Metabotropic glutamate type 5 receptor binding availability during *d*-amphetamine sensitization in mice and humans

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SUPPLEMENTAL MATERIALS AND METHODS

MOUSE STUDIES
Subjects
A total of 51 wild-type (WT) mice were used in behavior and autoradiography. Mice were housed in groups of two to four animals per cage under standard conditions: 22±2 °C and a 12 h light/dark cycle (7:00-19:00 light period) with food and water provided *ad libitum*. All precautions were taken to minimize the number of animals used and their suffering. Animals were randomly allocated to experimental groups.

Mouse locomotor activity and sensitization procedures.
Locomotor response was measured in an Omnitech Digiscan activity monitor. Animals were placed in Plexiglas open-field chambers with photocells on bottom and side walls. Horizontal activity was measured in 5-minute bins. Following two consecutive days of habituation sessions in activity chambers, animals were assigned to receive either amphetamine or saline. On experimental days immediately consecutive to habituation, animals were placed in the boxes for habituation for two hours, followed by injection of saline or *d*-amphetamine. Activity was measured for 90 minutes following treatment and quantified as total distance travelled in 90 minutes.

Autoradiography procedures.
Brains from mice used for sensitization experiments were dissected frozen and sections (12μm) were taken on a Cryostat (Leica CM3050S), slide mounted and stored at -80°C. Autoradiography was performed as previously described [1]. Slide-mounted sections were pre-incubated in N_2 Hepes buffer (30mmol/L, pH 7.4 containing 110mmol/L NaCl, 5mmol/L KCl, 2.5mmol/L $CaCl_2$, and 1.2mmol/L $MgCl_2$) for 20 minutes at room temperature. Next, sections were incubated in buffer containing 2nmol/L [^{3}H]ABP688 (American Radiolabeled Chemicals, Saint Louis, MO, USA) for 90 minutes (room temperature). To assess nonspecific binding, slides were incubated in medium with 10μmol/L MPEP. Sections were rinsed rapidly in incubation buffer (4 rinses of 5 minutes each, 4°C) and dried at room temperature overnight.

Labeled sections were exposed on BAS-TR Fuji Imaging screens (Fuji Film Photo, Tokyo, Japan) for 6 days. Screens were scanned with a Fuji Bioimaging Analyzer BAS-5000 (Fuji Film Photo). Digitized images were analyzed with MCID software (Imaging Research, St. Catharine's, Canada). Receptor density was quantified relative to radioactive standards in dorsal striatum, nucleus accumbens, and prelimbic cortex. Results were expressed as the mean ± S.E.M. of [^{3}H]ABP688 density in ng receptor per fmol tissue.

Immunofluorescence procedures

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Coronal sections of fresh frozen mouse brains (WT or mGlu5-KO mice) were taken at the level of the striatum with a Cryostat (Leica CM3050S) and mounted on glass slides. Slide-mounted sections were postfixed in -20°C methanol for 6 minutes then washed 3 times in phosphate buffered saline (PBS) and air dried. Sections were pre-incubated for 45 minutes at room temperature in PBS with gelatin (2gL) and Triton-X100 (0.25%), then incubated with primary antibodies overnight at 4°C. Slides were then washed in PBS-gelatin-Triton (3 x 15 minutes) and incubated with secondary antibodies for two hours at room temperature. In all experiments, primary antibodies were detected with secondary anti-mouse IgG coupled to Alexa Fluor 488, anti-guinea pig IgG coupled to Alexa Fluor 555, and anti-rabbit IgG coupled to Alexa Fluor 647, all 1:2000. Slides were then washed in PBS (1x10'), rinsed in distilled water, and mounted with Fluoromount.

To visualize DRD1R and mGlu5, *DrD1-tomato transgenic m*ice were anesthetized and intracardially perfused with phosphate-buffered saline containing paraformaldehyde (4%). Brains were collected, post-fixed in 4% PFA and cryoprotected in phosphate-buffered saline containing 10% sucrose. Coronal sections (20μm) at the level of striatum were taken using a cryostat and immunofluorescence was performed as above described.

Acquisition and analysis

Images were acquired using a Zeiss Axio Observer.Z1 inverted fluorescence microscope with Axiocam MRm camera and Apotome.2 attachment (Carl Zeiss, Canada). Triple-label and DRD1tomato mouse images were acquired on a laser scanning confocal microscope, Zeiss LSM 880 with airyscan detector using a 63x, 1.4 NA oil immersion objective with pixel size 42nm and z-step of 180nm. Airyscan images were automatically processed using Zen software (Blue Edition v2.3, Carl Zeiss, 2011).

Colocalization and distance analysis were performed on 4 sites from 3 animals: two NAc images taken from one animal, 1 NAc image taken from a second animal, and one dorsal striatum image taken from a third animal. Fluorescent labeling was rendered as surfaces using Imaris software version 8.0.2 (Bitplane AG, Zurich, Switzerland). Colocalization analysis was performed in Imaris using automatically-determined thresholds [2], manually inspected and corrected where necessary.

Percent colocalization was calculated between mGlu5 and PSD95 or VGLUT1 labelling on the full image. A mask was then applied to the image to examine sites of co-occurring PSD95-VGLUT1 labelling only. Analysis of mGlu5 colocalization with PSD95 and VGLUT1 were then repeated on the masked image. Distance analysis was performed using the DiAna plugin [3] for ImageJ software [4, 5] to further quantify relationships among mGlu5, PSD95, and VGLUT1. Median edge-to-edge distance between closest pairs of each marker (mGlu5-PSD95 and mGlu5-VGLUT1).

HUMAN STUDY

Screening procedures

Personal and family medical histories were assessed in screening sessions by unstructured interview and the Structured Clinical Interview for DSM-IV, Non-Patient edition [6]; a medical examination by a physician; and routine blood work.

Inclusion and exclusion criteria

- **Inclusion:**

  - o Men and women age 20-40 years.
  - o Tridimensional Personality Questionnaire (TPQ, [7]) novelty-seeking subscale ≥ 20 (based on a mean score of approximately 15±5 in previous studies of healthy Canadian adults [8, 9]).
- **Exclusion:**
  - o Current or past major medical illness
  - o Abnormalities in EKG or bloodwork (reviewed by a physician)
  - o Personal or first-degree relative history of psychiatric disorders including (but not limited to) mood disorders, substance abuse or dependence, schizophrenia or psychotic symptoms, and attention-deficit hyperactivity disorder
  - o Current or past use of psychiatric medication including stimulants, antidepressants, antipsychotics
  - o Beck Depression Inventory score >10
  - o Regular use of cigarettes (>5 cigarettes per day)
  - o Lifetime use of psychostimulants (excluding cigarettes) >5 exposures
  - o Any use of psychostimulants within the past year
  - o Positive urine toxicology screen for illicit drugs (amphetamine, benzodiazepines, buprenorphine, cannabis, cocaine, 3,4-methylenedioxymethamphetamine, methamphetamine, methadone, or opioids; Express Diagnostics, MN, USA) at screening or (excluding amphetamine) on test days
  - o Positive urine pregnancy test at screening or study sessions
  - o Contraindications for MRI or PET scanning, including participation in another PET study within the previous year

Protocol deviations

Due to schedule changes after the first test session, in two cases participants in the placebo group could not commit to in the full 4+ hours of post-drug monitoring on a subsequent test days. To ensure safety without excessive participant burden, in one of these cases the participant was run single-blind with researchers aware of treatment status and in the other, researcher blind was removed between the third and fourth test sessions. Due to equipment failure, one participant in the Amph group completed the follow-up PET scan one day before the challenge drug session instead of on the same day, with an extra sham scan performed before the final dose.

As noted in the main text, the baseline scan from one subject (Amph group) was excluded due to injected activity more than 3 S.D. below mean across all scans (248 MBq; mean across scans, 370 ± 28.5 MBq). The day 21 scan from another subject (placebo group) was excluded due to injected radiotracer mass more than 3 S.D. above the mean (0.92 μg/kg; mean across scans, 0.16 ± 0.17 μg/kg).

Behaviour and physiological assessments

Behavior assessment. Alertness, mood and subjective effects of Amph were assessed using the Profile of Mood States (POMS), Visual Analog Scales (VAS), and the Addiction Research Center Inventory (ARCI), Amphetamine subscale [10]. Eye-blink rate and motor activity (Actiwatch AW-16, Philips Respironics, USA) were assessed as objective indices of psychomotor response, and the Interpersonal Speech Task [11] was used to assess talkativeness. The ARCI and the speech task were each completed at a single time point post-Amph or placebo on each test day in order to minimize habituation. For all other assessments, responses were recorded throughout the post-drug period and outcome measure was area under the curve from baseline to three hours post-pill.

Psychophysiology. Heart rate and blood pressure were measured prior to pill administration and every thirty minutes thereafter. Blood samples were drawn from an indwelling catheter at baseline and at 45, 90, and 120 minutes after pill administration for analysis of serum cortisol and plasma Amph levels.

MRI and PET processing
High-resolution T1-weighted structural magnetic resonance imaging (MRI) scans were acquired for PET co-registration. MR images were pre-processed with the CIVET pipeline version 2.0.0 (http://www.bic.mni.mcgill.ca/ServicesSoftware/CIVET/). ROI masks were applied to each summed radioactivity image in PET space using non-linear registration. BP_{ND} values were extracted from each ROI using tools developed by Turku PET Centre (http://www.turkupetcentre.net/). Voxel-wise BP_{ND} values were compared at baseline vs. follow-up within each treatment group using SPM12 (Wellcome Functional Imaging Laboratory).

ROI definition.
BP_{ND} values were estimated in three striatal subregions (defined as in [13]), three prefrontal cortex subregions (defined as in [14]), and four limbic regions (cingulate, insula, amygdala, and hippocampus; PickAtlas software). As previously [15,16], striatal subregions comprise the *associative striatum* (including dorsal caudate and anterior putamen, corresponding to dorsomedial striatum in mice), *sensorimotor striatum* (posterior putamen, corresponding to the mouse dorsolateral striatum), and *ventral striatum* (corresponding to the mouse NAc). For comparison with animal data in exploratory analyses, when results did not differ between subregions the voxel-weighted mean of the associative and sensorimotor striatum was computed and described as the dorsal striatum.

Statistical analysis
In mice, locomotor responses to drug were assessed using 2 session × 2 treatment repeated measures ANOVAs (rmANOVA) and post-hoc paired *t*-tests comparing dose 1 to challenge within each treatment group. Independent sample *t*-tests were used to compare receptor density in saline and Amph groups within 3-dose and 5-dose experiments separately. Relationship between [^{3}H]ABP688 binding and extent of behavioral sensitization (defined as distance travelled at challenge minus distance travelled at dose 1) was assessed using Pearson's *r*.
In humans, behavioral responses to Amph were assessed using 4 session × 2 treatment rmANOVAs followed by planned pairwise comparisons within each treatment group. Sensitization was operationally defined as greater responses to the last drug dose on day 21 as compared to the first dose on day 1. Changes in BP_{ND} were assessed using 2 session × 2 treatment × subregion rmANOVA. For measures on which sensitization was observed, relationship between BP_{ND} and drug response and between BP_{ND} and change in drug response were assessed using Pearson's *r*.

RESULTS

Psychophysiological effects of Amph in humans.
Plasma drug concentrations increased over time within each session (main effect of time, $F_{3,15}=168$, $p<0.001$) with no difference between sessions (session effect $F_{3,15}=0.87$ $p=0.48$). On average, the peak plasma Amph concentration was observed at 120 minutes post-administration. Mean peak plasma Amph concentration on each day did not change significantly and ranged from 41.6 ± 14.0 ng/mL after dose 2 to 46.3 ± 8.50 ng/mL after dose 3. Systolic and diastolic blood pressure readings, heart rate, and serum cortisol levels were significantly elevated following each acute drug administration session but did not differ across sessions (time $\times$ treatment interaction, systolic BP $F_{4,64}=13.8$, $p<0.001$, diastolic BP $F_{4,64}=9.3$, $p<0.001$, heart rate $F_{4,64}=4.6$, $p=0.002$; session $\times$ time $\times$ treatment interactions ps > 0.25).

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Table S1 corresponding to Figure 2

	MICE (panels A-B)			
	3 pre-treatment doses		**5 pre-treatment doses**	
	Two-way ANOVA			
	F	p	F	p
Session	$F_{1,25} = 13$	0.001	$F_{1,21} = 27$	<0.001
Treatment	$F_{1,25} = 9.0$	0.006	$F_{1,21} = 60$	<0.001
Session × Treatment	$F_{1,25} = 13$	0.001	$F_{1,21} = 30$	<0.001
	Post-hoc within-group paired *t*-test, dose 1 vs. challenge			
	t	p	t	p
Amph	$t_{11}=-3.2$	0.008	$t_{10} = -5.2$	<0.001
Saline	$t_{14} = -0.22$	0.83	$t_{11} = 0.97$	0.36
	HUMANS (panels C-D)			
	Speech Task			
	Two-way ANOVA			
	F		p	
Session	$F_{3,48} = 1.6$		0.20	
Treatment	$F_{1,16}= 0.80$		0.38	
Session × Treatment	$F_{3,48} = 4.8$		0.005	
	Post-hoc within-group paired *t*-test, dose 1 vs. challenge			
	t		p	
Amph	$t_9=-4.0$		0.003	
Placebo	$t_8=-0.61$		0.56	

	Visual Analog Scales							
	Three-way ANOVA							
	Activating [†]		Euphoric [†]		Anxiety		Motivation	
	F	p	F	p	F	p	F	p
Session	$F_{1,17}=2.8$	0.12	$F_{1,17}=0.64$	0.43	$F_{3,51}=2.2$	0.10	$F_{1,17}=3.6$	0.073
Treatment	$F_{1,17}=9.7$	0.006	$F_{1,17}=14$	0.001	$F_{1,17}=0.023$	0.88	$F_{1,17}=0.19$	0.67
Subscale	$F_{2,34}=0.26$	0.78	$F_{2.2,51}=1.3$	0.28			$F_{1,17}=48$	<0.001
Session × Treatment	$F_{1,17}=0.40$	0.54	$F_{1,17}=0.035$	0.85	$F_{1,17}=0.62$	0.44	$F_{1,17}=0.023$	0.88
Session × Subscale	$F_{1.6,34}=0.19$	0.79	$F_{2.1,51}=2.7$	0.077			$F_{1,17}=2.0$	0.18
Treatment × Subscale	$F_{2,34}=4.3$	0.021	$F_{3,51}=1.1$	0.34			$F_{1,17}=0.021$	0.89
Session × Treatment × Subscale	$F_{1.6,34}=0.84$	0.42	$F_{2.1,51}=0.31$	0.74			$F_{1,17}=0.50$	0.49
	Post-hoc within-group paired *t*-test, dose 1 vs. challenge (activating subscales)							
	Alertness		Mind Racing			Energy		
	t	p	t	p		t		p

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Amph	$t_9=-1.1$	0.31	$t_9=-2.5$	0.035	$t_9=-1.7$	0.12
Placebo	$t_8=-1.1$	0.32	$t_8=-0.26$	0.80	$t_8=-0.40$	0.70

[†] Huynh-Feldt correction applied where appropriate (Mauchly's test of sphericity, ps < 0.02).

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Sensitization to Amph in mice

Animals pre-treated with 3 doses of Amph had greater locomotor response to a challenge dose of Amph (Amph-Amph) compared to animals pre-treated with 3 doses of saline given a challenge dose of Amph (Sal-Amph) or animals pre-treated with saline who received an injection of saline at challenge (Sal-Sal) (rmANOVA after challenge, main effect of treatment, $F_{2,30}=7.4$, $p=0.0024$; post-hoc Tukey tests, Amph-Amph vs. Sal-Amph $p < 0.05$, Amph-Amph vs. Sal-Sal, $p < 0.05$).
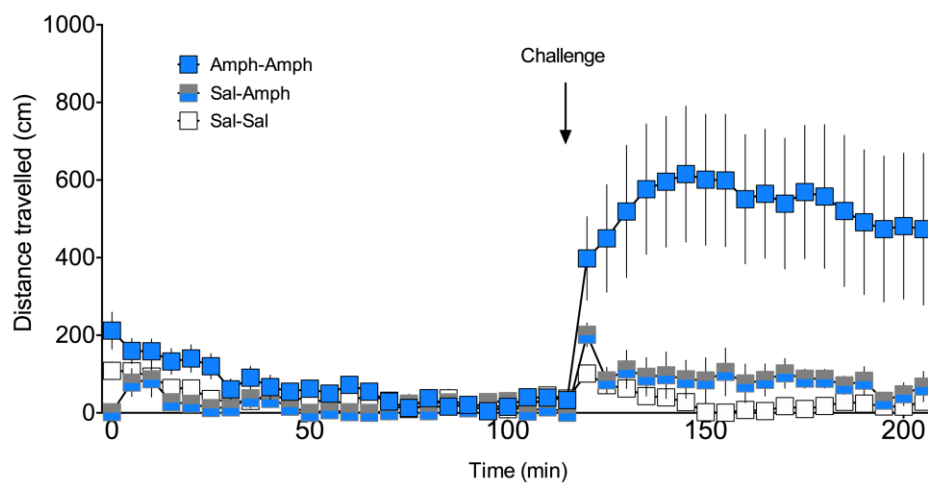
Supplementary Figure S1, related to Figure 2. Behavioural response to challenge dose of saline or Amph in animals pre-treated with 3 doses of saline or Amph (all doses 2mg/kg). Plot shows mean ± S.E.M. distance travelled in 5-minute bins before and after challenge dose.
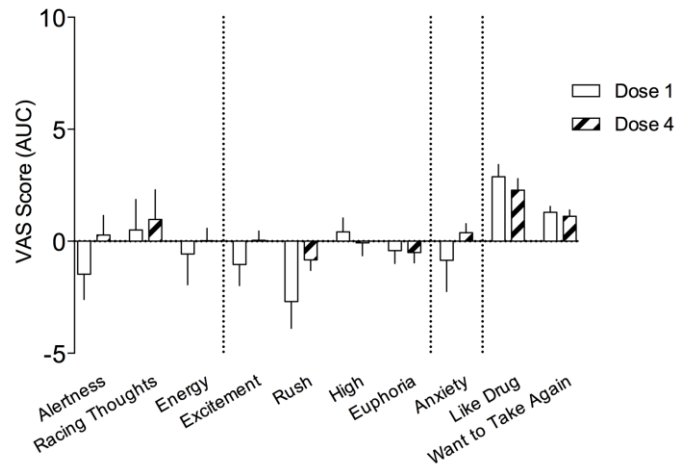
Placebo group self-report ratings

Placebo group self-report ratings of mood and drug responses (visual analog scales) did not change from baseline to follow-up (Like Drug, t=2.1, $p=0.066$; other subscales, ps > 0.18).

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Supplemental Figure S2, related to Figure 2. Visual analog scale ratings of mood and drug effects at baseline and day 21 in placebo group. Values are mean ± S.E.M. for the area under the curve across 8 time points (pre-pill baseline to 150 minutes after administration).

Effects of acute Amph on [^{3}H]ABP688 density in mice

[^{3}H]ABP688 binding was measured in animals pre-treated with saline who received a single challenge dose of Amph after saline pre-treatment (Saline-Amph) did not differ from that in saline pre-treated, saline-challenged (Saline-Saline) animals.
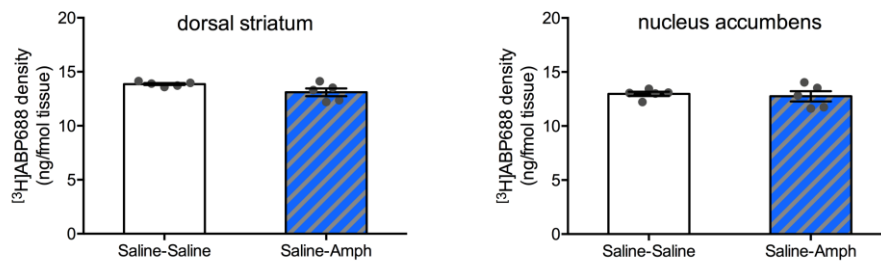
Supplemental Figure S3. No difference in mGlu5 binding between animals treated with 3 saline injections followed by saline challenge and those treated with 3 injections of saline followed by 2mg/kg Amph challenge in the dorsal striatum (t=0.81, p=0.44) or nucleus accumbens (t=-0.41, p=0.69).

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Table S2 corresponding to Figure 3

	MICE			
	Independent samples *t*-test			
	Dorsal striatum		Nucleus accumbens	
	t	*p*	*t*	*p*
3 pre-treatment doses	$t_{25}=-0.31$	0.76	$t_{25}=-0.68$	0.50
5 pre-treatment doses	$t_{19}=2.2$	0.038	$t_{19}=1.8$	0.086
	HUMANS			
	Three-way ANOVA			
	Striatum		Cortex	
	F	*p*	*F*	*p*
Session	$F_{1,15}=4.1$	0.060	$F_{1,15}=1.6$	0.23
Treatment	$F_{1,15}=0.14$	0.72	$F_{1,15}=0.61$	0.45
Subregion	$F_{2,30}=94$	<0.001	$F_{4,60}=103$	<0.001
Session × Treatment	$F_{1,15}=0.10$	0.76	$F_{1,15}=0.024$	0.88
Session × Subregion	$F_{2,30}=0.93$	0.41	$F_{4,60}=1.5$	0.22
Treatment × Subregion	$F_{2,30}=0.98$	0.39	$F_{4,60}=1.5$	0.22
Session × Treatment × Subregion	$F_{2,30}=1.6$	0.22	$F_{4,60}=0.93$	0.45

	Post-hoc *t*-tests					
	Dorsal striatum		Ventral striatum		Prefrontal cortex	
	t	*p*	*t*	*p*	*t*	*p*
Amph	$t_8=-1.4$	0.21	$t_8=-0.92$	0.39	$t_8=-0.91$	0.39
Placebo	$t_7=-1.5$	0.17	$t_7=-2.0$	0.085	$t_7=-0.81$	0.44
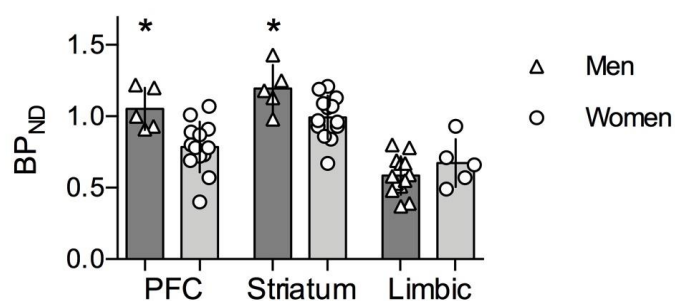
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Supplemental Figure S4, related to Figure 3. BP_{ND} was higher at baseline in men (n=5) than women (n=13) in the PFC (t=-3.0, *p*=0.009) and striatum (t=-2.4, *p*=0.026). There was no difference between sexes in subcortical limbic regions (t=-1.1, *p*=0.28). Limbic BP_{ND} represents the mean of BP_{ND} in the amygdala and hippocampus. PFC, prefrontal cortex.

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Table S3 corresponding to Figure 4

MICE							
Correlations							
Dorsal striatum				Nucleus accumbens			
r		*p*		*r*		*p*	
-0.56		0.007		-0.53		0.01	
HUMANS							
Correlations							
Dorsal striatum		Ventral striatum		Prefrontal cortex		Occipital cortex	
r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
-0.73	0.016	-0.66	0.037	-0.70	0.023	-0.29	0.91
BP_{ND} comparisons (associative striatum)							
Wilcoxon signed ranks test (baseline vs. follow-up)				Mann-Whitney U tests (sensitizers vs. non-sensitizers)			
		Z	*p*			*U*	*p*
Placebo		-0.84	0.40	Baseline		6.0	0.41
Amph, non-sensitizers		-2.0	0.043	Follow-up		2.0	0.032
Amph, sensitizers		0.0	1.0				

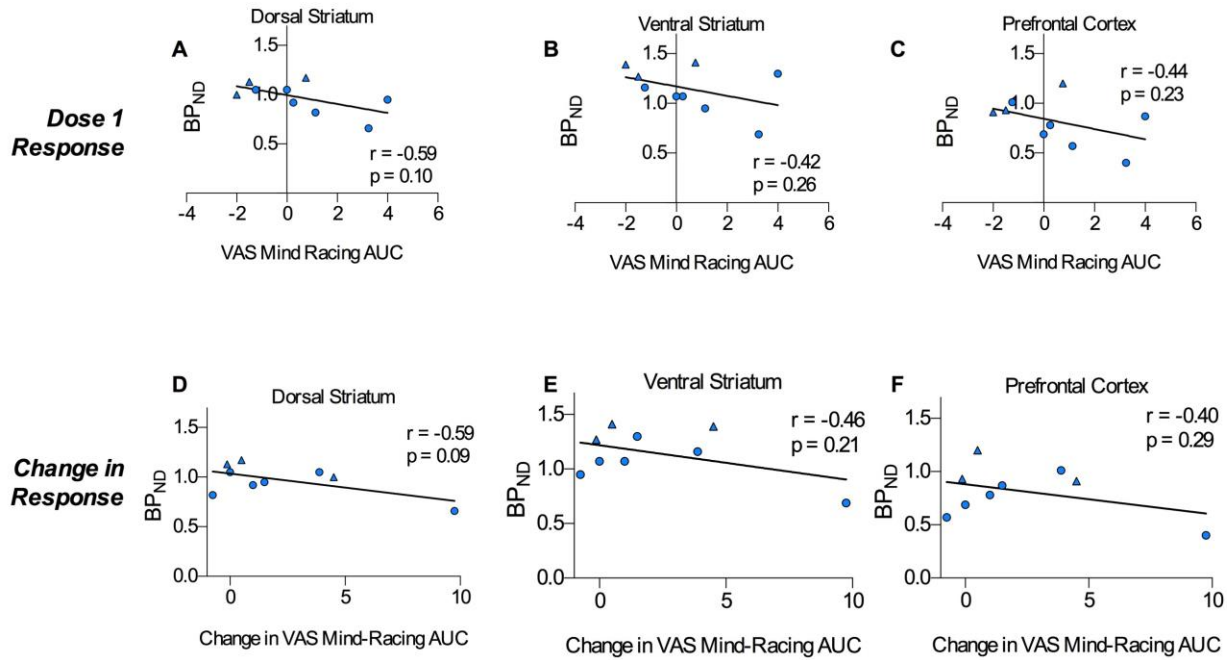**Relationships between baseline [^{11}C]ABP688 BP_{ND} and behavioral response**

mGlu5 binding availability at baseline in humans was not statistically associated with behavioral response (VAS Mind Racing rating) following first dose of Amph or with subsequent change in behavioral response at follow-up.

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Supplementary Figure S5, related to Figure 4. Relationship between baseline BP_{ND} and drug response at baseline (panels A-C) and change in drug response (panels D-F) on VAS Mind Racing ratings.
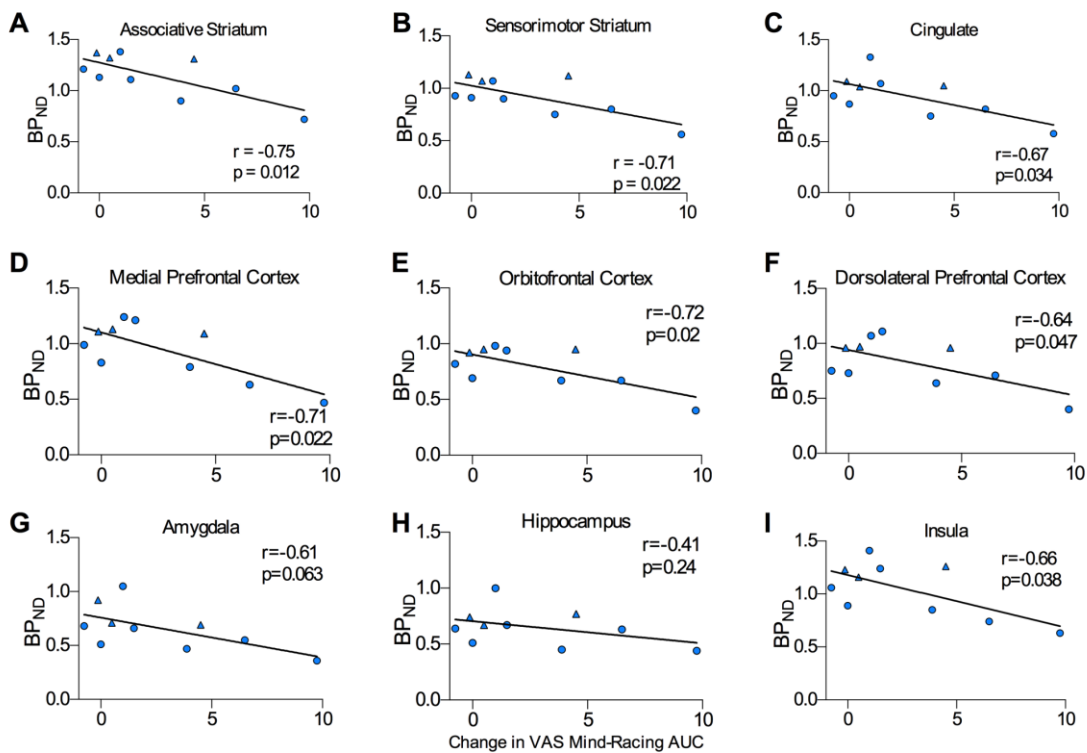
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Relationships between change in behavioral response and regional [^{11}C]ABP688 BP$_{ND}$

Binding availability of mGlu5 was negatively correlated with increase in behavioural response (VAS Mind Racing ratings) across the striatum, prefrontal cortex, cingulate, and insula.

Supplementary Figure S6, related to Figure 4. Relationship between scan 2 BP$_{ND}$ and sensitization of psychoactivating drug effects (VAS Mind Racing) across all studied brain regions.

Relationship between [¹¹C]ABP688 BP_{ND} and other behavioral measures

The relationship between BP_{ND} and drug response was assessed on each measure on which a sensitization effect was seen. BP_{ND} was negatively correlated with VAS Mind Racing ratings (main text) but was not associated with change in ARCI Amph scale scores or speech rate. Limbic BP_{ND} was computed as the mean of BP_{ND} in the amygdala and hippocampus.

Supplemental Figure S7, related to Figure4. No correlation between BP_{ND} at follow-up and ARCI Amphetamine scores (panels A-C) and change in speech rate (panels D-F).

Appendix 1 to Smart K, Nagano-Saito A, Milella MS, et al. Metabotropic glutamate type 5 receptor binding availability during d-amphetamine sensitization in mice and humans. *J Psychiatry Neurosci* 2020.
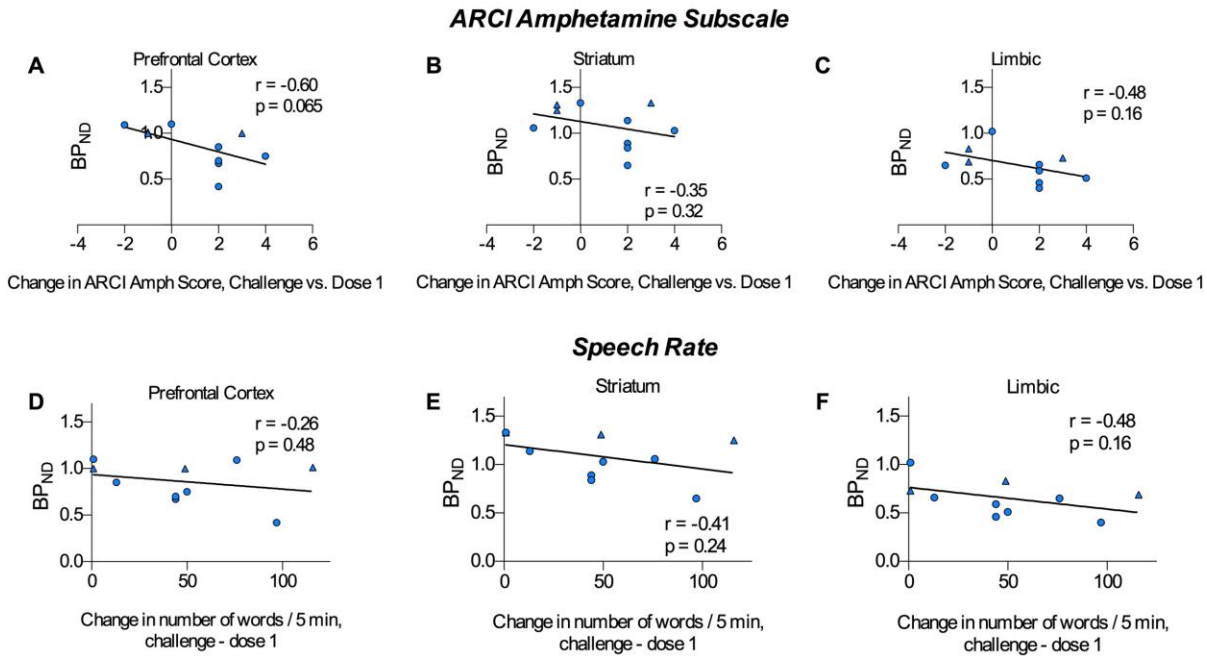
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