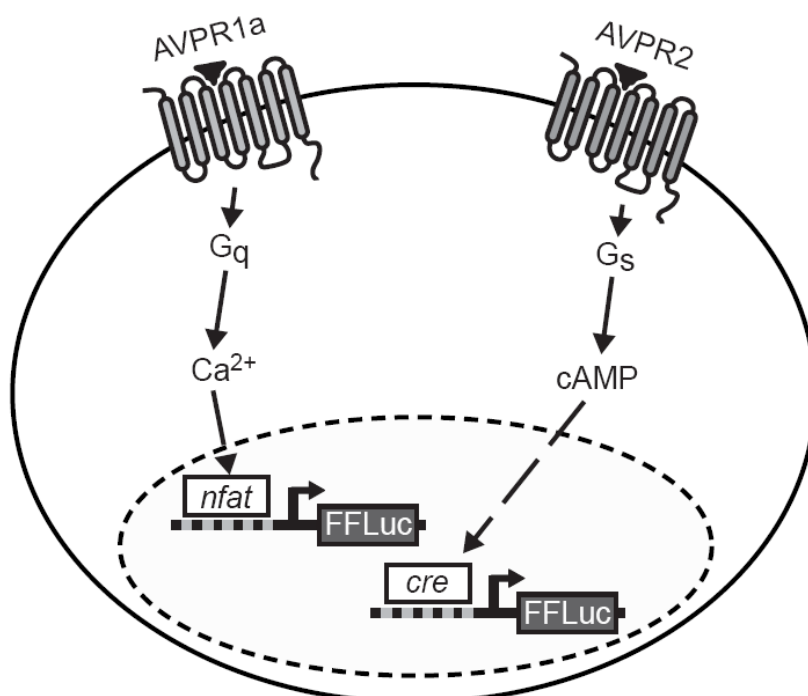


Fig. 14. Principle of *cis*-reporter assays to monitor cellular signaling.

Ca^{2+} signaling of the AVPR1a receptor via G_q was monitored by using an NFAT-driven *cis* element (*nfat*) coupled to the firefly luciferase. cAMP signaling of the AVPR2 receptor was analyzed by using a CREB-driven *cis* element (*cre*) coupled to the luciferase gene. *Cis*-reporter assays were used to analyze signaling properties of differently tagged versions of these receptors. FFLuc: Firefly luciferase gene.

An *nfat*-luciferase reporter construct driven by a NFAT-responsive *cis*-element was used to indirectly monitor a rise in Ca^{2+} levels and therefore serve as a readout for the G_q pathway. In analogy to this, a *cre*-luciferase reporter driven by a CREB-responsive *cis*-element was used to indirectly monitor changes in cAMP levels as a readout for the G_s pathway (Fig. 14).

Functionality of *cis* reporters was tested by applying well characterized substances which are known to lead to a substantial activation of the respective *cis* element (Fig. 15). Dibutyl cAMP (db-cAMP) is a cell-permeable cAMP analogue which can transactivate *cre* (Montminy et al. 1986). Phorbol esters like phorbol myristate acetate (PMA) activate protein kinase C (Castagna et al. 1982) whereas the ionophore ionomycin increases intracellular calcium levels. Phorbol esters and ionophors act synergistically to activate NFAT (Boss et al. 1998).

Pathway-specific responsiveness of *cre*- and *nfat*-luciferase reporters was further tested by doing cross-controls in which the effect of PMA-ionomycin on *cre*-luciferase and of db-cAMP on *nfat*-luciferase was monitored (Fig. 15). DMSO served as a carrier for PMA and ionomycin and was therefore added to the control for PMA-ionomycin (Mock + DMSO).

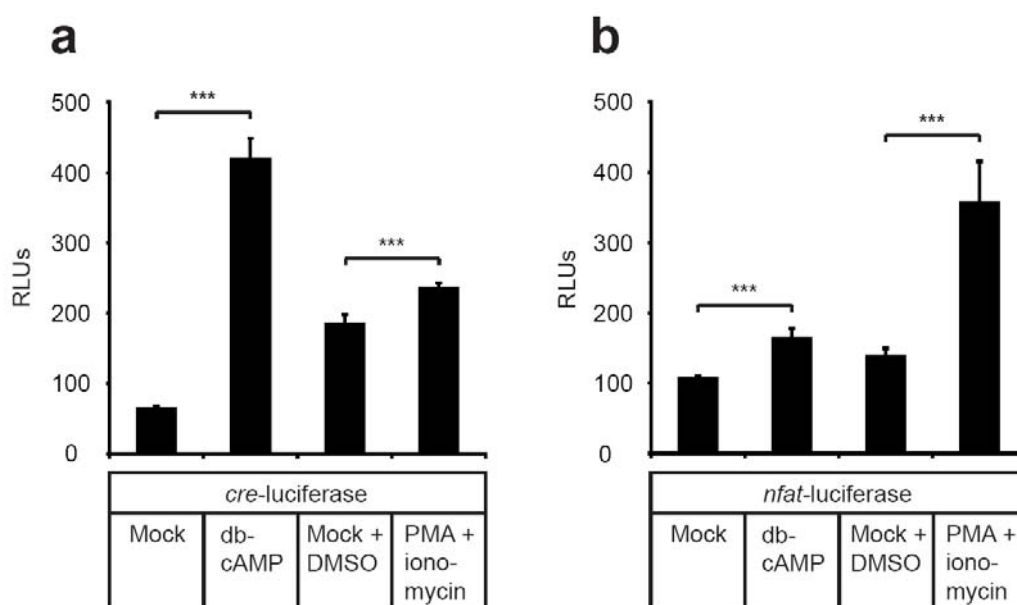


Fig. 15. Specific responsiveness of the *cre*-luciferase and *nfat*-luciferase reporter for db-cAMP and PMA/ionomycin responses.

(a,b) PC12 cells were transfected with *cre*- (a) or *nfat*-luciferase (b), respectively, treated with 1mM cAMP or 10ng/ml PMA + 1 μ M ionomycin after 16 h, and lysed after another 24 h. Mock + DMSO served as control for PMA-ionomycin treatment. Mock: pcDNA backbone vector. Data are given as means of $n=6 \pm$ s.d. *: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$, two-sided t-test.

Treatment of PC12 cells with 1mM db-cAMP led to a 6.6-fold activation of *cre*-luciferase compared to controls ($p < 4 \times 10^{-11}$, Fig. 15a). 10ng/ml PMA + 1 μ M ionomycin induced a 2.6-fold activation of *nfat*-luciferase ($p < 5 \times 10^{-6}$, Fig. 15b). However, addition of DMSO to Mock led to a pronounced activation of *cre*-luciferase and treatment with PMA-ionomycin slightly but significantly enhanced this (1.3-fold, $p < 10^{-5}$, Fig. 15a). Likewise, a significant activation of *nfat*-luciferase by db-cAMP was observed (1.5-fold, $p < 5 \times 10^{-6}$, Fig. 15b), although to a lesser extent than by PMA-ionomycin. Thus, the tested *cis* reporters clearly respond in a pathway-specific way with preferred activation of *cre*-luciferase by cAMP and of *nfat*-luciferase by calcium-mediated signals. However, cross-activation by other intracellular signals occurs to some extent. Additional application of AVP did not affect the db-cAMP or PMA-ionomycin mediated effects indicating the absence of endogenous vasopressin receptors, as seen by ratios between AVP-stimulated and non-stimulated conditions of about 1 (Fig. 16a,b).

The AVPR1a receptor construct carrying solely a small V5 epitope tag activated *cre*-luciferase 3.3-fold upon stimulation with AVP ($p < 2.3 \times 10^{-8}$, Fig. 16a). This AVP effect was similar for all other AVPR1a fusion constructs regardless of whether N-TEV-tevS-GV, VC-N-TEV-tevS-GV or C-TEV were attached to the receptor (Fig. 16a). Stimulation of AVPR2 receptor constructs, however, resulted in 9- to 12-fold signal increases for all fusion proteins proving the preferential $G_s/cAMP$ coupling (Fig. 16a).

A 2.5-fold increase of *nfat*-luciferase activity was achieved by AVP stimulation of the AVPR1a receptors carrying the V5 tag ($p < 6.3 \times 10^{-7}$, Fig. 16b). Tagging of AVPR1a with N-TEV-tevS-GV or C-TEV resulted in a less pronounced transcriptional activation of *nfat*-luciferase (about 1.7-fold, $p < 2 \times 10^{-6}$, for both constructs). Interestingly, AVP-dependent *nfat*-reporter activation by AVPR1a-VC-N-TEV-tevS-GV was only subtle (1.3-fold, $p < 0.0002$, Fig. 16b). This construct differs from the corresponding N-TEV-tevS-GV variant only by the C-terminal domain of AVPR2 (VC). In contrast to AVPR1a constructs, none of the AVPR2 fusion constructs showed *nfat*-reporter activities which were significantly enhanced by AVP (Fig. 16b). These data validate the preferred coupling of AVPR1a receptors to G_q/Ca^{2+} but also indicate that the AVPR2 C-tail modification of AVPR1a receptors can alter signaling properties.

Finally, the dose-response performance of AVPR2- and AVPR1a-N-TEV-tevS-GV constructs was examined in *cre*- and *nfat*-reporter assays (Fig. 16c,d). *Cre* activation in response to AVPR2-N-TEV-tevS-GV-mediated signaling yielded a sigmoidal dose response with an EC_{50} of 89.5 pM (Fig. 16c).

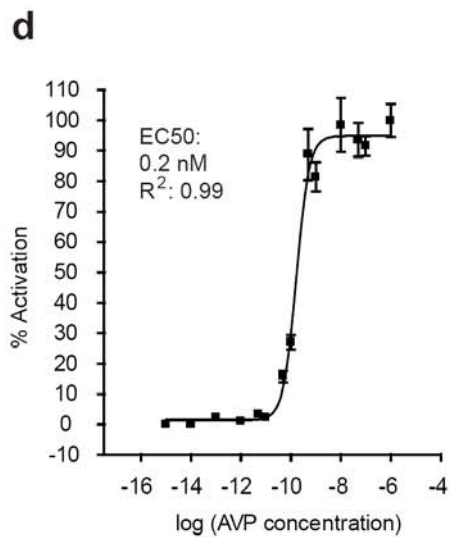
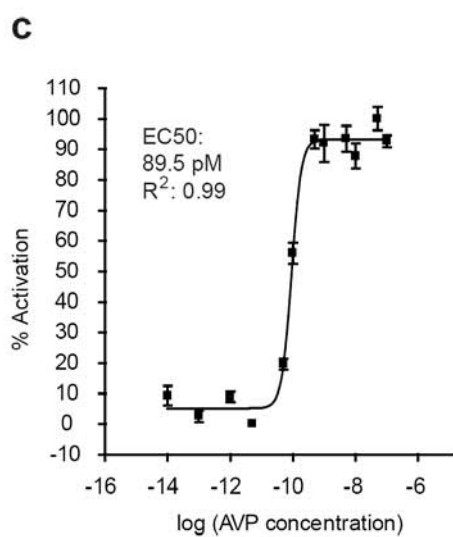
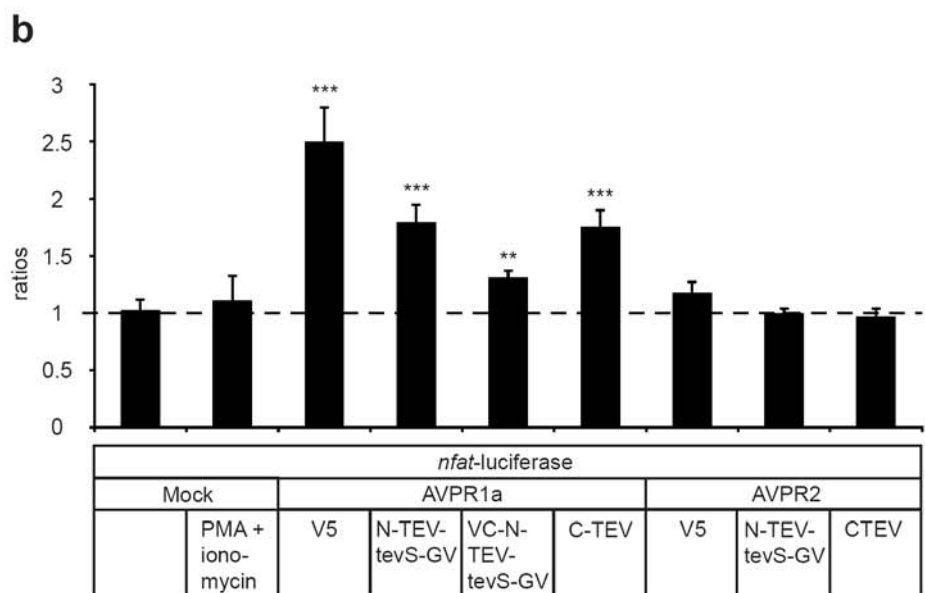
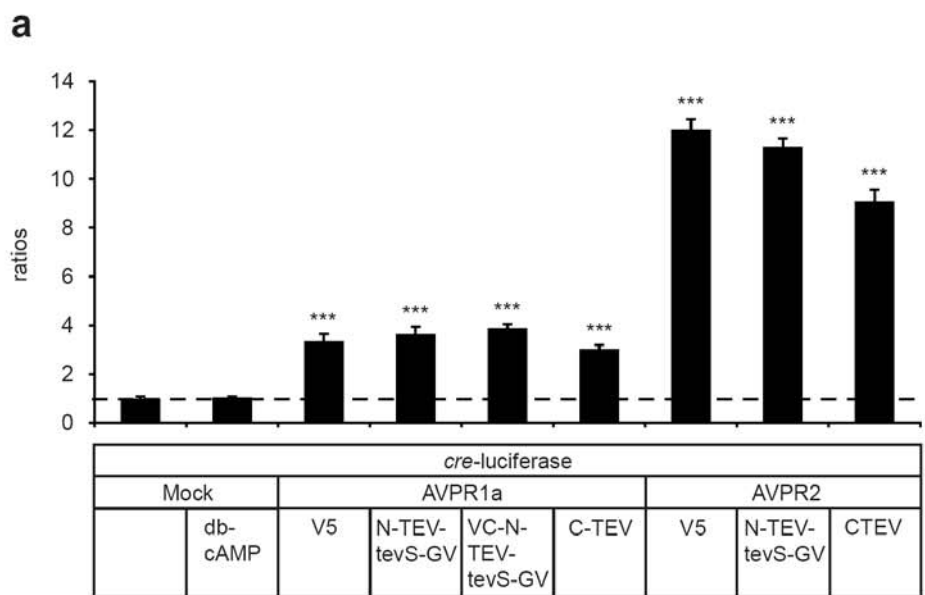


Fig. 16. Cellular signaling by differently tagged AVPR1a and AVPR2 receptors.

(a,b) Reporter gene assays in PC12 cells monitoring *cre*-luciferase (a) and *nfat*-luciferase (b) activation in response to control stimulation with 1mM db-cAMP (a) and 10ng/ml PMA + 1 μ M ionomycin (a) as well as stimulation of different AVPR1a (a,b) and AVPR2 (a,b) fusion proteins with 1 μ M AVP. Data are displayed as ratios between AVP-stimulated and non-stimulated conditions. Data are given as means of $n=6 \pm$ s.d. *: $p<0.01$, **: $p<0.001$, ***: $p<0.0001$, two-sided T-test. (c,d) Reporter gene assays in PC12 cells monitoring dose-response curves *cre*-reporter activities of AVP stimulated AVPR2-N-TEV-tevS-GV (c) and NFAT-reporter activities of AVPR1a-N-TEV-tevS-GV (d). Mock: pcDNA backbone vector. Indicated EC_{50} values and R^2 Pearson's correlation coefficients were determined by sigmoidal curve fitting algorithm using GraphPad Prism[®]. Data are given as means of $n=6 \pm$ s.d.

Nfat activation as a result of AVPR1a-N-TEV-tevS-GV signaling using the AVPR1a-N-TEV-tevS-GV construct was as well dose-dependent following a typical sigmoidal response curve with an EC_{50} of 0.16 nM (Fig. 16d). This further substantiates that the C-terminal N-TEV-tevS-GV tag *per se* does not dramatically modify the signaling properties of AVPR1a and -2 receptors.

5 Discussion

In this thesis, a method is presented to measure GPCR activation through the stimulation-dependent interaction between GPCRs and β Arr2. It represents a modified split-TEV assay which uses the recombination of TEV protease fragments upon protein-protein interaction and combines it with a transcription-coupled readout. Components of GPCR split-TEV assays are 1) GPCR constructs which carry the GPCR, the N-terminal TEV fragment, the TEV cleavage site and the transcription factor GV, 2) β Arr2 constructs where β Arr2 is attached to the C-terminal TEV fragment, and 3) reporter constructs. To evaluate the assay system, cleavage of the receptor constructs, use of different TEV cleavage sites and transcription-coupled reporters as well as the physiological behaviour of GPCR constructs and applicability of the assays in different cell types were tested. Split-TEV assays were furthermore compared to the previously described full-TEV/Tango assays which rely on the induced proximity of a full-length TEV protease with its substrate.

5.1 Cleavage of GPCR constructs by TEV protease

GPCR constructs containing the natural cleavage site ENLYFQ'G (tevS) were efficiently cleaved by cytosolic TEV protease, proving sterical availability of the TEV cleavage site despite the rather big C- and N-terminally attached N-TEV and GV. However, variable levels of background were observed which were especially high for ADRB2. Thus, transcriptional activation of the reporter gene by GV seems to occur at a distinct level even in the absence of TEV protease. Possible scenarios are the cleavage of a C-terminal fragment of the receptor by a membrane-associated or cytosolic protease followed by translocation to the nucleus or nuclear localization of the receptor itself. Evidence for a role of both scenarios in GPCR biology exists in the literature. For example, it has been shown that the angiotensin II type 1 receptor undergoes cleavage and that the resulting C-terminal fragment accumulates in the nucleus, eventually regulating gene expression (Cook et al. 2007). On the other hand, localization at nuclear membranes and signaling by a couple of GPCRs including beta-adrenergic receptors has recently emerged though this could not be established for ADRB2 (Boivin et al. 2006; Boivin et al. 2008).

Cleavage of GPCR constructs by TEV was site-specific, as the low-affinity ENLYFQ'L (tevS*) was not cleaved by cytosolic TEV, as previously described by Barnea et al. (Barnea et al. 2008). It seems that random presentation of tevS* to TEV is not sufficient to induce efficient cleavage.

5.2 Influence of the cleavage site on split-TEV and full-TEV assay performance

Split-TEV assays sensitive for the stimulation-dependent interaction of GPCRs with β Arr2 in transient transfections were established for vasopressin and dopamine receptors as well as the beta-adrenergic receptor 2. In contrast, the full-TEV approach was in our hands not sufficiently sensitive to monitor activation of most of the tested GPCRs, and less sensitive than split-TEV in the case of AVPR1a. Different TEV cleavage sites have been used in split-TEV and full-TEV assays to obtain optimal performance, as evaluated in a separate experiment. The high-affinity tevS was used in split-TEV assays where it provided a sensitive readout which could not be obtained with tevS*. Loss of specificity due to the high-affinity tevS is not an issue in split-TEV assays because specificity is mainly given by the functional complementation of inactive fragments which occurs only upon protein-protein interaction. In contrast, the low-affinity cleavage tevS* was used for full-TEV assays where no specific readouts could be obtained with tevS (Barnea et al. 2008). As cleavage of tevS by cytosolic TEV provides a high background, unspecific random interactions between GPCR constructs carrying tevS and β Arr2-TEV might affect signal-to-noise ratios in such way that no window is left to discriminate real stimulation-dependent interactions. In this case, use of a low-affinity cleavage site would provide the necessary specificity. On the other hand, use of the low-affinity tevS* might at least partially explain why full-TEV assays do not reliably work in transiently transfected cells. In a recent report by Hanson et al., however, it was shown that the TEV-cleavage site with a leucine carboxyterminally of the cleavage site (position P1') is less efficient compared to another version with a methionine at position P1' (ENLYFQ'M) (Hanson et al. 2009). Therefore, it cannot be excluded that full-TEV assays, when performed with reporters harbouring a methionine at P1', may also be successfully adapted for transient transfection approaches.

5.3 Use of a β Arr2 truncation mutant for split-TEV and full-TEV assays

Split-TEV and full-TEV assay performance was compared when using either a full-length or a β Arr2 deletion mutant (β Arr2 Δ) lacking the entire C-terminal tail (aa 383-410) in fusion with C-TEV. The C-terminus contains essential parts of the clathrin binding sites and is likely to be important in keeping β Arr2 in its inactive conformation thereby improving phosphorylation-dependent recruitment (Han et al. 2001; Koo et al. 1999). The use of β Arr2 Δ substantially improved split-TEV assays by increasing ratios mainly due to less

constitutive recruitment reducing the baseline values. In contrast, full-TEV assay performance was even further reduced by β Arr2 Δ which is most likely due to the enhanced selectivity of the mutant β Arr2 Δ version and the lower overall sensitivity of full-TEV assays.

5.4 Flexibility of split-TEV assay readout

The modular nature of split-TEV assays provides a high grade of flexibility, e.g. by allowing to choose the most convenient readout out of a toolbox. Therefore, fluorescence readouts were evaluated in addition to the mostly used luciferase readout. Taking an AVPR2 split-TEV assay as example, we could show that analysis of number of fluorescent cells carrying the reporter gene and of total fluorescence by flow cytometry were very similar to what was measured in luciferase assays. Likewise, cleavage of GPCR constructs was analyzed both in luciferase assays and by flow cytometry, and e.g. the more efficient cleavage of AVPR2 compared to DRD2 and ADRB2 was reflected in both readouts. However, assessment of cleavage in luciferase assays was done in separate experiments for each GPCR and with slightly differently GPCR constructs and can thus not be directly compared to the simultaneously performed fluorescence assays. In general, fluorescence readout could nicely allow quick assessment of assay performance under a fluorescence microscope, however, high throughput assays will pose a challenge on quantification by flow cytometry.

5.5 Cell type-dependent differences in split-TEV assay performance

Split-TEV was previously described to be applicable in even hard-to-transfect primary cells such as various neuronal cell types (Wehr et al. 2006; Wehr et al. 2008). Here, we could show that split-TEV-based GPCR activation assays are applicable in different heterologous cell types as well as in primary cultured neuronal and glial cells. The high flexibility of split-TEV assays may be a helpful feature since contextual specificity, which is mainly determined by the cell type used for a particular assay, appears to be of high importance when studying GPCR pharmacology (Kenakin 2005; Kenakin 2009; Thomsen et al. 2005). In line with the importance of considering the cell type used, marked differences in assay performance were observed between different cellular systems. For example, vasopressin receptor activation assays performed robustly in PC12 cells and primary neurons, but not in U2OS cells. In contrast, dopamine receptor assays performed well in U2OS cells and primary neurons, but did not reveal ligand-dependent differences in reporter activation in PC12 cells. PC12 cells are known to secrete dopamine (Schubert

and Klier 1977) and it seems possible that the endogenous dopamine is already sufficient to activate the exogenous dopamine receptors. This assumption is supported by the high background levels of DRD1 receptor constructs when co-transfected with β Arr2-C-TEV expression cassettes. Assays in primary neurons and astrocytes showed higher signal-to-noise ratios and more variability among biological replicates. This was possibly due to lower efficiency of transfection with lipofectamine and higher vulnerability of primary cells as well as a higher diversity among individual cells compared to immortalized cell lines.

In conclusion, assay performance does not only depend on the functionality of the sensor system *per se* but can be influenced by the endogenous equipment of the cells. The expression of endogenous ligands but also receptors as well as the set of G proteins, GRKs and β -arrestins may influence the measured response.

5.6 Split-TEV dose-response assays for GPCR activation

GPCR/ β Arr2-interactions were dose-dependent in split-TEV assays for all GPCRs tested. EC_{50} s as a measure of agonist potency were comparable to those reported from radioligand-binding or other GPCR activation assays (Barnea et al. 2008; Breit et al. 2004; Hammer et al. 2007; Hanson et al. 2009; Harmar et al. 2009; Oakley et al. 2002). Importantly, very similar EC_{50} s were obtained across different cell types, as shown for ADRB2 in U2OS cells and primary cultured astrocytes.

5.7 Cellular signaling by differently tagged GPCRs

To improve assay sensitivity via enhanced β Arr2 recruitment, Barnea et al. added the C-terminal intracellular domain of AVPR2 to the GPCR reporter constructs (Barnea et al. 2008). This strategy was adopted for split-TEV assays and indeed led to an improved assay performance for some receptors. It has, however, not been addressed so far whether these modified receptors may be altered with respect to their signaling properties. Therefore, downstream signaling of differently tagged AVPR1a and AVPR2 constructs was monitored with cAMP- and calcium-responsive *cis*-element driven reporters. Preferential coupling of AVPR1a to $G\alpha_q$ with elevation of intracellular Ca^{2+} and of AVPR2 to $G\alpha_s$ with increase of cAMP was validated in these assays. Interestingly, stimulation of AVPR1a constructs led to a significant activation of *cre*-luciferase, though to a minor extent than AVPR2 did. In cross-controls, *cre*-luciferase showed a slight but significant response to stimulation with PMA + ionomycin, as did *nfat*-luciferase when stimulated with db-cAMP. Indirect effects like cross-activation of downstream signaling by effector

molecules or direct activation of the respective *cis* element might explain this. Concerning cre activation by AVPR1a, coupling of AVPR1a to G_s to a certain extent should not be excluded.

The different split-TEV tags (N-TEV-tevS-GV and C-TEV) appeared to have no effect on the cAMP signaling but seemed to reduce the AVPR1a mediated Ca²⁺-response. With an additional AVPR2 C-terminal domain fused to the AVPR1a receptor to enhance βArr2 recruitment, however, the Ca²⁺-response was nearly completely abolished. It may therefore be concluded that constructs harbouring potential signaling-competent modifications should not be used to monitor cellular effects beyond receptor activation.

5.8 Advantages and disadvantages of split-TEV assays for GPCR activation

Split-TEV assays for GPCR activation show high sensitivity for GPCR/βArr2 interactions and transient transfections are sufficient to obtain robust readouts. Specificity is acquired by recombination of the TEV protease so that coincidental proximity of the proteins of interest would not be displayed in the final readout. Readouts of split-TEV assays can be flexibly chosen. Transcription-coupled reporters can be employed to amplify weak signals. Additionally, fluorescent reporters can be useful for measurements in living cells without the requirement of cell lysis.

However, transcription-coupled readouts are relatively slow compared to the transient nature of many signaling events and are not suited to monitor online kinetics. Measurements reflect an integral of those events which led to transcriptional changes in the time window between stimulation and measurement. Events which happen for only a short time after stimulation, e.g. due to fast degradation of an agonist, might not turn up as a significant change in reference to a control condition. Furthermore, no subcellular resolution of signaling events within microdomains can be obtained, as could e.g. be the case in FRET measurements (Gao X and Zhang 2010).

Split-TEV assays require tags containing the inactive protease fragments as well as the transcription factor, and this could have an influence on receptor behaviour. In the presented experiments, fusion proteins were properly expressed and reflected GPCR activation including pharmacological properties, but downstream signaling might have been compromised by the use of tagged receptors.

Split-TEV assays can in principle be applied in any cell type that allows genetic modification and this was exemplarily shown in different heterologous cell lines as well as

primary cultured neurons and astrocytes. Especially the possibility to use primary cultured cells which cannot be subjected to stable transfections is an attractive option if one wants to assess GPCR pathology in the primary biological context. GPCR split-TEV assays themselves were dependent on cellular context but this might not necessarily be a drawback if it reflects actual biology.

Activation profiles of an array of GPCRs in response to a given substance would be an interesting application of GPCR-split-TEV assays. As already mentioned, many drugs affect more than one target and many targets are affected by more than one drug, so receptor activation profiling could be useful to estimate therapeutic and adverse effects (Boran and Iyengar 2010; Yildirim et al. 2007).

Additionally, split-TEV assays might be used to study orphan GPCRs for which no ligand has been identified yet. Libraries of putative ligands could thus be screened independently of the downstream signaling employed by these receptors (Wise et al. 2004).

Assays can be carried out in a 96-well format and are relatively easy to handle in terms of expertise and equipment. They could moreover be adjusted to a high-throughput scale.

5.9 Outlook: Possible implementation of GPCR split-TEV assays into multiplexed assays

Split-TEV assays for GPCR activation could be integrated into higher-level analyses of GPCR signal transduction. Parallelized analyses e.g. of GPCR activation, internalization and intracellular signaling including transcriptional regulation would mainly require a high number of reporters of which each one stands for a specific process or signaling component. Barcode reporters present a solution for this challenge by using unique expressed oligonucleotide tags which can be coupled to transcription-encoded split-TEV assays as well as to a variety of cis elements or potentially to split-TEV and be analyzed with microarrays or high-throughput sequencing (Botvinnik et al. 2010).

6 Summary

G protein-coupled receptors (GPCRs) constitute the largest receptor family in mammals and represent important drug targets. Signaling through GPCRs mediates physiological effects which are strongly dependent on the cellular context. Therefore, the availability of assays monitoring GPCR activation applicable in different cell types could help to better understand GPCR functions and to realize the potential of known as well as novel substances. Here, we introduce a split-TEV assay to monitor GPCR activation through the stimulation-dependent recruitment of β -arrestin 2. Inactive N- and C-terminal fragments of the TEV protease are coupled to a GPCR and β -arrestin 2, respectively. Ligand-dependent interaction of the two fusion proteins leads to functional complementation of the TEV protease, followed by cleavage of an artificial transcription factor and successive reporter gene activation. The presented split-TEV assay system is highly sensitive and was successfully applied in heterologous cell lines as well as in primary cultured neuronal and glial cells. We show that assay performance strongly depends on the endogenous properties of different cell types. The sensitivity and flexibility makes split-TEV assays a valuable tool to analyze GPCR activation in different cell types in a rapid and cost-effective way.

7 References

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Curriculum vitae

I was born on 7th October 1984 in Wilhelmshaven where I grew up and attended school until 2000. I then changed to CJD Christophorus School, Braunschweig, from where I obtained the “Abitur” in 2002. In 2002 and 2003, I presented results of two lab rotations in Braunschweig and Köln in the “Jugend forscht” young scientists competition and won 3rd prizes on a federal country level as well as a special prize by the “Bundesministerium für Bildung und Forschung” (BmBF) in the BundesUmwelt-Wettbewerb 2002.

I started Medical School at the University of Göttingen in 2002 and accomplished the “Physikum” in 2004. In 2005, I joined the MSc/PhD/MD-PhD program “Neurosciences” (International Max Planck Research School) organized by the University of Göttingen and the Max Planck Institutes in Göttingen where I achieved a “Master of Science” (MSc) in October 2007. From 2005 to 2006, I obtained a stipend by the International Max Planck Research School. From August 2008 to July 2009, I spend my “Practical Year” in the Departments of Neurology and Surgery at the University Clinic Göttingen and in Internal Medicine subspecialties at the University of San Diego, California. Throughout my studies, I was pursuing own scientific projects in the Department of Neuroanatomy (2003 to 2005) and the Department of Medical Psychology (2005 to 2006) at the University of Göttingen and the Department of Neurogenetics (since 2006) at the Max Planck Institute for Experimental Medicine in Göttingen.

I graduated from Medical School in December 2009 and afterwards proceeded with my MD thesis in the lab of PD Dr. Moritz Rossner (Dept. of Neurogenetics) at the Max Planck Institute for Experimental Medicine in Göttingen.