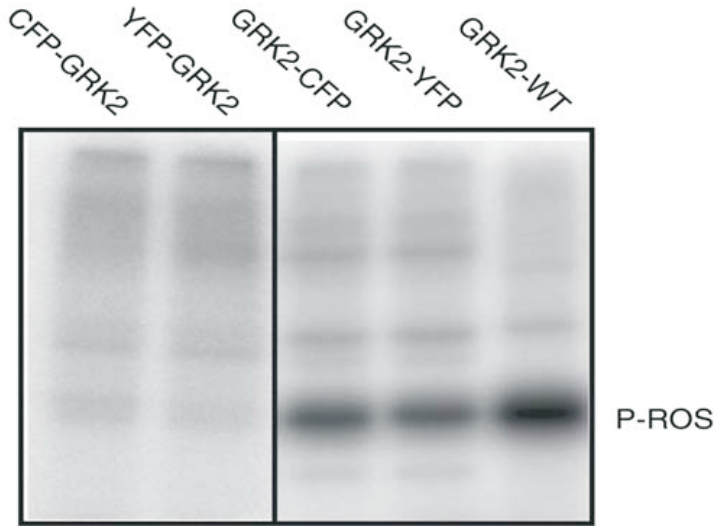
Valerie Wolters, Cornelius Krasel, Jörg Brockmann, Moritz Bünemann:
Influence of $G\alpha_q$ on the dynamics of M_3-ACh-receptor interaction,
Molecular Pharmacology



Supplemental Figure 1 Construction of fluorescent GRK2 mutants and investigation of their Rhodopsin phosphorylation. Human GRK2 was tagged either at the N-terminus or at the C-terminus with CFP or YFP (as described in the Methods section). All constructs were cloned into pcDNA3 and expressed in HEK293 cells. Expression was verified by observing cytosolic fluorescence (data not shown). To verify the functionality of the expressed constructs HEK293 cells expressing wild-type GRK2 or the various constructs were harvested and lysed by osmotic shock. The lysate was clarified by centrifugation.
The clarified lysate was used to phosphorylate light-activated urea-treated rod outer segments in vitro in the presence of 3 µM of G-protein βγ subunits and of [32P]-ATP (Krasel et al., 2001). Phosphorylated proteins were resolved by SDS polyacrylamide gel electrophoresis and phosphate incorporation was visualized by exposure to film.
As shown in Fig. S1 only wild-type GRK2 and the constructs, which had fluorescent proteins attached to the C-terminus, were able to phosphorylate rhodopsin in a light-dependent manner, whereas the constructs tagged with fluorescent proteins at the N-terminus were inactive.

Acknowledgements
We are grateful to Christian Dees for purifying G-protein βγ subunits.

Reference
Krasel C, Dammeier S, Winstel R, Brockmann J, Mischak H, Lohse MJ (2001) Phosphorylation of GRK2 abolishes ist inhibition by calmodulin. *J Biol Chem* **276**: 1911-1915.

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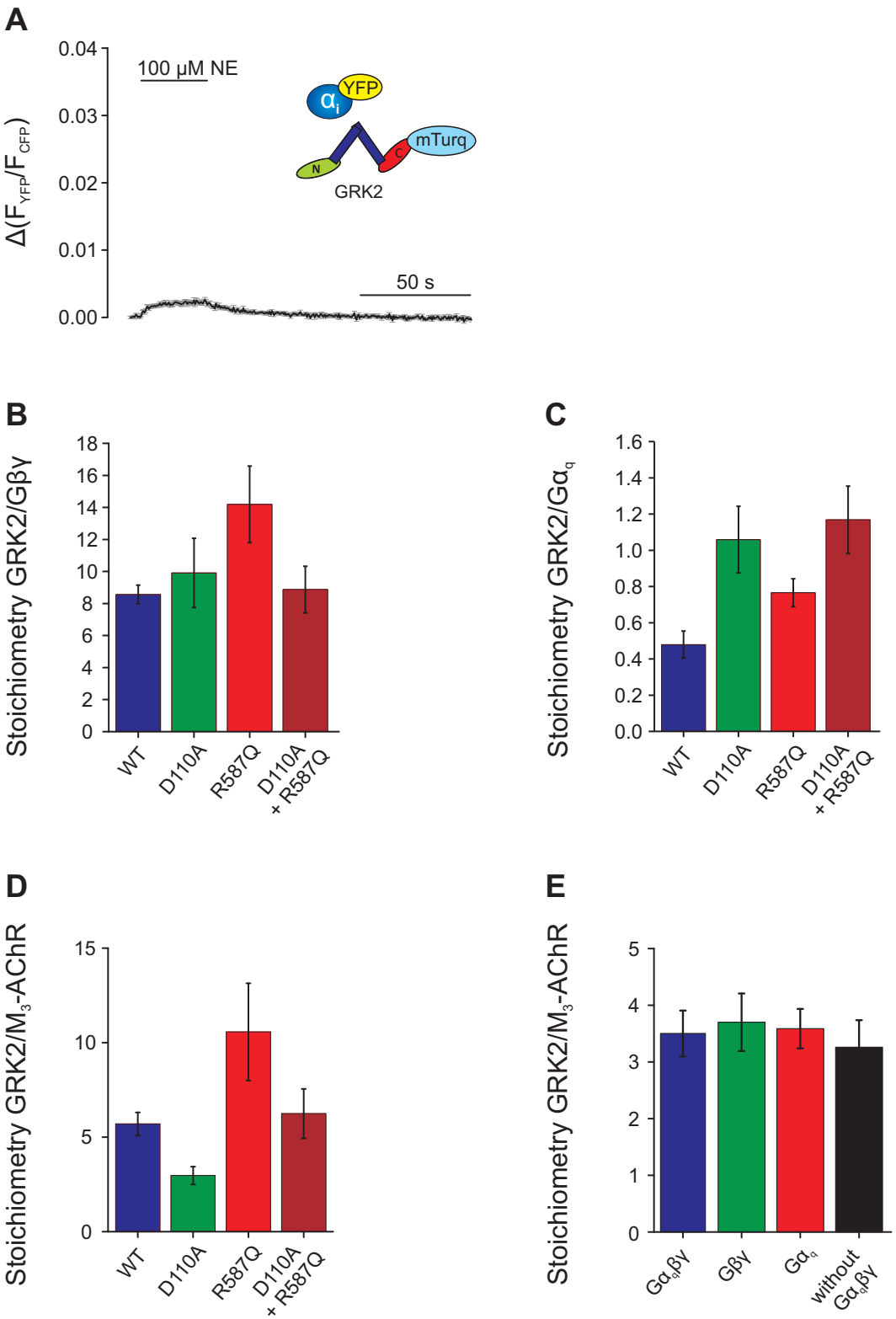

Supplemental Figure 2

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Supplemental Figure 2 (A) HEK293T cells transiently transfected with $G\alpha_i$-YFP, GRK2-mTurq and unlabelled α_{2A}-AR, Gβ and Gγ were subjected to single-cell FRET imaging recorded at 2 Hz. Individual single-cell FRET recordings were averaged (mean ± S.E.M.; n=12) and displayed as absolute alterations in FRET. α_{2A}-AR stimulation with 100 µM NE as indicated induced only a minor rise in FRET.
(B-E) Relative expression levels of the fluorescently tagged proteins in the assays shown in Fig. 2C **(B)**, Fig. 2D **(C)**, Fig. 3B **(D)** and Fig. 4E **(E)** were equal or revealed an excess of the FRET acceptor YFP.

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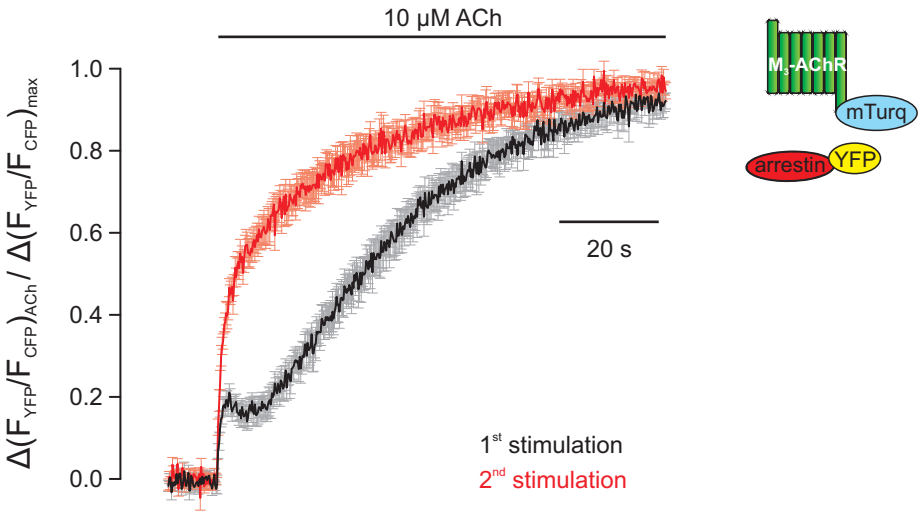

Supplemental Figure 3
HEK293T cells transiently transfected with M_3-AChR-mTurq, arrestin3-YFP, unlabelled $G\alpha_q$, Gβ, Gγ and GRK2 were subjected to single-cell FRET imaging recorded at 5 Hz. The cells were sequently stimulated with 10 µM ACh for two times. Individual single-cell FRET recordings were corrected for bleaching, averaged (mean ± S.E.M.; n=15) and displayed as data normalised to the individual maximal agonist-induced response in order to compare onset-kinetics of the agonist-induced effect.

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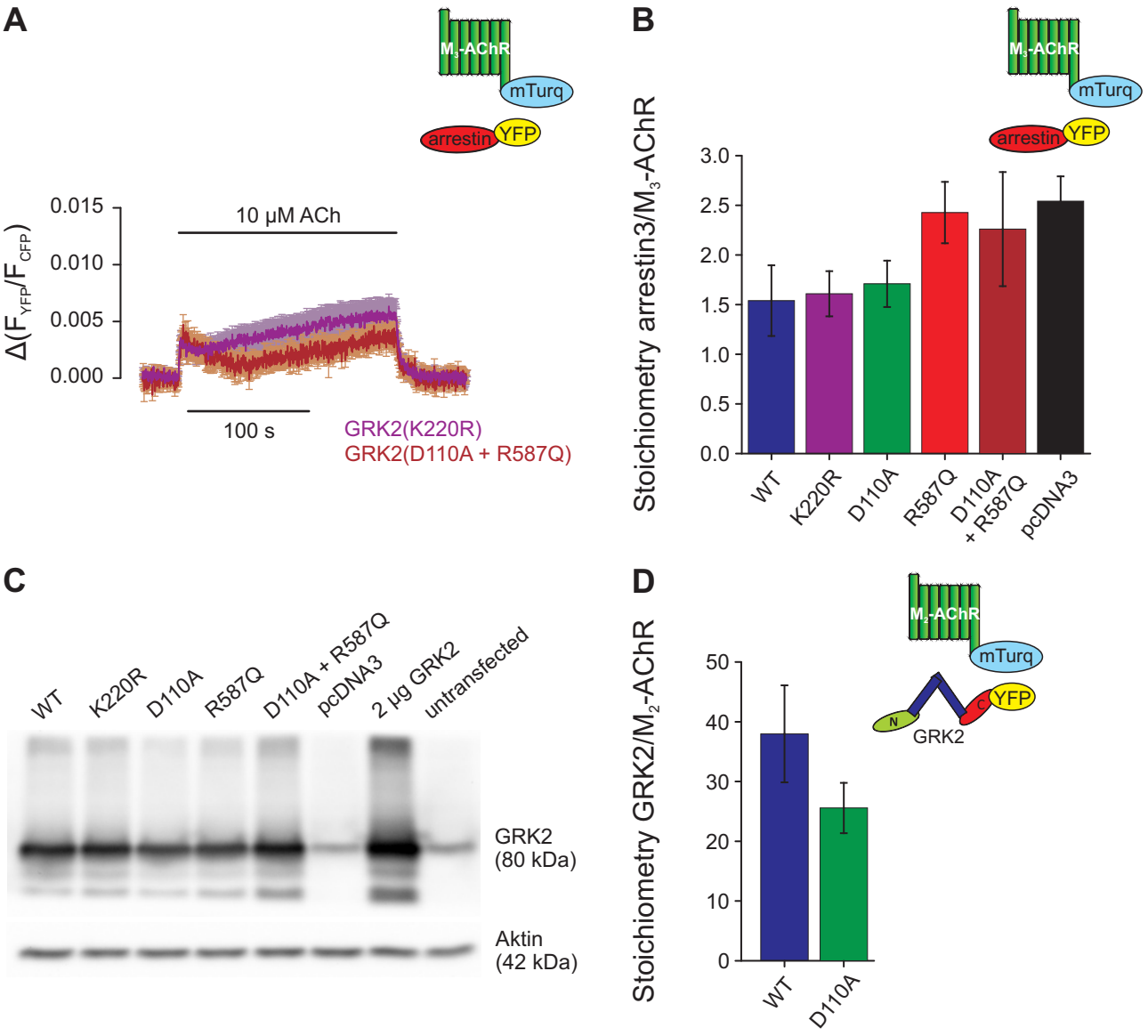

Supplemental Figure 4
(A) HEK293T cells transiently transfected with M_3-AChR-mTurq, arrestin3-YFP, unlabelled $G\alpha_q$, Gβ, Gγ and the different GRK2 mutants were subjected to single-cell FRET imaging recorded at 5 Hz. Individual single-cell FRET recordings were corrected for bleaching, averaged (mean ± S.E.M.; n≥11) and displayed as absolute alterations in FRET. Statistics are given for analysis of absolute amplitudes by ANOVA with Bonferroni posthoc test (*: $p<0.05$). **(B)** Relative expression levels of the fluorescently tagged proteins in the assay shown in Fig. 6A and A were not significantly different and revealed an excess of the FRET acceptor YFP. **(C)** Representative western blot out of three, confirming an equal expression level of the different GRK2 mutants in the assay shown in Fig. 6A and A. **(D)** Relative expression levels of the fluorescently tagged proteins in the assay shown in Fig. 6B were not significantly different and revealed an excess of the FRET acceptor YFP.

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Supplemental Figure 4

Methods: Western Blot
HEK293T-cells were harvested 40 h after transfection in 0.5 ml lysis buffer (20 mM Tris (pH 7.4), 50 mM NaCl and proteinase inhibitor mix (Complete ULTRA Tablets Mini EDTA-free, EASYpack; Roche)). After sonification for 2 s, the suspension was centrifuged for 30 min at 13300 rpm. The supernatant was used for the further process. The protein amounts of the different samples were analysed by a BCA-assay (Pierce) and equalised with lysis buffer. Loading buffer was added (50% (w/v) glycerine, 312.5 mM Tris/HCl (pH 6.8), 10% (w/v) SDS, 25% (w/v) 2-mercaptoethanol, 0.1% Bromophenol Blue, in water) and the samples were heated to 95 °C for 5 min. The proteins were separated with a 10% SDS-gel and transferred to a PVDF membrane (Roche) by wet blotting for 4h at 110 mA. The membrane was blocked with blocking milk (5% skimmed milk powder in TBST (50 mM Tris pH 7,5, 150 mM NaCl, 0,05% Tween 20, in water)) for 1h and cut at 60 kDa. The part with the larger proteins was incubated with the primary antibody anti-GRK2 C-15, sc-562 (Santa Cruz) 1:200 in blocking milk and the part with the smaller proteins with the primary antibody anti-actin clone C4 mouse (MB Biomedicals) 1:100000 in blocking milk at 4 °C over night. As secondary antibodies were used HRP conjugated anti-rabbit (Cell Signaling) 1:2500 and HRP conjugated anti-mouse (Cell Signaling) 1:4000 in blocking milk, respectively. The membrane was incubated for 3 min in ECL-solution (Applichem) and luminescence was detected with a Chemidoc (Bio-Rad Laboratories).