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2.1 In vivo recordings- single units and local field potentials (LFPs)

2.1.1 Subjects and experimental groups: Eleven adult, male C57Bl/6 mice (26.5 – 33.5 g) were surgically implanted with a micro-drive each and subsequently housed individually in transparent plastic cages. The mice housing room was maintained on a 12-h light/ 12-h dark cycle with ad libitum access to food and water. After 6-8 days of post-surgery recovery, these mice were randomly assigned to either the Stress (n=5) or the Control (n=6) groups.

2.1.2 Chronic immobilization stress: Animals were subjected to complete immobilization (2h/day) for 10 consecutive days in mice immobilization bags (Harvard apparatus), without access to either food or water (Vyas et al., 2002). Control mice remained undisturbed in their home cages.

2.1.3 Micro-drive: Micro-drives that weighed ~4.0 gm, 3.5 cm high; (fig 2.1) and had eight independently movable tetrodes were prepared by obtaining a prefabricated base from a 3 D printer with the in-house workshop facility (RIKEN, Japan). Tetrodes were constructed from four individually insulated nichrome wires (~13 μm diameter), twisted together and heat bonded to form a single entity that was approximately 35 μm in diameter. Electrode tips were trimmed to a blunt end and gold plated to reduce electrode impedances to between 100-300 kΩ at 1 kHz.

2.1.4 Surgery: On the day of surgery, mice were anesthetized by i.p. injection of tribromoethanol (Avertin; 15 ml/kg of 2.5% w/v) solution. After shaving the head, the mouse was fixed to the stereotaxic unit and an incision was made to expose the skull. Four jeweler’s screws were inserted into the skull to provide support to micro-drive implant while an additional screw provided as ground. A small hole (approx 1.5 mm) was drilled over right hemisphere at position 1.8 mm posterior and 1.4 mm lateral to the bregma. The micro-drive implant was secured with dental acrylic and skin was sutured back in place to cover the wound.
Mice recovered within a day and after which tetrodes were slowly lowered to reach to the hippocampus.

2.1.5 Pre recording measures to reach to area CA1: The micro-drive on mice head was connected to head stage pre-amplifier and connected to the 32 channel recording system (Neuralynx, Tuscon, AZ). Over the period of one week, tetrodes were independently lowered to reach to the area CA1 of hippocampus. One tetrode was placed in overlying white matter and served as the reference channel. The presence of enhanced theta during movement, sharp-wave ripples during quiet wakefulness and presence of high amplitude, isolatable clusters of cells served as the marker for arrival of tetrodes in the cell layer (Fig 2.2). Neurophysiological signals (units and LFP) were continuously acquired at 32 kHz by using the Cheetah data acquisition system (Neuralynx, Tuscon, AZ). During this screening process, animals rested in a high walled small square sleep box. In order to maintain stable recordings, on a given day, the tetrodes were not moved prior to a recording session. For acquisition of single units, a threshold of 60 µV was enforced and the wide-band signals were digitally band-pass filtered (600Hz – 6 kHz).

2.1.6 Experimental protocol and recording apparatus: All the recordings were carried out in a square enclosure made up of black curtains on three sides, while the fourth side was a white wall with diffused red light as distal cue. This whole apparatus was located inside a room which was shielded and worked as faraday cage. The mice were brought into the recording room and remained undisturbed for ~ 30 min before the data acquisition began.

2.1.6.1 Experimental design: Neuronal recordings were obtained during track exploration (RUN) that was always bracketed by baseline activity (Rest) periods during which the animal rested in a small sleep box. In addition, the data was also obtained during the 2h immobilization period. For the sake of ease of analysis, two hour immobilization stress period was divided into four 30 minute periods: 0-30 min (First-30’ or early-phase), 30-60 min, 60-90 min and 90-120 (Last-30’ or late-phase). Since we were interested in knowing if 2h duration causes habituation of neuronal firing, here we only took into consideration First-30’ and Last-30’epochs. This data during stress was compared with an earlier ~ 15 min Rest data (Chapters 3 and 4). While mice were always immobilized and kept in an area away from recording room, on D-01, D-06 and D-10, immediately after the start of immobilization, they were brought into the recording room for neuronal recordings. It must be noted that 2h duration of Rest data recorded in Control group was obtained from only four out of six mice. To assess the effect of chronic stress on previously formed spatial representations, on D-01, mice were exposed to a Linear track-1 (LT-1) before stress. Subsequently on D-06 (after 5 days of stress) and D-11 (after 10 days of stress), animals were re-exposed to LT-1 and RUN data was recorded. In addition, to assess the effect of stress
on subsequent encoding of spatial representations, mice were also exposed to a novel C-maze on D-06. Further, to assess the effects of stress on smaller contextual differences (context discrimination), on D-11, the neuronal data was recorded while animals were exposed to another Linear track-2 (LT-2) which was similar in shape and size to LT-1. After every run session (~15 min), tracks were wiped clean with ethanol solution to minimize odor cues. To avoid complications stress-induced modulation of reward pathway (Dias-Ferreira et al., 2009) and its influence on place cell activity, animals were not provided with any food reward at the end of tracks.
2.1.6.2 Track dimensions

**LT-1**: Metallic, black, perforated, rectangular track which was 68 cm long, 8 cm wide and 11 cm in high, with two 14 cm brown colored cardboard square boxes attached at either ends.

**C-maze**: Silver coloured, aluminum, rectangular rack which was 44 cm long and 14 cm wide partitioned along the most of the length by placing a 2 cm wide and 34 cm long cardboard in the middle. One side of the partition had eight purple strips (paper tapes) at an inter strip interval of 4 cm. This configuration provided a 6 cm wide c-shaped linear track.

**LT-2**: Plastic, brown, mildly trapezoid shaped track which was 75 cm long, 5 cm wide at base and 11 cm wide at the top. Similar to LT-1, two 14 cm brown colored cardboard boxes were attached to either end of LT-2.

2.1.7 Data analysis

2.1.7.1 Histology: At the conclusion of the experiment mice were injected with an overdose of Avertin anesthesia and a small electrical current (50µA) was run down each tetrode for 8 seconds to create a small lesion at the tip of the probe. Animals were then transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB) and were decapitated. The skull was cut open and brain was gently removed and stored in 4% PFA solution for 24 hr. The brains were then transferred to 20% followed by 30% sucrose solution till coronal sections (50µm) were obtained using cryostat. The sections were then stained with
cresyl violet stain and mounted on the slides. The brain sections were then viewed and the location of the lesion was confirmed using light microscopy.

2.1.7.2 Cell sorting: Since each action potential is recorded on each wire of a tetrode, the waveforms detected on four wires are relatively unvarying while those from different cells are often distinct. Therefore by comparing the amplitude and waveform on various combination of pairs of wires of the tetrode, a cluster of spikes emerged which were assigned to a single cell. This was achieved by employing an automatic program KlustaKwik (Harris et al., 2000), followed by manual adjustment of the clusters.

2.1.7.3 Criteria to accept a complex-spike cell for analysis: The data obtained from the pyramidal layer (str.pyr.) contains both pyramidal cells as well as inhibitory neurons (fig 2.4.). In the current experiments, I have only analysed the effect of stress on pyramidal neurons (complex-spike cells). A cell had to pass following criteria to be called a complex-spike cell. Spike width >250 µS, Complex Spike Index (CSI) ≥ 5, average firing rate 0-4 Hz with a minimum of 50 spikes during ~15 min recording period. Since we analyzed immobilization stress data for ~30 min, to avoid sampling bias, for a cell to be considered for analysis it had to fire spikes greater than 100.

2.1.7.4 Criteria for a complex-spike cell to be called a place cell: Only RUN data when animal was involved in exploration at a speed > 2cm/s was used for place cell analysis. During this exploratory behaviour, local field potentials display a rhythmic 8-12 Hz theta activity (See fig 1.4 C). For a cell to be called place cell, it had to pass the following additional criteria
Place field size | \( \geq 6 \) contiguous pixels
---|---
Mean firing | > 0.2 Hz
Peak firing | > 1.0 Hz
Signal to noise | +ve

### 2.1.8 Place Cell parameters, what they mean and how they are calculated:
Each spike on the maze was assigned a position by tracking mouse’ head position by recording the location of a pair of infrared diodes by sampling at 30 Hz with help of an overhead camera.

**Figure 2.5** A representative of color coded place/rate map of a place cell in the linear track. The linear track is divided into 1 cm\(^2\) bins and place cell’s firing in each bin is plotted to give rise to colour coded pixels. The highest firing pixels are shown in dark red with firing rate descereasing from red, yellow to sky blue. Dark blue pixels represent firing rate of zero. The scale on right side displays corresponding firing rate values (Hz) for a given color. Number of contiguous pixels around maximum (peak) firing rate pixel together make a place field.

**2.1.8.1 Firing rat map of a place cell:** The linear track area is binned into 1 cm\(^2\) pixels. The camera provides the dwell time of the mice, during a recording session, in each pixel, while recording setup give the information about the number of spikes fired by the place cell in each pixel. Putting these two types of information together, a firing rate of a given pixel is achieved. For visualization purpose, the firing rates in each pixel is color coded such that highest firing rate is represented by red colour while a lesser firing rate is represented by yellow colour. The dark blue coloured pixels indicate that place cell did not discharge at all in those pixels. At this stage, we have a pixilated **rate map** of the place cell (Fig 2.5).
2.1.8.2 **Place field size:** The pixel with maximum (peak) firing is identified; let us for the sake of convenience call it “maximum firing pixel”. All the contiguous pixels that display a firing rate, not less than 10% of maximum firing pixel, together make a place field. The pixelated place field is then smoothed by using a Gaussian function (in MATLAB). Since we chose a pixel of 1 cm², a place field with 50 pixels will cover 50 cm square of the actual area on the linear track. In case if a place cell displayed more than one place field on the maze, the place field with maximum peak firing rate was taken into the consideration (not necessarily the biggest place field). However, “place representation” (percentage of track encoded), is a parameter that included all the pixels above threshold and provided a measure of percentage of track encoded by a place cell.

2.1.8.3 **Complex spike index:** CA1 pyramidal cells often display bursting activity and hence are also called complex-spike cells (Ranck, 1973). These neurons often display spike bursts, each containing 2-6 spikes of decreasing amplitude at inter-spike-interval (ISI) ranging from 5-12 ms (McHugh et al., 1996). Neuronal bursting has been hypothesized to cause supralinear summation of EPSPs in synapses such that postsynaptic targets more reliably display spiking activity by a burst of spikes than the same number of single spikes separated by longer intervals. Complex Spike Index (CSI) combines a measure of neuronal bursting with a measure of the likelihood that later spikes in bursts are smaller in amplitude than spikes earlier in bursts.

\[
\text{CSI} = 100 \times (\text{pos} - \text{neg})
\]

**pos:** ISIs that contribute positively to CSI i.e. preceding ISIs with spike amplitude difference > 0 and following ISIs with spike amplitude difference < 0

**neg:** ISIs that contribute negatively to CSI i.e. preceding ISIs with spike amplitude difference < 0 and following ISIs with spike amplitude difference > 0

2.1.8.4 **Spatial information content (bits/spike):** Information content is another parameter that is used to predict animal’s location from the firing of a place cell. Information content measures the amount of information carried by a single spike about the location of the animal. Of course, the higher the spatial information content, the more reliably a single spike can predict the animal’s location. Information content is calculated by using Skaggs’ formula (Skaggs et al., 1993):

\[
\text{Spatial information content} = \sum P_i \left( \frac{R_i}{R} \right) \log 2 \left( \frac{R_i}{R} \right)
\]

Where: \(i\) is the bin/pixel number, \(P_i\) is the probability for occupancy of bin \(i\), \(R_i\) is the mean firing rate for pixel \(i\) and \(R\) is the overall firing mean rate.
2.1.8.5 Spatial coherence: An ideal place field has highest firing rate in the centre which gradually decreases away from the center of place field. Coherence measures the extent to which the firing rate in a pixel is predicted by the rates in its neighbouring pixels. Thus an abrupt change in firing rates of neighbouring pixels will make the place field less coherent. Coherence is calculated by correlating the firing rate in a pixel with the average firing rate of its neighbouring 8 pixels. The rate for the neighbouring pixels is the sum of all spikes divided by the sum of dwell times in these pixels. Single correlation values obtained from the rate array thus measures the local smoothness of firing rate contours.

2.1.9 Sharp-wave associated ripple activity: Raw LFP signal recorded from the tetrodes were first down sampled (rate-reduced) to 1.627 kHz and were digitally filtered (1-600 Hz) and smoothed with a Gaussian kernel. Ripples were defined as LFP that stayed 5SDs above the mean power in the ripple frequency band (150-200Hz) for at least 30ms. CA1 ripples exhibit

![Figure 2.6 A screenshot of local field potential (LFP) activity at different depths in the brain.](image)
maximum amplitude in pyramidal cell layer (Fig 2.6) and decreases with distance away from the cell body layer (Buzsáki et al., 1992; O’Keefe and Nadel, 1978). The farther away the recording tetrode is from the cell body layer, the smaller the ripple amplitude will be. Averaging the raw values (of ripple amplitude) across animals thus can potentially induce greater variability and may eclipse stress-induced changes in ripples characteristics. Therefore, in analysis of ripple activity, values from each animal were transformed into a percentage of its Rest value and then compared between Control and Stress groups.

**2.1.10 Statistics:** All the codes for place cell analysis were run in MATLAB. All statistical comparisons were performed in Origin 8 (Originlab software). Since, in many cases (Rest, First-30’ and Last-30’), data were not normally distributed (according to a Kolmogorov-Smirnov test), nonparametric statistical tests were used whenever possible. In these cases Mann Whitney U (MWU) test was employed to compare medians from two groups. When comparing more than two groups, a Kruskal-Wallis ANOVA was used, followed by post hoc comparisons based on MWU test for multiple comparisons. For normal data, t-test or one-way ANOVA followed by post hoc comparisons based on Tukey’s test for multiple comparison was used. For repeated comparison within a group, repeated-measure ANOVA followed by post hoc comparison based on paired t-test were used. In addition, for comparison of activity of same place cells across two tracks on either D6 or D11, paired-t-test was employed. Nonparametric data was displayed by box plots with its inter-quartile-range as boundaries, median of the data is represented by horizontal lines, the small square inside the box plot represent the mean of the data. Normal data was displayed by bar graphs with Mean ±SEM. All the plots were made in Origin 8 (Originlab software) and then copied into CS5 for final figure preparation.

**2.2 Behavioural experiments - auditory fear conditioning, contextual fear conditioning and elevated plus maze test**

**2.2.1 Stress protocol**

**2.2.1.1 Chronic immobilization stress:** Animals were subjected to complete immobilization (2h/day) for 10 consecutive days in plastic bags, without access to either food or water (Vyas et al., 2002). Control mice remained undisturbed in their home cage.

**2.2.1.2 Acute immobilization stress:** Animals were subjected to complete immobilization for a 2 hour duration in immobilization bags without access to either food or water (Mitra et al., 2005). Control mice remained undisturbed in their home cage.

**2.2.2 Animals and housing protocol:** Male rats (~1.5-2.0 months old) were used for fear conditioning and anxiety experiments. All animals were housed in groups of two or three with *ad libitum* access to food and water and were maintained in a temperature-controlled room with...
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a 14h/10h day/night cycle. Notice that contextual fear conditioning experiments were done in Long Evans rats as they are the most favoured rodent strain to be used in visual tasks and also because my experiments were motivated by Fanselow’s research that routinely uses Long Evans rats (Fanselow, 1990; Maren et al., 1996; Wiltgen et al., 2006). Auditory fear conditioning and EPM experiments involved the usage of Sprague-Dawley rats as they are the most commonly used strain in AFC (Bauer et al., 2002; Quirk, 2002).

2.2.3 Contextual fear conditioning paradigm

Figure 2.7 Experimental design for contextual fear conditioning. On Conditioning day, rats receive a foot-shock (1.5mA for 2 s) after placement into the conditioning context (placement to shock interval) at one of the four intervals 0-10s, 20-30s, ~3 min or ~5 min. Next day, animals are again placed into the same context and assessed for context fear memory by analysing freezing response for 5 min duration.
2.2.3.1 **Apparatus:** Both Conditioning and testing were done in the same conditioning arena (Context-A) which was made of aluminum (side walls and roof) and Plexiglas (front and back walls) with a floor consisting of a removable stainless steel grid (see fig 2.7). An overhead yellow light illuminated the arena. This whole setup was placed inside an Isolation cubicle (30"W x17.75"D x18.5"H) that works as a sound attenuator (Coulbourn instruments, Allentown, PA). The foot-shock delivery (1.5mA for 2s) to the animals was delivered by a shock unit (Habitest Line) that was controlled by an adjacent computer with Graphic State 2 software. The Conditioning chamber including the floor was thoroughly cleaned with alcohol between sessions. Animals were considered to be freezing if no obvious movement except that of breathing was detected.

2.2.3.2 **Experimental procedure:** Contextual fear conditioning (CFC) is a two day protocol (except in preexposure experiments). First day, rats receive a single foot-shock (1.5mA for 2 s) after a set interval of exposure to the context. This interval between animal’s entry to the conditioning context and foot-shock delivery is termed as placement to shock interval (PSI). 24 hour later, rats are again exposed to the same context (fear recall) and freezing behaviour is analysed for 5 min duration. The data between any two groups was compared by employing two sample-unpaired t-tests. The legend under the relevant figures provides the statistical significance (p-values).

2.2.3.3 **Experimental groups**

Based on PSI both Control and Stress groups can be subdivided into four subgroups:

- **0-10 PSI:** received foot-shock within 10 s of placement into the Context
- **20-30 PSI:** received foot-shock between 20-30 s of placement into the context
- **3 min PSI:** rats received foot-shock at approximately 3 min after the placement into the context
- **5 min PSI:** rats received foot-shock at approximately 5 min after the placement into the context

2.2.4 **Auditory fear conditioning paradigm**

2.2.4.1 **Apparatus:** The apparatus consisted of a conditioning arena (Context-A: 12"W x10"D x12"H) and a testing box (Context-B). Each box was placed inside a sound attenuation chamber (Coulbourn instruments, Allentown, PA). The conditioning arena (Context-A) was the same as described earlier in CFC experiment. The fear testing box (Context-B) was a rat housing box.
(off white colored), with transparent Plexiglas walls, husk as bedding, white incandescent light, white coloured walls and contained peppermint odour.

Conditioning arena and testing box were always placed inside an Isolation cubicle that works as sound attenuator (30"W x17.75"D x18.5"H) (Coulbourn instruments, Allentown, PA). The delivery of the auditory tone (CS) and foot-shock (US) were controlled by Graphic state 2 software (Coulbourn Instruments, Allentown, USA). Rat’s behaviour during conditioning and fear recall was videotaped and subsequently analysed to measure fear behaviour by assessing freezing response. After recording from each rat, the rat-housing box was changed while the transparent plexiglas walls were cleaned with alcohol.

2.2.4.2 Fear conditioning procedure: Auditory fear conditioning consisted of a three day protocol. On first day, rats receive a 10 min habituation session each in a “to-be-conditioning context” and also in a “to-be testing box” (without peppermint odour). Next day, after a 3 min habituation to the context, rats received either a paired or an unpaired conditioning protocol. 24 hours later, animals are placed in the testing chamber and after a 2 min context habituation, receive the same auditory tone (CS) thrice (for 30 s each) with an inter-trial-interval (ITI) varying between 60 and 120 s. Freezing response (CR) during tone presentation was later analysed to assess their conditioned fear. Animals were considered to be freezing if no obvious movement except that of breathing was detected.

2.2.4.3 Experimental groups and conditioning protocol: Based on the type of conditioning protocol that rats received, they belonged to:

Naïve group: animals did not receive a conditioning protocol.

Paired group: During conditioning procedure, after a 3 minute acclimatization to the context, animals received 5 pairings of a 20s auditory tone (CS: 5 kHz, 75dB) that each time co-
terminated with a 0.5 s foot-shock (US: 0.5mA for weak conditioning and 1.0mA for strong conditioning). The inter-tone-interval during conditioning varied between 60-120 s.

**Unpaired group:** During this “pseudoconditioning” procedure, after a 3 min acclimatization period, animals received five presentations each of a 20 s auditory tone as well as 0.5 s foot-shock (0.5mA for weak conditioning and 1.0mA for strong conditioning) in a staggered manner such that they never overlapped temporally. The inter stimulus-interval (ISI) varied from 60-120 sec. The data between any two groups was compared by employing two sample unpaired t-tests.

![Experimental design of auditory fear conditioning](image)

**Figure 2.9 Experimental design of auditory fear conditioning.** On habituation day, rats receive 10 min exposure each to Context-A and Context-B. Next day, during conditioning, animals receive five tone-foot-shock pairings in Context-A. One day later, during fear recall (testing) rats again experience the same auditory tone (thrice) in Context-B. The freezing response during tone delivery provides a measure of conditioned fear.

**2.2.5 Elevated plus maze test for anxiety:** Elevated plus maze test (EPM) is a standard test used to measure rodent’s anxiety-like behaviour. EPM consists of two open arms and two closed arms and the whole maze is elevated to a height of 75 cm above the ground. Both open
and closed arms were 60 cm long and 15 cm wide. Closed arms also had 15 cm high metal walls around it (except top) while open arms are bounded by 1 cm Plexiglas edges. Open arms were illuminated such that at the end of the arms the light intensity was 120 lx while at the center it was 80 lx. Black curtains were drawn around the maze to avoid any visual cues/attractant. Animals were placed at the central square (15 cm x 15 cm) of the maze, facing an enclosed arm, and were tested for anxiety-like behaviour for 5 min duration. Overhead CCTV camera recorded rat’s behaviour for future analysis. Between sessions, the maze was cleaned with 40% ethanol. An arm entry was defined as the entry of all four paws inside it. Time spent in open arms, closed arms as well as entries to the arms were used to assess animal’s anxiety. The data between Control and Stress groups was compared by employing two sample unpaired t-tests.