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RESEARCH REPORT

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Reactivity to conditioned threat cues is distinct from exploratory drive in the elevated plus maze

Joe R. Hilton[1] | **Susannah R. Simpson**[1] | **Emily R. Sherman**[1] | **Will Raby-Smith**[1] | **Keemia Azvine**[1] | **Maite Arribas**[1] | **Jiaqi Zhou**[1] | **Serena Deiana**[2] | **Bastian Hengerer**[2] | **Emma N. Cahill**[1,3]

[1]Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK

[2]CNS Discovery Research, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany

[3]School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol, UK

Correspondence
Emma N. Cahill, School of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol BS8 1TD, UK.
Email: emma.n.cahill@bristol.ac.uk

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Abstract

Fear and anxiety are adaptive states that allow humans and animals alike to respond appropriately to threatening cues in their environment. Commonly used tasks for studying behaviour akin to fear and anxiety in rodent models are Pavlovian threat conditioning and the elevated plus maze (EPM), respectively. In threat conditioning the rodents learn to associate an aversive event with a specific stimulus or context. The learnt association between the two stimuli (the 'memory') can then be recalled by re-exposing the subject to the conditioned stimulus. The elevated plus maze is argued to measure the agoraphobic avoidance of the brightly lit open maze arms in crepuscular rodents. These two tasks have been used extensively, yet research into whether they interact is scarce. We investigated whether recall of an aversive memory, across contextual, odour or auditory modalities, would potentiate anxiety-like behaviour in the elevated plus maze. The data did not support that memory recall, even over a series of time points, could influence EPM behaviour. Furthermore, there was no correlation between EPM behaviour and conditioned freezing in independent cohorts tested in the EPM before or after auditory threat conditioning. Further analysis found the production of 22 kHz ultrasonic vocalisations revealed the strongest responders to a conditioned threat cue. These results are of particular importance for consideration when using the EPM and threat conditioning to identify individual differences and the possibility to use the tasks in batteries of tests without cross-task interference.

KEYWORDS
anxiety, elevated plus maze, exploratory behaviour, fear, memory

1 | INTRODUCTION

Tasks to measure rodent behaviour motivated by aversive experience are commonly used to model features of symptoms present in anxiety disorders. Nonetheless, there is much discussion on how to best describe and validate what the tasks are detecting in terms of emotionally driven behaviour, and what the tasks might predict for

translation (for review, see Bach, 2021; Steimer, 2011). An influential system of classification of rodent defensive responses places the response along a theoretical axis of psychological or physical predator imminence versus escapability of the context (Fanselow et al., 1988). The predator imminence model has been used to categorise specific readouts, such as freezing or avoidance, as more akin to states of fear or anxiety, respectively (Blanchard & Blanchard, 1969; McNaughton & Corr, 2004). However, whether a clear distinction between emotional states of fear or anxiety is fully warranted for effective translation has recently come under debate given the overlap in mechanisms recruited by the behaviour related to each state (Daniel-Watanabe & Fletcher, 2022).

In rodents, innate avoidance of open arm (OA) exploration in the elevated plus maze (EPM) has been argued to reflect an anxiety-like agoraphobia (Walf & Frye, 2007). The production of 22 kHz ultrasonic vocalisations (USVs), or so-called alarm calls, has been posited to also reflect a state akin to anxiety (Anderson, 1954; Schwarting & Wöhr, 2012). Interestingly, the production of 22 kHz is not typically reported in the EPM (Borta et al., 2006), but is more reliably triggered by exposure of rodents to a conditioned threat stimulus (CS) or mild shocks such as during Pavlovian conditioning (Wöhr & Schwarting, 2008). Conditioned freezing to threat cues has more recently been rebranded from a description as a fear-related behaviour (LeDoux, 2014) but is often classed as distinct from the anxiety-like behaviours recorded in maze tasks such as the EPM. Despite widespread use of the EPM and conditioned freezing tasks, sometimes in tandem, there is little consensus on whether the tasks do measure truly independent behaviours or indeed how recent experience impacts performance in these tasks (File, 1993). One could hypothesise that recall of a recent aversive experience would lead to heightened anxiety responses within the window of that memory reactivation. This hypothesis is consistent with the notion that the EPM is sensitive to acute behavioural states.

On the other hand, the EPM has been used as a measure of so-called trait like, anxiety, which suggests it could be stable over time and potentially reveal individual differences that translate into resilience or risk for pathological responses (Richter-Levin et al., 2019; Shumake et al., 2014). Considering the rising debate on the distinction of rodent defence responses as fear like or anxiety like, we examined whether a potential interaction between unconditioned behaviour in the EPM and conditioned freezing might exist. In addition, we report on the production of 22 kHz USVs in response to auditory threat conditioning cues. To better characterise potential impacts of prior aversive experience on EPM performance, a series of experiments were carried out that systematically varied the time since stimulus presentation and the modality of the conditioning stimulus. Furthermore, we examined whether the EPM measurements taken days before or after threat conditioning measurements held any relationship.

2 | MATERIALS AND METHODS

2.1 | Subjects

Subjects were 104 adult male Lister-Hooded rats (Charles River and Envigo, UK) weighing approximately 250–300 g at the start of experiments. All animals were housed in groups of four per cage and kept under a reversed 12 h light/dark cycle (lights off 07:00 h until 19:00 h) and were provided with food and water ad libitum, except for during behavioural procedures, which were conducted during the rats' dark cycle. The rats were randomly assigned to each group and experimenters were blind to group allocation for subsequent analysis. This research was conducted on Project Licence 70/7548 and has been regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

2.2 | Behavioural procedures

For conditioned freezing, four identical operant boxes (29.5 × 32.5 × 23.5 cm, MedAssociates) were used with a Plexiglass rear wall, hinged door and roof, with aluminium side walls. The boxes also contained a house light (2.5 W, 24 V), a speaker (3 kHz tone, 75 dB), ultrasonic microphones (Metris, Netherlands) and a CCTV video camera (Spy Camera model CMOSNC76). The boxes were positioned within sound attenuating chambers and each also contained a ventilation fan, which provided background noise (approximately 60 dB). At Time 0, the house light was turned on. The same box was used for each rat throughout the experiments. The unconditioned stimulus was a mild footshock (.5 mA, .5 s). All training and test sessions were video recorded for off-line behavioural analysis. The percentage of time freezing (absence of movement except for breathing) during 1 min before (Pre-CS) and during the CS was manually scored from the videos by observers blind to the groups.

2.3 | Contextual conditioning

Rats were individually placed in the conditioning boxes, which they had not previously been habituated to. A mild

electric foot shock (.5 mA, .5 s) was administered after the rats had been in the context for 2 min to allow the contextual representation to first be encoded (Fanselow, 1990). The context–shock pairing was repeated two more times with 2 min intervals between. Following the last shock and 2 min interval, the house light remained on for a further 2 min. Following this, the house light was turned off, and the rats were removed from the conditioning boxes and then returned to their home cage. No Shock controls were placed in the context for the same duration. For the recall test, rats were placed in the context for 3 min.

2.4 | Olfactory conditioning

Rats were individually habituated to the context for 1 h on Day 0 and Day 1. Rats were then conditioned to associate the foot shocks with the odour acetophenone (Sigma Aldrich). The odour, acetophenone, was prepared in a separate room and gloves were changed by the experimenters before handling the rats. The odour was diluted to 10% in mineral oil and 200 μl of the diluted odour was placed onto a cotton pad. Following a 4 min baseline period, the odour was introduced into the shock box in the waste tray and the conditioned group received three foot shocks with a 2 min intertrial interval. The control odour exposure group (No Shock) was exposed to the odour for the same duration but did not receive foot shocks. Between sessions, a fan was run for 15 min to ventilate the room. For the recall test, rats were placed back in the context and after 3 min had passed the odour was introduced on a cotton pad as described above. The rats remained in presence of the odour for 3 min.

2.5 | Auditory conditioning

On Day 0, rats were habituated to the context for 1 h. On Day 1, after a baseline period of 20 min, three pairings of the 1 min tone (3 kHz, 75 dB) coterminous with the foot shock were presented with 5 min interstimulus intervals. At recall test, after a 5 min baseline, the tone was presented for 1 min, and then, the rats remained in the context for a further 5 min.

2.6 | EPM

Each rat was tested individually on a plus maze made from black Perspex with four arms of 50 cm long and 10 cm wide, at a height of 50 cm from the floor, with raised sides of 30 cm (ViewPoint, France). The maze was situated in a room with many external cues located around the maze, and these cues remained the same throughout training and testing of each cohort. At the start of each session, a rat was placed on the end of the same OA facing away from the middle region. The experimenter triggered the recording and left the room to observe from an adjacent room. After 5 min, the video recording was stopped, and the experimenter re-entered the room and removed the rat from the maze. Rats were then returned to their home cage. Between sessions, the maze was cleaned with water and dried. In the singular case where a rat jumped off the maze, the experimenter re-entered the room, returned the rat to the home cage and this rat was excluded from statistical analyses. Time spent in the open and closed arms and in the centre zone was manually scored; each arm time was then calculated as a percentage of the total time (exploring all three zones). In addition, the absolute number of entries into open and closed arms and the latency to first enter the OAs were scored. The rat was scored to have entered a particular arm when its hind legs passed the border.

2.7 | USV analysis

An ultrasound microphone (Metris, Netherlands) was placed through a hole in the middle of the operant box roof about 30 cm above the shock floor. The microphones were sensitive to frequencies of 15–125 kHz. Vocalisation was recorded and analysed using the Sonotrack software (Metris, Netherlands). Call detection was provided by an automatic threshold-based algorithm. Experimenters manually screened the calls detected and classified based on the mean frequency as 22 kHz calls or 55+ kHz calls or cage noise. The number of USV calls and total calling time were analysed for the 22 kHz calls only.

2.8 | Statistical analysis

Data points represent individual rats and are presented as mean ± *SEM*, unless otherwise stated. Statistical analyses included two-tailed *t* tests, repeated-measures ANOVA and planned Sidak comparisons for more than three groups were made using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA, version 9.3.0). Where Mauchly's test of sphericity indicated the assumption of sphericity had been violated, degrees of freedom were Greenhouse–Geisser corrected. The Pearson correlation coefficient was calculated between given measures in order to explore their potential relationship. For comparison of categorical data, that is, the proportion of rats that produced 22 kHz vocalisation or not, a

Fisher's exact test was performed. The significance level was set at $p < .05$. Graphs and figures were generated in GraphPad Prism 9.3.0.

3 | RESULTS

3.1 | Recall of a Pavlovian aversive memory had no impact on subsequent EPM behaviour

3.1.1 | Contextual conditioning

Whether recall of an aversive experience would impact behaviour in the EPM tested shortly after memory recall was assessed. The responses of a contextual threat conditioned group (Shock) were compared with a control group of rats that were exposed to the context for the same duration of time but did not receive any foot shocks (No Shock, Figure 1b). As expected, at the context recall test the shocked group showed a strong conditioned freezing response relative to the No Shock controls across the conditioning session ($F_{\text{group}(1,10)} = 23.33$, $p = .0007$) and at the recall test (two-tailed t test, $t_{10} = 4.807$, $p = .0007$, Figure 1c). Ninety minutes later, all rats were tested for arm exploration and entries in the EPM. The time spent in the closed arms was not different between the groups (two-tailed t test, $t_{10} = 1.024$, $p = .3302$, Figure 1d). Of note, one rat in the Shock group did not enter the closed arm after initiation of the test at all and showed intermittent freezing responses in the OA. When the conditioned freezing at recall was compared with the extent of time spent in the closed arm there was no significant correlation between the measurements ($r_{(12)} = -.3241$, $p = .3040$). Previous results in the literature have demonstrated that 90 min after stress or recall of a stressful experience, EPM behaviour was negatively impacted (Mechiel Korte et al., 1999). Using independent conditioned groups, a series of time points after recall were further examined to see if any temporal impact of memory recall on the EPM could be detected (Figure 2). None of the time points examined influenced the time spent in the closed arms, most notably even for rats that were introduced to the EPM as directly as possible after context recall (0′ group, Figure 2b).

3.1.2 | Olfactory conditioning

It could be argued that a contextual conditioning protocol might be prone to extinction during the recall test of the memory, as the cue (i.e., the context) is constantly present during the recall trial in absence of the US. Although no significant within session extinction during contextual recall was observed, we proceeded to test if a more discrete modality of aversive memory recall would impact the EPM behaviour. Pavlovian olfactory conditioning was performed, and a control group (No Shock) were exposed to the neutral odour for the same duration as those that received paired footshocks (Shock, Figure 3). Conditioned freezing increased across the conditioning session for the Shock group relative to the No Shock controls ($F_{\text{group}(1,10)} = 56.98$, $p < .0001$). To check that exposure to the odour during recall would not result in complete extinction of the association, a second recall

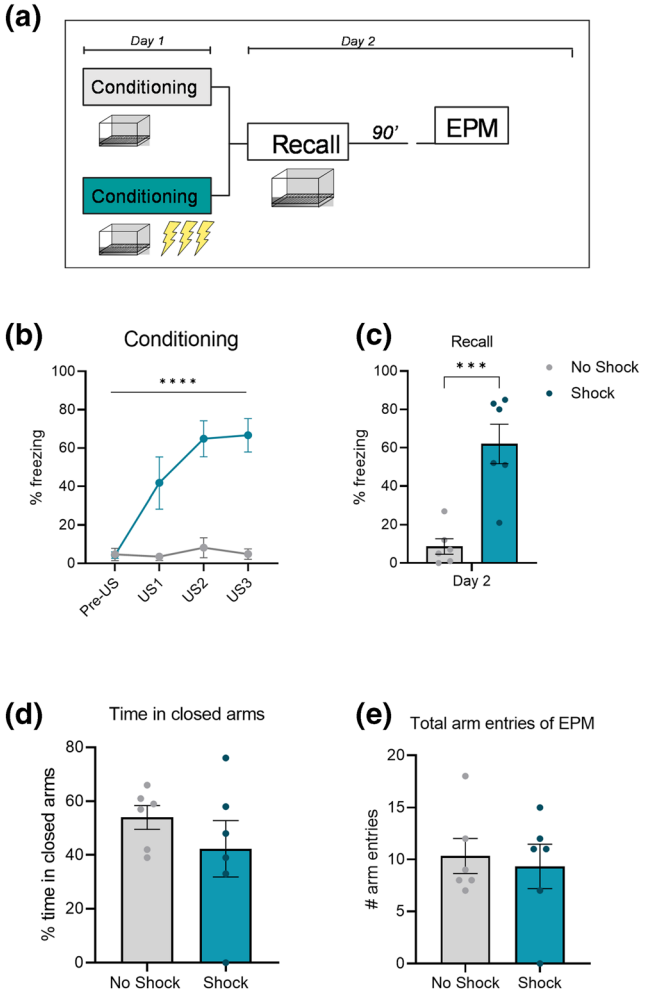


FIGURE 1 (a) Rats were either threat conditioned by pairing mild foot shocks with a contextual environment (Shock) or placed in the context in absence of foot shock (No Shock). A memory recall test was performed, and then, 90′ later, they were tested in the EPM. (b) The Shock group acquired conditioned freezing across training. (c) The Shock group showed robust conditioned freezing to the context at the recall test. (d) 90′ later, the time spent in the closed arms of the EPM was equivalent between the two groups. (e) Total arm entries were equivalent between the groups. $^{****}p < .0001$, $^{***}p < .001$

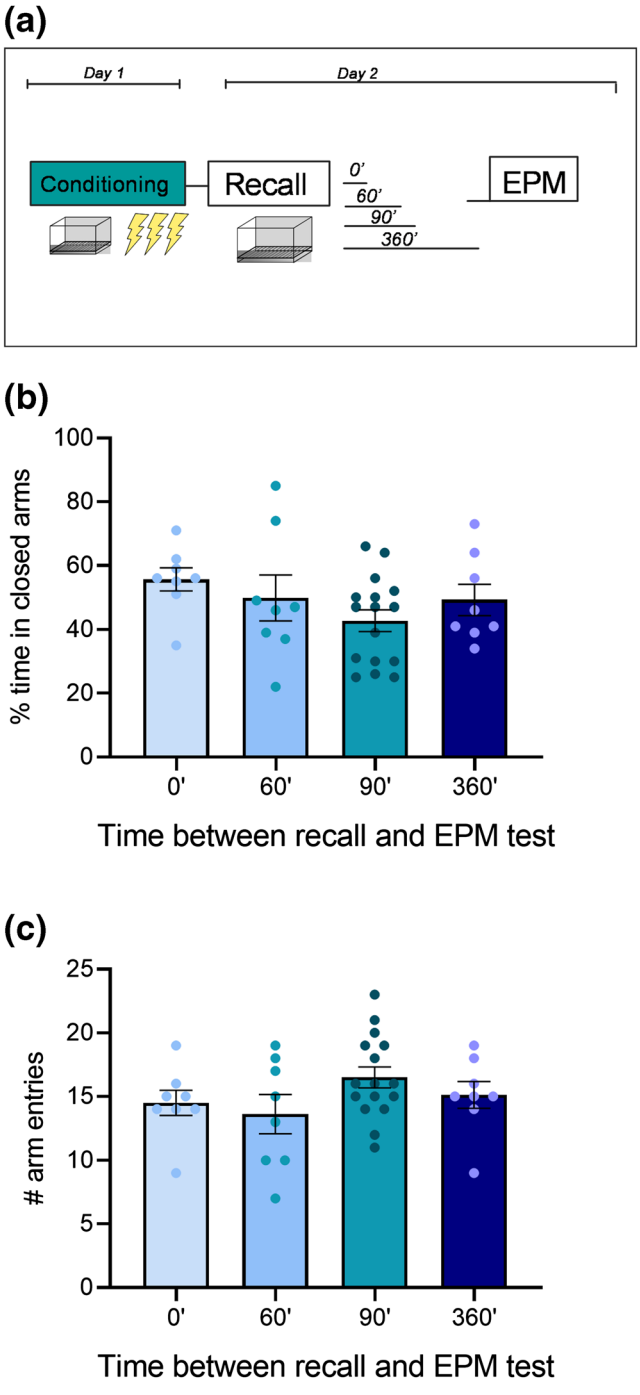


FIGURE 2 (a) Independent cohorts were tested at a series of time points after recall of conditioned threat context memory to investigate any temporal effect of threat memory recall on EPM measures. (b) None of the time points investigated showed increased closed arm exploration after threat memory recall. (c) Total arm entries were unaffected by recent or remote memory recall.

test was performed 8 days later, and the rats were tested in the EPM 90 min after that recall session (Figure 3a). Significant levels of freezing were detected in the Shock group, relative to the No Shock group ($F_{\text{group}(1,10)} = 76.28$, $p < .001$, Figure 3b) and still at the Day 9 s recall test ($p = .0240$, Figure 3c). Both groups were tested in the EPM 90 min after recall. There was no significant difference between the time spent in the closed arms of the EPM nor total arm entries ($t_{10} = .9865$, $p = .3472$, Figure 3d). Furthermore, the conditioned freezing behaviour did not correlate to time spent in the closed arms ($r_{(12)} = .04270$, $p = .8952$). This suggests that recent recall of an olfactory aversive memory did not impact subsequent behaviour in the EPM.

3.1.3 | Auditory conditioning

The final modality of conditioning examined was with an auditory tone as the CS. As before, a control group was presented the tone in absence of foot shocks (No Shock). A conditioned group (Shock) were trained with paired tone foot shock presentations and presented conditioned freezing relative to the No Shock group after the second pairing of CS + US ($F_{\text{interaction}(3,57)} = 5.467$, $p = .0023$, Figure 4b). The next day, a recall test was performed 90 min before testing in the EPM. As seen with the contextual and olfactory modalities, the conditioned freezing was specific to the paired presentation ($F_{(1,19)} = 7.288$, $p = .0142$, Figure 4c). The tone memory recall had no impact the time spent in the closed arm of the EPM ($t_{19} = .2282$, $p = .8219$, Figure 4d) nor arm entries ($t_{19} = .005984$, $p = .9953$, Figure 4e).

The independence of EPM measures from experience of conditioned threat was further tested across a longer period. We performed a follow-up retest in the EPM (EPM Test 2) 7 days after the first EPM test (EPM Test 1, Figure S1). The time spent in the closed arm did not significantly change between test and retest ($t_{12} = .2754$, $p = .7877$) although total arm entries increased slightly ($t_{12} = 2.184$, $p = .0495$). We also examined whether the time spent in the closed arm related to CS-evoked freezing, or vice versa whether CS-evoked freezing would relate to subsequent EPM behaviour in two independent cohorts (Figure 5). In one cohort, rats were screened in the EPM to examine if this behaviour related to subsequent conditioned freezing. At the recall test of auditory cued conditioning, 8 days after the EPM test, there was no relationship between time spent in the closed arm and the freezing during the test CS presentation ($r_{(9)} = .2934$, $p = .4107$, Figure 5a). In another cohort, the order of testing was reversed, and rats underwent auditory cue conditioning; then, 7 days later, they were tested in the EPM. Conditioned freezing at test during the CS presentation did not relate to subsequent closed arm time in the EPM ($r_{(9)} = -.1546$, $p = .6698$, Figure 5b). There were no significant differences in the mean levels of CS freezing nor

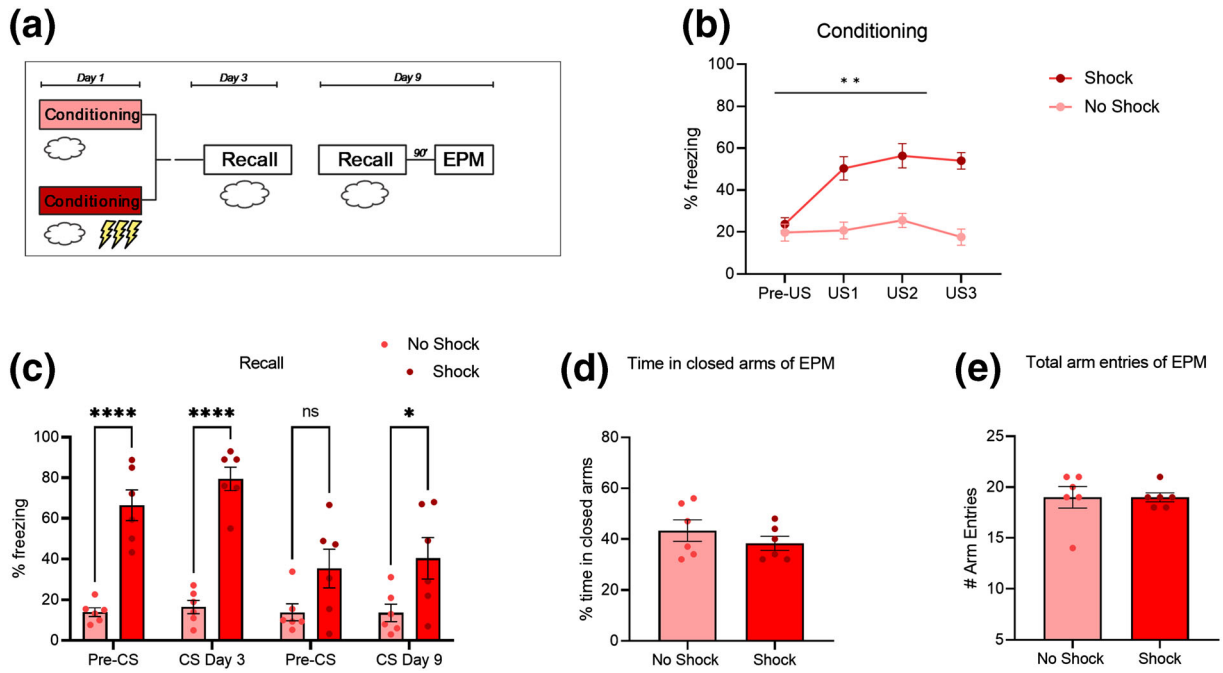
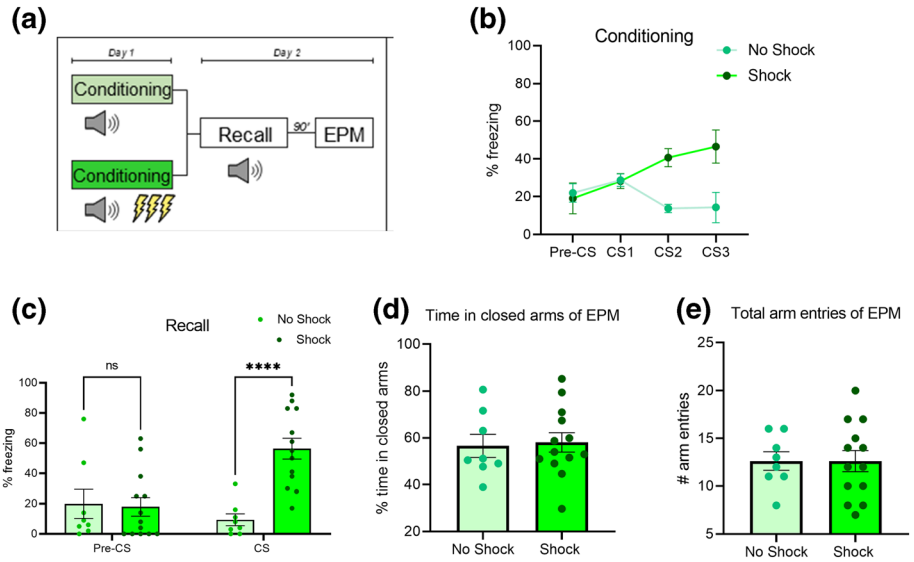


FIGURE 3 (a) Rats were conditioned by pairing mild foot shocks with a neutral odour acetophenone (Shock), or presented the odour in absence of foot shock (No Shock). (b) Conditioned freezing to the odour increased across training. (c) An initial test of consolidation was performed at Day 3, followed by a subsequent test at Day 9 to assess the stability of the olfactory cue memory. The Shock group showed persistent conditioned freezing at recall to the odour CS and freezing in the context (Pre-CS). (d) 90′ after memory recall the time spent in the closed arms of the EPM was equivalent between the two groups. (e) Total arm entries were equivalent between the groups. $^{****}p < .0001$, $^{*}p < .05$

FIGURE 4 (a) Rats were conditioned by pairing mild foot shocks (Shock) with an auditory tone or presented the tone in absence of foot shock (No Shock). A memory recall test was performed and then 90′ later they were tested in the EPM. (b) The Shock group acquired conditioned freezing to the tone across the training session. (c) The Shock group showed conditioned freezing at recall to the CS. (d) Time spent in the closed arms of the EPM was equivalent between the two groups. (e) Total arm entries were equivalent between the groups. $^{****}p < .0001$



time in the closed arms between the cohorts. Differences in conditioned freezing recall test have been reported to be revealed by performing median splits of rats into the extreme of responses on OA activity performed around a month before the recall test (Borta et al., 2006). In agreement with the prior study (Borta et al., 2006), when we performed a median split of the group EPM tested a week before threat conditioning, there was a trend that the low OA (LOA) explorative rats had higher levels of freezing behaviour during the CS presentation of the recall test session (two-tailed t test, $t_8 = 2.088$, $p = .0702$) despite equivalent freezing during conditioning training. However, when taken as a whole group the arm exploration values did not significantly correlate to freezing

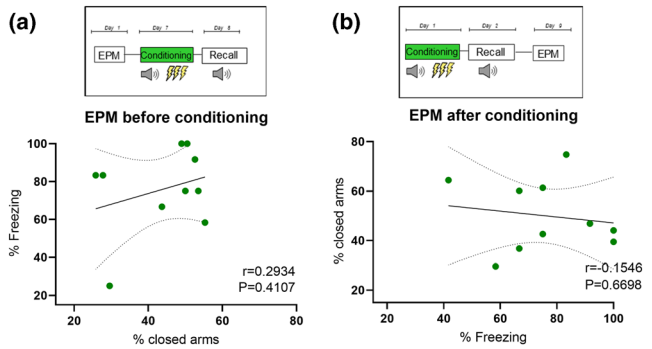


FIGURE 5 (a) The time spent in the closed arm of the EPM did not relate to subsequent conditioned freezing at tone CS test 8 days later. (b) Conditioned freezing at CS test did not relate to the time spent in the closed arm of the EPM 7 days later. $N = 10$ for each independent cohort

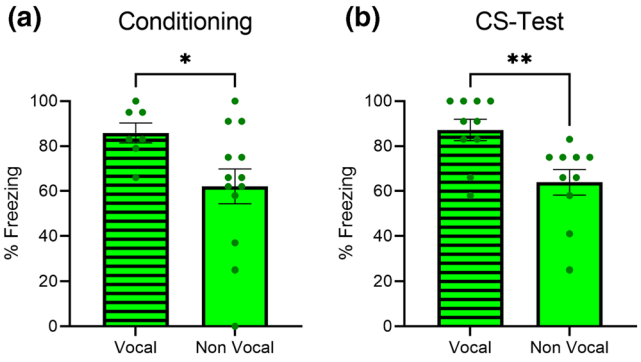


FIGURE 6 (a) Rats that produced 22 kHz vocalisations had higher levels of freezing by the end of conditioning than those not producing calls. (b) 24 h later, the rats that produced 22 kHz vocalisations in response to the tone CS at recall were those with significantly higher levels of freezing. $^{**}p < .005$, $^{*}p < .05$

behaviour. We also performed the same split analyses of the cohort tested with the EPM 1 week after cued-threat conditioning. The levels of freezing during conditioning or during the CS test did not differ between the subsequently segregated LOA and High OA groups ($p = .1788$ and $p = .3495$, respectively). Taken together, the cohort split by OA behaviour before or after threat conditioning revealed no significant differences between their conditioned freezing, so our analyses were performed without any median split segregation of the rats by EPM behaviour to capture the full range of responses.

The levels of conditioned threat detection behaviour (i.e., conditioned freezing) in these experiments did not impact EPM performance. Together this may suggest that the EPM behaviour was independent of the recent aversive experience tested under these strengths, modalities and time points.

3.1.4 | Rats that produced 22 kHz USVs displayed stronger conditioned freezing

The production of 22 kHz USVs calls during the auditory threat cue conditioning and CS test sessions were analysed in both cohorts of rats screened with the EPM before or after cue conditioning. In the cohort tested in the EPM before cue conditioning, the time spent in the closed arm did not correlate with the total duration of USV calls through subsequent conditioning ($r_{(10)} = .3126$, $p = .3792$) nor at CS test ($r_{(10)} = .2103$, $p = .5599$). Also, the duration of USVs made during conditioning ($r_{(10)} = -.04174$, $p = .9089$) or during the CS test ($r_{(10)} = -.1376$, $p = .7045$) of the cohort examined with the EPM afterwards did not relate to closed arm behaviour at that time. In the 'Vocal' group, the duration of 22 kHz calls USVs produced in the sessions varied largely across individuals (min 11 ms, max 307 s). While it must be acknowledged that there is considerable inter-individual variability in the propensity to produce USVs, from the present data, the performance in the EPM did not seem to reflect the presence or absence of calling during conditioned freezing. On the other hand, we observed that the production of 22 kHz USVs was tightly coupled to conditioned freezing behaviour and so hypothesised these rats would show the strongest freezing response to the threat cue. In both cohorts, rats that produced USVs displayed higher levels of freezing than those that did not vocalise during CS conditioning (two-tailed t test, $t_{18} = 2.127$, $p = .0475$, Figure 6a). The duration of USVs produced during conditioning did not significantly correlate with freezing taking all vocal and non-vocal rats together ($r_{(20)} = .3896$, $p = .0895$). However, at the test day, the correlation of USVs duration and conditioned freezing became significant ($r_{(20)} = .6110$, $p = .0042$). At CS test, the difference in freezing between vocal and non-vocal rats was also significant (two-tailed t test, $t_{18} = 3.138$, $p = .0057$, Figure 6b). As such, the production of 22 kHz calls triggered by a threat cue is useful to reveal the heightened state of the most reactive individuals.

4 | DISCUSSION

Presentation of an aversive CS at a recall test has been shown to activate brain circuits shared with those activated by EPM experience, somewhat depending on the modality of the associative stimulus (Steimer, 2002; Tovote et al., 2015). Therefore, it is possible that there may be some overlap between the brain mechanisms recruited by experience of the EPM and threat

conditioning (Jimenez et al., 2018; Wang et al., 2011; Zhang et al., 2014). Although the conditioning modalities examined herein, namely contextual, olfactory and auditory, recruit stimulus-specific circuits, they have all been shown to require intact amygdala function for the associative learning of unconditioned stimulus to CS pairings (Cousens & Otto, 1998; Kim & Fanselow, 1992). Thus, the potential interaction between the mechanisms underlying EPM behaviour and conditioned freezing makes it interesting to compare how these two measures may relate to each other.

The series of experiments described herein established that the EPM was insensitive to recent aversive memory recall and the exploratory behaviour seen in the EPM did not relate to conditioned freezing, across the modalities examined. These findings might be considered surprising given that others have reported negative effects on OA activity, sometimes taken as a measure of 'state anxiety', following re-exposure to contexts associated with foot shocks (Marinzalda et al., 2014; Mechiel Korte et al., 1999). Those studies demonstrated an effect of 'fear-potentiation' in the EPM by context re-exposure, which was gone after a 180 min interval postcontext exposure compared with a no stressed (No Shock) 0 min exposure group. Here, we found no effect on the EPM across the Cxt Shock group time points evaluated, but we limited our focus to the 90 min time point for our comparison with a No Shock group. Although Mechiel Korte et al. did not report conditioned freezing data, it is unlikely that the lack of a 'fear-potentiation' effect of context re-exposure reported here was simply due to some weaker associative learning because no correlation was detected between freezing at memory recall and the time spent in the closed arms across the experiments, where a wide range in strength of conditioned responding was seen at the recall test. Most notably, the levels of freezing induced by olfactory conditioning were stronger at the first recall test than those elicited by auditory or contextual cues paired with equivalent level and number of footshocks and considerable generalisation to the context was seen at the subsequent test. It could be argued that the aversive state induced by recall of aversive memory is perhaps too weak to impact EPM behaviour, yet others have reported similar observations even with a relatively strong (controllable or uncontrollable) tail shock, in that they did not influence EPM arm exploration measures 2 h later (Grahn et al., 1995). Of course it is possible that methodological differences in EPM conditions may underlie the different findings (Wall & Messier, 2001), nonetheless, the levels of exploration seen herein do not differ vastly from those reported elsewhere in the literature (Duvarci et al., 2009; Grahn et al., 1995). A possible interpretation of these findings is then that the EPM is not acutely sensitive to recall of an aversive experience and measures a more trait-like level of OA exploration.

Given an interpretation that the EPM measures may reflect something more akin to a stable trait measure, two cohorts were screened in the EPM prior to or after conditioning to see if closed arm exploration would correlate to the acquisition or expression of conditioned freezing or *vice versa* across a longer time window. There were no correlations detected between pre-EPM or post-EPM screening and conditioned freezing. Furthermore, in a subset of experiments, a follow-up retest of the rats in the EPM did not reveal any sensitisation or habituation to the maze itself. This suggests on a short time scale at least, the behaviour is consistent across testing in our set-up. Nonetheless, there are studies that have demonstrated changes in EPM behaviour from retest which suggest learning can occur in the EPM itself and influence measures, which would be problematic with a 'trait' account of its measurements (Carobrez & Bertoglio, 2005). Studies have also demonstrated that rats that were poor at discrimination of a CS+ from a CS− at a recall test were statistically prone to spend more time in the closed arms of the 2 days after the recall (Duvarci et al., 2009). These findings might suggest that in the extremes of memory precision the EPM may reveal meaningful individual differences. Overall, there appears to be limited relationship of the EPM measures to conditioned freezing if a full range of responses are taken without thresholds to segregate groups of rats based on responses. Determination of what would constitute a robust threshold that can pull apart reliable differences in behaviour is a challenge for the field in many tasks not just the EPM.

Throughout the experiments, the production of 22 kHz USVs was consistently seen to be present in the rats showing the strongest conditioned freezing responses. These two behaviours, in contrast to arm avoidance in the EPM and freezing, seem to be closely tied together. The interplay of 22 kHz USVs and conditioned freezing represents an interesting means to reveal the most reactive individuals in a threat conditioning task. If freezing is taken as an active 'fear-like' response to evade detection by an inescapable predator (McNaughton & Corr, 2004), the progression to 22 kHz calls by an individual in isolation may represent a shift to an active response to deter a predator stalking attack (Blanchard et al., 1991). Notably, the levels of USVs did not correlate to prior or subsequent EPM behaviour, again tying the production of such alarm calls to sensitive conditioned threat cue detection. Nonetheless, the variability in production of USVs again points to important individual differences in propensity to react to a threat cue.

Variations of mazes that expose rodents to open areas are routinely used to measure behaviour argued to be akin to anxiety symptoms (Cryan & Holmes, 2005). Interestingly, in mice, there is evidence that while the open field task correlated to levels of conditioned contextual freezing, the EPM did not correlate to either baseline nor conditioned contextual freezing (Ahn et al., 2013), which mirrors our findings here with auditory conditioning. In the same study, it was also remarked that the performance in the open field task did not correlate with the EPM behaviour, perhaps surprising given how these tasks are thought to both track an agoraphobia driven behaviour, but other work has substantiated these differences in measures taken in each task (Carola et al., 2002). Indeed, in the early studies of 'fear-like' responses that preceded the now widespread use of a traditional EPM set-up, Montgomery and Monkman (1955) argued in a series of papers a distinction between fear and exploratory behaviour in mazes that was driven more by novelty than 'fear'. Despite the potential issues with the EPM as a translational task (Ennaceur, 2014), the independence of measures in the EPM from conditioned freezing may simply indicate that they measure different forms of aversively motivated behaviours that are context appropriate. This may be advantageous for studies that use the tests in series in a battery of tasks. Given the prospect that individual differences in performance at the extremes proves interesting for further exploration, these presented findings call for caution in generalisation of interpretations from the typical range of behaviour in the EPM across other aversively motivated tasks.

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CONFLICT OF INTEREST

The authors declare no financial or personal relationship which could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Joe R. Hilton: Data curation; formal analysis; writing-original draft; writing-review and editing. **Susannah R. Simpson:** Formal analysis; writing-review and editing. **Emily R. Sherman:** Formal analysis; writing-original draft; writing-review and editing. **Will Raby-Smith:** Formal analysis. **Keemia Azvine:** Formal analysis. **Maite Arribas:** Formal analysis. **Jiaqi Zhou:** Formal analysis. **Serena Deiana:** Writing-original draft; writing-review and editing. **Bastian Hengerer:** Writing-original draft; writing-review and editing.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data are available on request to the corresponding author.

ORCID

Emma N. Cahill https://orcid.org/0000-0003-3054-1708

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