Supplementary Material:

Opioid analgesics activate μ-opioid receptors in a voltage dependent manner

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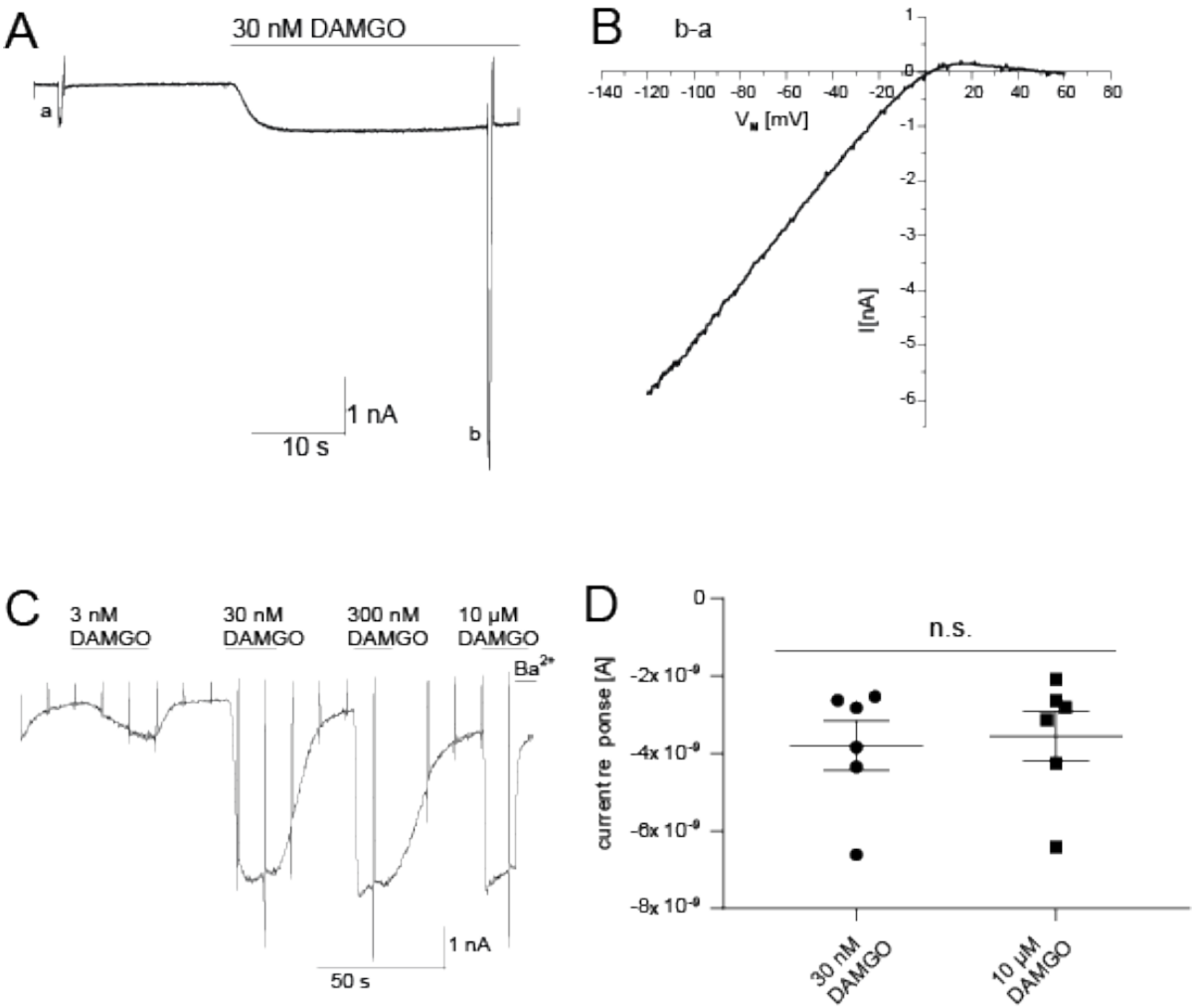


Figure S1: Membrane potential regulates MOP-induced outward GIRK currents. ***A,*** Magnification of the excerpt, which was used for calculation of the background substracted I/V curve (shown in B). Voltage ramps which were used for calculation are marked as a and b. ***B***, Background subtracted I/V-curve (b-a, obtained from the voltage ramps a and b), as indicated in *fig. S1A* show strong inward rectification***.*** ***C,*** Representative recording (n = 6) of GIRK current responses to increasing concentrations of DAMGO. ***D,*** Statistical comparison of GIRK current responses to 30 nM and 10 µM DAMGO. As responses were compared withon the same cell statistics were performed using a paired t-test (n.s.: $p > 0.05$, n = 6).

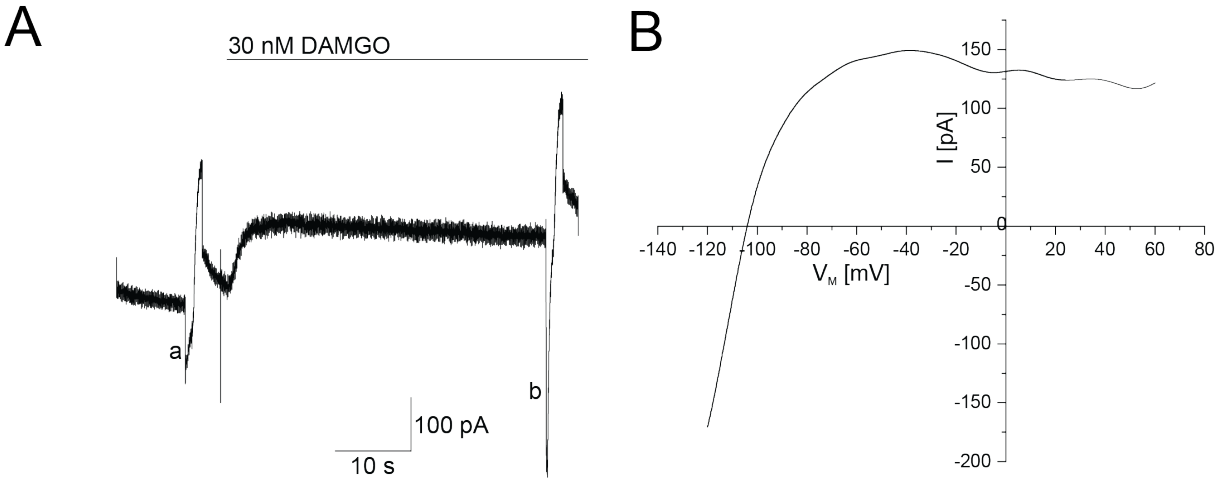


Fig. S2: The observed voltage sensitivity occurs also in physiological K^+ concentrations. ***A,*** Magnification of the excerpt, which was used for calculation of the background substracted I/V-curve (shown in ***B***). Voltage ramps which were used for calculation are marked as a and b. ***B***, Background subtracted I/V-curve (b-a, obtained from the voltage ramps a and b), as indicated in *fig. S2A***.**

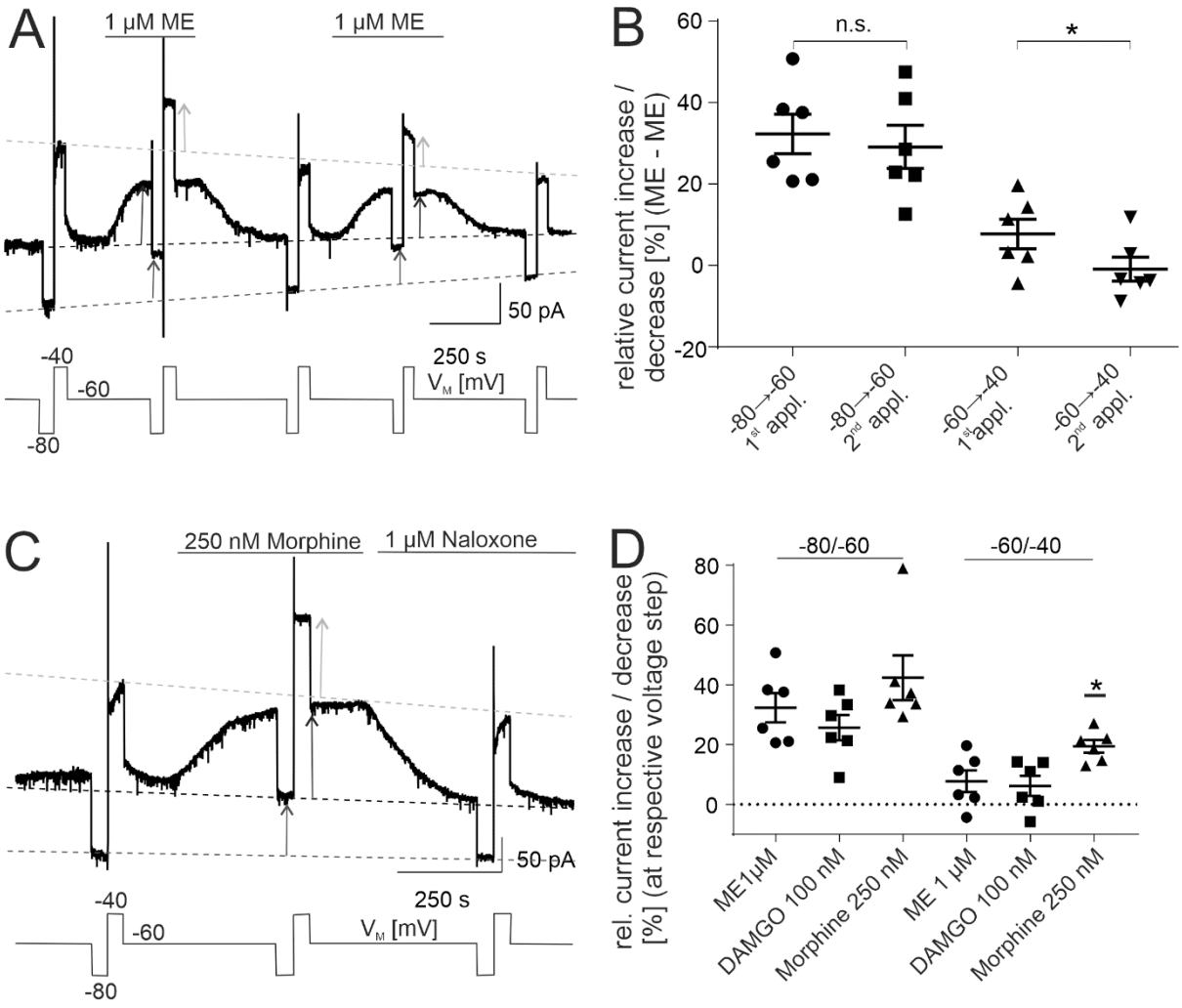


Figure S3: Comparison of voltage induced current responses in LC neurons. ***A,*** Representative recording of outward K^+ currents in LC neurons. GIRK currents were evoked by non-saturating concentration of Met-enkephalin (ME; 1 µM). During measurements, cells were kept at -60 mV and briefly clamped to -80 mV and -40 mV in turn (voltage protocol is depicted at the bottom, note that depolarization steps shift both basal and active currents due to the current-voltage relationship of the channel). Baseline current responses at each membrane potential (dashed lines) were determined by voltage-steps in control aCSF. The magnitude of respective agonist induced current responses are depicted as arrows (dark grey: -80 mV; black: -60 mV, light grey: -40 mV). ***B,*** Statistical analysis in voltage induced current change for 2 subsequent applications of Met-enkephalin (ME) shows that second applications of ME cause significantly smaller current responses (**p:* < 0.05; n = 6; paired t-test). ***C,*** Representative recording of morphine (250 nM) induced responses upon single ligand application in LC neurons. During measurements, cells were kept at -60 mV and briefly clamped to -80 mV and -40 mV in turn (voltage protocol is depicted at the bottom, note that depolarization steps shift both basal and active currents due to the current-voltage relationship of the channel). Baseline current responses at each membrane potential (dashed lines) were determined by voltage-steps in control aCSF. To accelerate wash out of morphine, naloxone (1 µM) was applied afterwards. The magnitude of respective agonist induced current response is depicted as arrows (dark grey: -80 mV; black: -60 mV, light grey: -40 mV). ***D*** Voltage-induced current changes during single agonist applications of Met-enkephalin (ME), DAMGO or morphine (as shown in fig. S3C, *: $p < 0.05$, ordinary one-way ANOVA with Tukey's multiple comparison test; n = 6 respectively) show that relative increase of currents in the -60 to -40 depolarization step are significantly higher during morphine application. Note, that due to the current voltage relationship a general increase in ligand-evoked currents can be seen upon depolarization, from which the increase seen upon morphine application differs statistically significantly.

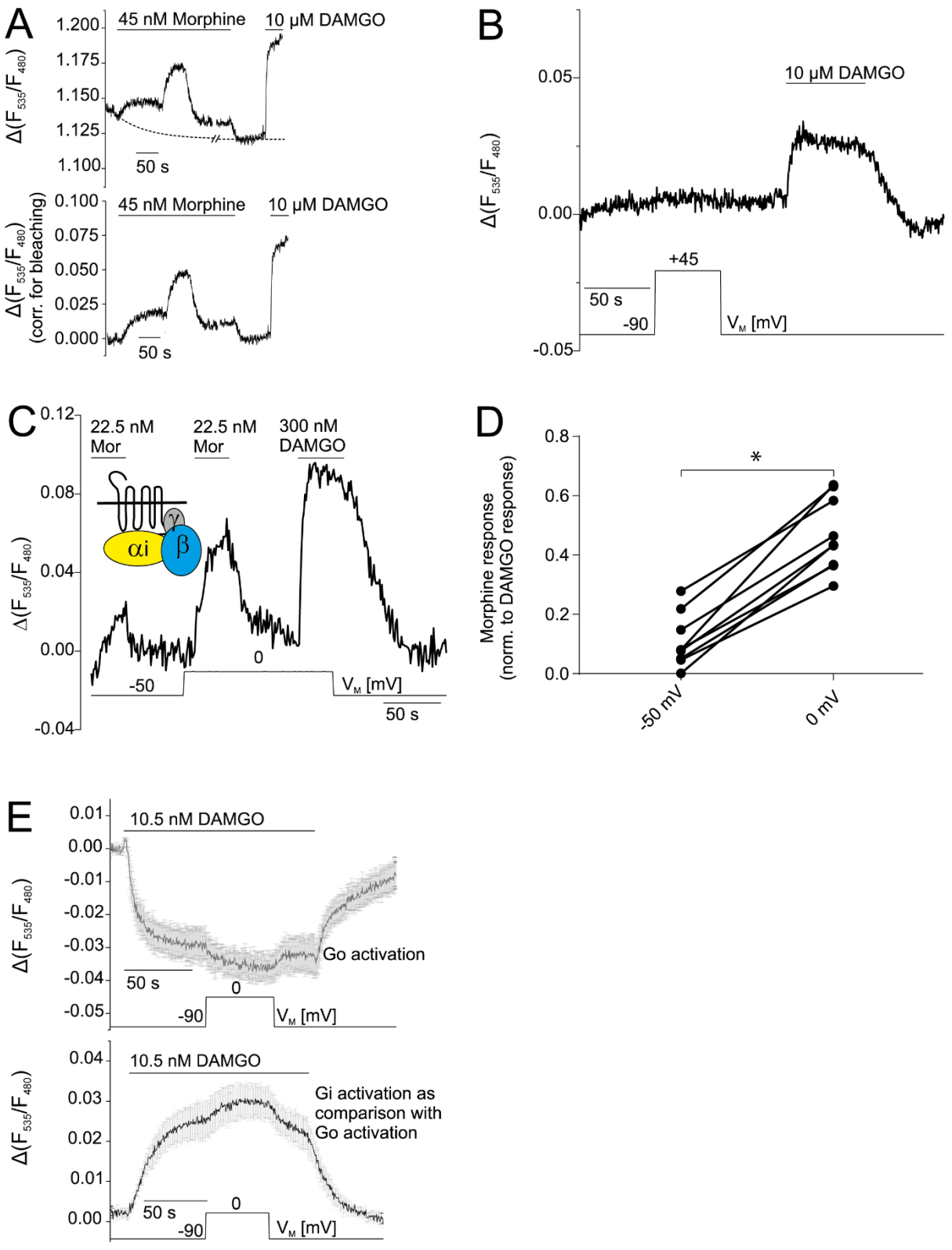


Figure S4: Bleach correction of FRET recordings, depolarization in the absence of agonist, Reproduction of the protocol from GIRK recordings in FRET recordings from G-protein activation, comparison of voltage induced changes in Go- and Gi-protein activation assay, *A,* Representative FRET trace of a cell that was transiently transfected with unlabeled MOP, Gαi-YFP, Gß$_1$-mTur2 and Gγ without correction for photobleaching (above) and after subtraction with a mono-exponential function (below). ***B,*** Representative cell (out of n = 5) that was transfected with unlabeled

MOP, Gαi-YFP, Gß$_1$-mTur2 and Gγ. A depolarization step from a holding potential of -90 mV to +45 mV was performed upon buffer application and for control reasons, a saturating concentration of agonist (McPherson et al., 2010) was applied subsequently. ***C,*** A representative FRET recording (out of n=9) from cells that were transfected with MOP-wt, Gα$_i$-YFP, Gß$_1$-mTur2, Gγ$_2$-wt is illustrated. Morphine- or DAMGO-mediated G-protein activation is shown by an increase in FRET (Bünemann et al., 2003) measured at -50 mV and 0 mV. Traces were corrected for photobleaching as indicated in *fig. S3A*. ***D,*** In analogy to GIRK current measurements (as conducted in Fig. 2), the same protocol was used to determine G-protein activation evoked by an non-saturating morphine concentration (22.5 nM) was normalized to the maximum response (300 nM DAMGO) at respective membrane potentials (n=9) and the relative voltage-dependent effect induced by morphine was compared at -50 mV and 0 mV (*$p <$ 0.05 between responses at -50mV and 0mV; paired t-test, as responses were compared within the same cell). ***E,*** Averaged (mean ± S.E.M., n = 9 for Gi, n = 3 for Go) preliminary traces of agonist/voltage evoked alterations in FRET are shown and compared for cells expressing the Go –FRET sensor (upper panel) or the Gi-protein-FRET sensor (Bünemann et al., 2003; Frank et al., 2005). ***F,***

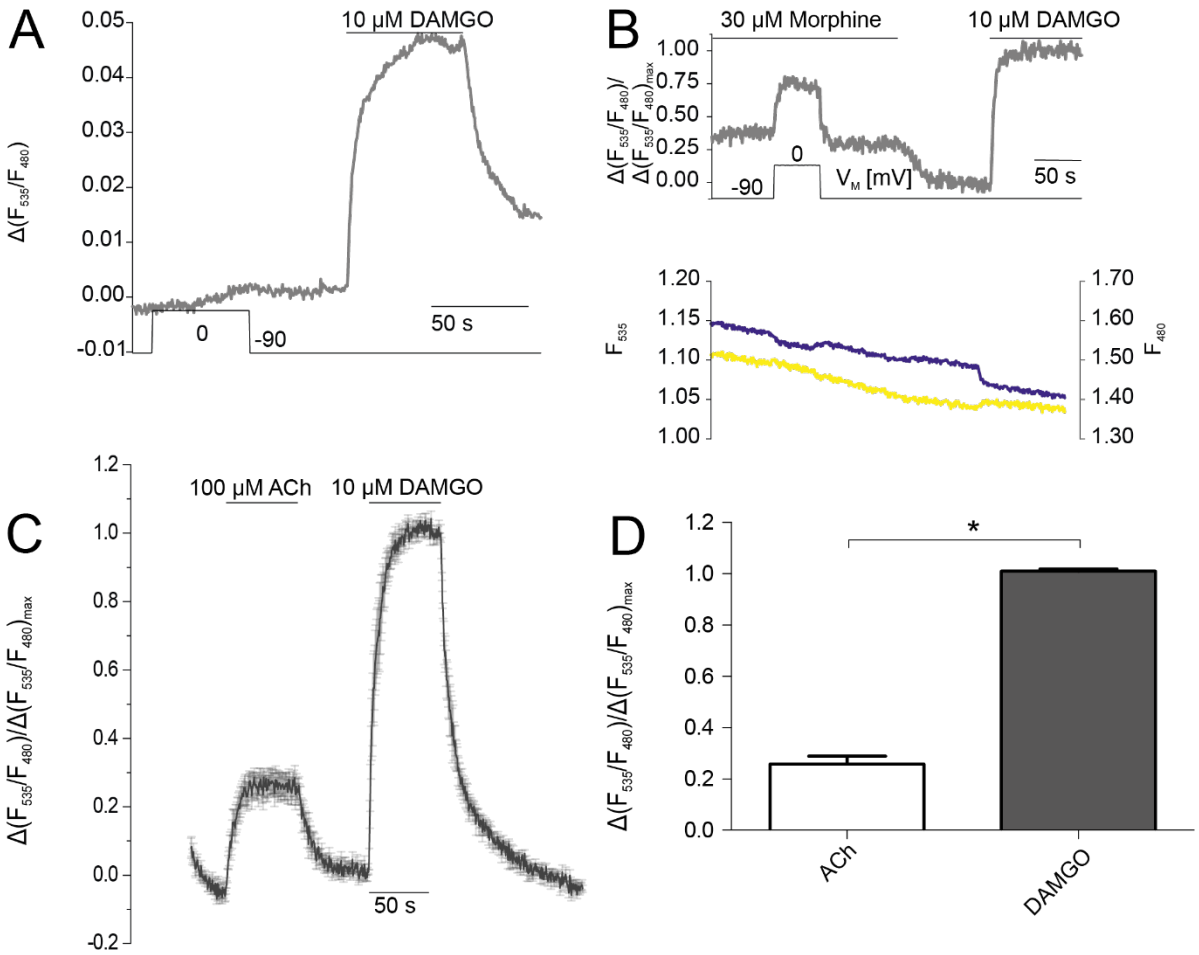
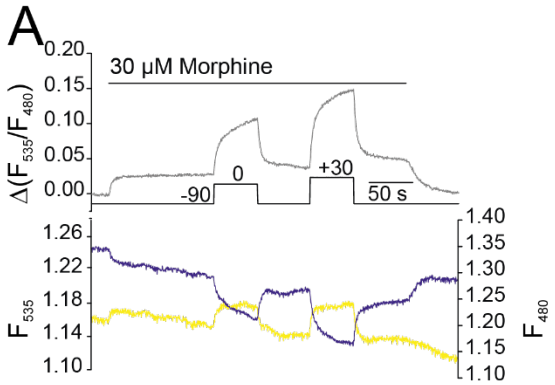
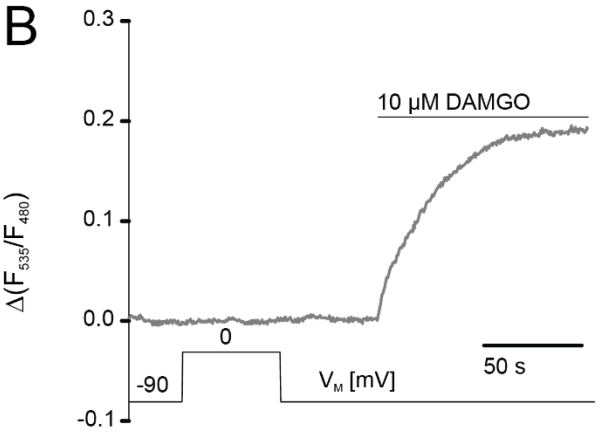
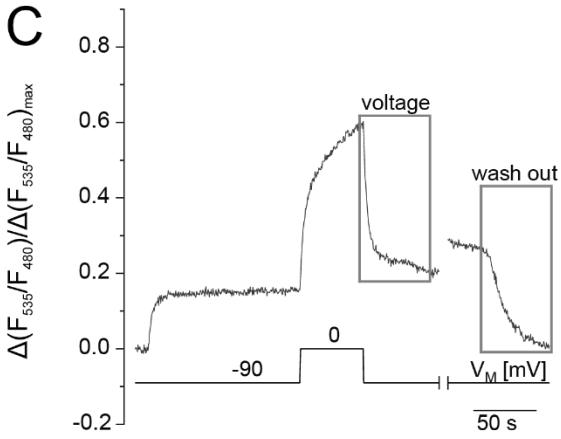
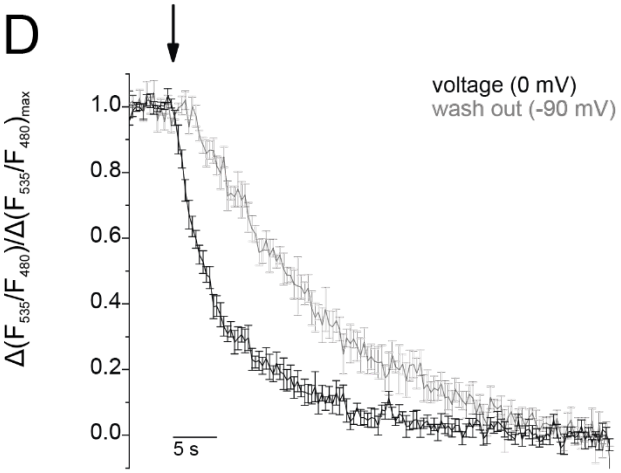


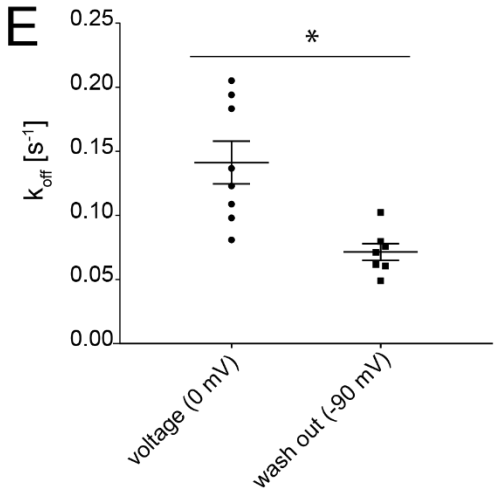
Figure S5: Depolarization in the absence of agonist, single emission traces and Bystander FRET experiments in a GRK2-recruitment assay. ***A***, Representative FRET (out of n = 6) recording from cells that were transfected with sYFP2-labelled MOP and mTur2-labelled GRK2, as shown previously. Depolarization in the absence of agonist has no effect on MOP – GRK2 interactions. ***B***, Representative FRET recording plotted as relative agonist evoked alterations in the sYFP2/mTur2 emission ratio and corresponding recordings of CFP (F_{480}) and YFP (F_{535}) emission. ***C***, Bystander FRET between MOP-sYFP2 and GRK2-mTur2 evoked by 100 µM ACh at non-labelled muscarinic M_3R leads to 26 % ± 3 % (mean ± S.E.M.) non-specific FRET (compared to a maximum DAMGO response (n = 9), ***D***, Statistical analysis of FRET amplitudes following non-specific, ACh-evoked response and DAMGO-induced response. To compare responses within the same cell, a paired t-test was used (*: $p < 0.05$).

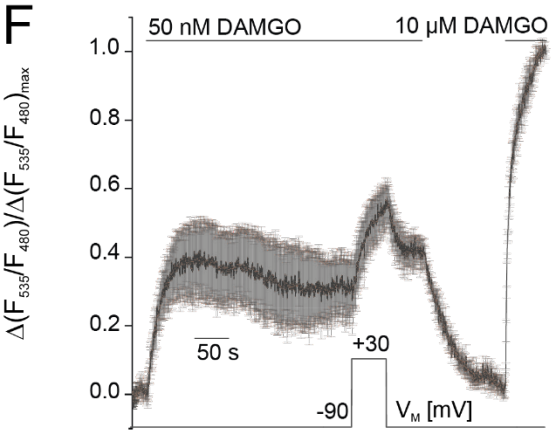

A
30 µM Morphine
0.20
0.15
0.10
0.05
0.00
Δ(F535/F480)
0
+30
-90
50 s
1.26
1.22
1.18
1.14
1.10
F535
1.40
1.35
1.30
1.25
1.20
1.15
1.10
F480


B
0.3
0.2
0.1
0.0
-0.1
Δ(F535/F480)
10 µM DAMGO
0
-90
VM [mV]
50 s


C
0.8
0.6
0.4
0.2
0.0
-0.2
Δ(F535/F480)/Δ(F535/F480)max
voltage
wash out
0
-90
VM [mV]
50 s


D
1.0
0.8
0.6
0.4
0.2
0.0
Δ(F535/F480)/Δ(F535/F480)max
voltage (0 mV)
wash out (-90 mV)
5 s


E
0.25
0.20
0.15
0.10
0.05
0.00
koff [s-1]
*
voltage (0 mV)
wash out (-90 mV)


F
1.0
0.8
0.6
0.4
0.2
0.0
Δ(F535/F480)/Δ(F535/F480)max
50 nM DAMGO
10 µM DAMGO
50 s
+30
-90
VM [mV]

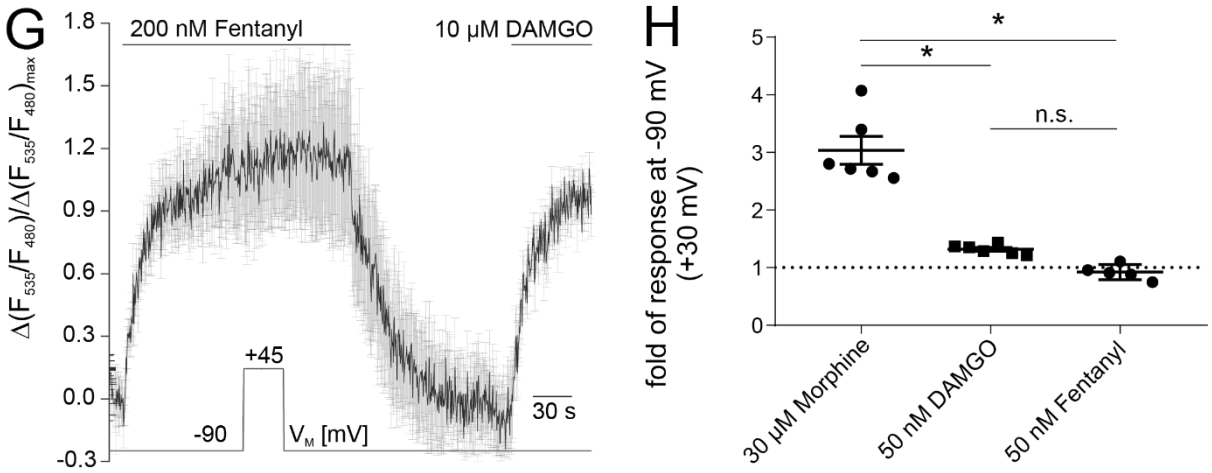


Figure S6: Single emission traces and depolarization in the absence of agonists, kinetics of voltage-induced changes, characterization of voltage induced effects on DAMGO- or fentanyl-mediated Arrestin3 recruitment at equieffective concentrations to morphine. ***A***, Representative FRET recording plotted as relative agonist evoked alterations in the sYFP2/mTur2 emission ratio and corresponding recordings of CFP (F_{480}) and YFP (F_{535}) emission. ***B***, Voltage does not influence MOP – Arrestin3 interactions in the absence of agonist (representative FRET trace, n = 7). ***C***, Exemplary trace (of n = 7) indicates which excerpts were used for determination of kinetic analysis (see fig. S6D, E) of depolarization-induced or wash-out induced termination of MOP-Arrestin interaction. ***D***, Average (mean ± S.E.M.) recordings showing termination of the MOP-Arrestin3-interaction upon wash-out (light grey trace, n = 7) of 30 µM morphine at holding potential (-90 mV) or following repolarization (black trace, n = 8) from 0 mV to holding potential (-90 mV) in continued application of 30 µM morphine. The arrow indicates the time point of agonist withdrawal or repolarization respectively. ***E***, Comparison and statistical analysis of k_{off}-values (for data see *C*). Statistical significance was determined by a paired t-test as responses were compared within same cells (*: $p < 0.05$; n = 7). ***F,*** Average (mean ± S.E.M.; n = 6) of MOP-Arrestin interaction upon application of 50 nM DAMGO (sub-saturating concentration). Cells were depolarized from -90 mV to +30 mV. ***G***; Average (mean ± S.E.M.; n = 4) of MOP-Arrestin interaction upon application of 200 nM fentanyl, saturating concentration. Cells were depolarized from -90 mV to +45 mV.***H,*** Statistical analysis of changes in MOP-Arrestin3 interaction upon depolarization from -90 mV to +30 mV under application of a saturating concentration morphine or a non-saturating concentration of DAMGO or fentanyl (Data shown in Fig. 6E). To confirm statistically significant differences in voltage dependent response for morphine, DAMGO and fentanyl an ordinary one-way ANOVA with Tukey's multiple comparison test was used (*: $p < 0.05$; n.s.: $p > 0.05$; n = 5 - 6).