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Exposure to an alarm pheromone combined with footshock stress enhances responsivity of the medial amygdala-hippocampus circuit

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Abstract: Alarm substances are released under stressful situations and may constitute signals that prevent other members of the group from encountering dangerous situations by producing fear. 2-Heptanone is an alarm pheromone that increases the neuronal firing rate in temporal lobe structures that are related to fear in the rat, such as the basal amygdala. A single stress session of unavoidable electric footshock or 2-heptanone sniffing increases the responsivity of the medial amygdala-hippocampus circuit, but unknown is the timing of action of simultaneous exposure to both stressors on the firing rate and responsivity of CA1-CA3 neurons identified by their connections with the medial amygdala nucleus. Twenty-four or 48 h after a single stress session, we obtained single-unit extracellular recordings. The firing rate was higher in the 48 h group. The peristimulus histogram showed an increase in the responsivity of amygdala-hippocampus neurons, which was more pronounced 48 h after a single stress session. The present results suggest an increase in the sensitivity of this circuit after a single stress session, seemingly representing a first step in the formation of emotional memories related to a conditioned response to fear.

Keywords: 2-heptanone, Fear, Footshock, Amygdala, Hippocampus

1. Introduction

When a subject suffers from anxiety or fear, frequently other individuals who are placed in the surrounding environment may also display signs of anxiety or fear [1, 2], which can lead to dangerous collective panic attack [3, 4] through an unknown process. Several studies have used positron emission tomography to demonstrate changes in the activity of deep temporal lobe structures when subjects are exposed to photographs of faces that contain expressions of fear [5]. Exposure to odors also produces the activation of brain structures that are related to emotional processing [6]. In mammals, the olfactory system, rather than thalamic projections, is anatomically associated with structures related to emotional memory processes, such as the amygdala and hippocampus [7, 8], in which olfactory nuclei project to amygdala nuclei [9]. These anatomical and functional features open the possibility that some substances that are detected by the olfactory system may produce behavioral changes, such as those related to fear.

Alarm pheromones are substances that are emitted by an animal that experiences a dangerous situation. The alarm pheromones are then perceived by other members of the same species to inform them about the presence of danger [10]. Among alarm pheromones, 2-heptanone is a ketone that is delivered in several fluids by mammals, including humans [11]. Its abundance increases in the urine of physically stressed rats and produces anxiety-like behavior in a receptor rat [12]. Repeated 2-heptanone exposure causes the development of behavioral despair [13]. Acute 2-heptanone exposure increases the neuronal firing rate of basal amygdala nuclei, possibly reflecting the preparation to cope with a threatening situation [14, 15].

Amygdala nuclei modulate memory processing in the hippocampus [16]. During emotional experiences, the

basolateral amygdala is activated, with the strengthening of synapses on neurons that are reciprocally interconnected with the hippocampus [17, 18]. The medial amygdala nucleus plays an important role in the modulation of unconditioned fear behavior elicited by stimuli associated with predators, such as cat odor [19], trimethylthiazoline (an odor derived from fox feces) [20], and ferret odor [21].

A useful approach to study the responsivity of a given anatomical circuit is based on the stimulation of one nucleus while simultaneously recording neuronal activity from a second nucleus [22]. Using this approach, the isolated application of footshock or 2-heptanone increases the responsivity of the medial amygdala-hippocampus circuit 24 h after a single stress session [23], but unclear is whether medial amygdala-hippocampus circuit activity is modified by the combination of these stressors. Therefore, we tested the hypothesis that a single session of 2-heptanone exposure combined with footshock facilitates the long-term responsivity of medial amygdala-hippocampus connections.

2. Materials and Methods

The animal procedures were performed in strict accordance to National Institutes of Health guidelines and international and institutional standards for the care and use of animals in research [24]. Authorization was obtained from the Biomedical Research Institute (Universidad Nacional Autónoma de México) Ethical Committee to perform the study.

2.1. Animals and Housing Conditions

The study included male Wistar rats (n = 32), 3 months of age and weighing 350-400 g at the beginning of the experiments. The rats were housed in groups of five in Plexiglas boxes (45 x 30 x 30 cm) in local housing facilities and maintained under a 12 h/12 h light/dark cycle (lights on at 7:00 AM) with ad libitum access to food and water. Each rat was handled daily for 5 min each day for 1 week before the tests. All of the procedures were performed during the light period between 10:00 AM and 3:00 PM.

2.2. Apparatus

We used a Plexiglas box (30 cm length × 25 cm width × 30 cm height) that had a grid floor (stainless steel bars: 0.5 cm diameter, 1.3 cm spacing; Model E10-10R, Coulbourn Instruments, Lehigh Valley, PA, USA). The box was placed inside a noise-isolated box (56 × 46 × 40 cm; Coulbourn Instruments, Whitehall, PA, USA).

2.3. Experimental Groups

Two different groups of rats were subjected to a 16-min single aversive stimulation session. Each rat was individually placed in the Plexiglas box that was odorized with 2-heptanone by adding 0.4 ml to a small piece of cotton that was placed beneath the grid floor [13]. This concentration corresponds to the olfactory detection range reported for mice (10^{-11}-10^{-10} M) [25]. The grid floor was connected to an electronic stimulator (Grass Instruments S44, Quincy, MA, USA) coupled in series to a stimulus isolation unit (Grass Instruments SIU5, Quincy, MA, USA) and constant-current unit (Grass Instruments CCUIA, Quincy, MA, USA SIU5) that allowed the application of a total of 10 random-interval unavoidable electric footshocks (0.6 mA direct current, 1.0 s duration, 0.5 c/s) over 16 min [26, 27]. One group of rats (n = 11) underwent stereotaxic surgery 24 h after the single session (24 h group). A second group (n = 11) underwent stereotaxic surgery 48 h after an identical single session (48 h group). A non-footshocked group and non-2-heptanone-exposed group (n = 10) were also placed in a similar box for 16 min and underwent the same stereotaxic procedures as the experimental groups, at equivalent time than experimental groups. After each experimental session, the box was carefully cleaned and deodorized with a cleaning solution (0.5% ammonia, 15% ethanol, 10% extran, 5% isopropyl alcohol, 19% Pinol, and 50.5% water).

2.4. Stereotaxic Surgery

The rats were profoundly anaesthetized with ethyl carbamate (1 g/kg urethane, i.p.; Sigma Chemical, St. Louis, MO, USA). The rat's head was fixed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) for surgery. Cardiac pulse and a parietal cortical surface electroencephalogram were continuously monitored on a polygraph (Grass 79, Grass Instruments, Quincy, MA, USA). During recording, we added one-tenth of the initial dose of urethane upon the detection of any signs of alertness, such as respiratory acceleration, movements of the vibrissae, or blinking, or sudden changes in cardiac pulse. A midline incision uncovered the skull. Through a small trepanation, we lowered a glass micropipette filled with 1 M KCl (4-5 MΩ) that contained pontamine blue (Chicago Sky Blue, Sigma Chemicals, St. Louis, MO, USA) as a dye with a final concentration of 4% [28] using a hydraulic micromanipulator (Trent Wells, South Gate, CA, USA) toward the hippocampus (CA1-CA3; coordinates: anterior/posterior, -6 mm; lateral, -3.9 mm; dorsal/ventral, -2 to -3 mm) [29]. At the end of recordings a mark in the last hippocampal recorded site was left by depositing the dye with the aid of cathodic current of 20 microamperes for 20 minutes. Another trepanation was made at coordinates that corresponded to the medial amygdala (anterior/posterior, -3.0 mm; lateral, -3.3 mm; dorsal/ventral, -9.0 mm), where a stainless-steel bipolar electrode was placed (~100 kΩ resistance, 1 mm insulation uncovered at the inner tip, 100 μm diameter).

2.5. Single-Unit Extracellular Recordings

The micropipette signal was connected in series to a 7P511L Grass amplifier (Quincy, MA, USA; bandwidth pass filters: 300 Hz-3 KHz) and oscilloscope (model 5111A, Tektronix, Beaverton, OR, USA) that received a filtered signal free from background noise through a window discriminator and in parallel to an audio amplifier. The

absence of sudden changes in the amplitude of the firing rate over 300 s verified a stable recording. Afterward, each spike that was detected by the amplifier was fed to a Grass S88 stimulator (Quincy MA, USA) that delivered spike-corresponding square pulses of constant amplitude and duration (4 V, 0.6 ms). The signal was then sent to an interphase (CED MICRO 1401; Cambridge Electronic Design, Cambridge, England) that transformed the analog signal to a digital signal. The Spike2 program delivered digital data for the statistical analysis. The firing rate was analyzed using frequency histograms and peristimulus histograms (base, 50 ms; bin width, 0.1 ms).

After 1 min of basal spontaneous activity recording of hippocampal neurons, medial amygdala stimulation began (1 ms, 0.3 Hz, 1 min). For amygdala stimulation, the bipolar electrode was connected to a stimulator (Grass S88, Quincy, MA, USA) coupled to an isolation unit (Grass SIU 5a, Quincy, MA, USA). After medial amygdala stimulation was completed, hippocampal recording commenced, which lasted 1 additional minute.

2.6. Histological Analysis

To mark the last recorded point, we passed a direct current (1 min for each polarity) through the recording micropipette. After a lethal dose of pentobarbital, we perfused the rats intracardially with 0.9% saline (200 ml), followed by 30% formaldehyde (200 ml). After removal, the brain was frozen at -22°C, cut into 40 µm thick sections with a cryocut microtome (Leica-Jung, Nussloch, Germany), and dyed using the Nissl technique to reconstruct the path followed by the micropipette with the aid of stereotaxic coordinates [29]. After sectioning, only the brains for which we recognized a clear mark left by the electrodes in the hippocampus (CA1-CA3) and medial amygdala were included in the data analysis.

2.7. Statistical Analysis

We analyzed (SigmaStat 3.5) the neuronal firing rate of the hippocampus (CA1-CA3) using two-way repeated-measures analysis of variance (ANOVA), with group (Control, 24 h, and 48 h) as the first factor and recording period (basal, electrical stimulation, and post-stimulation) as the second factor. Values of $p \leq 0.05$ in the ANOVA were followed by the Dunnett post hoc test.

Based on the peristimulus histograms (25 ms), a complete database was constructed with all of the recorded neurons. Therefore, the cumulative peristimulus histograms included all of the recorded neurons. For the peristimulus analysis, we also calculated the unit activity ratio of all cells in each experimental group by comparing the number of unit spikes during the post-stimulation and pre-stimulation periods divided by the sum of the two (Post-Pre / Post + Pre). This method allowed the symmetrical depiction of excitation and inhibition around a zero point. Negative values reflected the suppression of unit activity that resulted from stimulation. Positive values reflected the facilitation of unit activity. The values varied from −1.00 (total inhibition) to +1.00 (maximum increase in firing), with zero indicating no change. This derivation compensates to a large extent for differences in baseline rates of unit firing [30]. One-way ANOVA was used to compare the results.

3. Results

3.1. Histological Control

We recorded a total of 79 neurons from the CA1-CA3 related to the medial amygdala. Of these, 20 neurons came from the control group (n = 10 rats), 29 neurons came from the 24 h group (n = 11 rats), and 30 neurons from the 48 h group (n = 11 rats). The histological analysis allowed identification of the hippocampus (CA1: 2.0-3.0 mm beneath the cortical surface) as the recording location and medial amygdala (9.0 mm beneath the cortical surface) as the stimulation location (Figure 1). The neuronal recording points were located between -2.0 and -3.0 mm beneath the cortical surface.

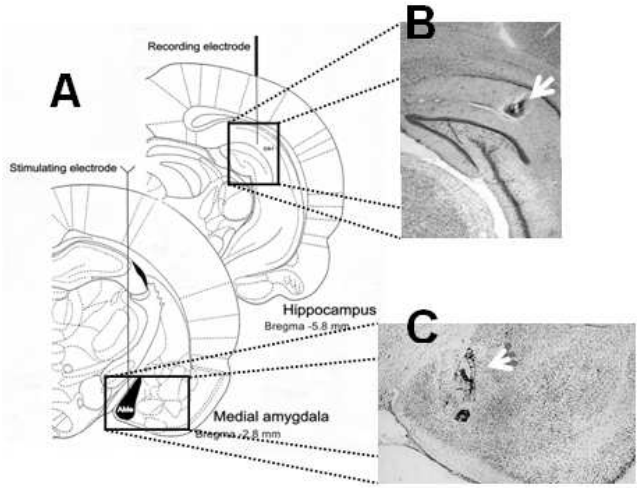

Figure 1. *(A) Schematic illustration of two coronal sections of the rat brain showing the stimulating electrode trajectory to the medial amygdala nucleus (AMe) and hippocampus (CA1). Rectangles indicate the amplified location (4x). (B) Coronal sections stained using the Nissl technique from a rat that illustrate the marks left by the recording electrode (arrow). (C) Coronal sections stained using the Nissl technique that illustrate the marks left by the stimulating electrode (arrow). The numbers indicate stereotaxic coordinates anterior to bregma.*

3.2. Single-Unit Extracellular Recording

3.2.1. Spontaneous Activity

The two-way ANOVA indicated a significant effect of group ($F_{2,225} = 5.64$, $p < 0.004$). Independent of the recording period, the neuronal firing rate was higher in the 48 h group (mean spikes, 10.31/10 s ± 0.68) than in the 24 h group (mean spikes, 7.06/10 s ± 0.69) and control group (mean spikes, 9.05/10 s ± 0.85; $p < 0.05$, Student-Newman-Keuls). No effect of recording period was found ($F_{2,225} = 0.58$, $p = 0.55$), with no group X recording period interaction ($F_{4,225} = 0.17$, $p = 0.95$).

3.2.2. Peristimulus Histogram Analysis (25 ms)

The peristimulus histogram showed that all of the experimental groups exhibited an increase in the neuronal activity of hippocampal cells after amygdala stimulation, but the response was higher in the 48 h group. The duration of the effect was longer in the 48 h group (14 ms duration) and 24 h group (15 ms duration) than in the control group (11 ms duration; Figure 2).

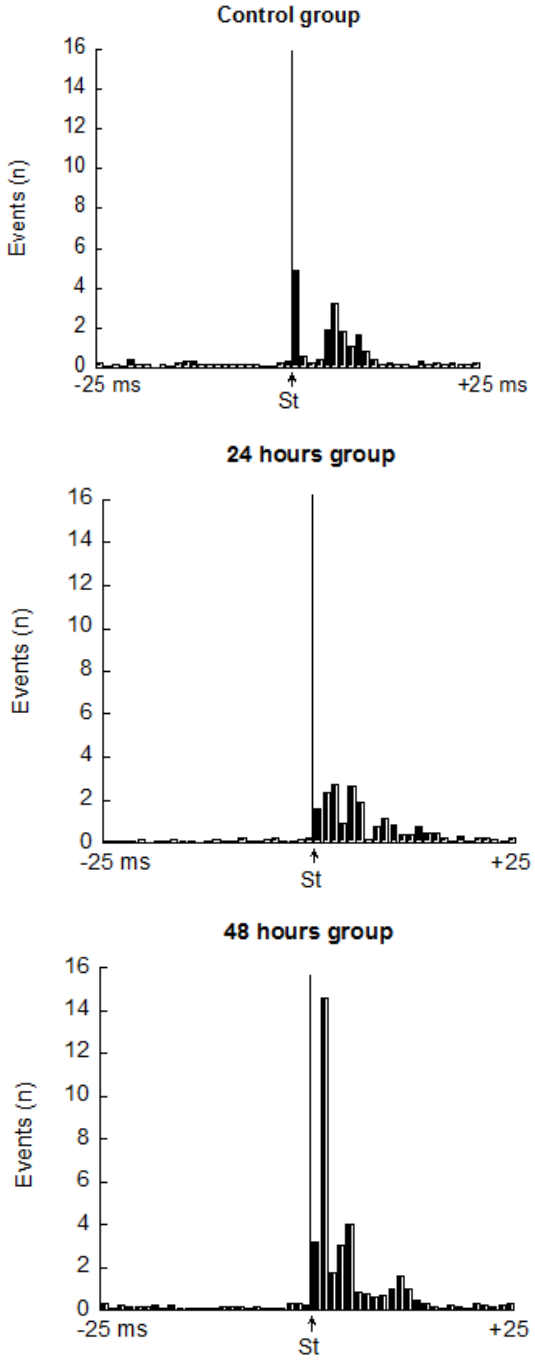

Figure 2. *Peristimulus histograms (25 ms before and after electrical stimulation). The histograms are the mean of the data from all of the recorded neurons in each experimental group. The hippocampal responses to amygdala stimulation were larger in the 48 h group than in the control group after a single stress session. Ordinate, number of events. Abscissa, time of recording before and after medial amygdala stimulation.*

3.2.3. Unit Activity Ratio

Medial amygdala stimulation had an overall excitatory effect on hippocampal neurons (Figure 3). The one-way ANOVA revealed significant differences between the experimental groups ($F_{2,76} = 7.05$, $p < 0.002$). The post hoc analysis indicated that the unit ratio was higher in the 48 h group than in the control group ($p < 0.05$, Dunnett's test).

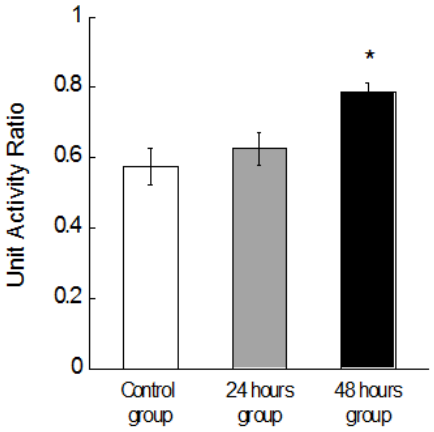

Figure 3. *Unit activity ratio. Electrical stimulation of the amygdala increased the neuronal activity of hippocampal cells in the 48 h group. Ordinate, unit activity ratio. Abscissa, experimental groups. *p < 0.05, Dunnett post hoc test.*

4. Discussion

The aim of the present study was to investigate whether a single session of stress that consisted of a combination of unavoidable footshock and an environment impregnated with the alarm pheromone 2-heptanone changes the long-term responsivity of amygdala-hippocampus connections. The results indicated that a single session of aversive stimulation increased the responsivity of hippocampal neurons to amygdala stimulation, which was evident 48 h later.

The amygdala and hippocampus encode emotionally charged experiences [16, 31], including olfactory memories, leading to the concept that the medial amygdala integrates chemosensory stimuli [32, 33], and the hippocampus is involved in processing olfactory information related to threatening situations. Exposure to predator odors, such as 2,3,5-trimethyl-3-tiazoline (a substance that is secreted in fox feces) and 2-propylthietane (a substance contained in weasel gland secretions), elicits fast waves in the dentate gyrus [34]. Exposure to cat odor produced an innate avoidance response while enhancing hippocampal long-term potentiation [35].

The amygdala is a temporal lobe structure that confers emotional valence to sensory information [36]. The medial amygdala nucleus receives direct inputs from the accessory nucleus and indirect inputs from the main olfactory bulb [32, 37] and integrates olfactory information [33], thus allowing the detection of alarm substances that derive from predators [38] or conspecifics [39]. Alarm substances increase Fos expression in the medial, lateral, and basal amygdala [40]. The medial amygdala processes unconditioned fear related to chemical signals, such as pheromones and predator odors [19].

Exposure to acute stress induces changes in glutamatergic transmission that facilitate the induction of long-term potentiation in the amygdala and ventral hippocampus [41, 42]. Acute stress that consisted of a combination of two different stressors (movement restraint and tail shock) increased the excitability of hippocampal pyramidal neurons (1 and 24 h after stress exposure) and promoted the acquisition of associative learning [43]. Amygdala-hippocampal connections, among other structures, are involved in the modulation of unconditioned fear, defense reactions, goal-directed behavior, and the formation of episodic representations of emotional situations [36, 40, 44].

Current anatomical concepts divide the human hippocampus into two parts. The anterior portion is related to memory, and the posterior portion is related to emotional processes [45]. In rats, the corresponding portions are the dorsal and ventral hippocampus, which are related to memory and emotional processing, respectively [46]. We obtained single-unit extracellular recordings from dorsal hippocampal neurons that were identified by their connections with the amygdala. The responsivity of these neurons increased after a single session of stress, suggesting some changes in memory processes.

The mammalian olfactory system processes a wide range of volatile molecules that carry vital information about the animal's environment [47]. In a previous study, an odor (amyl acetate) that was paired with footshock (one session of five odor-shock presentations) potentiated the startle response in rats [48]. Coffee odor, which is associated with neither preference nor aversion [49], produced defensive behavior in rats when they were reexposed to the conditioned odor alone, but only after five session of pairing the odor with footshock [50]. Additionally, the use of a predator odor instead of footshock has been an important tool to establish contextual conditioned stimulus associations in the brain [51, 52].

Predator odor and footshock stimulation provoked different activation patterns. Predator odor was shown to activate the dorsomedial periaqueductal gray and medial amygdala [53], whereas footshock induced broad activation in the amygdala, anterior hypothalamus, ventral hypothalamus, premamilar hypothalamus, lateral septum, dorsal periaqueductal gray, and ventral hippocampus [44]. These structures activate the amygdala, conferring an emotional meaning to any sensorial stimulus.

Amygdala activation modulates memory storage processes in the hippocampus [16]. Long-term potentiation reflects the enduring enhancement of synaptic efficacy that is induced in seconds and last hours and even days [54]. Long-term potentiation has two phases. Early potentiation lasts 1-3 h, and late potentiation lasts more than 6 h and requires protein synthesis [55]. After periods of single stimulation trains (15/s for 15-20 s), long-term potentiation in the dentate gyrus lasts from 1 h to 3 days [56]. High-frequency stimulation in the perforant path enhances activity in the dentate gyrus for 24 h, and subsequent trains of stimulation increase population spikes at 48, 72, and 96 h [57]. We obtained similar results using olfactory stimulation with an alarm pheromone combined with light physical stress (footshock). The stress session seemingly caused changes in amygdala neurons that were connected with the hippocampus that resembled the process of long-term potentiation, an effect that requires time (48 h) to be expressed.

5. Conclusion

In conclusion, a single simultaneous exposure to footshock and 2-heptanone evoked a time-dependent increase in medial amygdala-hippocampus responsivity, possibly representing an initial step in the formation of fear memory.

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