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AMYGDALA-DEPENDENT AND AMYGDALA-INDEPENDENT PATHWAYS FOR CONTEXTUAL FEAR CONDITIONING

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Abstract—The basolateral amygdala (BLA), consisting of the lateral and basal nuclei, is considered to be essential for fear learning. Using a temporary inactivation technique, we found that rats could acquire a context-specific long-term fear memory without the BLA but only if intensive overtraining was used. BLA-inactivated rats' learning curves were characterized by slow learning that eventually achieved the same asymptotic performance as rats with the BLA functional. BLA inactivation abolished expression of overtrained fear when rats were overtrained with a functional BLA. However, BLA-inactivation had no effect on the expression of fear in rats that learned while the BLA was inactivated. These data suggest that there are primary and alternate pathways capable of mediating fear. Normally, learning is dominated by the more efficient primary pathway, which prevents learning in the alternate pathway. However, alternate pathways compensate when the dominant pathway is compromised. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: amygdala, fear conditioning, anxiety, freezing, learning, memory.

The currently dominant view of the neurobiology of learning and memory is that there are specialized sets of independent circuits dedicated to particular functions such as spatial learning, declarative memory and emotional learning (Gold, 2004; Poldrack and Rodriguez, 2004; Squire, 2004; White, 2004). However, this "multiple memory systems" view conflicts with anatomical evidence that the brain is a highly plastic complex recurrent network or dynamical system, with multiple pathways between any two structures (Young et al., 1995). The present series of experiments seek to reconcile these two very different positions applying them to pavlovian contextual fear conditioning.

The most widely accepted view of fear is based on the assumption that there is an essential neuroanatomical circuit for fear learning and behavior that is centered on the amygdala (Fanselow and LeDoux, 1999; Davis, 2000; Pare et al., 2004; Maren, 2005). Applying this view to contextual fear conditioning (e.g. Maren and Fanselow, 1995), contextual information is encoded by the hippocampus (HPC) and converges with aversive information at the basolateral amygdaloid complex (BLA) which contains the lateral, basolateral, and basomedial nuclei. Plasticity in the BLA supports the formation and storage of an association between environmental information and shock and passes this information to the central nucleus of the amygdala (CeA), whose efferents to the ventral periaqueductal gray (vPAG) trigger the expression of fear as indexed by conditional freezing (Fanselow, 1991; LeDoux et al., 1991; Davis, 1992; Kim and Davis, 1993; Fanselow and LeDoux, 1999; Pare et al., 2004). There is a tremendous amount of evidence from lesion, pharmacological, genetic and electrophysiological studies that supports this role for the BLA in fear memory and its expression. For instance, reversible inactivation or lesions of the BLA prior to training block the acquisition of fear, while inactivation or lesions of the BLA prior to testing completely abolish the expression of fear (e.g., Miserendino et al., 1990; Helmstetter and Bellgowan, 1994; Maren et al., 1996a; Wilensky et al., 1999). Moreover, a recent study demonstrated that lesions of the BLA made either 1 day or 1.3 years following fear learning completely abolished the expression of fear, suggesting that the BLA is a site of formation and permanent storage of CS–US associative learning (Gale et al., 2004). All of these data have led to the widely held view that the BLA is an essential component of a specialized HPC→BLA→CeA→vPAG circuit that is necessary for contextual fear learning.

Contradicting this view that the BLA is essential for fear learning, Maren (1999, 2001) reported that learning deficits normally produced by BLA lesions can be mitigated with extensive over-training (see also Gale et al., 2004). However, post-training BLA lesions, regardless of the amount of training completely abolish the expression of conditional fear. Together these results suggest that if BLA function or the primary neural pathway is compromised an alternate neural pathway has the capacity to acquire conditional fear. However, since, post-training lesions abolished fear in rats it appears that plasticity in this alternate pathway does not normally play a role in fear learning. Rather, the alternate pathway plays a compensatory role only when the primary fear learning circuitry (i.e. BLA) fails.

It is not known why this compensatory circuitry is recruited when the BLA is damaged. There are a number of possibilities. The stress of the overtraining session may result in a generalized and nonspecific response that does not occur when the normal systems for dealing with fear are functioning properly. Perhaps freezing in the lesioned and overtrained rats reflects this sort of nonspecific stress response rather than context-specific fear (Fanselow, 1980). Alternatively, it is possible that the permanent le-

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Abbreviations: ACSF, artificial cerebrospinal fluid; BLA, basolateral amygdaloid complex; CeA, central nucleus of the amygdala; HPC, hippocampus; MUSC, muscimol; vPAG, ventral periaqueductal gray.

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sion-induced denervation of the structures communicating with the amygdala generated some degree of rewiring or new patterns of network activity in the rest of the fear circuit during the week that intervened between lesion and training. A third possibility is that the animal's inability to predict shock on so many trials may have provided sufficient error-correction signals to establish plasticity in a slower learning neural circuit. To address these issues, the current experiments used temporary inactivation of the BLA that only compromised neural activity, not the neurons themselves, and did so only during training and/or testing in both the trained and a novel context. We also examined the context specificity of the overtrained fear.

EXPERIMENTAL PROCEDURES

Subjects

Male Long-Evans rats initially weighing 250–280 g were obtained from a commercial supplier (Harlan, Indianapolis, IN, USA). After arrival, the rats were housed individually in standard stainless-steel cages on a 12/-h light/dark cycle and were provided free access to food and tap water. After being housed, the rats were handled daily (60–90 s per rat) for 5 days to acclimate them to the experimenter. All procedures conformed to the U.S. National Research Council Guide to the Care and Use of Laboratory Animals and were approved by the UCLA Animal Research Committee. The number of animals used was the minimum required to ensure reliability of the results, and every effort was made to minimize animal suffering.

Surgery

Under aseptic conditions, animals were given atropine methyl nitrate (0.04 mg/kg, i.p.), anesthetized with sodium pentobarbital (65 mg/kg, i.p.), and mounted into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The scalp was incised and retracted, and head position was adjusted to place bregma and lambda in the same horizontal plane.

Cannula implantation. Small burr holes were made to implant 26 gauge guide cannulas (Plastics One, Roanoke, VA, USA) bilaterally into the amygdala (from bregma: anteroposterior, −3.1 mm; mediolateral, ±5.2 mm; dorsoventral, −7.6 mm). Implanted cannulas were cemented to the skull using two anchoring screws to stabilize the dental acrylic. After surgery the cannulae were kept patent by inserting "dummy cannulae." These dummies were replaced daily with clean ones. This adapts the rat to handling during the 12–13 day recovery period making it easy to insert the injectors in awake animals at the time of muscimol (MUSC) or ACSF infusion. For drug infusion, 33-g injectors were inserted so that they extended 1 mm below the guide cannula.

MUSC infusion

MUSC free base (Sigma-Aldrich, St. Louis, MO, USA), dissolved in artificial cerebrospinal fluid (ACSF) (1 mg/mL, pH 7.4) was micro-infused into the BLA (bilaterally) by back loading the drug up a 33 gauge infusion cannula into polyethylene (PE 20) tubing connected to 10 μl Hamilton microsyringes (Hamilton Company, Reno, NV, USA). The infusion cannula protruded 1 mm beyond the guide cannula. An infusion volume of 0.25 μl/side was delivered using a Harvard #22 syringe pump (Harvard Apparatus, South Natick, MA, USA) at a rate of 0.1 μl/min. The injector was replaced with a dummy cannula 1 min after completion of the injection.

Because our intra-amygdalar MUSC infusion parameters are similar to those used in previous fear-conditioning studies (Helmstetter and Bellgowan, 1994; Muller et al., 1997; Wilensky et al., 1999, 2006; Maren et al., 2001) the extent of MUSC diffusion in the amygdala should be comparable. Based on studies that examined 3H-MUSC spreading (Krupa et al., 1996; Arikan et al., 2002) in the cerebellum in which a 1 μl volume infusion diffused a radius of 1.6–2.0 mm, it was estimated that 0.25 μl of MUSC used in the present study would spread within a radius of 0.5–0.7 mm from the infusion needle tip. Hence, it is likely that infused MUSC would have diffused to the lateral, and basal nuclei of the amygdala and possibly to portions of central nucleus and adjacent neighboring structures.

Conditioning apparatus

Context A. The context A environment consisted of aluminum (side walls) and Plexiglas (front, back, and top) chambers (28×21×22 cm; Lafayette Instruments, Lafayette, IN, USA). The floor of each chamber had 18 stainless steel rods (4 mm diameter, 1.5 cm apart) connected to a shock scrambler and generator (which, along with internal ventilation fans, supplied background noise of 70 dB, A scale). The chambers were cleaned with 5% ammonium hydroxide solution and scented with 0.1% benzaldehyde in 100% ethanol. These computer-controlled (Med-Associates, Lafayette, IN, USA) chambers were in a well lit room separate from the observers.

Context B. The context B environment was in a separate room. These chambers (same size as above) had a white rear wall inserted and two white plastic side walls (24×21 cm) placed at 60° to the floor, forming a triangular enclosure. The floors consisted of 17 staggered rods (two rows, 1 cm vertically apart; in each row, each rod was 2.6 cm apart). Background noise (70 dB) was supplied by a white-noise generator, and the chambers were cleaned and scented with 1% acetic acid solution. This room was illuminated by a 30 W red light bulb.

Experimental design

Experiment 1: The effect of BLA inactivation on learning and expression of over-trained fear. The goal of experiment 1 was to determine whether animals can learn and express fear while the BLA was inactivated. To test this, the $GABA_A$ receptor agonist MUSC was used to inhibit amygdalar neurons before overtraining and/or testing. We used freezing as a measure of fear. Freezing during overtraining and during testing was scored from videotape by an observer blind to the treatment conditions. The design was a 2 (Training Drug Treatment)×2 (Testing Drug Treatment) factorial. Half the rats received MUSC injected bilaterally into the BLA 20 min prior to overtraining. To equate MUSC experience in all animals, the other half received the same MUSC injection 20 min after training. It has been previously shown that post-training infusions of MUSC do not disrupt cue fear memory consolidation (Wilensky et al., 1999). Three days later, half of each condition received MUSC and other half received the ACSF 20 min prior to an 8 min context test in the training context.

Overtraining procedure: After a 2 min exposure to the chamber, rats were given 76 unsignaled shocks (1 mA, 2 s) with a 64 s ITI. Two minutes after the final shock, rats were removed from the chamber and returned to the home cage. Three days after training they were again infused with MUSC and tested in the original training context.

Experiment 2: BLA inactivation on the context specificity of overtrained fear. Rats were given the same overtraining as experiment 1, except that all rats received pretraining MUSC. To test for context specificity these rats were tested twice (3 days and 6 days after training), once in the training chamber and once in a novel chamber (test order was counterbalanced between animals). Freezing was measured as described for experiment 1.

Experiment 3: Intra-BLA MUSC infusions at two different time points with normal training. Rats were trained with five shocks under MUSC and the specificity of context fear was tested in the

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