Supplementary Materials for

Distinct phosphorylation sites in a prototypical GPCR differently orchestrate β-arrestin interaction, trafficking, and signaling

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Figs. S1 to S8
Figure S1

A

Surface expression (% normalized)

βarr1  βarr2

V2R WT   V2R TT/AA  V2R SSS/AAA  V2R TSS/AAA  V2R SS/AA  V2R T347A  V2R S350A  V2R S357A  V2R T359A  V2R T360A  V2R S362A  V2R S363A  V2R S364A

0 40 80 120 160 200

AVP (min)

0 30 0 30 0 30 0 30

-20

0

20

40

60

80

100

120

βarr1/2 signal in co-IP (% normalized)

B

βarr1

IP: HA  IP: FLAG

AVP (min)  0 30 0 30

54kDa

54kDa

WB: βarr

WB: V2R

βarr2

IP: HA  IP: FLAG

AVP (min)  0 30 0 30

54kDa

54kDa

WB: βarr

WB: FLAG

Lysate

Lysate
**Figure S1. Surface expression of V2R mutants and specificity of co-immunoprecipitation assay.**

A. Surface expression of V2R mutants as measured by whole-cell ELISA under co-expression conditions of βarr1 or 2. Values are normalized with respect to V2RWT treated as 100%, and represent mean±SEM from at least three independent experiments, each performed in duplicate. 

B. The specificity of our anti-Flag agarose based co-immunoprecipitation assay is established using anti-HA antibody agarose as a negative control on HEK-293 cells expressing Flag-tagged V2RWT and βarrs. Upon agonist-stimulation (100nM, AVP), we observe pull-down of βarrs with anti-Flag M2 antibody agarose through Flag-tagged V2R but not with anti-HA antibody agarose. Cellular lysate taken before DSP cross-linking were probed for βarr1/2 and Flag-V2R to verify comparable input in co-IPs. Representative images from three independent experiments, and densitometry-based quantification of data (mean±SEM), normalized with the signal at 30min agonist-stimulation for V2RWT (treated as 100%) are shown.
Figure S2. Quantification of agonist-induced βarr translocation as measured by confocal microscopy. A-B. Agonist-induced localization of βarrs for different V2R mutants were measured by confocal microscopy as described earlier, and βarr localization in cells from multiple fields in at least three independent experiments were manually scored. Confocal images captured during 1-8min and 9-60min post-agonist stimulation were grouped under early and late time-frames, respectively. The localization of βarrs was scored as surface and internalized depending based on YFP fluorescence in the plasma membrane and punctate structures in the cytoplasm, respectively. Data are plotted as % of βarr localization pattern from more than hundred cells counted for each condition.
Figure S3

A. 

**V₂R³⁶²A**

AVP (min) | 0 | 5 | 30 | 0 | 5 | 30
54kDa | | | | | | |
54kDa | | | | | | |

βarr1 recruitment (% normalized)

βarr1 recruitment (% normalized)

B. 

**V₂R³⁶³A**

AVP (min) | 0 | 5 | 30 | 0 | 5 | 30
54kDa | | | | | | |
54kDa | | | | | | |

βarr1 recruitment (% normalized)

βarr2 recruitment (% normalized)

C. 

**V₂R³⁶⁴A**

AVP (min) | 0 | 5 | 30 | 0 | 5 | 30
54kDa | | | | | | |
54kDa | | | | | | |

βarr1 recruitment (% normalized)

βarr2 recruitment (% normalized)
Figure S3. Mutation of the individual sites in the SSS cluster differentially affects βarr recruitment. A-C. Mutation of S362 and S363 robustly inhibits agonist-induced (100nM AVP) βarr recruitment, however, S364 has a relatively smaller effect, as measured by co-immunoprecipitation (co-IP) experiment in HEK-293 cells. Representative images from three independent experiments (four for V2R5364A+βarr1 and five for V2R5364A+βarr2), and densitometry-based quantification of data (mean±SEM), normalized with the signal at 30min time-point for V2RWT is shown. Data are analyzed using Two-Way ANOVA (ns, non-significant; p<0.05; **p<0.01; ***p<0.001).
Figure S4

A

0 min 2-10 min 30-50 min

βarr1

βarr2

V2R362A

B

0 min 2-10 min 30-50 min

βarr1

βarr2

V2R363A

C

0 min 2-10 min 30-50 min

βarr1

βarr2

V2R364A
Figure S4. Mutation of the individual sites in the SSS cluster does not affect the overall pattern of βarr trafficking.

A-C. Agonist-induced trafficking of βarrs for the V2R^{S362A}, V2R^{S363A} and V2R^{S364A} are qualitatively similar to that of V2R^{WT} as assessed by confocal microscopy in HEK-293 cells expressing the indicated receptor mutant and βarr-mYFP. Cells were stimulated with 100nM AVP and representative images from three independent experiments at indicated time-points are shown (scale bar = 10μm).
Figure S5

A

V2R<sub>SSS/AAA</sub> 341CCARGRTPPSLGPQDESCTTAASLAKDTSS<sup>371</sup>

| V2R<sub>WT</sub> | V2R<sub>SSS/AAA</sub> |
|-----------------|------------------------|
| AVP (min)       | 0 5 30 0 5 30         |
| 54kDa           | 54kDa                  |

B

V2R<sub>SSS/AAA</sub> 341CCARGRTPPSLGPQDESCTTAASLAKDTSS<sup>371</sup>

| V2R<sub>WT</sub> | V2R<sub>SSS/AAA</sub> |
|-----------------|------------------------|
| AVP (min)       | 0 5 30 0 5 30         |
| 54kDa           | 54kDa                  |
Figure S5. Double and triple phosho-site mutations in the SSS cluster ablate βarr recruitment. A-B. Mutation of S^{362} and S^{363} (i.e. V_{2R}^{SS/AA}) and S^{362}, S^{363} and S^{364} (i.e. V_{2R}^{SSS/AAA}) together nearly eliminate agonist-induced (100nM AVP) βarr recruitment as measured by co-immunoprecipitation (co-IP) experiment in HEK-293 cells. Representative images from three independent experiments, and densitometry-based quantification of data (mean±SEM), normalized with the signal at 30min time-point for V_{2R}^{WT} is shown. Data are analyzed using Two-Way ANOVA (**p<0.01; ***p<0.001; ****p<0.0001).
Figure S6

A

V2R WT V2R T359A

AVP (min) 0 5 30 0 5 30

54kDa

54kDa

βarr1 recruitment (% normalized)

ns

ns

0 min 5 min 30 min

0 30 60 90 120 150

V2R WT

V2R T359A

βarr1 recruitment (% normalized)

B

V2R WT V2R TT/AA

AVP (min) 0 5 30 0 5 30

54kDa

54kDa

βarr1 recruitment (% normalized)

****

* ns

0 min 5 min 30 min

0 30 60 90 120 150

V2R WT

V2R TT/AA

βarr1 recruitment (% normalized)

C

0 min 2-10 min 30-50 min

D

AVP (min) 0 5 15 0 5 15

43kDa

pERK1/2

ns

43kDa

tERK1/2

ERK1/2 phosphorylation (% normalized)

ns

0 min 5 min 15 min

V2R WT

V2R TT/AA

ERK1/2 phosphorylation (% normalized)

****

** ns
Figure S6. T^{359} in the TT cluster is dispensable but double phospho-site mutation inhibits βarr recruitment. A-B. Mutation of T^{359} does not significantly influence but the double phospho-site mutation (i.e. T^{359+360}) nearly eliminates agonist-induced (100nM AVP) βarr recruitment as measured by co-immunoprecipitation (co-IP) experiment in HEK-293 cells. Representative images from four independent experiments (three for V_{2R}^{TT/AA}), and densitometry-based quantification of data (mean±SEM), normalized with the signal at 30min time-point for V_{2R}^{WT} is shown. Data are analyzed using Two-Way ANOVA (*p<0.01; ****p<0.0001). C. Mutation of T^{359} does not significantly alter agonist-induced βarr trafficking pattern as assessed by confocal microscopy in HEK-293 cells expressing the receptor mutant and βarr-mYFP. Cells were stimulated with 100nM AVP and representative images from three independent experiments at indicated time-points are shown (scale bar = 10μm). D. Double phospho-site mutation (i.e. T^{359+360}) results in a significant decrease in ERK1/2 activation, compared to V_{2R}^{WT}, at 5min after agonist-stimulation (100nM AVP). Representative images from five independent experiments, and densitometry-based quantification of data (mean±SEM), normalized with respect to the signal at 5min time-point for V_{2R}^{WT} (treated as 100%) is shown. Data are analyzed using Two-Way ANOVA (ns = non-significant; **p<0.01).
Figure S7

(A) cAMP response (% normalized) over time post-AVP stimulation (min) for V2R<sup>WT</sup>, V2R<sup>SSH/AAA</sup>, V2R<sup>T360A</sup>.

(B) cAMP response (% normalized) vs. Log (AVP) M for V2R<sup>WT</sup> and V2R<sup>T360A</sup>.

(C) cAMP response (% normalized) over time post-AVP stimulation (min) for V2R<sup>WT</sup> and V2R<sup>T360A</sup>.
Figure S7. Agonist-induced cAMP response for the V$_{2}$R$^{T360A}$ and V$_{2}$R$^{SSS/AAA}$ mutants. A. Time-course analysis of agonist-induced cAMP response for the V$_{2}$R$^{WT}$ and V$_{2}$R$^{SSS/AAA}$ constructs at 10pM concentration of AVP reveals relatively sustained level of cAMP for the mutants as expected based on their lack of βarr recruitment. Data (mean±SEM) from six independent experiments (dose response presented in Figure 4F), each performed in duplicate and normalized with respect to the signal at 60min post-stimulation for V$_{2}$R$^{WT}$, are shown. B-C. Dose response and time-course analysis of agonist-induced cAMP response for V$_{2}$R$^{T360A}$ as measured in HEK-293 cells using the GloSensor assay. Data (mean±SEM) from four independent experiments, each performed in duplicate, are presented here. Data are normalized with respect to the response at 1μM concentration of AVP for V$_{2}$R$^{WT}$ (treated as 100%) for the dose response curve, and with respect to the signal at 60min post-stimulation for V$_{2}$R$^{WT}$ for the time-course analysis. The arrows in panels A and C indicate the addition of agonist.
Figure S8. Binding profile of the V_{2}Rpp and mutated versions to βarr1. Classical unbiased simulation was used to monitor the dynamics of the V_{2}Rpp and the phospho-site mutants investigated in this study. A quantitative measure of V_{2}Rpp dynamics is obtained by computing root mean square fluctuation (RMSF) per residue. We find that the V_{2}Rpp as well as the studied mutants show a similar fluctuation profile (see RMSF plots). Not surprisingly, more fluctuation (i.e. higher RMSF) is found for the N- (346 to 348) and C-terminal ends (366 to 372). In contrast, stable fragments are found at distinct sections of the V_{2}Rpp, namely residue 349 to 352 and 360 to 365 (i.e. low RMSF). Note, both sections adopt an extended β-strand that pack against existing strands in the βarr1 via backbone-backbone interactions (indicated in green in the panel above). This secondary structural arrangement is the reason for stability and low RMSF values in these positions. We observed that the most stable residue is consistently observed at T^{360} for all simulated systems (highlighted with a green arrow in plots). T^{360} is part of a β-strand and additionally interacts with K^{294} in the lariat loop via a strong electrostatic interaction (top left). Interestingly, we observed the same stability pattern even for the T^{360}A mutant which we ascribe to its backbone-backbone interaction with βarr1.