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Supporting Online Material for

Neurokinin 1 Receptor Antagonism as a Possible Therapy for Alcoholism

David T. George, Jodi Gilman, Jacqueline Hersh, Annika Thorsell, David Herion, Christopher Geyer, Xiaomei Peng, William Kielbasa, Robert Rawlings, John E. Brandt, Donald R. Gehlert, Johannes T. Tauscher, Stephen P. Hunt, Daniel Hommer, Markus Heilig*

*To whom correspondence should be addressed. E-mail: markus.heilig@mail.nih.gov

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Correction (14 March 2008): The authors have made added the corrected type I error rate to the fMRI analysis. In Table S3, they have revised the descriptions of brain regions, Talairach coordinates, and *t*-scores for the first 12 rows.

Materials and Methods

Experiments with NK1R null mice

MF1 x 129/sv x C57BL/6 NK1R null mutants were generated and described previously (1). Here, they were used after back-crossing into the C57BL/6 line for 10 generations, because genetic background plays a decisive role in determining voluntary alcohol consumption, and C57/BL6 mice consume sufficient amounts of alcohol to be potentially sensitive to a suppressive effect of the mutation (2). Animals were maintained in a temperature and humidity controlled vivarium on a regular light cycle (on 6 am, off 6 pm), and standard food pellets were available *ad libitum*.

To measure voluntary alcohol intake, we used male mice in a two-bottle free choice model with increasing alcohol concentration (3-15%) and alcohol continuously available. Animals were single-housed for the drinking study and bottle-position alternated daily in order to avoid side-preference. Alcohol and water intake was measured daily at 11 am.

Alcohol solutions may in part be consumed due to non-specific motivation for fluid ingestion, or for their taste, a composite of sweet and bitter (3). To exclude that genotype effects on alcohol intake and preference were confounded by differences in thirst or taste preference, male mice (-/-: $n = 12$; +/-: $n = 13$; +/+: $n = 11$) were first evaluated for water consumption (1 week, consumption measured every second day), and then assessed for taste preference of sucrose (5% w/v; 2 consecutive days) and quinine (0.1 mM; 2 consecutive days). None of these control behaviors was affected by genotype (water consumption: $F_{2,33} = 0.5$, $P = 0.60$; sucrose: $F_{2,33} = 0.01$, $P = 0.99$; quinine: $F_{2,33} = 1.7$, $P = 0.20$).

Loss of righting reflex (LORR) was performed by intraperitoneal (i.p.) injection of 3.5 g/kg of alcohol and animals placed on their back in a V-shaped trough. Time from unconsciousness until waking (being able to turn over after being placed on the back 3 times within 30 sec) was measured.

Human study: Recruitment and Subjects

Participants (25 / arm) were recruited among patients admitted to the NIAAA inpatient care unit at the NIH Clinical Research Center (CRC) in Bethesda, MD, under a general protocol for assessment and treatment of alcohol use disorders. Seven subjects required benzodiazepines for withdrawal treatment on admission (of these, 4 subsequently randomized to placebo and 3 to LY686017). Withdrawal treatment was first completed if needed, and subjects entered the NK1R study after a minimum of 2 days without measurable withdrawal symptoms. Subjects underwent a structured clinical interview for diagnosis (SCID; (4)). They were eligible if they were 21-65 years of age, had a diagnosis of alcohol dependence based the SCID interview, reported alcohol problems as their primary complaint, had used alcohol within the last month, and scored >39 on the Spielberger Trait Anxiety Inventory (STAI) (5). Women were required to use an effective method of contraception, and were not eligible if pregnant or lactating. Random drug testing was carried out to exclude the possibility of drug use during the study. Subjects were excluded if they had complicated medical or psychiatric problems, had received

psychotropic medication other than withdrawal treatment in the last 4 weeks, were court ordered to treatment, or were likely to become incarcerated during the course of the study. Detailed eligibility criteria are available at www.clinicaltrials.gov (trial identifier NCT00310427). Upon completion, subjects received a total compensation with \$600. Based on a clinical case-by-case assessment and in dialogue with each subject, every attempt was made to disburse these funds so that they would not be used for procurement of alcohol or illicit drugs. For example, funds could partially or fully be applied toward the cost of a half-way house after discharge from the hospital, or other therapeutically beneficial goal. The study was approved by the Institutional Review Board (IRB), and informed consent was obtained according to IRB approved procedures. A CONSORT flowchart is provided in Figure S1, and baseline characteristics of subjects are given in Table S1.

Overall design

Sixty-six subjects were screened for this study, and 50 were ultimately analyzed. All subjects began a 1 week single-blind placebo lead-in period, during which they underwent a baseline alcohol cue reactivity session. Subjects were assessed for cue-reactivity as described previously (6) (i.e. they were considered non-reactive if their craving ratings in response to the neutral water stimulus was equal to or higher than in response to alcohol cue). Eight out of 66 subjects were non-reactive, and did not continue into the randomized phase of the study. Subjects who did continue were randomized to receive active drug or placebo during weeks 2 through 4. Ratings of spontaneous cravings, clinicians global impression, and general psychopathology were obtained throughout the 4 week study as described below. During week 4, a second cue reactivity session was carried out, in which presentation of the alcohol cue was preceded by a standardized stressor, the Trier Social Stress Test (TSST) (7). Also during week 4, and a minimum of 48h after the second cue reactivity session, an fMRI scan was obtained to assess brain responses to emotional stimuli and alcohol cues. Throughout hospitalization, all subjects participated in standard-of-care behavioral treatment aimed at facilitating abstinence. Random urine screens were used from admission and throughout the study to exclude ongoing drug use.

Drug treatment

LY686017 (8) is a high-affinity, selective, non-peptide, orally available NK1R antagonist. Its preclinical pharmacology, safety and human pharmacokinetics will be reported separately. LY686017's brain penetrance and NK1R receptor occupancy (RO) were established in a human positron emission tomography (PET) study of 8 healthy volunteers. Displacement of the specific NK1R receptor antagonist PET ligand [¹¹C]GR205171 in frontal cortex was determined after 7 days of once daily dosing (QD) with 1, 10, 30 and 100 mg LY686017. RO was determined at steady state approximately 24 hours (trough) after the 7th dose of LY686017. Modeling of trough plasma concentrations ($C_{\text{trough, ss}}$) and RO assessments estimated the E_{max} to be 98%, and indicated that LY686017 was able to block brain NK1R receptors in a concentration and dose-dependent manner. Preclinical data suggest that near maximal target blockade is desirable with respect to exploring the clinical safety and efficacy of LY686017. A PK/RO model was developed to guide dose selection. Simulations were conducted to

predict $C_{\text{trough,ss}}$ and $RO_{\text{trough,ss}}$ assuming doses ranging from 1-100 mg QD for 12 weeks, and revealed that 30 mg QD should lead to sustained target occupancy of >80% with 90% confidence. In order to provide added assurance for near maximal target occupancy in patients, 50 mg QD was given in the current study. LY686017 has been demonstrated to be safe and well tolerated at this dose level in previous clinical studies. The modeling results show that doses in excess of 50 mg QD would not lead to more extensive target occupancy.

Assessment instruments and rating scales

To assess spontaneous alcohol cravings over time on the inpatient unit, the self-report based Alcohol Urge Questionnaire (AUQ) (9) was collected twice weekly in the evening. For global assessment of alcohol related pathology over time, the clinician rated clinical global impression (CGI) was obtained weekly (10). The self-report version of the Comprehensive Psychopathological Rating Scale, Self-Affective (CPRS-SA) (11) was collected twice weekly to obtain measures of general psychopathology. This instrument provides subscales for symptoms of anxiety and depression.

Challenge sessions

On challenge session days, subjects consumed a standard hospital lunch on the inpatient unit, and were allowed ad libitum nicotine use until 1 hour prior to the sessions. Challenge sessions were carried out from 4 to 5 pm in a test room outside the unit. The subjective craving responses to smelling and manipulating an alcohol stimulus (Cue Reactivity, CR) were assessed as described (6). Briefly, subjects reported their craving on the AUQ, and also on 0–100 mm visual analog scales (VAS) after a 3-min period of relaxation, a 3-min presentation of a neutral (water) stimulus, a 3-min presentation of the alcohol stimulus, and an additional 3-min interval of relaxation. On the combined stress and alcohol cue session (see below), VAS ratings of subjective distress / anxiety were also obtained prior to and after the stressor, and then after the stimuli described above. On the combined stress and alcohol cue session (see below), subjects were also asked to rate, on a 0 – 10 scale, their degree of subjective distress / anxiety prior to and after the stressor, and then after the stimuli described above.

Each subject participated in two sessions. A baseline session during placebo lead-in was designed to determine the subject's cue-reactivity without additional manipulations. The second, medicated session was aimed at evaluating the ability of NK1R antagonism to modulate craving and neuroendocrine responses to a combined stimulus consisting of the TSST, a stressor, followed by alcohol associated stimuli. Subjects received an i.v. line, and were allowed to rest for at least 30 min. Baseline blood samples were obtained at 20 min prior to the start of the TSST and again at the start of the TSST. Immediately after the second of these blood draws, the TSST was carried out over 10 min as described (7). Briefly, participants performed a 5-min impromptu speech followed by 5 min of serial subtraction in front of three unfamiliar individuals in white coats. Immediately after completion of the TSST, cue reactivity was assessed as described for challenge session 1.

This procedure was not designed to independently assess the individual contribution of the stress and the cue exposures components, respectively. Instead, it combined the two

types of stimuli in order to simulate a situation prone to high relapse risk, and produce a craving response of a magnitude that would be sufficient for modulation by potential medication effects. As intended, the challenge procedure resulted in a robust craving response on the AUQ, with a magnitude very similar to that recently reported by others using a multimodal craving induction (12).

Serial blood draws were obtained for cortisol analysis throughout the combined challenge session. Adrenocorticotrophic Hormone (ACTH) responses were not analyzed, because this measure peaks prior to completion of the TSST challenge and declines rapidly thereafter (7), while our behavioral paradigm did not allow sampling until much later. The cortisol analyses were carried out using standard clinical methods by the CLIA accredited laboratory at the NIH Clinical Center.

fMRI study

To evaluate the ability of NK1R antagonism to modulate brain responses to emotional stimuli and alcohol associated cues in alcoholics, blood oxygen-level dependent (BOLD) activity was registered after presentation of stimuli composed of negative and positive emotional stimuli from the International Affective Picture Series (IAPS) (13) and alcoholic or neutral beverage cues. This task had been used in our lab to show greater BOLD response to fearful images among alcoholics and reduction of this response by alcohol beverage images (14).

Visual stimulation and task: Fifty-five high-arousal negative pictures and 55 high-arousal positive pictures from the IAPS were presented. We presented the same images to both groups. We chose high-arousal negative images containing scenes of violence, injuries, surgeries, car crashes etc. and positive images of animals, nature, children, nudes etc. Negative images had a valence of 2.4 ± 0.7 (mean \pm SD) and an arousal of 6.02 ± 0.88 ; positive images had a valence of 7.1 ± 0.63 and arousal of 5.92 ± 0.95 . Negative and positive images were not significantly different in arousal ratings.(1) These pictures were paired with pictures of alcoholic beverages and neutral beverage pictures (i.e. milk, orange juice) The IAPS pictures and the beverage pictures appeared simultaneously, side-by-side. This recently developed composite fMRI task allows independent assessment of responses to affective stimuli, alcohol associated cues, and the interaction of these two factors, as well as direct comparisons between conditions. Scrambled images were used as the control condition and were displayed during the inter-stimulus-interval (ISI). The scrambled images were derived from the IAPS images using a script that introduced a random phase shift into Fast Fourier Transformations (FFT) of each image, which preserved overall brightness and color but did not contain recognizable features. The inter-stimulus interval (ISI) ranged from 0 to 15 sec. The pictures were presented in a random order in one run lasting 9 min and 30 sec. Stimuli were presented using a Linux laptop computer with in-house stimulus delivery software. They were projected using an Epson MP 7200 LCD projector onto a screen placed at the foot of the MRI scanner bed and were viewed using a mirror mounted on the head coil. Each stimulus presentation lasted 800 msec. Participants were instructed to attend to the pictures.

fMRI acquisition: Imaging was performed using a 3 T General Electric MRI scanner (General Electric, Milwaukee, WI) and a 16 channel head coil. In-plane resolution was 3.75 x 3.75 mm. Functional scans were acquired using a T2*-sensitive echoplanar sequence with a repetition time (TR) of 400 msec, echo time (TE) of 40 msec, and flip equal to 30°. We collected eight 5.0 mm contiguous axial slices drawn from the base of the orbitofrontal cortex upward to the level approximately at the top of the corpus callosum, which allowed us to image most of the temporal and ventral frontal lobe, as well as the ventral visual stream. Eight slices was the maximum number we could collect at a TR of 400 msec, which was chosen because it allowed for selective filtering of noise due to the cardiac cycle (15). A total of 1430 volumes were collected. Structural scans were acquired using a T1-weighted MP-RAGE (magnetization-prepared rapid gradient echo) sequence (TR, 100 msec; TE, 7 msec; flip, 90°), which facilitated localization and co-registration of functional data.

fMRI analysis: Analyses focused on changes in BOLD signal contrast (hereafter, activation) that occurred as the participants viewed the positive and negative pictures. Analyses were conducted using Analysis of Functional Neural Images (AFNI) software (16). Echoplanar image volumes were preprocessed as follows: (1) voxel time series were interpolated to correct for non-simultaneous slice acquisition within each volume (using sinc interpolation and the most inferior slice as a reference), and (2) volumes were corrected for motion in three-dimensional space. Motion-correction estimates indicated that no participant's head moved >1.0 mm in any dimension from one volume acquisition to the next. We imposed a 6 mm full-width half-maximum (FWHM) smoothing kernel in the spatial domain. (3) We created a mask so that all of the background values outside of the brain were set to zero, so that we could calculate the percentage signal change in each voxel. This analysis was conducted in two stages. First, statistical maps were generated for each individual separately by linear contrasts of the regressors of interest and the scrambled condition. Preprocessed time series data for each individual were then analyzed by multiple regression, which allowed co-variation of variables related to head motion and scanning run. The regression model consisted of the regressors of interest and six regressors of no interest modeling residual motion. Regressors of interest were convolved with a gamma-variate function that modeled a prototypical hemodynamic response before inclusion in the regression model (17). Idealized signal time courses were time-locked to image onset. Anatomical maps of *t* statistics representing each of these regressors of interest were spatially normalized by warping to Talairach space and combined into a group map. We applied a family-wise error rate correction (using a Monte Carlo simulation) to rule out false positives. Only activated volumes greater than 703 mm³, or clusters larger than 10 voxels were considered significant, yielding a corrected type I error rate < 0.05. Second, we calculated a statistical map of the activation within each group (LY686017-treated and placebo) for each stimulus condition. Each condition was compared to the baseline scrambled image. We then performed voxel-wise *t*-tests of the event-related β -coefficients calculated from the general linear model to test for differences between the LY686017-treated group and the placebo group for each condition.

Statistical analysis

Behavioral and endocrine data were analyzed using Statistica (Statsoft, Tulsa OK) general linear model or SAS (SAS Inst Inc, Cary NC) procedure MMRM. Dependent variables were assessed for homogeneity of variance. AUQ and CPRS-SA data collected on the unit, which violated this assumption, were rank transformed. Ratings on the AUQ, CGI and CPRS-SA collected on the unit were analyzed by mixed model ANOVAs. Repeated ratings for a subject over the 3 week long randomized placebo-controlled phase (weeks 2-4) were a within-subjects factor, and drug treatment a between-subjects factor. Because in different subjects, AUQ and CPRS ratings were obtained on different days relative to randomization, this independent variable was treated as a continuous measure, with the day of relative randomization as its value. Approximately 10% of the data points on these variable were missing, in part because 7 subjects requested to leave the unit once the fMRI session was completed. No imputation was used for missing values. According to a predefined analytical plan, baseline (the last rating obtained during the placebo lead-in phase) for the respective scale, age, sex and the body mass index (BMI) were entered into the model as covariates, and were retained if they reduced the residual variance, or were otherwise left out. For craving and cortisol responses to the combined stress and alcohol cue challenge session, a similar mixed model ANOVA was applied to evaluate the responses to the challenge as a main effect of the within-subjects time factor, the possible effects of treatment as the main between-subjects effect, and the interaction between the two as an ability of treatment to modulate the respective challenge response. The same co-variates were evaluated, except pre-challenge baseline values were not used as a co-variates in these analyses, because they were obtained while subjects were already on medication, and might be contaminated by main treatment effects. Frequencies of adverse events were compared using two-tailed Fisher's Exact test.

Table S1. Baseline characteristics of subjects. Data are given as counts, or as means±SEM. BMI: Body Mass Index. TLFB: Time Line Follow Back (18). Heavy drinking refers to 5 or more standard drinks in a day for males, and 4 or more for females. A standard drink is 10 – 12g alcohol. STAI: Spielberger Trait Anxiety Inventory (possible score range: 20 – 80). ADS: Alcohol Dependence Scale (19) (possible score range: 0 – 47); AUQ: Alcohol Urge Questionnaire (9) (possible score range 8 – 56). CPRS-SA: Comprehensive Psychopathological Ratings Scale, Self-Affective version (11); the depression and anxiety subscales each have a possible score range of 0 - 27. FTND: Fagerstrom Test of Nicotine Dependence (20) (possible range 0 – 10). The groups were not significantly different on any of the baseline variables assessed. *With the exception of one person reporting use of chewing tobacco, all nicotine users were smokers.

Characteristic	Placebo (n = 25)	LY686017 (n = 25)
Males / Females	16 / 9	21 / 4
Age	40.9 ± 2.0	42.0 ± 2.0
BMI	26.3 ± 1.1	26.2 ± 1.1
Trait anxiety (STAI)	54.5 ± 2.5	54.0 ± 1.6
Alcohol Dependence Severity (ADS)	24.1 ± 1.8	21.1 ± 1.6
Heavy drinking days in preceding 90 days (TLFB)	65.6 ± 4.8	62.5 ± 2.1
Average drinks per drinking day (TLFB)	12.4 ± 1.2	12.8 ± 1.6
Baseline cravings (AUQ)	19.1 ± 2.4	19.7 ± 2.3
Baseline depression (CPRS-SA)	6.2 ± 0.8	6.0 ± 0.7
Baseline anxiety (CPRS-SA)	11.9 ± 1.6	11.5 ± 1.3
Nicotine use / non-use*	19 / 6	21 / 4
Degree of nicotine dependence (FTND)	3.2±0.7	3.5±0.6
Cannabis use / non-use	5 / 20	8 / 17
Cocaine use / non-use	10 / 15	7 / 18

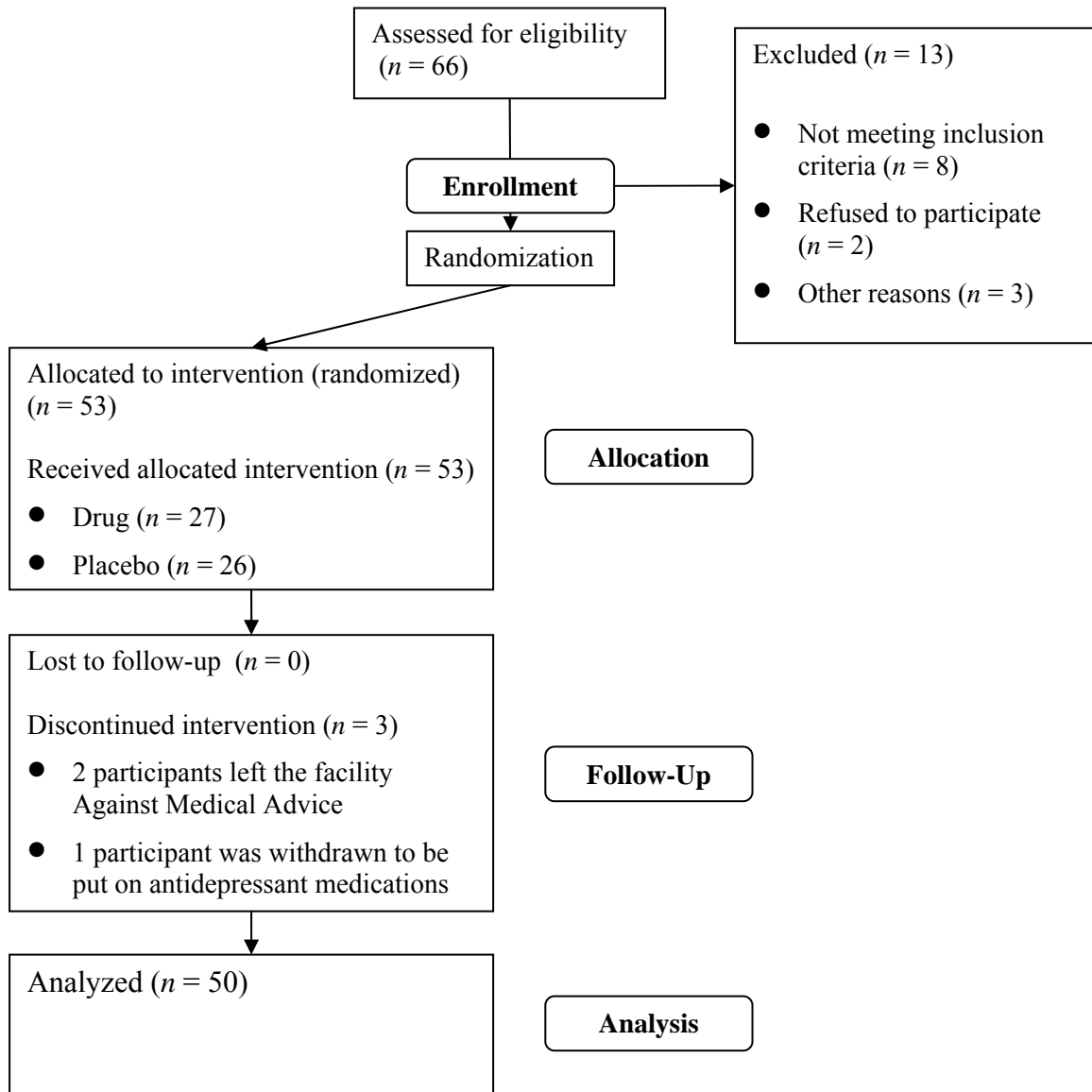
Table S2. Absolute and relative frequencies of treatment emergent adverse events (AE). A frequency comparison across these 26 categories, uncorrected for multiplicity of tests to avoid type II errors, did not reveal a higher incidence in the active treatment group for any category. A weak trend ($P = 0.13$) was present for increased incidence of fatigue in the active treatment group. This was mild in all cases. *indicates that xerostomia was present prior to the start of treatment in one of the two individuals exhibiting this adverse event (i.e. it was not a treatment emergent adverse event).

Adverse Event	Placebo			LY686017			
	Pre Rx %	On Rx	%	Pre Rx	%	On Rx	%
Abdominal cramps	0.0%	2	8.0%	-	0.0%	1	4.0%
Acne	0.0%	1	4.0%	-	0.0%	-	0.0%
Back pain	0.0%	1	4.0%	-	0.0%	1	4.0%
Blurred vision	0.0%	-	0.0%	-	0.0%	1	4.0%
Constipation	0.0%	1	4.0%	-	0.0%	1	4.0%
Decreased appetite	0.0%	-	0.0%	-	0.0%	2	8.0%
Diarrhea	14.0%	2	8.0%	-	0.0%	3	12.0%
Dizziness	0.0%	2	8.0%	-	0.0%	3	12.0%
Dysgeusia	0.0%	1	4.0%	-	0.0%	-	0.0%
Eructation	0.0%	1	4.0%	1	4.0%	1	4.0%
Fatigue	0.0%	5	20.0%	-	0.0%	11	44.0%
Flatulence	0.0%	2	8.0%	-	0.0%	1	4.0%
Headache	28.0%	6	24.0%	2	8.0%	6	24.0%
Hiccoughs	0.0%	-	0.0%	-	0.0%	1	4.0%
Irritability	0.0%	-	0.0%	-	0.0%	1	4.0%
Leg numbness	0.0%	-	0.0%	-	0.0%	1	4.0%
Myasthenia	0.0%	-	0.0%	-	0.0%	1	4.0%
Myiodesopsia	0.0%	1	4.0%	-	0.0%	-	0.0%
Nausea	0.0%	2	8.0%	-	0.0%	3	12.0%
Neck pain	0.0%	-	0.0%	-	0.0%	1	4.0%
Odynophagia	0.0%	1	4.0%	-	0.0%	-	0.0%
Otalgia	0.0%	1	4.0%	-	0.0%	-	0.0%
Rash or pruritus	0.0%	1	4.0%	-	0.0%	2	8.0%
Sleep disturbance	0.0%	1	4.0%	-	0.0%	-	0.0%
Tinnitus	0.0%	-	0.0%	-	0.0%	1	4.0%
Xerostomia	0.0%	-	0.0%	1	4.0%	2 *	8.0%

Table S3. Brain regions activated in response to negative and positive affective stimuli. The table lists contiguous clusters (activated volumes greater than 703 mm³ or larger than 10 voxels) with a difference between groups sufficient to obtain a family-wise corrected type I error rate of $P < 0.05$ using Monte Carlo simulation as implemented by the Alpha-Sim module of the AFNI software (16). Voxel coordinates given are at the peak of the respective cluster. T-scores indicate the effect size of the respective comparison.

Condition	Group	Region	Left / Right	Talairach coordinates			t-score		
				x	y	z			
Negative		Precuneus	L	-5	-62	3	-3.418		
		Precuneus	L	-35	-3	26	3.121		
		Fusiform gyrus	L	-49	3	-6	3.656		
		Fusiform gyrus	R	41	-55	6	3.124		
		Insula	R	21	-61	6	-2.928		
		Insula	L	-40	-29	21	3.463		
		Parahippocampal gyrus	L	-35	-19	-11	3.309		
		Middle temporal gyrus	R	53	-41	0	3.939		
		Caudate	R	49	25	6	3.892		
		Caudate	L	-31	-39	6	3.412		
		Putamen	R	19	5	0	3.155		
		Putamen	R	15	-5	0	2.849		
			LY686017 > Placebo	No clusters detected					
			Placebo > LY686017	Middle temporal gyrus	L	-57	-41	-6	3.375
				Middle temporal gyrus	R	61	-27	0	2.861
				Cuneus	R	7	-75	32	2.995
		Insula	L	-45	-31	22	3.244		
		Middle occipital gyrus	L	-53	-77	2	2.976		
		Inferior frontal gyrus	R	45	37	-16	3.187		
Positive	LY686017	Anterior cingulate	R	1	1	-3	4.284		
		Thalamus	R	1	-25	2	3.35		
			Lingual gyrus	L	-9	-59	-2	2.881	
	Placebo	Anterior cingulate	L	-23	35	6	-4.076		
		Superior frontal gyrus	R	19	51	-2	-3.042		
		Inferior frontal gyrus	L	-57	11	16	-3.052		
		Precuneus	L	-27	-61	30	3.194		
		Medial frontal gyrus	R	3	55	-14	-3.738		
			Inferior temporal gyrus	L	-61	-47	-14	-3.396	
		LY686017 > Placebo	Thalamus	R	1	-25	4	2.916	
			Caudate	R	1	-1	2	3.07	
			Lingual gyrus	L	-1	-91	-12	2.57	
			Inferior temporal gyrus	L	-51	-25	-18	2.64	
			Middle temporal gyrus	R	59	-37	-8	3.009	
			Middle temporal gyrus	L	-57	-17	-4	3.097	
		Placebo > LY686017	No clusters detected						

Figure S1. CONSORT flow chart of subjects.



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