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Retrieval of Contextual Memories Increases Activity-Regulated Cytoskeleton-Associated Protein in the Amygdala and Hippocampus

David A. Figge, IhteshamUr Rahman, Philip J. Dougherty, and David J. Rademacher

Department of Cellular and Molecular Pharmacology, Rosalind Franklin University of Medicine and Science, Chicago Medical School, North Chicago, IL, USA

Abstract

Activity-regulated cytoskeleton-associated protein (Arc) integrates information from multiple intracellular signaling cascades and, in turn, regulates cytoskeletal proteins involved in structural synaptic modifications. The purposes of the present study were: (a) to determine if the retrieval of contextual memories would induce Arc in hippocampal and amygdalar neurons; and (b) use unbiased stereology at the ultrastructural level to quantify synapses contacting Arc-labeled (Arc+) and unlabeled (Arc−) postsynaptic structures in brain regions in which the amount of Arc integrated density (ID) correlated strongly with the degree of amphetamine conditioned place preference (AMPH CPP). The retrieval of contextual memories increased the Arc ID in the dentate gyrus, cornu ammonis (CA)1, and CA3 fields of the hippocampus and the basolateral, lateral, and central nuclei of the amygdala but not the primary auditory cortex, a control region. Stereological quantification of Arc+ and Arc− synapses in the basolateral nucleus of the amygdala (BLA) was undertaken because the strongest relationship between the amount of Arc ID and AMPH CPP was observed in the BLA. The retrieval of contextual memories increased the number and density of asymmetric (presumed excitatory) synapses contacting Arc+ spines and dendrites of BLA neurons, symmetric (presumed inhibitory or modulatory) synapses contacting Arc+ dendrites of BLA neurons, and multisynaptic boutons contacting Arc+ postsynaptic structures. Thus, the retrieval of contextual memories increases Arc in the amygdala and hippocampus, an effect that could be important for approach behavior to a drug-associated context.

Keywords

amphetamine; classical conditioning; conditioned place preference; activity-regulated cytoskeleton-associated protein; amygdala; hippocampus

Introduction

Environmental cues and contexts associated with drug use play a critical role in the acquisition and maintenance of drug-taking behavior and subsequent relapse to drug-seeking behavior (see Robinson and Berridge 2000 for review). In the conditioned place preference (CPP) behavioral assay of reward, rapidly formed associations between the environmental context (conditioned stimulus, CS) and the drug effects (unconditioned stimulus, UCS)

Address correspondence to: David J. Rademacher, Ph.D., Michigan State University, College of Human Medicine, Department of Translational Science and Molecular Medicine, 333 Bostwick Avenue NE, Grand Rapids, MI 49503, Phone: 1-616-234-0970, Fax: 1-616-234-0992, david.rademacher@hc.msu.edu.

Conflict of interest

The authors declare that they have no conflict of interest.

depend on *de novo* protein synthesis (Kuo et al. 2007), are long-lasting (Sadler et al. 2007; Schroeder and Packard 2003), and are resistant to extinction (Mueller and Stewart 2000; Mueller et al. 2002). The expression of CPP is dependent on memory processes including retrieval and consolidation mechanisms (White 1996). Furthermore, this type of classical conditioning induces experience-dependent structural synaptic changes in neuronal circuits (Geinisman 2000; Rademacher et al. 2010). In fact, amphetamine CPP (AMPH CPP) increases a presynaptic marker of structural alterations of synapses in the basolateral nucleus of the amygdala (BLA), dentate gyrus (DG), cornu ammonis (CA)1 and CA3 fields of the hippocampus, events that are strongly correlated with the degree of CPP (Rademacher et al. 2006). Importantly, the amygdala and hippocampus play a key role in the formation of associations between environmental context and the rewarding effects of drugs (Ferbinteanu and McDonald 2001; Helmstetter and Bellgowan 1994; Hsu et al. 2002; Olmstead and Franklin 1997).

The immediate early gene, activity-regulated cytoskeleton-associated protein (Arc), has been characterized as a 'master regulator' of synaptic plasticity (see Shepherd and Bear 2011 for review) that integrates information from multiple intracellular signaling cascades (Pintchovski et al. 2009; Teber et al. 2004; Wuang et al. 2008) and, in turn, regulates cytoskeletal proteins involved in synaptic remodeling (Fujimoto et al. 2004; Mokin et al. 2006). Arc is unique in that neuronal activity causes a rapid and specific delivery of newly synthesized Arc mRNA to dendrites (Link et al. 1995; Lyford et al. 1995). Unlike other well-studied immediate early genes such as c-fos and c-jun (Hung et al. 2000; Nikam et al. 1995), Arc is expressed in neurons but not glia (Vazdarjanova et al. 2006). Moreover, synaptic activity causes Arc mRNA to localize selectively at synapses that have been activated (Steward et al. 1998) and Arc protein is concentrated in the postsynaptic density (Moga et al. 2004; Steward and Worley 2001). Thus, Arc is a marker for recently activated synapses. The induction of Arc gene expression, delivery of mRNA to dendrites, and protein synthesis occur during the first few hours after the inducing event and correlate well to the occurrence of protein synthesis-dependent synaptic modifications (Guzowski et al. 1999; Wallace et al. 1998). Arc is increased by acute psychostimulant administration (Tan et al. 2000) and behavioral experience that leads to long-lasting synaptic modifications (Kelly and Deadwyler 2002; 2003). That infusion of Arc antisense oligodeoxynucleotides into the hippocampus can impair the maintenance of long-term potentiation and spatial learning performance without affecting short-term plasticity and short-term memory suggests that Arc plays a role in memory consolidation (Guzowski et al. 2000). Interestingly, there is a correlation between Arc protein expression in dendritic spines and the onset of synaptogenesis during development (Wang and Pickel 2004). The finding that the protein translational machinery is present in the distal dendrites at the base of spines (Steward 1995) supports the hypothesis that Arc protein is synthesized locally and selectively at activated portions of the dendrite (Lanahan and Worley 1998).

We have previously demonstrated that, in the BLA, AMPH CPP increased the number and density of single synaptic boutons (SSBs; i.e., boutons that form synapses with a single postsynaptic element) and multisynaptic boutons (MSBs; i.e., boutons that form separate synapses with two or more postsynaptic elements) (Rademacher et al., 2010). The latter is an ultrastructural index of the remodeling of existing synapses (Geinisman et al. 2001; Nicholson and Geinisman 2009; Toni et al. 1999; 2001). With regard to SSBs, AMPH CPP increased the number and density of asymmetric (Gray Type I, presumed excitatory) axospinous and axodendritic synapses contacting BLA neurons (Rademacher et al., 2010). Although this learning-induced 'rewiring' of the BLA was not accompanied by changes in synapse strength or the probability of glutamate release in the BLA *ex vivo* (Hetzl et al. 2012), it was reflected in increases in BLA neuronal excitatory drive *in vivo* (Rademacher et al. 2010). In the present study, we hypothesized that re-exposure of animals to the CPP

apparatus during the CPP test, which would cause retrieval of context-drug and context-vehicle memories, would: (a) induce Arc in neurons of the hippocampus and amygdala; (b) increase the number and density of Arc-labeled (Arc+) asymmetric and symmetric (Gray Type II, presumed inhibitory or modulatory) synapses contacting Arc+ postsynaptic structures; and (c) increase the number and density of MSBs contacting Arc-labeled postsynaptic structures of BLA neurons.

Materials and methods

Animals

Thirty male Sprague Dawley rats (Harlan, Indianapolis, IN, USA), weighing 175–199 g (44–48 d) at the start of the experiment, were housed in groups of three. Each animal was handled for 5 days before behavioral conditioning. Food and water were available *ad libitum* in their home cages. Rats were maintained on a 12 h light:dark cycle with lights on at 0700 h. All studies were carried out in accordance with the Declaration of Helsinki and with the Institute for Laboratory Animal Research of the National Academy of Science *Guide for the Care and Use of Laboratory Animals* (8th edition, revised 2011) and were approved by the Rosalind Franklin University of Medicine and Science Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their pain or discomfort.

Drugs

D-amphetamine (AMPH) sulfate (Sigma, St. Louis, MO, USA) was dissolved in sterile 0.9% saline. Injections were administered at a concentration of 1 mg/kg intraperitoneal (i.p.) and doses refer to the drug base.

Behavioral conditioning

Behavioral conditioning was performed in a three-chambered apparatus (AccuScan Instruments, Inc., Columbus, OH, USA). The two larger, outer chambers (25 × 30 × 32 cm) were separated by a central chamber (10 × 25 × 32 cm) and differed in both visual and tactile cues. One outer chamber had white vertical stripes on the clear walls and the other, white horizontal stripes. The chambers had white floors with different textures. The central chamber had white walls and a Plexiglas floor, and allowed free movement between the two outer chambers unless barred by two white partitions. Infrared sensors along all four sides of the conditioning apparatus allowed for the detection and measurement of the movement and location of the animals within the apparatus.

Conditioning sessions were conducted under conditions of dim illumination and in the presence of white noise. Conditioning occurred according to previously published protocols (Rademacher et al. 2006; 2010; Shen et al. 2006). Approximately 72 h before conditioning, each rat was placed into the center chamber and allowed free access to the entire apparatus for a 15 min pretest. Animals that spent more than 70% of their time in either of the two outer chambers during the pretest were excluded from the experiment. Animals that did not show a strong initial preference for one chamber over the other during the pretest were randomly assigned to one of the three groups. Rats were randomly assigned to receive drug or vehicle in one or the other outer chamber. Approximately 72 h after the pretest, conditioning began and took place over a five-day period (days 1–5). Conditioning sessions lasted for 45 min. Between conditioning sessions, the walls of the place conditioning apparatus were thoroughly washed and the floors of the chambers were replaced. For one group (n=10), AMPH (1.0 mg/kg, i.p.) was administered on days 1, 3, and 5 immediately before placement into one of the two outer chambers. On days 2 and 4, animals were injected with saline (1 ml/kg, i.p.) and immediately confined to the opposite outer chamber

for 45 min. A second group of animals (n=10) received saline injections before being placed into one outer chamber on days 1, 3, and 5 or the other on days 2 and 4. To distinguish the effects of drug-induced learning from the effects of drug exposure alone, a third group of animals, a delayed pairing group (n=10), received AMPH as above, but the time interval between the UCS (i.e., the effects of AMPH) and the CS (i.e., the environmental context) was lengthened to 4 h so that no association could be made between the drug effects and the environmental context. Four h was selected, as this is sufficient for this dose of i.p. AMPH to be largely cleared from the rat brain (Honecker and Coper 1975). Accordingly, on days 1, 3, and 5 rats received AMPH (1.0 mg/kg, i.p.) and were returned to their home cage for 4 h before placement into one of the two outer chambers. On days 2 and 4, animals were injected with saline (1 ml/kg, i.p.) and immediately confined to the opposite outer chamber for 45 min. Approximately 72 h after the last conditioning session, drug- and vehicle-free animals were tested for their preference. For that test, rats were placed into the central chamber and the dividing partitions were removed. Rats were then allowed to explore the entire place conditioning apparatus for 30 min. The time spent in each chamber was recorded and the degree of place preference (i.e., the amount of time spent in the AMPH-paired chamber minus the time spent in the unpaired chamber during the CPP test) was calculated. We have previously demonstrated that this conditioning regime produces motor sensitization and conditioned place preference for animals conditioned with 1.0 mg/kg AMPH and habituation for animals conditioned with saline (Rademacher et al. 2006; 2007; Shen et al. 2006).

Arc immunohistochemistry for light microscopy

One half of the animals that completed the CPP test (n=15, n=5/group) were randomly selected to be used for light microscopic analysis of Arc immunoreactivity in the amygdala and hippocampus. These animals were euthanized 60 min after completing the test session for CPP, since Arc protein levels increase up to 60 min after behavioral conditioning (Ramírez-Amaya et al. 2005), and their brains processed for Arc immunohistochemistry (IHC). All rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.1M phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde (PF) in 0.1M phosphate buffer (PB, pH 7.4) for 20 min. All solutions were made at the same time and the volume of each perfusate was kept uniform. The brains were removed and postfixed in buffered 4% PF for 2.5 h at 4°C, rinsed, and transferred to 30% sucrose in 0.1M PB (pH 7.4) at 4°C until saturation. Coronal sections (35 µm) were cut on a cryostat at -22°C and immunoreacted immediately or stored in cryoprotectant (ethylene glycol and glycerol in 0.1M phosphate buffer) at -20°C until immunohistochemical processing.

For Arc IHC, free-floating sections were rinsed in 0.1M PBS (pH 7.4), blocked in 0.1M PBS containing 5% normal goat serum (NGS, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and 0.5% Triton X-100 (Tx) for 1 h at room temperature, then incubated in 0.1M PBS containing rabbit anti-Arc antibody (1:400, Synaptic Systems GmbH, Goettingen, Germany), 0.5% Tx, and 5% NGS for 48 h at 4°C. Following extensive rinsing, sections were incubated in 0.1M PBS containing biotinylated goat anti-rabbit IgG (1:200, Vector Labs, Burlingame, CA, USA), 0.5% Tx, and 3% NGS for 2 h at room temperature and, again after rinsing, were incubated with avidin-biotin peroxidase complex (Vectastain kit; Vector Labs, Burlingame, CA, USA) for 1 h at room temperature. The sections were reacted with 0.05% 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) containing 0.01% H₂O₂ for 15 min. The sections were mounted onto slides, dried, dehydrated, and topped with coverslips. Three different controls were performed to demonstrate that the rabbit anti-Arc antibody was Arc specific: (1) An Arc blocking peptide (control protein) was mixed with the primary antiserum; (2) The sections were incubated in

normal rabbit IgG; and (3) The primary serum was omitted. Arc staining was absent after all three control procedures (data not shown).

Quantification of Arc immunoreactivity

An observer blind to the treatment groups conducted image capture using a Nikon Eclipse E600 microscope and digital camera (Optronics, Goleta, CA, USA). A rectangular counting frame of the same size (0.1464 mm^2) was placed over each region under investigation to quantify Arc immunoreactivity. Cells and punctate staining were captured at 20X magnification in the amygdala, hippocampus, and the primary auditory cortex (Aud), a control region (Fig. 1). Note that in the hippocampus, ~10% of imaging field contained somata. The BLA and lateral nucleus (LA) of the amygdala, hippocampal CA1 and CA3 fields, and DG were sampled at three different levels in the coronal plane, bregma -2.12 mm (rostral), -2.80 mm (intermediate), and -3.80 mm (caudal) (Paxinos and Watson 1998). The central nucleus (CeA) of the amygdala was sampled at two levels in the coronal plane, bregma -2.12 mm (rostral) and -2.80 mm (intermediate) (Paxinos and Watson 1998). Aud, a control region, was sampled at one level in the coronal plane (-3.80 mm , caudal) (Paxinos and Watson 1998), as no rostral-caudal differences in the amount of Arc immunoreactivity were expected in this brain region. Digital photos were analyzed with ImageJ software for Macintosh (Scion Corp, Frederick, MD, USA). The thickness of each section was estimated using a Nikon E400 microscope equipped with a motorized stage in 3 axes, video camera, and Stereo Investigator software (MBF Bioscience, Williston, VT, USA). Arc immunoreactivity was analyzed according to published methods (Rademacher et al. 2006; 2007) with minor modifications. Briefly, the mean optical density of pixels within a cell/puncta was computed based on a scale of 0 to 256 relative units. Background values were taken from a white matter structure (corpus callosum) and subtracted from the mean optical density of grey level. The image was quantified as an integrated density (ID) score (mean density $\times$ the number of labeled pixels in the region) (Wang and McGinty 1995; Zhou et al. 2004). The optical density for sections at different levels in the coronal plane (BLA, LA, CA1, CA3, and DG, bregma -2.12 , -2.80 , and -3.80 mm ; CeA, bregma -2.12 and -2.80 mm ; Aud, bregma -3.80 mm) was measured and averaged in each rat. Statistical tests were performed on ID data that were pooled for each group ($n=5/\text{group}$).

Electron microscopy and stereology

The half of the animals not used for the Arc light microscopy study that completed the CPP test ($n=15$, $n=5/\text{group}$) were used for the quantification of synapses contacting Arc-labeled and unlabeled (Arc $-$) postsynaptic structures of neurons in the BLA. Procedures to prepare brain tissue for electron microscopy were modified from previous studies (Morshedi et al. 2009; Rademacher et al. 2010). Briefly, since Arc protein levels increase up to 60 min after behavioral conditioning (Ramírez-Amaya et al. 2005), 1 h after the test for CPP, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg , i.p.) and transcardially perfused with 10 ml of 0.1M PBS (pH 7.4), followed by 50 ml of 3.75% acrolein (Polysciences Inc, Warrington, PA) in buffered 2% PF (pH 7.4) and 200 ml of buffered 2% PF. All solutions were made at the same time and the volume of each perfusate was kept uniform. The brains were removed, post-fixed for 30 min in buffered 2% PF at room temperature, and then blocked to include the entirety of the BLA. Coronal sections through the rostrocaudal extent of the BLA (relative to the bregma, anterior-posterior, -1.8 mm to -4.8 mm , Paxinos and Watson 1998) (see Gundersen et al. 1988 for review) were cut coronally at $60 \mu\text{m}$ thickness on a vibratome (The Vibratome Co., St. Louis, MO) and were collected serially in 0.1M PB into numbered 24-well plates. An unbiased stereological approach (i.e., the physical disector) was used to estimate the total number of synapses contacting Arc $+$ and Arc $-$ postsynaptic structures. Systematic random sampling was used to select sections for the physical disector analysis and to estimate the total volume of the BLA (as described in

Mouton 2002). After a random start, every 5th section was selected, yielding a series of 10 equally spaced sections. A second series of 10 equally spaced sections were collected to estimate the total volume of the BLA (V_{ref}) using a modified Cavalieri method (as described in Bothwell et al. 2001). The series of sections selected to estimate the total volume of the BLA (V_{ref}) were adjacent to the series of sections used for the physical disector analysis. Sections selected for physical disector analysis were rinsed in 0.1M PBS (pH 7.4), blocked in 0.1M PBS containing 5% NGS (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and 0.03% Tx for 1 h at room temperature, and incubated in 0.1M PBS containing a rabbit anti-Arc antibody (1:200, Synaptic Systems GmbH, Goettingen, Germany), 0.03% Tx, and 5% NGS for 48 h at 4°C. Following extensive rinsing, sections were incubated in 0.1M PBS containing biotinylated goat anti-rabbit IgG (1:200, Vector Labs, Burlingame, CA, USA) and 3% NGS for 2 h at room temperature and, again after rinsing, were incubated with avidin-biotin peroxidase complex (Vectastain kit; Vector Labs, Burlingame, CA, USA) for 1 h at room temperature. The sections were reacted with 0.05% DAB (Sigma, St. Louis, MO, USA) containing 0.01% H₂O₂ for 15 min. Sections were fixed in 1% osmium tetroxide diluted in 0.1M PB for 30 min at room temperature in the dark, dehydrated, and flat embedded in epoxy resin (comprised of a mixture of EMBED 812, nadic methyl anhydride, dodecenyl succinic anhydride, and benzyldimethylamine; Electron Microscopy Sciences, Hatfield, PA, USA) between two sheets of aclar fluorohalocarbon film (Ted Pella, Inc., Redding, CA, USA). The epoxy resin was allowed to polymerize at 60°C for 72 h. An area of known size (0.5 × 0.5 mm) was selected from the sections, chosen by systematic random sampling (Calverley et al. 1988). Ultrathin serial sections (silver, ~60–70 nm) were cut (~10 per grid; EM UC6; Leica Microsystems Inc., Bannockburn, IL), mounted on formvar-coated copper slot grids (Electron Microscopy Sciences, Hatfield, PA, USA), and stained with uranyl acetate and lead citrate prior to viewing. All electron microscopic images were captured on a JEOL JEM-1230 electron microscope equipped with a Hamamatsu ORCA HR CCD camera. A disector analysis was performed on serial ultrathin sections from each block. Using a systematic sampling method, whereby micrographs are taken at least two widths of the electron microscope screen apart, 15 images of the same region in four adjacent sections of the same thickness were taken and used as the reference and look-up sections (Hunter and Stewart 1993; Ingham et al. 1998; Day et al. 2006). We used 240 disectors for each animal for each treatment group (1,200 total disectors per group). As previously described (Morshedi et al. 2009) and in accord with well recognized criteria (Peters et al. 1991), a synapse was identified by the accumulation of at least three presynaptic vesicles and by the presence of a widened synaptic cleft with parallel, thickened pre- and postsynaptic membranes. Consistent with a previous study (Rademacher et al. 2010) synapses were classified as either SSBs or MSBs. SSBs and MSBs were classified as either asymmetric or symmetric synapses. Asymmetric synapses had postsynaptic densities 2–3 times thicker than the presynaptic densities, whereas the pre- and postsynaptic densities of symmetric synapses were of equal thickness. The associated postsynaptic targets, which were either Arc+ or Arc–, were identified as somata, dendrites, spines, or unclassified as previously described (Morshedi et al. 2009) and in accord with well-established criteria (Peters et al. 1991). Somata were identified by the presence of a nucleus. Dendrites were identified by the presence of structures postsynaptic to the axon terminals, such as mitochondria, microtubules, and, with larger dendrites, rough endoplasmic reticulum. Dendritic spines often contained a spine apparatus and were usually smaller than dendrites, typically lacking mitochondria, microtubules, and rough endoplasmic reticulum. Synapses were determined to be ‘unclassified’ if it was not possible to unambiguously identify the postsynaptic target. A rectangular, unbiased counting frame (32.02 μm^2), containing 2 inclusion and 2 exclusion lines, was centered on each of the micrographs. The area of each of the micrographs was equal to 35.04 μm^2 . Synapses that were within the counting frame or on the inclusion but not the exclusion lines were counted in the reference section only if the same synapse was not found in the look-up section

(referred to as “tops”). Sections were then reversed so that the look-up section served as the reference section. Estimation of the mean synaptic density ($N_{v\text{ syn}}$) (synaptic number per μm^3) was calculated using the formula $N_{v\text{ syn}} = \Sigma Q_{\text{-syn}} / (h \times A)$ adapted from Sterio (Stereo 1984) and de Groot and Bierman (de Groot and Bierman 1986), where $Q_{\text{-syn}}$ = number of synapses present in the reference section but not the look-up section (tops), h = distance (μm) between disector planes (section thickness), and A = sample area (μm^2). Finally, two folds from each section used for analysis were photographed. Section thickness (h) was estimated as half the mean width of the measured folds (De Groot 1988; Small 1968). The following formula was used to estimate the absolute number of synapses (N) onto postsynaptic structures: $N = N_{v\text{ syn}} \times V_{\text{ref}}$. For physical disectors, estimate precision was expressed as the coefficient of error for the group. The series of 10 equally spaced sections collected to estimate the total volume of the BLA (V_{ref}) was mounted onto poly-L-lysine-coated slides from deionized water and dried at 60°C overnight. Sections were then stained with cresyl violet, dehydrated in an ascending series of alcohols, cleared in xylene, and coverslipped. V_{ref} was estimated using a Nikon E400 microscope equipped with a motorized stage in 3 axes, video camera, and Stereo Investigator software (MBF Bioscience, Williston, VT, USA). Using the Stereo Investigator software, the BLA was outlined for each section. The following formula was then used to determine the reference volume (V_{ref}) (μm^3): $V_{\text{ref}} = a \times t \times s$ where a = mean area (μm^2) of the BLA, t = mean thickness (μm) of the Vibratome sections, and s = total number of sections through the BLA (~50).

Statistical analysis

Locomotor data were analyzed by two-way ANOVA [behavioral conditioning (saline, delayed pairing, AMPH CPP) $\times$ conditioning day [days 1–5]] followed by *post hoc* Bonferroni's multiple comparison tests. Changes in locomotor activity (day 5 - day 1) were analyzed with one-sample *t*-tests with a theoretical mean equal to 0. The linear relationship between synaptic variables and CPP scores were determined by calculating the Pearson correlation coefficient (*r*). CPP, synaptic, and Arc ID data were analyzed with one-way ANOVA [behavioral conditioning (saline, delayed pairing, AMPH CPP)] with *post hoc* Student-Newman-Keuls (SNK) tests. Synaptic connectivity data were analyzed by *z*-tests with Yates correction. Prior to statistical analysis, the normality of distribution was determined with Kolmogorov and Smirnov test; equality of variance was tested using Barlett's test. The α level was set at 0.05. All data is represented as mean $\pm$ *S.E.M.*

Results

Locomotor behavior and conditioned place preference

Repeated administration of AMPH (1.0 mg/kg, i.p.) resulted in a stepwise increase in locomotor activity (main effect of behavioral conditioning, $P < 0.0001$, main effect of conditioning day, $P < 0.0001$, and a behavioral conditioning $\times$ conditioning day interaction, $P < 0.0001$; Fig. 2a). The number of infrared beam breaks was greater for animals conditioned with AMPH compared to the delayed pairing and saline-treated control groups on conditioning days 1 ($P < 0.001$), 3 ($P < 0.001$), and 5 ($P < 0.001$; Fig. 2a). For animals conditioned with AMPH, the number of beam breaks was greater on day 5 than on day 1 ($P < 0.05$; Fig. 2a). Whereas repeated AMPH administration produced motor sensitization ($P < 0.05$; Fig. 2b), repeated saline administration and repeated AMPH administration with a long (4 h) delay in pairing (delayed pairing) produced habituation of the motor response ($P < 0.05$; Fig. 2b). Animals conditioned with AMPH spent more time during the CPP test in the AMPH-paired than the unpaired chamber ($P < 0.05$; Fig. 2c). Neither a long (4 h) delay in pairing (delayed pairing) after AMPH ($P > 0.05$) nor treatment with saline alone ($P > 0.05$) resulted in preference for one chamber over the other (Fig. 2c).

Arc immunoreactivity

Qualitative analysis of Arc immunoreactive cells indicated that Arc expression was greater for rats that exhibited AMPH CPP compared to saline-treated or delayed pairing control rats (compare Fig. 3c to Fig. 3a and b; compare Fig. 3f to Fig. 3d and e). Quantitative analyses revealed a significant effect of behavioral conditioning with AMPH on Arc ID in all regions of the hippocampus and amygdala but not the Aud, a control region (Fig. 4). Specifically, behavioral conditioning with AMPH increased Arc ID in the DG ($P < 0.05$ versus saline and delayed pairing), CA1 field ($P < 0.01$ versus saline and delayed pairing), CA3 field ($P < 0.01$ versus saline and delayed pairing), CeA ($P < 0.01$ versus saline and delayed pairing), BLA ($P < 0.01$ versus saline and delayed pairing), and LA ($P < 0.01$ versus saline and delayed pairing). Behavioral conditioning with AMPH had no effect on Arc ID in the Aud ($P > 0.05$; Fig. 4). We also investigated whether Arc ID is related to the 'degree of AMPH CPP'. To do so, the amount of time spent in the AMPH-paired chamber during the CPP test was correlated with Arc ID. Pearson's r tests indicated significant positive relationships between the time spent in the AMPH-paired chamber during the CPP test and Arc immunoreactivity for all regions of the hippocampus and amygdala except the LA and the Aud, the control region (Fig. 5).

SSBs in the BLA: asymmetric synapses contacting Arc+ and Arc- postsynaptic structures

Since Arc protein levels increase up to 1 h after behavioral conditioning (Ramírez-Amaya et al. 2005), 1 h after the completion of the CPP test, the number of synapses that contacted Arc+ and Arc- postsynaptic structures of BLA neurons were quantified stereologically at the electron microscopic level by using a physical disector design. As previously described, synapse were classified as either SSBs or MSBs. SSBs and MSBs were classified as either asymmetric (Gray Type I, presumed excitatory) or symmetric (Gray Type II, presumed inhibitory or modulatory) synapses. There was no difference in the BLA volume between the saline, delayed pairing, and AMPH CPP groups 1 h after the CPP test ($P > 0.05$). With regard to SSBs, 295.1 ± 17.7 asymmetric (presumed excitatory) synapses per rat were analyzed. The number and density of asymmetric synapses in the BLA of each group were significantly increased for the AMPH CPP group compared to the saline and delayed pairing control groups (number, $P < 0.01$; Fig. 6e; density, $P < 0.01$; Table 1). There was no difference between the saline and delayed pairing groups in the number and density of asymmetric synapses ($P > 0.05$; Fig. 6e; Table 1). The number and density of excitatory synapses was related to their postsynaptic target (i.e., spines, dendrites, or unclassified). Note that synapses were determined to be 'unclassified' if it was not possible to unambiguously identify the postsynaptic target. We analyzed 235.1 ± 14.1 asymmetric synapses per rat contacting spines, 31.6 ± 4.4 asymmetric synapse per rat contacting dendrites, and 27.3 ± 3.5 asymmetric synapses per rat contacting unclassified postsynaptic structures. There was no difference between the saline and delayed pairing groups in the number and density of asymmetric axospinous ($P > 0.05$), axodendritic ($P > 0.05$), or unclassified ($P > 0.05$) synapses (Fig. 6e, Table 1). In contrast, the number and density of asymmetric axospinous, axodendritic, and unclassified synapses was significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups (number of asymmetric axospinous synapses, $P < 0.05$; number of asymmetric axodendritic synapses, $P < 0.05$; number of asymmetric unclassified synapses, $P < 0.05$; Fig. 6e; density of asymmetric axospinous synapses, $P < 0.05$; density of asymmetric axodendritic synapses, $P < 0.05$; density of asymmetric unclassified synapses, $P < 0.05$; Table 1).

Asymmetric synapses were subclassified based on the presence or absence of Arc immunoreactivity in the postsynaptic structures (i.e., spines, dendrites, or unclassified). We analyzed 136.5 ± 8.7 asymmetric synapses per rat contacting Arc+ postsynaptic structures. The number and density of asymmetric (presumed excitatory) synapses contacting Arc+

postsynaptic structures of neurons in the BLA of each group were significantly increased for the AMPH CPP group compared to the saline and delayed pairing control groups (number, $P < 0.01$; Fig. 6f; density, $P < 0.01$; Table 2). There was no difference between the saline and delayed pairing groups in the number and density of asymmetric synapses contacting Arc+ postsynaptic structures in the BLA ($P > 0.05$; Fig. 6f; Table 2). We analyzed 98.5 ± 6.5 asymmetric synapses per rat contacting Arc+ spines, 22.3 ± 2.8 asymmetric synapses per rat contacting Arc+ dendrites, and 15.5 ± 2.1 asymmetric synapses per rat contacting Arc+ unclassified postsynaptic structures. There was no difference between the saline and delayed pairing groups in the number and density of asymmetric synapses contacting Arc+ spines ($P > 0.05$), dendrites ($P > 0.05$), or unclassified postsynaptic structures ($P > 0.05$; Fig. 6f; Table 2). In contrast, the number and density of asymmetric synapses contacting Arc+ spines and dendrites was significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups (number of asymmetric synapses contacting Arc+ spines, $P < 0.05$; number of asymmetric synapses contacting Arc+ dendrites, $P < 0.05$; Fig. 6f; density of asymmetric synapses contacting Arc+ spines, $P < 0.05$; density of asymmetric synapses contacting Arc+ dendrites, $P < 0.05$; Table 2). The total number and density of asymmetric synapses contacting Arc+ unclassified postsynaptic structures was significantly increased in the AMPH CPP group compared with the delayed pairing group ($P < 0.05$) but not the saline group ($P > 0.05$; Fig. 6f). The density of asymmetric synapses contacting Arc+ unclassified postsynaptic structures was significantly increased in the AMPH CPP group compared with the delayed pairing group ($P < 0.001$) and the saline group ($P < 0.001$). Moreover, the density of asymmetric synapses contacting Arc+ unclassified postsynaptic structures was increased in the delayed pairing group compared with the saline group ($P < 0.001$; Table 2).

SSBs in the BLA: symmetric synapses contacting Arc+ and Arc– postsynaptic structures

With regard to SSBs, we analyzed 52.3 ± 3.9 symmetric (Gray Type II, presumed inhibitory or modulatory) synapses per rat. The number and density of symmetric synapses were significantly increased for the AMPH CPP group compared to the saline and delayed pairing control groups (number, $P < 0.01$; Fig. 7e; density, $P < 0.01$; Table 1). There was no difference between the saline and delayed pairing groups in the number and density of symmetric synapses ($P > 0.05$; Fig. 7e; Table 1). The number of inhibitory or modulatory synapses was related to their postsynaptic target (i.e., spines, dendrites, or somata). We analyzed 5.3 ± 0.6 symmetric synapses per rat where it was not possible to unambiguously identify the postsynaptic target (i.e., unclassified synapses), 5.2 ± 0.6 symmetric synapses per rat contacting spines, 40.3 ± 3.5 symmetric synapses per rat contacting dendrites, and 1.8 ± 0.4 symmetric synapses per rat contacting somata. No differences between the three groups were detected in the number and density of symmetric axosomatic synapses and those symmetric synapses where it was not possible to unambiguously identify the postsynaptic target (i.e., unclassified synapses) (number of symmetric axosomatic, $P > 0.05$; number of symmetric unclassified, $P > 0.05$; Fig. 6e; density of symmetric axosomatic, $P > 0.05$; density of symmetric unclassified, $P > 0.05$; Table 1). There was no difference between the saline and delayed pairing groups in the number and density of symmetric axospinous ($P > 0.05$) or axodendritic ($P > 0.05$) synapses. In contrast, the total number and density of symmetric axodendritic synapses was significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups (number of symmetric axodendritic synapses, $P < 0.01$; Fig. 7e; density of symmetric axodendritic synapses, $P < 0.01$; Table 1). The number and density of symmetric axospinous synapses was greater for animals in the AMPH CPP group compared to those conditioned with saline ($P < 0.05$) but not the delayed pairing controls ($P > 0.05$; Fig. 7e; Table 1).

Symmetric synapses were subclassified based on the presence or absence of Arc immunoreactivity in postsynaptic structures (i.e., spines, dendrites, somata, or unclassified).

We analyzed 2.3 ± 0.4 symmetric synapses per rat contacting Arc+ spines, 29.7 ± 2.5 symmetric synapses per rat contacting Arc+ dendrites, 1.8 ± 0.4 symmetric synapses per rat contacting Arc+ somata, and 3.1 ± 0.6 symmetric synapses per rat contacting Arc+ unclassified postsynaptic structures. The number and density of symmetric (presumed inhibitory or modulatory) synapses contacting Arc+ postsynaptic structures of neurons in the BLA of each group were significantly increased for the AMPH CPP group compared to the saline and delayed pairing control groups (number, $P < 0.01$; Fig. 7f; density, $P < 0.01$; Table 2). The number and density of symmetric synapses contacting Arc+ dendrites of neurons in the BLA of each group were significantly increased for the AMPH CPP group compared to the saline and delayed pairing control groups (number, $P < 0.01$; Fig. 7f; density, $P < 0.01$; Table 2). In contrast, no difference between the three groups was detected in the total number and density of symmetric synapses contacting Arc+ spines, somata, and unclassified postsynaptic structures (number of symmetric synapses contacting Arc+ spines, $P = 0.09$; number of symmetric synapses contacting Arc+ somata, $P > 0.05$; number of symmetric synapses contacting Arc+ unclassified postsynaptic structures, $P > 0.05$; Fig. 7f; density of symmetric synapses contacting Arc+ spines, $P = 0.07$; density of symmetric synapses contacting Arc+ somata, $P > 0.05$; density of symmetric synapses contacting Arc+ unclassified postsynaptic structures, $P > 0.05$; Table 2). There was no difference between the saline and delayed pairing groups in the number and density of symmetric synapses contacting Arc+ dendrites in the BLA ($P > 0.05$; Fig. 7f; Table 2).

MSBs contacting Arc+ and Arc− postsynaptic structures of BLA neurons

We analyzed 5.9 ± 0.8 MSBs per rat. The MSBs were never symmetric. There was evidence for remodeling, as the total number and density of MSBs was significantly increased in the AMPH CPP group compared to the delayed pairing ($P < 0.01$) and saline controls ($P < 0.01$; Fig. 8c; Table 1). There was no difference between the saline and delayed pairing groups in the number or density of MSBs ($P > 0.05$). MSBs were subclassified based on the presence or absence of Arc immunoreactivity in postsynaptic structures (i.e., spines or dendrites). We analyzed 2.7 ± 0.4 MSBs per rat contacting Arc+ postsynaptic structures. The number and density of MSBs contacting Arc+ postsynaptic structures of neurons in the BLA were significantly increased for the AMPH CPP group compared to the saline and delayed pairing control groups (number, $P < 0.01$; Fig. 8c; density, $P < 0.05$; Table 2).

Map of synapses activated by the retrieval of contextual memories

The distribution of asymmetric, symmetric, and MSB inputs contacting BLA neurons can be compared to a 'map' of recently activated asymmetric, symmetric, and MSB inputs contacting BLA neurons produced by the retrieval of contextual memories. To do so, we first determined whether there were between groups differences in the proportion of the total number of synapses of each type and in the proportion of the total number synapses of each type contacting Arc+ postsynaptic structures. No differences between the three groups were detected in the proportion of the total number of synapses classified as asymmetric ($P > 0.05$), symmetric ($P > 0.05$), or MSBs ($P > 0.05$). Similarly, no differences between the three groups were detected in the proportion of the total number of synapses classified as asymmetric ($P > 0.05$), symmetric ($P > 0.05$), or MSBs ($P > 0.05$) that contacted Arc+ postsynaptic structures. Because between groups differences were not detected, the data were combined across the groups (saline, delayed pairing, AMPH CPP). Significant differences were found between the proportion of the total number of synapses classified as asymmetric (0.8354 ± 0.0077) versus the proportion of the total number of synapses classified as asymmetric that contacted Arc+ postsynaptic structures (0.3882 ± 0.0144 , $P < 0.05$; Fig. 9a). No differences were detected in the proportion of the total number of synapses classified as symmetric (0.1471 ± 0.0076) versus the proportion of the total number of synapses classified as symmetric that contacted Arc+ postsynaptic structures.

(0.1040 ± 0.0056 , $P > 0.05$; Fig. 9a), and in the proportion of the total number of synapses classified as MSBs (0.0175 ± 0.0014) versus the proportion of the total number of synapses classified as MSBs that contacted Arc+ postsynaptic structures (0.0081 ± 0.0011 , $P > 0.05$; Fig. 9a). Thus, there was a significant difference in the proportion of excitatory but not inhibitory/modulatory or MSB synapses activated by the retrieval of contextual memories versus the proportion of the total synapses in the BLA classified as excitatory, inhibitory/modulatory, or MSBs.

Next we compared the proportion of asymmetric synapses that contacted spines, dendrites, or unknown postsynaptic structures (unclassified synapses) to the proportion of asymmetric synapses activated by the retrieval of contextual memories that contacted spines, dendrites, or unknown postsynaptic structures (unclassified synapses). No differences between the three groups were detected in the proportion of the total number of asymmetric synapses that contacted spines ($P > 0.05$), dendrites ($P > 0.05$), or unclassified postsynaptic structures ($P > 0.05$). No differences between the three groups was detected in the proportion of the total number of asymmetric synapses that contacted Arc+ spines ($P > 0.05$), Arc+ dendrites ($P > 0.05$), or Arc+ unclassified postsynaptic structures ($P > 0.05$). Because no differences between groups were detected, we combined data across the groups (saline, delayed pairing, AMPH CPP). Significant differences were found between the proportion of the total number of synapses classified as asymmetric axospinous (0.8027 ± 0.0134) versus the proportion of the total number of synapses classified as asymmetric that contacted Arc+ spines (0.3376 ± 0.0154 , $P < 0.05$; Fig. 9b). No difference was detected in the proportion of the total number of synapses classified as asymmetric axodendritic (0.0985 ± 0.0070) versus the proportion of the total number of synapses classified as asymmetric that contacted Arc+ dendrites (0.0692 ± 0.0050 , $P > 0.05$; Fig. 9b), and in the proportion of the total number of asymmetric synapses that could not be classified (0.0988 ± 0.0115) versus the proportion of the total number of asymmetric synapses that could not be classified yet contacted Arc+ postsynaptic structures (0.0572 ± 0.0078 , $P > 0.05$; Fig. 9b).

The distribution of inhibitory/modulatory inputs contacting spines, dendrites, or unknown postsynaptic structures (unclassified synapses) was compared to the distribution of inhibitory/modulatory inputs contacting spines, dendrites or unknown postsynaptic structures (unclassified synapses) produced by the retrieval of contextual memories. No differences between the three groups were detected in the proportion of the total number of symmetric synapses that contacted spines ($P > 0.05$), dendrites ($P > 0.05$), somata ($P > 0.05$), or unclassified postsynaptic structures ($P > 0.05$). No differences between the three groups was detected in the proportion of the total number of symmetric synapses that contacted Arc+ spines ($P > 0.05$), Arc+ dendrites ($P > 0.05$), Arc+ somata ($P > 0.05$), or Arc + unclassified postsynaptic structures ($P > 0.05$). Because no between groups differences were detected, we combined data across the groups (saline, delayed pairing, AMPH CPP). No difference was detected between the proportion of the total number of synapses classified as symmetric axospinous (0.0964 ± 0.0092) versus the proportion of the total number of synapses classified as symmetric that contacted Arc+ spines (0.0416 ± 0.0050 , $P > 0.05$; Fig. 9c), the proportion of the total number of synapses classified as symmetric axodendritic (0.7655 ± 0.0197) versus the proportion of the total number of synapses classified as symmetric that contacted Arc+ dendrites (0.5754 ± 0.0281 , $P > 0.05$; Fig. 9c), the proportion of the total number of symmetric synapses that could not be classified (0.1111 ± 0.0135) versus the proportion of the total number of symmetric synapses that could not be classified yet contacted Arc+ postsynaptic structures (0.0677 ± 0.0119 , $P > 0.05$; Fig. 9c), and the proportion of the total number of synapses classified as symmetric axosomatic (0.0368 ± 0.0082) versus the proportion of the total number of synapses classified as symmetric that contacted Arc+ somata (0.0368 ± 0.0082 , $P > 0.05$; Fig. 9c).

Discussion

This is the first study to demonstrate that conditioned reward, in the form of AMPH CPP, alters the number and density of symmetric (Gray Type II, presumed inhibitory or modulatory) synapses contacting BLA neurons. Moreover, the stereological quantification of synapses contacting Arc+ postsynaptic structures allowed for the creation of the first ever ‘map’ of recently activated synapses. Our earlier work showed that AMPH CPP increased the number of SSBs and MSBs (Rademacher et al., 2010). With regard to the SSBs, AMPH increased the total number of asymmetric (Gray Type I, presumably excitatory) synapses contacting spines and dendrites of BLA neurons (Rademacher et al. 2010). The present stereological analysis fully supports those findings but also augments them by providing evidence for increases in asymmetric (Gray Type I, presumably excitatory) and symmetric (Gray Type II, presumably inhibitory or modulatory) inputs contacting Arc+ and Arc− postsynaptic structures.

Consistent with our previous studies (Rademacher et al. 2006; 2010; Shen et al. 2006), behavioral conditioning with AMPH (1.0 mg/kg, i.p.) induced a robust CPP 72 hours after conditioning. Moreover, repeated AMPH administration produced motor sensitization, and both repeated saline administration and repeated AMPH administration with a long (4 hour) delay in pairing (delayed pairing) produced habituation of the motor response (Rademacher et al. 2007). Hearing and colleagues (2010) reported that re-exposure of animals to a drug-associated context increased Arc mRNA in the BLA and hippocampal regions DG, CA1, and CA3, suggesting a role for Arc in the retrieval and/or reconsolidation of long-term memories. Similarly, in the present study, re-exposure of animals to the CPP apparatus during the CPP test, which presumably would cause the retrieval of context-drug and context-saline memories, increased the Arc ID in the DG, CA1, and CA3 regions of the hippocampus and BLA, LA, CeA nuclei of the amygdala. Due to the inclusion of both saline-treated and delayed pairing control groups, the increase in Arc immunoreactivity in the hippocampus and amygdala was not due to either exposure to AMPH or to a previously experienced context, both of which induce Arc (Tan et al. 2000; Zhang et al. 2005).

The hippocampus and amygdala are thought to subserve different roles in the associative learning that underlies CPP. During place conditioning, it is thought that the formation of associations between discrete CS and the drug UCS occur in the BLA (Everitt et al. 1991; Ito et al. 2006) whereas spatial context is represented in the hippocampus (Ito et al. 2006). Spatial context is thought to be result of the integration of elemental, discrete CS (Holland and Bouton 1999; Jeffery et al. 2004; Rudy and Sutherland 1995) as well as place and movement-related CS (Ito et al. 2006). Exposure to both drug-paired and saline-paired contexts, which occurs during the test for place conditioning, produces synaptogenesis (Rademacher et al. 2006; 2010) and increases Arc immunoreactivity in the hippocampus and amygdala, as shown in the current study. Arc is increased when animals are returned to a previously experienced context (Zhang et al. 1998), following a change in context (Guzowski et al. 1999), and by acute psychostimulant administration (Tan et al. 2000). Additionally, there is greater Arc induction in the CA3 and CA1 fields of the hippocampus and the BLA, LA, and CeA subregions of the amygdala in animals exposed to a footshock-paired context compared to a neutral context (Zhang et al. 2005). Exposure to a nicotine-paired context in the absence of nicotine elevates Arc mRNA in several brain regions including the amygdala (Schiltz et al. 2005). Exposure to the drug-paired context leads to retrieval and reactivation of contextual CS-drug UCS memories. When a stabilized memory is recalled or reactivated, it becomes sensitive to disruption, its maintenance requires protein synthesis, and it has been proposed that these reactivated memories must be reconsolidated to be preserved (see Dudai and Eisenberg 2004 for review). Although it is not yet clear whether reconsolidation is simply a repetition of consolidation or, instead, reflects a distinct

process, Arc null mice show impairments in the consolidation of long-term amygdalar- and hippocampal-dependent memories (Plath et al. 2006). We hypothesize that Arc plays a role in the consolidation or reconsolidation contextual CS-drug UCS memories following retrieval; additional studies are required to test this hypothesis.

The strong positive correlation observed between the degree of Arc expression in the hippocampus and spatial learning performance in the Morris water maze led Guzowski and colleagues (2001) to hypothesize that the positive correlation between the degree of Arc expression and the degree of learning reflects the involvement of a specific brain region in a specific task. This hypothesis was supported by the finding that the degree of Arc expression in the dorsomedial but not dorsolateral striatum was positively correlated with learning performance on a reversal task (Daberkow et al. 2007). The positive correlations between the amount of Arc ID in the hippocampal subregions DG, CA1, and CA3 and amygdalar subregions BLA and CeA provides additional support for this hypothesis and is consistent with the notion that both the hippocampus and amygdala play an important role in contextual learning, such as CPP (Helmstetter and Bellgowan 1994; Hsu et al. 2002; Muller et al. 1997; Olmstead and Franklin 1997; Ferbinteanu and McDonald 2001; Shen et al. 2006). The BLA, however, was the only brain region in which the immunoreactivity for the immediate early gene protein product, Fos, was positively correlated with the degree of AMPH CPP (Rademacher et al. 2006). The BLA was also one of two brain regions in which the amount of immunoreactivity for the presynaptic structural plasticity marker, synaptophysin, was strongly correlated with the degree of AMPH CPP (Rademacher et al. 2006), and the brain region in which the strongest positive correlation was observed between the amount of Arc ID and degree of AMPH CPP [present results]. Moreover, strong positive correlations were observed between the number of excitatory inputs contacting BLA neurons and the degree of AMPH CPP and the frequency of spontaneous synaptic events recorded from BLA neurons *in vivo* and the degree of AMPH CPP (Rademacher et al. 2010). Based on these results, we speculate that the BLA is a site in which structural and functional synaptic plasticity changes occur in order to 'drive' approach behavior to a context previously paired with drug.

We hypothesize that the learning-induced changes in BLA synaptic connectivity reported herein 'drives' approach behavior to a drug-associated context. Consistent with our previous study (Rademacher et al. 2010), context-drug learning increased the number and density of MSBs and SSBs. All of the MSBs were asymmetric (Gray Type I, presumably excitatory). With regard to the SSBs, context-drug learning increased the number of asymmetric axospinous and axodendritic synapses contacting BLA neurons. Thus, this type of appetitive associative learning resulted in an increase in the formation of new synapses and the remodeling of existing synapses. In addition, context-drug learning increased the number and density of symmetric (Gray Type II, presumed inhibitory or modulatory) synapses contacting BLA neurons. The BLA contains glutamatergic pyramidal neurons (~85% of all neurons) and gamma-aminobutyric acid (GABA) interneurons (~15% of all neurons), which are local circuit cells (McDonald and Betette 2001; Muller et al. 2005; 2007). The GABA interneurons, just as in the cerebral cortex, provide a dense innervation of the perisomatic compartment of the pyramidal neurons (McDonald and Mascagni 2001). Such proximal synapses place these inhibitory neurons in an ideal position to regulate BLA outflow and participate in feed-forward inhibition of the pyramidal cells (Woodruff et al. 2006). In addition, these interneurons have gap junctions between their dendrites (Muller et al. 2005), which increases the probability that excitatory drive will be robust due to their intercellular coupling (O'Donnell and Grace 1997). Because the vast majority of spines are found on pyramidal projection neurons (Brinley-Reed et al. 1995) and asymmetric specializations are the ultrastructural hallmark of glutamatergic synapses, the learning-induced increase in the number of asymmetric axospinous synapses likely resulted in increased excitatory synaptic

'drive' of BLA pyramidal neurons. However, because cell-type specific markers were not used in the present study, it is difficult to speculate about the functional consequences within the BLA circuitry of the learning-induced changes in the other types of synaptic specializations.

We hypothesize that synaptic activation induced by the retrieval of contextual memories leads to approach to the drug-associated context. The use of Arc immunoelectron microscopy with a physical disector design allowed for the quantification of recently activated synapses (i.e., synapses contacting Arc+ postsynaptic structures). Moreover, the evaluation of the pattern of Arc immunoreactivity at the ultrastructural level to provide a 'map' of recently activated synapses is analogous to the evaluation of Fos to provide a 'map' of activated neurons (Dragunow et al. 1987). For animals conditioned with AMPH, re-exposure to the CPP apparatus increased the number and density of recently activated, remodeled synapses (i.e., MSBs contacting Arc+ postsynaptic structures), recently activated asymmetric axospinous and axodendritic synapses (SSBs), and recently activated symmetric axodendritic synapses (SSBs). Interestingly, the 'map' of recently activated synapses did not differ between groups. For instance, the number of MSBs activated by re-exposure to the CPP apparatus during the CPP test accounted for ~0.8% of the total synapses in the BLA. The number of excitatory and inhibitory/modulatory synapses activated by re-exposure to the CPP apparatus during the CPP test accounted for ~39% and ~10% of the total synapses in the BLA, respectively. Thus, for animals conditioned with AMPH, the retrieval of context-drug and -vehicle memories activated a greater number rather than a greater proportion of remodeled (MSBs), excitatory (SSBs), and inhibitory/modulatory (SSBs) synapses which, in turn, promoted approach behavior to the AMPH-associated context. These findings indicate that it is the degree rather than the pattern of synaptic activation in the BLA that is important for 'driving' approach behavior to a context paired with drug.

There are several caveats to the present study. First, according to the functional Gray synapses hypothesis, asymmetric (Gray Type I) synapses are excitatory and symmetric (Gray Type II) synapses are inhibitory. In support of this hypothesis, immunoreactivity for the primary excitatory neurotransmitter in the CNS, glutamate, and the primary inhibitory neurotransmitter in the CNS, GABA, has been observed in axon terminals that form asymmetric and symmetric synapses, respectively (Beaulieu and Somogyi 1990; Kozell and Meshul 2001; 2004; Somogyi and Sotlész 1986). Contrary to this hypothesis, GABA immunoreactivity has been observed in axon terminals that form asymmetric synapses (Pinard et al. 1991) and glutamate immunoreactivity has been observed in axon terminals that form symmetric synapses (van den Pol 1991). Importantly, BLA neurons receive substantial inputs from neuromodulatory systems including dopaminergic, cholinergic, and noradrenergic systems. Since the axon terminals of these neuromodulatory inputs form symmetric synapses with BLA neurons (e.g., Carlsen and Heimer 1986; Li et al. 2001; Muller et al. 2009; Pinard et al. 2008) we refer to symmetric synapses as inhibitory or modulatory. Thus, it seems difficult to ascribe a function to a synapse based solely on its ultrastructural characteristics. Second, the immunoreactivity for Arc in the larger postsynaptic structures such as dendritic shafts and somata, which together comprise ~18% of the total number of synapses contacting Arc+ postsynaptic structures, is diffuse and, therefore, may not be related to the activity of any particular axon terminal. Thus, in these particular cases, Arc may not be a specific marker of recently activated synapses. Third, it is possible that the learning-induced alterations in the landscape of synapses contacting BLA neurons might be unique to the developmental stage of the rats. Specifically, the changes reported herein may be observed during adolescence but not adulthood (greater than postnatal day 63 in the rat; McCutcheon and Marinelli 2009) since the 'plastic capacity of the brain' may be higher during adolescence than adulthood (e.g., Kolb and Teskey 2012).

Further studies are required to determine the relationship between the degree of structural synaptic plastic changes and age in the CPP paradigm.

Arc has been characterized as a ‘master regulator’ of synaptic plasticity (see Shepherd and Bear 2011 for review) that integrates information from multiple intracellular signaling cascades (Pitchovski et al. 2009; Teber et al. 2004; Waung et al. 2008) and, in turn, regulates cytoskeletal proteins involved in synaptic remodeling (Fujimoto et al. 2004; Mokin et al. 2006). To our knowledge, this is the first report of the use of Arc immunoelectron microscopy combined with the physical disector approach to create a ‘synaptic map’ underlying implicit long-term memories. The findings reported herein raise the interesting possibility that the blockade of Arc induction in the BLA, due to the retrieval of long-term implicit memories, may reduce the impact of drug-paired environmental stimuli to elicit craving and relapse.

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Fig. 1.

The areas analyzed for Arc immunoreactivity (indicated by the shaded boxes). Stereotaxic maps were reproduced, with permission from Elsevier Science, from a brain atlas (Paxinos & Watson, 1998). Abbreviations: DG, dentate gyrus; CA1, cornu ammonis 1; CA3, cornu ammonis 3; CeA, central nucleus of the amygdala; BLA, basolateral nucleus of the amygdala; LA, lateral nucleus of the amygdala; Aud, primary auditory cortex

Fig. 2.

Effects of behavioral conditioning on locomotor behavior and CPP. **a**, Repeated administration of AMPH (1.0 mg/kg, i.p.) to rats resulted in a stepwise increase in locomotor activity. The number of infrared beam breaks was greater for animals conditioned with AMPH compared to the delayed pairing and saline-treated control groups on conditioning days 1, 3, and 5. For animals conditioned with AMPH, the number of beam breaks was greater on day 5 than on day 1. *** $P < 0.001$ versus delayed pairing and saline groups, # $P < 0.05$ versus AMPH CPP animals on day 1. **b**, Whereas repeated AMPH administration produced motor sensitization, repeated saline administration and repeated AMPH administration with a long (4 h) delay in pairing (delayed pairing) produced habituation of the motor response. * $P < 0.05$ versus a theoretical mean equal to zero. **c**, Animals conditioned with AMPH spent more time during the CPP test in the AMPH-paired than the unpaired chamber. Neither a long (4 h) delay in pairing (delayed pairing) after AMPH nor behavioral conditioning with saline alone resulted in preference for one chamber over the other. Each bar in the graph represents a mean of 10 rats; vertical lines represent *S.E.M.* Significantly different from the delayed pairing and saline groups, * $P < 0.05$

Fig. 3. Brightfield photomicrographs depicting the effect of AMPH conditioning on Arc immunoreactivity in the CA3 field and the BLA. Arc immunoreactivity in the CA3 field **a**, after behavioral conditioning with saline; **b**, after delayed pairing with 1.0 mg/kg AMPH; and **c**, after behavioral conditioning with 1.0 mg/kg AMPH. Arc immunoreactivity in the BLA **d**, after behavioral conditioning with saline; **e**, after delayed pairing with 1.0 mg/kg AMPH; and **f**, after behavioral conditioning with 1.0 mg/kg AMPH. The scale bar in **f** is valid for **a–f** and equals 50 μm

Fig. 4.

Effects of the retrieval of contextual memories on Arc ID in hippocampal and amygdalar subregions and in the primary auditory cortex. Each bar represents a mean of 5 rats; vertical lines represent *S.E.M.* Significantly different from the delayed pairing and saline-treated groups, * $P < 0.05$; ** $P < 0.01$

Fig. 5.

The relationship between the amount of Arc ID and the degree of CPP for all brain regions investigated. There was a significant positive correlation between Arc ID and degree of CPP for the DG, CA1, CA3, CeA, and BLA but not the LA and the Aud

Fig. 6.

Effect of AMPH CPP and the retrieval of contextual memories on the number of SSBs that form excitatory contacts with BLA neurons. **a**, An electron micrograph depicting an example of an asymmetric synapse contacting the head of a large Arc- spine of a BLA neuron. **b**, An electron micrograph depicting an example of an asymmetric synapse contacting the head of an Arc+ spine of a BLA neuron. The parent dendrite is also Arc+. **c**, An electron micrograph depicting an asymmetric synapse contacting an Arc- dendrite of a BLA neuron. **d**, An electron micrograph depicting an Arc+ dendrite of a BLA neuron. This Arc+ dendrite is contacted by two asymmetric synapses. **e**, The total number of asymmetric synapses and the number of asymmetric axospinous, axodendritic, and unclassified synapses were greater for animals in the AMPH CPP group compared to animals in the delayed pairing and saline groups. There were no differences in the total number of asymmetric synapses and the number of asymmetric axospinous, axodendritic, and unclassified synapses for rats in the delayed pairing group compared to the saline group. Data are expressed as the mean $\pm$ S.E.M. ** $P < 0.01$, * $P < 0.05$ versus the delayed pairing and saline groups. **f**, The number of asymmetric synapses contacting Arc+ postsynaptic structures, spines and dendrites was greater for animals in the AMPH CPP group compared to animals in the delayed pairing and saline groups. The number of asymmetric synapses contacting unclassified postsynaptic structures was greater for rats in the AMPH CPP group compared

to rats in the delayed pairing group. There was no difference in the number of asymmetric synapses contacting Arc+ postsynaptic structures for rats in the delayed pairing group compared to the saline group. Data are expressed as the mean $\pm$ *S.E.M.* ** $P < 0.01$, * $P < 0.05$ versus the delayed pairing and saline groups; # $P < 0.05$ versus the delayed pairing group. Abbreviations: as, asymmetric synapse; sp-, unlabeled spine; sp+, Arc-labeled spine; d-, unlabeled dendrite; d+, Arc-labeled dendrite; Total, total number of synapses; Spin, axospinous; Dend; axodendritic; Som, axosomatic; Unclass; unclassified. The scale bar in **d** is valid for **a–d** and equals 250 nm

Fig. 7.

Effect of AMPH CPP and the retrieval of contextual memories on the number of SSBs that form inhibitory or modulatory contacts with BLA neurons. **a**, An electron micrograph depicting a symmetric synapse contacting an Arc- dendrite of a BLA neuron. **b**, An electron micrograph depicting a symmetric synapse contacting an Arc+ dendrite of a BLA neuron. **c**, An electron micrograph depicting a symmetric synapse contacting an Arc- soma of a BLA neuron. **d**, An electron micrograph depicting a symmetric synapse contacting an Arc+ soma of a BLA neuron. **e**, The total number of symmetric synapses and the number of symmetric axodendritic synapses were greater for animals in the AMPH CPP group compared to animals in the delayed pairing and saline groups. The number of symmetric axospinous synapses was greater for animals in the AMPH CPP group compared to animals in the saline group. There were no differences in the total number of symmetric synapses and the number of symmetric axodendritic and axospinous synapses for rats in the delayed pairing group compared to the saline group. Data are expressed as the mean $\pm$ S.E.M. **f**, The number of symmetric synapses contacting Arc+ postsynaptic structures and the number of symmetric synapses contacting Arc+ dendrites was greater for rats in the AMPH CPP group compared to rats in the delayed pairing and saline groups. Data are expressed as the mean $\pm$ S.E.M. ** $P < 0.01$ versus the delayed pairing and saline groups; # $P < 0.05$ versus the saline group. Abbreviations: ss, symmetric synapse; d-, unlabeled dendrite; d+, Arc-labeled dendrite; som -, unlabeled soma; som+, Arc-labeled soma; Total, total number of synapses; Spin, axospinous; Dend, axodendritic; Som, axosomatic; Unclass, unclassified. The scale bar in **d** is valid for **a-d** and equals 250 nm

Fig. 8.

Effect of AMPH CPP and the retrieval of contextual memories on an index of remodeling. **a**, An electron micrograph depicting a MSB contacting two Arc⁻ spines of BLA neurons. **b**, An electron micrograph depicting a MSB contacting two Arc⁺ spines of BLA neurons. **c**, The number of MSBs was greater for animals in the AMPH CPP group compared to animals in the delayed pairing and saline groups. There were no differences in the number of MSBs for rats in the delayed pairing group compared to the saline group. Data are expressed as the mean $\pm$ *S.E.M.* $** P < 0.01$ versus the delayed pairing and saline groups. **d**, The number of MSBs contacting Arc⁺ postsynaptic structures was greater for animals in the AMPH CPP group compared to animals in the delayed pairing and saline groups. There were no differences in the number of MSBs for rats in the delayed pairing group compared to the saline group. Data are expressed as the mean $\pm$ *S.E.M.* $** P < 0.01$ versus the delayed pairing and saline groups. Abbreviations: msb⁻, multisynaptic bouton contacting Arc⁻ postsynaptic structures; msb⁺, multisynaptic bouton contacting Arc-labeled postsynaptic structures; sp⁻, unlabeled spine; sp⁺, Arc-labeled spine

Fig. 9.

A comparison between the distribution of synapses contacting BLA neurons and those synapses activated by the retrieval of contextual memories. **a**, The proportion of the total number of synapses classified as asymmetric was greater than the proportion of the total number of synapses classified as asymmetric that contacted Arc+ postsynaptic structures. No difference was detected in the proportion of the total number of synapses classified as symmetric versus the proportion of the total number of synapses classified as symmetric that contacted Arc+ postsynaptic structures. No difference was detected in the proportion of the total number of synapses classified as MSBs versus the proportion of the total number of synapses classified as MSBs that contacted Arc+ postsynaptic structures. Data are expressed as the mean $\pm$ *S.E.M.* * $P < 0.05$ versus the proportion of activated synapses. **b**, The proportion of the total number of synapses classified as asymmetric axospinous was greater than the proportion of the total number of synapses classified as symmetric that contacted Arc+ spines. No difference was detected in the proportion of the total number of synapses classified as asymmetric axodendritic versus the proportion of the total number of synapses classified as asymmetric that contacted Arc+ dendrites. No difference was detected in the proportion of the total number of asymmetric synapses that could not be classified versus the proportion of the total number of asymmetric synapses that could not be classified yet contacted Arc+ postsynaptic structures. Data are expressed as the mean $\pm$ *S.E.M.* * $P < 0.05$ versus the proportion of activated synapses. **c**, No difference was detected in the proportion of the total number of synapses classified as symmetric axospinous versus the proportion of the total number of synapses classified as symmetric that contacted Arc+ spines. No difference was detected in the proportion of the total number of synapses classified as symmetric axodendritic versus the proportion of the total number of synapses classified as symmetric that contacted Arc+ dendrites. No difference was detected in the proportion of the total number of synapses classified as symmetric axosomatic versus the proportion of the

total number of synapses classified as symmetric that contacted Arc+ somata. No difference was detected in the proportion of the total number of symmetric synapses that could not be classified versus the proportion of the total number of symmetric synapses that could not be classified yet contacted Arc+ postsynaptic structures. Data are expressed as the mean $\pm$ *S.E.M.* Abbreviations: MSB, multisynaptic boutons; Unclass, unclassified

Table 1

The density of excitatory and inhibitory/modulatory synapses in the BLA for each group measured 1 h after the CPP test.

Synapse Type	Behavioral Conditioning Regimen		
	Saline	Delayed Pairing	AMPH CPP
Total Asymmetric	1.189 ± 0.063	1.262 ± 0.064	1.878 ± 0.192 **
Asymmetric Axospinous	0.934 ± 0.071	1.018 ± 0.039	1.437 ± 0.174 *
Asymmetric Axodendritic	0.102 ± 0.014	0.133 ± 0.007	0.180 ± 0.026 *
Asymmetric Unclassified	0.131 ± 0.012	0.088 ± 0.032	0.212 ± 0.032 *
Total Symmetric	0.204 ± 0.015	0.207 ± 0.014	0.342 ± 0.020 **
Symmetric Axospinous	0.014 ± 0.003	0.025 ± 0.003	0.033 ± 0.006 [#]
Symmetric Axodendritic	0.156 ± 0.020	0.150 ± 0.014	0.273 ± 0.023 **
Symmetric Axosomatic	0.006 ± 0.001	0.011 ± 0.004	0.008 ± 0.001
Symmetric Unclassified	0.028 ± 0.004	0.021 ± 0.003	0.028 ± 0.013
Multisynaptic Boutons	0.022 ± 0.004	0.023 ± 0.003	0.049 ± 0.008 **

Data are expressed as mean synapses per $\mu\text{m}^3 \pm S.E.M.$,

* $P < 0.05$,

** $P < 0.01$ versus the delayed pairing and saline groups;

[#] $P < 0.05$ versus the saline group

Table 2

The density of excitatory and inhibitory/modulatory synapses contacting Arc+ postsynaptic structures of neurons in the BLA for each group measured 1 h after the CPP test.

Synapse Type	Behavioral Conditioning Regimen		
	Saline	Delayed Pairing	AMPH CPP
Total Asymmetric	0.630 ± 0.034	0.734 ± 0.066	1.129 ± 0.050 **
Asymmetric Axospinous	0.423 ± 0.041	0.392 ± 0.038	0.610 ± 0.065 *
Asymmetric Axodendritic	0.122 ± 0.017	0.159 ± 0.008	0.216 ± 0.044 *
Asymmetric Unclassified	0.074 ± 0.006	0.174 ± 0.013 #	0.281 ± 0.027 ***
Total Symmetric	0.158 ± 0.015	0.147 ± 0.014	0.256 ± 0.020 **
Symmetric Axospinous	0.009 ± 0.002	0.008 ± 0.002	0.014 ± 0.001
Symmetric Axodendritic	0.124 ± 0.017	0.106 ± 0.013	0.206 ± 0.013 **
Symmetric Axosomatic	0.006 ± 0.001	0.011 ± 0.004	0.008 ± 0.001
Symmetric Unclassified	0.019 ± 0.003	0.022 ± 0.002	0.028 ± 0.013
Multisynaptic Boutons	0.011 ± 0.002	0.009 ± 0.002	0.022 ± 0.004 *

Data are expressed as mean synapses per $\mu\text{m}^3 \pm S.E.M.$,

* $P < 0.05$,

** $P < 0.01$,

*** $P < 0.001$ versus the delayed pairing and saline groups,

$P < 0.001$ versus the saline group