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Context and topography determine the role of basolateral amygdala metabotropic glutamate receptor 5 in appetitive Pavlovian responding

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Abstract

Preclinical data have shown that the excitatory metabotropic $G\alpha_q$-coupled glutamate receptor, mGluR5, has a role in substance abuse and relapse. However, little is known about the contribution of mGluR5 to the expression of conditioned responding elicited by appetitive Pavlovian cues. We investigated this question in rats that were trained to associate a discrete, auditory conditioned stimulus (CS) with a fructose-glucose solution (5.5% fructose/4.5% glucose; 'sugar'). In subsequent tests for the expression of conditioned responding without sugar delivery, CS-elicited fluid port entries were elevated in a context associated with sugar, relative to an equally familiar, neutral context. Inhibiting mGluR5 via systemic injections of a negative allosteric modulator (MTEP; 5 mg/kg) reduced CS port entries in both the sugar context and neutral context. Targeting MTEP microinjections (3 µg/side; 0.3 µl/min) to the nucleus accumbens (Acb) core had no effect on CS port entries at test, whereas the same manipulation in the basolateral amygdala (BLA) produced effects that were topographically dependent. Specifically, microinjecting MTEP in the posterior BLA had no effect on behavior, whereas inhibiting mGluR5 in the anterior BLA enhanced the contextual discrimination of CS port entries. These data are the first to show a role of mGluR5 in the context-dependent expression of appetitive Pavlovian conditioned responding, with a topographically defined arrangement of mGluR5 in the BLA being particularly important for context-based responding to a discrete, appetitive cue.

Introduction

Altered glutamate homeostasis is hypothesized to play a major role in substance abuse [1], prompting considerable research on the role of the glutamatergic system in addiction [2, 3]. The excitatory metabotropic $G\alpha_q$-coupled glutamate receptor, mGluR5 (nomenclatures: IUPHAR = $mGlu_5$; HGNC = GRM5), has been studied in both human and non-human animals, and holds promise as a potential therapeutic target [4-12].

Preclinical animal models of relapse suggest that mGluR5 in the nucleus accumbens (Acb) core and basolateral complex of the amygdala (BLA) mediates operant drug-seeking behavior that is reinforced by drug-predictive cues. For example, inhibiting mGluR5 through microinjection of the negative allosteric modulator 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) in the Acb core or BLA reduced cue-induced reinstatement of alcohol-seeking [7], and MTEP microinjections into the Acb core reduced context-induced reinstatement (renewal) of cocaine seeking [13]. Less is known about the role of mGluR5 in conditioned responding that is acquired through Pavlovian conditioning and triggered by appetitive Pavlovian cues. This is an important question because drug-predictive cues can evoke craving and physiological responses that may precipitate relapse in people with substance use disorders [14].

Animal models of aversive Pavlovian conditioning suggest a role for mGluR5 in the acquisition and expression of this form of learning. For example, MTEP administration impaired the acquisition of aversive conditioning and acute administration prior to test reduced the expression of conditioned responding to an aversive conditioned stimulus [15, 16]. Similarly, intra-BLA microinjections of MTEP during the acquisition of conditioned taste aversion rendered the conditioned taste aversion resistant to extinction [17]. In appetitive Pavlovian conditioning studies, inhibiting mGluR5 during acquisition prevented a cue from subsequently functioning as a conditioned reinforcer, suggesting that mGluR5 is needed for appetitive cues to acquire incentive properties [18]. These studies provide precedent for the hypothesis that glutamate transmission at mGluR5 plays a central role in behavior that is acquired through appetitive Pavlovian conditioning.

To test this hypothesis, we investigated the role of mGluR5 in the expression of conditioned responding to an appetitive Pavlovian conditioned stimulus (CS). Rats were trained to associate a discrete, auditory CS with the delivery of a 10% fructose-glucose solution (5.5% fructose/4.5% glucose; 'sugar'). We used this monosaccharide ratio because it is common in commercial foods and sweetened beverages and can alter dopamine function [19]. Moreover, palatable foods and sugar can induce neural adaptations and promote addiction-like behaviors [3, 20]. Because mGluR5 has been implicated in processing contextual information [12], we used a task that allowed us to examine the necessity of mGluR5 in responding to a CS that predicted sugar in a context that was associated with sugar as well as in a different, neutral context [21-23].

First, we examined the impact of the sugar-associated context on the expression of CS-elicited fluid port entries. Next, we examined the effect of systemically inhibiting mGluR5 (with MTEP) or NMDA glutamate receptors on CS port entries in both the sugar context and the neutral context. We chose MTEP because it is widely used to inhibit mGluR5 activity in studies of appetitive motivation [24]. Previous studies using MK-801 ((5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine) have implicated NMDA receptors in Pavlovian learning [25], and have shown interactions between NMDA receptors and mGluR5 [26-28]. We then investigated the Acb core and BLA as neuroanatomic loci for MTEP-mediated reductions in CS port entries. The BLA emerged as an important region, with an anteroposterior (AP) gradient within the BLA defining the impact of MTEP.

Methods

Animals

We used 122 experimentally-naïve, male, Long-Evans rats (Charles River, QC, Canada). Housing conditions are described in the supplementary materials and methods. Male rats were used to build on prior research on the role of the glutamate system in appetitive Pavlovian behaviour [21]; however, future studies will extend this work to female rats. Rats had unrestricted access to food (Teklad, Envigo, QC, Canada) and water throughout the experiments. All procedures were

approved by the Animal Research Ethics Committee at Concordia University and performed in accordance with guidelines from the Canadian Council on Animal Care.

Surgery

Using standard stereotaxic procedures [21], rats in separate microinjection experiments received bilateral, 26 ga cannulae (Plastics One, Roanoke, VA, USA) targeting the Acb core, the BLA, or a more anterior portion of the BLA. Coordinates in mm from bregma were: Acb core, +1.5 AP, ± 3.23 ML on a 10° angle, and -4.3 mm DV; BLA, -2.54 AP, ± 5 ML, and -5.5 DV; anterior BLA, -2.1 AP, ± 4.9 ML, and -5.5 DV.

Drugs and Solutions

MTEP hydrochloride (Cat# A15174, CAS#: 1186195-60-7, Lot# L15174B001) and MK-801 (Cat# A12761, CAS#: 77086-22-7, Lot# L12761B001) were obtained from Adooq Bioscience (Irvine, CA, U.S.A.). Drugs were dissolved in a vehicle solution of 5% DMSO/0.9% sterile saline. A 10% fructose-glucose solution (hitherto referred to as 'sugar') was prepared by dissolving 55 g/L fructose and 45 g/L glucose in tap water (Cat#: FRC180 and GLU501, CAS#: 57-48-7 and 50-99-7, BioShop, ON, Canada).

Apparatus

Behavioral training was conducted using 12 identical conditioning chambers (30.5 x 31.8 x 29.2 cm, Cat#: ENV-009A, Med Associates, St Albans, VT, USA) that are described in the supplementary materials and methods.

General Behavioral Procedures

Home-cage exposure to sugar. Rats were pre-exposed to sugar for 48 h in their home-cages. A pre-weighed fluid receptacle containing 90 mL of sugar was placed on the home-cage. This bottle was re-weighed 24 h later, refilled to 90 mL and then weighed again after 24 h. Rats consumed all, or nearly all, of the sugar.

Pavlovian conditioning with context discrimination. Rats were habituated to transport and the conditioning chambers over 3 days (see supplementary materials and methods).

They were then assigned to one of two contexts for Pavlovian conditioning sessions (the sugar context), while the remaining context served as the familiar, neutral context (see Table 1 for description of contexts). Discrete stimuli were a 10 s, continuous white noise or 10 s of a 5 Hz clicker. Rats were assigned one stimulus (the conditioned stimulus or CS) to be paired with sugar delivery in the sugar context and the other (the neutral stimulus or NS) to be presented without sugar in the neutral context. The purpose of the NS was to equate the acoustical salience of both contexts. Rats were counterbalanced across contexts, stimuli, and session order such that there were no differences in home-cage sugar consumption or bodyweight. Rats were then given one training session a day that alternated between each context until they had received 10 sessions of Pavlovian conditioning in the sugar context and 10 sessions of exposure to the NS in the neutral context.

During training sessions, rats received 10 stimulus presentations (either CS or NS as per the appropriate context) with intervals of 120, 240, or 360 s between trials (mean inter-trial interval (ITI) = 240 s), with each trial consisting of a 10 s Pre-CS/NS interval, 10 s CS/NS presentation, and 10 s post-CS/NS interval. In the sugar context, presentations of the CS co-terminated with 6 s of syringe pump operation to deliver 0.2 mL of fructose-glucose ('sugar') solution. In the neutral context, NS presentations also co-terminated with 6 s of syringe pump operation, but no syringes were present and thus no sugar was delivered.

Testing. At 24 h after the last training session, the expression of conditioned responding elicited by the CS was tested in the absence of sugar. Tests occurred in the sugar context and the neutral context for each rat, with 1-2 sessions of retraining in each context between tests. At test, the CS was presented as during prior Pavlovian conditioning sessions and the syringe pump was activated for 6 s, but no syringes were present and thus no sugar was delivered. The NS was never presented at test. Moreover, our preliminary data indicate that the NS does not elicit port entries when presented alone in either the sugar or neutral contexts [29].

Four separate experiments (described in detail in the supplementary materials and methods) were conducted using this behavioral procedure.

Table 1 Description of contexts used for Pavlovian conditioning with context discrimination.

Modality	Context 1	Context 2
Visual	Black cardboard-covered sides Brown paper in waste pan	No covers (clear acrylic) White paper in waste pan
Tactile	Acrylic glass floor	Wire grid floor
Olfactory*	10% lemon oil	10% almond odor (benzaldehyde)

* Sprayed onto a clear petri dish located in the waste pan beneath the chamber floor.

Histology

Standard histological procedures [21] were used to visualize placements of the microinjectors within targeted brain regions (see supplementary materials and methods).

Data analyses and availability of materials

Statistical analyses were performed using SPSS 24 (IBM, NY, USA), and included paired t-tests, repeated measures ANOVA, mixed-design ANOVA, Bonferroni-corrected post-hoc comparisons, and Pearson correlations. For repeated measures ANOVA, Greenhouse-Geisser sphericity corrections were used when $\varepsilon < 0.75$. The non-parametric Friedman's Two-Way ANOVA was used when data violated assumptions of homogeneity of variance.

The behavior we measured was entries into the fluid port during different intervals of the session. These intervals included 10 s before each CS/NS (Pre-CS/NS), the 10 s CS/NS, and the variable inter-trial interval (ITI). Conditioned responding is depicted as an elevation score, calculated by subtracting Pre-CS port entries from CS port entries [30, 31].

Each experiment was run as a single replicate. The underlying raw data and Med-PC code are available on Figshare [32].

Results

CS port entries were selectively elevated in the sugar context

We previously reported a reliable and selective elevation in port entries elicited by a CS that predicted alcohol in an alcohol context, relative to a neutral context [21]. The impact of context on port entries elicited by a CS that predicted sugar is unknown. To examine this question, rats (n=17) were trained and tested as described above (Fig. 1a). At test, CS port entries were elevated in the sugar-associated context, relative to the equally familiar, neutral context. Normalized CS port entries (Fig. 1b) were significantly higher at test in the sugar context than the neutral context ($t_{16} = 4.268$, $p = 0.001$), and the latency to make a port entry after CS onset was significantly shorter in the sugar context than in the neutral context (Fig. 1c; $t_{16} = -6.235$, $p < 0.001$). Context had no effect on port

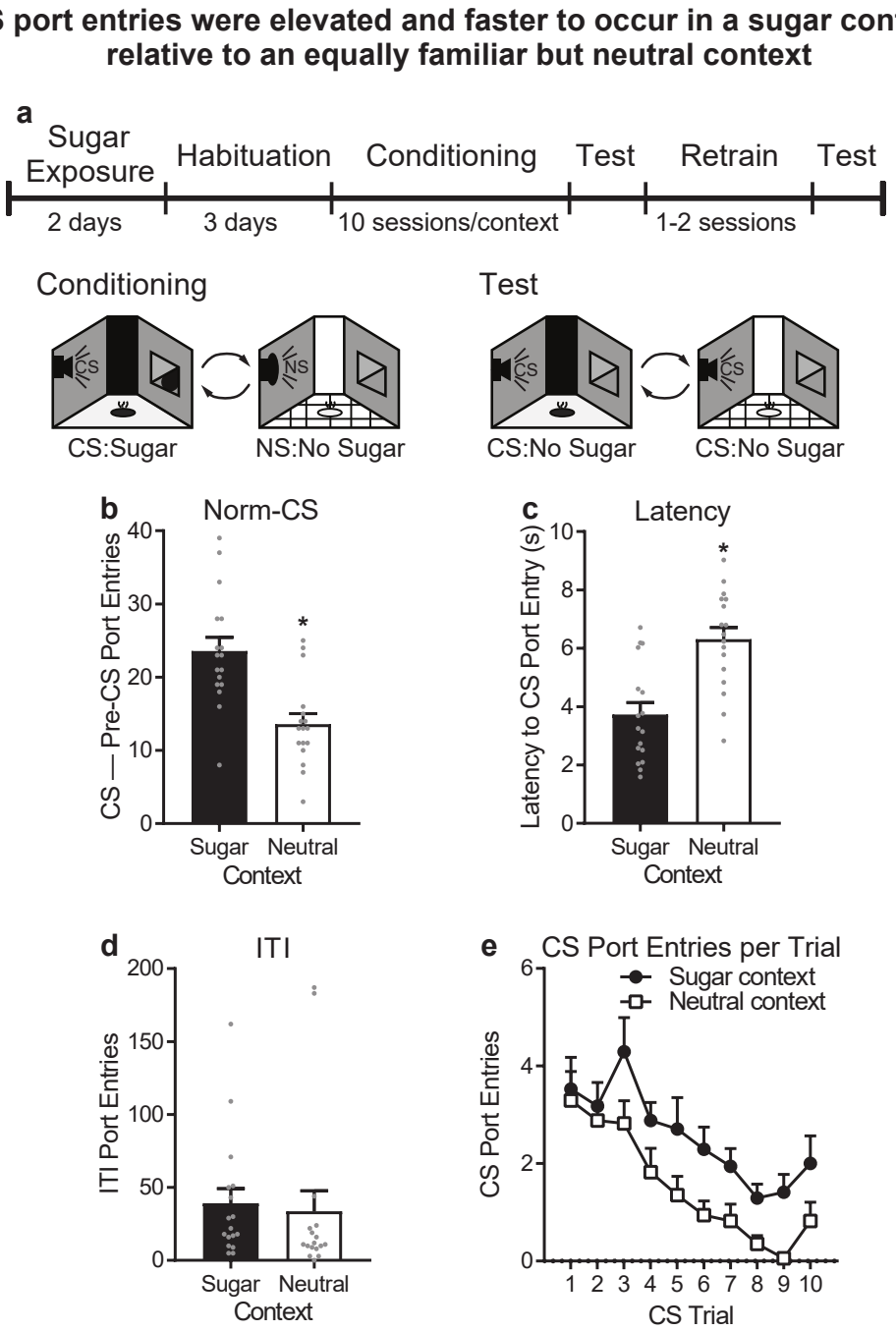

Fig. 1 CS port entries were elevated and faster to occur in a context associated with sugar compared to an equally familiar, neutral context. (a) Rats (n=17) were exposed to sugar in the home-cage and then habituated to transport and contexts over 3 days. Rats were then trained to associate one auditory conditioned stimulus (CS) with sugar in one context (sugar context) and given equal exposure to a distinct, neutral context where a different, neutral auditory stimulus (NS) was presented without sugar. Contextual configurations were counterbalanced between the sugar and neutral contexts (see description in Table 1). At test, the CS was presented without sugar in both contexts, with retraining between tests. (b) At test, normalized CS port entries (CS – Pre-CS port entries) were elevated in the sugar context compared to the neutral context. (c) Latency to the first CS port entry was shorter in the sugar context than the neutral context. (d) There was no effect of context on the number of port entries made during the inter-trial intervals (ITI). (e) Non-normalized port entries in each CS trial decreased across test, but did not differ between contexts. Data are presented as means ± SEM. * $p < 0.05$ for paired t-tests. Statistical tests were paired t-tests (b-d) and repeated measures ANOVA (e). Data from individual rats are depicted as grey dots (b-d).

entries made during the ITI (Fig. 1d; $t_{16} = 0.349$, $p = 0.699$), indicating a selective influence of context on CS port entries.

An analysis of within-session responding at test found that the number of port entries made during each CS trial decreased across trials (Fig. 1e; Trial, $F_{9,144} = 9.876$, $p < 0.001$), with more overall CS port entries in the sugar context (Context, $F_{1,16} = 23.14$, $p < 0.001$) but a comparable decrease across CS trials in both contexts (Context × Trial interaction, Greenhouse-Geisser corrected, $\varepsilon = 0.537$; $F_{4.836,77.378} = 0.567$, $p = 0.72$).

Systemic administration of MTEP, but not MK-801, reduced CS port entries in both contexts

In the same rats, we assessed the contribution of NMDA receptors and mGluR5 in the expression of CS port entries in both contexts. Prior to tests in either context, rats received an injection of vehicle, 0.1 mg/kg MK-801, or 5 mg/kg MTEP according to a within-subjects, Latin Square design. These doses have been shown previously to affect dopamine release in the prefrontal cortex [27] and reinstatement of methamphetamine and cocaine seeking [33, 34]. At test, normalized CS port entries were significantly higher in the sugar context than in the neutral context (Fig. 2a; Context, $F_{1,16} = 45.122$, $p < 0.001$). ANOVA indicated a significant main effect of Treatment ($F_{2,32} = 23.166$, $p < 0.001$) that did not differ across contexts (Context × Treatment, $F_{2,32} = 2.157$, $p = 0.132$). Bonferroni-corrected post-hoc comparisons revealed a significant reduction in CS port entries following MTEP, relative to vehicle or MK-801 ($p < 0.001$ for both comparisons), suggesting that inhibiting mGluR5 reduced CS port entries regardless of the context in which the CS was presented.

Latency to the first CS port entry followed the same pattern. At test, CS port entries were initiated more rapidly in the sugar context (Fig. 2b; Context, $F_{1,16} = 37.477$, $p < 0.001$) and there was a significant main effect of Treatment (Greenhouse-Geisser corrected, $\varepsilon = 0.69$, $F_{1.381,22.09} = 32.484$, $p < 0.001$). The effect of MTEP did not differ between contexts (Context × Treatment, $F_{2,32} = 1.283$, $p = 0.291$). Bonferroni-corrected post-hoc comparisons indicated a significant increase in latency following MTEP, relative to vehicle ($p = 0.001$) or MK-801 ($p < 0.001$).

Systemic MTEP but not MK-801 reduced CS port entries

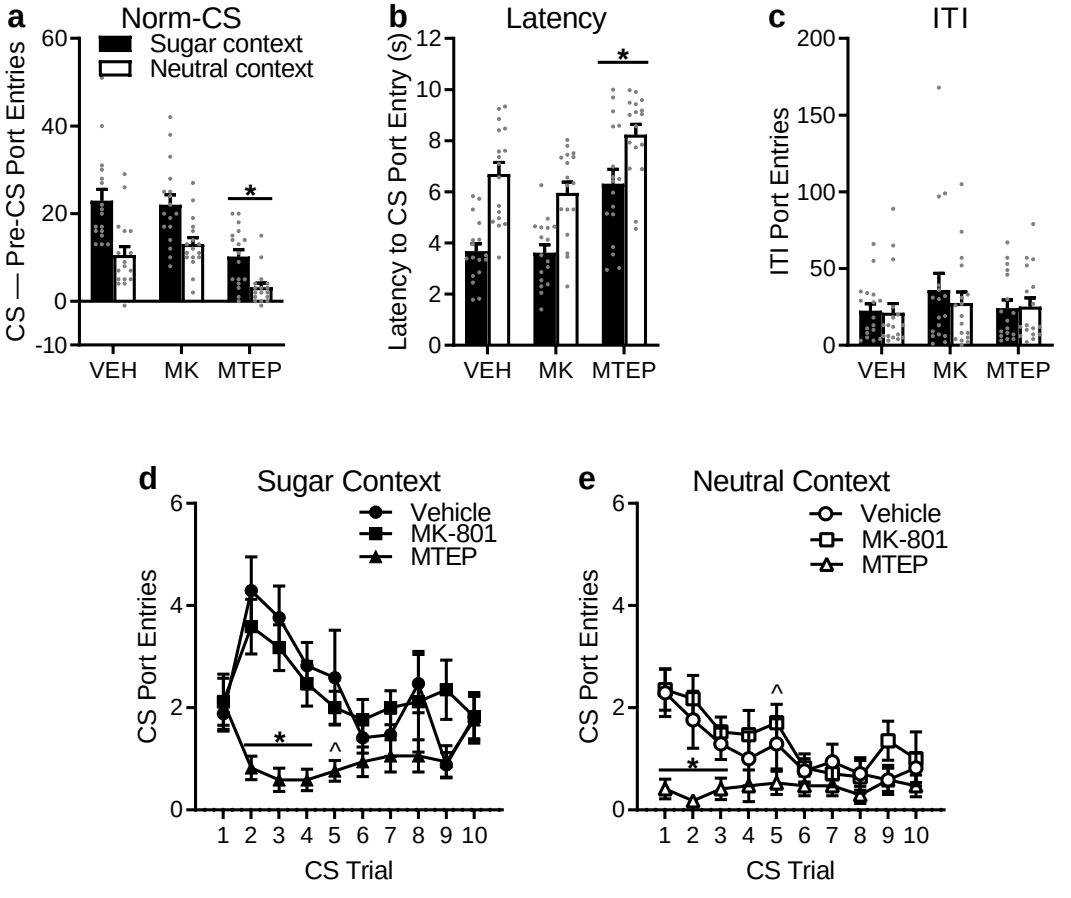

Fig. 2 Systemic MTEP but not MK 801 reduced CS port entries. (a) We tested the expression of CS port entries in well-trained rats (n=17) following systemic injections of vehicle, 0.1 mg/kg MK-801, or 5 mg/kg MTEP in a counterbalanced, within-subjects design. Normalized CS port entries (CS – Pre-CS port entries) were elevated in the sugar context relative to the neutral context, and reduced following MTEP, but not MK-801, in both contexts. (b) The first CS port entry took longer to occur in both contexts following MTEP, but not MK-801. (c) MTEP had no effect on port entries during the ITI. (d) In the sugar context, non-normalized CS port entries were significantly reduced by MTEP in the first half of the session, beginning in the second CS trial. (e) In the neutral context, MTEP reduced CS port entries in the first half of the session, but beginning on the first trial. Data are presented as means ± SEM. * $p < 0.05$ Bonferroni post-hoc comparisons for MTEP compared to vehicle and MK-801. ^ $p < 0.05$ Bonferroni post-hoc comparisons for MTEP compared to MK-801. Statistical tests were repeated measures ANOVAs. Data from individual rats are depicted as grey dots (a-c).

There was no impact of Context ($F_{1,16}$ = 1.056, p = 0.319) or Treatment ($F_{2,32}$ = 1.494, p = 0.24) on ITI port entries (Fig. 2c), and no Context × Treatment interaction (Greenhouse-Geisser corrected, ε = 0.719, $F_{1,439,23.019}$ = 0.31, p = 0.664).

We examined the number of port entries in each CS trial to determine if inhibiting NMDA receptors or mGluR5 influenced the pattern of responding that emerged at test (Fig. 2d and 2e). The number of port entries was higher at test in the sugar context relative to the neutral context (Context, $F_{1,16}$ = 39.086, p < 0.001), and decreased as a function of CS trial (Trials, $F_{9,144}$ = 4.465, p < 0.001), comparably in both contexts (Context x Trial, $F_{9,144}$ = 1.495, p = 0.155). This analysis recapitulated a significant main effect of Treatment ($F_{2,32}$ = 23.642, p < 0.001), with no Context x Treatment interaction ($F_{2,32}$ = 2.149, p = 0.133). Interestingly, however, the effect of MTEP differed as a function CS trial (Treatment × Trial, $F_{18,288}$ = 3.038, p < 0.001) in the sugar and neutral contexts (Context × Treatment × Trial, $F_{18,288}$ = 1.862, p = 0.019). In the sugar context (Fig. 2d), MTEP reduced CS port entries in trials 2, 3 and 4 compared to vehicle and MK-801 ($p \leq 0.006$), and in trial 5 compared to MK-801 (p = 0.02). In the neutral context (Fig. 2e), MTEP reduced CS port entries in trials 1, 2 and 3 ($p \leq 0.042$) compared to vehicle and MK-801, and in trial 5 compared to MK-801 (p = 0.049).

Although MK-801 had no effect on responding in this experiment, we found in a separate experiment that prior repeated exposure to 0.1 mg/kg MK-801 induced a selective sensitization of CS port entries, indicating that it was a behaviourally effective dose (Fig. S1).

Additional experiments in separate rats indicated that systemic injection of MTEP (5 mg/kg) had no impact on locomotor behaviour in an open field test (Fig. S2) or on the consumption of fructose-glucose solution in the home cage (Fig. S3)

Thus, blocking NMDA glutamate receptors had no impact on the expression of CS port entries. However, inhibiting mGluR5 selectively reduced responding to the appetitive CS without causing a non-specific reduction in locomotor behaviour or a change in the hedonic properties of

sugar. Moreover, MTEP reduced CS port entries immediately in the neutral context, but this effect only emerged in the second CS trial in the sugar context.

MTEP in the nucleus accumbens core had no effect on CS port entries

In separate rats (n=21), we examined the effect of MTEP microinjection into the Acb core on CS port entries in the sugar and neutral contexts (Fig. 3a), at a dose previously shown to reduce cue-induced reinstatement of alcohol seeking [7]. At test, CS port entries were significantly elevated in the sugar context relative to the neutral context (Context, $F_{1,13} = 31.338$, $p < 0.001$). However, MTEP in the Acb core did not affect CS port entries (Treatment, $F_{1,13} = 0.013$, $p = 0.909$) in either context (Context × treatment, $F_{1,13} = 1.361$, $p = 0.264$).

MTEP in the Acb core had no effect on latency to the first CS port entry (Fig. 3b). While responses occurred more rapidly in the sugar context than the neutral context (Context, $F_{1,13} = 112.742$, $p < 0.001$), MTEP did not affect latency (Treatment, $F_{1,13} = 0.067$, $p = 0.799$) in either context (Context × treatment, $F_{1,13} = 0.026$, $p = 0.874$). There was also no effect of MTEP in the Acb core on CS port entries on a per trial basis (Fig. S4a).

An analysis of ITI port entries (Fig. 3c) revealed no effect of context ($F_{1,13} =0.081$, $p = 0.78$) and no impact of intra-Acb core MTEP on ITI port entries (Treatment, $F_{1,13} = 1.042$, $p = 0.326$) in either context (Context x Treatment, $F_{1,13} = 1.078$, $p = 0.318$).

The placements of microinjector cannulae for all rats are depicted in Fig. 3d (see also Fig. S5a). Two rats were excluded from the analyses due to lost head mounts and 5 rats were excluded following histology (final n=14).

Thus, mGluR5 in the Acb core did not appear necessary for the expression of CS port entries.

MTEP in the basolateral amygdala – support for more anterior basolateral amygdala targeting

We then examined the effect of MTEP microinjection into the BLA (total n=20) on CS port entries in the sugar and neutral contexts (Fig. 4a). Normalized CS port entries were higher in the sugar context than in the neutral context (Context, $F_{1,14} = 29.383$, $p < 0.001$). MTEP microinfused into

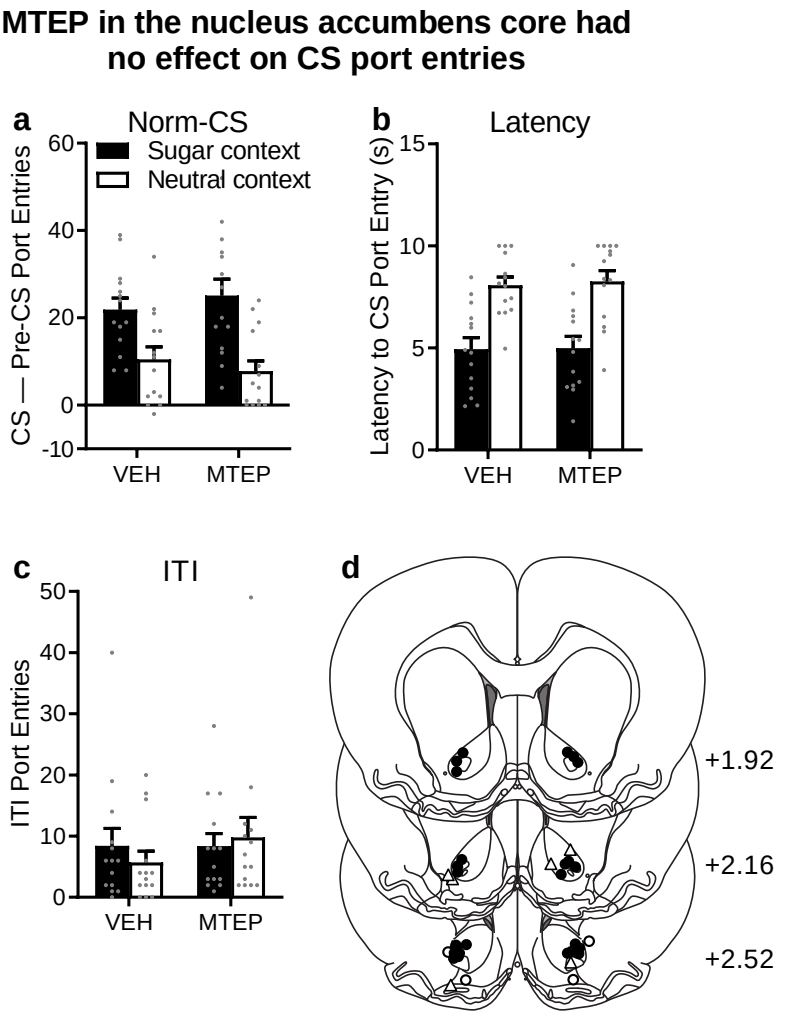

Fig. 3 MTEP microinjections targeting the nucleus accumbens (Acb) core had no effect on CS port entries. (a) Normalized CS port entries (CS – Pre-CS port entries) at test were elevated in the sugar context compared to the neutral context; however, there was no effect of 3 µg/side MTEP in the Acb core on CS port entries in either context. (b) The latency to the first CS port entry was unaffected by MTEP. (c) MTEP had no effect on port entries during the ITI. (d) Histological verification of microinjection sites. Two rats that lost head mounts, and 5 rats had misplaced cannulae (open circles, o; open triangles for unilateral placement, Δ). Final n = 14 (black circles, •). Data are presented as means ± SEM. Anteroposterior coordinates are given in mm from bregma. Statistical tests were repeated measures ANOVAs. Data from individual rats are depicted as grey dots (a-c).

MTEP in the anterior basolateral amygdala enhanced context-based differences in CS port entries

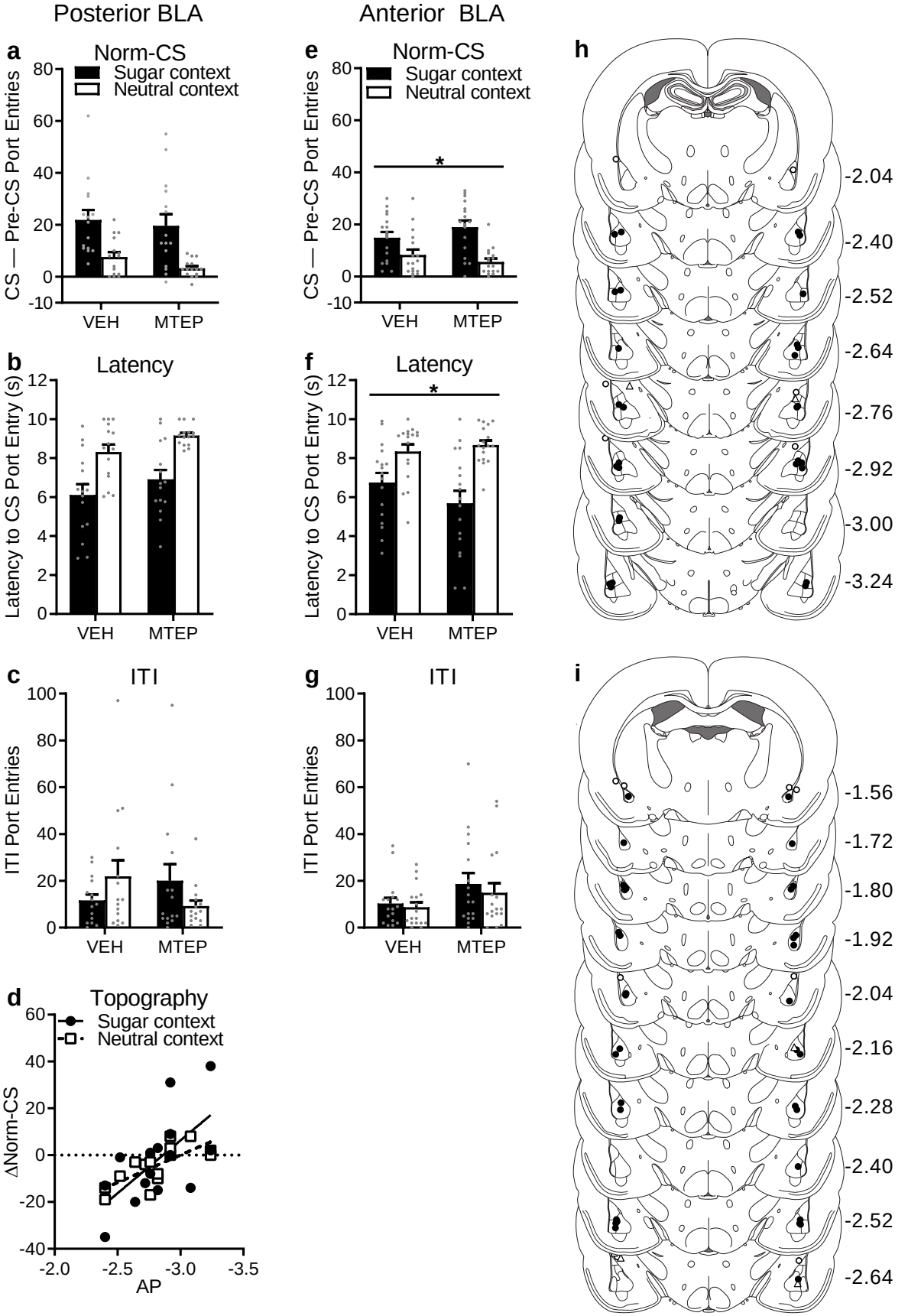

Fig. 4 Anterior targeting of the basolateral complex of the amygdala (BLA) enhanced context-based differences in CS port entries. (a) In rats with cannulae targeting the posterior BLA, CS port entries were elevated at test in the sugar context compared to the neutral context. However, there was no effect of MTEP on normalized CS port entries (CS – Pre-CS port entries), (b) latency to the first CS port entry, or (c) ITI port entries. (d) Exploratory analysis showed that more anterior BLA cannula placements were associated with greater reductions in normalized CS port entries following 3 μg/side MTEP (Δnorm-CS = norm-CS_{MTEP} – norm-$CS_{Vehicle}$). Therefore, a separate experiment targeting the anterior BLA was conducted. (e) In this separate experiment, normalized CS port entries at test were elevated in the sugar context compared to the neutral context and MTEP significantly increased the context-based differences in CS port entries, (f) as well as the latency to the first CS port entry. (g) There was no effect of MTEP on ITI port entries. (h) Histological verification of posterior BLA microinjection sites. One rat died in surgery and 4 rats had misplaced cannulae (open circles, o; open triangles for unilateral placement, Δ). Final n = 15 (black circles, •). (i) Histological verification of anterior BLA microinjection sites. One rat died in surgery and 6 rats had misplaced cannulae (open circles, o). Final n = 17 (black circles,•). Data are presented as means ± SEM. * $p < 0.05$ for a Treatment × Context interaction. All statistical tests were repeated measures ANOVAs except the Pearson correlation in panel d. Data from individual rats are depicted as grey dots (a-c, e-g).

the BLA had no impact on CS port entries (Treatment, $F_{1,14} = 1.193$, $p = 0.293$) in either context (Context × Treatment, $F_{1,14} = 0.25$, $p = 0.625$).

Similarly, CS port entries were initiated more rapidly in the sugar context (Fig 4b; Context, $F_{1,14} = 33.277$, $p < 0.001$). However, there was no effect of MTEP on this measure (Treatment, $F_{1,14} = 4.045$, $p = 0.064$) in either context (Context × Treatment, $F_{1,14} = 0.004$, $p = 0.95$).

There was also no effect of intra-BLA MTEP on CS port entries on a per trial basis (Fig. S4b).

There was also no effect of intra-BLA MTEP on port entries made during the ITI (Fig. 4c). Kolmogorov-Smirnov tests detected significant violations of the assumption of normality in the sugar context following MTEP ($D_{15} = 0.294$, $p = 0.001$) and in the neutral context following vehicle ($D_{15} = 0.246$, $p = 0.015$). We therefore performed a non-parametric Friedman's two-way ANOVA, which was not significant ($Q_3 = 5.834$, $p = 0.12$).

Despite these null results, an exploratory analysis of the data suggested that MTEP in the BLA might suppress CS port entries in a topographically-dependent manner. In the anteroposterior (AP) axis, the bulk of the rat BLA encompasses approximately -1.56 mm to -3.36 mm from bregma, with some small subnuclei extending to nearly -5 mm [35, 36]. Several studies have shown differential behavioral and neurophysiological effects depending on AP BLA topography [22, 37-39]. Based on these reports, we examined the possibility that the effect of MTEP in the BLA might vary as a function of topography. For this we plotted the change score for normalized CS port entries (Δnorm-CS = norm-CS_{MTEP} minus norm-$CS_{Vehicle}$) as a function of the AP coordinates of the microinjection placement (Fig. 4d). A negative change score in this analysis reflects a reduction in CS port entries following MTEP, relative to vehicle. Interestingly, we found significant correlations between the AP coordinates of the microinjection site, and Δnorm-CS in the sugar context ($r_{13} = -0.629$, $p = 0.012$) and neutral context ($r_{13} = -0.729$, $p = 0.002$). These results suggest that mGluR5 located more anteriorly in the BLA might preferentially contribute to CS port entries in both contexts.

Histological verification of microinjection sites (Fig. 4h; see also Fig. S5b) resulted in the exclusion of 4 rats with inaccurate cannula placements and 1 rat that died in surgery (final n=15).

MTEP in the anterior basolateral amygdala enhanced the context-based discrimination of CS port entries

In separate rats (n=24), we tested the hypothesis that mGluR5 in the anterior BLA might be preferentially involved in CS port entries. As in all prior experiments, normalized CS port entries at test were elevated in the sugar context relative to the neutral context (Fig. 4e; Context, $F_{1,16}$ = 29.037, $p < 0.001$). MTEP microinjections targeting the anterior aspect of the BLA had no overall impact on CS port entries (Treatment, $F_{1,16}$ = 0.164). However, a significant Context x Treatment interaction ($F_{1,16}$ = 8.771, p = 0.009) showed that there was a larger difference between CS port entries in the sugar and neutral contexts following MTEP, relative to vehicle. Although MTEP enhanced discrimination between contexts, Bonferroni-corrected post-hoc tests found no significant differences between vehicle and MTEP in either the sugar (p = 0.074) or neutral (p = 0.234) contexts

Rats were faster to respond to the CS (Fig. 4f) in the sugar context, relative to the neutral context (Context, $F_{1,16}$ = 32.946, $p < 0.001$). Although MTEP did not impact latency to make a CS port entry overall (Treatment, $F_{1,16}$ = 1.06, p = 0.318), there was a significant Context x Treatment interaction ($F_{1,16}$ = 4.58, p = 0.048), indicating a larger difference in latency scores between sugar and neutral contexts following MTEP, compared to vehicle. Although MTEP enhanced discrimination between contexts, Bonferroni-corrected post-hoc tests found no significant differences between vehicle and MTEP in either the sugar (p = 0.094) or neutral (p = 0.335) contexts.

CS port entries on a per trial basis were no different following vehicle or MTEP in either context (Fig. S4c).

There was no effect of Context ($F_{1,16}$ = 0.99, p = 0.335) on ITI port entries (Fig. 4g), and no effect of MTEP microinjection in the anterior BLA on ITI port entries (Treatment, $F_{1,16}$ = 3.41, p = 0.083) in either context (Context × Treatment, $F_{1,16}$ = 0.184, p = 0.673).

The placements of microinjector tips for this study are shown in Fig. 4i (see also Fig. S5c). One rat died in surgery and 6 rats were excluded following histological verification (final n=17).

Thus, inhibiting mGluR5 in the anterior BLA appeared to enhance the context-based discrimination of CS port entries, resulting in a modest increase and more rapid onset of CS port entries in the sugar context, and a modest decrease and slower onset of CS port entries in the neutral context.

Discussion

The present study produced several notable findings. We established that port entries elicited by a CS that predicted fructose-glucose solution ('sugar') were significantly elevated in a context associated with sugar, relative to a neutral context. Systemic administration of the NMDA glutamate receptor antagonist, MK-801, had no impact on behaviour. However, systemic administration of the mGluR5 inhibitor, MTEP, selectively reduced CS port entries in the sugar and neutral contexts. The neural locus of this MTEP effect was not the Acb core; however, mGluR5 in the BLA was involved in the expression of CS port entries in a topographically dependent manner. Specifically, MTEP microinjections in the anterior BLA enhanced the context-based discrimination of CS port entries. These results show that mGluR5 is involved in the expression of appetitive Pavlovian conditioned responding, and identify a topographical gradient within the BLA that defines a context-dependent role for mGluR5 in this behavior.

In well-trained rats, systemic injection of MTEP, but not MK-801, selectively reduced the number of CS port entries and increased the latency to respond to the CS in both sugar-associated and neutral contexts. The lack of effect of MTEP on ITI port entries in all our experiments suggests that inhibiting mGluR5, either systemically on within the Acb core or BLA, did not cause a non-specific decrease in locomotion. We also found that systemic MTEP (5 mg/kg) had no impact on locomotor activity in an open field test or on the consumption of a fructose-glucose solution. Taken together, these results highlight a novel role for mGluR5 in the expression of conditioned responding

elicited by appetitive Pavlovian cues, which complements the necessity of this receptor for the expression of operant behavior that is reinforced by drug-predictive cues [7-10].

We also observed an interesting difference in the within-session pattern of CS port entries at test. In the neutral context, systemic injection of MTEP produced an immediate reduction in CS port entries, suggesting that under these conditions, mGluR5 might be required for motivation to respond to a discrete CS, or for retrieving memories of the motivational value of an appetitive Pavlovian CS [18]. In the sugar context, there was no difference between MTEP and vehicle in the first CS trial, but a reduction following MTEP emerged thereafter. The latter results are consistent with the interpretation that inhibiting mGluR5 in the sugar context accelerated within-session extinction of CS port entries. mGluR5 has been implicated in extinction [12, 13]; however, results from operant studies suggest that mGluR5 receptor activation, rather than mGluR5 inhibition, facilitates extinction [40, 41]. There may therefore be a difference in the role of mGluR5 in the extinction of appetitive Pavlovian and operant learning, an intriguing hypothesis that requires further research.

In the literature on operant drug reinforcement, sucrose self-administration is often used as a control for reinforcer specificity. In several of these studies, neither systemic administration of MTEP nor intracerebral microinjection of MTEP into various brain regions had an effect on relapse to sucrose-seeking [7, 13, 42]. In contrast, we observed a significant effect of systemic and intra-BLA MTEP on conditioned responding elicited by a cue that predicted a non-drug, fructose-glucose solution. Again, this intriguing difference could be related to the contribution of mGluR5 in the expression of behavior acquired through Pavlovian or operant learning strategies.

Based on our results, mGluR5 within the Acb core did not contribute to the expression of appetitive Pavlovian conditioning. In addition to no change in CS port entries relative to vehicle, there was no significant association between the effect of MTEP on CS port entries and the AP placement of microinjections (Fig. S6). These results were unexpected, because previous studies have shown that intra-Acb core microinjections of MTEP reduced operant drug-seeking behavior in

relapse models [7, 13]. Moreover, cocaine-primed reinstatement upregulated Acb core mGluR5 [43], and mGluR5 in the Acb core is necessary for the interoceptive effects of alcohol to be expressed [44]. One explanation for why our data differ from the operant relapse studies is that in our task the CS was not systematically extinguished prior to test, whereas in operant relapse models instrumental responding is rigorously extinguished across consecutive sessions before reinstatement tests. Extinction learning induced by this protocol might engage mGluR5 in the Acb core and influence the role of this receptor in subsequent reinstatement tests. This hypothesis is supported by the finding that MTEP in the Acb core did not impact on-going operant alcohol self-administration that had not previously been extinguished [45]. Notably, in the present procedure CS port entries were incompletely extinguished in each test, and one or two Pavlovian conditioning sessions were conducted between tests.

In separate experiments, MTEP microinjections that encompassed more posterior BLA coordinates had no overall effect on CS port entries. A follow-up experiment with placements that covered more anterior aspects of the BLA and overlapped only with the most anterior coordinates from our first BLA experiment found that MTEP microinjections had a differential impact on CS port entries in the sugar and neutral contexts. Specifically, inhibiting mGluR5 in more anterior aspects of the BLA enhanced the context-based discrimination of CS port entries, resulting in a modest increase and more rapid onset of CS port entries in the sugar context, and a modest decrease and slower onset of CS port entries in the neutral context. One caveat here is that following vehicle microinjections, CS port entries in the sugar context were lower for rats with anterior BLA placements relative to other experiments. However, this anomaly is countered by the within-subject experimental design, which accommodates for differences in overall levels of behaviour that may emerge between experiments. Indeed, rats in each experiment made a number of CS port entries in the sugar context following vehicle microinjection that was comparable with their performance in session 10 of Pavlovian conditioning (posterior BLA targeting, M = 20.9 ± 4.15 SEM; anterior BLA targeting, M = 15.3 ± 2.15 SEM).

The anatomic correlation found when targeting more posterior BLA coordinates predicted an MTEP effect with more anterior targeting, but did not predict the enhanced contextual discrimination. This outcome highlights the importance of confirming exploratory analyses. One explanation for the present results could be related to the topographical distribution of mGluR5 in the BLA: while this is consistently dense throughout the BLA [46] the smaller size of the anterior BLA may mean that fewer neurons overall express mGluR5 in the anterior BLA relative to the posterior BLA (Fig. S7). Another consideration is the relation between the present data and prior research suggesting that excitatory pyramidal neurons in the anterior and posterior BLA are important for aversive and appetitive behaviours, respectively [47]. While these findings do not appear consistent with the present data, additional studies are needed to evaluate the effect of MTEP on these specific neuronal subpopulations on context-dependent responding to appetitive Pavlovian cues.

The present results suggest that mGluR5 in anterior BLA normally suppresses CS port entries in a sugar-associated context, but is necessary for this behaviour in a neutral context. These differential effects could be related to the modulation of glutamate release in the anterior BLA by context, which could signal expectancy of sugar, potentially through hippocampal inputs to the BLA. The observed results in the anterior BLA are consistent with reports that inactivation of the anterior but not posterior BLA reduced cue-induced reinstatement of cocaine-seeking [39], and that prelimbic projections to the anterior but not posterior BLA were recruited during the acquisition of appetitive Pavlovian learning [38]. Circuits involving the anterior BLA were also required for cue-induced reinstatement of cocaine-seeking [48] and extinction learning [49]. The BLA has topographically defined projections, with the anterior BLA projecting more to the Acb core [50] and posterior BLA projecting to the shell [51]. The density of projections from the BLA to the ventral hippocampus, central amygdala, and Acb also varies along the AP axis [52]. Further research is necessary to evaluate the contribution of distinct topographically-defined BLA circuits to the context-dependent expression of appetitive Pavlovian responding.

In conclusion, the present results reveal an influential role for environmental context in responding to an appetitive Pavlovian cue. The finding that CS port entries were elevated in a sugar context relative to a neutral context was replicated in four separate experiments, highlighting the importance of considering context in experimental design. mGluR5 emerged as critical for the expression of CS port entries, as this behavior was reduced by systemic MTEP administration in both a sugar context and a neutral context. In contrast, a behaviourally effective dose of the NMDA receptor antagonist, MK-801, had no impact on behavior. mGluR5 in the anterior BLA had differing, context-based functions in CS port entries, whereas mGluR5 in the Acb core or posterior BLA did not contribute to this behaviour. These novel data add to our overall understanding of glutamatergic processes in appetitive Pavlovian conditioning, and contribute to a growing literature on the nuances of amygdala topography in emotional behaviour [22, 37-39, 47, 52, 53].

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Context and topography determine the role of basolateral amygdala metabotropic glutamate receptor 5 in appetitive Pavlovian responding

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Supplementary materials and methods

Animals

We used 122 experimentally-naïve, male, Long-Evans rats (Charles River, QC, Canada). On arrival, rats were initially pair-housed in plastic cages (44.5 x 25.8 x 21.7 cm) containing Teklad Sani Chip bedding (Cat# 7090, Envigo, QC, Canada), a nylabone (Cat#: K3580, Bio-Serv, NJ, USA), a tunnel (Cat#: K3245 or K3325, Bio-Serv), and shredded paper in a climate-controlled (21°C) vivarium on a 12 h: 12 h light/dark cycle (lights on at 07:00). After 3 days, rats were then singly-housed in otherwise identical conditions and handled for 7 days. Rats had unrestricted access to food (Teklad, Envigo, QC, Canada) and water throughout the experiments. All procedures were approved by the Animal Research Ethics Committee at Concordia University and performed in accordance with guidelines from the Canadian Council on Animal Care.

Surgery

Rats were anesthetized using isoflurane and stereotaxic surgery was performed as previously described [1]. Rats' heads were shaved and they were placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) for bilateral cannulation. The head was swabbed with iodine and an incision of approximately 2.5 cm was made to expose the skull. Bilateral 26 ga guide cannulae (Plastics One, Roanoke, VA, USA) were then implanted targeting the Acb core, the BLA, or a more anterior portion of the BLA. Coordinates in mm from bregma were: Acb core, +1.5 AP, ± 3.23 ML on a 10° angle, and -4.3 mm DV; BLA, -2.54 AP, ± 5 ML, and -5.5 DV; anterior BLA, -2.1 AP, ± 4.9 ML, and -5.5 DV. Cannulae were secured in place with the aid of four skull screws and acrylic dental cement.

Dummies, cut flush to the cannula, were then inserted and secured in place with dust caps. During microinjections, injectors projected 3 mm beyond the cannula. Rats were given 5 mg/kg ketoprofen and 0.05 mg/kg buprenorphine (s.c.) for post-operative analgesia, 0.9% saline (s.c.) for rehydration and prophylactic procaine penicillin (60,000 IU, i.p.). Rats were given at least 7 days for recovery during which time they were monitored and weighed daily.

Apparatus

Behavioral training was conducted using 12 identical conditioning chambers (30.5 x 31.8 x 29.2 cm, Cat#: ENV-009A, Med Associates, St Albans, VT, USA). Each chamber was contained within a sound-attenuating cubicle with a fan to provide ventilation and background noise (70-75 dB). Each chamber had a white houselight (ENV-215M) in the centre near the ceiling of the left wall, next to a white noise generator (ENV-225SM, calibrated to 8 dB above background) and a clicker (ENV-135M). The right wall had a fluid port (ENV-200R3AM) located 2 cm above the floor, which was connected to a 20 mL syringe via polyethylene tubing. A syringe pump (PHM-100, 3.3 RPM) that was located outside the sound-attenuating cubicle controlled the syringe. A PC running Med-PC IV controlled presentation of stimuli and recorded entries into the port as measured by infrared beam breaks (ENV-254CB).

General Behavioral Procedures

Home-cage exposure to sugar. Rats were pre-exposed to sugar (a 10% fructose-glucose solution, composed of 55 g/L fructose and 45 g/L glucose) for 48 h in their home-cages. A pre-weighed fluid receptacle containing 90 mL of sugar was placed on the home-cage. This bottle was re-weighed 24 h later, refilled to 90 mL and then weighed again after 24 h. Rats consumed all, or nearly all, of the sugar.

Pavlovian conditioning with context discrimination. Rats were habituated to experimental training procedures over 3 days. On day 1, rats were transported from the vivarium to the behavior room in their home-cages on a trolley. Rats were briefly handled in the behavior room and then left

there for 20 min before being returned to the vivarium. On days 2 and 3, rats were placed into the conditioning chambers located in the behavior room. Chambers were set up as two distinct contexts, which were composed of different visual, olfactory, and tactile stimuli. In context 1, the transparent sides and ceiling of the conditioning chamber were covered with black cardboard, and a petri dish with approx. 2.5 mL of a 10% lemon oil suspension (Cat#: W262528, CAS#: 8008-56-8, Sigma-Aldrich, ON, Canada) was placed on brown paper in the waste pan beneath an acrylic glass floor. In context 2, the sides of the chamber were uncovered, 10% bitter almond odor was used (Benzaldehyde, Cat#: B6259, CAS#: 100-52-7, Sigma-Aldrich), the waste pan was lined with white benchcoat, and a metal grid floor was used. Rats were habituated to one context on each day in counterbalanced order, with the houselight switched on during the 20 min session and port entries recorded.

Rats were then assigned to one of two contexts for Pavlovian conditioning sessions (the sugar context), while the remaining context served as the familiar, neutral context (see Table 1 in the accompanying article for a description of contexts). Discrete stimuli were a 10 s, continuous white noise or 10 s of a 5 Hz clicker. Rats were assigned one stimulus (the conditioned stimulus or CS) to be paired with sugar in the sugar context and the other (the neutral stimulus or NS) to be presented without sugar in the neutral context. The purpose of the NS was to equate the acoustic salience of both contexts. Rats were counterbalanced across contexts, stimuli, and session order such that there were no differences in home-cage sugar consumption or bodyweight. Rats were then given one training session a day that alternated between each context until they had received 10 sessions of Pavlovian conditioning in the sugar context and 10 sessions of exposure to the NS in the neutral context.

During training sessions, rats received 10 stimulus presentations (either CS or NS) with intervals of 120, 240, or 360 s between trials (mean inter-trial interval (ITI) = 240 s), with each trial consisting of a 10 s Pre-CS/NS interval, 10 s CS/NS presentation, and 10 s Post-CS/NS interval. In the sugar context, presentations of the CS co-terminated with 6 s of syringe pump operation to deliver 0.2 mL of 10% fructose-glucose solution (sugar). In the neutral context, NS presentations also co-

terminated with 6 s of syringe pump operation, but no syringes were present and thus no sugar was delivered.

Testing. At 24 h after the last training session, the expression of conditioned responding elicited by the CS was tested in the absence of sugar. Tests occurred in the sugar context and the neutral context for each rat, with 1-2 sessions of retraining in each context between tests. At test, the CS was presented as during prior Pavlovian conditioning sessions and the syringe pump was activated for 6 s, but no syringes were present and thus no sugar was delivered. The NS was never presented at test. Moreover, our preliminary data indicate that the NS does not elicit port entries when presented alone in either the sugar or neutral contexts [2].

Experiment 1. Impact of context on CS port entries and effect of MTEP and MK-801 on CS port entries in both sugar *and neutral contexts.*

We have previously reported a reliable and selective elevation in port entries elicited by a CS that predicted alcohol in an alcohol context, relative to a neutral context [1]. The impact of context on port entries elicited by a CS that predicted sugar is unknown. To examine this question, rats (n = 17) were trained and tested as described above.

Next, in the same rats we examined the contribution of NMDA and mGluR5 glutamate receptors in the expression of CS port entries at test in the sugar and neutral contexts. Following 2 sessions of re-training, rats were tested 20 min after an intraperitoneal (i.p.) injection of vehicle, 0.1 mg/kg MK-801, or 5 mg/kg MTEP. These doses have been shown previously to affect dopamine release in the prefrontal cortex [3] and reinstatement of methamphetamine and cocaine seeking [4, 5]. Treatment order was counterbalanced using a Latin square design, and 2 sessions of re-training in either context occurred between tests.

Experiment 2. Effect of MTEP in the nucleus accumbens core on CS port entries

In the previous experiment, systemic administration of MTEP but not MK-801 reduced CS port entries. Here, we determined if mGluR5 in the Acb core was the neural locus for this effect. Rats (n=21) received bilateral cannulation, home-cage exposure to sugar, and Pavlovian conditioning with context discrimination as described above. Over the last 4 training sessions they were habituated to the microinjection procedure and received a probe test in the neutral context.

To habituate rats to microinjection procedures they received sham microinjections with injectors that did not extend beyond the cannula. After their final training session, full length injectors projecting 3 mm beyond the cannula were inserted and removed in the colony room to prevent side-effects from doing microinjections in fresh brain tissue. The following day, rats received a probe test to habituate them to a full microinjection day. Immediately prior to the probe test, rats received microinjections of 0.3 µL/side 0.9% sterile saline over 1 min, with injectors left in place for a further 2 min. They were then subjected to a session in which they were presented with the CS in the neutral context without sugar delivery to examine whether they would respond normally following microinjections.

All rats were tested in both contexts using a within-subjects design, following intra-Acb core microinjections of vehicle or 3 µg/side MTEP in volumes of 0.3 µL/side. The order of receiving a given treatment in a particular context was randomly allocated. Doses were chosen based on previous studies that have found effects of 1-5 µg/side of MTEP in the Acb core and BLA [6, 7].

Experiment 3. Effect of MTEP in the basolateral amygdala on CS port entries

We showed previously that AMPA glutamate receptors in the BLA are required for port entries elicited by a CS that predicted alcohol [1]. Here, we examined the involvement of mGluR5 receptors in the BLA in CS port entries in rats that were trained with sugar. Rats (n=20) with cannulae targeting the BLA were trained and tested in procedures identical to those used for experiment 3.

Experiment 4. Effect of MTEP in the anterior basolateral amygdala on CS port entries

Results from experiment 3 suggested that more anterior targeting of the BLA may be associated with a larger MTEP-mediated decrease in CS port entries. We tested this hypothesis in a separate cohort of rats (n = 24) cannulated using a more anterior set of BLA coordinates and trained and tested in procedures identical to those used for experiments 3 and 4.

Experiment 5. Effect of MTEP on locomotor activity and home-cage consumption of fructose-glucose solution

To examine whether MTEP produced non-specific locomotor deficits, we tested a separate cohort of rats (n = 16) in a 39 x 42 x 50 cm open field monitoring system (Coulbourn Instruments, Whitehall, PA, USA) housed in sound attenuating boxes and connected to a computer running Tru Scan 2.0. On day 1, rats were placed on a trolley, taken to the locomotor room, weighed, handled, and left in the locomotor room for 20 min to habituate them to transport. On day 2, rats were transported to the locomotor room and given habituation injections of 0.9% saline (1 mL/kg, i.p.), 20 min before being placed in the locomotor boxes for a 45 min session to familiarise them to the context. On day 3, rats were randomly allocated to receive 1 mL/kg 5% DMSO/saline vehicle or 5 mg/kg MTEP (i.p.) 20 min before a 45 min locomotor test.

Next, to examine any possible reduction in the hedonic value of 10% fructose-glucose solution (sugar), we tested the effect of MTEP on home-cage sugar consumption. Across days 4 – 6, rats received 48 h of exposure to sugar as described above. On day 7, their access was reduced to 1 h of sugar. On day 8, they received habituation injections of 0.9% saline (1 mL/kg, i.p.). On day 9, rats were randomly allocated to receive 1 mL/kg 5% DMSO/saline vehicle or 5 mg/kg MTEP 20 min before 1 h of access to sugar.

Histology

After testing, cannulated rats were euthanised using an overdose of >100 mg/kg sodium pentobarbital combined with lidocaine to reduce abdominal irritation [8]. To help visualise the

microinjection site, rats received a 0.3 µL microinjection of 4% fast green. They were then transcardially perfused with 0.1 M phosphate buffered saline and 4% paraformaldehyde. Brains were dissected, post-fixed in 30% sucrose/4% paraformaldehyde overnight and then coronally sectioned at 40 µm in a cryostat at -20°C. Sections were stained with cresyl violet and visualised under a light microscope. Decisions on exclusion and inclusion from overall analyses were based on histology by a person who was blind to the data.

Supplementary Results

MK-801 during acquisition of appetitive Pavlovian conditioning produced sensitization

To validate the dose of MK-801 used in experiment 1, we examined the impact of this treatment on the acquisition and expression of Pavlovian conditioning [9, 10]. Following home-cage exposure to 10% fructose-glucose solution (sugar), rats (n = 24) were habituated to the conditioning chambers (devoid of added contextual cues) in a single 20 min session following a systemic injection of 0.9% saline (1 mL/kg, 20 min prior to session, i.p.). Rats were then randomly allocated (n = 8 per group) to receive 0.9% saline vehicle, 0.1 mg/kg MK-801, or 0.3 mg/kg MK-801 20 min prior to each of 7 Pavlovian conditioning sessions. These sessions were structured as in experiment 1, except that the CS consisted only of the clicker stimulus. On sessions 8 and 9, we examined CS port entries in the absence of sugar delivery to evaluate if MK-801 during acquisition had an impact on the expression of CS port entries or had induced sensitization to MK-801 [11, 12]. In both tests, the CS was presented as before but without sugar. At test on session 8, no injections were administered, in keeping with previous studies that avoided administering injections due to stress-related sensitization from repeated injections [13]. At test on session 9, rats were administered with the same dose of MK-801 that they had experienced during training, 20 min before the test.

During the acquisition phase of this experiment the 0.3 mg/kg MK-801 dose, but not the 0.1 mg/kg dose, produced non-specific elevations in ITI and pre-CS port entry behavior. Port entries during the pre-CS, CS intervals are depicted in Fig. S1a. In rats receiving pre-session treatment with vehicle or MK-801 (0.1 mg/kg or 0.3 mg/kg), port entries increased across the 7 training sessions (Session, Greenhouse-Geisser, $\varepsilon = 0.509$, $F_{3.052,64.091} = 12.423$, $p < 0.001$). The number of port entries made was higher overall during the CS than the pre-CS (Interval, $F_{1,21} = 32.024$, $p < 0.001$), and increased faster across session during the CS than the pre-CS (Interval x Session, Greenhouse-Geisser corrected, $\varepsilon = 0.427$, $F(2.564,53.837) = 14.781$, $p < 0.001$). Bonferroni post-hoc tests showed discrimination between the pre-CS and CS in sessions 3-7 (all *p's* < 0.001). Blocking NMDA receptors had no overall impact on port entries (Treatment, $F_{2,21} = 1.157$, $p = 0.334$). However, MK-801

differentially affected port entries during pre-CS and CS intervals (Interval x Treatment, $F_{2,21} = 4.74$, $p = 0.02$). Post-hoc comparisons found a significant elevation in port entries during the pre-CS interval following 0.3 mg/kg MK-801 relative to vehicle ($p = 0.002$). Thus, rats learned to associate the CS with sugar across 7 Pavlovian conditioning sessions, but rats receiving 0.3 mg/kg MK-801 had elevated port entries during the pre-CS interval, suggesting a non-specific increase in responding.

Supporting this interpretation, pre-treatment with 0.3 mg/kg MK-801 also elevated port entries during the ITI, relative to other groups (Fig. S1b). Mixed-design ANOVA revealed a significant main effect of Treatment ($F_{2,21} = 7.175$, $p = 0.004$), with post-hoc comparisons showing a significant difference between vehicle and 0.3 mg/kg MK-801 ($p = 0.003$). ITI port entries did not change across Sessions (Greenhouse-Geisser, $\varepsilon = 0.532$, $F_{3.195,67.09} = 2.115$, $p = 0.103$) in any group (Session × Treatment, $F_{6.389,67.0} = 1.967$, $p = 0.079$).

At 24 h after the last Pavlovian conditioning session, we examined the effect of prior MK-801 treatment on the expression of CS port entries in the absence of sugar delivery. The expression test occurred without pre-treatment. A sensitization test occurred 24 h later and rats were pre-treated with the same dose of MK-801 that they had received previously (Fig. S1c).

A mixed-design ANOVA revealed more overall port entries during the CS than the pre-CS (Interval, $F_{1,21} = 31.091$, $p < 0.001$) and in the sensitization test than the expression test (Test, $F_{1,21} = 11.396$, $p = 0.003$). There was no significant main effect of Treatment ($F_{2,21} = 0.996$, $p = 0.386$) or Interval x Treatment interaction ($F_{2,21} = 2.82$, $p = 0.082$). However, ANOVA indicated significant Test x Treatment ($F_{2,21} = 6.56$, $p = 0.006$), Interval x Test ($F_{1,21} = 6.75$, $p = 0.017$), and Interval x Test x Treatment ($F_{2,21} = 5.078$, $p = 0.016$) interactions. Post-hoc comparisons showed that compared to the expression test, CS port entries were significantly elevated following 0.1 mg/kg MK-801 in the sensitization test ($p = 0.002$). In contrast, pre-treatment with 0.3 mg/kg MK-801 significantly increased pre-CS ($p < 0.001$) and CS ($p = 0.014$) port entries in the sensitization test, relative to the expression test. Thus, prior repeated exposure to 0.1 mg/kg of MK-801, which was the dose used in experiment 1, produced a sensitization of CS port entries in the sensitization test.

Finally, non-specific effects of the 0.3 mg/kg MK-801 dose were also seen in the ITI at test (Fig. S1d). There were differential effects of the MK-801 doses during the sensitization test (Test × Treatment interaction, $F_{2,21} = 5.576$, $p = 0.011$). There appeared to be generally higher ITI responding in the sensitization test than expression test (Test, $F_{1,21} = 8.345$, $p = 0.009$), and the dose of MK-801 also affected ITI responding (Treatment, $F_{2,21} = 11.424$, $p < 0.001$). Post-hoc tests showed that only the 0.3 mg/kg MK-801 dose significantly increased ITI port entries in the sensitization test relative to the expression test ($p < 0.001$). Neither the vehicle or 0.1 mg/kg MK-801 sensitization tests were associated with significant differences compared to their respective expression tests ($p = 0.931$ and 0.599 respectively).

These results demonstrate the behavioral efficacy of 0.1 mg/kg MK-801, consistent with previous studies that have used this dose of MK-801 [9, 10].

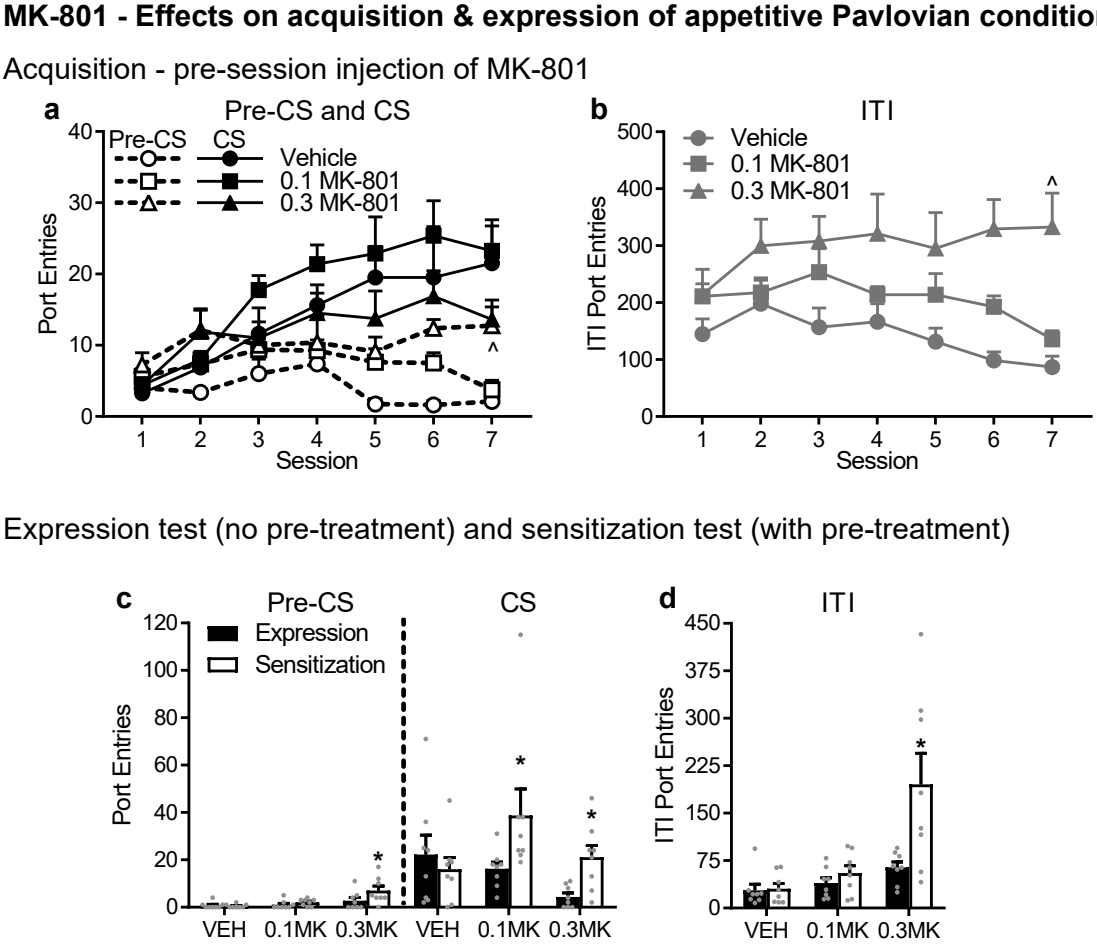

Fig. S1 Systemic MK-801 during training produced behavioral sensitization to the CS but a high dose had non-specific effects. (a) During acquisition, rats were trained in daily sessions in which a CS was paired with fructose-glucose solution ('sugar'), following injections of vehicle, 0.1, or 0.3 mg/kg MK-801 (n = 8 per group). While CS port entries increased over the course of training, Pre-CS port entries also increased in rats receiving 0.3 mg/kg. (b) ITI port entries for rats receiving 0.3 mg/kg MK-801 were elevated during acquisition. (c) Rats were tested for the expression of CS port entries and then tested for sensitization the following day after receiving the same dose they received during acquisition. Both tests occurred in the absence of sugar. Pre-treatment with 0.1 mg/kg or 0.3 mg/kg MK-801 in the sensitization test produced an elevation in port entries that was confined to the CS. (d) Pre-treatment with 0.3 mg/kg MK-801 also elevated port entries during the ITI. Data are presented as means ± SEM. ^ $p < 0.05$ for differences between 0.3 mg/kg MK-801 and vehicle across acquisition. * $p < 0.05$ Bonferroni post-hoc tests for differences between the expression and sensitization test. Statistical tests were mixed-design ANOVAs. Data from individual rats are depicted as grey dots (c-d).

MTEP had no effect on open field locomotor behavior

Rats tested for open field locomotor behavior following vehicle (n = 8) or 5 mg/kg MTEP (n = 8) showed no differences in behavior. Based on their performance during the locomotor habituation session on day 2, there were no pre-existing differences in bodyweight (t_{14} = 0.362, p = 0.723), number of floor plane moves (t_{14} = 0.276, p = 0.787), the amount of time spent moving in the floor plane (t_{14} = 0.021, p = 0.983), total distance travelled (t_{14} = 0.424, p = 0.678), the amount of time spent in the center of the arena (t_{14} = -0.022, p = 0.983), or the number of stereotypic movements (t_{14} = 0.541, p = 0.597). At test, MTEP had no effect on any of these measures of open field activity. Independent t-tests showed there was no significant difference in number of moves (t_{14} = 0.197, p = 0.847, Fig. S2a), movement time (t_{14} = 1.106, p = 0.287, Fig. S2b), the total distance travelled (t_{14} = 1.109, p = 0.286, Fig. S2c), center time (t_{14} = 0.449, p = 0.66, Fig. S2d), or number of stereotypic movements (t_{14} = 1.094, p = 0.292, Fig. S2e). Moreover, there was no effect on the pattern of activity during the course of the session (Fig. S2f), as a mixed-design ANOVA showed no effect of MTEP treatment ($F_{1,14}$ = 1.231, p = 0.286) on velocity (cm/min). The average velocity of movement decreased over the course of the locomotor session, as shown by a main effect of time ($F_{8,112}$ = 53.13, p < 0.001), but this appeared unaffected by MTEP because there was no significant treatment × time interaction ($F_{8,112}$ = 0.722, p = 0.671).

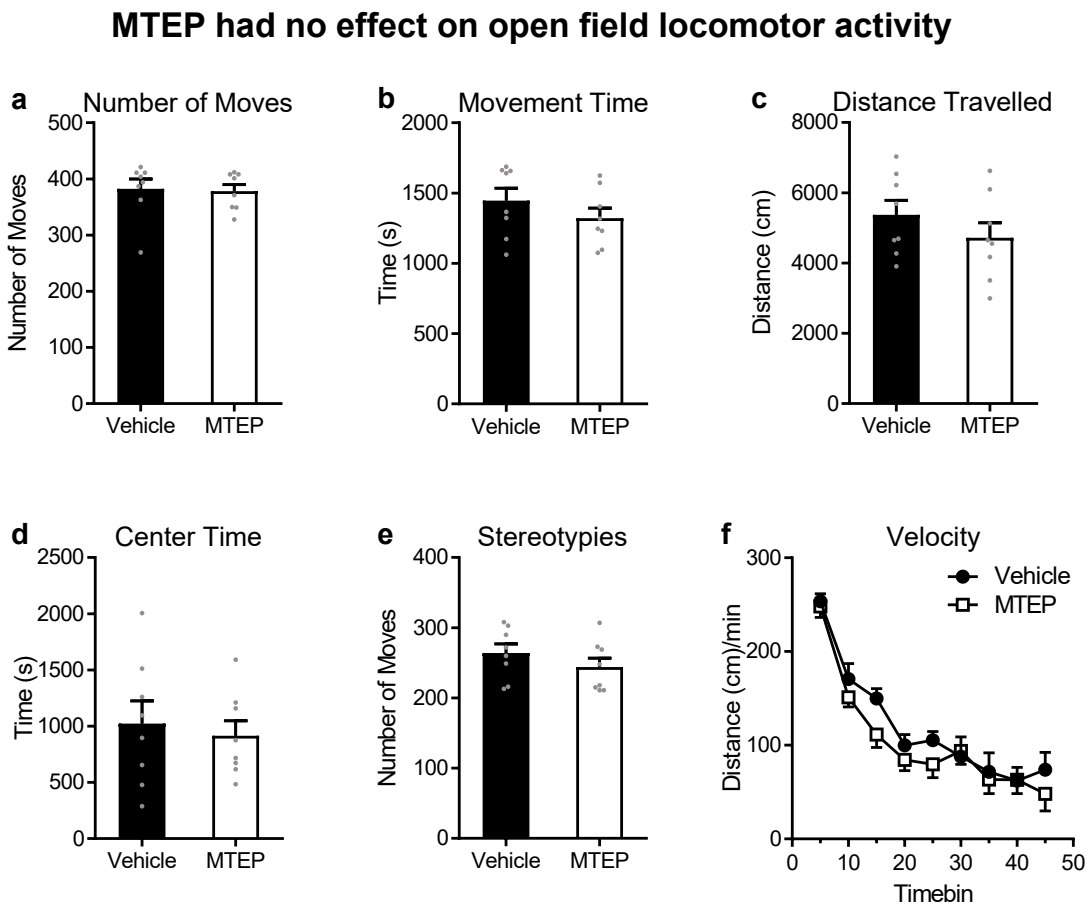

Fig. S2 MTEP had no effect on open field locomotor activity. Rats that received 5% DMSO/0.9% saline vehicle (n = 8) or 5 mg/kg MTEP (n = 8) did not differ on (a) the number of moves, (b) the amount of time spent moving, (c) the total distance travelled, (d) the amount of time spent in the center of the arena, (e) the number of stereotyped movements, or (f) velocity (cm/min) for each 5 min timebin. Data are means ± SEM. Statistical tests were independent t-tests (a-e) or mixed-design ANOVA (f). Data from individual rats are depicted as grey dots (a-e).

MTEP had no effect on home-cage 10% fructose-glucose solution (sugar) consumption

Rats were then exposed to sugar in their home-cage for 48 h. On days 7 and 8 they were given 1 h of access and consumed a mean ± SEM of 6.54 ± 0.55 mL and 6.57 ± 0.68 mL respectively, calculated using an empirically determined density of 1.023 g/mL for 10% FGS. Because home-cage consumption was immediately stable and high compared to the 2 mL available during Pavlovian conditioning sessions, rats were tested on day 9. Randomisation to treatment conditions produced no pre-existing differences in bodyweight (t_{14} = 0.663, p = 0.518) or volume of sugar consumed (t_{14} = 0.121, p = 0.905). At test, rats that received vehicle (n = 8) did not significantly differ from rats that received 5 mg/kg MTEP (n = 8) in terms of the volume of sugar consumed (t_{14} = 0.1637, p = 0.872, Fig. S3a). As shown in Fig. S3b, vehicle and MTEP-treated rats also did not differ in terms the amount of fructose/glucose consumed (t_{14} = 0.163, p = 0.873). Water consumption during the 1 h test was negligible across both groups, with rats consuming a mean ± SEM of 0.02 ± 0.01 mL of water.

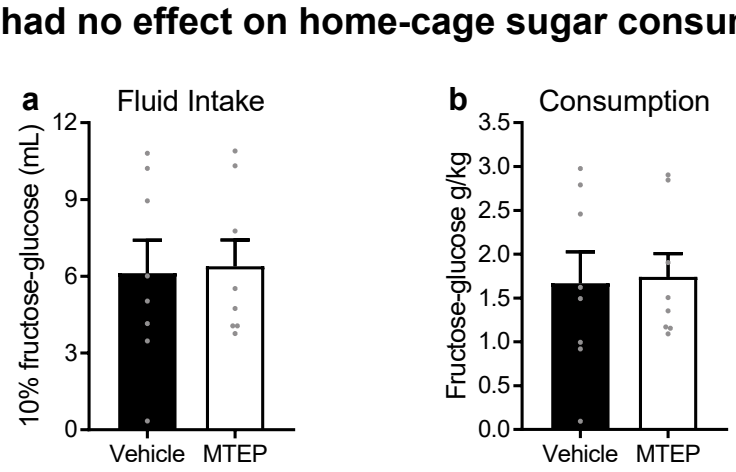

Fig. S3 MTEP had no effect on home-cage consumption of 10% fructose-glucose solution (sugar). Rats received either 5% DMSO/0.9% saline vehicle (n = 8) or 5 mg/kg MTEP (n = 8) 20 min before sugar was made available. (a) Over the course of 1 h, rats showed a significant preference for drinking sugar, but MTEP had no impact on the volume of fluid consumed. (b) MTEP had no effect on the amount of fructose/glucose consumed per kg of bodyweight. Data are means ± SEM. Statistical tests were independent t-tests. Data from individual rats are depicted as grey dots.

The number of CS port entries on a per CS trial basis at test for microinjection studies

Nucleus accumbens core. Analysis of the time course of non-normalized CS port entries (Fig. S4a) found higher overall levels of CS port entries in the sugar context (Context, $F_{1,13} = 31.56$, $p < 0.001$). The number of CS port entries decreased across CS trials (Trial, $F_{9,117} = 14.935$, $p < 0.001$) similarly in both contexts (Context × Trial, Greenhouse-Geisser, $\varepsilon = 0.349$, $F_{3.141,40.835} = 0.888$, $p = 0.459$). MTEP had no effect overall (Treatment, $F_{1,13} = 0.002$, $p = 0.962$), within a particular context (Treatment × Context, $F_{1,13} = 1.541$, $p = 0.236$), or within particular trials (Treatment × Trial, $F_{9,117} = 0.744$, $p = 0.668$). The number of CS port entries did not differ across trials as a function of context and MTEP administration (Treatment × Context × Trial interaction, Greenhouse-Geisser, $\varepsilon = 0.445$, $F_{4.007,52.09} = 0.377$, $p = 0.825$). These results suggest that MTEP in the Acb core does not alter CS port entries either overall or in how responding is structured during the session.

Posterior basolateral amygdala. As shown in Fig. S4b, overall CS port entries were elevated in the sugar context (Context, $F_{1,14} = 32.237$, $p < 0.001$), and decreased across CS trials (Trial, $F(9,126) = 6.767$, $p < 0.001$) comparably in both contexts (Context x Trial, Greenhouse-Geisser, $\varepsilon = 0.413$, $F(3.717,52.035) = 1.606$, $p = 0.19$). There was no main effect of Treatment ($F(1,14) = 1.516$, $p = 0.239$) in either context (Treatment × Context, $F(1,14) = 0.3$, $p = 0.592$), and no differential effect of MTEP on port entries as a function of trial (Treatment × Trial, $F(9,126) = 0.432$, $p = 0.916$) in either context (Treatment × Context × Trial, Greenhouse-Geisser, $\varepsilon = 0.408$, $F(3.668,51.351) = 0.736$, $p = 0.561$).

Anterior basolateral amygdala. Visual inspection of the number of CS port entries as a function of trial (Fig. S4c) suggested that MTEP caused an immediate reduction in CS port entries in the neutral context, but not the sugar context. However, repeated measures ANOVA did not support this observation. At test, CS port entries were higher in the sugar context than in the neutral context (Context, $F_{1,16} = 27.187$, $p < 0.001$), and decreased as a function of trial (Trial, Greenhouse-Geisser, $\varepsilon = 0.337$, $F_{3.03,48.485} = 9.18$, $p < 0.001$). Interestingly, ANOVA indicated a significant Context x Trial interaction (Greenhouse-Geisser, $\varepsilon = 0.402$, $F_{3.618,57.882} = 2.702$, $p = 0.044$). Bonferroni-corrected post-

hoc comparisons showed overall differences between the sugar context and the neutral context on trial 1 (p = 0.026), trial 2 (p = 0.019), trials 4-7 (p's ≤ 0.006), and on trial 9 (p = 0.014). Intra-aBLA microinjection of MTEP had no overall effect on CS port entries (Treatment, $F_{1,16}$ = 0.429, p = 0.522), although there was a greater difference in CS port entries between the sugar and neutral contexts following MTEP relative to vehicle (Treatment × Context, $F_{1,16}$ = 8.823, p = 0.009). Moreover, the effect of MTEP did not vary as a function of trial (Treatment × Trial, Greenhouse-Geisser, ε = 0.458, $F_{4.12,65.927}$ = 1.896, p = 0.12), nor did it vary as a function of trial within specific contexts (Treatment × Context × Trial, $F_{9,144}$ = 1.061, p = 0.395).

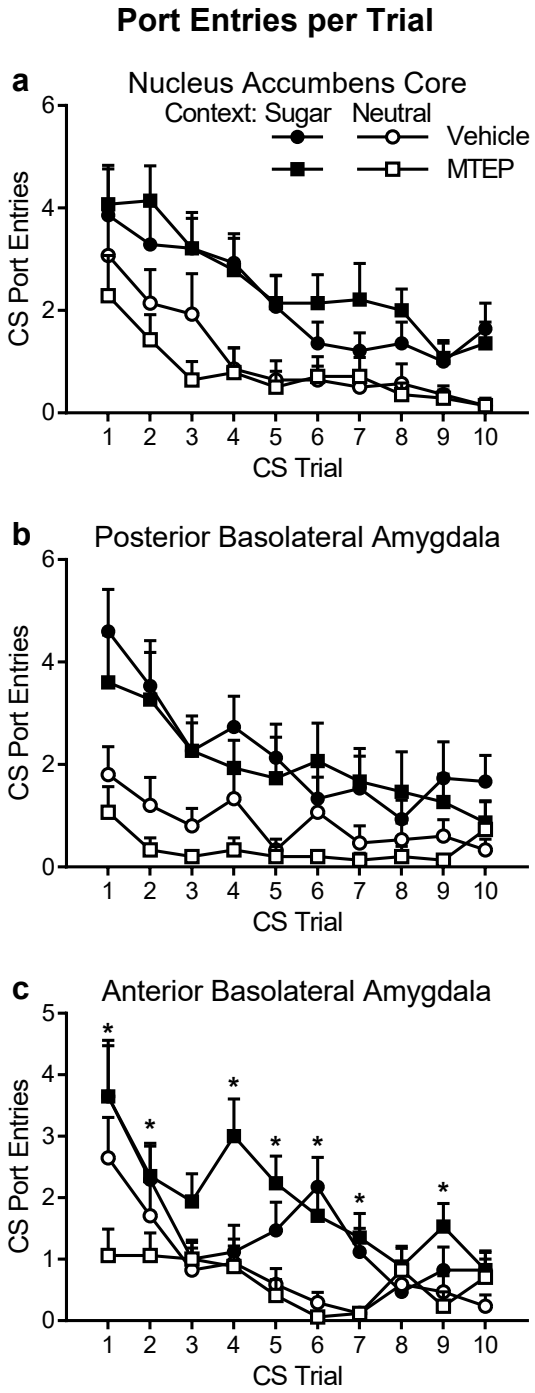

Fig. S4 MTEP had no effect on the within-session pattern of CS port entries when microinjected to the nucleus accumbens core or basolateral amygdala. (a) In the nucleus accumbens core, MTEP did not affect the pattern of non-normalized CS port entries in either context. (b) Similarly, MTEP microinjection into the basolateral amygdala using a more posterior set of coordinates had no effect on CS port entries in either context. (c) In the anterior basolateral amygdala, MTEP significantly increased context-based differences in CS port entries, but there was no effect on the within-session pattern of CS port entries. However, the difference between sugar context and neutral context varied as a function of trial. Data are presented as means ± SEM. * $p < 0.05$ Bonferroni corrected post-hoc comparing the sugar and neutral contexts. Statistical tests were repeated measures ANOVAs.

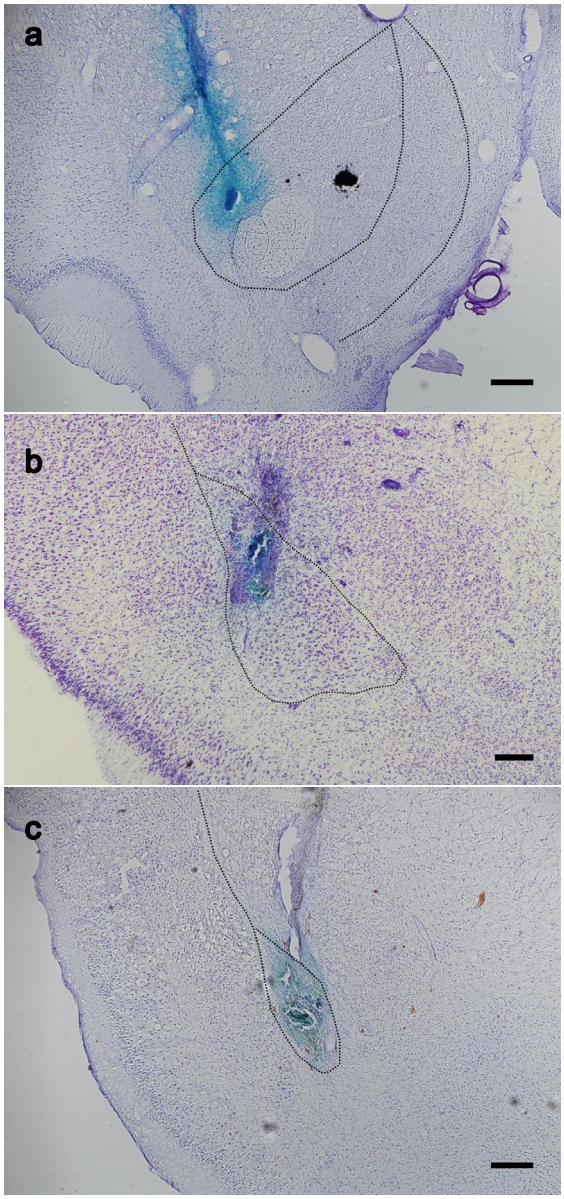

Fig. S5 Photomicrographs of microinjection sites. (a) A deposit of fast green can be seen adjacent to the anterior commissure in the nucleus accumbens core (AP +2.28 mm from bregma). (b) An example microinjection targeting posterior coordinates in the basolateral amygdala (AP -2.76 mm from bregma). (c) An example microinjection targeting anterior coordinates in the basolateral amygdala (AP -1.80 mm from bregma). Scale bars represent 250 µm.

Topographical analysis of MTEP effects in the nucleus accumbens core

The nucleus accumbens has been reported to have both anatomic and neurochemical gradients. For example, the amygdala and thalamus preferentially project to the anterior nucleus accumbens [14] and previous studies have shown enkephalin synthesis in the anterior nucleus accumbens was more sensitive to lesions of dopamine neurons [15]. In the nucleus accumbens shell, there are anteroposterior gradients that affect both appetitive and aversive conditioning [16-19]. Therefore, we examined whether there were any anteroposterior correlations between the effect of MTEP and the AP coordinates of the microinjections. As shown in Fig. S6, there was no significant correlation between AP coordinates and ΔNorm-CS (Norm-CS_{MTEP} minus Norm-$CS_{Vehicle}$) in either the sugar context (r_{12} = 0.345, p = 0.227) or the neutral context (r_{12} = 0.042, p = 0.885).

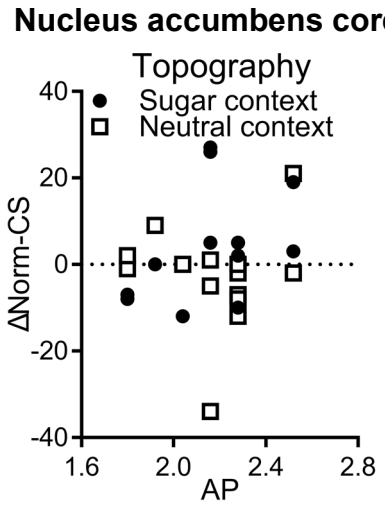

Fig. S6 There was no association between the anteroposterior coordinates of MTEP microinjection in the nucleus accumbens core and the effect of MTEP on Norm-CS port entries (ΔNorm-CS = Norm-CS_{MTEP} minus Norm-$CS_{Vehicle}$) in either the sugar or neutral contexts.

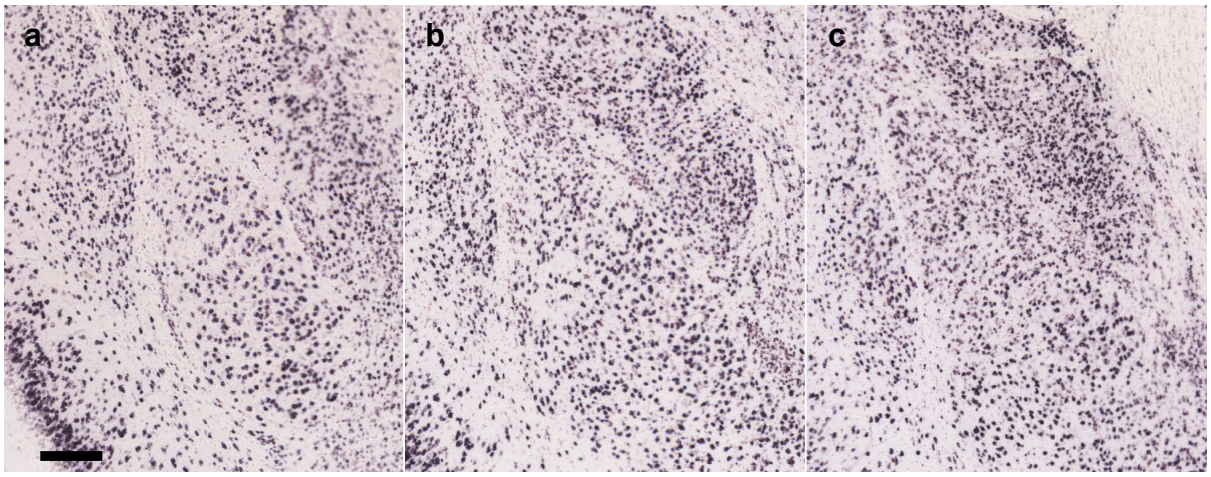

Fig. S7 Expression of mGluR5 in basolateral amygdala of the mouse brain. In situ hybridization for GRM5 in the mouse brain shows expression of mGluR5 throughout the AP-axis of the BLA. Images from the Allen Mouse Brain Atlas present (a) anterior coordinates (atlas image 25), (b) intermediate coordinates (atlas image 27), and (c) posterior coordinates (atlas image 29). Scale bar represents 210 µm. Images used in accordance with the Allen Institute's terms of use and license. © 2004 Allen Institute for Brain Science. Available from: mouse.brain-map.org/gene/show/72233

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