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Effect of amphetamine place conditioning on excitatory synaptic events in the basolateral amygdala ex vivo

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Abstract

The basolateral amygdala (BLA) plays an important role in the formation of associations between context and drug. BLA activity and BLA-dependent drug-seeking behavior are driven by excitatory inputs. Drug-seeking behavior driven by context involves participation of the BLA, and plasticity of excitatory inputs to the BLA may contribute to this behavior. In this study, amphetamine conditioned place preference (AMPH CPP) was used to model the formation of context-drug associations. Learning-induced changes of excitatory synapses within the BLA were examined. Male Sprague Dawley rats were assigned to one of three groups, the experimental group (AMPH CPP) or one of two control groups (saline or AMPH delayed pairing). Approximately 24 hours after testing their preference, spontaneous and miniature excitatory postsynaptic currents (s and mEPSCs, respectively) in BLA pyramidal neurons were investigated using whole-cell patch-clamp recordings. There were no between-groups differences in the amplitude or frequency of sEPSCs or mEPSCs. In a higher osmolarity solution to increase release, there was a significantly greater frequency of the mEPSCs in neurons from AMPH CPP animals compared to controls. This was observed with no change detected in the probability of glutamate release. Together these data demonstrate no evidence for increased synaptic strength, but are consistent with an increase in the number of synapses in the BLA after AMPH CPP. These findings may underlie increased excitatory drive of the BLA after AMPH CPP, and contribute to the animals' preference for the AMPH-paired compartment.

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Disclosure

The authors have no conflict of interest, financial or otherwise, to disclose. Conditioned place preference was done by A.H., *in vitro* recordings were done by J.A.R. and A.H., histology was done by A.H. The study was designed by G.E.M., J.A.R. and D.J.R. The manuscript was written by G.E.M., D.J.R., J.A.R. and A.H. All authors have approved the final article.

Keywords

associative learning; spontaneous EPSC; miniature EPSC; osmolarity; excitatory synapses

Repeated drug use in a specific environment results in the formation of long-lasting cue and context-drug associations. In drug abstinent individuals, re-exposure to environmental contexts and cues often leads to craving, drug-seeking, and relapse (O'Brien et al. 1998). Place conditioning is a behavioral assay for measuring the rewarding or aversive aspects of natural stimuli and chemical agents, including drugs of abuse (Reicher and Holman 1977; Bardo et al. 1999; Bardo and Bevins 2000; Tzschentke 1998, 2007). On a drug-free test day, a significant increase in the time spent in the drug-paired context is referred to as a conditioned place preference (CPP) and is interpreted as a rewarding effect of the drug. Place conditioning involves the formation of context-drug associations, requires memory consolidation, and produces experience-dependent morphological changes in neuronal circuits (Geinisman 2000; Rademacher et al. 2010). Because approach to a drug-associated context typically sets the occasion for drug-taking behavior, environmental context associated with drug use play a critical role in acquiring and maintaining drug-taking behavior.

The basolateral amygdala (BLA) is involved in assigning affective value to stimuli (Cardinal et al. 2002; LeDoux 2000). Afferents conveying multimodal sensory information about conditioned and unconditioned stimuli from the ventral tegmental area and substantia nigra (Asan 1997, 1998; Muller et al. 2009), cerebral cortex (Ottersen 1982; McDonald and Jackson 1987; Mascagni et al. 1993), thalamus (LeDoux et al. 1985, 1990; Turner and Herkenham 1991) and hippocampus (Canteras and Swanson 1992) converge in the BLA. Associative learning of the link between the rewarding effects of a drug and environmental stimuli requires activity in the BLA (Everitt et al. 1991; White and McDonald 1993), as does reinstatement of drug-seeking behaviors after re-exposure to drug-associated cues (Meil and See 1997; McLaughlin and See 2003; Peters et al. 2008). Functional inactivation of the BLA interferes with several forms of appetitive associative learning (Kruzich and See 2001; See et al. 2003; Gabriele and See 2010), disrupts contextual fear conditioning (Helmstetter and Bellgowan 1994; Muller et al. 1997) and blocks amphetamine-induced CPP (AMPH CPP; Hsu et al. 2002).

It is known that associative learning can facilitate the strength of excitatory inputs *in vitro*. For example, the formation of conditioned fear memory is accompanied by modifications in synaptic plasticity and synaptic strength in afferent pathways to the lateral amygdala (Schroeder and Shinnick-Gallagher 2004, 2005). Furthermore, *in vivo* and *ex vivo* experiments have shown that cue-reward learning induced an increase in the strength of thalamic synapses in the lateral amygdala, whereas the strength of cortical synapses remained unchanged (Tye et al. 2008). Previous work from our lab has shown that the formation of context-amphetamine associations is accompanied by an increase in the number of multisynaptic boutons and asymmetric (Gray Type I, presumably excitatory) synapses within the BLA (Rademacher et al. 2010), presumably contacting pyramidal neurons. Furthermore, there was an increase in excitatory synaptic drive to BLA pyramidal neurons after AMPH CPP, measured by *in vivo* intracellular recordings from BLA neurons approximately 20 hours after the test for AMPH CPP (Rademacher et al. 2010).

The aim of this study was to examine the cellular mechanisms for the increased excitatory drive that accompany the learning-induced increase in the number of excitatory inputs to the BLA. Here we specifically test whether the increased number of asymmetric synapses can be measured as an increase of functional synaptic activity and whether the previously

observed increased synaptic drive is caused by an increase in functional synapses or a change in synaptic release. We performed *ex vivo* whole-cell electrophysiological recordings from horizontal rat brain slices to measure excitatory synaptic inputs to the BLA approximately 24 hours after the AMPH CPP test.

2 Experimental Procedures

2.1 Animals

Male Sprague Dawley rats (Harlan, Indianapolis, IN) with an average group weight ranging from 230 to 240 g and an average group age ranging from 53 to 55 days at the beginning of the experiment were housed in groups of two per cage. They were handled for two days prior to behavioral studies. Food and water were available *ad libitum*. Rats were maintained on a 12 h light/dark cycle with lights on at 0700 hours. All studies were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* issued by the National Institute of Health, and were approved by the Rosalind Franklin University of Medicine and Science Institutional Animal Care and Use Committee.

2.2 Conditioned Place Preference

The place conditioning apparatus and behavioral conditioning were performed as previously described (Shen et al. 2006; Rademacher et al. 2006, 2010). Rats were placed in the center compartment of a three compartment apparatus. The compartments differed in visual and tactile cues (Rademacher et al. 2006) and were separated by removable guillotine-doors. Rats had free access to the entire apparatus for a 15 minutes pretest. The 5-day conditioning began 72 hours after the pretest. Each conditioning session lasted for 45 minutes. Rats were randomly assigned to one of three treatment groups. The experimental group received amphetamine (AMPH) on days 1, 3 and 5 immediately before being paired with one outer compartment. D-amphetamine sulfate was dissolved in sterile 0.9% saline (vehicle) and injected at a dose of 1 mg/kg, i.p. Doses refer to the drug base. On days 2 and 4 rats were injected with saline (1 ml/kg) and placed into the other outer compartment. Animals in the AMPH delayed pairing (AMPH DP) group were used to control for repeated AMPH exposure. They were subjected to the same conditioning schedule with AMPH as the experimental group, but remained in the home cage for 4 hours after the injection, followed by being placed into a conditioning chamber. The four-hour delay is sufficient to ensure that AMPH has cleared the brain (Honecker and Coper 1975). The second control group received saline injections before being placed in one outer compartment on days 1, 3 and 5 and in the other on days 2 and 4. Animals were tested drug- and vehicle-free for their compartment preference 72 hours after the last conditioning session. Rats were placed into the center compartment with free access to the entire apparatus for 30 minutes. The time spent in each compartment was recorded, and the place preference was calculated (the time spent in the drug-paired compartment minus the time spent in the saline-paired compartment during the test).

2.3 Ex vivo Electrophysiology

Compounds were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. KCl, NaCl, NaH₂PO₄, NaHCO₃, dextrose and MgCl₂ were purchased from Fisher Scientific (Pittsburgh, PA).

Animals were anesthetized (90 mg/kg Ketamine, Ketavet and 10 mg/kg Xylazine, Anased, Webster Veterinary Supply, Sterling, MA) 20-28 hours following the CPP test. Rats were perfused transcardially using ice cold, aerated (95% O₂ / 5% CO₂) high sucrose artificial cerebrospinal fluid (ACSF) containing (in mM) 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 7 dextrose, 7 MgCl₂, 0.5 CaCl₂, 210 sucrose, 1.3 ascorbic acid, 3 sodium pyruvate, with an

osmolality of approximately 300 mOsm. After decapitation, the brain was removed quickly and sectioned horizontally at 300 μm in a vibratome (Ted Pella, Inc., Redding, CA) in ice-cold high sucrose ACSF. Brain slices recovered for approximately 1 h at 34°C in physiological extracellular ACSF containing (in mM) 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , 10 dextrose, 1 MgCl_2 and 2 CaCl_2 , with the addition of 1.3 mM ascorbic acid and 3 mM sodium pyruvate, before being placed into the recording chamber. Recordings were performed at 30–33°C in submerged slices in physiological extracellular ACSF. (+)-bicuculline (10 μM ; Ascent Scientific, Princeton, NJ; dissolved in dimethyl sulfoxide) and picrotoxin (10 μM ; Tocris Cookson, Inc., Ellisville, MO; dissolved in ethanol) were both added in all experiments to the ACSF to block GABA_A receptor-mediated currents and isolate spontaneous or evoked excitatory postsynaptic currents (EPSCs). A subset of neurons was recorded in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM ; dissolved in dimethyl sulfoxide), to block α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA receptors). The sodium channel blocker tetrodotoxin citrate (TTX, 1 μM ; Ascent Scientific, Princeton, NJ) was added to pharmacologically isolate miniature EPSCs (mEPSCs). In a subset of experiments, mEPSCs were then recorded in the presence of 100 mM sucrose to increase external osmolality and increase synaptic glutamate release (Stevens and Tsujimoto 1995; Bekkers and Stevens 1995; Henze et al. 2002). All solutions were aerated with 95% O_2 / 5% CO_2 . Electrodes (1.6 – 6 $\text{M}\Omega$ open tip resistance) were filled with an intracellular solution containing (in mM) 150 CsCl, 0.2 EGTA, 10 HEPES, 2 NaCl, 4 ATP-Mg, 0.3 GTP-Tris, 7 tris-phosphocreatine, 5 QX314 chloride (Ascent Scientific, Princeton, NJ) and 0.2% neurobiotin (Vector Laboratories, Inc., Burlingame, CA). Whole-cell voltage-clamp recordings were performed from visually identified pyramidal neurons within the BLA, held at -90 mV. The holding potential of -90 mV was chosen to increase the driving force for synaptic events. However, in many neurons, periods of activity were recorded at -90 mV to -80 mV and -70 mV. If a neuron displayed any indication of instability at -90 mV, a holding potential of -80 or -70 mV was used for analysis. Only neurons that displayed a change of $<10\%$ in the current required to maintain the holding potential across the entire experiment were included in the analysis. For comparisons within a single neuron, the same holding potential was used. The mean holding potential did not differ between groups (saline -79.4 ± 2.1 mV, AMPH DP -76.3 ± 1.8 mV, AMPH CPP -78.8 ± 1.2 mV). In a subset of neurons, mEPSCs were recorded at -70 mV and -90 mV for comparison. There was no difference in the frequency of mEPSCs when comparing both holding potentials (-70 mV: saline 2.8 ± 0.4 Hz, AMPH DP 3.0 ± 0.7 Hz, AMPH CPP 3.7 ± 1.3 Hz; -90 mV: saline 2.8 ± 0.4 Hz, AMPH DP 2.9 ± 0.7 Hz, AMPH CPP 3.6 ± 1.3 Hz). Recordings were performed using an Axopatch 200B Capacitor Feedback Patch Clamp Amplifier or an Axoclamp 2A Amplifier (Molecular Devices, Inc., Sunnyvale, CA). Signals were low-pass filtered at 3–5 kHz and digitalized at 10–50 kHz.

A stimulator (Master-8, A.M.P.I., Jerusalem, Israel) and stimulus isolator (ISO-Flex, A.M.P.I., Jerusalem, Israel) were used for local stimulation experiments. Local electrical stimulation was performed by placing a self-made bipolar stimulation electrode into the BLA approximately 50 to 100 μm away from the recorded cell. Single and paired-pulse (PP) stimulation was applied at varying stimulation intensities (0.07–2 mA). For analysis of the coefficient of variation (CV) of EPSC amplitude a single stimulation was given 10–20 times at 0.2 Hz using a stimulation intensity that evoked EPSCs between 50 and 100 pA. The CV was calculated using the GraphPad Prism software. The CV equals the standard deviation of the EPSC amplitude divided by the mean of EPSC amplitude, multiplied by 100 ($\text{CV} = (\text{SD} / \text{mean}) \times 100$). To analyze the PP ratio (PPR), two consecutive stimuli were applied 20–30 times at 0.2 Hz with a stimulation intensity that evoked EPSCs > 100 pA. The inter-stimulus-interval (ISI) for PP stimulation was 25, 50 or 100 ms. The PPR was calculated by dividing the amplitude of the second evoked response (A2) by the amplitude of the first response (A1). The amplitude of the second EPSC was determined from just prior to the

second stimulation. Only evoked responses with monosynaptic EPSCs were analyzed as determined by a monotonic rise and singular peak.

For each experiment, approximately equal numbers of neurons from the respective group were measured. Only cells with series resistance below 20 M Ω were analyzed. All electrophysiology data were recorded and analyzed off-line with AxoGraph X software version 1.3.5 (Axograph Scientific) and stored on a computer (Mac Pro, Apple). For the spontaneous postsynaptic currents (sPSCs), sEPSCs and mEPSCs, the total number of events that occurred during one-minute recording epochs was analyzed. The frequency and amplitude of all EPSCs were measured and compared. EPSCs were analyzed by using a variable amplitude sliding template with a shape of an average synaptic current (Axograph X). The detection threshold for the events was set at 2.5 to 3 times baseline noise standard deviation. In the presence of hypertonic solution, mEPSCs were analyzed after a stabilization of the effects was observed (approximately 3-6 min after effects of sucrose began).

2.4 Histology

After recordings, slices were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for up to two weeks at 4°C. Sections were rinsed several times with PBS, treated with Triton X-100 (VWR international, Radnor, PA; 1% in PBS) for 6 to 8 hours and then incubated in the Vectastain ABC Reagent (Vector Laboratories, Burlingame, CA) in PBS at room temperature overnight. After several rinses with PBS, they were reacted with diaminobenzidine (DAB) and H₂O₂ (Peroxidase Substrate Kit DAB, Vector Laboratories, Burlingame, CA) in water to visualize the neurobiotin-filled neurons. Slices were washed in PBS repeatedly to stop the reaction. Sections were then mounted, dried and coverslipped. Stained slices were used to localize the recording sites, verified by the position of the filled neurons. Neurons were considered BLA pyramidal neurons if they were histologically confirmed to lie within the BLA and had a morphology consistent with pyramidal neurons.

2.5 Statistical analysis

All data were analyzed using the GraphPad Prism statistical package version 5.0d (GraphPad Software, Inc., La Jolla, CA). Statistical significance was determined by using one-way ANOVA followed by *post hoc* Tukey-Kramer test. Before statistical analysis, normality of distribution was determined with Kolmogorov-Smirnov test and homogeneity of variance was tested with Bartlett's test. The α -level was set at 0.05. Unless stated otherwise, data are expressed as mean $\pm$ SEM.

3 Results

3.1 Repeated pairings of AMPH with a context produces AMPH CPP

During place conditioning rats received either an AMPH or saline injection paired with one outer compartment of the CPP apparatus. The other outer compartment was paired with saline injections. Rats were tested for compartment preference 72 hours after the last conditioning session. The AMPH place preference was calculated by subtracting the time spent in the saline-paired compartment from the time spent in the drug-paired compartment during the test. Consistent with previous studies (Rademacher et al. 2006, 2010), neither the saline-treated animals (76.9 ± 92.1 s preference for the drug-paired chamber, $n = 23$ rats), nor the AMPH DP animals (-113.6 ± 82.9 s preference for the drug-paired chamber, $n = 22$ rats) showed a significant preference for one compartment over the other during the test (Figure 1; one-way ANOVA, saline versus AMPH DP $p > 0.05$). However, animals treated with AMPH and immediately paired with one compartment (AMPH CPP) showed a significant preference for the AMPH-paired compartment compared to the two control

groups (654.6 ± 77.3 s preference for the drug-paired chamber, $n = 23$ rats; one-way ANOVA, $F = 22.5$, $p < 0.0001$; saline versus AMPH CPP $p < 0.05$; AMPH DP versus AMPH CPP $p < 0.05$). The preference exhibited by the AMPH CPP group for the AMPH-paired compartment reflects the context-drug learning and is not caused by AMPH treatment alone, as the preference is absent in the AMPH DP group.

3.2 No effect on spontaneous EPSCs

Animals were anesthetized and the brains were prepared for *ex vivo* electrophysiology 20 to 28 hours after the CPP test. Animals of each treatment group (saline $n = 21$ rats, AMPH DP $n = 17$ rats, AMPH CPP $n = 21$ rats) were used to record sPSCs by holding the cell at -70 to -90 mV. Bath application of $10 \mu\text{M}$ bicuculline and $10 \mu\text{M}$ picrotoxin was used to pharmacologically isolate sEPSCs (Figure 2). Those currents were completely blocked by the addition of $10 \mu\text{M}$ CNQX when tested in a subset of cells at the end of the experiment, indicating they were mediated by AMPARs (data not shown). The amplitudes (saline 17.0 ± 0.8 pA, $n = 45$ neurons, AMPH DP 17.1 ± 0.8 pA, $n = 42$ neurons, AMPH CPP 18.1 ± 1.1 pA, $n = 49$ neurons; one-way ANOVA, $p > 0.05$) as well as the frequency of the sEPSCs (saline 3.4 ± 0.3 Hz, $n = 45$ neurons, AMPH DP 4.2 ± 0.6 Hz, $n = 42$ neurons, AMPH CPP 4.0 ± 0.4 Hz, $n = 49$ neurons; one-way ANOVA, $p > 0.05$) were comparable between the groups (Figure 2). Additionally, no differences in the rise time and half-width of the sEPSCs were detected between the three groups (Table 1). Thus, AMPH CPP had no significant effect on the spontaneous EPSC parameters measured from BLA pyramidal neurons.

3.3 No effect on strength of synaptic release

To determine whether context-drug learning altered the strength of excitatory synapses within the BLA, electrical stimulation experiments were performed. To do so, a bipolar stimulation electrode was placed within the BLA and the evoked excitatory response was recorded. The excitatory response was isolated by blocking inhibitory GABA_A receptor currents using $10 \mu\text{M}$ bicuculline and $10 \mu\text{M}$ picrotoxin. A single stimulation was repeated 10 to 20 times at 0.2 Hz and the coefficient of variation (CV) of the EPSC amplitude was calculated (Figure 3A,B). No significant difference in the CV was observed between the three treatment groups (saline $18.3 \pm 2.5\%$, $n = 12$ neurons from 10 rats; AMPH DP $19.9 \pm 1.8\%$, $n = 11$ neurons from 10 rats; AMPH CPP $17.7 \pm 2.3\%$, $n = 16$ neurons from 11 rats; one-way ANOVA, $p > 0.05$). The evoked EPSCs decayed exponentially with similar time constants between the groups (saline 15.3 ± 1.8 ms; AMPH DP 18.2 ± 2.9 ms; AMPH CPP 15.9 ± 2.1 ms; one-way ANOVA, $p > 0.05$). In a subset of cells $10 \mu\text{M}$ CNQX was added at the end of the experiment. CNQX blocked the evoked EPSCs completely, indicating that they were mediated by AMPARs (data not shown).

In addition to the measurement of the CV, the response to paired-pulse (PP) stimulation is another measure that reflects release probability. The PP ratio (PPR) was recorded at different inter-stimulus-intervals (ISIs; 25, 50 and 100 ms). The PP stimulation was repeated 20-30 times at 0.2 Hz (Figure 3C). There were no differences in the PPRs among the treatment groups at any of the ISI used (25 ms: saline 1.02 ± 0.07 , $n = 8$ neurons; AMPH DP 0.85 ± 0.09 , $n = 5$ neurons; AMPH CPP 0.96 ± 0.06 , $n = 14$ neurons; 50 ms: saline 1.06 ± 0.06 , $n = 9$ neurons; AMPH DP 0.95 ± 0.06 , $n = 6$ neurons; AMPH CPP 0.99 ± 0.04 , $n = 14$ neurons; 100 ms: saline 0.98 ± 0.04 , $n = 9$ neurons; AMPH DP 0.81 ± 0.07 , $n = 8$ neurons; AMPH CPP 0.94 ± 0.04 , $n = 14$ neurons; from 5 saline rats, 6 AMPH DP rats, 8 AMPH CPP rats; one-way ANOVA, $p > 0.05$). Thus, a drug-context learning-induced change in the probability of glutamate release within the BLA was not detected.

3.4 AMPH CPP increases the frequency of sucrose-evoked miniature EPSCs

The measurement of miniature excitatory postsynaptic currents (mEPSCs) is a classical method to determine the locus of synaptic changes. Since spontaneous EPSCs represent both action potential-dependent and independent glutamate release, 1 μ M of the sodium channel blocker TTX was added to the bath solution, to eliminate the contribution of the action potential-mediated release of glutamate. These pharmacologically isolated mEPSCs reflect spontaneously released vesicles of glutamate. When the mEPSCs from BLA pyramidal neurons were recorded, no significant differences in either the amplitude (saline 16.2 ± 1.1 pA, $n = 17$ neurons from 8 rats; AMPH DP 16.5 ± 1.2 pA, $n = 15$ neurons from 7 rats; AMPH CPP 15.4 ± 0.8 pA, $n = 16$ neurons from 7 rats; one-way ANOVA, $p > 0.05$) or the frequency (saline 2.5 ± 0.3 Hz, $n = 17$ neurons from 8 rats; AMPH DP 2.6 ± 0.3 Hz, $n = 15$ neurons from 7 rats; AMPH CPP 3.0 ± 0.6 Hz; $n = 16$ neurons from 7 rats; one-way ANOVA, $p > 0.05$) of mEPSC-events were detected between the groups (Figure 4).

To induce greater synaptic release, and potentially recruit release in otherwise low-probability synapses, mEPSCs were recorded in the presence of 100 mM sucrose to increase extracellular osmolarity, and boost action potential-independent glutamate release. This low sucrose concentration was used to avoid fast neurotransmitter depletion (Stevens and Tsujimoto 1995; Rosenmund and Stevens 1996; Henze et al. 2002). Application of 100 mM sucrose increased the frequency of mEPSCs in all groups. However, a 1.55-fold greater frequency of mEPSCs was observed in the AMPH CPP group (AMPH CPP 7.3 ± 1.1 Hz, $n = 13$ neurons from 7 rats; saline 4.7 ± 0.5 Hz, $n = 16$ neurons from 8 rats; AMPH DP 4.7 ± 0.4 Hz, $n = 13$ neurons from 6 rats; one-way ANOVA, $F = 4.45$, $p = 0.018$; saline versus AMPH CPP $p < 0.05$; AMPH DP versus AMPH CPP $p < 0.05$), compared to the control groups. No difference was detected in the frequency of events between the saline and AMPH DP group (Figure 5; one-way ANOVA, saline versus AMPH DP $p > 0.05$). Additionally, in the presence of sucrose there was no significant difference in the amplitudes of the mEPSCs between the three groups (saline 16.0 ± 1.1 pA, $n = 16$ neurons, AMPH DP 16.7 ± 1.0 pA, $n = 13$ neurons, AMPH CPP 16.0 ± 1.4 pA, $n = 13$ neurons; one-way ANOVA, $p > 0.05$). The absence of a difference in mEPSC amplitude or kinetics implies that the observed effects were likely due to presynaptic factors. The increase in the frequency of mEPSCs in the presence of sucrose in the AMPH CPP group compared to controls, suggest either an elevated probability of glutamate release under those conditions or an increase in the number of synapses. The absence of group differences in baseline mEPSC frequency, PPR and CV would indicate that the most parsimonious explanation for our results is an increase in the number of excitatory synapses.

4 Discussion

Surprisingly, our data show no evidence for a learning-induced increase in synaptic strength. However, when more glutamatergic release events were induced by adding sucrose, we observed a 1.55-fold increase in the frequency of mEPSCs from AMPH CPP animals compared to controls. These new findings, an increase in the frequency of high osmolarity-induced release of glutamate after AMPH CPP, in the absence of a change in release probability, are consistent with an increase in the number of functional excitatory synapses contacting BLA pyramidal neurons.

Other studies have shown an increase in afferent drive to the lateral amygdala after cue specific reward learning (Tye et al. 2008) as well as to the BLA after drug-induced learning (Tye et al. 2010; Rademacher et al. 2010). Any differences are unlikely to be caused by an overshadowing impact of handling or injection-induced stress, as the frequency and kinetics of the EPSCs measured in control groups were similar to control groups of other studies (Läck et al. 2007; Tye et al. 2008, 2010), and were similar to untreated animals in the same

age range (data not shown). A few studies also examined the effect of other stimulants on the modification of BLA synapses, mostly focusing on the lateral amygdala (LA). Tye et al. (2010) have shown that acute administration of methylphenidate facilitated the strengthening of cortico-amygdala synapses to the LA after drug-induced learning through a postsynaptic increase in AMPAR-mediated currents. In addition, Numachi et al. (2007) found a significant increase in EphA5 mRNAs in the amygdala 9 and 24 h after acute methamphetamine treatment. They therefore suggested that methamphetamine could affect patterns of synaptic connectivity. Goussakov and colleagues (2006) showed that withdrawal (21 h) after repeated cocaine injections was accompanied by enhancements of glutamatergic synaptic transmission within the LA.

The absence of a change in spontaneous release is inconclusive by itself. To further analyze possible changes in synaptic strength to BLA pyramidal neurons after AMPH CPP, we analyzed the PPR as well as the CV of evoked EPSC amplitudes. These measures reflect potential differences in release probability. The PPRs were comparable to previous work, recorded in diverse brain regions (Schlüter et al. 2006; Tye et al. 2010). There was no difference in the CV or the PPR among the groups. Combined with the absence of an effect on EPSCs, those data suggest no pre- or postsynaptic changes in synaptic strength to BLA pyramidal neurons after AMPH CPP. This complements the results of our previous finding of increased excitatory drive of BLA neurons *in vivo* (Rademacher et al. 2010). However, that study was not designed to dissociate differences arising from the number of synapses versus the strength of synapses. The current results indicate that the changes observed *in vivo* likely arise by a change in synapse number and not an increase of synaptic strength. Other factors may also contribute to the changes observed *in vivo* that contrast with *ex vivo* conditions, including the presence of extracellular synaptically released GABA that can modulate release of glutamate, and the *in vivo* study focused on afferents arising from the hippocampal formation, while in this current study we used local stimulation within the BLA, activating diverse excitatory inputs, independent of their source. It is therefore possible that selective changes, which might occur only at afferents from the hippocampal formation, are masked in the presence of other inputs that might be unchanged. In addition, methodological differences exist between these studies, including the loss of a significant amount of synaptic connectivity in slices compared to *in vivo*, the differences in temperature, intracellular versus whole-cell recording techniques, and the presence of an abundance of neuromodulators *in vivo* that are absent in the slice.

While we did not find evidence for increased synaptic strength following the CPP testing, previous studies support a role for an increase of synaptic strength immediately following a single conditioning in the amygdala (Tye et al. 2008), and over the course of several days in other brain regions (Stuber et al. 2008). Additionally, further studies describe a potentiation of glutamatergic synapses to neurons of other brain areas caused by an exposure to drugs of abuse (Ungless et al. 2001, Saal et al. 2003, Faleiro et al. 2004, Mameli and Lüscher 2011). It is possible that there is a similar transient increase in synaptic strength that occurs during early components of CPP that is then translated into long-term structural changes. Furthermore, an increase of synaptic strength may occur in a subset of inputs, but these changes might be overshadowed by the relative absence of a change in the remaining inputs, masking the change of synaptic strength in this subset of inputs.

Taken together, our data indicate that it is unlikely that AMPH CPP leads to changes of synaptic release probability. A change in the number of excitatory synapses may be more readily observed in conditions when more synaptic terminals release their contents. Action potential-independent exocytosis can be stimulated in a manner that is independent from internal or external calcium concentration by increasing the extracellular osmolarity with sucrose (Rosenmund and Stevens 1996). There was no change in the amplitude of the

sucrose-evoked mEPSCs, similar to other studies, and consistent with a presynaptic site of effect (Stevens and Tsujimoto 1995; Rosenmund and Stevens 1996). Elevating the osmolarity of the extracellular saline increased the mEPSC frequency in each group, but neurons from AMPH CPP treated animals showed a mEPSC frequency in the presence of sucrose that was significantly higher than that of the two control groups. These results suggest a presynaptic change that might be caused by either a modification in release probability (Rosenmund and Stevens 1996) or by an increase in the number of excitatory synapses per neuron. The difference in the sucrose-evoked frequency of mEPSCs alone does not allow one to discriminate between these two possibilities. But, considering the increased frequency of sucrose-evoked mEPSCs in the absence of a change in the PPRs and the CV of EPSC amplitude, as well as no changes in the frequency of spontaneous or mEPSCs, a modification in release probability seems to be unlikely. Consequently, together, those data strongly support the hypothesis that AMPH CPP leads to an increase in the number of excitatory synapses rather than a change in the release probability. Thus, these data support our previous results. The 1.55-fold greater increase in the frequency of sucrose-evoked excitatory events after AMPH CPP fits very nicely with the previously described learning-induced 1.6-fold increase in the total number of asymmetric synapses contacting BLA neurons (Rademacher et al. 2010). We suggest that the lack of changes in the frequency of excitatory events after AMPH CPP without sucrose might be due to the low release probability of these new synapses.

During the preference test the animals were exposed to the drug-paired environmental context, but in the absence of the drug. A CPP memory can be extinguished, as repeated drug-free exposure to a drug-associated context reduces CPP behavior (Bardo et al. 1986; Schroeder and Packard 2003). Extinction is an active process that involves new memory formation (Rescorla 2001; Schroeder and Packard 2003). The BLA plays an important role in the extinction of CPP. For instance, excitotoxic lesions of the BLA prior to extinction sessions attenuated the extinction of CPP (Fuchs et al. 2002). Due to the importance of the BLA in extinction of CPP, and the possibility that this form of learning induces synaptic changes, we cannot rule out a potential contribution of extinction to our findings.

4.1 Conclusion

This study demonstrates that changes induced by AMPH CPP occurred at the level of mEPSC, not spontaneous or evoked EPSCs *ex vivo*. Therefore, these changes are unlikely to involve global activity of neuronal networks that provide input to the BLA. Instead, our *ex vivo* data reveal a change that is consistent with an increased number of synapses after AMPH CPP. Combined with previous studies, this indicates that several changes contribute to the potency of drug-associated contexts in the BLA-dependent relapse to drug-seeking. Factors include specific enhancement of glutamatergic inputs from the hippocampal formation, in the absence of a generalized increase of synaptic strength. This is coupled to, and perhaps caused by, an increase in the number of excitatory synapses in the BLA. Perhaps, in the context associated with drug availability, the BLA is under a steady stream of excitatory drive, and the potent hippocampal inputs overwhelmingly drive the BLA. The expected result would be a BLA that is readily controlled by hippocampal inputs at the expense of other afferents. This imbalance may underlie the strong context-driven drug-seeking behavior seen in those individuals addicted to drugs.

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Abbreviations

ACSF artificial cerebrospinal fluid	AMPA
α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors	AMPH
amphetamine	BLA
basolateral amygdala	CNQX
6-cyano-7-nitroquinoxaline-2,3-dione	CPP
conditioned place preference	CV
coefficient of variation	DAB
diaminobenzidine	DP
delayed pairing	EPSC
excitatory postsynaptic current	ISI
inter-stimulus-interval	LA
lateral amygdala	mEPSC
miniature excitatory postsynaptic current	PBS
phosphate-buffered saline	PP
paired-pulse	PPR
paired-pulse ratio	sEPSC
spontaneous excitatory postsynaptic current	sPSC spontaneous postsynaptic current
TTX	tetrodotoxin citrate

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Highlights

- no changes in the spontaneous or miniature EPSCs in BLA after AMPH CPP
- no presynaptic changes in the probability of glutamate release after AMPH CPP
- significant increase in frequency of mEPSCs in high sucrose solution after AMPH CPP
- drug-seeking behavior associated with increased BLA synapse number, not strength

Figure 1. Contextual paired amphetamine exposure produces amphetamine conditioned place preference (AMPH CPP)

AMPH CPP was performed using three different behavioral treatments. One group of animals received saline injections on all conditioning days immediately before being placed in the CPP apparatus. For a second group (AMPH DP), the AMPH injections were paired with one outer compartment and saline injections with the other, but pairing occurred delayed, four hours after the injection. A third group (AMPH CPP) received AMPH injections immediately before being paired with one outer compartment and saline injections before being placed in the other one. Only the AMPH CPP group expressed a significant place preference compared to saline and AMPH DP (one-way ANOVA, * $p < 0.0001$). Those animals spent significantly more time in the amphetamine-paired compartment than in the saline-paired context during the CPP test. There was no difference between the saline and AMPH DP group ($p > 0.05$). Animals from both groups spent nearly equal time in both compartments during the test. Data are expressed as mean $\pm$ SEM.

Figure 2. AMPH CPP does not affect the spontaneous EPSCs in BLA pyramidal neurons
A. sEPSCs were recorded in the presence of 10 μ M bicuculline and 10 μ M picrotoxin in voltage-clamp from BLA pyramidal neurons (inset) at a holding potential of -70 to -90 mV. Representative traces from neurons of the different treatment groups illustrate that there was no difference in the amplitude and frequency of the spontaneous events. **B.** Histograms and cumulative probability (prob.) plots of the amplitudes and frequencies recorded from all neurons (saline $n = 45$ cells from 21 rats, AMPH DP $n = 42$ cells from 17 rats, AMPH CPP $n = 49$ cells from 21 rats). Data are expressed as mean $\pm$ SEM.

Figure 3. Evoked EPSCs of single or paired-pulse electrical stimulation

Single and paired-pulse local electrical stimulation were performed in the presence of 10 μ M picrotoxin and 10 μ M bicuculline on BLA pyramidal neurons of saline (10 rats), AMPH DP (10 rats) and AMPH CPP (11 rats) treated animals. **A.** Representative overlay (grey) and average (black) traces of evoked excitatory responses to a single stimulation are shown. **B.** The mean coefficient of variation (CV) of the EPSC-amplitude was calculated. There was no difference in the CV between the treatment groups (saline $n = 12$ neurons, AMPH DP $n = 11$ neurons, AMPH CPP $n = 16$ neurons). **C.** Example traces (overlay and average) of evoked responses following PP stimulations at 50 ms inter-stimulus-intervals (ISIs) from each treatment group were measured and the PPR was calculated. **D.** There was no difference in the PPR at any ISI recorded between the treatment groups (saline $n = 9$ neurons, AMPH DP $n = 6$ neurons, AMPH CPP $n = 14$ neurons). Data are expressed as mean $\pm$ SEM.

Figure 4. AMPH CPP does not affect the miniature EPSCs in BLA pyramidal neurons

A. mEPSCs were recorded in the presence of 10 μ M bicuculline, 10 μ M picrotoxin and 1 μ M TTX in voltage-clamp from BLA pyramidal neurons at a holding potential of -70 to -90 mV. Representative traces from neurons of each group showing that there was no difference in the amplitude and frequency of the miniature events. **B.** Histograms and cumulative probability (prob.) plots of the amplitudes and frequencies recorded from all neurons (saline $n = 17$ cells from 8 rats, AMPH DP $n = 15$ cells from 7 rats, AMPH CPP $n = 16$ cells from 7 rats). Data are expressed as mean $\pm$ SEM.

Figure 5. Sucrose-evoked mEPSCs show a higher frequency after AMPH CPP
mEPSCs were recorded in the presence of 10 μ M bicuculline, 10 μ M picrotoxin, 1 μ M TTX and 100 mM sucrose in voltage-clamp from BLA pyramidal neurons at a holding potential of -70 to -90 mV. **A.** Representative traces of the mEPSCs before (left panel) and after the addition of 100 mM sucrose to the bath solution (middle panel). **B.** Histograms and cumulative probability (prob.) plots of the amplitude and frequency of the sucrose-evoked mEPSCs show that the mEPSC-amplitude was similar among the groups, but the mEPSC-frequency was significantly increased in AMPH CPP animals compared to controls (one-way ANOVA, $p < 0.05$; saline $n = 16$ cells from 8 rats, AMPH DP $n = 13$ cells from 7 rats, AMPH CPP $n = 13$ cells from 7 rats). There was no difference in the frequency between the saline and AMPH DP groups. **C.** The time-course of the frequency of mEPSCs before and during sucrose shows that sucrose increased the frequency in all groups, with a greater effect in the AMPH CPP group. Data are expressed as mean $\pm$ SEM.

Table 1
Basic properties of spontaneous, miniature and sucrose-evoked excitatory postsynaptic currents

	saline			AMPH DP			AMPH CPP		
	sEPSC	mEPSC	mEPSC + sucrose	sEPSC	mEPSC	mEPSC + sucrose	sEPSC	mEPSC	mEPSC + sucrose
amplitude (pA)	17.0 ± 0.8	16.2 ± 1.1	16.0 ± 1.1	17.1 ± 0.8	16.5 ± 1.2	16.7 ± 1.1	18.1 ± 1.1	15.4 ± 0.8	16.0 ± 1.4
frequency (Hz)	3.40 ± 0.28	2.51 ± 0.32	4.68 ± 0.57	4.20 ± 0.56	2.60 ± 0.32	4.68 ± 0.43	4.03 ± 0.37	2.99 ± 0.58	7.32 ± 1.08
rise time (ms)	1.47 ± 0.08	1.61 ± 0.06	1.87 ± 0.08	1.40 ± 0.09	1.36 ± 0.09	1.43 ± 0.09	1.59 ± 0.11	1.52 ± 0.08	1.69 ± 0.07
half-width (ms)	4.11 ± 0.20	4.13 ± 0.27	4.57 ± 0.27	4.13 ± 0.25	3.92 ± 0.25	4.33 ± 0.23	4.21 ± 0.18	3.86 ± 0.21	4.25 ± 0.17

Data are expressed as mean ± SEM. sEPSCs - spontaneous excitatory postsynaptic currents, mEPSCs - miniature excitatory postsynaptic currents