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AMYGDALA KINDLING DISRUPTS TRACE AND DELAY FEAR CONDITIONING WITH PARALLEL CHANGES IN FOS PROTEIN EXPRESSION THROUGHOUT THE LIMBIC BRAIN

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Abstract—Amygdala kindling is well known to increase unconditioned fear and anxiety. However, relatively little is known about whether this form of kindling causes functional changes within the neural circuitry that mediates fear learning and the retrieval of fear memories. To address this issue, we examined the effect of short- (i.e., 30 stimulations) and long-term (i.e., 99 stimulations) amygdala kindling in rats on trace and delay fear conditioning, which are aversive learning tasks that rely predominantly on the hippocampus and amygdala, respectively. After memory retrieval, we analyzed the pattern of neural activity with Fos, the protein product of the immediate early gene *c-fos*. We found that kindling had no effect on acquisition of the trace fear conditioning task but it did selectively impair retrieval of this fear memory. In contrast, kindling disrupted both acquisition and retrieval of fear memory in the delay fear conditioning task. We also found that kindling-induced impairments in memory retrieval were accompanied by decreased Fos expression in several subregions of the hippocampus, parahippocampus, and amygdala. Interestingly, decreased freezing in the trace conditioning task was significantly correlated with dampened Fos expression in hippocampal and parahippocampal regions whereas decreased freezing in the delay conditioning task was significantly correlated with dampened Fos expression in hippocampal, parahippocampal, and amygdaloid circuits. Overall, these results suggest that amygdala kindling promotes functional changes in brain regions involved in specific types of fear learning and memory.

Key words: kindling, temporal lobe epilepsy, amygdala, hippocampus, fear conditioning, memory.

INTRODUCTION

Epileptic seizures are known to dramatically affect behavior and cognition. Patients with temporal lobe epilepsy often experience interictal (between seizure) behavioral and cognitive comorbidities that manifest as elevations of fear and anxiety, as well as memory problems (Strauss et al., 1982; Dodrill and Batzel, 1986; Schwarcz and Witter, 2002; Mula, 2013). These comorbidities have a significant impact on quality of life and can be more debilitating for the patients than the seizures themselves (Perrine et al., 1995; Cramer, 2002; Johnson et al., 2004). Unfortunately, heterogeneity among patient populations is a major obstacle for understanding the neural mechanisms that underlie alterations in behavior and cognition in epileptic patients. To overcome many of these issues, researchers have adopted the use of animal models that can investigate these topics directly.

Kindling is an animal model that has frequently been used to study the pathophysiology of temporal lobe epilepsy. Kindling refers to the gradual development and intensification of motor seizures that result from daily electrical stimulation of a discrete brain site (Goddard et al., 1969). In addition to its epileptogenic effects, kindling is particularly useful for studying the aberrant neural plasticity that promotes interictal behavioral and cognitive comorbidities (Kalynchuk, 2000; Kalynchuk and Meaney, 2003; Kalynchuk et al., 2006). In contrast to the well-described effects of kindling on unconditioned fear and anxiety responses (Botterill et al., 2012), relatively little is known about the effects of kindling on learned fear responses. Fear conditioning is a form of Pavlovian conditioning that pairs a neutral conditioned stimulus (i.e., an auditory tone; CS) with an aversive unconditioned stimulus (i.e., a footshock; US) (LeDoux, 1995). Upon presentation(s) of the CS and US, the CS predicts an aversive outcome and comes to elicit a conditioned response (CR), such as defecation, piloerection, tachycardia, and freezing behavior (LeDoux, 1995). Lesion and pharmacological studies have revealed that cued (i.e., tone) and contextual fear learning are heavily reliant on the amygdala and hippocampus, respectively (Selden et al., 1991; Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Specifically, delay fear conditioning involves a

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Abbreviations: CR, conditioned response; CS, conditioned stimulus; PBS, phosphate-buffered saline; US, unconditioned stimulus.

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co-terminating CS–US association, recruiting circuits that converge on the lateral amygdala and project to the central amygdala to elicit a CR (LeDoux, 2000). In contrast, trace fear conditioning involves a temporal gap between the CS–US presentations. Hippocampal projections containing contextual information converge on the basolateral amygdala, which then project to the central amygdala to elicit a CR (LeDoux, 2000; O'Reilly and Rudy, 2001). The distinct neuroanatomical circuitry involved in trace and delay fear conditioning therefore provides an opportunity to investigate the functional consequences of amygdala kindling on fear memory and retrieval.

We have recently shown that long-term kindling to 99 stimulations impairs fear learning in rats subjected to trace fear conditioning (Fournier et al., 2013). However, we did not evaluate whether these deficits occur at an earlier time point (i.e., short-term kindling or 30 stimulations) or under different fear learning paradigms (i.e., delay fear conditioning). The issue of short-term vs. long-term kindling is relevant because previous work has clearly shown that the magnitude of kindling-induced changes in fear and cognitive behaviors increases substantially with increasing numbers of stimulations (Kalynchuk, 2000). We therefore sought to characterize the effects of short- and long-term amygdaloid kindling on trace and delay fear conditioning. Rats were sacrificed following memory retrieval and cell counts were conducted on postmortem brain tissue immunostained for the presence of Fos protein. As Fos is a marker of behaviorally relevant neuronal activity (Morgan and Curran, 1991; Robertson, 1992; Guzowski et al., 2005), we hypothesized that the pattern of Fos immunoreactivity within the hippocampus and amygdala would parallel performance on these tasks.

EXPERIMENTAL PROCEDURES

Animals

Male Long-Evans rats, weighing approximately 200–250 g (7–8 weeks old) at the time of arrival (Charles River, Quebec, Canada) were used in this experiment. Rats were individually housed in rectangular polypropylene cages with standard laboratory bedding. Purina rat chow and water was provided *ad libitum* in a colony room maintained at an ambient temperature of 20 ± 1 °C with a 12:12 h light–dark cycle (lights on at 8 a.m.). All experimental procedures were conducted during the light period of the light-dark cycle. Experimental manipulations were in accordance with the guidelines of the Canadian Council on Animal Care and a protocol approved by the University of Saskatchewan Committee on Animal Care and Supply. We made all possible efforts to minimize the number of animals used. A total of five rats were removed from the study due to incorrect electrode placement or head cap loss during kindling.

Surgery

All rats received daily handling and a minimum 1-week habituation to the colony room prior to surgery. To begin the surgery, each rat was individually anesthetized with isoflurane (5%) and injected with a preoperative analgesic (Anafen, Ketoprofen, 10 mg/kg, s.c.) to reduce pain and inflammation. Once the rat was secured in a stereotaxic apparatus, a mixture of isoflurane and oxygen (5% initial, 2.5% maintenance) was provided through a mouth tube to maintain the anesthesia. A small incision was made down the scalp and surrounding connective tissue was excised. A single stainless steel bipolar stimulating electrode (MS-303-2-B-SPC, Plastics One, Roanoke, VA, USA) was chronically implanted into the left basolateral amygdala using the coordinates 2.8 mm posterior, 5.0 mm lateral, and 8.5 mm ventral to bregma in flat skull position (Paxinos and Watson, 1998). The electrode was secured to the skull with stainless steel screws (2 anterior, 2 posterior; 0-80 X 3/32, Plastics One) and dental acrylic. To minimize the risk of post-surgical infection, all rats received daily topical administration of Hibitane antibacterial-antifungal ointment (Chlorhexidine acetate B.P. 1% (w/w) around the incision for a minimum of 1 week.

Kindling

The experimental outline of the study is shown in Fig. 1A. All rats received a post-surgical recovery period of 10–14 days prior to the onset of kindling. The rats were then randomly divided into three separate groups such that the rats in each group began the experiment with approximately equal body weights. The three groups were long-term kindled (99 kindling stimulations, $n = 20$), short-term kindled (69 sham stimulations followed by 30 kindling stimulations, $n = 15$) and sham stimulated (99 sham stimulations, $n = 17$). All stimulations were delivered in a procedures room separate from the room in which the rats were housed. Rats received three stimulations per day, 5 days per week, with a minimum of 3 h between consecutive stimulations. The kindling stimulations were delivered using an isolated pulse stimulator (Model 2100, A-M Systems, Sequim, WA, USA) and comprised a 1 s, 60-Hz train of square-wave pulses, with each pulse lasting 1 ms with a biphasic amplitude of 800 μA (peak-to-peak). The sham stimulations were similar except that no electrical current was passed through the stimulation lead. Rats were returned to their home cage once all motor convulsions had ceased or after 30 s for sham stimulations. To control for handling effects, all rats received a total of 99 kindling or sham sessions.

The behavioral convulsion elicited by each stimulation was scored using a revised eight class extension (Pinel and Rovner, 1978) of Racine's original five class scale (Racine, 1972). The classes were operationally defined as: Class 0: immobility, Class 1: orofacial automatisms, Class 2: orofacial automatisms with head nodding, Class 3: unilateral forelimb clonus, Class 4: rearing with bilateral forelimb clonus, Class 5: rearing with bilateral forelimb clonus followed by falling, Class 6: multiple class five convulsions and falling episodes, Class 7: previous classes with running fit, Class 8: previous classes with intermittent muscle tonus. Using this classification system, rats are considered to be "kindled" after three consecutive Class 5 convulsions (Pinel and Rovner, 1978).