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].

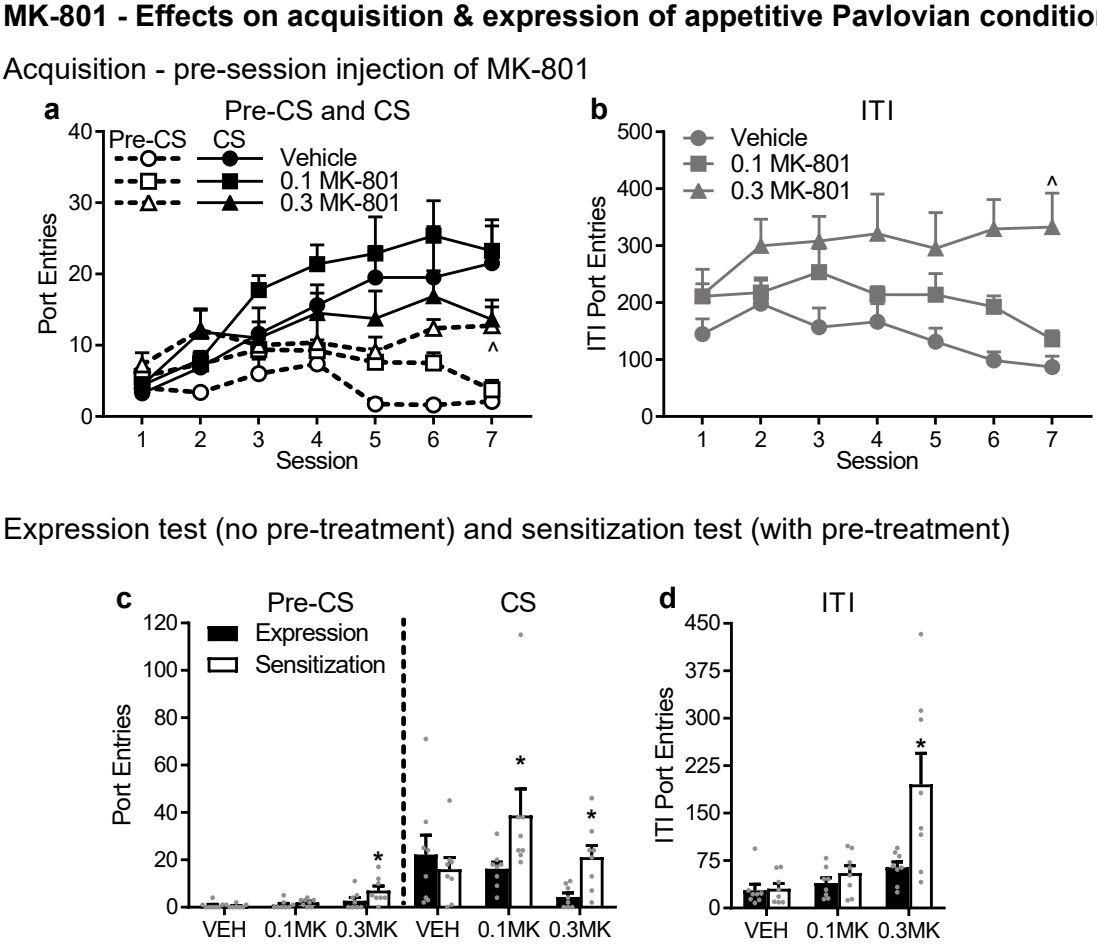

Fig. S1 Systemic MK-801 during training produced behavioral sensitization to the CS but a high dose had non-specific effects. (a) During acquisition, rats were trained in daily sessions in which a CS was paired with fructose-glucose solution ('sugar'), following injections of vehicle, 0.1, or 0.3 mg/kg 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).

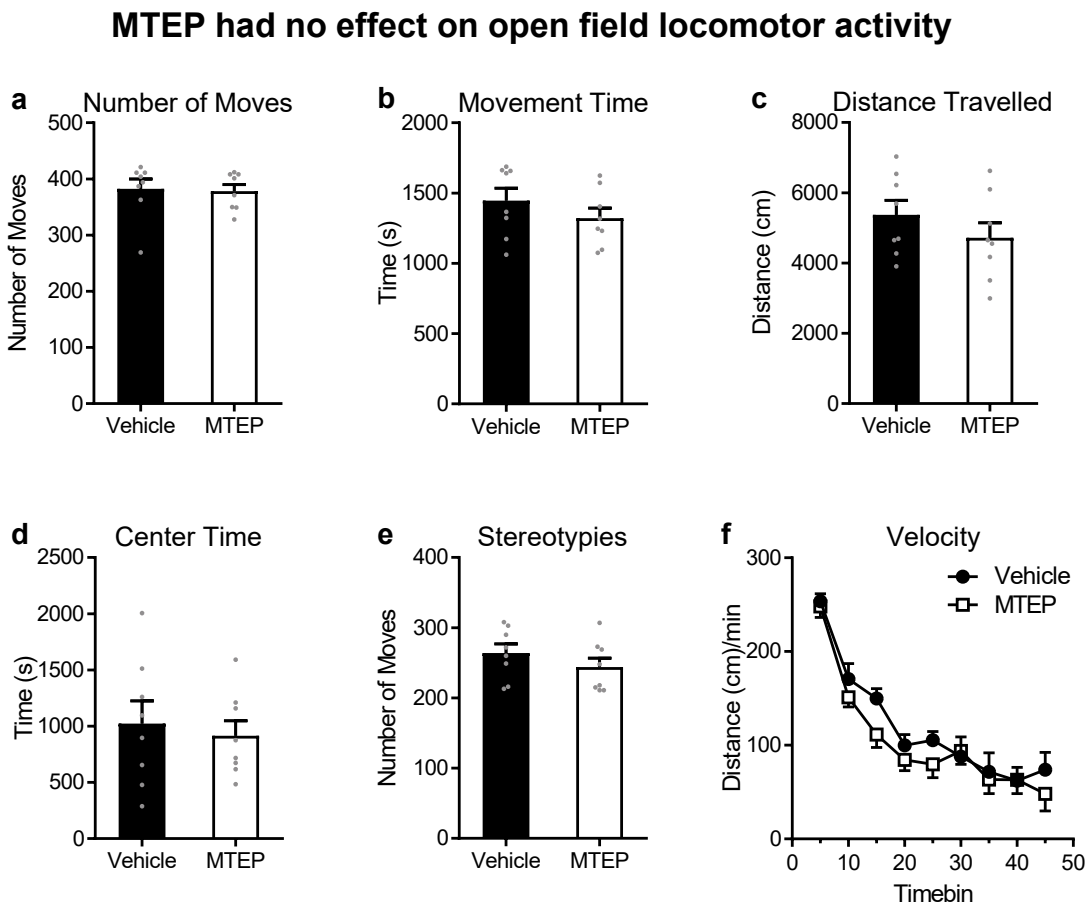

Fig. S2 MTEP had no effect on open field locomotor activity. Rats that received 5% DMSO/0.9% saline vehicle (n = 8) or 5 mg/kg MTEP (n = 8) did not differ on (a) the number of moves, (b) the amount of time spent moving, (c) the total distance travelled, (d) the amount of time spent in the center of the 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.

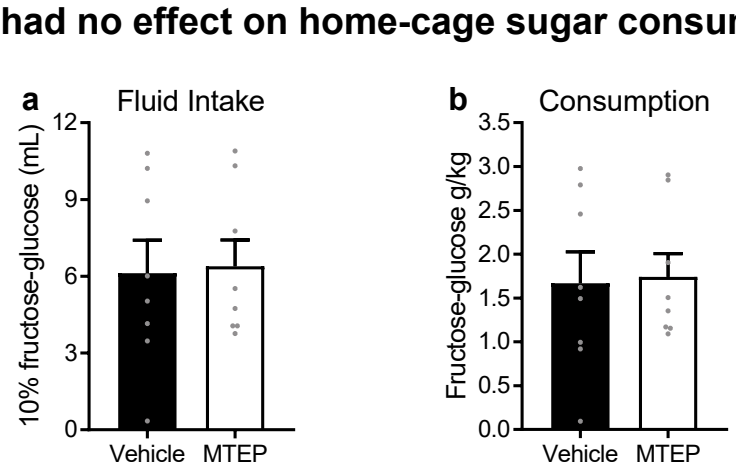

Fig. S3 MTEP had no effect on home-cage consumption of 10% fructose-glucose solution (sugar). Rats received either 5% DMSO/0.9% saline vehicle (n = 8) or 5 mg/kg MTEP (n = 8) 20 min before sugar was made available. (a) Over the course of 1 h, rats showed a significant preference for drinking sugar, but 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).

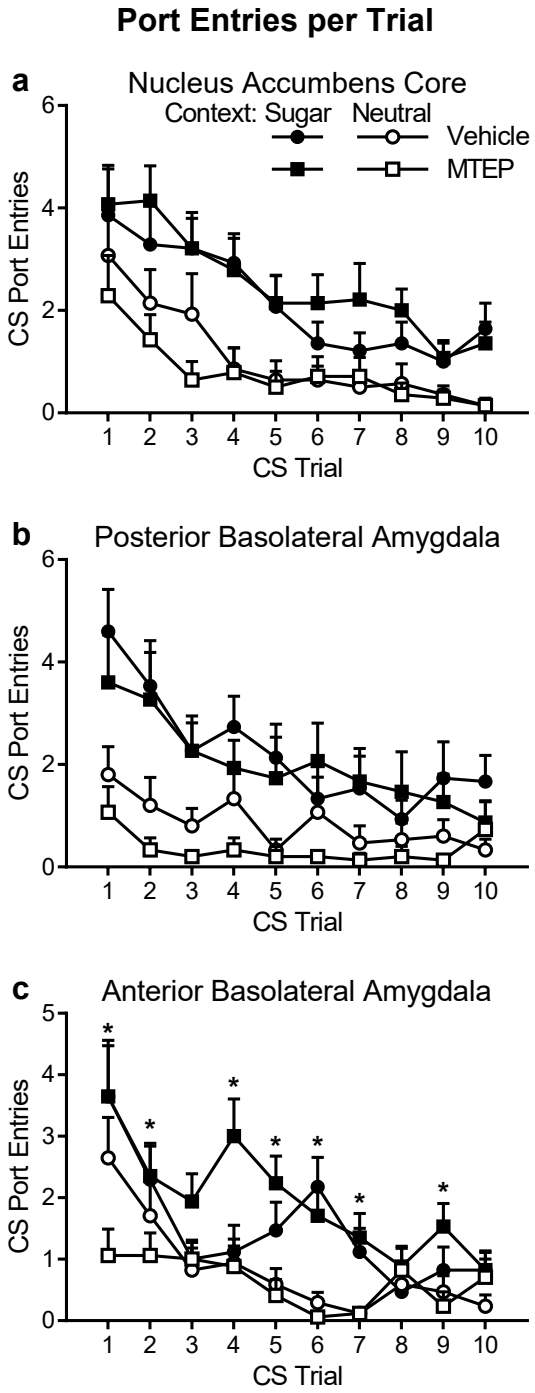

Fig. S4 MTEP had no effect on the within-session pattern of CS port entries when microinjected to the nucleus accumbens core or basolateral amygdala. (a) In the nucleus accumbens core, MTEP did not affect the pattern of non-normalized CS port entries in either context. (b) Similarly, MTEP microinjection 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.

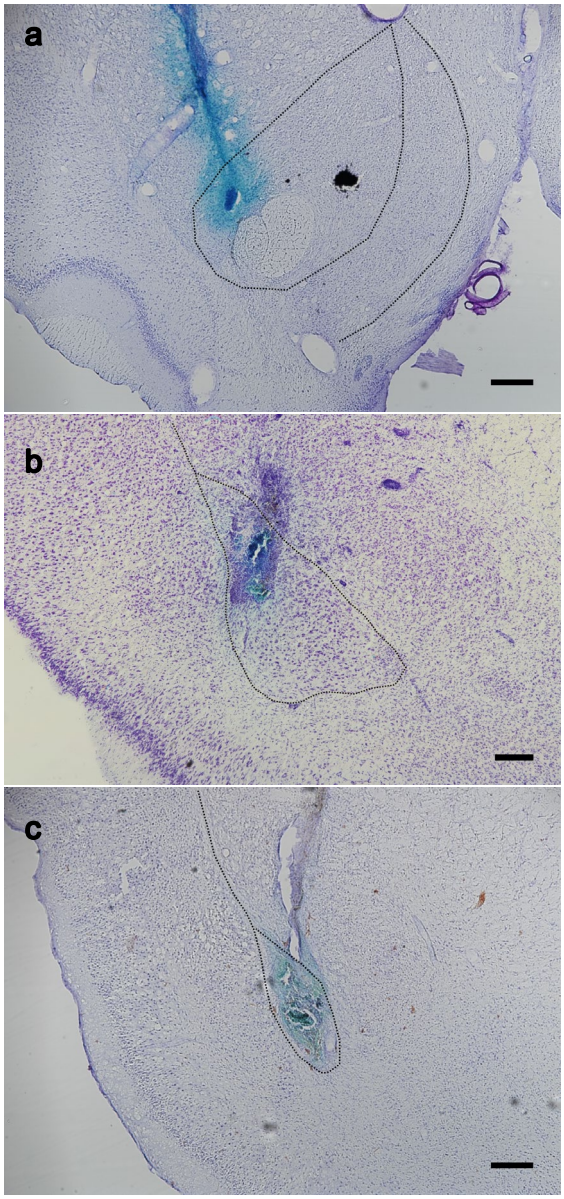

Fig. S5 Photomicrographs of microinjection sites. (a) A deposit of fast green can be seen adjacent to the anterior commissure in the nucleus accumbens core (AP +2.28 mm from bregma). (b) An example microinjection targeting posterior coordinates in the basolateral amygdala (AP -2.76 mm from bregma). (c)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

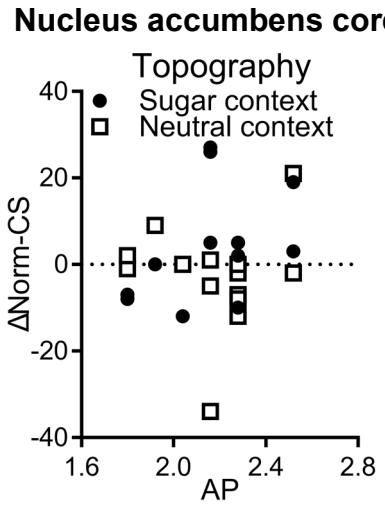

Fig. S6 There was no association between the anteroposterior coordinates of MTEP microinjection in the nucleus accumbens core and the effect of MTEP on Norm-CS port entries (ΔNorm-CS = Norm-CS_{MTEP} minus Norm-$CS_{Vehicle}$) in either the sugar or neutral contexts.

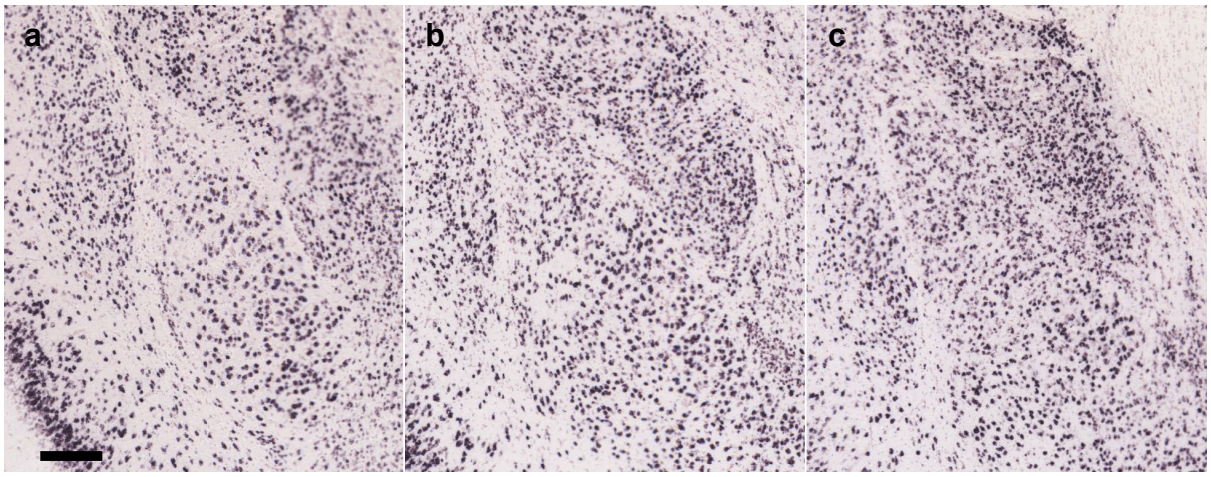

Fig. S7 Expression of mGluR5 in basolateral amygdala of the mouse brain. In situ hybridization for GRM5 in the mouse brain shows expression of mGluR5 throughout the AP-axis of the BLA. Images from the Allen Mouse Brain Atlas present (a) anterior coordinates (atlas image 25), (b) intermediate coordinates (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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