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Case Series

The Role of Acid Sensing Ion Channels (ASICs) in the Acquisition of Contextual Fear Memory in Rat

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Abstract

With the recent discovery that the protons are neurotransmitters [1] they have become a novel target of study in their role in the regulation of different physiological functions. The acid-sensing chemosensory machinery in the brain plays an important role in the modulation of several physiological functions including breathing, pain, synaptic plasticity, learning, memory, etc [2]. Also, some studies suggest that chemosensory and sleep circuitries are closely linked [3,4]. The respiratory rate remains low during NREM sleep, it, however, significantly increases during REM sleep [3]. Why breathing rate remains low during NREM sleep and increases during REM sleep remains an enigma. We have recently proposed that REM sleep possibly acts as a sentinel to keep CO_2 level within a physiological limit by increasing breathing rate and thus helps maintain longer sleep duration [3]. Also, studies have demonstrated that mouse, lacking acid-sensing channels (ASICs), exhibits a deficit in the consolidation of conditioned fear memory [2]. These studies suggest that acid-sensing chemosensory system plays a modulatory role in memory consolidation. However, the underlying chemosensory circuitries involved in cognitive functions are unknown. Here we propose to study the modulatory role of brainstem chemosensory systems in cognitive functions.

Case: I have already completed my master M.SC. in life sciences from School of Life Sciences, Jawaharlal Nehru University in the sleep analysis laboratory and my work dissertation was under the title '' The Role of Acid-Sensing Ion Channels (ASICs) of the Locus Coeruleus in the acquisition of Contextual Fear Memory in the rat'' i had worked for more than one year under the supervision the professor Sushil K Jha sir, i had worked with animal models specifically Wister rats (250_300gm). I hypothesized that Apetx1 microinjection in the Locus Coeruleus would alter the acquisition of hippocampal-dependent contextual fear memory.

Conclusions: The LC, apart from playing a significant role in sleep-wake and hydrogen ion regulation, is also involved in learning and memory [5,6]. The LC neurons fire at a low rate during NREM sleep but show a transient increase in their activity during NREM sleep after an intensive learning experience [5]. Although the amygdala and hippocampus play a primary role in the consolidation of fear-conditioned memory [7], the LC also has a major role in the consolidation of fear-conditioned memory [6]. It has been observed that the LC modulates neuronal activity in the amygdala as well as the freezing response to a fearful stimulus [8]. Interestingly, it has been recently reported that the consolidation of fear-conditioned memory requires the ASICs dependent induction of long-term potentiation at multiple amygdala synapses [9]. All these studies suggest that LC and ASICs in the amygdala are independently involved in the consolidation of fearful memory. Although, all these studies suggest that LC and ASICs may be playing an important role in the consolidation of contextual fear memory, however, our results suggest that it the ASICs in the LC does not have any role in the acquisition of contextual fear memory. We did not find memory deficit in APETX1 group compared to the vehicle group; rather both exhibited comparable freezing response.

Keywords: Locus Coeruleus; Chemoreceptor; Amygdala; Hippocampus; Fear Memory; Acquisition; Consolidation; Apetx1

Background

It has been recently identified that protons act as a neurotransmitter. However, their role in the regulation of physiological and cognitive functions are not known. The proton-sensing neurons are located in some of the brainstem areas, and they are involved in the regulation of bodily CO_2 level and breathing. Also, studies suggest

that proton-sensing channels, located outside the chemosensory areas, are involved in the modulation of several functions such as pain, neurological disorders, and cognitive functions. However, it is not known, if the brainstem chemosensory neurons can modulate learning and memory. We intend to investigate if the acid-sensing ion channels modulate hippocampal-dependent contextual fear-conditioned memory.

Hypothesis and Specific Aims

We hypothesized that Apetx1 microinjection in the Locus Coeruleus would alter the acquisition of hippocampal-dependent contextual fear memory.

Specific Aims

To study the role of Apetx1 in the acid-sensing ion channel in the Locus Coeruleus in the acquisition of fear memory.

Subjects

Two–three months old male Wistar rats (250-300gm) were used in the study. The animals were obtained from Jawaharlal Nehru University's animal house facility and brought to the school's central animal room facility, a week before the commencement of experiments, to acclimate the animals to colony conditions. All rats were housed in a plastic cage in groups of three to four animals. They were maintained on a 12:12 light-dark (L:D) cycle (lights on at 7:00 hrs and off at 19:00 hrs) at 24°C room temperature with ad libitum access to food and water. All behavioral procedure was conducted during the light phase of the cycle. The bedding of the animals was changed periodically. Animals were trained by the dedicated personal. All animals used, and experimental procedures used in this study were approved by the Institution's Animal Ethical Committee (IAEC) of Jawaharlal Nehru University, New Delhi, India.

Surgical Procedures

The animals were surgically prepared for cannula implantation for microinjection of APETX-1. Animals were anesthetized using isoflurane inhalation anesthesia. The head of the animal was cleaned and shaved. The animal was then fixed in the stereotaxic instrument and was maintained on isoflurane anesthesia through a face mask. A midline incision was made on the scalp, and the skin was cut aside to expose the skull. Bregma and lambda were aligned on the same horizontal plane. The double-barrel bilateral guide cannulas (24 gage, stainless steel) were implanted in the locus coeruleus through small holes drilled on each side of the skull at the following coordinates: AP = 9.7mm from the bregma, ML= ± 1.3 to ± 1.3 mm and DV = 2.6 mm (the rat brain atlas by Paxinos and Watson, 2007). The stainless steel obturators were placed inside the guide cannula to prevent occlusion. The guide cannula was fixed to the skull with dental cement, and the skin was sutured. The anesthesia mask was detached, and the animal was removed from the stereotaxic apparatus. The animal was treated with dexamethasone to reduce brain inflammation and nebasulfantibiotic powder at the surgical site to prevent infection for a minimum of 4-5 days. Ibuprofen was also used post –operatively to reduce pain. The animal was fed on the soft food postoperatively. The animals were allowed to recover from surgery for 5-7 days.

Contextual fear conditioning (cxfc)

Animals were engaged in the contextual fear conditioning experiments after complete recovery from surgery. The animal as initially habituated (day 1 and day 2) in the behavioral chamber (shock chamber, Coulbourn Inc, USA) for 5 minutes, between 1:00 - 2:00 PM) and after that, it was brought back to the animal colony. On the day 2, the animal was again placed in the neutral behavioral chamber during the time matched hour, and the spontaneous freezing behavior was recorded for 5 minutes in a computer using freezing frame software and CCTV camera (Sen Tech, USA).The next day (day 3) the baseline freezing behavior was recorded using freezing frame software and CCTV camera. On day 4, the animal was randomly assigned to either of two groups, vehicle (n = 6) or Apetx1 (n = 6) groups. In the vehicle group, normal saline 2µl and Apetx1 group, Apetx1, 2µl volume at 1µl/min rate were injected into the locus coeruleus using micro-infusion pump, with amicrosyringe, 30 minutes before CXFC.After that, animal was trained for CXFC using the standard protocol as mentioned below. Induced freezing behavior was recorded during the training.The animal was brought back to the animal colony immediately after training. The animal was handled and trained by an unfamiliar person to the animals. Next day (on day 5), the animal was tested for the contextual fear conditioned memory in the shock chamber, but no shock was presented. The induced freezing behavior was recorded during testing in the computer using freeze frame software and CCTV camera.The freezing response during testing was compared with spontaneous freezing behavior at the baseline statistically (one-way RM ANOVA followed by Tukey post hoc test).

Analysis of Freezing behavior

Freezing is a reliable measure of fearful memory and is defined as the cessation of all movement, except that is necessitated by

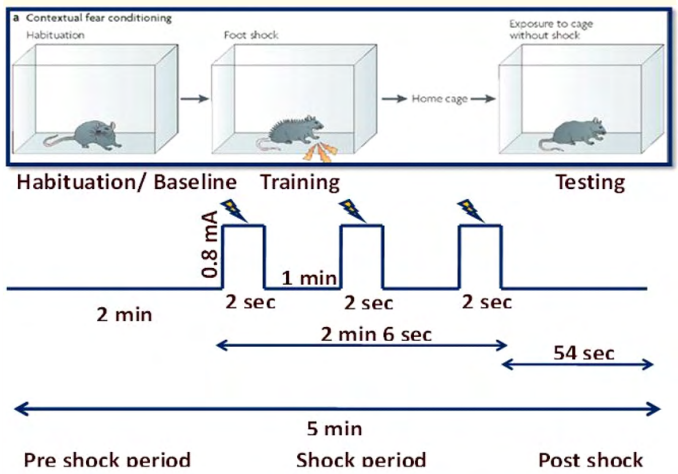

Figure 1: Contextual Fear Conditioning protocol. Image Source: Nature Reviews Neuroscience 9, 46-56 (January 2008).

breathing. The freezing behavior was analyzed offline by using Freeze software (Coulbourn Inc, USA). The percent freezing response for entire 5 minutes was calculated for baseline, training and testing days. Bout length of the motion index was set at 2 seconds where an event was recorded as a freezing response when the animal remained static for 2 seconds or more. The freezing threshold was set at 10%, at which the freezing bout peak falls around 25-75 seconds motion index. The freezing response was thus, analyzed by using computer software having a stringent criterion, with no manual intervention. The average percent freezing values were calculated for animals in each group. The changes in the freezing response in different groups were compared statistically (one –way ANOVA followed by Tukey post hoc test).

Microinjection

For Microinjection of APETX1 and vehicle, the animals were restrained by hand, and the obturators were removed from the cannula. The injector cannula (30-gauge;17 mm length) connected to the Hamilton micro –Syringe was introduced through the guide cannula. The length of the injector cannula was 1mm more than the guide cannula, so when it was inserted it protruded 1mm beyond the guide cannula. The micro-syringe filled with either APETX1 or vehicle was fitted in the micro-syringe pump (Kent Scientific, USA). A total of 0.4µlvehicle or APETX1was infused in the respective group bilaterally at 0.1µl/min rate using microsyringe pump 30 minutes prior to the training. The injector cannula was removed from the guide cannula 2 minutes after the microinjection of the drug/vehicle.

Histology

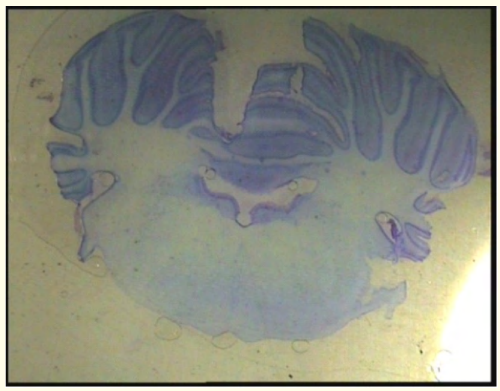

Figure 2: Cresyl violet stained brain section showing the location of cannula insertion above the locus coeruleus.

At the end of the experiment, the animals were anesthetized using thiopentone sodium (40 mg/kg IP) and trans-cardially perfused with 0.9%normal saline followed by 10% formaldehyde solution. The brains were immediately removed and stored in 10% formalin for 3 days and there after transferred into 30% sucrose solution. The brain was kept in the sucrose solution until it sank completely and then was used for histology. Using a cryostat, 40µm thick sections were obtained, which were then stained with 3%cresyl violet. The sites of injection were confirmed under the microscope.

Statistical Analysis

The overall freezing response for all the groups was plotted as mean ± S.E.M. Statistical analysis were performed using Sigma Stat3 software. The significant differences in the freezing response within the group were determine using one-way ANOVA followed by Tukey post hoc test which between groups was determined using one-way ANOVA followed by Tukey Post hoc test.

Results

The effects of Apetx1 in the Locus Coeruleus in the acquisition of contextual fear -conditioned memory:

The microinjection of APETX1 before training did not affect the acquisition of contextual fear memory. Using RM ANOVA, we found that the animals in the vehicle group (n = 6) exhibited a significant increase in freezing response compared to the baseline ($F(1,11) = 54.517$; $P < 0.001$) and the training ($F(1,11) = 17.925$; $P = 0.008$) days (Fig10). Similarly, the animals in the APETX1 microinjected group (n = 6), exhibited a significant increase in the freezing response on the testing day compared to the baseline ($F(1,11) = 48.141$; $P < 0.001$) and training ($F(1,11) = 27.586$; $P = 0.003$) days.

Although APETX1 group of animals demonstrated increased freezing response compared to the vehicle group of animals on testing day, it was, however, not statistically significant (One way ANOVA) ($F(1,11) = 1.32; P = 0.302$). Further, we tested the changes in the cumulative freezing response during five mints periods on the testing day between the vehicle and APETX1 groups. We observed that the APETX1 animals demonstrated a consistent trend towards an increase in cumulative freezing response compared to the vehicle group. Our results suggest that acquisition of contextual fear conditioned memory remains unaltered after blocking ASIC in the LC.

Percent freezing in the vehicle group

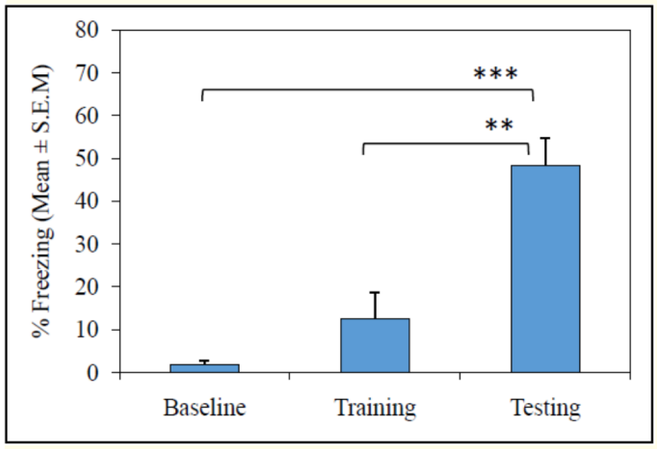

Figure 3: The percent freezing response in the vehicle group on baseline, training, and testing days. The percent freezing significantly increased on the testing day compared to baseline ($P < 0.001$) and training ($P < 0.01$) days, suggesting that animals were conditioned to contextual fear memory.

Percent freezing in the APETX1 group

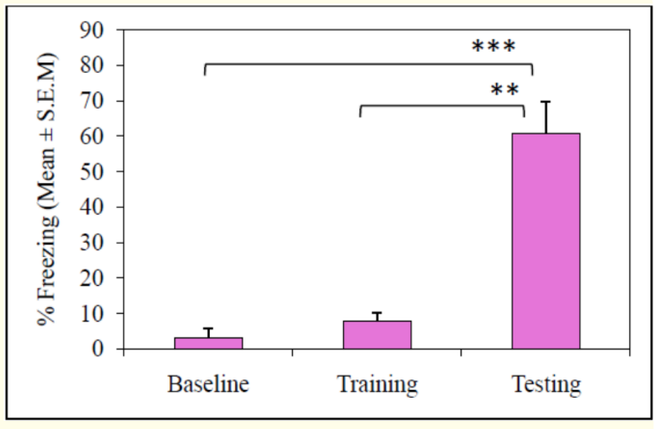

Figure 4: The percent freezing response in the APETX1on baseline, training, and testing days. The percent freezing significantly increased on testing days compared to the baseline ($P < 0.001$), and training ($P = 0.003$) days. Similar to the vehicle group, percent freezing significantly increased on testing days suggesting that animals were conditioned to contextual fear memory.

Comparative freezing responses in the vehicle and APETX1 group

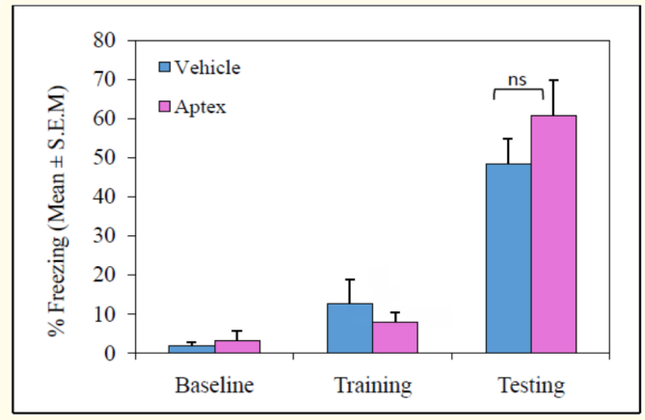

Figure 5: The changes in percent freezing response between the vehicle and APETX1 groups on the baseline, training and testing days. The animals in the APETX1 group, however, showed a comparable freezing response to the vehicle group on baseline and training days. Compared to the vehicle group, a trend towards an increase in the percent freezing response was observed in the APETX1 group, it was, however, statistically not significant.

Cumulative freezing response in the vehicle and Drug-treated groups

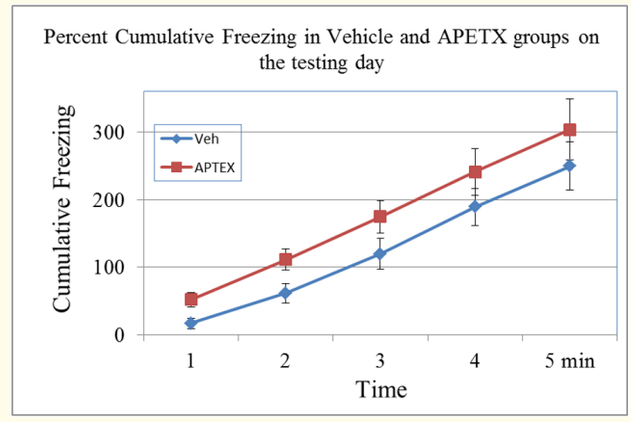

Figure 6: The changes in the cumulative freezing response of vehicle and APETX1 microinjected groups during the 5 minutes conditioning period. Both groups exhibited a comparable change in the cumulative freezing response on the testing day. However, the APETX1 microinjected group showed a trend towards an increase in their freezing response on the testing day.

Conclusions

The LC, apart from playing a significant role in sleep-wake and hydrogen ion regulation, is also involved in learning and memory [5,6]. The LC neurons fire at a low rate during NREM sleep but show a transient increase in their activity during NREM sleep af-

ter an intensive learning experience [5]. Although the amygdala and hippocampus play a primary role in the consolidation of fear-conditioned memory [7], the LC also has a major role in the consolidation of fear-conditioned memory [6]. It has been observed that the LC modulates neuronal activity in the amygdala as well as the freezing response to a fearful stimulus [8]. Interestingly, it has been recently reported that the consolidation of fear-conditioned memory requires the ASICs dependent induction of long-term potentiation at multiple amygdala synapses [9]. All these studies suggest that LC and ASICs in the amygdala are independently involved in the consolidation of fearful memory. Although, all these studies suggest that LC and ASICs may be playing an important role in the consolidation of contextual fear memory, however, our results suggest that it the ASICs in the LC does not have any role in the acquisition of contextual fear memory. We did not find memory deficit in APETX1 group compared to the vehicle group; rather both exhibited comparable freezing response.

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