Supporting Information

SI Results

Pharmacodynamic Properties of ADX88178, ADX104608 and ADX104583. Positive allosteric modulators (PAMs) are receptor ligands that bind to a site topographically distinct from that bound by the orthosteric agonist and enhance the potency and/or efficacy of the orthosteric agonist. Pharmacodynamic properties of PAMs are typically tested *in vitro* by evaluating their potentiating effect on a low orthosteric agonist concentration such as EC_{20} (i.e., effective concentration causing 20% of the maximal effect). ADX88178 is an already proven selective PAM of metabotropic glutamate receptor 4 (mGluR4) (**Fig. 1**)(1), whereas ADX104608 and ADX104583 are novel constrained, structurally-related analogues of ADX88178 (structures not revealed for intellectual property protection). To test the potency of ADX88178, ADX104608, and ADX104583, we determined their effects at increasing concentrations on the response to Glu EC_{20} (6 μM) in HEK293 cells stably expressing the human or rat mGlu4R-encoding gene. Co-transfection with the gene coding for Gα16, a protein that can redirect the signalling of non-Gq-coupled receptors to the phospholipase C-mediated calcium pathway, was used to detect mGluR4 activation by fluorescent cell based-Ca^{2+} mobilization assay (as described in **SI Materials and Methods**). In HEK293 cells expressing either human or mGluR4, the addition of ADX88178 or ADX104608 at concentrations in the range of 1-50 nm greatly potentiated the effect of Glu. Specifically, in cells expressing human mGluR4, ADX88178 and ADX104608 increased the effect of Glu EC_{20} up to 92% and 102% of the maximal Glu response, respectively, showing a half-maximal effective concentration (EC_{50}) of 3.5 ± 0.3 nM and 39 ± 7.8 nM (mean ± SD), respectively. Similarly, in rat $mGluR4^{+}$ cells, ADX88178 and ADX104608 potentiated the response to Glu EC_{20} up to 119% and 121% of the maximal Glu response with an EC_{50} value of 9.0 ± 3.1 nM and 30 ± 7.2 nM, respectively. In contrast, ADX104583 did not display any modification in Ca^{2+} mobilization in either human or rat mGluR4-expressing cells and was thus used as negative control in subsequent studies. Since neither ADX88178 (2) nor ADX104608 (unpublished data) modulated Glu responses in HEK2093 cells expressing group I, group II or other group III mGluRs (mGluR6, mGluR7, and mGluR8), these data indicate that ADX104608 and more so ADX88178 are potent and selective mGluR4 PAMs.

SI Materials and Methods

Generation of stable cell lines expressing human or rat mGluR4. The cDNA encoding human or rat mGluR4 (hmGluR4 and rmGluR4, respectively) were subcloned into an expression vector

containing also the hygromycin resistance gene. In parallel, the cDNA encoding a Gα16 protein allowing redirection of the activation signal to intracellular calcium flux was subcloned into a different expression vector containing the puromycin resistance gene. Transfection of both vectors was performed into HEK293 cells with the PolyFect reagent (Qiagen), according to the supplier's protocol, and hygromycin and puromycin were used to select antibiotic resistant cells which had integrated stably both plasmids. Positive cellular clones, i.e., expressing mGluR4, were identified in a functional assay measuring changes in calcium fluxes in response to the EC_{20} of Glu, the mGluR4 orthosteric agonist, in the presence or absence of different concentrations of PAMs. HEK293 cells expressing human or rat mGluR4 and Gα16 were maintained in media containing DMEM, dialyzed fetal calf serum (10 %), GlutamaxTM (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), geneticin (100 µg/ml), hygromycin-B (40 µg/ml) and puromycin (1 µg/ml), at 37°C with 5% CO_2 in a humidified atmosphere.

Fluorescent cell based-Ca^{2+} mobilization assay. Assays were performed in a pH 7.4 buffered-solution containing 20 mM HEPES, 143 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1 mM $CaCl_2$, 0.125 mM sulfinpyrazone, and 0.1 % glucose. Twenty four hours prior to the experiment, human and rat mGluR4-transfected HEK293 cells were plated out at a density of 2.5 x 10^4 cells/well in black-well/clear-bottomed and poly-L-ornithine-coated 384-well plates in a glutamine/glutamate free DMEM medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. On the day of the assay, cells were loaded with a 3 µM solution of Fluo4-AM (LuBioScience) in an assay buffer containing 0.03% pluronic acid. After 1 h at 37°C with 5% CO_2 in a humidified atmosphere, the extracellular dye was removed by washing the cell plate with the assay buffer and calcium flux was measured using a FLIPR (Molecular Devices). After 10 sec of basal fluorescence recording, various concentrations of the compounds to be tested were added to the cells. Changes in fluorescence levels were first monitored for 180 sec in order to detect any agonist activity of the compounds. Then, cells were stimulated by Glu EC_{20} for an additional 110 sec in order to measure enhancement activity of the compounds.

SI References

1. Kalinichev M*, et al.* (2014) Characterization of the Novel Positive Allosteric Modulator of the Metabotropic Glutamate Receptor 4 ADX88178 in Rodent Models of Neuropsychiatric Disorders. *The Journal of pharmacology and experimental therapeutics* 350(3):495-505.
2. Le Poul E*, et al.* (2012) A potent and selective metabotropic glutamate receptor 4 positive allosteric modulator improves movement in rodent models of Parkinson's disease. *The Journal of pharmacology and experimental therapeutics* 343(1):167-177.

Supplementary Table S1. Plasma Levels of ADX88178 in mice with Relapsing-Remitting Experimental Autoimmune Encephalomyelitis (RR-EAE; doses of 10 and 60 mg/kg) and naive SJL mice (dose of 30 mg/kg).

Dose 10 mg/kg						
	Day 1		Day 7		Day 14	
Time (h)	mouse #	conc. (ng/ml)	mouse #	conc. (ng/ml)	mouse #	conc. (ng/ml)
0	m1	BLQ	m1	BLQ	m1	4.20
	m2	BLQ	m2	2.40	m2	9.60
1	m3	188	m3	17.4	m3	168
	m4	188	m4	39.6	m4	144
6	m1	7.83	m1	156	m1	21.9
	m2	51.7	m2	38.3	m2	46.9
24	m3	BLQ	m3	BLQ	m3	BLQ
	m4	BLQ	m4	BLQ	m4	5.90
Dose 60 mg/kg						
	Day 1		Day 7		Day 14	
Time (h)	mouse #	conc (ng/ml)	mouse #	conc (ng/ml)	mouse #	conc (ng/ml)
0	m5	BLQ	m5	110	m5	77.0
	m6	BLQ				
1	m7	251	m7	203	m7	330
	m8	782	m8	270	m8	303
6	m5	122	m5	188	m5	326
	m6	115				
24	m7	36.9	m7	64.3	m7	102
	m8	30.5	m8	20.9	m8	77

Time (h)	**Dose (mg/kg)**	Terminal sampling	
		mouse #	conc (ng/ml)
1	10	m1	65.7
		m2	48.0
		m3	72.0
		m4	81.1
1	60	m5	200
		m7	235
		m8	299

Dose 30 mg/kg	Day 1	
Time (h)	mouse #	conc (ng/mL)
1	m1	230
	m2	481
	m3	550
6	m4	123
	m5	87,1
	m6	25,8
24	m4	15,2
	m5	12,5
	m6	7,86

Table S2. Statistical Analysis of RR-EAE experiments of PHCCC- and ADX88178- *vs.* vehicle-treated groups.

ENTIRE PERIOD:

	Significance		
Comparison	*min-P*[a]	*global-P*[b]	*adjusted-P*[c]
PHCCC vs. *vehicle*	0.0306 *	0.1763	0.3526
ADX88178 (10 mg/kg) vs. *vehicle*	0.1405	0.4513	0.4513
ADX88178 (30 mg/kg) vs. *vehicle*	0.0149 *	0.0853	0.2559
ADX88178 (60 mg/kg) vs. *vehicle*	0.0008 ***	0.0095 **	0.0380 *

ON TREATMENT:

	Significance		
Comparison	*min-P*	*global-P*	*adjusted-P*
PHCCC vs. *vehicle*	0.0306 *	0.1459	0.2918
ADX88178 (10 mg/kg) vs. *vehicle*	0.1405	0.3883	0.3883
ADX88178 (30 mg/kg) vs. *vehicle*	0.0149 *	0.0646	0.1938
ADX88178 (60 mg/kg) vs. *vehicle*	0.0008 ***	0.0058 **	0.0232 *

POST TREATMENT:

	Significance		
Comparison	*min-P*	*global-P*	*adjusted-P*
PHCCC vs. *vehicle*	0.2768	0.4833	0.9666
ADX88178 (10 mg/kg) vs. *vehicle*	0.3539	0.5411	0.9666
ADX88178 (30 mg/kg) vs. *vehicle*	0.0292 *	0.0868	0.2764
ADX88178 (60 mg/kg) vs. *vehicle*	0.02132 *	0.0691	0.2764

[a]minimum *P*-value obtained from Wilcoxon-Mann-Whitney tests

[b]*P*-value resulting from the Westfall & Young maxT method

[c]multiplicity-adjusted *P*-value obtained by the Bonferroni-Holm method

Table S3. Statistical Analysis of RR-EAE experiments of PHCCC- *vs*. ADX88178-treated groups.

ENTIRE PERIOD:

	Significance		
Comparison	*min-P*[a]	*global-P*[b]	*adjusted-P*[c]
ADX88178 (10 mg/kg) vs. *PHCCC*	0.3646	0.7967	0.7967
ADX88178 (30 mg/kg) vs. *PHCCC*	0.0437 *	0.2173	0.4346
ADX88178 (60 mg/kg) vs. *PHCCC*	0.0106 *	0.0961	0.2883

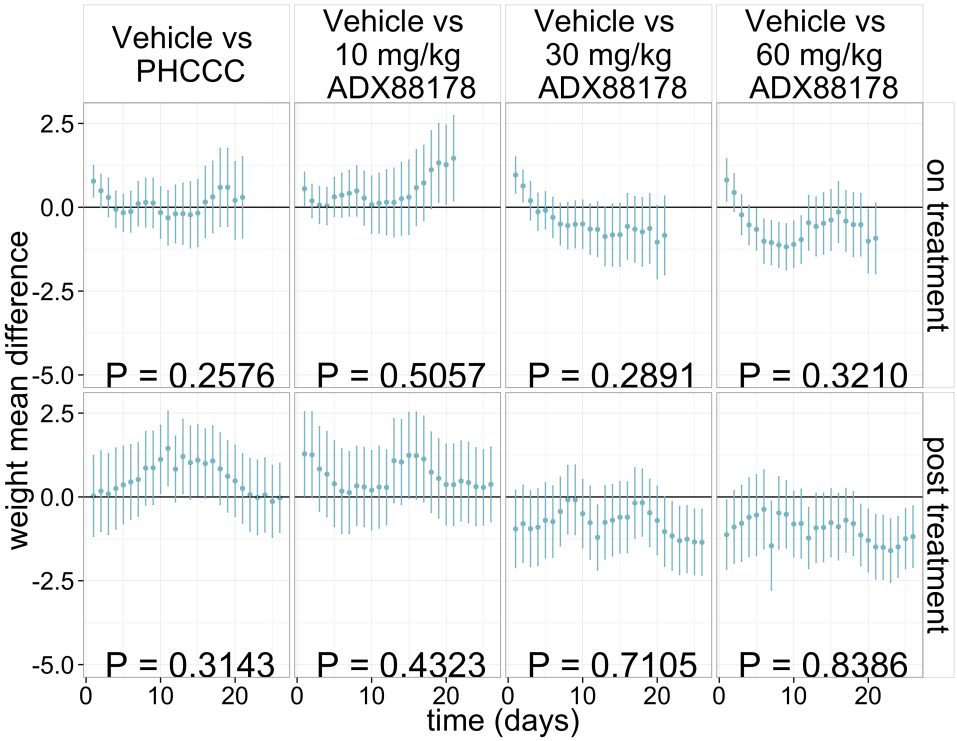
ON TREATMENT:

	Significance		
Comparison	*min-P*	*global-P*	*adjusted-P*
ADX88178 (10 mg/kg) vs. *PHCCC*	0.5492	0.8606	0.8606
ADX88178 (30 mg/kg) vs. *PHCCC*	0.0468 *	0.1972	0.3944
ADX88178 (60 mg/kg) vs. *PHCCC*	0.0106 *	0.0730	0.2190

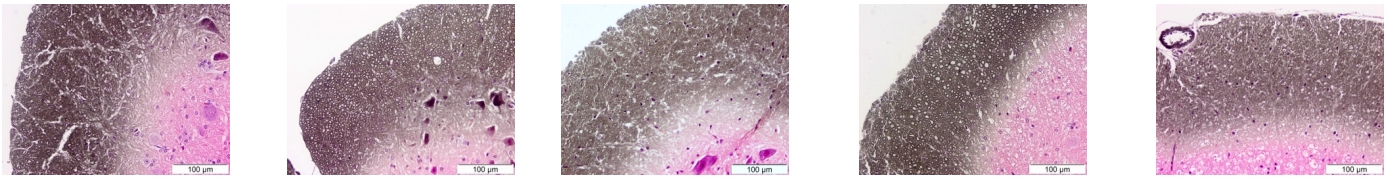
POST TREATMENT:

	Significance		
Comparison	*min-P*	*global-P*	*adjusted-P*
ADX88178 (10 mg/kg) vs. *PHCCC*	0.3646	0.5793	0.5793
ADX88178 (30 mg/kg) vs. *PHCCC*	0.0437 *	0.1219	0.3657
ADX88178 (60 mg/kg) vs. *PHCCC*	0.0360 *	0.1246	0.3657

[a]minimum *P*-value obtained from Wilcoxon-Mann-Whitney tests
[b]*P*-value resulting from the Westfall & Young maxT method
[c]multiplicity-adjusted *P*-value obtained by the Bonferroni-Holm method



Supplementary Fig. S1. Body weight mean differences during (on treatment; day 0 corresponding to day 10 post-PLP sensitization) or after drug treatment (post-treatment; day 0 corresponding to day 31 post-PLP sensitization). Indicated *P*-values represent the global *P*-values for each group in each time period.



Supplementary Fig. S2. Histopathological analysis of spinal cord sections of one representative mouse from each group (indicated) at 60 d after immunization. Spinal cord sections were immunostained for myelin-binding protein (MBP) to evaluate myelin depletion and stained with H&E to assess inflammation.