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Pairing Neutral Cues with Alcohol Intoxication: New Findings in Executive and Attention Networks

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Abstract

Rationale: Alcohol-associated stimuli capture attention, yet drinkers differ in the precise stimuli that become paired with intoxication.

Objectives: Extending our prior work to examine the influence of alcoholism risk factors, we paired abstract visual stimuli with intravenous alcohol delivered covertly and examined brain responses to these Pavlovian conditioned stimuli in fMRI when subjects were not intoxicated.

Methods: Sixty healthy drinkers performed task-irrelevant alcohol conditioning that presented geometric shapes as conditioned stimuli. Shapes were paired with a rapidly rising alcohol limb (CS+) using intravenous alcohol infusion targeting a final peak breath alcohol concentration of 0.045 g/dL or saline (CS−) infusion at matched rates. On day two, subjects performed monetary delay discounting outside the scanner to assess delay tolerance and then underwent event-related fMRI while performing the same task with CS+, CS−, and an irrelevant symbol.

Results: CS+ elicited stronger activation than CS− in frontoparietal executive/attention and orbitofrontal reward-associated networks. Risk factors including family history, recent drinking, sex, and age of drinking onset did not relate to the [CS+ > CS−] activation. Delay-tolerant choice and [CS+ > CS−] activation in right inferior parietal cortex were positively related.

Conclusions: Networks governing executive attention and reward showed enhanced responses to stimuli experimentally paired with intoxication, with the right parietal cortex implicated in both

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alcohol cue pairing and intertemporal choice. While different from our previous study results in 14 men, we believe this paradigm in a large sample of male and female drinkers offers novel insights into Pavlovian processes less affected by idiosyncratic drug associations.

Keywords

classical conditioning; cue reactivity; associative conditioning; laboratory task; ethanol; BA 40; intertemporal choice; alcoholism; addiction

INTRODUCTION

Stimuli associated with intoxication attract attention and provoke activation in limbic and reward/motivation nodes such as orbitofrontal cortex, ventral striatum, and ventromedial prefrontal cortex (Chase et al. 2011; Kuhn and Gallinat 2011; Schacht et al. 2013), in addition to executive/attention areas in the medial and lateral frontal lobe and parietal cortex (Vollstädt-Klein et al. 2012). The vast majority of alcohol/drug cue reactivity studies to-date utilize familiar drug cues, such as images, odors, and tastes. However, a problem with such stimuli is that they can invoke unique conditioning histories and circumstances that vary by subject, and which can evoke behavioral changes related to alcohol, drinking history, and expectancies about alcohol's influence on behavior (Freeman et al. 2010; Friedman et al. 2007; Kramer and Goldman 2003). To eliminate the effects of idiosyncratic associations previously linked to intoxication, we sought to pair alcohol intoxication with neutral cues—novel, abstract figures possessing no intrinsic conditioning history. Similar approaches have successfully elicited conditioned brain and behavioral responses using neutral cues paired with stimulants (Mayo and de Wit 2015; Mayo et al. 2013; Van Hedger et al. 2018) or alcohol (Mayo and de Wit 2016), demonstrating the utility of classical conditioning to intoxication in humans.

In a previous proof-of-concept study in a small sample of men (Kareken et al. 2012), we covertly paired alcohol infusion with geometric images (conditioned stimulus; CS) in a reaction time task that aimed to conceal the intent to associate the stimuli with intoxication. These conditioned stimuli were then tested for brain responses during functional magnetic resonance imaging (fMRI). We did not detect a behavioral indication of conditioning, as quantified by attentional bias (differential response time) to drug-paired cues. Expecting greater activation to the CS+ (when compared to the CS−) in limbic reward/motivation areas, we instead found *reduced* activation to the CS+ in dorsomedial PFC. Insofar as the CS were tested in the absence of alcohol intoxication, we interpreted the reduced CS+ activation as a prediction error signal, consistent with previous work showing reduced neural response in reward areas to unmet expectations (Schultz et al. 1997), including such as can occur in medial PFC/anterior cingulate (Alexander and Brown 2017).

Extending this previous work in a considerably larger and more heterogeneous sample of men and women, we employed here the same alcohol-cue pairing technique to: 1) replicate the originally published effects in a larger sample, and 2) determine if responses to the alcohol-paired CS+ are associated with alcoholism risk factors (familial alcoholism, recent drinking, self-reported personality measures, impulsive choice behavior). Utilizing a sample

containing both sexes and a wider range of drinking should enable greater power to detect correlations with risk factors. As our original findings suggested prediction error effects in heavy drinkers, we therefore sought to test the hypothesis that elevated alcohol risk factors would correlate with larger prediction error effects (a depressed CS+ signal) in dorsomedial prefrontal areas. However, as is apparent in the results below, we did not replicate reduced activation to the CS+ in dorsomedial PFC, as in the original report. To better understand our current results, we performed whole-brain analyses to search for CS+ effects in brain circuits involved in reward, attention, and associative conditioning in a system comprising cortical (orbitofrontal, dorsolateral, and parietal), and striatal regions (Jarbo and Verstynen 2015). We then related alcoholism risk factors to the observed CS+ responses.

MATERIALS AND METHODS

Subjects

Subjects signed informed consents prior to study procedures, all of which were approved by the Indiana University Institutional Review Board. Sixty right-handed drinkers (Table 1) were tested in what they were told was a study of how alcohol and other drugs affected reaction time (RT). We largely recruited heavy drinkers, defined as drinking harmful amounts (Gunzerath et al. 2004) at least twice/month, although some moderate drinkers were included to provide a range of drinking for correlational analyses. Exclusions included contraindications for MRI, pregnancy, head injury with loss of consciousness, DSM-IV Axis I diagnoses or current psychotropic treatment, desire for alcohol use disorder treatment, current or prior dependence on illicit drugs, or a positive urine screen for commonly abused drugs (exception: one subject tested positive for marijuana, but showed no acute manifestation of altered mental state). The 35-day Timeline Followback self-report (TLFB; Sobell et al. 1986) from the in-person interview was used to estimate recent drinking, and the Alcohol Use Disorders Identification Test (AUDIT; Saunders et al. 1993) assessed alcohol-related problems. Drinks per week and drinks per drinking day were scaled by total body water to account for differences in body weight. The Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA; Bucholz et al. 1994) captured a lifetime DSM5 alcohol use disorder (AUD) symptoms, with a cutoff of 3 or more symptoms used to classify probable AUD. Personality traits relevant to AUDs were assessed with the I_7 Impulsiveness Questionnaire (Eysenck et al. 1985), the Sensation Seeking Scale-V (Zuckerman et al. 1978), and an antisocial behavior count derived from the SSAGA (Oberlin et al. 2012). Eleven subjects (18.3%) met criteria for a lifetime diagnosis of AUD. All had breath alcohol (BrAC) measurements of 0.000 upon admission for the study. Given the 1½ day procedure, the three participating smokers were allowed to smoke between experimental periods to avoid nicotine withdrawal. One subject's age of first intoxication was missing from the SSAGA interview, and was excluded from the principal components analysis described below.

Procedures

On study day one, subjects underwent conditioning comprised of two infusion sessions during which they performed what they were told were RT tasks. BrAC of 0.000 was verified before both conditioning sessions. The instructions noted that these tests were to

determine the RT effects of several drugs: alcohol, saline, caffeine, or antihistamine. In actuality, only alcohol or saline was administered, with caffeine and antihistamine mentioned solely to obfuscate expectations (Conrad et al. 2012). After conditioning to alcohol and saline in separate counterbalanced sessions (9:30AM and 2:30PM), subjects stayed overnight in the Indiana Clinical Research Center, and underwent fMRI the next morning (9:15AM).

Intravenous infusion.—Alcohol and saline infusion employed physiologically based pharmacokinetic modeling (O'Connor et al. 1998), which used height, weight, gender, and age to create standardized alcohol exposure profiles for all subjects. The infusions (6% alcohol in half-normal saline or saline alone) were conducted with the subject alone in a sound-proof booth while seated in a recliner; communications with experimenter used a microphone and speaker. A video camera placed in the booth allowed monitoring of the subject during conditioning. Infusion pulses were interleaved with jittered 16-min resting periods, during which the subject could read. Subjects were alerted 1–2 min in advance of the upcoming infusion pulse. Each pulse (six per conditioning run) targeted an especially rapid (and thus salient) breath alcohol concentration (BrAC) ascent of 0.017 g/dL in 200 sec, and a final peak of 0.045 g/dL in 83.5 minutes (see Figure 1a for modeled profiles). BrAC was measured at the peak of the sixth infusion pulse with a Dräger Alcotest® 6510 breath meter.

RT Visual probe task (cue conditioning).—Visual stimuli were conditioned (paired) with alcohol or saline during the disguised RT task, although subjects were not informed of the true nature of the stimulus-infusion pairing. The sole purpose of conditioning was to associate a visual stimulus with either intoxication or saline. Cues were presented during the infusion ascending to each peak, such that CS+ cues were always paired with the ascending limb of the BrAC curve (and CS− cues with saline infusion), but not the BrAC peak *per se*. During the task (e.g., see Field et al. 2004), subjects saw a CS and an adjacent asterisk, both of which disappeared to reveal a green arrow (response probe) that required a timed button-press to identify the arrow's direction; see Figure 1b and Kareken et al., (2012). During each conditioning session subjects saw only two cue types: the cue associated with that condition, (CS+ or CS−) and the asterisk distractor. Conditioned stimuli (CS+ paired with alcohol, CS− with saline) were presented 20 times in a given conditioning run during a single infusion pulse. Six infusion pulses were delivered per infusion session, yielding a total of 120 individual cue presentations per infusion session. An irrelevant CS (CS*i*) was employed for a 20 trial task practice prior to infusion, and the symbols assigned to CS+, CS−, and CS*i* (Figure 1c) were counterbalanced across subjects. The side of the CS+ and CS−, the side of the response probe, and whether the probe was hidden by a CS or asterisk were counterbalanced and matched for frequency. Conditioning was performed with a trackball device similar to the MRI-compatible fiber-optic response box (Current Designs, Philadelphia, PA) used during imaging. The trackball was oriented 90° such that 'up' and 'down' indications corresponded to the left and right buttons, respectively to mimic how the trackball was held during the subsequent fMRI.

Subjective perceptions.—At the peak of each of the six infusion pulses, subjects rated craving and perceived drug effects. Subjects rated “How intoxicated do I feel right now?”, “How stimulated do I feel right now?”, and “How sedated do I feel right now?” on a visual analog scale (VAS) anchored by “Not At All” at zero to “Extremely” at 10. Subjects also rated subjective drug effects, i.e. “How many alcoholic beverages do I feel I have had?”, “How many caffeinated beverages do I feel I have had?”, and “How many antihistamine pills do I feel I have had?” with responses incremented in units of 0.5 drinks or pills ranging from 0 to 10+. These scales were displayed again during imaging. All subject responses were logged by a trackball device during conditioning (Logitech, Newark, CA) and fMRI (Current Designs, Philadelphia, PA). After fMRI, subjects reported which infusate they thought they received during each of the conditioning sessions, and fMRI. Two subjects did not report assessments of what they thought to be in their infusates (post-scan).

Delay discounting.—Impulsive choice was quantified with a monetary discounting task, which subjects performed while seated in the lab at 9:00AM on Day 2. Delayed money amounts of $20 and $200 were used with delays of 2 days, 1 week, 1 month, 6 months, 1 year, and 5 years. Subjects were told that one of their choices would be chosen at random and paid according to their selection. For each delay, the immediate amounts were initially half the delayed, and adjusted on subsequent trials by halves (Du et al. 2002). For example, a trial is presented as, “Which would you prefer: $10 today OR $20 after 6 months”. If the subject chose the delayed option in the current example, the options for the next trial (within that amount/delay combination) would be “$15 today OR $20 after 6 months”. Amount/delay combinations and the side of the screen on which the options were presented were pseudorandomized. Subjects completed sixty trials—five trials for each of the six delays, duplicated for the two amounts. The task took approximately 10 minutes to complete, and choice behavior could not shorten the task length. Forty-six subjects yielded usable discounting data. Among the remaining fourteen subjects, three were excluded for nonsystematic discounting (per Johnson and Bickel 2008), two were lost to technical problems, and nine completed their study participation before the task implementation was feasible.

fMRI Methods

RT probe task presentation.—During fMRI, subjects performed a probe task similar to the conditioning paradigm with one major difference: all three CS symbols were presented during each scan (6:19 min). This task was designed to elicit a conditioned response to the symbols previously paired with alcohol. Each symbol was presented 16 times per scan (16 * 3 = 48 symbol presentations per scan), with ordering and inter-stimulus time jittering (3–13 sec) scheduled by OptSeq2 (http://surfer.nmr.mgh.harvard.edu/optseq/), a tool to optimize rapidpresentation event-related fMRI design. Stimuli were back-projected onto a screen at the rear of the scanner bore, which subjects viewed with the head coil mirror. To preserve expectations as during conditioning, subjects retained their IV cannulae during imaging and were told that they could again receive any of the drugs. However, no infusion was delivered during the imaging, and the IV pumps remained off. Implicit visual attention biases were inferred by assessing differential RTs between cue conditions (e.g. shorter RTs when the CS + appeared on the same side as the arrow probe, relative to the CS−).

Image acquisition.—Four blood oxygenation level dependent (BOLD) functional scans were acquired on a Siemens 3T Magnetom Skyra (Erlangen, Germany) scanner using a 20-channel head coil array: echo planar imaging (EPI), gradient echo, 183 BOLD volumes, repetition/echo time TR/TE=2100/29ms, flip angle 77°, matrix 80×80, 35 interleaved 3.2mm thick slices, 2.75 × 2.75 × 3.2mm^3 voxels, and GRAPPA acceleration factor 2. A high resolution anatomical scan (1.05 × 1.05 × 1.2mm^3 voxels, 3D magnetization prepared rapid gradient echo; MPRAGE) was used to position slices for subsequent BOLD scans. A gradient echo field mapping scan (TR=500ms, TE1/TE2=5.00/7.46ms) with an imaging volume and voxel size identical to BOLD EPI was acquired immediately before the first BOLD scan. This 1:31 min scan with an advanced B0 shim mode adjustment optimized the field homogeneity and facilitated the BOLD EPI volume distortion evaluation and unwarping, allowing improved localization most notably in frontal areas. Subjects' head movement and motion-related artifacts were minimized by using foam pads and real time prospective acquisition motion correction (Thesen et al. 2000), with additional steps accounting for head motion as detailed in the image analysis section.

Image analysis.—Image preprocessing and all analyses utilized SPM8 (Wellcome Department of Imaging Neuroscience, University College, London, UK), except for BOLD volume unwarping and motion outlier tagging performed in FSL (FMRIB Software Library v 5.0.9, Oxford, UK; Smith et al. 2004). Image preprocessing of functional EPI scans included BOLD volume unwarping using FSL's topup/applytopup with slice-time acquisition correction, rigid-body realignment, and co-registration implemented in SPM8. Each subject's MPRAGE structural MRI was segmented in SPM8, with those transformation parameters used to convert BOLD volumes into Montreal Neurological Institute (MNI) stereotactic space. The resulting normalized volumes were interpolated to 2mm/side isotropic voxels and smoothed by a 6mm full-width at half-maximum isotropic Gaussian kernel. Within-subject fixed effects of BOLD response to CS trials were estimated using SPM's canonical hemodynamic response function. CS+, CS− , and CS*i* conditions were modeled to have an onset coinciding with the symbol appearance and duration of 3 sec. The model regressors included six head motion parameters from the SPM8 realignment step and two FSL metrics (frame displacement and DVARS from fsl_motion_outliers) recommended for tagging outlier BOLD volumes corrupted by large motion (Power et al. 2012). An autoregressive AR(1) model accounted for serial correlations with a high-pass filter set to (1/128 Hz) to remove low-frequency noise. Random effects SPM models focused on CS+ and CS− in a 2 (CS type) × 4 (scan) SPM factorial model.

Statistics

Non-imaging statistics employed SPSS 24 (IBM Corp., Armonk, NY). Alpha was set to 0.05 for non-imaging analyses; in-text means are ± standard deviation, unless noted.

Component extraction of self-report scales.—To condense risk factors, principal components analysis (PCA) with Varimax rotation and the Kaiser criterion was used for variable reduction on recent drinking (normalized by total body water to account for body size differences), drinking problems, early use risk, and personality inventories. Individual measures were assigned to components based on loadings > 0.6.

RT probe task: conditioning.—A 2 (order; alcohol first, saline first) × 2 (infusion; alcohol, saline) × 6 (run) mixed ANOVA assessed alcohol or order effects on accuracy and median RT.

RT probe task: imaging.—To assess for effects of classical conditioning on implicit attention, we performed a 2 (CS/probe side; same, opposite) × 3 (CS type; CS+, CS− , and CS*i*) within-subjects ANOVA on median RT and accuracy of responses to probes during scanning. Subjects' correct identification of alcohol or saline infusates (reported after imaging) was determined with χ^2 tests of goodness of fit against chance (25%).

Delay discounting.—Discounting behavior was quantified using hyperbolic regression:

$$V = \frac{A}{1 + kD}$$

The subjective value (indifference point) V equals the amount of the delayed option A divided by the denominator (1 + fitted parameter k times delay D); k indexes slope such that larger values indicate greater preference for immediate rewards (Mazur 1987). Values of k were calculated for both $20 and $200, and normalized by natural-log transformation.

Subjective perceptions.—A 2 (infusion) × 7 (timepoint: baseline and six infusion pulses) repeated-measures ANOVA assessed differences in perceptions during infusion; significant interactions were followed by paired *t*-tests to identify differing time points.

fMRI: We examined alcohol conditioned responses within a reward/attention/executive system likely to be engaged by a reward conditioning task requiring attentiveness and executive function (dorsolateral frontal, parietal, and orbitofrontal cortices and striatum; Jarbo and Verstynen 2015). The frontoparietal network (comprised of dorsolateral frontal and parietal cortices) is implicated in a range of functions; flexibility, inhibition, working memory, initiation, planning, and vigilance (meta-analysis of 193 studies; Niendam et al. 2012); i.e., abilities required by the present task. The orbitofrontal network forms associations between sensory representations of stimuli and value outcomes for drug (and non-drug) reward, illustrated by reinforcer devaluation studies with food (Kringelbach et al. 2003; Kringelbach and Rolls 2004). Regarding alcohol specifically, the flavor of a preferred beer (i.e., a naturalistic alcohol CS) activates bilateral OFC relative to an appetitive control (Oberlin et al. 2016), demonstrating orbitofrontal involvement in conditioned drug reward. The striatum, especially the ventral striatum, responds to drug-conditioned stimuli (Oberlin et al. 2013; Schacht et al. 2013), is key to reinforcement learning (Schultz et al. 1997), and heavily connected with cortical regions important to reward, attention, executive function, and action (Haber and Knutson 2010; Jarbo and Verstynen 2015). Together, these linked networks form a system adapted to detecting, attending to, and acting on reward (Sesack and Grace 2010), and which should respond to classically conditioned alcohol stimuli. We selected cortical regions of interest defined in resting state fMRI (17 networks; Yeo et al. 2011); Frontoparietal (networks 12 and 13), and Orbitofrontal (network 10). The striatal ROI was defined by Mawlawi et al. (2001).

fMRI:BOLD responses to alcohol conditioned stimuli.—The contrasts of greatest interest involving infusion-paired stimuli, i.e. [CS+ > CS−], were included in the random effects SPM model. Statistical significance was inferred at the cluster level (p_{FWE}<0.05) using family wise error (FWE) correction for multiple comparisons across the whole brain, with cluster forming height threshold set to p= 0.001, uncorrected (Eklund et al. 2016). To assess the possible contributions from smokers or subjects who satisfied criteria for a lifetime DSM AUD diagnosis, we performed the analysis with those subjects removed, for ns = 57 and 49, respectively. Potential extinction effects were assessed using within-subjects ANOVA (4 scans) on mean [CS+ > CS−] values from significant clusters of activation. As alcohol-attenuated encoding of the CS+ could make it novel relative to the CS− cue, cue novelty effects were separately examined by comparing the CSi to CS−. Effects of infusion order were assessed with t-test in subjects receiving alcohol first (n = 31) vs. saline first (n = 29).

fMRI:BOLD correlation with other factors.—We tested for relationships between alcoholism risk factors and BOLD contrasts of interest using voxelwise correlations with family history, AUD risk factors/personality (from PCA), and delay discounting behavior. Family history was tested as both a correlation (ordinal ranking of genetic risk by degree of relatedness; zero AUD relatives = 0, 2nd degree AUD relatives only = 1, 1st degree AUD relatives = 2) and as extremes (subjects with zero AUD relatives vs. subjects with first degree AUD relatives). To capture correlations relevant to the conditioned response to alcohol-paired stimuli, we limited the search region to responding voxels from the [CS+ > CS−] contrast, within network regions.

Comparison with previous conditioning study.—To address the possibility of sample effects, we assessed the similarity of the current sample population relative to our prior study (Kareken et al. 2012) by testing for the equivalence of demographic and drinking variables. Sex, family history of alcoholism, and race were assessed with χ^2 tests of independence; age and education with t-tests; and recent drinking (drinks per week, heavy drinking days per week, drinks per drinking day, AUDIT) and drinking age (age of first drink, age of first intoxication, age of regular drinking) were tested with mixed ANOVA using study as the between-subjects factor. Means are reported collapsed across levels in the absence of interactions; main effects or interactions with study are summarized in Supplemental Table S1. Factors that differed were then tested in the current neuroimaging data to assess their possible influence on non-replication.

RESULTS

Pavlovian Conditioning

Ethanol infusion and perceptions.—The measured BrAC mean of 0.044 ± 0.007 g/dL (at the end of alcohol conditioning) closely matched the target peak BrAC of 0.045 g/dL. Significant main effects and interactions were detected for subjective intoxication, sedation, and number of drinks (Fs(6,354) > 2.66, ps < 0.018). Successive alcohol infusion pulses increased the number of alcoholic drinks perceived (paired tests: ts(59) > 5.4, ps < 0.001), feelings of intoxication (ts(59) > 5.0, ps < 0.001), and sedation (ts(59) > 2.4, ps < 0.018 for

the last two infusion pulses); only a main effect of infusion was present for stimulation ($F(1,354) = 15.1$, $p < 0.001$); Figure 2. Perceived effects from other drugs (caffeine or antihistamine) were not present, interaction ps > 0.3. Post-scan, 69% of subjects correctly identified alcohol as the infusate for the CS+ conditioning session (χ^2 (3, N=58) = 144.5, $p < 0.001$ relative to chance), but subjects identified saline at chance rates ($p > 0.2$), with only 36% of subjects correctly naming saline as the infusate for the CS− session (52% named caffeine or antihistamine).

RT probe task: conditioning.—Neither infusate nor infusion order affected response accuracy during cue pairing (main effects and interactions, $n = 60$, ps > 0.19), with mean accuracies of 97.2 ± 2.3% and 97.5 ± 1.9% for alcohol and saline, respectively.

RT probe task: imaging.—Neither conditioning (CS) nor side congruence (CS on the same or opposite side as probe) affected median RT or accuracy ($n = 60$, ps > 0.24 and 0.38, respectively), with the interactions of CS × side congruence being non-significant (ps > 0.33). Collapsed across side, mean RTs were 491 ± 92, 491 ± 92, and 489 ± 94 msec for CS +, CS−, and CS*i* respectively; with mean accuracy of 97.6 ± 2.0%.

Personality scales and drinking data

Component extraction.—Fifteen self-reported drinking and personality factors reduced to five components with eigenvalues > 1.0; Recent drinking/drinking problems, Impulsive/antisocial, Age of drinking onset, Thrill-seeking, and Experience seeking ($n = 59$); Table 2.

fMRI

Differential activation from geometric symbols.—The symbols, without regard to CS assignment, showed no main effect of symbol type in $n = 60$. Thus, the individual symbols alone, independent of CS assignment, were equivalent in regional activation.

Activation to alcohol-conditioned cues.—CS+ (relative to the CS−) elicited distributed activation in the frontoparietal, attentional, and orbitofrontal networks (Table 3 and Figure 3a,b; also see Supplemental Table S2 for extended results). There was no significant activation either in the striatum, or as in our prior report (Kareken et al. 2012), in the [CS− > CS+] contrast. Mean activation did not differ by scan in the full sample, $n = 60$, $F(3,177) = 1.45$, $p = 0.23$. The effect of CS+ by scan and cluster are illustrated in Supplemental Figure S1. Removing AUD subjects had little effect: the right supramarginal gyrus result dropped to trend level ($n = 49$, peak voxel at [46, −38, 54], $Z = 3.71$, $p < 0.001$, extent = 113, $p_{FWE} = 0.102$), but the other five clusters remained significant. Interestingly, the left orbitofrontal cortex exceeded the significance threshold, (peak voxel at [−26, 36, −18], $Z = 4.67$, $p < 0.001$, extent = 156, $p_{FWE} = 0.032$). Removing smokers yielded similar results, with all six clusters from Table 3 meeting significance, and an additional significant cluster in the left middle frontal gyrus ($n = 57$, peak voxel at [−34, 52, 12], $Z = 3.88$, $p < 0.001$, extent = 157, $p_{FWE} = 0.031$). There were no effects of infusion order, p_{FWE}s > 0.99.

Novel cue effects.—The [CS*i* > CS−] revealed no activation overlapping with the [CS+ > CS−] results, and no results were detected in the [CS− > CS*i*] contrast.

Correlation of activation with delay discounting.—Preference for delayed money (*ln k*, $20) correlated with a cluster of [CS+ > CS−] activation in the right inferior parietal cortex (supramarginal gyrus, BA 40, frontoparietal network, $n = 46$, peak voxel at [46, −44, 46], $Z = 3.35$, $p < 0.001$, extent = 13; Figure 4). The $200 amount was qualitatively similar (peak [46, −44, 46], $Z = 3.40$, $p < 0.001$, extent = 9). Preference for immediate money was uncorrelated with [CS+ > CS−] activation.

Correlation of activation with alcoholism risk factors.—Family history, either as a continuum or extremes, was uncorrelated with activation. None of the five AUD risk/personality components derived from PCA correlated with [CS+ > CS−], either positively or negatively ($n = 49$).

Comparison of factors differing by study and imaging.—Although our previous study showed significant [CS− > CS+] medial frontal activation, the present study did not replicate that finding. Factors that differed between studies were sex, χ^2 (1, N=74) = 8.29, $p = 0.004$; heavy drinking days per week, $t(72) = 2.67$, $p = 0.009$; AUDIT, $t(72) = 2.70$, $p = 0.009$; drinking age, $t(71) = 2.04$, $p = 0.045$; FH, χ^2 (1, N=73) = 6.29, $p = 0.012$ (one subject was FH unknown), age, $t(72) = 3.11$, $p = 0.003$; education, $t(72) = 2.38$, $p = 0.020$; and race , χ^2 (1, N=68) = 7.23, $p = 0.007$, see Supplemental Table S1 for means and summary. These factors were tested in the current (larger) imaging data set ($n = 60$) to evaluate their possible role in explaining the differing results. As examined in *t*-tests of the [CS+ > CS−] contrast, the factors of sex, family history, or race (white vs. others [n=11]) did not affect the direction of the CS effect, nor did the BOLD contrast effect correlate with, or change according to, heavy drinking days/week, AUDIT, drinking age, education, or age.

DISCUSSION

The principal value of a method for creating conditioned associations between novel, neutral stimuli and alcohol intoxication is the capacity to control learning with greater experimental precision. This avoids varied, uncertain learning histories and idiosyncratic associations that exist with previously conditioned stimuli (e.g., a picture of a beer glass). Our small (n=14) proof of concept study (Kareken et al. 2012) suggested that when the BOLD fMRI response to stimuli experimentally paired with the rapidly rising limb of intravenously infused alcohol was measured in extinction, there was drop in CS+ activity in medial frontal cortex. This suggested a kind of prediction error from CS+ presentation during non-reinforcement (Schultz et al. 1997). Using virtually identical methods, this considerably larger (n=60) sample including both sexes did not replicate that preliminary result. Using a strict whole-brain corrected statistic with a robust cluster definition (Eklund et al. 2016), we show here that, in and of themselves, the geometric forms used for stimuli induced essentially equivalent activation. However, those stimuli that were associated with alcohol intoxication (CS+) elicited significantly greater activation in a fronto-parietal attentional network. By contrast, there were no regions in which CS-activation induced greater activation than CS+ stimuli. The resulting activation associated with the CS+ was nevertheless unrelated to drinking and alcoholism risk factors. The outcomes of this line of work offer some perspective on the challenges of measuring the human brain response to stimuli associated with alcohol intoxication in the laboratory.

As in the original study, we covertly paired novel visual stimuli with alcohol and saline infusions. Our current findings showed CS+ eliciting significant BOLD responses widely in frontoparietal and orbitofrontal regions relative to the CS−, suggesting a possible conditioned response. However, cue pairing did not result in differential speed of target identification as a function of CS interference, as would be predicted by behavioral conditioning. Subjects experienced the unconditioned stimuli (alcohol and saline) as intended, as evidenced by their subjective ratings and BrACs. Delayed money preference correlated with CS+ activation within the frontoparietal network. Alcoholism risk factors such as family history and other drinking and personality factors did not appear to mediate [CS+ > CS−] activation. As the symbols were presented in extinction, we might expect reduced [CS+ > CS−] by scan. We nevertheless failed to detect such effects. An alternative explanation for our findings is that the CS+ cue possessed some novelty due to attenuated encoding (intoxication during conditioning) relative to the CS− cue (conditioned while sober), but we did not detect novelty effects with the more novel (less frequently presented) CS*i* cue in the [CS*i* > CS−] contrast.

Stimuli successfully conditioned to intoxication should engage brain systems involved in: 1) object recognition/classification, 2) attention, 3) conditioned reward response, and 4) salience marking. Our CS+ elicited activation within frontoparietal and orbitofrontal, as well as salience networks (anterior cingulate and insula). The resulting frontoparietal [CS+ > CS −] activity, especially in parietal cortex, suggests “top-down” attention in which subjects conduct a goal-directed search for visual stimuli (Corbetta et al. 2008; Corbetta and Shulman 2002) as required by the conditioning task. In particular, the brain’s attentional system has distinct dorsal and ventral elements, with the dorsal element thought to manage goal directed searching and executive activity, and the ventral dedicated to attentional orientation to unexpected events (the “bottom-up system”; Corbetta and Shulman 2002). More broadly, frontoparietal regions also strongly interconnect with other brain networks (Spreng et al. 2013), and govern a range of executive functions, including flexibility, inhibition, working memory, initiation, planning, and vigilance (Niendam et al. 2012; Seeley et al. 2007; Sridharan et al. 2008). While the stimuli paired with alcohol infusion provoked activation in the dorsal attention systems (including the frontal eye fields), saline paired stimuli showed no such BOLD increases (relative to CS+). Therefore, although the cue pairing was never made explicit to subjects, and CS presentation preceded the goal (response probe), we believe that the greater BOLD activity from the CS+ symbols suggests that these cues elicit a greater degree of attentional neural processing than the CS− symbols. While there was no separate control group lacking alcohol conditioning, equivalent activation across all stimuli in these areas (collapsing across infusion condition) lends credence to this interpretation.

The orbitofrontal network is deeply involved in valuation, evidenced by its correlated activation with imagined reinforcer value (see Sescousse et al. 2013 for meta-analysis) and subjective liking of food reinforcers (Kringelbach et al. 2003; Plassmann et al. 2010). Associative conditioning also involves orbitofrontal regions, as evidenced by activation to conditioned visual cues presented in the absence of unconditioned stimuli (Cox et al. 2005). We previously demonstrated similar bilateral orbitofrontal activation to a different alcohol CS; the taste of a favorite beer (Oberlin et al. 2016). These previous results were similar,

with the right OFC peak from that study [22 36 −14] falling within the current right OFC cluster. That prior study relied on drinking history to create the cue pairing (conditioning) as do most cue reactivity studies, while in the present context we experimentally induced the conditioning. Our current results within the salience network (Uddin 2015), i.e. the anterior cingulate cortex and anterior insula , comports with its known role in modulating attention by integrating sensory information and interoceptive states (Craig 2002; Damasio 1994). This enables organisms to respond correctly to stimuli (Seeley et al. 2007) by co-activating with frontoparietal systems, and assisting in keeping the most appropriate network active (Sridharan et al. 2008). The current paradigm might then be expected to engage such attention-modulating systems, as adaptive brains require efficient evaluation of reinforcers in the environment and the capacity to direct attention to the most salient features.

A large body of previous work implicates the inferior parietal lobule (IPL; including supramarginal gyrus/ BA 40) in intertemporal choice tasks (Clithero et al. 2009; Luhmann et al. 2008; McClure et al. 2004; Peters and Büchel 2010; Stoeckel et al. 2013; Wittmann et al. 2007). These tasks involve executive attention for valuation of abstract rewards. Our findings reveal that right IPL [CS+ > CS−] activation correlates with greater preference for delayed money in delay discounting. Insofar as we know the IPL to activate to all choice types during delay discounting tasks, the data here suggest that patient subjects (shallow discounters) may be those whose attentional networks respond more to salient stimuli—here, mental representations of reward either conditioned (CS+) or imagined (monetary). Exploring this hypothesis further would require imaging of both tasks.

Naturalistic cues for alcohol and other drugs activate a number of limbic and cortical foci in heavy users, including the striatum, amygdala, thalamus, pallidum, inferior parietal lobule, middle frontal gyrus, insula, anterior and posterior cingulate cortex, middle frontal gyrus, and others (meta analyses: Engelmann et al. 2012; Kuhn and Gallinat 2011; Schacht et al. 2013), with the anterior cingulate and ventral striatum being largely consistent across many studies. Our anterior cingulate result is analogous to the naturalistic cue findings, suggesting that the salience marking performed in the cingulate during drug associative conditioning may be unspecific to particular cue characteristics, such as context, odor, or visual properties. We did not observe effects in the ventral striatum, as might be expected from prior work, although we did find sub-threshold activation (peak [8, 14, −2], Z=3.00, p=.001, k = 4). However, the neutral cues used here are more similar to another conditioning study showing orbitofrontal, but not striatal, activation to CS+ presented in extinction (Cox et al. 2005).

While we detected activation in attentional brain systems, the CS+ did not induce a behaviorally detectable attentional bias in this sample relative to the CS−. The absence of conditioned behavioral responses suggests that we were unable to detect classical conditioning in the conventional sense. However, attentional biases in similar paradigms tend to be small—less than 20 msec reaction time enhancement by drug cues (Bradley et al. 2004; Lubman et al. 2000; Townshend and Duka 2001). The ideal behavioral assessment would measure conditioned responses under exactly the same conditions as during conditioning, however our primary goal of measuring neural responses precluded this design. Test-retest correlation of RT tasks in vs. out of the scanner can be modest (e.g.,

Weafer et al. 2017), likely due to noise and awkward positioning required by scanning. We might have increased sensitivity to conditioned responses with behavioral retesting after fMRI in the identical context as conditioning. Another possible explanation for our insensitivity to attentional bias with conditioned (intrinsically neutral) stimuli is simply the amount time spent in cue pairing. Our subjects experienced 120 2-sec CS presentations during alcohol infusion (4 min total cue pairing time), while naturalistic cues utilizing real-world conditioning, i.e. drinking (used in most studies) presumably involve many hours of CSUS pairing in heavy drinkers. Utilizing naturalistic cues, while uncontrolled, takes advantage of the robust CS-US pairing inherent to a long drinking history and likely enhances detecting attentional biases. Recent work utilizing neutral cues have demonstrated conditioning in just two CS+ conditioning sessions (Mayo and de Wit 2015; Mayo and de Wit 2016; Mayo et al. 2013; Van Hedger et al. 2018), but these studies used 60 minutes of CS+ cue pairing, which likely generated more robust conditioning than the current study. Another possible mediator of the lack of behavioral conditioning is explicit awareness of conditioning. Awareness of the conditioning contingencies could have affected behavioral and/or neural conditioned responses, but we did not explicitly ask subjects about their awareness.

The non-replication of the original study (Kareken et al. 2012) could be influenced, in theory, by different sample characteristics (detailed in Supplemental Table S1). The most notable demographic differences were sex (the previous study included only males) and drinking factors (we previously tested heavier drinkers with greater family history load). However, these do not obviously mediate the non-replication, as none of these factors changed the results in the current study when tested individually. There were minor experimental design changes in the current study: 1) we obfuscated the infusion type (subjects were instructed that they could receive ‘alcohol, saline, caffeine, or antihistamine’, while the prior study’s instructions exclusively referred to alcohol and saline), and 2) minor differences in data acquisition (3T Trio vs Skyra) and fMRI analyses (such as better accounting for BOLD outliers). However, we re-analyzed data from the prior study using the present fMRI methods and found that the results were qualitatively similar to the original processing stream. In the end, we are left with greater confidence in the larger (n=60 vs. n=14), more representative, and better characterized data set; the original study may have simply been underpowered.

This systems-level response to alcohol-paired cues, although differing from the original study, offers some perspective on this experimental approach to understanding the brain response to alcohol-associated stimuli. The present findings suggest (with the caveats above) that neutral stimuli might be covertly conditioned to alcohol intoxication, and that the resulting CS+ can induce differential activity in brain networks involving attentional/cognitive executive function (object recognition/classification, attention; frontoparietal network), reward/value perception (value magnitude and valence; orbitofrontal network), and stimulus importance (salience network). Further, activation within the frontoparietal network correlated with intertemporal choice behavior, implicating an area common to both alcohol cue conditioning and decision making, and suggesting BA 40’s involvement in two processes important to addiction—although we note that these neural processes may not be specific to addiction. We believe that addiction pathology involves conditioning mechanisms

in addition to learning, motivation, reward, and cognitive processes, and argue that studying drug cue conditioning processes in isolation might best be done using neutral cues (i.e. eliminating drug- or experience-specific features of conditioning). While we believe that controlled conditioning paradigms are key to parsing conditioned reward processes, future work will need to replicate these current findings, and test more directly for hypothesized regional attentional and salience mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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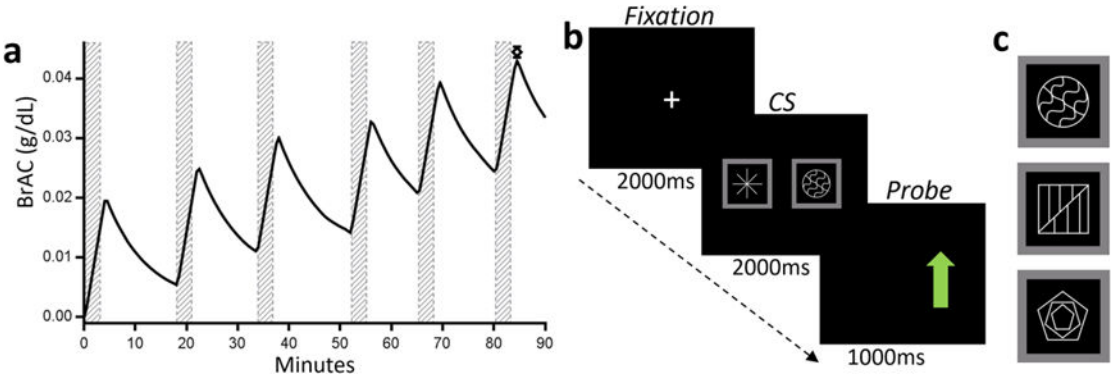

Fig. 1.

(a) *Alcohol infusion/conditioning.* CS symbol presentation (shaded bars) corresponded to rising brain alcohol concentration during six modeled infusion pulses; equivalent saline volume was infused during the saline session. Measured BrAC (mean ± SEM) at final peak for n=60 indicated as open diamond. (b) *RT probe task.* Subjects were repeatedly shown conditioned stimuli within a disguised "reaction time task". The CS+ was paired with alcohol infusion, and the CS− was paired with saline. CS*i* was used in a practice session, but was not paired with infusion. (c) The three symbols that were randomly assigned to CS+, CS −, or CS*i* conditions.

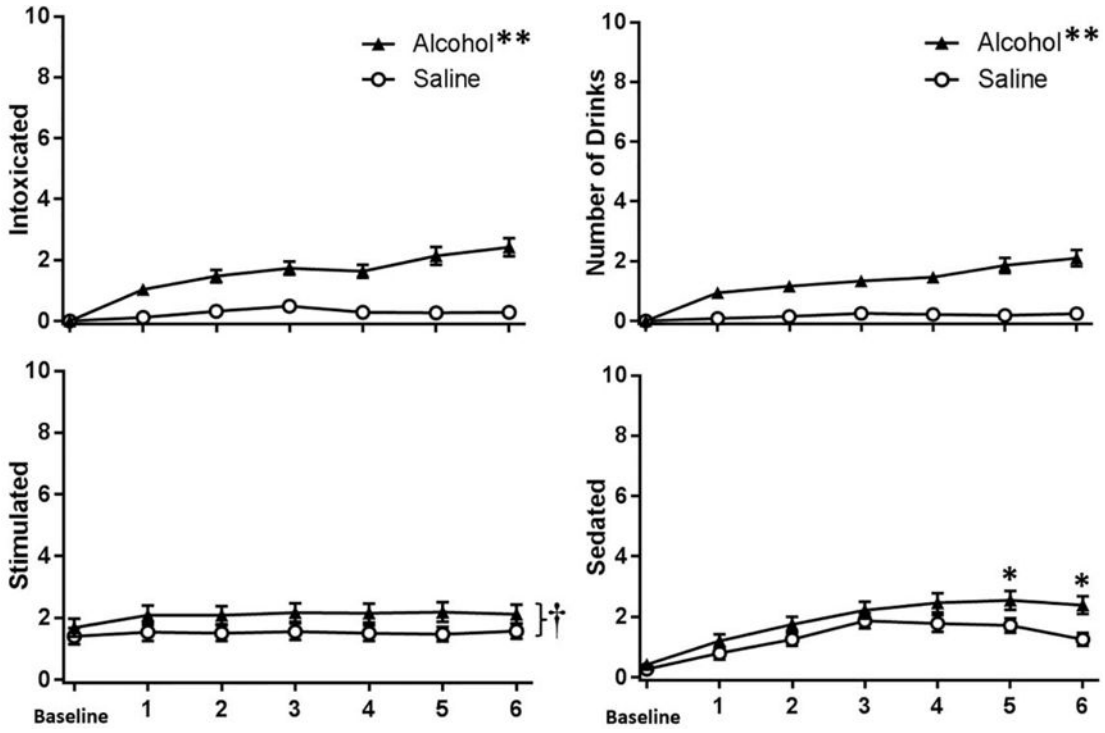

Fig. 2. Perception of alcohol effects during conditioning.

Plots showing significant main effects of infusion or interactions with time. Ratings obtained at the end of the six infusion pulses (see Figure 1A) corresponded with the peak alcohol concentration for that pulse. “Baseline”= pre-infusion ratings. *significant interaction and difference, **all non-baseline points differ, †main effect of infusion.

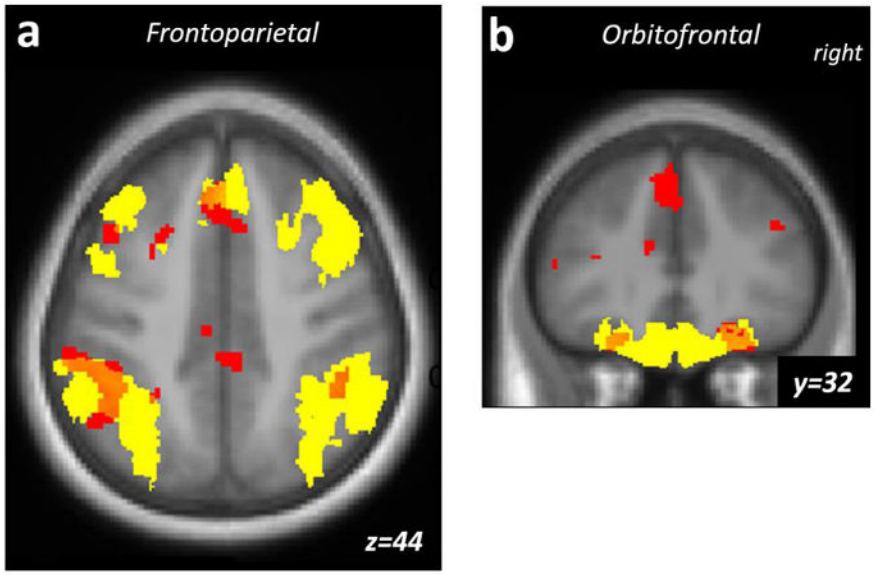

Fig. 3. Activation to alcohol conditioned stimuli [CS+ > CS−].

(a) Axial and (b) coronal activation maps illustrating BOLD response to alcohol conditioned (CS+) relative to saline-conditioned (CS−) stimuli in n=60 drinkers (red), relevant networks (yellow), and the overlap (orange); network labels on top. Display at $p_{uncorr} < 0.001$, extent = 20.

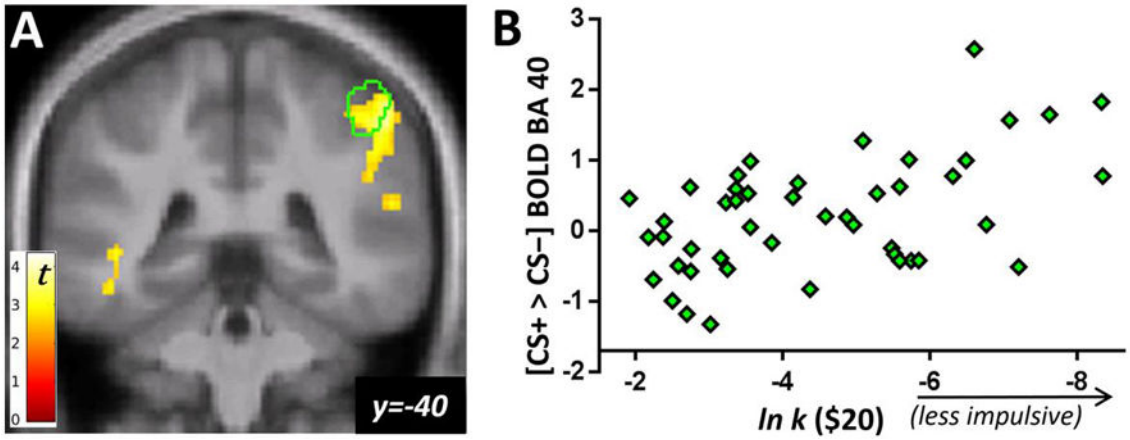

Fig. 4. Patient choice (less impulsive) and correlated activation to CS+.

(a) Activation map showing the region of correlation between preference for delayed money (*ln k*: $20) and activation to CS+ (relative to CS−) in right parietal cortex (BA 40) within the frontoparietal network, n=46. The main effect of [CS+ > CS−] BOLD activation in BA 40 is shown in green outline; displayed at $p_{uncorr} < 0.01$, extent = 200. (b) The nature of the correlation is illustrated by mean extracted values from the functional cluster in (a), indicating that greater BA 40 activation during CS+ presentation corresponds to less impulsive decision making.

Table 1

Subject Characteristics (*n*=60)

	Mean (SD)	Range	*n*(%)
Male			36(60)
Caucasian			50(83)[g]
Never smokers			15(25)
Recent smokers[a]			18(30)
Family history positive[b]			22(37)
Age	23.1 (1.6)	21–27	
Education (years)	15.2 (1.4)	12–19	
Drinks per week[c]	14.6 (9.6)	5–54	
Drinks per drinking day[c]	5.2 (2.6)	1.6–12.3	
Heavy drinking days per week[c,d]	1.4 (0.9)	0.4–4.2	
Age first drink[e]	16.4 (1.8)	12–20	
Age regular drinking[e]	18.9 (1.6)	15–22	
AUDIT[f]	10.3 (3.9)	4–21	

[a] Last exposure within 6 weeks.

[b] At least one first-degree relative with probable alcoholism.

[c] From the Alcohol Timeline Followback Interview (TLFB).

[d] Four/five or more drinks per day for female/male.

[e] From the SSAGA.

[f] Alcohol Use Disorders Identification Test.

[g] Others; *n*s=5 black, 5 asian, 3 mixed-race.

Table 2

Factor loadings by Component, n=59

Factor	Component				
	Recent drinking & problems	**Impulsive/ antisocial**	**Age of drinking onset**	**Thrill-seeking**	**Experience seeking**
Drinks per week[a,b]	**0.92**	0.04	−0.06	0.07	0.05
Heavy drinking days per week[a,c]	**0.90**	0.09	−0.06	0.19	−0.09
Drinks per drinking day[a,b]	0.59	0.14	0.00	0.22	−0.47
AUDIT[d]	**0.84**	0.12	−0.19	0.03	0.17
Age of first drink[e]	−0.04	−0.22	**0.88**	0.01	−0.19
Age of regular drinking[e]	−0.10	−0.17	**0.67**	−0.29	0.04
Age of first intoxication[e]	−0.16	−0.05	**0.87**	−0.17	−0.10
Antisocial behavior density[e]	0.12	**0.60**	−0.07	0.10	0.40
I_7 Impulsivity	0.16	**0.76**	−0.08	−0.01	0.15
I_7 Venturesomeness	0.15	0.24	−0.16	**0.86**	0.16
I_7 Empathy	0.07	**−0.71**	0.15	−0.10	0.16
SSS-V Thrill/Adventure	0.19	0.01	−0.21	**0.87**	0.13
SSS-V Experience seeking	−0.03	0.06	−0.14	0.27	**0.81**
SSS-V Disinhibition	0.42	0.35	−0.20	0.14	0.43
SSS-V Boredom susceptibility	0.16	**0.79**	−0.13	0.12	−0.01

[a] From the Timeline Followback Interview.

[b] Scaled by total body water.

[c] Four/five or more drinks per day for female/male.

[d] Alcohol Use Disorders Identification Test.

[e] From the SSAGA. Bold text: Factor loadings ≥ |0.6| in Varimax rotated solution.

Table 3

BOLD responses to alcohol conditioned stimuli [CS+ > CS−][1]

Region	Network[2]	Cluster size	Peak *Z*	MNI coordinate (mm)		
				x	*y*	*z*
Orbitofrontal cortex (R)	FP, OF	188	5.06	26	32	−20
Anterior cingulate gyrus/medial superior frontal gyrus; BA8, 24, 32	FP	293	4.80	−4	28	42
Middle/superior frontal gyrus (L); BA6, 8	FP	321	4.39	−26	18	50
Supramarginal/angular gyrus (L); BA39, 40	FP, Sal	611	4.15	−62	−32	46
Rostral cingulate/medial superior frontal gyrus; BA32	DMN	243	4.15	4	44	8
Supramarginal gyrus (R); BA40	FP	179	3.82	46	−38	54

Results named by the anatomical extent of the cluster; height threshold, $p_{\text{uncorr}} < 0.001$, cluster corrected, $p_{\text{FWE}} < 0.05$.

[1] The [CS− > CS+] contrast produced no significant results.

[2] Networks of primary interest: Frontoparietal (FP) and Orbitofrontal (OF). Other networks: Salience (Sal), Default Mode Network (DMN). MNI, Montreal Neurological Institute; R, right; L, left; BA Brodmann Area.