Dorsal hippocampus mediates light-tone sensory preconditioning task in mice

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Abstract

Daily choices are often influenced by environmental cues that are not directly linked to reinforcers. This process is known as higher-order conditioning and can be measured using sensory preconditioning tasks in rodents. This behavioral paradigm requires the repeated and simultaneous presentation of two low-salience stimuli, such as a light and a tone, followed by a devaluation phase where one stimulus is paired with an unconditioned stimulus, such as a mild footshock. The outcome is a conditioned response (i.e. freezing response) to both the conditioned stimulus (direct learning) and the non-conditioned stimulus (mediated learning). In our study, we set up a successful light-tone sensory preconditioning task in male and female mice. Sex differences were seen on the number of conditioning sessions required to acquire mediated learning and in the behavioral responses observed in certain control experimental groups. We used *in vivo* calcium imaging to characterize the activity of hippocampal neurons in the dorsal and ventral subregions of the hippocampus when associations between low-salience stimuli and reinforcers occur. Finally, we combined our sensory preconditioning task with chemogenetic approaches to assess the role of these two hippocampal subregions in mediated learning. Our results indicate that dorsal, but not ventral, CaMKII-positive cells mediate the encoding of low-salience stimuli during the preconditioning phase. Overall, we implemented a novel light-tone sensory preconditioning protocol in mice that allowed us to detect sex differences and to further elucidate the role of particular hippocampal subregions and cell types in regulating these complex cognitive processes.

Introduction

The brain mechanisms underlying associative learning have been classically elucidated through classical conditioning paradigms using strong salient stimuli as reinforcers such as an electric footshock paired with low-salience stimuli (e.g., light, tone, odor, etc). These important cognitive processes are not sufficient to fully understand animal behavior because daily choices are not always dictated by stimuli that have been directly associated with a potent reinforcer. Indeed, humans and animals frequently respond to cues that were never explicitly conditioned, but they happened to be previously associated with other stimuli paired with a specific aversive or rewarding meaning[1-5]. These higher-order conditioning processes, also known as mediated learning, can be captured in laboratory settings through sensory preconditioning procedures[2,6-11]. These tasks combine incidental associations between low-salience stimuli (e.g., odors, tastes, lights or tones) during a preconditioning phase followed by a classical conditioning of one of these stimuli with an aversive or appetitive unconditioned reinforcer[2,6-11]. As a result of these processes, subjects present aversion or preference to the stimulus never explicitly paired with the reinforcer, therefore allowing the evaluation of mediated learning[2,6-11]. Whereas brain circuits underlying classical direct associative memory between neutral cues and reinforcers have been largely studied, the brain mechanisms underlying incidental associations between low-salience sensory stimuli and their impact on behavior have been much less explored.

Hippocampus and other cortical regions (e.g., perirhinal, retrosplenial and orbitofrontal cortices) are involved in higher-order conditioned responses[6,8,10-14] assessed through different sensory preconditioning tasks using different sensory modalities. Previous findings suggest that the involvement of several brain regions in higher-order conditioning might depend on the different behavioral phases studied (i.e., preconditioning or testing) or the different sensory modalities used (visual, gustatory, olfactory or auditive cues). However, it is still unknown if there could be common brain locations where the different stimuli are integrated independently of the behavioral protocol or sensory modality used. In this sense, the hippocampus has been suggested to play an important role in different behavioral phases of sensory preconditioning procedures across species[1,4,11]. This brain region, which continuously exchanges information with other cortical areas, has been proposed as a possible integrator of previous experiences into several representations through a precise excitatory-inhibitory balance[15,16]. Indeed, previous studies pointed to a specific role of hippocampal mechanisms during the encoding of incidental associations[11]. However, the role of

different hippocampal subregions or cell types in the formation of mediated learning is still not understood. Indeed, the dorso-ventral axis of the rodent hippocampus is structurally and functionally segregated[17,18]. The dorsal hippocampus, which is connected with cortical regions and the thalamus, is mainly involved in cognitive functions such as navigation and exploration[17-21]. On the other hand, the ventral hippocampus is mainly connected to the amygdala, nucleus accumbens and hypothalamus, and is involved in motivated and emotional behavior[17,18,22]. In this context, our initial hypothesis was that dorsal and/or ventral hippocampus could be differentially involved in mouse sensory preconditioning.

We took advantage of chemogenetic and imaging approaches using an adapted light-tone sensory preconditioning[6] ($_{LT}$SPC) procedure in mice, to suggest that the activity of dorsal hippocampus, in particular the activity of CaMKII-positive cells, is crucial for the formation of incidental associations between lights and tones, eventually leading to mediated learning. Understanding the differential contribution of dorsal and ventral hippocampus to sensory preconditioning tasks provide valuable insights into the mechanisms underlying higher-order conditioning and the broader functioning of the hippocampus in associative processes.

Material and methods:

Animals

Male and female C57BL/6J mice were purchased from Charles River Laboratories and were used for behavioral and chemogenetic experiments. Male Parvalbumin (PV)-Cre mice were obtained by own breedings in our animal facility. All the mice used in this study were 8 weeks old at the beginning of the experiments and they were grouped-housed and maintained in a temperature (20–24°C) and humidity (40%–70%) controlled condition under a 12 h light/dark cycle and had ad libitum access to food and water. All behavioral studies were performed during the dark cycle (from 9am to 5pm) by trained researchers who were blind to the different experimental conditions.

All experimental procedures shown in this work have followed European guidelines (2010/62/EU) and were approved by the Committee on Animal Health and Care of Barcelona Biomedical Research Park (PRBB) and from the Generalitat de Catalunya. All experiments were performed in the animal facility of the PRBB, which has the full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Light-tone sensory preconditioning task ($_{LT}$SPC)

The task was performed using automatized conditioning chambers (Imetronic, France) and was divided in different phases (Figure 1A):

Habituation: Animals were exposed to the chambers with the presence of background noise (65db) (similar to white noise but allocated to different speakers to avoid that animals associate the sound with a location) during one session of 20 minutes.

Preconditioning Phase: This phase was composed by six sessions (2 per day; 3 hours of intersession interval) of 510 seconds each under background noise. These sessions started with an OFF period of 3 min and were followed by 5 simultaneous presentations of light (CS1, white LED light located in one side of the box) and tone (CS2, 65db,3000Hz) during 30 seconds with an intertrial interval of 30 seconds and a final OFF period of 1 minute. This protocol details apply to the Paired and No-Shock experimental groups. For the unpaired group, light and tone were never associated together during this phase. Indeed, animals were exposed to the same amount of time to light and tone but in different sessions (S1 light, S2 tone, S3 tone, S4 light, S5 light, S6 tone, in a pseudo-randomized way).

Conditioning Phase: This phase consisted of two training sessions in males and one training session in females. Each session last 510 seconds under background noise. In case of males, there was an inter-session interval of 3 hours. Specifically, the sessions started with an off period of 3 min and were followed by 5 presentations of a 10-seconds light stimulus (CS1) that co-terminate during 2 seconds with a mild footshock (0.4 mA, see results) with an intertrial interval of 1 min and a final OFF period of 1 min. This protocol details apply to the Paired and Unpaired experimental groups. For the No-Shock group, animals followed the same conditioning session but without the exposure to the electric footshock.

Probe Tests Phase:Mice were subjected to two probe test sessions on the same day where they were exposed to the tone (Mediated Learning, Probe Test 1) and the light (Direct Learning, Probe Test 2) in a new context (different from the previous phases). In particular, we changed 4 features of the context: floor texture, wall colors, smell (from 70% ethanol to a CR36 disinfectant solution), and absence of background noise to avoid fear responses elicited by the context itself. The two Probe Tests lasted 6 minutes and were separated for at least 1 hour. In this session, animals underwent an OFF period of 3 min followed by an ON period of 3 min where the Tone (Probe Test 1) or the Light (Probe Test 2) were continuously exposed. All experimental groups (Paired, Unpaired and No-Shock) perform the Probe Tests in the same manner. The presence of mediated and direct learning is shown by the freezing time duration during ON and OFF periods or with the percentage of change between these periods. This percentage of change of each individual comparing OFF and ON periods were calculated using the formula:

$$\%change = \frac{(time\ freezing\ during\ ON\ period - \ time\ freezing\ during\ OFF\ period)}{time\ freezing\ during\ OFF\ period} * 100$$

The behavior analysis was automatized using Deeplabcut to track the animal's position across time and a Python homemade script to compute all the different analyses, graphs, and statistics performed in the present paper. The main behavior measured was freezing response (i.e. immobility), which was defined as an euclidean distance lower than 0.02 cm per pair of frames for videos with 25 fps, resulting in a speed lower than 0.5 cm/s. We validated this automated behavioral counting by performing correlations with manual counts and another software dedicated to freezing scoring (EzTrack)[23], in which we found a high correlation (higher than 90%) (Supplementary Figure 1). The Deeplabcut project and all scripts will be available in the GitHub repository of the lab.

***In vivo* Fiber Photometry**

Male PV-Cre mice were anesthetized with a mixture of ketamine (75 mg/Kg, Imalgene 500, Merial, Spain) and medetomidine (1 mg/Kg, Domtor, Spain) by intraperitoneal (i.p.) injection. Then, animals were placed into a stereotaxic apparatus (World Precision Instruments, FL, USA) with a mouse adaptor and lateral ear bars. For viral intra-hippocampus delivery, AAV vectors were injected with a Hamilton syringe coupled with a nanofill attached to a pump (UMP3-1, World Precision Instruments, FL, USA). Mice were injected with a mixture of 200 nl of AAV.Syn.NES.jRCaMP1a.WPRE.SV40 (titer: 1×10^{13}, addgene 100848-AAV9) and 200nl of a Cre-dependent AAV-syn-FLEX-jGCaMP8f-WPRE (titer: 1×10^{13}, addgene 162379-AAV9) in order to monitor both the activity of the synapsin-positive cells in red and the PV interneurons in green. These viral vectors were infused (1 nl/s) directly into the dorsal (in one hemisphere) or ventral hippocampus (in the other hemisphere) with the following coordinates in mm: dorsal, AP ±1.5, ML ± 2, DV -1.5 and ventral, AP ±3.5, ML ± 3.3, DV -3.5, according to Paxinos and Franklin brain atlas (Paxinos and Franklin, 2001). After the AAV infusions, an optic Fiber (core 400 µm, N.A 0.5, RWD, China) was implanted using dental cement following the same coordinates except for DV that was placed 0.25 mm above viral infusions. Four weeks after this surgery, animals were used for *in vivo* calcium recordings where they undergo a $_{LT}$SPC and *in vivo* recordings were performed during Light-Tone associations (preconditioning phase) and Light-Shock associations (conditioning phase). Before this experiment, animals were habituated for 3 days to connect and disconnect the optic fibers and to be habituated to the cable in the same chamber where the behavioral experiment was performed. During these two behavioral phases, *in vivo* recordings were performed using a commercial Fiber Photometry equipment (RWD, China) where we used 470nM LED to excite the GCaMP sensor, and 560 nM for the RCaMP signal. In all mice used, after the recording experiments, the signal was checked to validate the viral infusions.

To analyze the Fiber Photometry experiments, a custom python code was used and the behavioral videos and photometry recordings were synchronised by TTL signals. In particular, raw calcium signals were pre-processed by removing the first minute of the recording to decrease the effect of the initial exponential photobleaching, and by removing point artifacts. The 470nM signal was fitted to the isosbestic 405nM using a linear fit and for each time point, ΔF/F was calculated as (F470nm – F405nm(fitted))/F405nm(fitted). This procedure is the same as described in previous works[24]. The codes used for the analysis will be found on the dedicated github repository.

Chemogenetic modulation

Stereotaxic surgeries were performed as described above for fiber photometry. In this case, C57BL/6J mice were injected with 500 nl AAV-CaMKII-mCherry (titer: 7×10^{12}, 114469-AAV5) and 500 nl AAV-CaMKII-hM4Di (titer:7×10^{12}, 50477-AAV2) directly into the hippocampus, with the following coordinates: dorsal, AP ±1.5, ML ± 2, DV -1.5 and ventral, AP ±3.5, ML ± 3.3, DV -3.5, according to Paxinos and Franklin brain atlas (Paxinos and Franklin, 2001). On the other hand, PV-Cre mice were infused with AAV-DIO-hM4Di using the same coordinates to target the dorsal hippocampus. Three control groups were performed for each subregion (dorsal and ventral hippocampus): animals infused with control virus (pAAV-CaMKIIa-mCherry) and injected with saline or JHU37160 dihydrochloride (J60, 0.1 mg/Kg, i.p., HelloBio, HB6261)[25], and animals infused with AAV-CaMKII-mCherry or AAV-DIO-hM4Di injected with saline. Animals were used for chemogenetics experiments four weeks after injections to get an optimal expression of the viruses. The injection of J60 was one hour before each preconditioning session (C57BL/6J and PV-Cre mice) or before the Probe Test 1 (C57BL/6J mice). In all mice used in the behavioral experiments, the signal was checked, and representative images are shown in Supplementary Figures.

Histology

After the chemogenetic and imaging experiments, mice were anesthetized i.p with a mixture of ketamine (50 mg/Kg) and xylazine (20 mg/Kg) in overdose (3-4x body weight), transcardially perfused with cold 4% paraformaldehyde to fix tissues. Brains were removed and sectioned in serial coronal sections of 20 um and collected directly to the slide for further analysis in the microscope. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, ref. 00-4959-52, Fluoromount-G w/DAPI, Life Technologies, USA) to visualize cell nuclei in the mounting medium. Slides were coverslipped and imaged by an epifluorescence Nikon Eclipse Ni-E. All animals were signal checked to guarantee the target of the hippocampus subregion.

Data collection and statistical analysis

Data collection

All mice were randomly assigned to experimental conditions. Researchers performing the experiments were always blind to the condition of the subject and we used an automated way to analyze behavioral responses throughout the study. Raw data was processed and analyzed using homemade python packages.

Statistical analysis

Graphs and statistical analysis were performed through homemade python scripts that will be openly shared. All data comes from distinct mice and is shown as independent

data points per animal ± standard error of the median (SEM). Normality and homoscedasticity of the data were assessed with the Kolmogorov-Smirnoff and Levene tests, respectively. Due to non-parametric properties of the data, a generalized linear model with gamma distribution was used for multivariable analysis (after a goodness of fit to select the appropriate distribution), followed by planned comparisons corrected with false discovery rate. For univariate analysis, the Kruskal-Wallis test were performed followed by planned comparisons corrected with false discovery rate., For simple comparisons the Mann-Whitney test were performed, and for comparisons of indexes against zero the Wilcoxon test with zero method were used. Detailed statistical data for each experiment can be found in Extended Table 1 (for Main Figures) and Extended Table 2 (for Supplementary Figures).

Results

Female and male mice show mediated learning using an auditory-visual sensory preconditioning task.

Sensory preconditioning tasks using olfactory and gustatory stimuli were already demonstrated in mice in several previous works[11,14]. However, other sensory modalities such as visual and auditory stimuli are also very relevant for animal daily choices. Whereas the use of these cues to assess aversive sensory preconditioning was also shown in rats[6,8,13], the use of auditory and visual cues in mice is limited[11], especially for aversive paradigms. The first aim of the present work was to set up a mouse sensory preconditioning task using auditory and visual cues to be able to assess mediated and direct learning in mice. In this regard, a sensory preconditioning protocol was established (see Methods, Figure 1A) together with the use of a novel automatized tool to analyze freezing response (i.e. immobility) in mice by combining DeepLabCut pose estimation and a new generated python script. This novel tool was validated and highly correlated with manual counting and the ezTrack[23] freezing scorings (Supplementary Figure 1).

Using male and female mice, we observed that the exposure to the Tone (Probe Test 1, ON period) induced an increased freezing time (Figure 1B) in relation to its absence (OFF period) and also a significant increase in the percentage of change (Figure 1C), suggesting the presence of mediated learning. When the light was presented (Probe Test 2), animals showed an increase in freezing response in the ON period in comparison with the OFF period (Figure 1D) and increased percentage of change in the ON period (Figure 1E), revealing the existence of direct learning. Both measures, percentage of time in freezing and percentage of change, revealed no significant changes between male and female mice. However, male mice needed to be trained with two conditioning sessions, whereas female mice were trained only with one session, to see a comparable mediated and direct learning in both sexes. During the set-up of this paradigm (Supplementary Figure 2), we realized that males with 1 training session presented lower expression of mediated learning in comparison with males that performed 2 training sessions or females with 1 training session. This data suggests potential sex-dependent differences in the sensory preconditioning task, where each protocol need to be adapted depending on the sex used.

Simultaneous light-tone associations during preconditioning are required for mediated learning formation.

At this point, we wanted to discard any bias due to direct behavioral effects induced by the exposition to certain sensory cues in order to fully validate our $_{LT}$SPC task. We performed a $_{LT}$SPC experiment with three different experimental groups (Figure 2A, see

Methods): Paired (exact protocol conditions as shown in Figure 1), Unpaired (same protocol as the paired group but light and tone were never associated together) and No-Shock groups (same protocol as the paired group but without the exposure to the electric footshock). Following this experimental design, male (Figure 2B and 2D) and female (Figure 2C and 2E) mice from the paired group showed mediated and direct learning, respectively. In particular, a higher amount of time exhibiting freezing was observed during the ON period when compared with the OFF period and the percentage of change was significantly different from zero. These results clearly reproduced the data shown above and confirmed the existence of mediated and direct learning in male and female mice. However, when light and tone were separated on time (Unpaired group), male mice were not able to exhibit mediated learning response (Figure 2B) whereas their response to the light (direct learning) was not affected (Figure 2D). On the other hand, female mice still present a lower but significant mediated learning response (Figure 2C) and a normal direct learning (Figure 2E). These results identified another sex difference between female and male mice regarding their performance in sensory preconditioning tasks. Finally, in the No-Shock group, both male (Figure 2B and 2D) and female mice (Figure 2C and 2E) did not present either mediated or direct learning, which also confirmed that the exposure to the tone or light during Probe Tests do not elicit any behavioral change by themselves as the presence of the electric footshock is required to obtain a reliable mediated and direct learning responses. Altogether, this data confirmed that we successfully set up a $_{LT}$SPC protocol in mice and that this behavioral paradigm can be used to further study the brain circuits involved in higher-order conditioning.

Hippocampal cells are engaged during $_{LT}$SPC

Previous findings with sensory preconditioning paradigms using other sensory modalities suggested a key role of the hippocampus during the processing of incidental associations between low-salience stimuli during the preconditioning phase[1,11]. To better characterize the involvement of dorsal and ventral hippocampus during our $_{LT}$SPC task, we conducted a simultaneous *in vivo* fiber photometry recordings with an optic fiber in each hippocampal subregion to monitor the activity of neurons in general (RCaMP activity) and the PV-positive interneurons in particular (GCaMP activity) during the preconditioning phase (*i.e.* pairing between light and tone) and the conditioning phase (*i.e.* pairing between light and footshock). Thus, we infused a mixture of an AAV-Syn-RCAMP and AAV-DIO-GCAMP (see Methods) into the dorsal (dHPC) or ventral (vHPC) subregion of the hippocampus of PV-Cre mice to be able to monitor the activity of different hippocampal cells. Four weeks after surgeries, animals underwent the $_{LT}$SPC

task (Figure 3A). Both the activity of dorsal and ventral hippocampal cells are measured by ΔF/F after the pairings in $_{LT}$SPC (dHPC, Figure 3B, left and vHPC, Figure 3C, left). Peri-event time histograms computed on the Z-scored ΔF/F show the average dynamics of activity in both hippocampal nuclei during light-tone associations (dHPC, Figure 3B, right and vHPC, Figure 3C, right). This quantifies, trial-by-trial, the response variability, which is lower in the dHPC, and its evolution over time, which is longer lasting also in the dHPC. Quantification of this increase is performed by measuring the peak value of the Z-scored ΔF/F in a 1-second window after the stimulus presentation and comparing it to the baseline value (Figure 3D). In addition, both activity markers in each hippocampal subregion clearly increase their activity during the conditioning sessions both at the onset of the light (*i.e.* conditioned stimulus) (Supplementary Figure 3) and when the footshock is applied (*i.e.* unconditioned stimulus) (Supplementary Figure 4). Altogether, these results demonstrate the engagement of hippocampal cells from both hippocampal subregions during the associative sessions (*i.e.* light-tone and light-shock pairings) in our $_{LT}$SPC task in mice.

The dorsal hippocampus mediates $_{LT}$SPC

The *in vivo* photometry results obtained suggest a major engagement of the dorsal hippocampal subregion with an increase on the activity of different hippocampal cells during the onset of light-tone presentations in the preconditioning phase. However, no study has explored so far, a potential causal distinct role between dorsal and ventral hippocampus in mouse sensory preconditioning. To test this idea, adeno-associated viral vectors carrying the expression of an inhibitory DREADD (hM4DGi, hereafter called DREADD-Gi)[26] under the CaMKII promoter were infused into the dHPC and vHPC of mice to be able to inhibit the activity of principal neurons from these two hippocampal sub-regions. Control animals injected with saline or the DREADD agonist J60 show a reliable mediated and direct learning in the $_{LT}$SPC task (Figure 4). Notably, the inhibition of CaMKII-positive neurons in the dHPC (*i.e.* J60 administration in DREADD-Gi mice) during preconditioning (Figure 4B), but not before the Probe Test 1 (Figure 4B), fully blocked mediated, but not direct learning (Figure 4D). In contrast, the inhibition of CaMKII-positive cells from the vHPC during preconditioning or Probe Test 1 did not impact mediated (Figure 4C) or direct (Figure 4E) learning. This data suggests that dHPC, but not vHPC, mediates the encoding of low-salience stimuli, such as light and tone, associations that are crucially involved in the formation of mediated learning. Once the dorsal hippocampus was identified as a key brain region modulating mediated learning, we wanted to investigate if the specific inhibition of PV interneurons, which were also engaged during stimuli presentation, was also impacting this particular behavior.

For that, we infused a Cre-dependent DREADD-Gi into the dHPC of PV-Cre mice. Notably, the inhibition of PV interneurons during light-tone associations was not impacting the mediated or direct learning formation (Supplementary Figure 5). Altogether, these results suggested that CaMKII-positive cells, but not PV interneurons, in the dHPC are key mediators of light-tone associations that lead to the formation of mediated learning.

Discussion

Our study provides novel insights into the involvement of the hippocampus in higher-order conditioning by validating a $_{LT}$SPC task in male and female mice, using *in vivo* calcium recordings to link the activity of particular hippocampal cells from different hippocampal subregions to different behavioral phases of the task and, finally, using chemogenetic approaches to causally link the dHPC, but not the vHPC, with the encoding of light-tone pairings, which are required for the formation of mediated learning.

Sex differences in classical fear conditioning have been previously found with several contradictory results as stronger conditioning in males or females or no differences[27-34] have been reported. However, previous experimental designs aimed at exploring sex differences undergoing sensory preconditioning paradigms have not been conducted. Our results indicate that both male and female mice can show mediated learning, but the protocol settings might differ between sexes as female mice require fewer conditioning sessions than male mice. This result could suggest that female mice are better in direct aversive conditioning by acquiring higher salience by its pairing with a reinforcer, which promotes mediated learning with less conditioning sessions than in male mice. Accordingly, previous works have shown significant differences in conditioned freezing responses between sexes [35-38].

When low salience stimuli were presented separated on time or when the electric footshock was absent, mediated and direct learning were abolished in male mice. In female mice, although light and tone were presented separately during the preconditioning phase, mediated learning was reduced but still present, which implies that female mice are still able to associate the two low-salience stimuli. No study has addressed sex differences in the capability of associating low-salience cues. However, although the salience of the pairing is different, it has been shown that female mice responded more than male mice to unpaired associations between a neutral stimulus and a reinforcer[32,39]. As female mice normally exhibit more generalization of fear[28,40,41], it is plausible that the sex differences observed in the unpaired group, could be related to this enhanced fear generalization in females and not to a better ability of female mice to associate stimuli that are presented separately[32].

Previous findings showed a crucial role of hippocampal cells in encoding location, context, cues, and memory expression associated with appetitive and aversive behaviors[42-46]. However, few studies have explored the causal involvement of these

hippocampal cells in mediating reinforcement learning[47] and, indeed, their possible role in the encoding of the associations between low-salience stimuli have not been explored. In this study, we have used *in vivo* fiber photometry to elucidate a novel role of hippocampal cells from the dorsal and ventral hippocampus underlying the pairings between two low-salience stimuli such as light and tone and between one of these stimuli and an electric footshock. According to previous findings[47], we demonstrated that both the conditioned stimulus (*i.e.* light paired with the footshcok) and the unconditioned stimulus (i.e. footshock) induced a large increase in the activity of hippocampal cells. The activation of particular neurons from the hippocampus are also engaged in reinforcement behavior[48,49]. In contrast with all these findings, it has been also proposed that the hippocampus is not required for learning simple cue relationships such as associating a conditional stimulus (CS) with an unconditional stimulus (US), which is mediated by other neural circuits (*e.g.,* the amygdala)[50].

According to the data obtained by our photometry recordings, the simultaneously exposure to different stimuli is encoded by the hippocampus, being important for its role in associative learning and for integrating sensory information from the environment[51]. Thus, it is not surprising that previous studies supported the function of this brain region during the associative phases (i.e. preconditioning and conditioning phases) of sensory preconditioning tasks both in humans and in animals[1,11,13,14,52]. However, none of these studies have explored the specific engagement of different hippocampal subregions or cell-types during these higher-order conditioning tasks. Indeed, our data suggests that when hippocampal activity is modulated by the specific manipulation of hippocampal subregions, this brain region is not involved during retrieval. In addition, the present results reveal that the dHPC, but not the vHPC, is involved in the encoding of light-tone pairings for future potential use, as it is the case of mediated learning processes. Specifically, our data provide an unforeseen role of the CaMKII-positive cells of the dHPC during associative memory between low-salience events. Overall, our results and previous studies[1,11,13,14,52] clearly underlined the role of the hippocampus in sensory preconditioning being important in different phases such as the encoding of the associations between low salience stimuli such as lights and tones or enabling the value of the reinforcer to spread across different stimuli.

Other brain regions, such as the orbitofrontal, retrosplenial, and perirhinal cortices, have been linked to the processing of pairings between low-salience sensory cues[6-8,10]. Interestingly, these regions anatomically interact with the hippocampus[53-55], suggesting unexplored brain circuits governed by the hippocampus allowing the brain to encode

associations between different external environmental cues. In this sense, the identification of the dHPC, but not the vHPC, as a crucial brain region to encode these associations might give some novel clues to further understand the brain network underlying higher-order conditioning. A plausible brain circuit involved in the processing of low-salience stimuli could be neuronal projections between the retrosplenial cortex and the dHPC. In line with this, the inhibition of CaMKII-positive neurons from the dorsal hippocampus, which has been shown to project to the restrosplenial cortex[56], blocked the formation of mediated learning. Future work will be performed to tackle this hypothesis and to further decipher how brain networks control higher-order conditioning.

Overall, our results provide new insights on how hippocampal circuits control mediated learning formation by encoding low-salience stimuli pairings during the preconditioning phase of a $_{LT}$SPC paradigm. It is relevant to fully understand how the brain allows the formation of mediated learning, which is a cognitive process that governs most of the animal and human daily decisions and could be involved in the basis of psychotic-like states[57,58].

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Author Contributions

A.B-G. and J.P. contributed to the conception of the project and performed and analyzed all the experiments. C.R-D. help on all surgical procedures and immunohistochemistry assays. I.M-S. help with behavioral and immunohistochemistry assays. A.B-G. and J.P wrote the manuscript. All authors have corrected and revised the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Figure Legends

Figure 1. Male and female mice performed auditory-visual induced mediated learning. A) Schematic representation of the $_{LT}$SPC task highlighting the experimental differences in male (2 training sessions during conditioning) and female (1 training session) mice. The percentage of time spent freezing during OFF and ON periods of the probe test 1 (Tone) (B) and probe test 2 (light) (D). The percentage of change of the OFF vs ON periods of the probe test 1 (Tone) (C) and probe test 2 (light) (E). GLM: generalized linear model fitted to gamma distribution with planned comparisons (B, D), * significant p-value (<0.05) after false discovery rate (FDR). Mann-Whitney between sexes (C, E) and # significant against 0 after tested by Wilcoxon test with zero method. See statistical details in Supplementary Table 1.

Figure 2. Simultaneous associations between light and tone are required for mediated learning formation. (A) Schematic representation of the $_{LT}$SPC task in males (2 training sessions) and females (1 training session) with a representation of paired, unpaired and no-shock experimental groups. The percentage of time spent freezing during OFF and ON periods of the probe test 1 (Tone) (B in males, C in females) and probe test 2 (light) (D in males, E in females). The percentage of change of the OFF vs ON periods of the probe test 1 (Tone) (B in males, C in females) and probe test 2 (light) (D in males, E in females). * significant p-value (<0.05) after false discovery rate (FDR). GLM: generalized linear model fitted to gamma distribution with planned comparisons. KW: Kruskal-Wallis across experimental groups: paired, unpaired, no-shock. # significant against 0 after Wilcoxon test with zero method. See statistical details in Supplementary Table 1.

Figure 3. Hippocampal cells during $_{LT}$SPC. A) Schematic representation of $_{LT}$SPC task while fiber photometry recordings of RCaMP (calcium sensor in synapsin-positive neurons) and Cre-dependent GCaMP (calcium sensor in PV-positive interneurons) in dHPC and vHPC of PV^{cre} mice. B) dHPC modulation during preconditioning of $_{LT}$SPC: on the left upper panel z scores of $\frac{\Delta f}{\mathrm{f}}$ (where f represents fluorescence) of GCaMP PV-positive interneurons on dHPC (green), left bottom z scores of $\frac{\Delta f}{\mathrm{f}}$ of RCaMP in neurons of dHPC (red), right upper number of events with a $\frac{\Delta f}{\mathrm{f}} > 2$ of GCaMP PV-positive interneurons on dHPC, and right bottom number of trials with a $\frac{\Delta f}{\mathrm{f}} > 2$ of Rcamp in

neurons of dHPC. C) vHPC modulation during $_{LT}$SPC: on left upper painel z scores of $\frac{\Delta f}{f}$ of GCaMP PV-positive inter neurons of vHPC (green), left bottom z scores of $\frac{\Delta f}{f}$ of RCaMP in neurons of vHPC (red), right upper number of trials with a $\frac{\Delta f}{f} > 2$ of Gcamp in PV-positive interneurons of vHPC, and right bottom number of trials with a $\frac{\Delta f}{f} > 2$ of RCaMP in neurons of vHPC. D) maximal value of $\frac{\Delta f}{f}$ in the first 1 second window after pairings compared with baseline, on the left (dHPC) and on the right (vHPC). * significant p-value (<0.05). See statistical details in Supplementary Table 1.

Figure 4. Chemogenetic modulation of dorsal and ventral hippocampus during $_{LT}$SPC. (A) Schematic representation of $_{LT}$SPC task combined with chemogenetic approaches, where an inhibitory DREADD was infused in dHPC (representative image on the left) and vHPC (representative image on the right). During the preconditioning (DPC) or the probe test (DPT), a DREADD agonist (J60) was injected intraperitoneally (controls where injected with the agonist in both phases) (diagram of DREADD agonist administration on the center). B, C) The percentage of time spent in freezing during OFF and ON periods (left) and the percentage of change (right) of the OFF vs ON periods of the Probe Test 1 (Tone) of animals infused in dHPC (B) and vHPC (C): controls (Controls_d_Off and controls_d_On, Controls_v_Off and controls_v_On), DPC (DPC_d_Off and DPC_d_On, DPC_v_Off and DPC_v_On) and DPT (DPT_d_Off and DPT_d_On, DPT_v_Off and DPT_v_On). D, E) The percentage of time spent in freezing during OFF and ON periods (left) and the percentage change (right) of the OFF vs ON periods of the probe test 2 (Light) of animals infused in dHPC (D) and vHPC (E): controls_d, DPC_d, DPT_d and controls_v, DPC_v, DPT_v). *significant p-value (<0.05) after false discovery rate (FDR). GLM: generalized linear model fitted to gamma distribution with planned comparisons (B, D, F, H). KW: Kruskal-Wallis across experimental groups: controls, DPC, DPT (C, E, G, I). # significant against 0 after Wilcoxon test with zero method. See statistical details in Supplementary Table 1.

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Figure 1

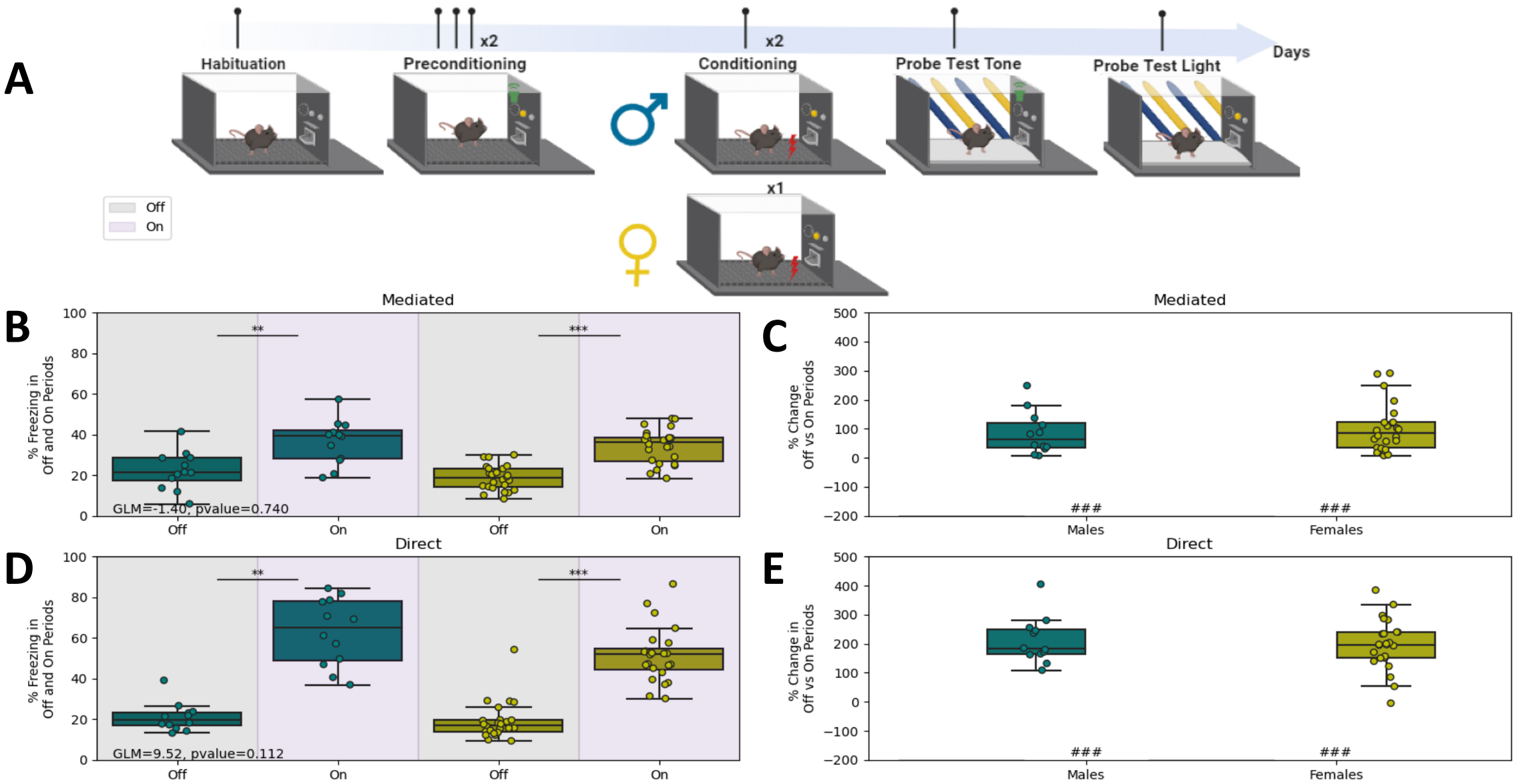
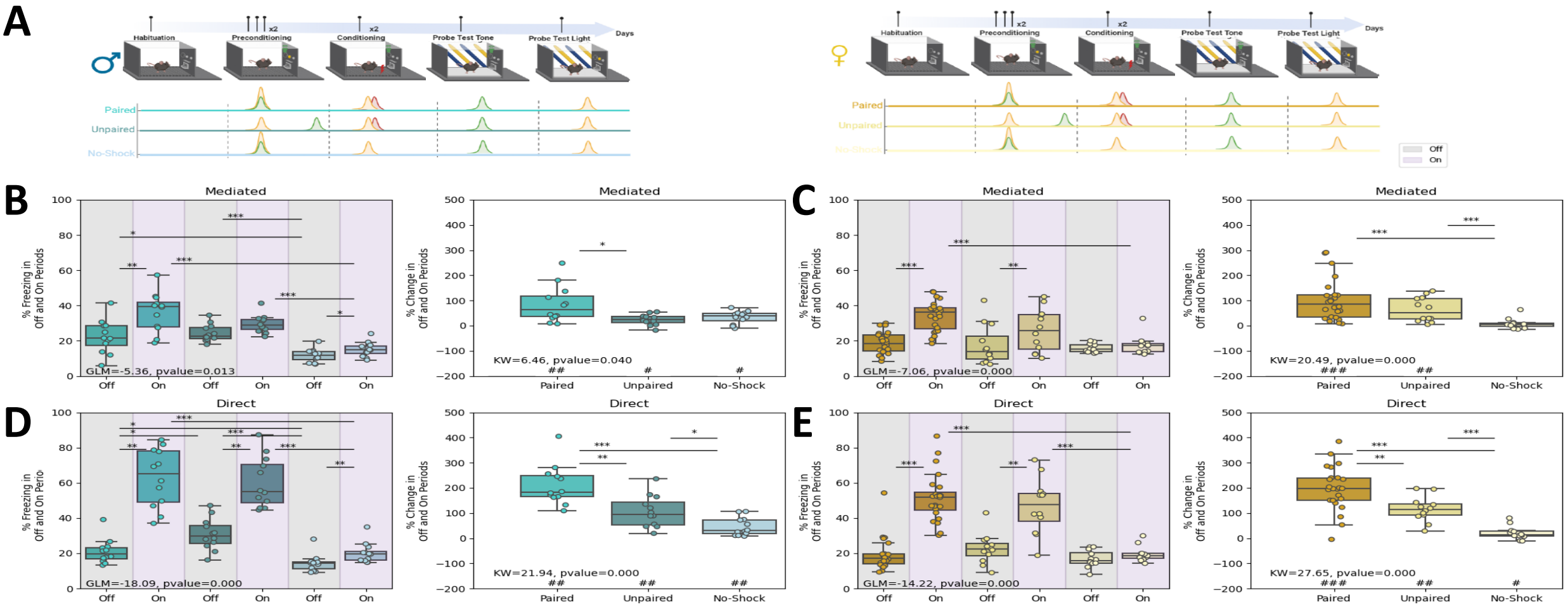

A
Habituation
Preconditioning
x2
Conditioning
x2
Probe Test Tone
Probe Test Light
Days
x1
Off
On
B
Mediated
% Freezing in Off and On Periods
GLM=-1.40, pvalue=0.740
Off
On
Off
On
C
Mediated
% Change Off vs On Periods
Males
Females
D
Direct
% Freezing in Off and On Periods
GLM=9.52, pvalue=0.112
E
Direct
% Change in Off vs On Periods

Figure 2


A
Habituation
Preconditioning
x2
Conditioning
x2
Probe Test Tone
Probe Test Light
Days
Paired
Unpaired
No-Shock
Off
On
B
Mediated
% Freezing in Off and On Periods
GLM=-5.36, pvalue=0.013
% Change in Off and On Periods
KW=6.46, pvalue=0.040
C
GLM=-7.06, pvalue=0.000
KW=20.49, pvalue=0.000
D
Direct
GLM=-18.09, pvalue=0.000
KW=21.94, pvalue=0.000
E
GLM=-14.22, pvalue=0.000
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Paired
Unpaired
No-Shock

Figure 3

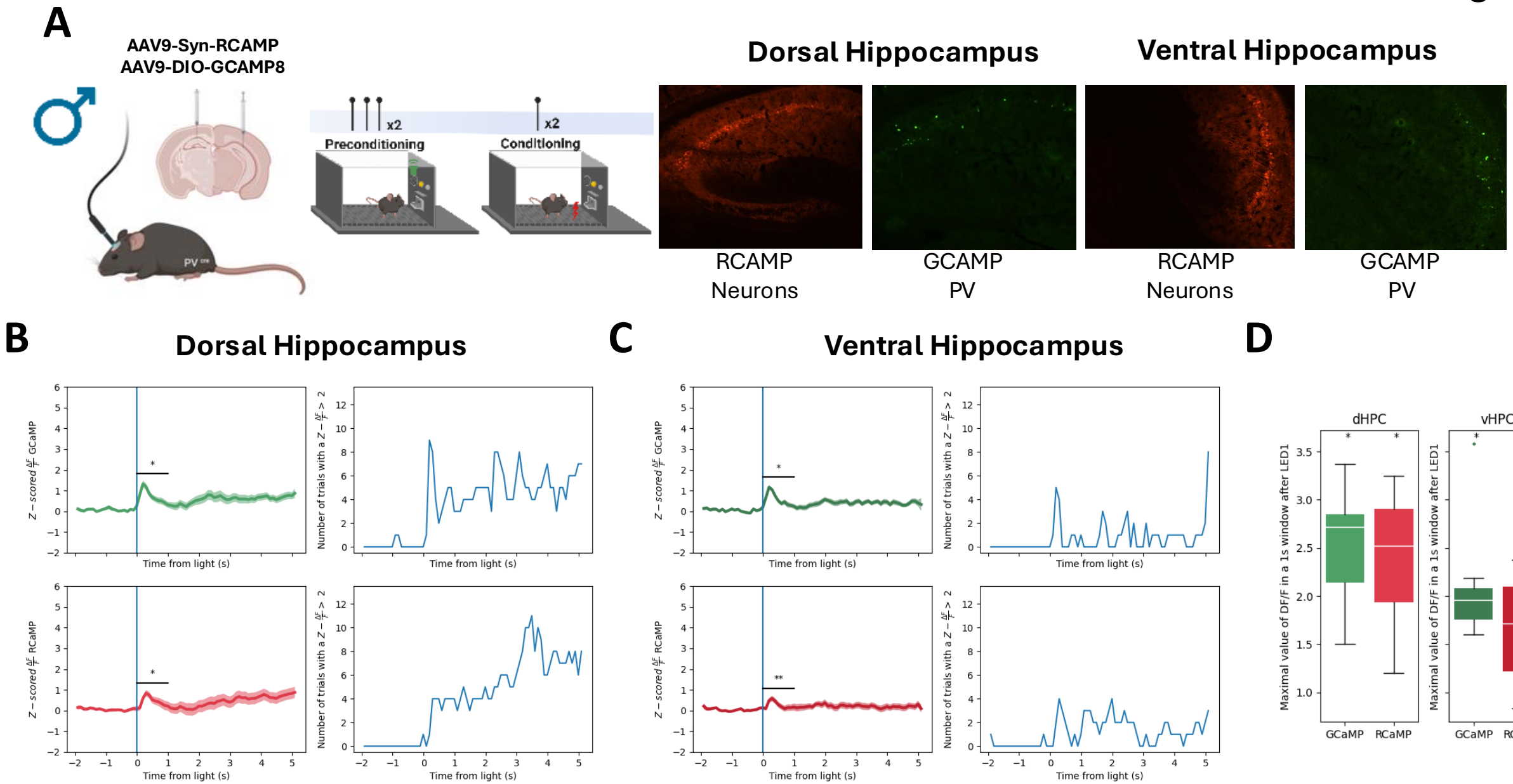
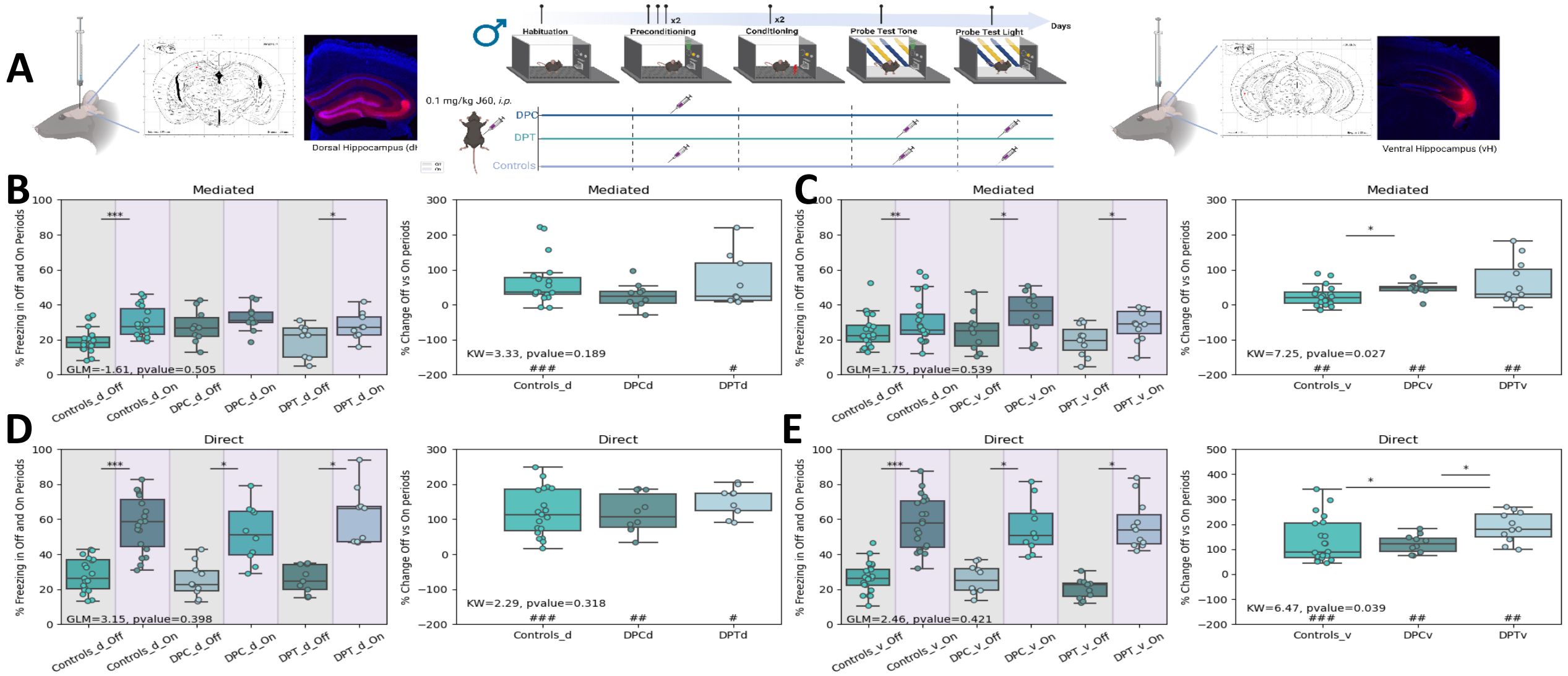

A
AAV9-Syn-RCAMP
AAV9-DIO-GCAMP8
PV cre
x2
Preconditioning
x2
Conditioning
Dorsal Hippocampus
Ventral Hippocampus
RCAMP
Neurons
GCAMP
PV
RCAMP
Neurons
GCAMP
PV
B
Dorsal Hippocampus
C
Ventral Hippocampus
D
Time from light (s)
Number of trials with a Z − ΔF/F > 2
dHPC
vHPC
Maximal value of DF/F in a 1s window after LED1
GCaMP
RCaMP

Figure 4


A
Habituation
Preconditioning
x2
Conditioning
x2
Probe Test Tone
Probe Test Light
Days
0.1 mg/kg J60, i.p.
DPC
DPT
Controls
Dorsal Hippocampus (dH)
Ventral Hippocampus (vH)
B
Mediated
% Freezing in Off and On Periods
GLM=-1.61, pvalue=0.505
Controls_d_Off
Controls_d_On
DPC_d_Off
DPC_d_On
DPT_d_Off
DPT_d_On
Mediated
% Change Off vs On periods
KW=3.33, pvalue=0.189
Controls_d
DPCd
DPTd
C
Mediated
GLM=1.75, pvalue=0.539
Controls_d_Off
Controls_d_On
DPC_v_Off
DPC_v_On
DPT_v_Off
DPT_v_On
Mediated
KW=7.25, pvalue=0.027
Controls_v
DPCv
DPTv
D
Direct
GLM=3.15, pvalue=0.398
Controls_d_Off
Controls_d_On
DPC_d_Off
DPC_d_On
DPT_d_Off
DPT_d_On
Direct
KW=2.29, pvalue=0.318
Controls_d
DPCd
DPTd
E
Direct
GLM=2.46, pvalue=0.421
Controls_v_Off
Controls_v_On
DPC_v_Off
DPC_v_On
DPT_v_Off
DPT_v_On
Direct
KW=6.47, pvalue=0.039
Controls_v
DPCv
DPTv