Supplementary Material

Detailed *in vitro* pharmacological characterization of clinically tested negative allosteric modulators of the metabotropic glutamate receptor 5 ($mGlu_5$)

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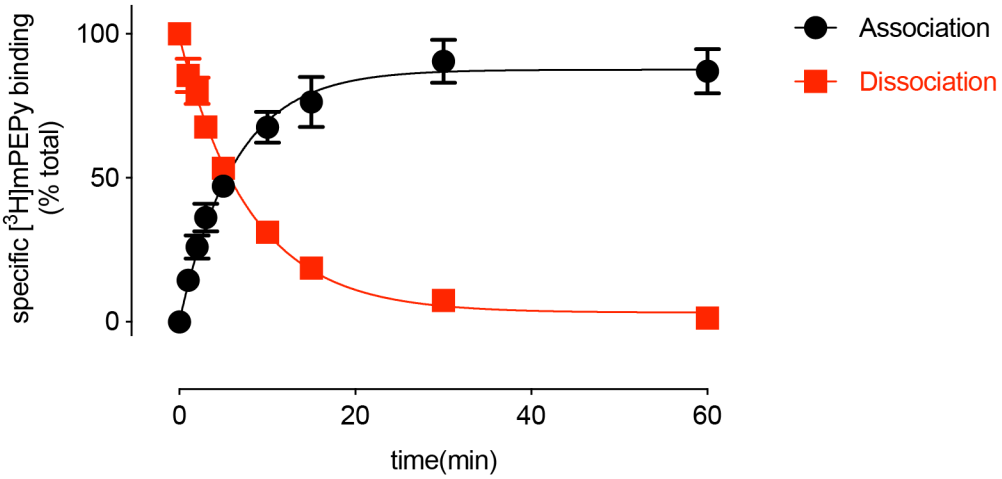
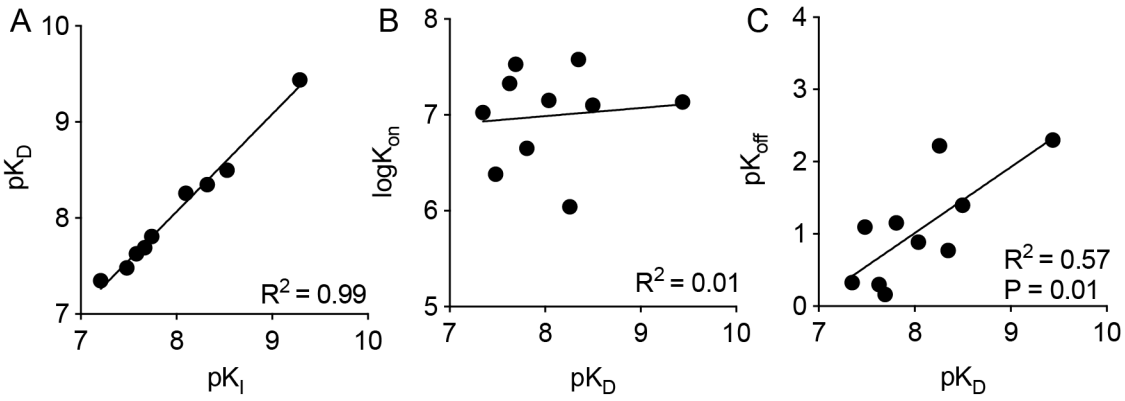
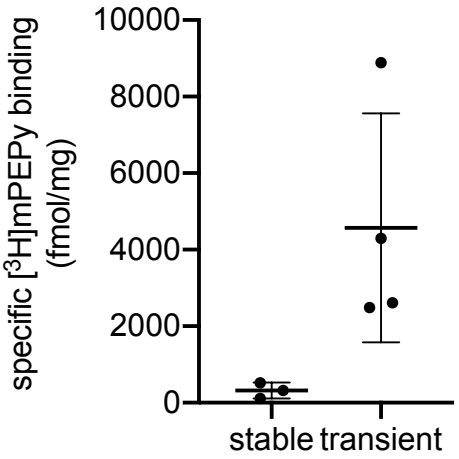


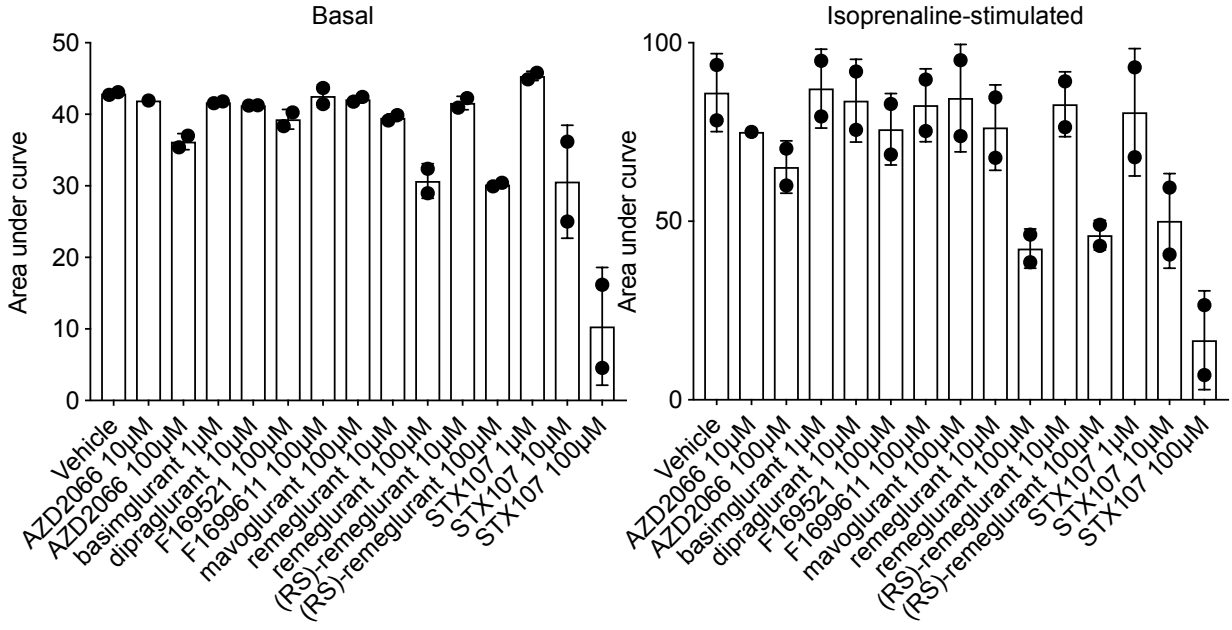
Supplementary Figure 1. Association and dissociation kinetics of [^{3}H]methoxy-PEPy binding in HEK293A-mGlu$_5$-low cell membranes. The dissociation rate was determined by simultaneous addition of a saturating concentration of unlabeled competitive ligand (1 μM MPEP). Data are represented as mean ± SEM of 7 independent experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.



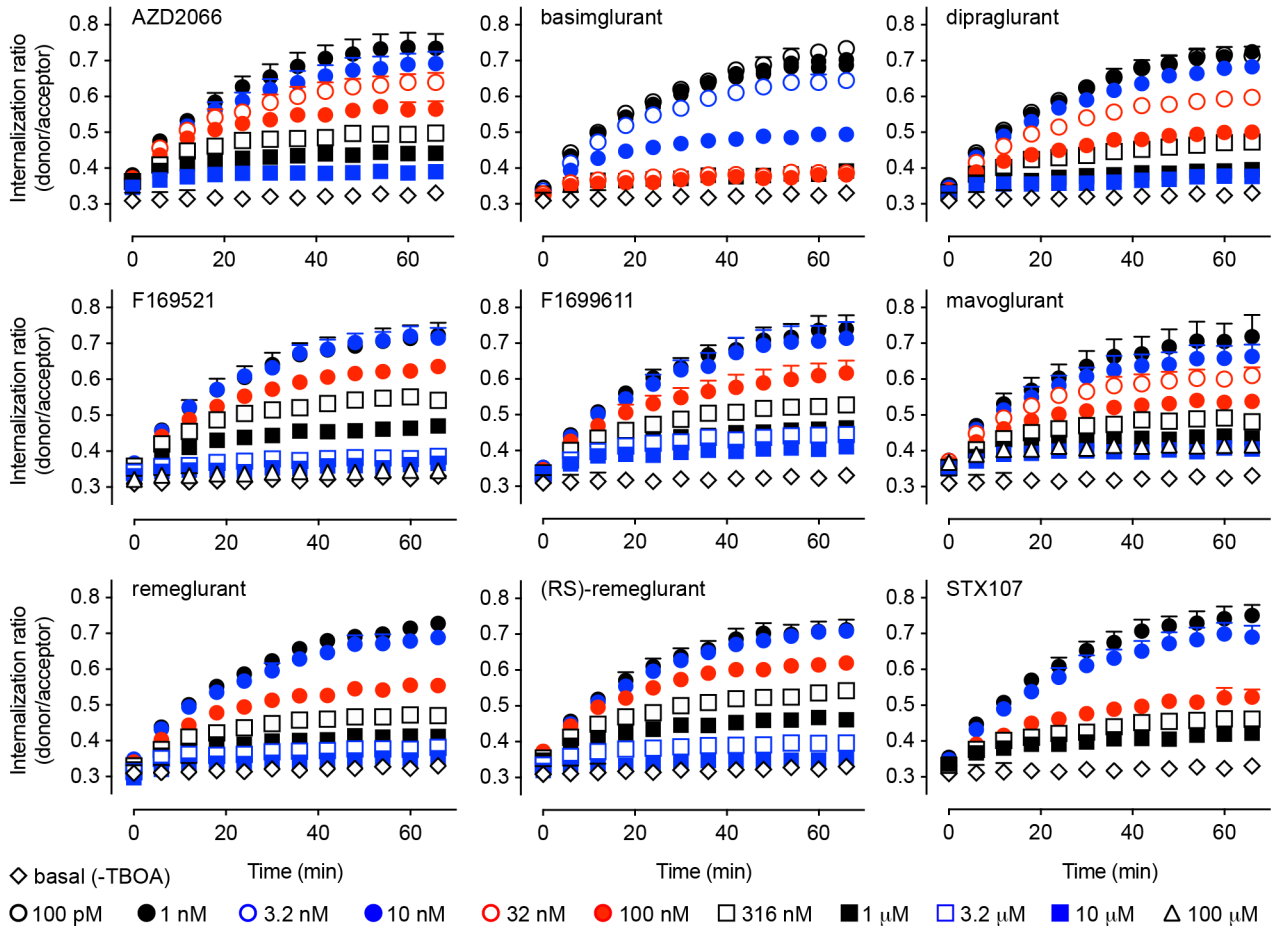
Supplementary Figure 2. Correlation plots of affinity, association rate, and dissociation rate of $mGlu_5$ NAMs. A) Affinities calculated from association (k_{on}) and dissociation (k_{off}) rates ($K_D = k_{off}/k_{on}$), where pK_D is the negative logarithm of K_D, versus pK_I estimates from inhibition binding experiments. **B**) Association rates do not correlate with pK_D. **C**) Dissociation rates correlate with pK_D.



Supplementary Figure 3. B_{max} estimates for [^{3}H]mPEPy binding to $mGlu_5$ in membranes from stable and transient transfected HEK293A cells. To estimate total receptor levels (B_{max}), two-point saturation binding experiments were performed using 2.6 ± 1.1 nM and 16.6 ± 3.6 nM [^{3}H]mPEPy and 10 μM MPEP to define non-specific binding. A saturation binding isotherm was fitted to the data to estimate B_{max}, with the assumption that the N-terminal SNAP-tag had no effect on the affinity of the allosteric radioligand ([^{3}H]mPEPy, reported in Table 1), which binds to the 7TM domain. Independent replicates for n=3-4 are shown, data are mean ± SD.



Supplementary Figure 4. High concentrations of select $mGlu_5$ NAMs interfere with FRET-based assay detection. The basal and agonist (isoprenaline)-stimulated internalization of SNAP-tagged β_2-adrenoceptor measured in presence of high concentrations of $mGlu_5$ NAMs or vehicle (1% DMSO). Area under the curve was calculated for real time internalization ratios (ratio of donor and acceptor emissions) measured for 66 min in 6 min intervals. Data points represent the mean of individual experiments and bars represent mean ± SEM from two independent experiments performed in triplicate.



Supplementary Figure 5. Real-time $mGlu_5$ internalization in response to 11.6 μM of L-glutamate is inhibited by $mGlu_5$ NAMs. Indicated NAMs were pre-incubated for 30 min prior to addition of L-glutamate (11.6 μM), fluorescein and DL-TBOA (to inhibit transport of glutamate). The ratio of donor (Lumi4-Tb-labeled SNAP-tagged $mGlu_5$) and acceptor (fluorescein) emissions (internalization ratio) increases over time when the receptor is internalized. Data are mean + SEM from 3 independent experiments performed in triplicate.

Supplementary Table 1: L-glutamate potency for $mGlu_5$ in four functional assays. Data are represented as mean ± SEM from the indicated number (n) of independent experiments performed in triplicate.

	pEC_{50}	n
Ca^{2+} mobilization	6.53±0.13	4
IP_1 accumulation	5.40±0.05	5
ERK1/2 phosphorylation	6.00±0.05	4
Receptor internalization	5.47±0.08 5.80±0.19[#]	3

[#] For receptor internalization data the concentration-response curve was re-fitted such that the bottom plateau was equal to the true basal level of the system, i.e. in the absence of DL-TBOA. In order to do so, the data points below the inflection point of the original curve were removed (100 nM and 1 μM).

Supplementary Table 2: Potency (pIC_{50}) values from functional assays for $mGlu_5$ NAMs, based on titrations in the presence of sub-maximal L-glutamate. Data was fitted to a four-parameter model of inhibition. Values are mean ± SEM from the indicated number (n) of independent experiments performed in triplicate.

	Ca^{2+} mobilization		**IP_1 accumulation**		**ERK1/2 phosphorylation**		**Receptor internalization**	
	pIC_{50}	**n**	**pIC_{50}**	**n**	**pIC_{50}**	**n**	**pIC_{50}**	**n**
AZD2066	7.29 ± 0.33	4	8.52 ± 0.04	4	7.88 ± 0.09	3	6.85 ± 0.03	3
basimglurant	9.13 ± 0.16	4	9.42 ± 0.07	4	8.99 ± 0.06	4	8.12 ± 0.04	3
dipraglurant	6.44 ± 0.20	4	7.37 ± 0.04	4	6.93 ± 0.12	3	7.14 ± 0.02	3
F169521	6.69 ± 0.12	4	7.07 ± 0.06	4	6.83 ± 0.03	3	6.34 ± 0.06	3
F1699611	7.16 ± 0.31	4	7.71 ± 0.10	4	7.21 ± 0.11	3	6.77 ± 0.10	3
mavoglurant	7.22 ± 0.27	4	8.43 ± 0.07	4	7.62 ± 0.05	3	7.09 ± 0.12	3
remeglurant	6.96 ± 0.29	4	7.92 ± 0.13	4	7.38 ± 0.14	3	6.78 ± 0.05	3
(RS)-remeglurant	6.63 ± 0.26	4	7.20 ± 0.04	4	7.00 ± 0.10	3	6.22 ± 0.05	3
STX107	7.81 ± 0.25	4	8.44 ± 0.08	4	8.00 ± 0.04	3	7.29 ± 0.09	3