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Effects of Context-Drug Learning on Synaptic Connectivity in the Basolateral Nucleus of the Amygdala in Rats

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Abstract

Context-drug learning produces structural and functional synaptic changes in the circuitry of the basolateral nucleus of the amygdala (BLA). However, how the synaptic changes translated to the neuronal targets was not established. Thus, in the present study, immunohistochemistry with a cell specific marker and the stereological quantification of synapses was used to determine if context-drug learning increases the number of excitatory and inhibitory/modulatory synapses contacting the gamma-aminobutyric acid (GABA) interneurons and/or the pyramidal neurons in the BLA circuitry. Amphetamine conditioned place preference (AMPH CPP) increased the number of asymmetric (excitatory) synapses contacting the spines and dendrites of pyramidal neurons and the number of multisynaptic boutons contacting pyramidal neurons and GABA interneurons. Context-drug learning increased asymmetric (excitatory) synapses onto dendrites of GABA interneurons and increased symmetric (inhibitory or modulatory) synapses onto dendrites but not perikarya of these same interneurons. The formation of context-drug associations alters the synaptic connectivity in the BLA circuitry, findings that have important implications for drug-seeking behavior.

Keywords

addiction; associative learning; basolateral amygdala; glutamic acid decarboxylase; electron microscopy; stereology; physical disector

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 & Berridge for review). In the conditioned place preference (CPP) paradigm, the affective properties of drugs are assessed by measuring approach behavior elicited by exposure to an environmental context previously associated with the drug (see

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Tzschentke, 1998; 2007 for review). Since the expression of CPP occurs on a drug-free test day, the behavior is dependent upon the retrieval of memories of an association between environmental context and the affective state produced by the drug (White & Carr, 1985; White, 1996). The basolateral nucleus of the amygdala (BLA) seems to mediate the link between the rewarding effects of a drug and the environment in which the drug was administered (Everitt *et al.*, 1991; White & McDonald, 1993). In support of this idea, contextual learning can be disrupted by inactivation of the BLA (Helmstetter & Bellgowan, 1994; Muller *et al.*, 1997; Hsu *et al.*, 2002), and the reinstatement of drug-seeking behaviors following re-exposure to the context where the drug was first administered could directly involve the BLA (Meil & See, 1997; McLaughlin & See, 2003; Peters *et al.*, 2008). Thus, the neural circuitry of the BLA is critically involved in conveying the sensory information required to activate the conditioned response during associative learning (see Blair *et al.*, 2001 for review).

The vast majority of neurons in the BLA are the pyramidal neurons, spiny projection neurons that utilize glutamate as a neurotransmitter (McDonald, 1992). The BLA also contains a moderate amount of small cholinergic interneurons (Carlsen & Heimer, 1986) and spine-sparse intrinsic gamma-aminobutyric acid (GABA) interneurons (McDonald & 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 pyramidal cells (McDonald & Mascagni, 2001). 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 the intercellular coupling (e.g., O'Donnell & Grace, 1997). The GABAergic synapses are, therefore, well situated to gate the responsiveness of BLA projection neurons to any large sensory input.

Our earlier work demonstrated that, in the BLA, amphetamine conditioned place preference (AMPH CPP) increased asymmetric (Gray Type I, excitatory) and symmetric (Gray Type II, inhibitory or modulatory) synapses contacting BLA neurons (Rademacher *et al.*, 2010; Figge *et al.*, 2013). 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* (Hetzel *et al.*, 2012), it was reflected in increases in BLA neuronal excitatory drive *in vivo* (Rademacher *et al.*, 2010). However, the data did not establish how the increase in excitatory drive translated to the neuronal targets. Thus, in the present study, immunohistochemistry with a cell specific marker and the stereological quantification of synapses was used to determine if context-drug learning increases excitatory and inhibitory/modulatory synapses contacting the GABAergic interneurons and/or the pyramidal cells in the BLA circuitry.

Materials and methods

Animals

This study was conducted with adult, male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA), weighing 200-224 g (49-52 d) at the start of the experiment. The rats were housed in groups of three and handled for five 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 performed in accordance 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 suffering.

Drugs

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

Behavioral conditioning

Conditioning followed our published procedures (Rademacher *et al.*, 2006; Rademacher *et al.*, 2010; Figge *et al.*, 2013; Hetzel *et al.*, 2012). Briefly, we used a three-chambered apparatus with two banks of infrared sensors that registered the location and movement of the animals (AccuScan Instruments, Inc., Columbus, OH, USA). The two larger, outer chambers (25 × 30 × 32 cm) were separated by a smaller central chamber (10 × 25 × 32 cm), and differed in both visual and tactile cues. The central chamber had white walls and a Plexiglas floor, and freely allowed movement between the two outer chambers unless barred by two guillotine-like partitions. Conditioning sessions were conducted during the lights on portion of the light: dark cycle, under conditions of dim illumination, and in the presence of white noise. Animals completed a pretest for place preference approximately 72 h before conditioning by placing each rat into the center chamber and allowing free access to the entire apparatus for 15 min. Any rat that spent more than 70% of their time in either of the two outer chambers was excluded from the experiment. Rats remaining in the experiment were randomly assigned to one of three groups for conditioning. 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 of rats (n=6), 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 for 45 min. On days 2 and 4, sterile saline (1 ml/kg, i.p.) was injected and the rat was immediately confined to the opposite outer chamber for 45 min. A second group of rats, the delayed pairing group (n=5), controlled for the effects of repeated AMPH exposure. Each of these rats was subjected to the identical conditioning schedule with AMPH (1.0 mg/kg, i.p.), but remained in the home cage for 4 h before being paired with the appropriate chamber. Four hours was selected, since this amount of time is sufficient to clear this dose of i.p. AMPH from the brain (Honecker & Coper, 1975). A third group of animals (n=4) received sterile saline injections before being placed into one outer chamber on days 1, 3, and 5 or the other chamber on days 2 and 4. Approximately 72 h after the last conditioning session, drug- and saline-free animals were tested for their preference by being placed into the central chamber with the dividing partitions removed. The time spent in each chamber was recorded and the degree of place preference (i.e., the time 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; Rademacher *et al.*, 2007; Hetzel *et al.*, 2012; Figge *et al.*, 2013).

Electron microscopic analysis

The procedures for preparing brain tissue for electron microscopy were modified from previous studies (Morshedi *et al.*, 2009; Rademacher *et al.*, 2010; Figge *et al.*, 2013). Within 24 h after the CPP test, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 10 ml of 0.1M phosphate-buffered saline (PBS), followed by 50 ml of 3.75% acrolein in buffered 2% paraformaldehyde (PF), pH 7.4, followed by 200 ml of buffered 2% PF. All solutions were made at the same time and the volume of the perfusate for each rat was kept constant. Each brain was removed, post-fixed in buffered 2% PF for 30 min 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, -1.80 mm to -4.80 mm (Paxinos & Watson, 1998; see Gundersen *et al.*, 1988 for review)) were cut coronally at 60 μ m thickness on a Vibratome (The Vibratome Co., St. Louis, MO, USA) and were collected serially in 0.1M phosphate buffer into numbered 24-well plates. An unbiased stereological approach (i.e., physical disector) was used to estimate the total number of synapses. After a random start, every 5th section was selected, yielding a series of 10 equally spaced sections. A second series of 10 equally spaced, adjacent 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. A low power photomicrograph (Fig. 1a) of a section used to estimate the total volume of the BLA was obtained by using a Nikon Eclipse TE2000 microscope equipped with a video camera linked to a computer running MetaMorph (Nashville, TN, USA) software. Sections selected for physical disector analysis were immunoreacted in primary antibodies against the two isoforms of glutamic acid decarboxylase (GAD), GAD 65 and GAD 67 with molecular weights of 65 and 67 kDa, respectively (GAD 65+67 antisera; Millipore, Billerica, MA, USA). This antibody has served as a reliable and specific marker for GABAergic interneurons (Wernig *et al.*, 2004; Scheffler *et al.*, 2005; Schechter *et al.*, 2007). Although immunoreactivity for GAD 65 and 67 was found in the somata, dendrites, and the puncta resembling axon terminals, GAD 65 immunoreactivity was preferentially located in axon terminals (Kohler & Chan-Palay, 1983; Panula *et al.*, 1984; Castañeda *et al.*, 2005), whereas GAD 67 was preferentially distributed the cytoplasm of somata (Esclapez *et al.*, 1994). The GAD 65+67 antibody was used here to reveal the GABAergic postsynaptic structures and the GABAergic terminals. Sections selected for physical disector analysis were rinsed in 0.1M PBS, blocked in 0.1M PBS containing 5% normal goat serum (NGS) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and 0.01% Triton X-100 (Tx) for 1 h at room temperature. The sections were rinsed and incubated in 0.1M PBS containing a rabbit anti-GAD 65+67 antibody (1:3000, Millipore, Billerica, MA, USA), 0.01% Tx, and 5% NGS for 48 h at 4°C. Following extensive rinsing, the sections were incubated in 0.1M PBS containing biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA) and

5% NGS for two h at room temperature, followed by rinsing and an incubation with avidinbiotin peroxidase complex (Vectastain Kit, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The sections were reacted with 0.05% 3'-diaminobenzidine tetrahydrochloride (DAB, St. Louis, MO, USA) containing 0.01% H₂O₂. Three different controls were performed to demonstrate that the rabbit anti-GAD 65+67 antibody was GAD specific: (1) A mixture of GAD 65+67 blocking peptides (control proteins) were mixed with the primary antiserum; (2) The sections were incubated in normal rabbit immunoglobulin; and (3) The primary serum was omitted. GAD staining was absent after all three control procedures (data not shown). A low power photomicrograph depicting GAD+ neurons in the BLA (Fig. 1b) was obtained by using a Nikon Eclipse TE2000 microscope equipped with a video camera linked to a computer running MetaMorph (Nashville, TN, USA) software. Sections were then processed for electron microscopy by fixation in 1% osmium tetroxide diluted in 0.1M phosphate buffer 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, USA), mounted on formvar-coated copper slot grids, 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 ultrathin sections of the same thickness were taken and used as the reference and look-up sections (Hunter & Stewart, 1993; Ingham *et al.*, 1998; Day *et al.*, 2006). We used 180 disectors for each animal per treatment group (total of 720-1080 disectors per group). As previously described (Morshedi *et al.*, 2009; Figge *et al.*, 2013) 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. Terminals were either GAD-labeled (GAD+) or GAD-unlabeled (GAD-). GAD+ terminals contacting postsynaptic structures were considered GAD+ terminals. GAD- terminals contacting postsynaptic structures were considered GAD- terminals. Consistent with previous studies (Rademacher *et al.*, 2010; Figge *et al.*, 2013) synapses were classified as either single synaptic boutons (SSBs; i.e., boutons that form synapses with a single postsynaptic element) or multisynaptic boutons (MSBs; i.e., boutons that form separate synapses with two or more postsynaptic elements). 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 GAD+ or GAD-, were identified as somata, dendrites, spines, or unclassified as previously described (Morshedi *et al.*, 2009; Figge *et al.*, 2013) and in accord with well-established criteria

(Peters *et al.*, 1991). Note that synapses were determined to be ‘unclassified’ if it was not possible to unambiguously identify the postsynaptic target. Somata were identified by the presence of a nucleus. Dendrites were identified by the presence of structures postsynaptic to the 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. A rectangular, unbiased counting frame ($32.02 \mu\text{m}^2$), containing 2 inclusion and 2 exclusion lines, was centered on each of the micrographs. The area of each of the micrographs was equal to $35.04 \mu\text{m}^2$. 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. The separate, adjacent 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, dried at 60°C overnight, and then stained with cresyl violet. Sections were then dehydrated in an ascending series of alcohols, cleared with xylene, and coverslipped. V_{ref} was estimated according to Cavalieri principles using a Nikon E400 microscope equipped with a stage motorized in 3 axes and a video camera linked to a computer running StereoInvestigator (MicroBrightField, Williston, VT, USA) software (Gundersen & Jensen, 1987). To estimate the total volume of the BLA (V_{ref} , expressed in μm^3), the following formula was used: $V_{\text{ref}} = a \times t \times s$, 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). Estimation of mean synaptic density ($N_{\text{v syn}}$) (synapse number per μm^3) was calculated using the formula $N_{\text{v syn}} = \Sigma Q_{\text{-syn}} / (h \times A)$, adapted from Stereo (Sterio, 1984) and de Groot and Bierman (de Groot & Bierman, 1986), where $Q_{\text{-syn}}$ = number of asymmetric 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). To estimate the absolute number of synapses (N) onto dendritic structures, the following formula was used: $N = N_{\text{v syn}} \times V_{\text{ref}}$. For physical disectors, estimate precision was expressed as the coefficient of error for the group.

Data analysis

CPP data were analyzed with one-sample *t*-tests. Synaptic data were analyzed with one-way ANOVA with post hoc Student-Newman-Keuls (SNK) tests. Prior to statistical analysis, the normality of distribution was determined with the Shapiro-Wilk test. The α level was set at 0.05. All data are represented as mean $\pm$ *S.E.M.*

Results

Amphetamine conditioned place preference

Two groups of rats were conditioned with AMPH and saline (experimental group), or saline alone (vehicle control), and exposed to chamber-specific contexts paired to treatment. Rats in a third group (delayed pairing control) were treated with AMPH and saline as the

experimental group but were delayed in their placement into the conditioning chambers. This group controlled for effects of AMPH exposure in the absence of novel learning by temporally separating the conditioned (environmental contextual cues) from the unconditioned (AMPH administration) stimuli. As expected, neither AMPH treatment with the delayed pairing nor treatment with saline alone resulted in preference for one chamber over the other (saline, one-sample *t*-test, $t_3 = 0.0631$, $P = 0.9530$; delayed pairing, one-sample *t*-test, $t_4 = -0.790$, $P = 0.494$; **Fig. 2**). In contrast, animals treated with AMPH and immediately paired with a specific chamber spent significantly more time in the AMPH-paired than the unpaired chamber during the CPP test (AMPH CPP, one-sample *t*-test, $t_5 = 8.472$, $P = 0.001$; **Fig. 2**). Thus, the increase in time spent in the AMPH-paired chamber was not due to repeated exposure to AMPH, but instead, to associating the context with the drug.

Synapses contacting GAD+ and GAD– structures

We analyzed the percentage of GAD+ and GAD– terminals and their postsynaptic targets in the BLA of animals in the saline-treated control group. Although the vast majority of terminals were GAD– (~99%), there was a greater percentage of GAD+ terminals with symmetric synapses (~6%) than GAD+ terminals with asymmetric synapses (<1%) (unpaired *t*-test, $t_{28} = 2.291$, $P = 0.0297$, **Table 1**). Moreover, there was a greater percentage of symmetric synapses contacting GAD+ spines (~23%) and dendrites (~77%) than asymmetric synapse contacting GAD+ spines (<2%) and dendrites (~45%) of BLA neurons (GAD+ spines, unpaired *t*-test, $t_{28} = 2.560$, $P = 0.0169$; GAD+ dendrites, unpaired *t*-test, $t_{28} = 3.9850$, $P = 0.0004$, **Table 1**). Although asymmetric synapses contacting GAD– somata were not detected, ~44% of the symmetric synapses contacted GAD+ somata (**Table 1**). Finally, although we did not encounter MSBs in which the MSB was GAD+, ~17% of the postsynaptic targets of MSBs were GAD+ (**Table 1**).

Volume of the BLA and relative percentage of synapses in the BLA

Approximately 24 h after the completion of the CPP test, the number of asymmetric (Gray Type I, excitatory; see Klemann & Roubos, 2011 for review) and symmetric (Gray Type II, inhibitory or modulatory; see Klemann & Roubos, 2011 for review) synapses were quantified with the transmission electron microscope by using the physical disector. We used the 10 Nissl-stained sections to calculate the volume of the BLA for each of the animals in the three groups. There was no difference in the Cavalieri volume between the saline, delayed pairing, and AMPH CPP groups 24 h after the CPP test (saline 2.90 ± 0.11 mm³, $n=4$ rats; delayed pairing 2.85 ± 0.22 mm³, $n=5$ rats; AMPH CPP 2.95 ± 0.17 mm³, $n=6$ rats; one-way ANOVA, $F_{2,14} = 0.61$, $P = 0.56$). No differences between the three groups were detected in the relative distributions of SSBs (one-way ANOVA, $F_{2,14} = 0.46$, $P = 0.64$) or MSBs (one-way ANOVA, $F_{2,14} = 1.27$, $P = 0.32$). No differences between the three groups were detected in the proportions of asymmetric axospinous (one-way ANOVA, $F_{2,14} = 0.26$, $P = 0.78$), axodendritic (one-way ANOVA, $F_{2,14} = 1.08$, $P = 0.37$), or unclassified (one-way ANOVA, $F_{2,14} = 0.43$, $P = 0.66$) synapses. Similarly, we found no differences in the number of symmetric axospinous (one-way ANOVA, $F_{2,14} = 1.11$, $P = 0.36$), axodendritic (one-way ANOVA, $F_{2,14} = 1.63$, $P = 0.24$), axosomatic (one-way

ANOVA, $F_{2,14} = 1.45$, $P = 0.27$), or unclassified (one-way ANOVA, $F_{2,14} = 0.05$, $P = 0.95$) synapses.

Asymmetric synapses contacting GAD+ and GAD– structures

The number of asymmetric synapses were significantly increased for the AMPH CPP group compared to the saline and delayed pairing control groups (one-way ANOVA, $F_{2,14} = 7.139$, $P = 0.009$, AMPH CPP versus saline $P = 0.011$; AMPH CPP versus delayed pairing, $P = 0.015$, **Fig. 3a-e**). There was no difference between the saline and delayed pairing groups in the number of axospinous (number, $P = 0.532$) or axodendritic (number, $P = 0.346$) synapses (**Fig. 3a-e**). In contrast, the number of asymmetric axospinous and axodendritic synapses was significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups (number of asymmetric axospinous synapses: one-way ANOVA, $F_{2,14} = 6.161$, $P = 0.014$, AMPH CPP versus saline $P = 0.031$; AMPH CPP versus delayed pairing, $P = 0.016$; number of asymmetric axodendritic synapses: one-way ANOVA, $F_{2,14} = 6.876$, $P = 0.010$, AMPH CPP versus saline, $P = 0.012$; AMPH CPP versus delayed pairing, $P = 0.022$, **Fig. 3e**).

Asymmetric synapses were classified as GAD+ or GAD– based on the presence or absence of GAD immunoreactivity in the terminal or in postsynaptic structures (i.e., spine, dendrite, or soma). While there were a few GAD+ terminals with asymmetric synapses and GAD+ spines, these were rare and there were no differences in the number of these structures between groups (data not shown). There was no difference between the saline and delayed pairing groups in the number of GAD– terminals with asymmetric synapses contacting GAD– spines ($P = 0.577$, **Fig. 3a, f**), GAD– dendrites ($P = 0.085$, **Fig. 3c, g**), or GAD+ dendrites ($P = 0.777$, **Fig. 3d**). In addition, no between groups differences were detected in the number of GAD– terminals with asymmetric synapses contacting GAD+ spines (one-way ANOVA, $F_{2,14} = 1.235$, $P = 0.325$, **Fig. 3b, f**). In contrast, the number of GAD– terminals with asymmetric synapses contacting GAD– dendrites and spines and the number of GAD– terminals with asymmetric synapses contacting GAD+ dendrites were significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups (number of GAD– terminals with asymmetric synapses contacting GAD– spines: one-way ANOVA, $F_{2,14} = 6.456$, $P = 0.012$, AMPH CPP versus saline, $P = 0.025$; AMPH CPP versus delayed pairing, $P = 0.014$, **Fig. 3a, f**; number of GAD– terminals with asymmetric synapses contacting GAD– dendrites: one-way ANOVA, $F_{2,14} = 10.390$, $P = 0.002$, AMPH CPP versus saline, $P = 0.002$; AMPH CPP versus delayed pairing, $P = 0.020$, **Fig. 3c, g**; number of GAD– terminals with asymmetric synapses contacting GAD+ dendrites: oneway ANOVA, $F_{2,14} = 7.890$, $P = 0.007$, AMPH CPP versus saline, $P = 0.013$; AMPH CPP versus delayed pairing, $P = 0.009$, **Fig. 3d, g**).

Symmetric synapses contacting GAD+ and GAD– structures

The number of symmetric synapses were significantly increased for the AMPH CPP group compared to the saline and delayed pairing control groups (one-way ANOVA, $F_{2,14} = 10.311$, $P = 0.004$, AMPH CPP versus saline $P = 0.007$; AMPH CPP versus delayed pairing, $P = 0.004$, **Fig. 4**). No between group differences were detected in the number of symmetric axospinous and axosomatic synapses (number of symmetric axospinous synapses: one-way

ANOVA, $F_{2,14} = 0.09$, $P = 0.913$; **Fig. 4h**; number of symmetric axosomatic synapses: one-way ANOVA, $F_{2,14} = 2.013$, $P = 0.176$, **Fig. 4e-h, k**). There was no difference between the saline and delayed pairing groups in the number of symmetric axodendritic synapses ($P = 0.19$). In contrast, the number of symmetric axodendritic synapses were significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups (one-way ANOVA, $F_{2,14} = 8.829$, $P = 0.005$, AMPH CPP versus saline, $P = 0.027$; AMPH CPP versus delayed pairing, $P = 0.005$; **Fig. 4a-d, h**).

There were no differences between the saline and delayed pairing groups in the number of GAD⁻ terminals with symmetric synapses contacting GAD⁺ dendrites ($P = 0.920$, **Fig. 4b, i**). We did not detect GAD⁺ terminals with symmetric synapses contacting GAD⁻ somata. Moreover, no difference was detected in the number of GAD⁻ terminals with symmetric synapses contacting GAD⁻ dendrites and somata, GAD⁺ terminals with symmetric synapses contacting GAD⁻ dendrites, GAD⁺ terminals with symmetric synapses contacting GAD⁺ dendrites, and GAD⁻ terminals with symmetric synapses contacting GAD⁺ somata (number of GAD⁻ terminals with symmetric synapses contacting GAD⁻ dendrites: one-way ANOVA, $F_{2,14} = 0.42$, $P = 0.67$, **Fig. 4a, i**; number of GAD⁻ terminals with symmetric synapses contacting GAD⁻ somata: one-way ANOVA, $F_{2,14} = 3.104$, $P = 0.082$, **Fig. 4g, k**; number of GAD⁺ terminals with symmetric synapses contacting GAD⁻ dendrites: one-way ANOVA, $F_{2,14} = 0.14$, $P = 0.87$, **Fig. 4c, j**; number of GAD⁺ terminals with symmetric synapses contacting GAD⁺ somata: one-way ANOVA, $F_{2,14} = 0.720$, $P = 0.507$, **Fig. 3f, k**; number of GAD⁺ terminals with symmetric synapses contacting GAD⁺ dendrites: one-way ANOVA, $F_{2,14} = 0.43$, $P = 0.66$, **Fig. 4d, j**; number of GAD⁻ terminals with symmetric synapses contacting GAD⁺ somata: one-way ANOVA, $F_{2,14} = 2.014$, $P = 0.176$, **Fig. 4e, k**). In contrast, the number of GAD⁻ terminals with symmetric synapses contacting GAD⁺ dendrites was significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups (one-way ANOVA, $F_{2,14} = 7.086$, $P = 0.011$, AMPH CPP versus saline, $P = 0.021$; AMPH CPP versus delayed pairing, $P = 0.010$, **Fig. 4b, i**).

Multisynaptic boutons (MSBs) contacting GAD⁺ and GAD⁻ structures

The number of MSBs, morphological hallmarks of the remodeling of existing synapses (Toni *et al.*, 1999; Geinisman *et al.*, 2001; Toni *et al.*, 2001; Nicholson & Geinisman, 2009), was significantly increased in the AMPH CPP group compared to the delayed pairing and saline controls (one-way ANOVA, $F_{2,14} = 4.379$, $P = 0.037$, AMPH CPP versus saline, $P = 0.035$; AMPH CPP versus delayed pairing, $P = 0.046$, **Fig. 5a-c**). There was no difference between the saline and delayed pairing groups in the number ($P = 0.727$) of MSBs. Most MSBs contacted spines.

The MSBs were never GAD⁺, but the postsynaptic structures were spines and dendrites and sometimes GAD⁺. Nevertheless, the number of GAD⁺ and GAD⁻ spines and dendrites contacted by MSBs was significantly increased in the AMPH CPP group compared to the delayed pairing and saline controls (number of MSBs contacting GAD⁺ targets: one-way ANOVA, $F_{2,14} = 4.420$, $P = 0.036$, AMPH CPP versus saline, $P = 0.034$; AMPH CPP versus delayed pairing, $P = 0.045$; **Fig. 5a, c**; number of MSBs contacting GAD⁻ targets: one-way ANOVA, $F_{2,14} = 4.387$, $P = 0.037$, AMPH CPP versus saline, $P = 0.035$; AMPH

CPP versus delayed pairing, $P = 0.046$ **Fig. 5b-c**). There was no difference between the saline and delayed pairing groups in the number of GAD⁻ and GAD⁺ postsynaptic structures contacted by MSBs (MSBs contacting GAD⁻ targets, $P = 0.736$; MSBs contacting GAD⁺ targets, $P = 0.725$, **Fig. 5c**).

Discussion

This is the first study to demonstrate that the formation of context-drug associations alters the synaptic connectivity of BLA pyramidal neurons and GABAergic interneurons. Our earlier work showed that AMPH CPP increased the total number of asymmetric (Gray Type I, excitatory) synapses contacting spines and dendrites of BLA neurons (Rademacher *et al.*, 2010; Figge *et al.*, 2013) and symmetric (Gray Type II, inhibitory or modulatory) synapses contacting dendrites of BLA neurons (Figge *et al.*, 2013) but did not distinguish the origin of these structures. The present stereological analysis fully supports those findings and augments them by providing evidence for increases in asymmetric (excitatory) and symmetric (inhibitory or modulatory) inputs contacting the GABAergic interneurons. Specifically, there is an increase of excitatory synapses onto dendrites of GABAergic interneurons and an increase in inhibitory or modulatory synapses onto dendrites but not somata of these same interneurons. These increases are seen exclusively in the AMPH CPP group and not in the delayed pairing group, suggesting that the changes are due to the formation of context-drug associations rather than repeated exposures to AMPH.

In addition to drug rewards, CPP is produced by conditioning with nondrug rewards, such as food, sex, and social interactions (Camacho *et al.*, 2009; Rubinow *et al.*, 2009; Yates *et al.*, 2013). Although AMPH produces a more robust CPP than the CPP induced by nondrug rewards, a context paired with social interaction eliminated AMPH CPP (Yates *et al.*, 2013). Research in our lab indicates that conditioning with 1.0 mg/kg AMPH produces a CPP score of ~500 seconds (Rademacher *et al.*, 2006; Rademacher *et al.*, 2010; Hetzel *et al.*, 2012; Figge *et al.*, 2013). In comparison, the CPP score produced by conditioning with food or sex ranges from 130-170 seconds (Suzuki *et al.*, 2003; Figlewicz *et al.*, 2004; Kudwa *et al.*, 2005). Interestingly, when rats are given a choice between spending time in a chamber previously paired with social interactions and a chamber previously paired with 1.0 mg/kg AMPH, they do not prefer one chamber over the other (Yates *et al.*, 2013). Thus, future research is needed to determine if CPP induced by nondrug rewards produces synaptic changes in the BLA that are similar to those produced by AMPH CPP.

The amygdala plays an important role in contextual learning, such as CPP (Helmstetter & Bellgowan, 1994; Muller *et al.*, 1997; Olmstead & Franklin, 1997; Hsu *et al.*, 2002). The BLA, however, was 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), was 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 immunoreactivity for the postsynaptic structural plasticity protein, activity-regulated cytoskeleton associated protein (Arc), and degree of AMPH CPP (Figge *et al.*, 2013).

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.

Through associative learning, previously neutral stimuli acquire incentive motivational value when previously paired with drug, and encounters with these conditioned stimuli may serve as environmental triggers of relapse to drug use (Wallace, 1989). In fact, consistent with stimulus substitution theory, after only a few pairings between drug and a conditioned stimulus, psychostimulant-paired conditioned stimuli can elicit many of the same subjective and reinforcing effects as the drug itself (Boileau *et al.*, 2007). Abstinent drug addicts often report intense craving when exposed to stimuli that have been previously associated with the drug of abuse (Wikler, 1973; Childress *et al.*, 1988; 1993; Ehrman *et al.*, 1992; Volkow *et al.*, 2006). One substrate of particular interest in the study of context-induced relapse is the BLA. Using brain-imaging techniques, several studies have noted an increase in amygdalar activity when abstinent individuals are presented with drug-associated conditioned stimuli (Grant *et al.*, 1996; Brieter *et al.*, 1997; Childress *et al.*, 1999; Kilts, 2001; Bonson *et al.*, 2002; Kilts *et al.*, 2004). Importantly, craving elicited by drug-paired conditioned stimuli (Fox *et al.*, 2005; Leyton *et al.*, 2005) predicts drug use on the street (Miller & Gold, 1994; Weiss *et al.*, 1995). According to our current model (Fig. 6), associative learning increased the number of excitatory synapses contacting spines and dendrites of BLA pyramidal neurons and increased the number of MSB contacting spines of BLA pyramidal neurons, which would produce an increase in excitatory drive. Additionally, context-drug learning increased the number of inhibitory or modulatory inputs contacting the dendrites GABAergic interneurons, which would result in disinhibition of pyramidal neurons. This structural change may be “balanced” or “offset” by increases in the number of MSBs and excitatory inputs contacting the dendrites of pyramidal neurons. The net functional effect of these synaptic changes would be an increase the excitatory drive of pyramidal neurons, craving, and drug-seeking behavior indexed by approach behavior to the context previously paired with AMPH.

There are several important 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 (Somogyi & Soltesz, 1986; Beaulieu & Somogyi, 1990; Kozell & Meshul, 2001; 2004). Contrary to this hypothesis, GABA immunoreactivity has been observed in terminals that form asymmetric synapses (Pinard *et al.*, 1991) and glutamate immunoreactivity has been observed in terminals that form symmetric synapse (van den Pol, 1991). Importantly, BLA neurons receive substantial inputs from neuromodulatory systems including dopaminergic, cholinergic, and noradrenergic systems (Ben-Ari *et al.*, 1977; Carlsen & Heimer, 1986; Fallon & Ciofi, 1992;

Asan, 1993; 1997; 1998; Li *et al.*, 2001; Pinard *et al.*, 2008; Muller *et al.*, 2009). The BLA receives one of the densest cholinergic innervations in the CNS (Ben-Ari *et al.*, 1977) and the major part of the innervation comes from the cholinergic projection neurons in the basal forebrain (Carlsen & Heimer, 1986). The BLA contains noradrenergic terminals that originate in the locus coeruleus (Fallon & Ciofi, 1992; Asan, 1993) and was one of the amygdalar regions that contained the densest innervation with noradrenergic fibers (Li *et al.*, 2001). The BLA also contains dopamine terminals that originate in the ventral tegmental area (Asan, 1997; 1998; Muller *et al.*, 2009). A large majority (~78%) of dopamine terminals in the BLA make synaptic contacts and the predominant targets are distal spines and dendrites of BLA pyramidal neurons (Muller *et al.*, 2009). Since the terminals of these neuromodulatory inputs (i.e., dopaminergic, cholinergic, and noradrenergic systems) form symmetric synapses with BLA neurons (e.g., Carlsen & 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, although most GAD⁺ terminals in the BLA are presumed to originate from GABAergic interneurons, there is electrophysiological evidence (e.g., Mello *et al.*, 1992) that some may arise from the GABAergic interneurons in the basal forebrain that are intercalated among the cholinergic interneurons. Third, it should be noted that the differences in the number and/or proportion of synapses observed in the present study compared to studies conducted by others can be explained by the sampling approach used. Specifically, the systematic-uniform-random sampling approach (see Mouton, 2002) was applied in the present study whereas various ‘biased’ sampling approaches have been applied by others. For instance, in the present study, in which systematic sampling was applied to each level of the sampling hierarchy (e.g., the selection of sections from the entirety of the BLA, 0.5 × 0.5 mm areas for physical disector analysis, ultrathin sections, areas of the selected ultrathin sections that were photographed), GAD⁺ terminals only formed ~1% of the total synapses. In contrast, Muller and colleagues (2005) found that parvalbumin immunoreactive terminals formed ~15% of the total synapses. The synaptic analysis in the BLA was performed on areas of the sections that had a high number of PV⁺ cell bodies (Muller *et al.*, 2005).

In summary, consistent with previous studies (Rademacher *et al.*, 2010; Figge *et al.*, 2013), context-drug learning increased the number of MSBs and asymmetric (excitatory) axospinous and axodendritic synapses contacting pyramidal neurons (GAD⁻ spines and dendrites) in the BLA. 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. Moreover, context-drug learning resulted in an increase in the formation of new synapses and the remodeling of existing synapses. The targets of the remodeled synapses are both intrinsic GABAergic interneurons (MSB/GAD⁺ target) and pyramidal neurons (MSB/GAD⁻ target). In addition, context-drug learning increased the number of GAD⁻ symmetric (inhibitory or modulatory) synapses contacting the dendrites of GABAergic interneurons (GAD⁺ dendrites). Future studies are needed to determine what

proportion of these GAD[−] symmetric synapses is cholinergic, noradrenergic, or dopaminergic.

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Abbreviations

AMPH	d-amphetamine sulfate
AMPH CPP	amphetamine conditioned place preference
Arc	activity-regulated cytoskeleton associated protein
BLA	basolateral amygdala
CPP	conditioned place preference
DAB	3'3-diaminobenzidine tetrahydrochloride
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GAD⁺	GAD-labeled
GAD[−]	GAD-unlabeled
MSB	multisynaptic bouton
NGS	normal goat serum
PBS	phosphate buffered saline
PF	paraformaldehyde
SNK	Student-Newman-Keuls
V_{ref}	reference volume

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Figure 1.

Low magnification images depicting GAD+ neurons and the boundaries of the BLA. a, A photomicrograph of a section stained with cresyl violet. The boundaries of the BLA are outlined by the black line. The scale bar equals 1 mm. b, A photomicrograph of a section immunoreacted for GAD 65+67 then processed for electron microscopy. The arrows point to GAD+ neurons in the BLA. The boundaries of the BLA are outlined by the black line. The scale bar equals 500 μm.

Figure 2.

Behavioral conditioning with AMPH produced AMPH CPP. Animals treated with AMPH and immediately paired with a specific chamber spent more time in the AMPH-paired than the unpaired chamber during the CPP test (n=6 rats). Animals treated with the delayed pairing regimen (n=5 rats) or with saline alone (n=4 rats) did not exhibit a preference for one chamber over the other during the CPP test. Data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ versus the delayed pairing and saline groups.

Figure 3.

AMPH CPP increased the number of excitatory synapses. **a**, An electron micrograph depicting a GAD- terminal forming an asymmetric synapse with a GAD- spine. **b**, An electron micrograph depicting a GAD- terminal forming an asymmetric synapse with a GAD+ spine. **c**, An electron micrograph depicting a GAD- terminal forming an asymmetric synapse with a GAD- dendrite. **d**, An electron micrograph depicting two GAD- terminals forming asymmetric synapses with the same GAD+ dendrite. Also depicted is a GAD- terminal forming an asymmetric synapse with a GAD- spine. **e**, The total number of asymmetric synapses and the number of asymmetric axospinous and axodendritic 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 and axodendritic 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 GAD- terminals with asymmetric synapses contacting GAD- spines was significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups. There was no difference in the number of GAD- terminals with asymmetric synapses contacting GAD- spines for rats in the delayed pairing group compared to the saline group. **g**, The number of GAD- terminals with asymmetric synapses contacting GAD- dendrites and the number of GAD- terminals with asymmetric synapses contacting GAD+ dendrites was significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups. There was no difference in the number of GAD- terminals with asymmetric synapses contacting GAD- dendrites and the number of GAD- terminals with asymmetric synapses contacting GAD+ dendrites for rats in the delayed pairing group compared to the saline group. Data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ versus the delayed pairing and saline groups. Abbreviations: as, asymmetric synapse; sp-, GAD- spine; sp+, GAD+ spine; d-, GAD- dendrite; d+, GAD+ dendrite. The scale bar in **d** is valid for **a-d** and equals 250 nm.

Figure 4.

AMPH CPP increased the number of inhibitory or modulatory synapses. **a**, An electron micrograph depicting a GAD⁻ terminal forming a symmetric synapse with a GAD⁻ dendrite. Also depicted is a GAD⁻ spine protruding from a GAD⁻ parent dendrite and a GAD⁻ terminal forming an asymmetric synapse with a GAD⁻ spine. **b**, An electron micrograph depicting a GAD⁻ terminal forming a symmetric synapse with a GAD⁺ dendrite. **c**, An electron micrograph depicting a GAD⁺ terminal forming a symmetric synapse with a GAD⁻ dendrite. **d**, An electron micrograph depicting a GAD⁻ terminal forming a symmetric synapse with a GAD⁺ dendrite. **e**, An electron micrograph depicting a GAD⁻ terminal forming a symmetric synapse with a GAD⁺ soma. **f**, An electron micrograph depicting a GAD⁺ terminal forming a symmetric synapse with a GAD⁺ soma. **g**, An electron micrograph depicting a GAD⁻ terminal forming a symmetric synapse with a GAD⁻ soma. **h**, The total number of symmetric synapses and the number of symmetric axodendritic but not axospinous or axosomatic 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 symmetric synapses and the number of symmetric axospinous, axodendritic, or axosomatic synapses for rats in the delayed pairing group compared to the saline group. Data are expressed as the mean $\pm$ S.E.M. **i**, The number of GAD⁻ terminals with symmetric synapses contacting GAD⁺ dendrites was significantly increased in the AMPH CPP group compared with the delayed pairing and saline control groups. There was no difference in the number of GAD⁻ terminals with symmetric synapses contacting GAD⁺ dendrites for rats in the delayed pairing group compared to the saline group. Data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ versus the delayed pairing and saline groups. **j**, AMPH CPP had no effect on the number of GAD⁺ terminals with symmetric synapses contacting GAD⁺ and GAD⁻ dendrites. Data are expressed as the mean $\pm$ S.E.M. **k**, AMPH CPP had no effect on the number of GAD⁻ terminals with symmetric synapses contacting GAD⁺ and GAD⁻ somata or the number of GAD⁺ terminals with symmetric synapses contacting GAD⁺ somata. GAD⁺ terminals with symmetric synapses contacting GAD⁻ somata were not detected. '0' in the graph is used to indicate that the group mean is equal to zero. Data are expressed as the mean $\pm$ S.E.M. Abbreviations: as,

asymmetric synapse; ss, symmetric synapse; sp-, GAD- spine; d-, GAD- dendrite; d+, GAD+ dendrite; som-, GAD- soma; som+, GAD+ soma. The scale bar in **g** is valid for **a-g** and equals 250 nm.

Figure 5.

AMPH CPP remodeled existing synapses. **a**, An electron micrograph depicting a GAD⁻ MSB contacting two GAD⁺ targets. **b**, An electron micrograph depicting a GAD⁻ MSB contacting two GAD⁻ targets. **c**, The total number of MSBs and the number of MSBs contacting GAD⁺ and GAD⁻ targets were greater for animals in the AMPH CPP group compared to animals in the delayed pairing and saline groups. There were no differences between the delayed pairing and the saline group for any of these three categories. Data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ versus the delayed pairing and saline groups. Abbreviations: GAD⁺, glutamic acid decarboxylase positive; GAD⁻, glutamic acid decarboxylase negative; MSB, multisynaptic bouton. The scale bar in **b** is valid for **a** and **b** and equals 250 nm.

Figure 6.

Context-drug learning alters the synaptic landscape of BLA pyramidal neurons and GABAergic interneurons. a, Behavioral output is dictated by the interaction between glutamatergic inputs (single gray circles) contacting distal spines of BLA pyramidal neurons (Pyr), dopaminergic (DAergic) input (gray triangles) originating in the ventral tegmental area and contacting proximal dendrites of pyramidal neurons, and GABAergic inputs (gray rectangles) which provide a dense innervation of the perisomatic region of pyramidal neurons. Also depicted is the dense innervation of the perisomatic region of neighboring GABAergic interneurons by the axon collaterals of BLA pyramidal neurons. b, After repeated context-drug associations, there is an increase in the number of excitatory and MSB (double gray circles) synapses contacting BLA pyramidal neurons and an increase in excitatory, inhibitory or modulatory, and MSB synapses contacting GABAergic interneurons. The net result is approach behavior to the drug-associated context.

Table 1

The percentage of GAD+ and GAD– terminals and postsynaptic targets in the BLA of saline-treated control animals. Data are expressed as mean percentages $\pm$ S.E.M.

Terminals/Synapses	Percent of whole	Synapses (onto)/postsynaptic structure	Percent of whole
Total GAD+	1.24 $\pm$ 0.29	Total synapses/GAD+ targets	18.29 $\pm$ 1.40
Total GAD–	98.76 $\pm$ 0.29	Total synapses/GAD– targets	81.71 $\pm$ 1.40
Asymmetric GAD+	0.66 $\pm$ 0.24	Asymmetric/GAD+ spine	1.26 $\pm$ 0.49
Asymmetric GAD–	99.34 $\pm$ 0.24	Asymmetric/GAD– spine	98.74 $\pm$ 0.49
Symmetric GAD+	5.68 $\pm$ 2.18	Asymmetric/GAD+ dendrite	45.28 $\pm$ 3.17
Symmetric GAD–	94.32 $\pm$ 2.18	Asymmetric/GAD– dendrite	54.72 $\pm$ 3.17
Total GAD– MSBs	100.00 $\pm$ 0.00	Symmetric/GAD+ spine	22.83 $\pm$ 9.46
		Symmetric/GAD– spine	77.17 $\pm$ 9.46
		Symmetric/GAD+ dendrite	76.89 $\pm$ 6.29
		Symmetric/GAD– dendrite	23.11 $\pm$ 6.29
		Symmetric/GAD+ soma	44.44 $\pm$ 17.54
		Symmetric/GAD– soma	56.56 $\pm$ 17.54
		MSB/GAD+ structure	16.67 $\pm$ 11.79
		MSB/GAD– structure	83.33 $\pm$ 11.79