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Thesis Paper

Population size matters: The cause & effect of heterogeneous β_1AR expression and its influence on receptor internalization.

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In cellular physiology, the impact of heterogeneous receptor expression is a topic that has recently started to receive much attention from the scientific community. However, much is still not understood about the specifics on the origin of this heterogeneity nor how it affects bulk measurements of heterogeneous population. While the literature often acknowledges this heterogeneity, it falls short of offering an explanation of what practical implications this variability may have. In this paper, we examine two methods for evoking protein expression of G Protein-Coupled Receptors, transient transfection and inducible cell lines, and show that these methods have markedly different expression variabilities. While the inducible cell lines have relatively homogeneous expression profiles, the transient transfected cells showed large variability to an extent that bulk measurements of transfected cells may yield misleading data. We hope that the results presented in this paper may inspire future research in the field to fully consider the effects of heterogeneous expression profiles.

Undoubtedly, it is a fair label to term the superfamily of the G-Protein-Coupled-Receptors (GPCRs) as the doorbells of the cell. These membrane receptors are involved in a wide range of varying signaling events initiated by extra-cellular ligands such as hormones, neurotransmitters, photons and growth factors, and are responsible for the continuation of these signals through the plasma membrane, a process they accomplish by activating G-proteins located on the inner leaflet of the membrane.[1,2] The presence of GPCRs is indeed so pervasive that they contribute to all known physiological processes in mammals.[3] As a direct consequence of this, the GPCRs as a group is by far the largest target for drugs and pharmaceutical-related research, with these drugs accounting for the largest amount of medicine being sold worldwide.[4] Of the GPCRs, a common target of study is the GPCR-subfamily of the β-adrenergic receptors, amongst which the subtype β_1AR is the most abundant in the mammalian brain.[5] This receptor is known to mediate interactions with the neurotransmitter norepinephrine and plays a crucial role in synaptic plasticity and memory regulation, including the formation of emotionally charged memories.[6,7]

An emerging field of study in cellular biology is protein heterogeneity. It has been long known that there exists a large variability in protein expression (the proteome), even for cells of completely identical genomes, but recent advancement in single-cell studies have allowed for even better characterization.[8,9] This natural variability is largely a consequence of stochasticity in genetic translation and transcription, and has the advantage that variable gene expression can broaden the range of stress resistance across a population of cells.[10] This advantage however, can quickly turn into a problem when it confers a resistance to diseased cells against various treatments such as antibiotics and cancer-therapy, and has even been speculated to be responsible for the process of aging.[11] Perhaps most importantly, there are cases in the literature where the functional characterization of the pharmaceutical properties of ligand-type drugs have yielded completely different results due to variance in protein expression levels.[12]

Despite this knowledge, a lot of research is still done without much consideration to the variability of protein expression in assays. A common method to evoke protein expression in cell lines is through transient transfection that introduce genes of interest into cells transiently.[13,14] Another less common method to evoke expression of protein is to develop or acquire an inducible monoclonal cell line, where the expression of a protein of interest is initially inhibited by a repressor, though expression of the protein can be activated and tuned by the addition of a correspond-

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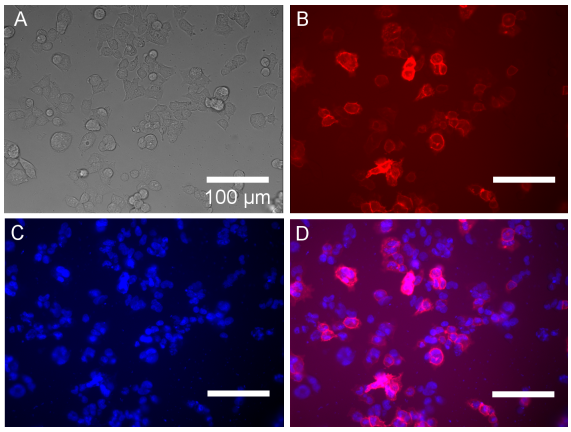

Fig. 1 Inverted light microscopy of transient transfected β_1AR T-REX^{TM} cells. One set of images of inverted light microscopy of transient transfected β_1AR T-REX^{TM} cells with **(A)** bright-field image, **(B)** β_1AR labelled with SNAP-surface BG549 and **(C)** cell nuclei labelled with NucBlue. **(D)** Overlaid image show co-localization and help determine the number of transfected cells. Scale bar: 100 μm.

ing effector. One such system is the tetracycline-controlled transcriptional activation system where tetracycline (TET) is used as effector to de-repress a promoter by binding to a corresponding inhibitor. [15,16]. Both of these methods has their own merits, but may result in different protein expression heterogeneities. If we take together the facts that GPCR expression may impact regulation and function as stated above, while a lot of current GPCR studies only has little or no consideration for variability of protein expression, a worrying trend begin to appear.

In this study, we investigated the variability of β_1AR expression across the transient transfection and the inducible cell line methods, as well as what impact varying receptor expression levels has on its own regulation, information that might prove crucial in order to make proper investigations of GPCRs in the future. As a model system, we employed the mammalian Flp-In^{TM} T-REX^{TM} 293 cell line with SNAP-tag derived from embryonic human kidney cells. We found that receptor expression variability is largely influenced by the chosen protein expression method and that it is possible for important parameters such as receptor internalization properties to be hidden in bulk measurements for very heterogeneous protein profiles. Essentially, protein expression methods evoking little heterogeneity, e.g. inducible cell lines, may be more desirable than a more heterogeneous counterpart.

Transient transfection yields heterogeneous expression

We imaged cells transient transfected with SNAP-β_1AR DNA (henceforth only referred to as β_1AR) with inverted light microscopy images (**Figure 1**. n=1, image shown is one representative image out of four). To determine if and how many cells were successfully expressing β_1AR receptor, the receptor and the cell nuclei were labelled with SNAP-Surface-BG549 and NucBlue respectively (see Materials and Methods). Manual data treatment revealed a transfection efficiency of 53.8 $\pm$ 5.1%. This is an estimate based on a manual count of the number of nuclei with overlapping receptor expression. Note then, that also dead, non-transfected cells contribute to the total number of nuclei counted, as these can be indiscernible from healthy cells. The imaging also revealed large cell-to-cell variability of expression levels of β_1AR for the cells with successful uptake (up to a factor 2.45 increase between low- and high-expression, found by comparing the average intensities of six cells of similar size showing low and high expression). Altogether, these results suggest that transient transfection yield heterogeneous expression of transfected protein, with a high amount of cells having low to no expression.

Monoclonal cell lines have more homogeneous expression than transient transfected cell lines

Next, we investigated the heterogeneity of transfected cell lines in a more quantitative manner. For this purpose, we used Flow Cytometry (FC) [17] to measure receptor expression of individual transfected cells (**Figure 2A-B**, n=1). For transfection, the total DNA concentration was always kept constant at 0.002 g/l (see materials and methods), with varying ratios of β_1AR DNA and pcDNA3 (Nonsense DNA with no coding properties). The point of keeping the total DNA concentration total (while increasing the [receptor:empty] DNA ratio), as opposed to simply increase the amount of transfected receptor DNA, is to make sure that approximately the same number of cells gets transfected for all the experiments. For all β_1AR DNA transfection quantities, there was a huge variance in receptor expression levels of individual cells, confirming the suggestion of the previous microscopy recordings. The transfection efficiency remained around a level close to approx. 55% for all receptor transfection quantities, which is close to the efficiency estimated with the microscope images. Note that the flow cytometry revealed much larger variance between low- and high-expressing cells than the inverted light microscopy did. While microscopy only revealed an intensity difference of almost a factor 2.5 between low- and high-expression, FC revealed expression differences of more than a factor 1000. This is likely a question of resolution: FC has a much higher signal-to-noise ratio, and thus much lower individual expression levels can be gathered. It is also important to note that the FC experiments each counted 10000 cells, while there only were a few hundreds cells in the microscope images from where we found our

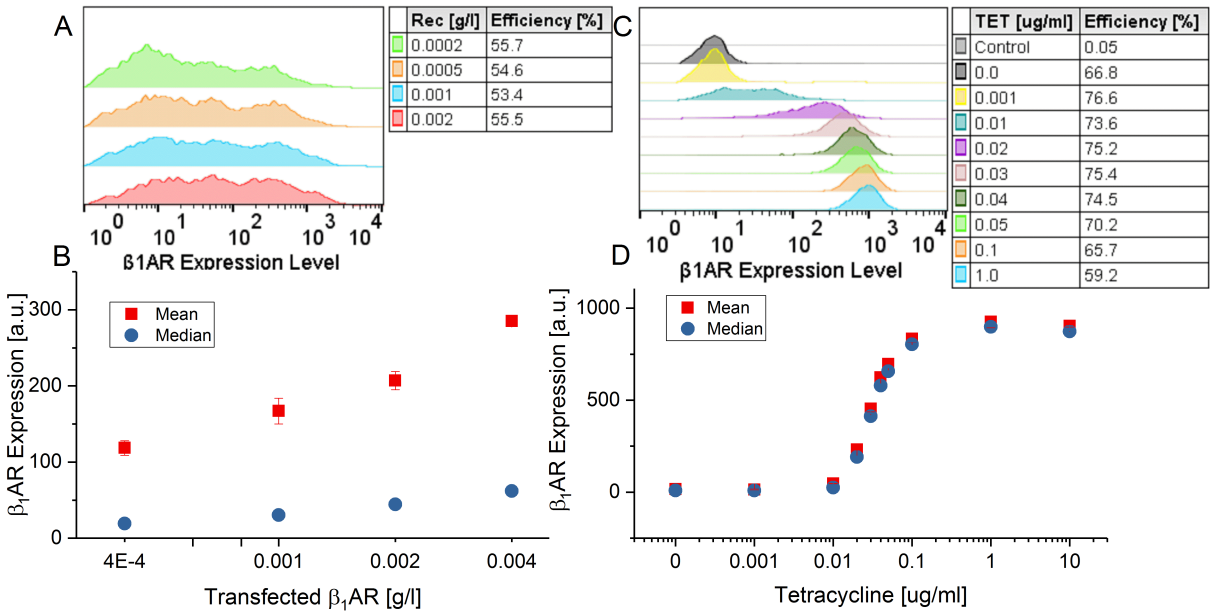

Fig. 2 Flow cytometry of cell lines reveal distinct heterogeneities (A) (n=1) Histograms of β_1AR expression for transfected cell lines for varying amounts of β_1AR DNA used for transfection. Experiment was carried out with duplicates. β_1AR expression is derived from fluorescence intensity of SNAP-BG649. Total DNA concentration was kept constant at 0.002 g/l per well for each condition, and the legend denotes how much of this total was made up of receptor DNA (while the rest was nonsense DNA). Efficiency refers to the transfection efficiency, and is the amount of cells of reasonable size and shape that express the receptor. Note that the size of the right 'tail' of expression increases as the quantity of transfected receptor DNA increases, but not the length. **(B)** Mean and median values ± S.D. of β_1AR expression for transfected cell lines. Note that for absolute values, the mean increases faster than the median for transfection, suggesting a skewing of the expression profile. **(C)** (n=1) Histograms of β_1AR expression for inducible cell lines for varying amounts of added tetracycline (TET). Except for a critical area between concentrations of 0.001-0.02 [ug/ml] tetracycline, the receptor expressions are much more confined for inducible cell lines than for the transfected cell lines. **(D)** Mean and median values of β_1AR expression from inducible cell lines. Though slightly lower, the median follows the mean throughout the increasing receptor expressions.

six extreme cell samples. While the overall receptor expression level rises as the β_1AR DNA ratios increases, the mean value of the expression levels rises more in absolute terms than the median does (with an increase of 166.5 as opposed to 42.9 [a.u.]). While the arithmetic mean is simply the average expression level of a cell, the median is the middle value of a population, and is therefore less affected by extreme outliers. The mean increase and the approximately constant median can be attributed to a skewing of the expression profile; more cells are expressing a large amount of protein and fewer cells are expressing a low amount of protein, while the extreme (both low and high) expression levels remain the same. This description fits the tendency shown by the expression profiles. To summarize, the data show the following: First, that transfection yield large $\beta_1$1AR expression heterogeneity, and secondly, that there is a skewing of the expression profile instead of just a shift to higher expressions for increased transfection quantities (for constant total DNA quantities).

In the search for a method to minimize $\beta_1$1AR heterogeneity, we tested further the intrinsic diversity of protein expression of inducible cell lines by performing a similar FC-recording on a β_1AR monoclonal inducible cell lines (**Figure 2C-D**, n=1), using tetracycline concentrations ranging from 0.0 to 10.0 ug/ml. Here tetracycline acts as an effector and activates expression of $\beta_1$1AR when used on our $\beta_1$1AR inducible cell line. Comparing the expression profile of the inducible cell lines with the transfected cell lines, there is a marked difference. First, the efficiency of receptor expression is higher than for transient transfection, with the amount of cells of appropriate size and shape expressing sufficient quantities of receptor being at around approx. 70%. In addition, most of the tetracycline concentrations resulted in, compared to the transfected cells, very homogeneous expression profiles. As all cells in a monoclonal cell line have identical genomes,[18] a relative homogeneous receptor expression is not unexpected. A possible contributor to variability is likely the binding of the effector to the inhibitor. If this is true, we predict that a constitutive monoclonal cell line (that is, one

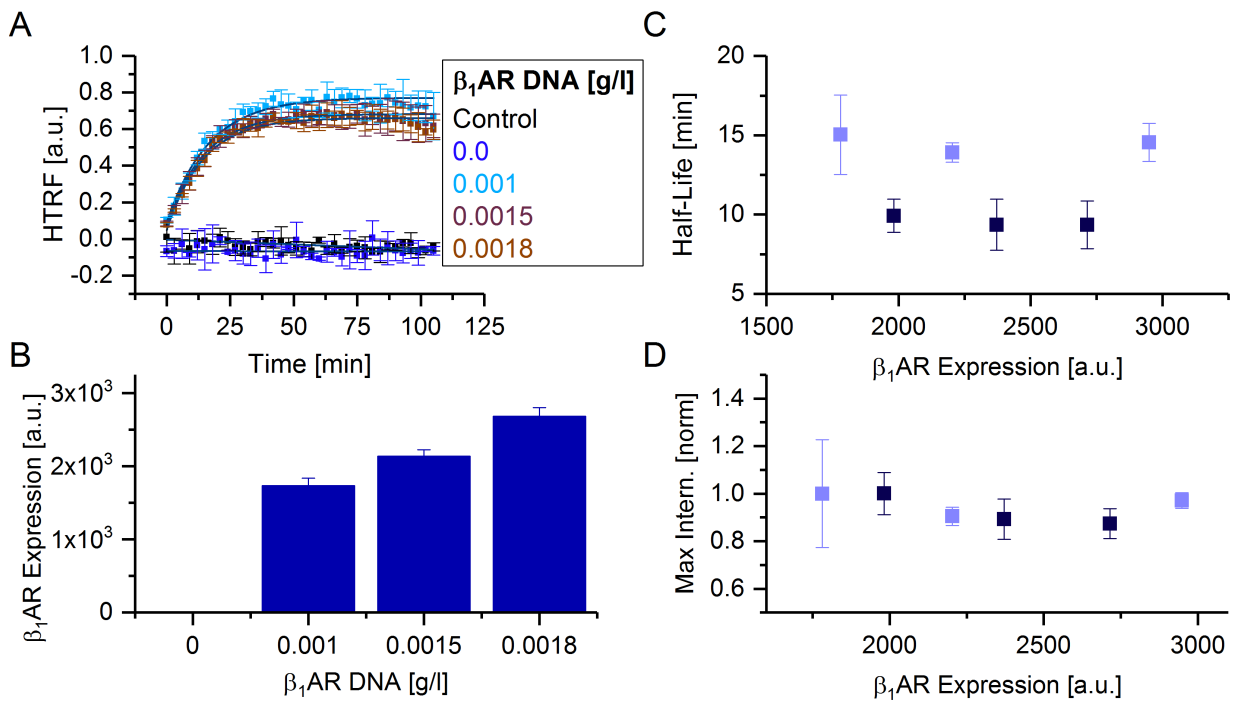

Fig. 3 Internalization of transient transfected cell lines (n=2) For C-D, each gradient denotes an independent experiment. **(A)** HTRF-value ± S.D. ($I_{Donor}/I_{Acceptor}$) evaluated over two hours. Experiment carried out in triplicates. Data shown is of one representative experiment out of two. Legend denotes how much of the total 0.002 [g/l] transfection DNA was made up of β_1AR DNA. The control group was non-transfected T-REX^{TM} 293 cells. **(B)** Mean ± S.E. receptor expression as a function of transfected receptor DNA quantity measured before internalization. Note that instead of transfection conditions, the internalization kinetics are described in regards to the receptor expression measured, as this gives a clearer view of the factual receptor concentration. **(C)** Half-life ± S.D. as a function of receptor level derived from the HTRF fits. **(D)** The maximal internalization (Max Intern.) ± S.D. of receptor as a function of receptor level derived from the HTRF fits. This data is normalized with regards the the maximum value of each dataset.

that does not have an inhibitor associated with the stably transfected gene) could be more homogeneous than an inducible one, though using a constitutive cell line means we loose control over the level of expression. We did not investigate this proposition any further. Supported by the shapes and position of the expression profiles, as well as the similar values of the median and mean, our results suggest that inducible cell lines are much more homogeneous than transfected cell lines in regards to protein expression, and that increased concentrations of effector lead primarily to a shift towards higher expression as opposed to a skewing of the expression profile. What comes next is an attempt to elucidate what effect a homogeneous protein expression has on experimental bulk measurements compared to the effects of a heterogeneous expression.

Internalization kinetic properties of transiently transfected cells could be hidden in bulk measurements

GPCRs are subject to a high degree of internal regulation, a process that is believed to finetune cellular receptor sensitivity and involves receptor desensitization by the GRK-β-arrestin system as well as internalization and recycling.[19,20] Due to the importance of this regulation, both constitutive and agonist-induced receptor internalization are typical subjects of study in cell physiology.[21,22] In order to study the practical implications of heterogeneous versus homogeneous receptor expression profiles, we investigate β_1AR internalization for different systems utilizing a novel Homogeneous Time Resolved Fluorescence (HTRF) assay.[23] This assay combines Fluorescence Resonance Energy Transfer (FRET)[24] with a Time Resolved measurement (TR). This HTRF assay works by labeling the receptor and solution with respectively a donor and an acceptor fluorophore capable of energy transfer, a so called FRET pair. Receptor internalization results in a quantifiable reduction of energy transfer that, if measured over time, gives an estimate of the amplitude of internalization. We will coin this estimate the HTRF-value.

First we tested the β_1AR internalization of cells transient transfected with increasing amount of receptor DNA using the transfection reagent Turbofect (**Figure 3**, n=2)

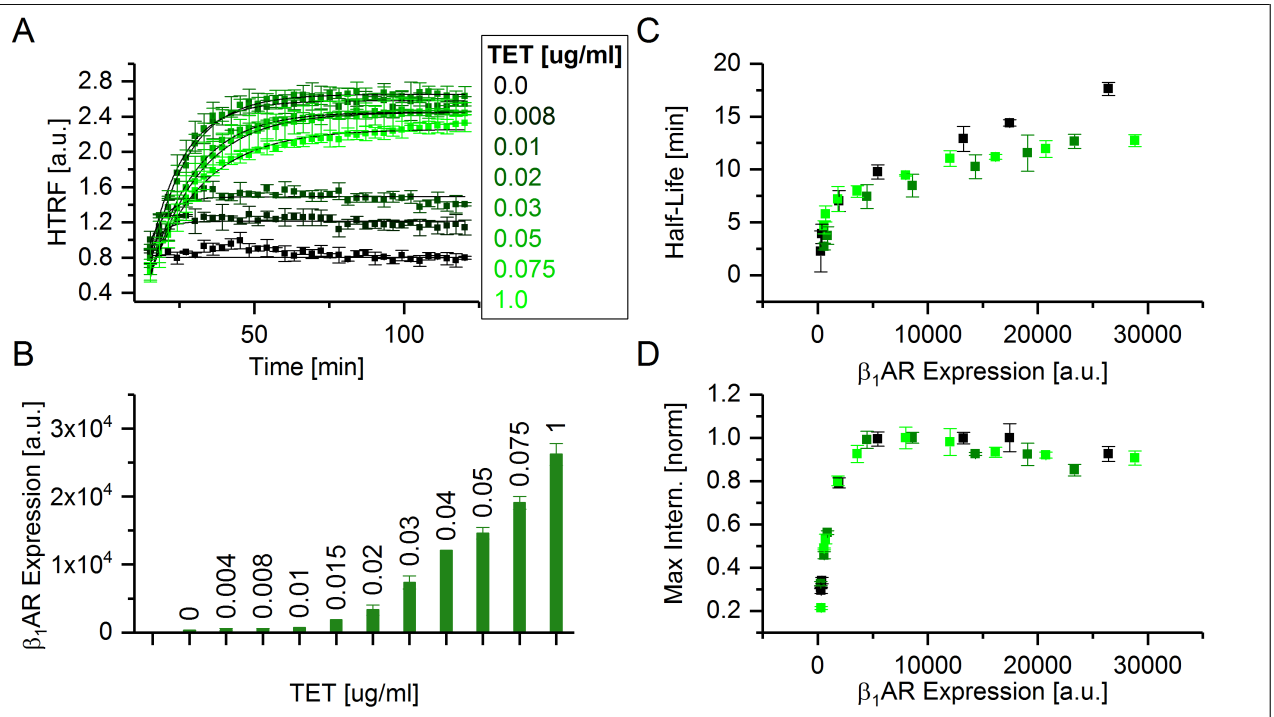

Fig. 4 Internalization kinetics of tetracycline-inducible cell lines (n=3) For C-D, each gradient denotes an independent experiment. **(A)** HTRF-value ± S.D. ($I_{Donor}/I_{Acceptor}$) evaluated over two hours. Experiment carried out in triplicates. Data shown is of one representative experiment out of three. Legend denotes how much tetracycline (TET) was used to induce receptor expression. The control group was non-transfected T-REX^{TM} 293 cells. **(B)** Average ± S.E. receptor expression as a function of tetracycline used for induction measured before internalization. Note that instead of induction conditions, the internalization kinetics are described in regards to the receptor expression measured, as this gives a clearer view of the factual receptor concentration. **(C)** Half-life ± S.D. as a function of receptor level derived from the HTRF fits. **(D)** The maximal internalization ± S.D. (Max Intern.) of receptor as a function of receptor level derived from the HTRF fits. This data is normalized with regards to the maximum value of each dataset.

with the total DNA concentration kept total. (See materials and methods). Note that the HTRF measurements are bulk measurement, and therefore the β_1AR expressions measured corresponds to the mean values of receptor expression. While the β_1AR expression rises steadily for larger quantities of receptor DNA, neither the half-life nor the maximal amount of internalization seems to be influenced by receptor expression for our transfected cells. It has been suggested by the literature that there exist a correlation between receptor regulation and receptor homo-oligomerization,[3,25] which would suggest that large receptor concentrations impacts receptor internalization. Another newer study also claim that self-regulation of receptor systems and inhibition of internalization are not necessarily due to oligomerization, but to depletion of the endocytic machinery.[26] The investigation of these mechanisms are far beyond the scope of this study, but no matter the cause, the scientific community suggests that high receptor density might inhibit the rate of internalization. As we do not observe this tendency of inhibited internalization in our transient transfection measurement, we speculate that it is caused by the extremely broad and heterogeneous protein expression profiles. Though increasing the receptor-DNA transfection quantity increases the average amount of protein the cells express, we maintain a large amount of cells expressing low quantities of the receptor (**Figure 2A**). The large quantity of low-expressing cells may well have a faster rate of internalization while making up for a large portion of the energy transfer. The large population of rapidly internalizing cells thus contributes to a large part of the bulk HTRF measurement in a way that limits the measurable HTRF-value difference between the different experimental conditions used. We describe this phenomena as *bulk-shielding*. Bulk-shielding could similarly explain why the maximal amount of internalization remains virtually unchanged even for larger averages of expressed receptor. Assuming this is true, we can further hypothesize that the marked difference in the measured half-life between the two transfection-experiments (**Figure 3C**) is due to variance in cell survivability at time of transfection. The increased half-life of the one experiment could be contributed to a transfection where less cells were transfected

with more receptor, possibly caused by a lower fraction of healthy cells at time of transfection. This condition would explain why for one experiment the receptor expression remains close to the other experiment while the internalization rate is slower. To repeat our findings, we suggest that the large heterogeneity of the receptor expression profile for transfected cell lines could influence HTRF measurements due to bulk-shielding. If this is true, then bulk measurements on cell lines with more homogeneous expression profiles should better show the hidden kinetic properties discussed.

Inducible cell line reveals distinct internalization kinetic properties compared to transfected cells

We now move on to investigate the more homogeneous expression profiles of inducible β_1AR cell lines with the HTRF-assay (**Figure 4**, n=3). The expression profiles for most of the induction condition were very homogeneous (**Figure 2C**), and as such we anticipate that far the majority of the cells to express a protein density comparable to the average that we measure with our HTRF machinery. Because of this, we expect that there would be little-to-no bulk-shielding taking place, and that the internalization kinetics are not independent of the receptor densities. We found that by increasing the receptor expression level by inducing with increasing concentrations of tetracycline (from 0.0 through 1.0 [ug/ml]), the half-lives and the maximal internalization amounts both increased in accordance with our expectations (**Figure 4C,D**). Note that the value of maximal internalization does not converge to a specific value, but slowly starts declining at large receptor densities. We contribute the peak level of the maximal internalization to saturation of the endocytosis.[27] This means that even though the amount of receptors on the cell surface increases, the amount of receptors that are internalized do not, which results in the slow decrease in the HTRF-value for high amounts of receptor expression. Our findings here point to the fact that homogeneous protein profiles allows bulk measurements to better seize the true properties of reviewed cells. Based on a comparison of our data between the transient transfection and induction β_1AR expression methods, we propose that properties of internalization kinetics are easier hidden in bulk measurements for very heterogeneous cell profiles, than it is for homogeneous populations. Note that there are some considerations towards the credibility of our proposition, and these are discussed in the Functional Relevance section further below.

The effect of post-synaptic density protein 95 on transfected cell lines kinetics

We have proposed so far how relatively simple kinetic assays might be influenced by the expression profile of the

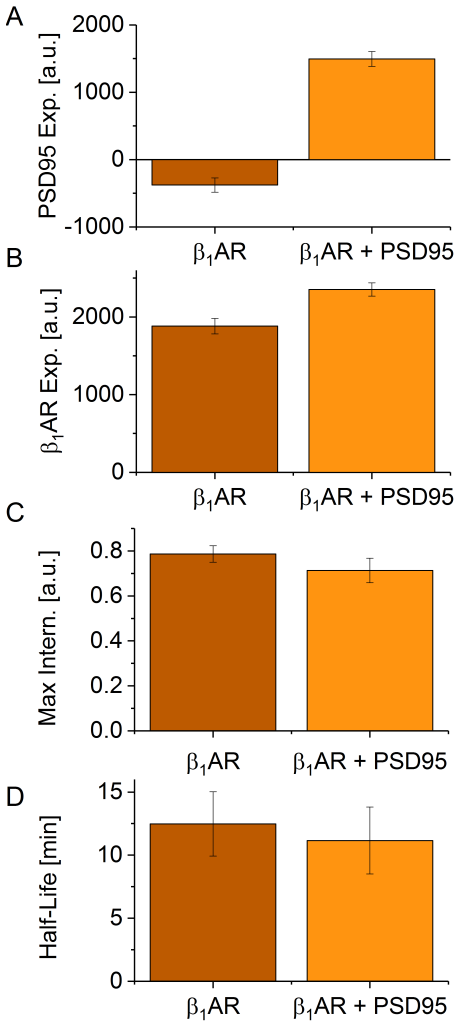

Fig. 5 Scaffold protein PSD95 stabilizes β_1AR on cell surface (n=2). Conditions carried out with the same total quantity of transfection DNA of 0.002 [g/l], the first being a 1:1 mix between β_1AR DNA and pcDNA3 DNA and the second an equal mix of β_1AR DNA and PSD95 DNA. The control was non-transfected T-Rex cells. **(A)** Mean PSD95-GFP expression $\pm$ S.E. **(B)** Mean receptor expression $\pm$ S.E., **(C)** mean max internalization $\pm$ S.E. and **(D)** mean half-life $\pm$ S.E. for β_1AR transfected cells transfected with and without PSD95.

receptors of interest. We suggest that a future step in the investigation of protein expression profiles could be to look into if and how receptor-interacting proteins influences the behavior of the receptor of interest dependent on receptor heterogeneity. Determining the practical implications of heterogeneity on important cellular functions could provide useful information on cell physiology. One such example is the post-synaptic density protein PSD95, a protein that specifically interacts with β_1AR[28] and is responsible for antagonizing receptor endocytosis in re-

sponse to agonist activation, stabilizing the receptor at cell surface.[29,30]

To illustrate how such a study might be undertaken, we investigated the effect of co-transfection of PSD95 with β_1AR for transiently transfected cell lines alongside the HTRF measurements (**Figure 5**, n=2). As before, the total quantity of transfected DNA remains constant, with cells being transfected either with a mix of β_1AR DNA and nonsense DNA, or β_1AR DNA and PSD95 DNA. This experiment supports what is already known in the literature; that PSD95 stabilizes β_1AR on the cell surface, as both indicated by increased receptor expression (more receptors on cell surface means more labeled receptors), and the decreased maximal internalization. No statistically significant enough change to the Half-life is observed to conclude anything, though the literature have demonstrated that PSD95 slows rate of agonist-induced β_1AR internalization.[7] This discrepancy can likely be blamed on the small number of experiments. Following a more in-depth study of PSD95's influence on transfected cell lines, a study of constitutive PSD95-expressing β_1AR inducible cell lines should follow, as to allow comparison between heterogeneous and homogeneous conditions.

Functional Relevance

A valid argument against our proposition of bulk-shielding is that the two cell lines investigated are not exactly identical due to the natural constraints of the experiment. This is a common problem for cross-cell line studies, but as both transfection and induction are methods used for modern research, it is nonetheless important to be able to pit them against each other. The practical implications of evoking receptor expression through the use of transient transfection compared to inducible cell lines has been presented in this article. We propose that it is primarily protein heterogeneity that is the cause of the disparity between the two methods, though we acknowledge the fact that this result cannot be completely unambiguous as per the use of differently treated cell lines.

Another troubling matter is the low number of independent replications of our experiments and the slightly varying β_1AR DNA and tetracycline concentrations between the FC- and the HTRF-measurements. The ramifications of this matter is ultimately that our conclusions and propositions should not be weighted too heavily, and instead of proposing a final truth, this paper should rather act as an inspiration for further protein studies to remember to consider protein heterogeneity as an important factor in experimental measurements. This heterogeneity-consideration is not only limited to the protein of interest, but should also cover natural variations of the proteome of interacting cells for in vivo experimentation. Even though some papers do acknowledge the heterogeneous nature of their samples, heterogeneity is also a matter of degree, and is something that could and should be characterized by future research.

Conclusional Remarks

Our study investigated what effect transient transfection and inducible cell lines might have on types of variability for receptor expression and how this influences receptor internalization. For this study, we have primarily utilized the novel homogeneous time resolved fluorescence measurement method as well as flow cytometry. Evoking β_1AR expression through tetracycline induction yielded markedly more homogeneous protein expression profiles than what transient transfection with β_1AR-DNA did (**Figure 2A,C**). Likewise, HTRF measurements on cells with the two forms of evoked β_1AR expression also revealed distinct tendencies in internalization kinetics (**Figure 3 & 4**). In accordance with the literature, the receptor expression was shown to drastically regulate both the half-life and maximal value of β_1AR internalization for the tetracycline-inducible cell line (**Figure 4C-D**), while these tendencies were completely omitted from the measurements on the transient transfected cell lines (**Figure 3C-D**. This, we propose, could be attributed to bulk-shielding, an indispensable phenomena of bulk measurements on heterogeneous cell profiles in which the change in a minority of cells is hidden by the large contribution of the unchanged majority. Altogether, our study concludes that it is imperative to take into account the heterogeneity of protein expression profiles when conducting bulk experiments. It should be noted however, that we admit the fact that our experiments are not altogether statistically valid, with many of the experiments only having been performed independently once or twice. Our conclusions should therefore only be considered a suggestion of the cause and effect of heterogeneous β_1AR expression and its influence on receptor internalization.

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Materials and Methods

Cell Culture and preparation Mammalian Flp-In^{TM} T-REX^{TM} 293 cells (Invitrogen), were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) 1:1 with GlutaMAX^{TM} and phenol red, supplemented with 10% FBS (henceforth referred to only

as DMEM, bought from Invitrogen). Antibiotics Zeocin (0.1 mg/ml, Gibco) and Blasticidin (0.015 mg/ml, Gibco) were mixed in the medium when used for cell growth but not for transfection. The tetracycline inducible stable cell line with inhibited expression of SNAP-β_1AR were obtained previous to the study by FLP-FRT recombination with pc3.1DNA/FRT/TO vectors (Genscript), and the antibiotics Blasticidin (0.015 mg/ml) and Hygromycin (0.1 mg/ml, Gibco) were used for cell growth but not for induction. When referring to β_1AR or the receptor(Rec) in regards to the practical experiments, we are actually referring to a SNAP tagged β_1AR variant. To prepare cells for experimentation, cells were seeded onto Perkin Elmer 384- or 24-well culture plates and incubated overnight in medium. The 384-well culture plates were incubated with 25 μg/well 0.001 % Poly-L-Lysine (Sigma-Aldrich) for 30 minutes before seeding. The next day, the medium was replaced by μl fresh DMEM with either transfection or induction reagent (see below). After 24 hour incubation, the cells were ready for HTRF, Microscopy and/or FC preparation and measurement.

Transfection and Induction Reagents Transient transfections were carried out using Turbofect Transfection Reagent (Life Technologies) using the transfection medium Opti-MEM (Life Technologies) and plasmids containing genes for β_1AR and/or PSD95-GFP-expression (Both from Cisbio). The total DNA concentration was always kept constant at 0.002 g/l DNA. When varying the quantity of β_1AR for transfection, pcDNA3 (Nonsense DNA plasmid that didn't, code for anything relevant, from Invitrogen) were added to the transfection mix in order to maintain a constant total DNA level. To prepare each transfection reagent, 2 μg DNA was suspended in 50 μl Opti-mem while 4 μl Turbofect was mixed also with 50 μl Opti-Mem. After 20 minutes of incubation, the two solutions were mixed and diluted 1:10 with DMEM. See Turbofect's online guide for additional information on successful transfection.[31] Induction reagent was fresh DMEM containing appropriate ligand (tetracycline) concentrations. Tetracycline was protected from light at all times.

Inverted Light Microscopy. Live cells were imaged using a Leica DMI6000B inverted light microscope (Leica Microsystems) with a HG-lamp. 2 mL of T-REX^{TM} cells transiently transfected with β_1AR (DNA concentration: 0.002 g/l DNA to 400.000 cells/ml. DNA was 1:1 mix between β_1AR and pcDNA3) were plated onto a glass-coverslip, and incubated with SNAP-Surface-BG549 (Bionordika) for 10 minutes. Subsequently, the plates were washed thrice with DMEM and twice with imaging medium (DMEM/F-12 (1:1) (1x) + L-glutamine + 15 mM HEPES (No Gluta-MAX and phenol red)). Finally, imaging medium and NucBlue^{TM} (Life Technologies) were added to the plates before measuring. The filter cubes EC3(SNAP-549), BGR(NucBlue) and TL-BF(Bright-Field)s were used for measurement. Image analysis was subsequently carried out using the software ImageJ.

Flow Cytometry. Flow Cytometry was carried out using a BD FACS Calibur Flow Cytometer, courtesy of the Biotech Research and Innovation Center (BRIC) of Copenhagen University. The heterogeneity of β_1AR expression was probed in T-REX^{TM} cells where expression was initiated by transient transfection, as well as with tetracycline activation on the inducible stable cell lines expressing β_1AR. Each experiment was done in duplicates and cells were grown on 24-well culture plates (300.000 cells/well). For labeling of cells for FC, the SNAP-Surface BG649 photostable fluoresent substrate (Bionordika) was added to the cells with appropriate medium containing 4 nM of the dye solution, and left to incubate for 10 minutes. The cells were then washed two times with labeling medium (DMEM/F-12 with L-glutamine and HEPES without phenol red (Gibco) and one time with FACS medium (phosphate buffered saline (PBS) with 1% FBS), carefully, as not to disturb the cells. After washing, the cells were suspended in FACS medium before commencing flow cytometry. A gating based on a side and forward scatter was used, as to only allow the cytometer to count cells of an appropriate size, that is, the living cells. The FC data was subsequently treated with FlowJo software.

HTRF Measurements. Homogeneous Time Resolved Fluorescence measurements were carried out using a Synergy H4 Hybrid Reader (BioTek Instruments) microplate reader. Measurements were carried out on either T-REX^{TM} cells transiently transfected with β_1AR, PSD95-GFP and/or pcDNA3, or on the β_1AR inducible cell line. Cells were transfected/induced and measured in a Perkin Elmer white CulturPlate-384 (0.07 cm^2/well growth area, 20.000 cells/well). Each experiment was done in triplicates. The ready cells were initially washed first with commercially available Tag-Lite buffer, pH = 7.4 (Cisbio), and then dyed with the donor, the long-lived fluorophore SNAP-Lumi4 Terbium Cryptate in DMSO solvent (Cisbio Bioassays, 100 μM stock solution diluted 1000 times in Tag-Lite buffer) that labels SNAP-β_1AR. The cells were from now on cooled on ice, inhibiting constitutive internalization. After a 3 hour long incubation the wells were washed four times with Tag-Lite buffer followed by addition of 50 μl Tag-Lite buffer to every well. The receptor expression was measured; donor intensities were collected using 340/30 nm excitation and 620/10 nm emission filters, sensitivity: 100. PSD95-GFP expression was measured with sensitivity 60. The Tag-lite medium was replaced by 25 μl of acceptor fluorophore biotin-4-Fluorescein (Cisbio) and 25 μl 2 μM of the ligand isoprotenerol to ini-

tiate internalization of the receptor. Immediately after, a new time-resolved measurement was started and carried out for two hours, with the intensity being gathered every third minute at 37^{o}C. Acceptor intensities were collected using 340/30 nm excitation and 520/10 emission filters, sensitivity: 120 for acceptor and 140 for donor. Measurements were taken with a 1500 μs time delay to account for short-lived background fluorescence. HTRF-values were obtained using the simple relationship between the measured donor- and acceptor-intensity: I_{HTRF} = I_{DONOR} / $I_{ACCEPTOR}$, and the data fitted with the equation $Y = A_1 - A_2 * e^{-kx}$. Half-Lives were found by taking $ln(2)/k$ while the maximal internalizations (Max Intern.) were the convergent values of the HTRF fits.

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