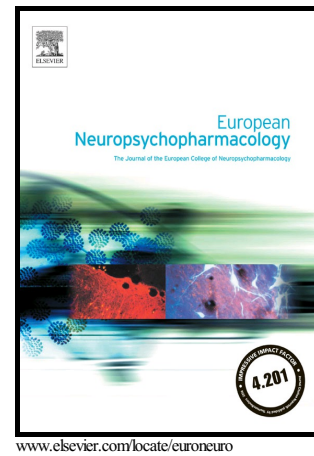


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Nitrous Oxide may Interfere with the Reconsolidation of Drinking Memories in Hazardous Drinkers in a Prediction-Error-Dependent Manner.

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ABSTRACT:

Weakening drinking-related reward memories by blocking their reconsolidation is a potential novel strategy for treating alcohol use disorders. However, few viable pharmacological options exist for reconsolidation interference in humans. We therefore examined whether the NMDA receptor antagonising gas, Nitrous Oxide (N₂O) could reduce drinking by preventing the post-retrieval restabilisation of alcohol memories in a group of hazardous drinkers. Critically, we focussed on whether prediction error (PE; a key determinant of reconsolidation) was experienced at retrieval. Sixty hazardous drinkers were randomised to one of three groups that retrieved alcohol memories either with negative PE (Retrieval + PE), no PE (Retrieval no PE) or non-alcohol memory retrieval with PE (No-retrieval +PE). All participants then inhaled 50% N₂O for 30 minutes. The primary outcome was change in beer consumption and alcohol cue-driven urge to drink from the week preceding manipulation (baseline) to the week following manipulation (test). The manipulation did not affect drinking following the intended retrieval+/- PE conditions. However, a manipulation check, using a measure of subjective surprise, revealed that the group-level manipulation did not achieve the intended differences in PE at retrieval. Assessment of outcomes according to whether alcohol-relevant PE was actually experienced at retrieval, showed N₂O produced reductions in drinking in a retrieval and PE-dependent fashion. These preliminary findings highlight the importance of directly testing assumptions about memory reactivation procedures in reconsolidation research and suggest that N₂O should be further investigated as a potential reconsolidation-blocking agent.

INTRODUCTION:

Many psychiatric disorders can be conceptualised in terms of maladaptive neural plasticity (Bernier et al., 2011; Gerhard et al., 2016; Nisticò et al., 2012; Pittenger, 2013). Such plasticity is usually in response to an environmental challenge that triggers neural adaptation, leading to an eventual hypoplastic disease state that precludes recovery and underlies the chronicity of many psychiatric illnesses (Tronson and Taylor, 2013). In substance and alcohol use disorders, neural and metabolic adaptations to repeated drug use produce allostatic states characterised by tolerance to the abused substance and withdrawal upon cessation (Koob and Le Moal, 2001). Concomitantly, extreme plasticity in mesocorticolimbic circuits produces powerful reward learning (Hyman, 2005), whereby environmental cues and contexts become associated with drug availability and reinforcement to form memory associations that promote a deleterious or harmful level of drug use. These maladaptive reward memories (MRMs) are thought to underlie craving, drug-seeking and relapse by triggering motivational processes in response to drug-associated cues and contexts (Shaham et al., 2003; Sinha et al., 2007). These MRMs support the non-homeostatically-mediated (i.e. post-withdrawal) long-term relapse susceptibility that typifies addiction.

Corrective measures to weaken MRMs have thus far generally eluded psycho- and pharmacotherapists, because of the putative permanence of memory traces once consolidated into stable, long-term storage (McGaugh, 1966). It has historically been thought that MRMs can be indirectly suppressed via extinction learning (the process underlying cue exposure therapy (Drummond et al., 1995) but not directly weakened, evidenced by spontaneous recovery, renewal and reinstatement (Bouton, 2002) of extinguished drug-seeking responses. In recent years, however, increasing attention has been paid to memory reconsolidation (Misanin et al., 1968); a phenomenon whereby consolidated memory traces become briefly unstable in order to update prior to restabilising (Przybylski and Sara, 1997). Reconsolidation may represent a core plasticity process throughout the CNS (Bonin and De Koninck, 2014, 2015) and therefore offers a unique opportunity for long-term or potentially permanent clinical benefit by directly updating or weakening MRMs (Milton and Everitt, 2012).

Genetic, pharmacological and behavioural interrogation has begun to elucidate the determinants and substrates of memory destabilisation and restabilisation (reconsolidation). The *N*-Methyl *D*-Aspartate receptor (NMDAR) and its downstream targets are central to these processes Lee and Everitt, 2008; von der Goltz et al., 2009) Importantly, GluN2b subunit-containing NMDARs are critical to destabilising memory traces (Mamou et al., 2006), while GluN2a subunit-containing NMDARs are needed for restabilisation (Milton et al., 2013). As such, antagonism of NMDARs after reactivation of conditioned drug memory traces is one of the most effective methods for weakening conditioned drug memories in laboratory animals (Milton et al., 2008; Milton et al., 2013; Milton et al., 2012; Das, Freeman & Kamboj, 2013). Post-reactivation NMDAR antagonism is thus a promising potential strategy for weakening maladaptive memory traces in substance and alcohol use disorders. However, it also presents considerable translational challenges, in terms of 1) drug selection and 2) drinking memory reactivation.

Options for tolerable, efficacious approved NMDAR antagonists with favourable pharmacokinetics for reconsolidation research (fast on- and off-set kinetics) are sparse in humans. Oral preparations suffer from an inability to accurately time peak plasma levels with the post-retrieval reconsolidation window (Das et al., 2015b) and if dosed pre-retrieval may prevent memory destabilisation through blockade of the GluN2b – dependent destabilisation cascade. One option is ketamine; a potent non-selective NMDAR antagonist that has shown striking efficacy in several psychiatric disorders (Collins et al., 2010; Feder et al., 2014; Murrough et al., 2013). Intravenous ketamine preparations allow plasma levels to be controlled relatively well (Absalom et al., 2007). However, ketamine produces profound psychological effects with increasing dose. Further, due to its abuse potential, ketamine is a

controlled drug in many countries and its administration for research purposes is limited to intravenous routes in hospital settings. While these issues do not necessarily preclude ketamine as a novel intervention in SUDs, they limit the reach of its clinical application to inpatient settings.

Nitrous Oxide gas (N₂O), also known as ‘laughing gas’, fulfils many of the criteria for a promising reconsolidation-blocker. It acts as an NMDAR antagonist (along with effects at opioid and dopaminergic sites) (Jevtović-Todorović et al., 1998; Ogata et al., 2006; Ori et al., 1989), has an excellent tolerability and safety profile, rapid on-off kinetics and can be administered simply and easily with minimal risk. Despite this, and its long and illustrious history as an analgesic, N₂O has received little attention outside of primary care settings. One recent study showed that N₂O produces similar rapid antidepressant effects to ketamine (Nagele et al., 2015) and we have recently shown that it can interfere with the consolidation of emotional memory traces (Das et al., 2016). Given these qualities, N₂O is the ideal candidate for investigation as a reconsolidation-blocker.

There are two possible mechanisms by which N₂O might affect destabilised memory expression. Firstly it could interfere with the reconsolidation of these memories via NMDAR antagonism; weakening these memories. Secondly N₂O might *update* the destabilised memories, either through incorporating an N₂O-specific internal context into the memory trace, which then becomes re-accessible only in the presence of the drug (Gisquet-Verrier et al., 2015). Alternatively, it may incorporate the subjective/affective effects N₂O itself into the motivational components of the memory trace, updating their strength or valence (Das et al., 2015a). We have previously shown that variation in the subjective response to N₂O is determined by family history of alcohol use disorders (Walsh, Das, & Kamboj, 2017) and its effects may therefore be a marker of endophenotypic variation in genetic/epigenetic substrates relevant to problematic alcohol use. The subjective response to N₂O is thus a potential key moderator its effects on the reconsolidation of drinking memories, particularly if it operates via memory updating and is found to be highly aversive or reinforcing.

The second key issue in leveraging reconsolidation blockade to weaken MRMs in the clinic is that retrieved memories do not always destabilise. Various ‘*boundary conditions*’ at retrieval determine whether memories are destabilised, and reconsolidation subsequently engaged. Of particular relevance to drug and alcohol use disorders is that strongly trained, older reward memories are more resistant to destabilisation at recall than younger, more weakly trained memories (Gräff et al., 2014; Robinson and Franklin, 2010). This has been proposed to be due to the lack occurrence of a prediction-error (PE) learning signal at retrieval, as experimental studies manipulating PE have shown reconsolidation-like effects only when PE occurs at retrieval (Sevenster et al., 2012, 2013, 2014). Current thinking is that reconsolidation is fundamentally a memory *updating* mechanism and *mismatch* between predicted and actual outcomes (PE) is important in initiating the process (Lee, 2008; Osan et al., 2011; Pedreira et al., 2004). Under this hypothesis, for MRMs which have been strengthened to asymptote in many contexts, the potential for PE is low and this may prevent memory destabilisation (Vousden and Milton, 2017).

Artificially generating cognitive PE during retrieval of MRMs may be a means to circumvent this problem. We have previously shown that this can be achieved through guided expectancy violation. By presenting drinkers with alcohol and telling them that they will drink it after showing prototypical alcohol cues (to retrieve alcohol memories), but then withholding the alcohol, a negative PE may be generated, which appears to destabilise even robust, old alcohol memory networks. Using this procedure, we have shown that aversive counterconditioning can be used to reduce the salience and evaluation of alcohol cues in a seemingly generalised manner, consistent with reconsolidation-based memory updating (Das et al., 2015a; Hon et al., 2016). Despite these findings, most reconsolidation studies do not specifically manipulate PE and yet have still shown reconsolidation-interference effects. In these cases, there may be implicit generation of PE but due to lack of measurement it is

currently impossible to determine whether PE is truly necessary for reconsolidation, nor whether it occurs in a binary manner.

In the current paper we therefore used a PE-generating procedure to attempt to destabilise alcohol MRMs in a cohort of *hazardous* beer drinkers, to assess whether subsequent N₂O could weaken MRMs and reduce drinking. Hazardous drinkers are at particularly high risk of transitioning to full alcoholism and are at elevated risk of alcohol-related harms such as mouth, oesophageal and stomach cancer, metabolic syndrome, stroke, psychiatric disorders and physical injury. We hypothesised that retrieval of alcohol MRMs with PE (Retrieval + PE) would cause memory destabilisation and that thirty minutes of 50% N₂O/O₂ (Entonox) administration following this procedure would interfere with reconsolidation of these memories. On the basis that MRMs contribute to drinking levels and cue-induced urge to drink, we hypothesised that this would produce reductions in these measures. If N₂O acted to *update* MRMs, rather than weaken them, we tentatively hypothesised that subjective responses to N₂O (reinforcing vs. dysphoric) would predict subsequent beer drinking levels.

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Participants and Design:

Sixty participants were randomly allocated to receive N₂O following cue-driven retrieval of drinking memories with prediction error (Ret + PE), retrieval of drinking memories with no prediction error (Ret no PE) or prediction error *without* drinking memory retrieval (No Ret + PE). Due to a technical error the wrong task condition at reactivation was deployed for one participant and final group Ns were: Ret + PE N = 21, Ret no PE N = 19, No Ret + PE N = 20. Inclusion criteria were ages >18<65, hazardous drinking as defined by scoring >8 on the Alcohol Use Disorder Identification Test (AUDIT) (Saunders et al., 1993); primarily drinking beer; drinking ≥4 days in 7; normal general physical health; normal or corrected to normal colour vision. Exclusion criteria were historical or current mental health issues requiring treatment; current alcohol use disorder as assessed by the Structured Clinical interview for DSM-IV (First et al., 2012); addiction to any drug other than nicotine, memory impairments, pregnancy or breastfeeding, regular (>1 times per month) recreational use of drugs other than alcohol, nicotine and caffeine, vitamin B12 deficiency and pneumothorax. All procedures were approved by the UCL research ethics committee and medically supervised.

Stimuli and apparatus:

Subjective assessments: The timeline follow-back (TLFB) for alcohol (Sobell and Sobell, 1992) was used to assess drinking levels across the study, the stages of change readiness and treatment eagerness scale (SOCRATES) (Miller and Tonigan, 1996) to assess desire to reduce drinking and the alcohol craving questionnaire (ACQ-NOW) (Singleton et al., 1994) to measure momentary urges to drink. The Spielberger State-Trait Anxiety Inventory: Trait version (STAI-T) was used to assess baseline and Positive and negative affect scale (PANAS) (Watson et al., 1988) to assess drug-induced affect changes. The Clinician-administered dissociative states scale (CADSS) (Bremner et al., 1998) measured drug-induced dissociation and the VAS-based bodily symptoms scale (BSS) (Bond and Lader, 1974) to measure subjective drug effects. We have previously shown stimulant/sedative ratio in response to N₂O calculated from the BSS to be a potential pharmaco-endophenotype for problematic alcohol use (Walsh, 2016).

Nitrous Oxide was supplied as Entonox (BOC, UK), a pre-mixed solution of 50% N₂O in oxygen. Drug was administered via an ultraflow-on demand valve regulator connected to a nose-and-mouth mask (BPR Medical, London, UK), that was tightly fitted to the participants to prevent rebreathing and minimise variation in the concentration of N₂O inhaled.

MRM retrieval and control stimuli:

Stimuli and retrieval procedures were the same as those used in our previous study (Das et al, 2015), with the addition of a ‘surprise’ rating following the retrieval +/- PE procedure. Briefly, alcohol ‘cue’ stimuli consisted of seven images of beer, four of which were to be MRM ‘retrieval’ cues, used in *Ret + PE* and *Ret no PE* groups on *Day 2* (*Beer Retrieval* cues). Three beer images were presented at baseline and test, but not during MRM retrieval on *Day 2* to assess within-category generalisation of any manipulation effects (*Beer Non-Retrieval* cues). Three pictures of wine were also rated on *Day 1* and *Day 3* assess generalisation of effects to other alcohol images (*Wine* cues). Non-MRM retrieval images for use in *No Ret + PE* on *Day 2* consisted of four pictures of orange juice (*OJ* cues). In all conditions, two control soft drink images of coffee and cola were used to assess generalisation of effects to reward stimuli unrelated to the memory networks being retrieved (*soft drink* cues).

Procedure:

A three-day testing protocol was utilised. All testing occurred mid-afternoon to early evening, during the period would normally be drinking, to prevent any time-of-day or state dependency effects.

Day 1 (Baseline)

After screening and completing informed consent, participants completed the SOCRATES, STAI-T, CEOA, HADS, TLFB and ACQ-NOW. Following this, participants rated all cue images (*Beer Retrieval*, *Beer Non-Retrieval*, *Wine*, *OJ* and *Soft drink* cues) to provide a baseline measure of subjective cue pleasantness and cue-induced urges to drink. Each cue image was presented for 10 seconds on-screen and participants rated out loud how pleasant they found the image from 0- 'Extremely Unpleasant' to 10 - 'Extremely pleasant' and how much the image affected their *urge to drink beer* from 0 - 'Greatly reduced urge to drink' to 10 'Greatly increases urge to drink beer'.

Day 2 (MRM Reactivation/No Reactivation)

Participants returned to the study centre 48-72 hours after *Day 1* and began the appropriate retrieval/PE task. In the *No Ret + PE* group, a 150ml glass of chilled orange juice was placed in front of the participants and they were instructed that they would '*drink this after rating some images*'. Participants were told they must consume the drink according to a series of on-screen prompts reading 'PICK UP DRINK', 'PREPARE TO DRINK' and 'DRINK NOW' and that they were not to drink until 'DRINK NOW' appeared. They then rated the orange juice and soft drink pictures for pleasantness and effects on urges to drink the juice (i.e. did not retrieve beer memories). After this, the prompt screens appeared, but the final screen unexpectedly read 'STOP! DO NOT DRINK', rather than 'DRINK NOW' in order to engender a negative PE.

The MRM reactivation procedure in *Ret + PE* was identical to *No Ret + PE*, except participants had a 150ml glass of beer placed in front of them while rating images. They rated the four *Beer Retrieval* images plus the two soft drink images prior to the PE generating procedure, which aimed produced an alcohol-specific PE in this group. In the *Ret no PE* group, the procedure was identical to *Ret + PE*, except the final drinking prompt screen read 'DRINK NOW' as expected and participants consumed the beer, putatively confirming expectancies and producing no PE. Immediately after these procedures, participants rated their '*Surprise as to what had just happened*' from 0 '*Completely Unexpected*' to 10 '*Completely Expected*' with a mid-point anchor of '*Neither Expected Nor Unexpected*'.

All participants then immediately completed verbal (letter M) and category (fruits) fluency tasks and Trailmaking A and B (Wechsler, 2008) to cognitively disengage them from MRMs, since rumination on MRMs may prevent destabilisation. The breathing mask was then fitted and participants completed baseline BSS and CADSS assessments immediately before beginning N₂O inhalation. After 10 minutes equilibration to the N₂O, participants again completed on-drug BSS and CADSS measures. Inhalation then continued such that total inhalation time was 30 minutes. After a further 10 minutes re-equilibration to normal air (with the mask still on), participants completed post-drug BSS and CADSS assessments. The mask was then removed. This completed *Day 2* testing, but participants were kept in the study centre for a further 30 minutes to ensure any residual drug effects had worn off. A schematic of the testing protocol on this day is presented in *Figure 1*.

Day 3 (Test)

The final testing day took place 7-10 days after *Day 2* and was identical to *Day 1*, with the same task order. Participants were then compensated and debriefed.

Data Analysis:

Data analyses were performed using IBM SPSS version 22 for Windows. All data were checked for normality, homogeneity of variance and sphericity (for repeated-measures with $K > 2$ comparisons). Where homogeneity of variance was violated in one-way ANOVA, Welch's F test is reported. The Greenhouse Geisser correction or multivariate equivalents used where sphericity was violated, depending on the value of ϵ and according to the recommendations of Stevens (2002). One-way ANOVA was used to assess group differences for baseline measures at $\alpha = 0.01$ owing to multiple measures being compared. For primary drinking-related DVs (beer drinking, craving), mixed ANOVA with a within-subjects factor of *Day* (Baseline vs. Test) and a between-subjects factor of *Group* (Ret + PE, Ret no PE, No Ret + PE) was used. For secondary outcome data (liking and wanting in response to cue images) a further within-subjects factor of *Cue Type* (*Beer Retrieval*, *Beer Non-Retrieval*, *Wine*, *OJ*, *soft drink*) was included. For GLMs including covariates, models were specified to include all main effects, and two and three-way interactions between terms. Significant $k > 2$ main effects and interactions in omnibus ANOVAs were investigated with simple effects analyses and paired tests on marginal means, where appropriate. Significance values for post-hoc these tests are Bonferroni- corrected to control Type I error.

Linear mixed models were run using the SPSS MIXED commands and estimated using maximum likelihood with unstructured working correlation matrices. Model fit was assessed using χ^2 tests on changes in log-likelihood and via minimisation of Akaike's Information criterion (AIC). Exploratory associations were investigated using Pearson's correlations in the case of parametric assumptions being met and using Kendall's τ otherwise.

RESULTS:

Baseline measures:

Groups did not differ in baseline measures of mood, drinking-related maladaptive behaviour or levels of drinking. Note an alpha level of .01 was adopted for these tests, owing to the multiple tests being conducted. Descriptive and inferential statistics are given in *Table 1*.

Drinking levels and craving

Changes in mean daily beer consumption pre-post intervention did not significantly differ between groups [Day x Group interaction: $F_{2,57} = 0.31, p = .726, \eta_p^2 = .011$], however in absolute terms, those in *Ret + PE* and *RET no PE* decreased their daily consumption by 153ml/ 1.63g EtOH (1079ml/ 80.9g EtOH/week) and 119 ml/0.988 g EtOH (852ml/50.4 g EtOH/week) respectively, while those in *No RET + PE* reduced their daily consumption by only 36.9ml/0.09g EtOH (284ml/5.6g EtOH/week). There was no significant Group effects $F_{2,57} = 2.433, p = .097, \eta_p^2 = .079$, nor Day x Group interaction for spirits consumption $F_{2,57} = 2.023, p = .142, \eta_p^2 = .066$. Similarly, there were no significant Group $F_{2,57} = 1.799, p = .175, \eta_p^2 = .059$ or Day x Group effects $F_{2,57} = 1.011, p = .37, \eta_p^2 = .034$, for wine consumption.

Total craving according to the ACQ did not significantly differ either pre-post intervention or between groups [Day x Group interaction: $F_{2,57} = 0.197, p = .659, \eta_p^2 = .003$]. Interestingly, there was a trend for an *increase* in the compulsivity subscale of the ACQ across days in all groups [Day main effect: $F_{1,57} = 3.28, p = .075, \eta_p^2 = .054$].

Liking/ of Alcohol Stimuli and induced urge to drink

A 2 (*Day*) x 5 (*Cue Type*) x 3 (*Group*) mixed ANOVA found a main effect of *Cue Type* [$F(4, 54) = 50.743, p < 0.001, \lambda = 0.79$] and a trend for a *Day x Group* interaction [$F_{2, 57} = 4.985, p = .086, \eta_p^2 = .082$]. The *Cue Type* effect revealed an unexpected pattern of cue liking; reflecting greater liking of *OJ* cues than all other cue types (all $ps < 0.0025$), equivalent liking of *Beer Retrieval* and *Soft Drink* cues ($p > 0.999$), and greater liking of *Beer Retrieval* and *Soft Drink* cues than *Wine* and *Beer Non Retrieval* cues ($ps < 0.001$), with the latter two being rated equivalently ($p > 0.999$). During debriefing it became clear that this pattern was driven by participants basing liking ratings on non-specific aesthetic properties of the *images themselves* (composition, brightness etc.). The trend-level *Day x Group* interaction was driven by an effect of *Day* in *Ret +PE* only [$F_{1, 57} = 4.465, p = .039, \eta_p^2 = .073$]; representing a general *increase* in rated pleasantness of cues from baseline to test in this group (see *Figure 2A*).

For ratings of urge to drink in response to cues, main effects of *Cue Type* [$F(4, 54) = 35.679, p < 0.001, V = .725$] and a *Day x Type* interaction [$F_{4, 54} = 3.025, p = .025, V = .183$] were found. Analysis of the interaction with simple effects of *Day* showed a decrease in urges to drink across days only for *Soft Drink* cues [$F_{1, 57} = 8.104, p = .006, \eta_p^2 = .124$]. Helmert contrasts on the *Cue Type* main effect showed that urges to drink followed the expected pattern; greater in response to *Beer Retrieval* cues than all other cues [$F_{1, 57} = 117.3, p < .001, \eta_p^2 = .673$], greater for *Beer Non-Retrieval* than *Wine, OJ & Soft drink* cues [$F_{1, 57} = 30.048, p < .001, \eta_p^2 = .345$], greater for *Wine* than *OJ & Soft drink* [$F_{1, 57} = 7.792, p = .007, \eta_p^2 = .12$], and no different between *OJ* and *Soft drink* cues [$F_{1, 57} = 0.01, p = .921, \eta_p^2 < .001$]. These effects are shown in *Figure 2B*. Analysis of the interaction with simple effects of *Day* showed a decrease in urges to drink across days only for *Soft Drink* cues [$F_{1, 57} = 8.104, p = .006, \eta_p^2 = .124$].

Measuring memory destabilisation: Manipulation check

Prediction Error: Explicitly rated surprise differed between groups following the Retrieval-drinking/PE procedure [$F_{2,57} = 4.455, p = .016, \eta^2 = .135$]. The *No Ret + PE* group showed significantly greater surprise following drink withholding than *Ret no PE* [$t(37) = 2.94, p = .005, r = .436$]. However, *Ret + PE* did not show greater surprise than *Ret no PE* [$t(38) = 1.95, p = .056, r = .3$]. The PE groups did not differ in surprise [$t(39) = 1.04, p = .301, r = .16$], indicating that the group-level prediction-error generation manipulation was only partially successful in producing surprise. During testing it became clear that several participants did not truly expect to be allowed to drink the beer placed in front of them. Since both unexpected omission of a reinforcer (negative PE) (Sevenster et al., 2013) and unexpected receipt of a reinforcer (positive PE) can induce memory destabilisation (Liu et al., 2014), prior expectancy of drink consumption would have determined level of PE and therefore MRM destabilisation. An examination of *Figure 3A* indicates that this was the case, with large variation and overlap in levels of surprise across groups.

As such, we re-examined changes in beer drinking, determined by *experienced* PE (surprise), rather than nominal ‘PE group’ since the latter was not a good approximation of the former. Firstly, given previous positive findings using retrieval tasks that do not explicitly model prediction error, we assessed whether drinking memory retrieval *per se*, regardless of PE (i.e. comparing both RET groups combined to No RET) affected beer drinking with a 2 (RET vs. No RET) \times 2 (Day) ANOVA. This showed no effect of retrieval (Group \times Day interaction $F_{1,58} = 0.611, p = .437, \eta_p^2 = .01$).

Since retrieval alone did not affect drinking outcomes, its combination with *achieved* PE was investigated by re-allocating participants into groups as follows: those who retrieved beer memories (*Ret + PE* & *Ret no PE*) were recoded as *Ret + PE* if their self-rated surprise at drinking or not drinking was > 5 out of 10 ($N = 29$) and were recoded as *RET no PE* if surprise was < 5 ($N = 11$). As can be seen from *Figure 4B*, this primarily involved re-allocation of participants from *RET no PE* to *RET + PE*, indicating a high incidence of positive PE in the former group. Baseline demographic and drinking-related metrics were re-analysed according to this new grouping using one-way ANOVAs and no significant differences were found. Importantly, the new groups were equivalent in baseline beer [$F_{2,57} = .37, p = .692, \eta^2 = .013$] and wine [$F_{2,57} = 1.776, p = .179, \eta^2 = .059$] consumption. The marginally significant baseline difference in spirit consumption became slightly less significant under the new grouping $F_{W 2,23,225} = 3.265, p = .056, \eta^2 = .073$], although the new *RET+PE* group still had the greatest baseline spirit consumption.

To assess whether this re-grouping by ‘*experienced PE*’ predicted drinking change better than ‘nominal PE group’ two linear mixed models were assessed, the first using the original grouping and the second using the new grouping. This was to directly assess the effects of re-specification of PE on model fit and parsimony. These used a random intercept by participant, with *Group* and *Day* as fixed effects factors and *surprise rating* as a covariate in a full-factorial model. The first model found a main effect of *Surprise* only [$F_{1,60} = 6.897, p = 0.011$], suggesting *experienced* PE was a primary determinant of beer drinking change under the original grouping. Using the new grouping, model fit was significantly improved compared to the same model using the previous grouping ($\Delta -2LL = -10.082; \Delta AIC = -14.08$). Importantly, re-specifying this model with *Surprise* as a continuous random effect did not improve model fit ($\Delta -2LL \chi^2(2) = 0.136, p = 0.467$) and reduced complexity-penalised model fit ($\Delta AIC = 3.864$). Subsequent analyses were therefore performed using GLM repeated measures ANOVA procedures in SPSS for consistency and comparison with previous analyses (these are equivalent to random-intercept only mixed models).

Beer drinking and craving as a function of PE:

Analysis of TLFB-scored beer drinking at baseline and test (*Day* factor) on the new *Groups*, including *Surprise* as a covariate and all interactions between *Group*, *Day* and *Surprise* found *Surprise* was no longer significant as a covariate main effect [$F_{1,54} = 1.117, p = .283, \eta_p^2 = .021$], however a significant *Day* x *Group* interaction [$F_{2,54} = 4.489, p = .01, \eta_p^2 = .156$] and a *Day* x *Group* x *Surprise* interaction [$F_{2,54} = 4.737, p = 0.013, \eta_p^2 = .149$] emerged. The *Day* x *Group* interaction indicated a significant reduction in beer drinking across days in the new *Ret + PE* group only [$F_{1,54} = 11.315, p = .001, \eta_p^2 = .173$]. These data are shown in *Figure 4C*. Investigation of the *Day* x *Group* x *Surprise* interaction through correlations between surprise and drinking levels on each *Day*, split by *Group* indicated that, after re-grouping of participants according to experienced PE, surprise was related to beer drinking at test only in *No Ret + PE* [$r(20) = -.395, p = .023$], potentially indicating a non-reconsolidation-specific arousal-based mechanism via which N_2O may impact beer drinking. The same analysis performed on spirit consumption revealed no significant *Day* or *Group* effects, nor interactions with surprise, however a *Day* x *Group* x *Surprise* interaction was found for wine consumption $F_{2,54} = 3.517, p = 0.037, \eta_p^2 = .115$.

No *Group* differences or changes across days in momentary craving as assessed by the ACQ-NOW were found.

Secondary Outcomes Grouped by PE: Cue Liking & Induced urge to drink

For cue liking data, a 2 (*Day*: Baseline, Test) x 5 (*Cue Type*: Beer Retrieval, Beer Non-Retrieval, Wine, Orange Juice, soft drink) x 3 (*Group*) mixed ANOVA, including *Surprise* as a covariate found a main effect of *Cue Type* [$F_{4,51} = 3.573, p = .012, V = 0.219, \eta_p^2 = .219$] and a *Day* x *Cue Type* x *Group* interaction [$F_{8,216} = 2.836, p = .005, \eta_p^2 = .095$]. Investigation of the simple effects of *Day* within each *Cue Type* in each *Group* showed an increase across days in liking of soft-drink stimuli in *No Ret + PE* only [$F_{1,54} = 4.4, p = .041, \eta_p^2 = .075$]. Changes in cue liking were not correlated with changes in drinking, indicating liking change was unlikely to be mechanistically responsible for the observed reductions in drinking.

The same ANOVA applied to cue-induced urge to drink ratings in response to cue images found a *Day* x *Group* interaction [$F_{2,54} = 3.752, p = .03, \eta_p^2 = .122$]. Descriptively, the interaction followed the pattern observed for drinking levels, with overall reductions in cue-induced urge in *Ret + PE* and *Ret no PE*, but a modest increase in urge in *No Ret + PE* (see *Figure 4D*). However, corrected simple-effects tests by group on this interaction did not approach significance. To assess whether urges to drink in response to beer cue images at test were related to changes in beer drinking, exploratory correlations were run between these two measures. These correlations were significant only in *Ret + PE* [Beer retrieval images $r(29) = .411, p = 0.027$; beer non-retrieval images; $r(29) = .39, p = 0.036$], tests on z-transformed correlation coefficients indicated that these values were significantly greater than those in *No Ret + PE* [$z = 2.82, p = 0.005$ and $z = 2.61, p = 0.009$, respectively] but not than those in *Ret no PE* ($ps > 0.5$).

Subjective responses to N₂O: Associations with drinking change

Correlations between CADSS-rated dissociation and stimulant/sedative ratio on calculated from the BSS in response to N_2O found no significant associations with beer drinking overall or at the group level (see *Table 2* for *r* values).

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DISCUSSION:

Two main obstacles impede our ability to pharmacologically weaken maladaptive reward memories (MRMs) for clinical benefit in alcohol use disorder: First is the potential resistance of alcohol-related MRMs to destabilisation. Second is the lack of drugs meeting the requirements for a clinically feasible reconsolidation-blocker; namely safety, tolerability, effective restabilisation prevention and rapid onset/offset kinetics. The current study aimed to address the latter, examining whether the NMDAR antagonist gas Nitrous Oxide (N₂O) could prevent restabilisation following a retrieval and prediction error (*Ret + PE*) procedure we have previously shown to effectively destabilise MRMs. The findings of the study were mixed, however we believe they highlight some highly promising avenues for translational work and key insights for the field moving forward.

Reconsolidation is thought to be a memory updating and maintenance process and mismatch at retrieval is proposed to be important for its initiation (Forcato et al., 2009; Forcato et al., 2010; Pedreira et al., 2004; Sevenster et al., 2012, 2013, 2014). As such, the ‘active’ memory destabilisation procedure used here relied on the generation of PE through guided expectancy violation. That is, participants were told they were going to drink beer and this expectancy was either putatively fulfilled (drinking consumption = no PE) or violated (beer withheld = PE) following retrieval of alcohol MRMs. This PE-generating procedure has previously been shown to be effective in MRM destabilisation (Das et al., 2015a; Hon et al., 2016). However in the current study, group-level differences in drinking were not observed following this procedure compared to control retrieval procedures. We should perhaps simply interpret these as null findings and conclude that N₂O was ineffective as a means of interfering with MRM reconsolidation. However this assumes that MRMs were successfully destabilised by the *Ret + PE* procedure. Human participants frequently anticipate subterfuge in psychological experiments. Successful PE generation (and, by extension, MRM destabilisation) therefore relies entirely upon participants’ expectancy of actually drinking the beer they are given. Examination of *experienced* PE as indexed by participant’s ratings of surprise following drinking or not drinking beer highlighted the importance of this consideration to the apparent success of the intervention. In this scenario, many participants who were presented with beer did not anticipate being able to actually consume it, despite the instructions. These participants, who were intended to experience no PE when beer was consumed were actually surprised at being ‘allowed’ to drink, such that reward was unexpected, or PE was positive. Critically, this type of PE (unexpected reinforcer, or positive PE) has been shown to be effective at destabilising memories (Liu et al., 2014; Sevenster et al., 2013).

In an exploratory analysis, where we re-assigned participants to groups based upon whether they actually experienced a meaningful level of surprise, those who experienced either positive *or* negative PE following brief retrieval of alcohol MRMs showed reductions in drinking from baseline to test when N₂O was subsequently inhaled. This effect would suggest that MRM retrieval with PE destabilised MRMs, allowing subsequent interference with memory reconsolidation, by N₂O. A similar pattern was found in cue-induced ‘urge to drink’ beer in response to alcohol cue images, but not ‘liking’ of these images. This ‘wanting’ effect was associated with reductions in beer consumption only in those who retrieved MRMs and experienced PE, suggesting the two may be mechanistically related or manifestations of a common process.

This disparity between ‘liking’ and ‘wanting’ is unsurprising in the light of incentive salience theory (Berridge et al., 2009; Robinson and Berridge, 1993, 2001), where the two processes are thought to diverge with continuing addiction-like behaviour. More simplistically, this disparity could be an artefact of the ratings themselves, since ‘liking’ ratings were regarding the cue images *per se* (and therefore assessed conditioned reinforcement), whereas ‘wanting’ ratings were regarding the effect of the cues on modulating urge to consume an actual reward (i.e. assessing conditioned motivation). Anecdotal reports from participants also suggested that non-alcohol-specific characteristics of the cue

images, such as colour, composition and context might have affected these ratings, based upon idiosyncratic aesthetic preferences. In future research, care should be taken in framing cue liking questions to circumvent these issues.

No effects of post-retrieval N_2O were observed on momentary craving, regardless of whether the original grouping or PE-based grouping were used. This may reflect the fact that momentary craving is subject to diurnal fluctuation and recency of drinking. Repeated ecological momentary assessment or measures of tonic craving may therefore have been better suited to assessing any effects of post-retrieval N_2O .

Intriguingly, under both the original and PE-based groupings, an absolute reduction in drinking was also observed in the *Ret no PE* group, although this did not reach significance. This may be due to the limited N in this group following group re-allocation (i.e. Type II error), an issue that is evident in the large standard errors relative to mean values in this group. Equally, the apparent reduction could be spurious (i.e. Type I error) and it is impossible to make strong inferences about effects in this group given the limited N and limited power to detect effects (Cohen, 1992). However, in our previous studies, we have also observed those retrieving MRMs without PE displaying a response to the subsequent challenge that falls somewhere between Ret + PE and non-retrieval groups (Das et al., 2015a). Given the current results, it is possible that expectancy effects and inter-individual differences in PE levels contributed to this effect, with even those in the nominal '*Ret No PE*' group experiencing some level of PE.

As we have speculated previously, these considerations suggest that destabilisation may not be an all-or-nothing process, but proportional to the magnitude of the PE 'updating signal', an idea for which contemporary computational models of reconsolidation (Gershman et al., 2017; Helfer and Shultz, 2017; Osan et al., 2011) and empirical evidence (Reichelt and Lee, 2013) provide some support. Importantly, this relationship between PE and destabilisation need not be linear. While the current dataset is too limited to properly explore relationships between levels of PE and destabilisation, this should be a focus of future research, as determining an 'optimum' level of PE may help develop more effective memory-updating manipulations. Further, the retrieval cues used to retrieve MRMs themselves likely have a large impact on the efficacy of subsequent interventions. We used four beer images here, selecting images that represented a spectrum of modes of beer consumption, hoping to destabilise a wider network of associations. However, there is no empirical evidence to support the use of four stimuli above one, two, three, five or ten for achieving destabilisation.

There are clear implications from the current study for the development of reconsolidation-based interventions for hazardous drinking and potentially addiction. Firstly, there is a pressing need to both understand the primary determinates of memory destabilisation and develop sensitive measures of destabilisation that can be employed in real-time to overcome the problematic interpretation of null results that plagues the field. Clinically-applied research into targeting maladaptive memory reconsolidation with both drugs and behavioural interventions has yielded both extremely promising (Das et al., 2015a; Germeroth, in press; Hon et al., 2016; James et al., 2015; Soeter and Kindt, 2015; Xue et al., 2017; Xue et al., 2012), and disappointing (Chan et al., 2010; Das et al., 2015b; Saladin et al., 2013; Treanor et al., 2017) results. Importantly, the majority of studies to date have utilised generic retrieval parameters that have become self-perpetuating in the literature. Largely these do not aim to generate PE, nor measure it, although they typically omit reinforcers during retrieval. Such procedures show very positive results in some cases and it is possible that these procedures *implicitly* incorporated PE due to reinforcer omission, but very rarely is this explicitly modelled or assessed. The generation of PE at retrieval is therefore entirely incidental and a function of individual expectancies and learning history. Variations in intervention-response may thus be expected. It is unclear to what extent detoxified alcoholic or heroin-using inpatients would actually expect to receive drug, for instance, although it is entirely possible that the strength of cue reactivity in these populations yields

explicit generation of PE less critical. Further, extended abstinence from exposure to the abused drug and associated cues may produce reactivation/destabilisation of MRMs regardless (Robinson and Franklin, 2010). While factors such as this may explain mixed findings when considered at the group level, there is currently too little research into the true importance of PE for destabilising memories of different modalities, ages and strengths. The current results highlight the importance of explicitly assessing such factors when trying to understand memory destabilisation independent of treatment effects.

Future research should therefore focus on candidate peri-retrieval metrics that index memory destabilisation. While measuring explicit PE will be informative, such metrics will likely include intrinsic neural signatures, arousal/craving levels, and length of time since last reinforcer exposure and state biological determinates such as cortisol and blood glucose levels. Objective assessment of the optimal number of cue exposures/duration for destabilisation of MRMs will be particularly informative. In general, a concerted effort is required from reconsolidation researchers towards the ultimate aim of the field; developing a general-purpose biomarker (or suite of biomarkers) of memory destabilisation. This could then be used to optimise memory retrieval procedures and examine the most effective post-retrieval interventions for clinical benefit.

Limitations:

The clear primary limitation of the current study was the disparity between intended and achieved levels of PE at retrieval and subsequent reliance on reallocation of participants to new groups. We therefore cannot make any firm conclusions about the efficacy of the procedures, or of N₂O itself without further data extending and replicating these effects. Although the re-grouping was based on hypothesised and empirically-based effects, it was necessarily post-hoc and the resulting groups were no longer random. The re-grouping of participants also led to a low N in the *Ret no PE* group, limiting power to detect effects in this group. The sample size of the study overall was too small to fully explore potential moderators of response to post-retrieval N₂O. It is likely that current levels of disordered drinking, family history of alcohol use disorders and acute responses to N₂O (Walsh et al., 2017) all determine level of response and these will need to be investigated further in future research. Overall, the findings based on group reallocation should therefore be considered exploratory and preliminary.

A further limitation is a lack of basis for mechanistic interpretation of observed effects. We have interpreted the N₂O effects as being due to blockade of NMDAR-mediated MRM reconsolidation (Jevtović-Todorović et al., 1998; Ogata et al., 2006). However, in the absence of pharmacodynamic data, it is impossible to infer that this was the true mechanism of action. N₂O has non-NMDAR sites of action including monoaminergic and opioid targets (Emmanouil and Quock, 2007; Ori et al., 1989; Quock et al., 1990; Zhu and Luo, 1992) which may have contributed to the effects. Alternatively, rather than MRM reconsolidation blockade, N₂O may have acted via memory *updating*. For example, depending upon the level of dysphoric subjective response to N₂O, this could have acted as a counterconditioning stimulus, pairing reactivated alcohol cues with a negative emotional state. Similarly, post-retrieval N₂O may have created a novel internal context, introducing state-dependency into MRM traces (Gisquet-Verrier et al., 2015). However, the state dependency of reconsolidation effects attributed to receptor blockade have yet to be explored in humans. Had we re-tested under N₂O, different effects on acute responses to cues might have been observed. The lack of correlations between measures of subjective response to N₂O suggest that this was not the case, however it is possible that the subjective measures we used did not capture the relevant components of experience that were relevant to the observed effects.

Beer drinking in the current study was entirely based on self-report and was not biologically verified. Indeed, accurate biological verification of alcohol consumption over the period of a week is difficult to achieve in a cost-effective manner. However, there is no reason to assume that reporting bias would

vary systematically between the groups. Related to this, spirits consumption at baseline was somewhat lower in the RET +PE group than the other two groups. As a group, they were the best conceptualised as ‘primary beer drinkers’ and this may have contributed towards the results in the current study, since the manipulation specifically targeted beer cues. The contribution of this inequality in spirit consumption to the findings is uncertain. As participants were randomised to conditions, this likely arose by chance there did not appear to be a systematic compensatory change in spirit or wine consumption to offset changes in beer consumption. Finally, although the target population here are certainly clinically-relevant and important from a public health perspective, they were not a clinical sample with alcohol use disorder. The decision to recruit this sample was made to balance clinical applicability while minimising the potential for iatrogenic harm from testing a novel experimental procedure in a clinical sample. This means the applicability of the current findings to a fully addicted sample remains to be established in future studies.

Conclusion:

We found no significant effects of N₂O on beer drinking when given after a prediction-error generating MRM retrieval procedure. However, analysis of N₂O effects as a function of *experienced* PE rather than *assumed* PE showed significant reductions in drinking following N₂O. These preliminary findings are the first, to our knowledge, demonstrating an effect of N₂O that is consistent with reconsolidation blockade. However, given the post-hoc nature of this finding, caution in interpretation is required. Future studies should directly assess indices of putative memory destabilisation mediators when assessing reconsolidation-based interventions.

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The authors declare that this research is original and that they are responsible the content herein.

TABLE AND FIGURE LEGENDS:

*Table 1: Baseline questionnaire-based measures of mood and maladaptive drinking behaviour. Data represent mean \pm SD, F-values are from 1-way ANOVA. For the TLFB-based daily drinking measure, figures refer to standard UK drinks. For beer this is 568ml (1 pint), for wine 175ml and spirits a single 25ml measure. For ease of international interpretation, equivalent grams of alcohol are also provided with each measure. **No Ret** = No retrieval, **Ret** = Retrieval, **PE** = Prediction Error.*

Table 2: Correlations of subjective responses to N₂O with changes in beer drinking. Values represent Pearson’s r values. No significant correlations emerged at the group level or overall.

Figure 1: Schematic of MRM retrieval and PE protocol prior to N₂O inhalation. Ret = Retrieval, PE = Prediction Error.

Figure 2(A): Liking of cue stimuli at baseline and test. Bars represent means \pm SEM (B): Urge to drink in response to different cue stimuli. Significance levels from Helmert contrasts. Bars represent means + SEM. (B): Urge to drink in response to different cue images. Significance levels are from Helmert contrasts. Bars represent means + SEM. '**Beer Ret**' = Beer Retrieval cues used on Day 2; '**Beer No Ret**' = Beer cues not retrieved on Day 2 but assessed at baseline and test; '**Wine**' = wine cues assessed at baseline and test; '**OJ**' = orange juice images used on day 2 in No RET +PE group.

Figure 3: Left panel: Heterogeneity in surprise generated by the retrieval/ PE conditions according to the original (nominal) grouping. Right panel: Reallocation of participants to conditions according to self-rated surprise following the RET + PE/no PE procedures

Figure 4: (A) Significant reductions in beer drinking from baseline (week preceding Day 1; pre manipulation) to test (week preceding Day 3, post-manipulation) in Ret + PE group. A large absolute reduction was observed in Ret no PE, however this did not reach significance. (B): Day x Group interaction on urge to drink ratings in response to cue stimuli when participants are grouped by self-rated surprise. Bars represent mean +SEM.

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Contributors statement.

Ravi Das, Katie Walsh, Josie Hannaford, Antonio Lazzarino and Sunjeev Kamboj confirm that we have contributed to the manuscript and that this has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript. The authors declare that this research is original and accept full responsible the content herein.

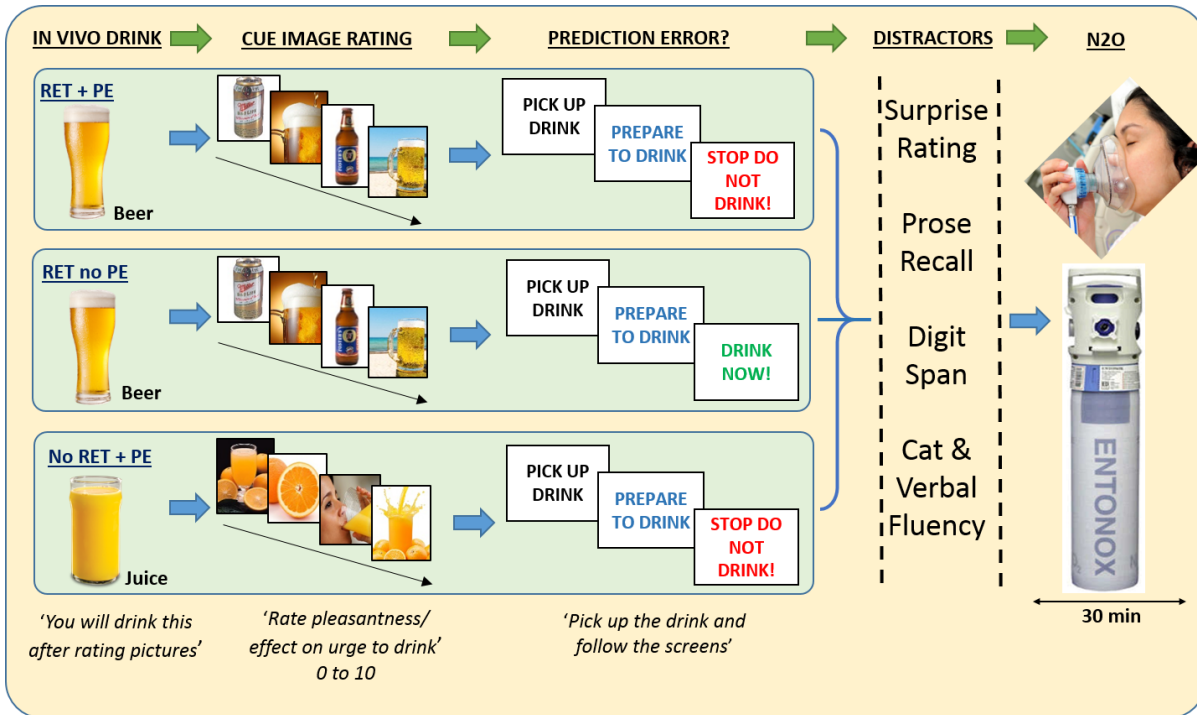
Table 1: Baseline questionnaire-based measures of mood and maladaptive drinking behaviour. Data represent mean \pm SD, F-values are from 1-way ANOVA. For the TLFB-based daily drinking measure, figures refer to standard UK drinks. For beer this is 568ml (1 pint), for wine 175ml and spirits a single 25ml measure. For ease of international interpretation, equivalent grams of alcohol are also provided with each measure. No Ret = No retrieval, Ret = Retrieval, PE = Prediction Error.

	No Ret +PE	Ret + PE	Ret no PE	F (2, 57)	Sig	η^2
AUDIT	15.6 \pm 4.95	14.66 \pm 3.69	13.78 \pm 5.30	0.731	0.486	0.03
HADS Depression	2.95 \pm 2.48	2.809 \pm 2.48	2.421 \pm 2.36	0.244	0.785	0.01
HADS Anxiety	5.85 \pm 2.90	6.523 \pm 3.84	6.368 \pm 3.70	0.205	0.816	0.01
ACQ GEN	2.905 \pm 0.64	2.566 \pm 0.63	2.773 \pm 0.70	1.373	0.262	0.05
ACQ COMP	1.125 \pm 0.87	0.8 \pm 0.60	0.952 \pm 0.80	0.919	0.405	0.03
ACQ XPECT	2.725 \pm 0.92	2.138 \pm 0.78	2.410 \pm 1.29	1.725	0.187	0.06
ACQ PURP	5.43 \pm 0.90	5.438 \pm 0.72	5.652 \pm 0.84	0.453	0.638	0.02
ACQ EMOT	2.35 \pm 1.01	1.861 \pm 1.20	2.057 \pm 1.25	0.916	0.406	0.03
PANAS +VE	34.65 \pm 6.83	35.42 \pm 4.82	34.42 \pm 5.17	0.176	0.839	0.01
PANAS -VE	17.45 \pm 5.20	18.33 \pm 6.80	18.89 \pm 7.40	0.244	0.785	0.01
STAI	55 \pm 4.63	52.26 \pm 8.16	53.66 \pm 4.41	0.984	0.381	0.04
Daily Pints Beer (g EtOH)	1.628 \pm 0.76 / (36.48 \pm 17.03)	1.624 \pm 0.73 / (36.39 \pm 16.36)	1.677 \pm 0.64 / (37.58 \pm 14.34)	0.032	0.968	0
Daily Glasses Wine/ (g EtOH)	0.493 \pm 0.69 / (8.85 \pm 12.39)	0.687 \pm 0.57 / (12.33 \pm 10.23)	0.829 \pm 0.90 / (14.88 \pm 16.15)	1.05	0.357	0.04
Daily Spirits/ (g EtOH)	1.339 \pm 1.29 / (10.56 \pm 10.18)	0.485 \pm 0.48 / (3.83 \pm 3.79)	1.1 \pm 1.37 / (8.68 \pm 10.81)	3.208	0.048	0.1
SOCRATES recognition	18.15 \pm 5.83	16.45 \pm 4.24	16.05 \pm 5.88	0.85	0.433	0.03
SOCRATES ambivalence	7.75 \pm 4.32	7.25 \pm 3.65	6.473 \pm 3.18	0.568	0.57	0.02
SOCRATES steps	15.1 \pm 5.26	14.5 \pm 5.35	14.89 \pm 5.32	0.066	0.936	0
CEOA Tension	6.7 \pm 1.97	6.619 \pm 1.49	6.736 \pm 1.93	0.022	0.978	0
CEOA Courage	12 \pm 2.91	11.85 \pm 2.81	12.31 \pm 3.46	0.115	0.891	0
CEOA Sexual	8.1 \pm 2.51	7.714 \pm 2.66	8.526 \pm 2.79	0.465	0.63	0.02
CEOA Impairment	19.85 \pm 5.12	19.28 \pm 4.94	20.36 \pm 4.09	0.259	0.773	0.01
CEOA Risk Aggression	10.75 \pm 2.89	10 \pm 3.71	11.10 \pm 3.05	0.607	0.548	0.02
CEOA Self percept	5.9 \pm 2.24	6.809 \pm 2.76	5.578 \pm 1.70	1.56	0.219	0.05

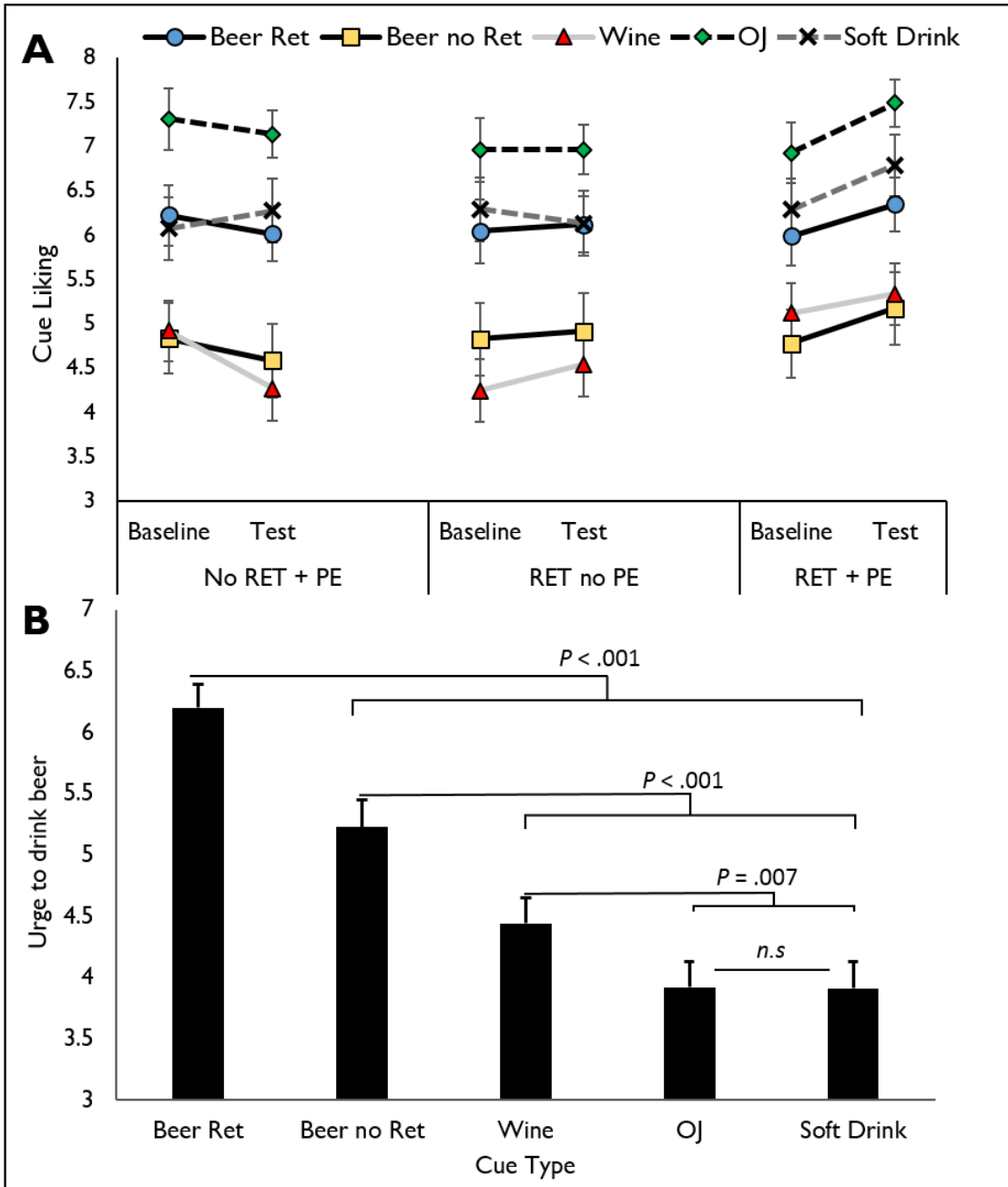
		Stim/Sed ratio	CADSS Pre N ₂ O	CADSS on N ₂ O
Beer drinking change	No Ret + PE (N = 20)	.01	-.22	-.216
	Ret no PE (N = 11)	-.085	.244	.123
	Ret + PE (N = 29)	.008	.039	.08
	Overall (N = 60)	-.022	.014	-.04

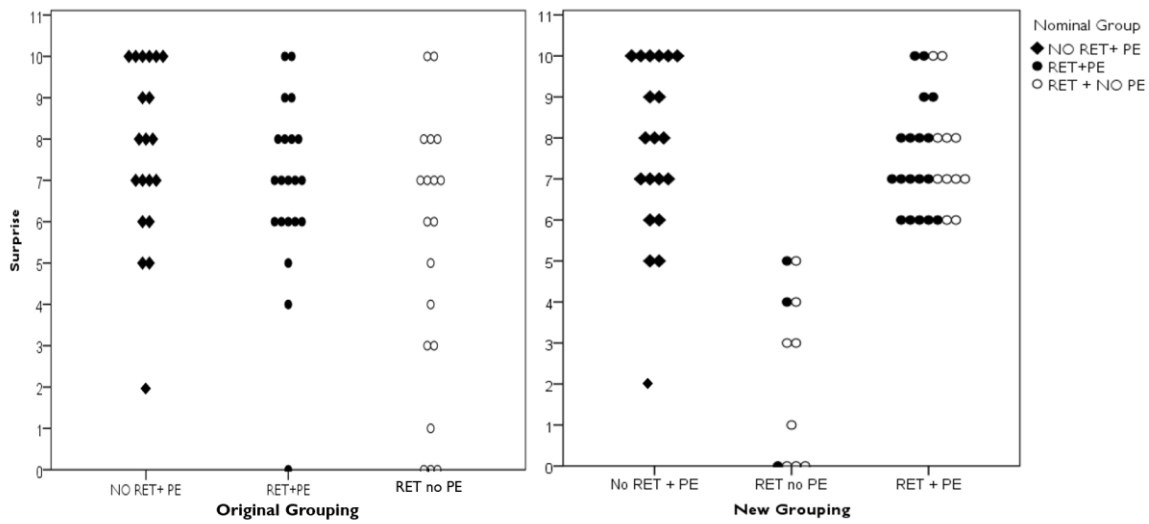
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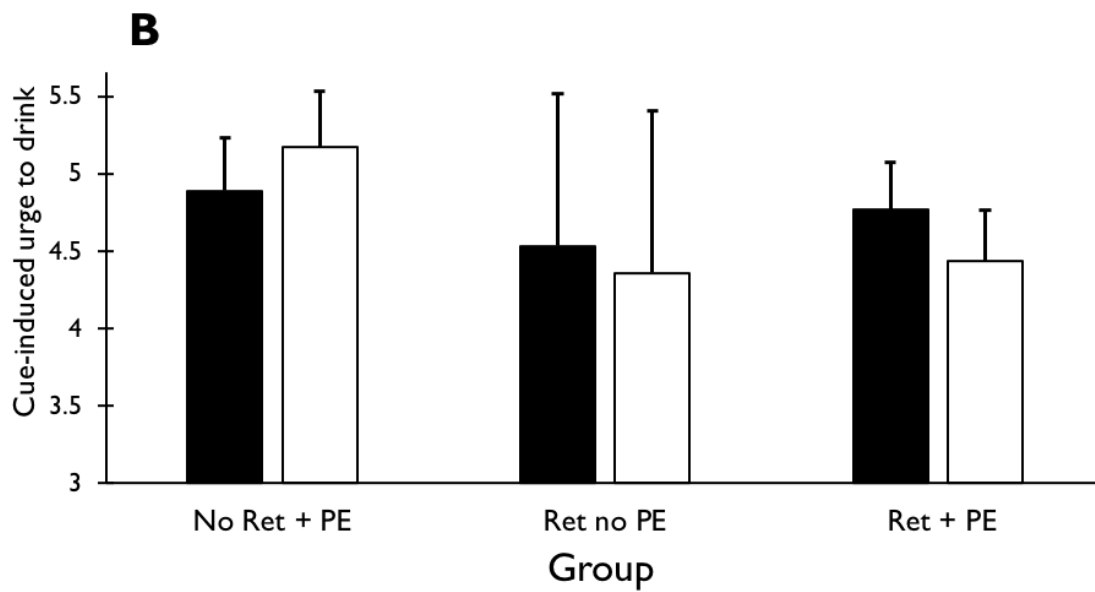
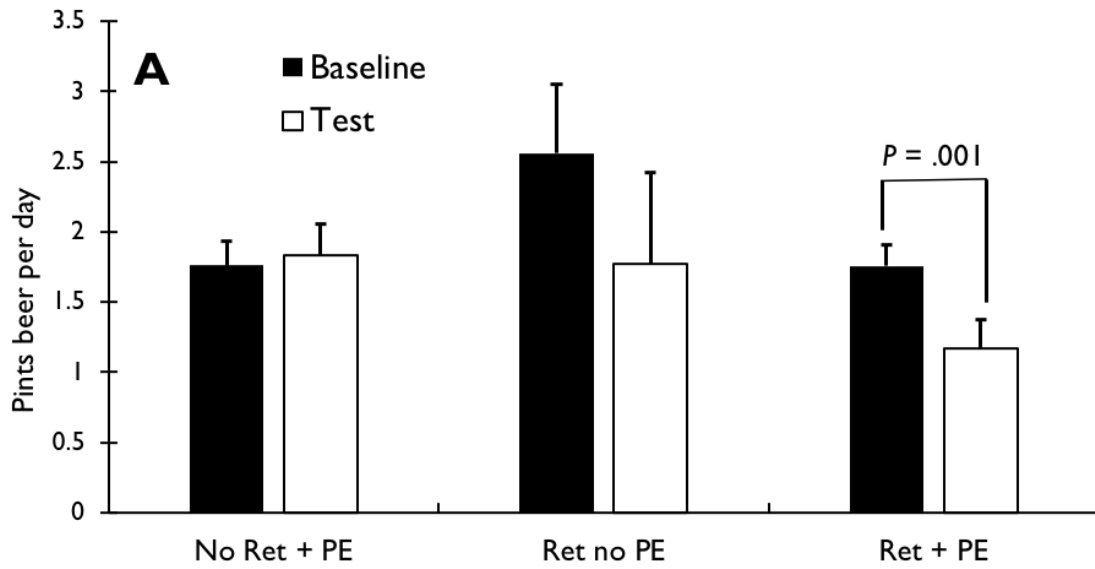


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