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Rapid Communication

Progression of activity and structural changes in the anterior cingulate cortex during remote memory formation

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ABSTRACT

The progression of activity and structural changes in the anterior cingulate cortex during remote contextual fear memory formation was measured by imaging c-fos expression and dendritic spines following retrieval tests administered at six post-training time points (days 1, 5, 7, 14, 21, 36). Here we report that conditioned mice exhibit robust freezing at each time point. C-fos expression starts to augment on day 5, showing a monotonic increase over the successive time points, and then stabilized in relation to the higher freezing scores. The first significant increase in mean spine density emerges on day 7. By day 14, the net number of spines remained stable, yet the distribution of single neuron spine density becomes progressively more homogeneous. Our findings reveal that activity changes precede structural remodeling of neurons in the neocortex while remodeling coherence develops gradually in cortical neuron ensembles.

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1. Introduction

Long term storage of fear memory traces involves sequential activation of hippocampal (HPC) and medial prefrontal cortical (mPFC) regions. Early recruitment of the hippocampus and its late disengagement in favor of mPFC is supported by data showing a training-induced and time-dependent inverted pattern of metabolic activity (Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999; Maviel, Durkin, Menzaghi, & Bontempi, 2004), early genes induction (Frankland et al., 2004) NMDA receptor expression (Takehara-Nishiuchi, Nakao, Kawahara, Matsuki, & Kirino, 2006), neural ensembles activation (Tayler, Tanaka, Reijmers, & Wiltgen, 2013) and synthesis of synaptic activity proteins Training also elicits time × region-specific structural remodeling of HPC and mPFC neurons. Remodeling consists of an increase in the number and size of dendritic spines that develop initially in the hippocampus and subsequently in the mPFC while concomitantly, the number and the size of HPC spines return to baseline (Restivo, Vetere, Bontempi, & Ammassari-Teule, 2009; Vetere et al., 2011a,b). Although computational theories of memory posit that remote memory requires a gradual transfer of memory traces from the hippocampus to the neocortex through progressive accumulation of neocortical changes (McClelland, 1998; McClelland, McNaughton, & O'Reilly, 1995; O'Reilly et al., 2014), experimental data depicting the actual progression of neuronal modifications occurring in the neocortex during remote memory formation are largely unexplored. In particular, to what extent c-fos activity and spine density, that respectively depict the number of activated neurons and the density of neuronal connections, follow the same pattern of modification is undetermined. To fill this gap, we trained mice for contextual fear conditioning (CFC) and then imaged c-fos expression and dendritic spines in the anterior cingulate cortex (aCC) after retrieval tests administered at subsequent time points (1, 5, 7, 14, 21, 36 days) from the conditioning phase.

2. Material and methods

2.1. Animals

Male C57BL/6J@Ico mice were purchased from Charles River Italia (Calco, Como). At the beginning of the experiments, animals were 9 weeks old and their weight ranged from 24 to 26 g. They were housed 5 per cage and maintained in a

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temperature-controlled facility (22 ± 1 °C) on a 12:12 h light-dark cycle with free access to food and water. All experimental procedures were conducted in accordance with the official European Guidelines for the care and use of laboratory animals (86/609/EEC).

2.2. Contextual fear conditioning protocol

Mice were first handled for three days in the conditioning room. Fear conditioning, consisting of one single 7 min session, began on the next day. Each mouse was placed in a transparent Plexiglas cage (28 × 28 × 10 cm) with a removable grid floor made of stainless steel rods. After 120 sec of free exploration, each mouse ($N = 78$, 13 per time point) was exposed to a series of 5 non-signaled footshocks (duration: 2 s; intensity: 0.7 mA; 60 s apart) delivered through the grid-floor. Pseudo-conditioned mice ($N = 54$, 9 per time point) were treated in the same manner except that they were not shocked. The choice to use a non-shocked control group (pseudo-conditioned) is motivated by the following reason. In contrast to tone fear conditioning where the control group cannot form a CS-US association because the CS (tone)-US (shock) sequence has been inverted (US-CS), CFC implicates as an aversive representation of that context. We have shown that mice exposed to a shock delivered in a given context show structural modifications in brain regions supporting the formation and consolidation of aversive contextual representations even when they were not re-exposed to the original context (Restivo et al., 2009). This means that running a control group with mice shocked in context A and then tested in context B would result in mice (i) showing minimal freezing in context B yet, as a consequence of the context A-shock association (ii) expressing the same structural modifications as the mice shocked and tested in context B. CFC tests were run by placing mice back into the conditioning chamber for 4 min in the absence of footshock either 1, 5, 7, 14, 21, or 36 days after conditioning. Behavior during conditioning and testing was recorded using an automated procedure. Briefly, activity was recorded by means of a video camera mounted 60 cm above the ceiling of the cage and connected to a computer equipped with Ethovision software (Noldus, Wageningen, The Netherlands). The time spent freezing (absence of all but respiratory movements) was used to score fear memory.

2.3. C-fos immunofluorescence labeling

Conditioned ($N = 23$, 4 or 3 per time point) and pseudo-conditioned mice ($N = 18$, 3 per time point) were deeply anesthetized with a cocktail of ketamine and xylazine (5.0 ml/kg body weight), and perfused transcardially with 0.9% saline (10 ml/min for 5 min) and 4% paraformaldehyde (10 ml/min for 10 min) 90 min following retrieval. Brains were dissected, immersed in PFA for 24 h, transferred in a 30% sucrose solution for 2 days, embedded in Tissue-Tek OCT (Sakura) and cut coronally (40-μm thick) at 20 °C using a cryostat. Sections were then stored in multi-well plates in a free floating condition. Immunofluorescence assays were carried out in at least 4 sections per mouse along the entire rostro-caudal extension of the anterior cingulate cortex. Sections were incubated overnight in a primary polyclonal c-fos antibody (Calbiochem, c-fos [AB-5] diluted 1:500 in phosphate buffer (PBS) containing 2% normal donkey serum, 1% albumin from bovine serum and 0.2% Triton X-100) at room temperature. On the following day, they were incubated for 2 h in the secondary antibody TRITC (tetrame-thylrhodamine isothiocyanate, diluted 1:200 in PBS containing 2% normal donkey serum, 1% albumin from bovine serum and 0.2% Triton X-100) anti-goat anti-rabbit (Jackson ImmunoResearch) for 2 h. To co-visualize c-fos expression with cell nuclei, sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/m for 3 min), mounted on polysined P glasses, and coverslipped with fluoromount.

2.4. C-fos image analysis

Immunofluorescence labeled cells were analyzed in aCC. All procedure was similar and previously described (Vetere et al., 2012). Briefly, images (xy: 2048 pxl) were obtained using a Leica confocal microscope (Obj, HC PL APO 20x, N.A. 0.70, Leica, Microsystem). Analysis of c-fos positive nuclei was performed by means of a computerized image-processing system, and cells in layers II/III of aCC were counted using a profile-counting method (constant background intensity across sections and animals). Counting frame (50 × 50 μm) was placed at an intersection of the lines forming a virtual grid which was moved within the region of interest. Measurements of c-fos-labeled cells were made by an observer blind to the treatment using the Image J (NIH) software. Only cells which satisfied the following criteria were considered c-fos positive: (1) Detection of well-defined cell outline; (2) detection of signal-to-noise ratio >2:1. The counts in the region of interest were obtained from the average of 3 representative coronal sections.

2.5. Golgi–Cox staining and tissue preparation

Conditioned ($N = 47$, 8 or 7 per time point) and pseudo-conditioned mice ($N = 35$, 6 per time point) were deeply anesthetized with a cocktail of ketamine and xylazine (5.0 ml/kg body weight) and perfused transcardially with a solution of 0.9% saline. Brains were dissected, immersed in the Golgi–Cox solution at room temperature for 6 days, transferred to a 30% sucrose solution for 2 days, and then sectioned using a vibratome. Coronal sections (100-μm thick) were mounted on gelatinized slides and coverslipped with Eukitt.

2.6. Spine density counting

Spine densities from pyramidal neurons in layers II/III of the anterior cingulate cortex were analyzed according to the Franklin and Paxinos (2001) mouse atlas. Measurements were performed on apical dendrites, which were previously found to undergo stronger CFC-induced structural remodeling (Restivo et al., 2009). Neurons were identified under low magnification (20×/NA 0.5) using a light microscope (Leica DMLB). Morphological measurements were then performed under higher magnification (100×/1.25 NA). Three neurons showing at least fourth order branches in apical dendrite compartments were selected within each hemisphere. Because no significant inter-hemispheric differences were found, measurements were pooled yielding a final sample of six neurons per animal. Spines were visualized using a video camera (Qimaging Qicam Fast1394) connected to the microscope. Neurons were drawn and analyzed using Neurolucida software. On each neuron and for each dendrite category, five 30–100 μm dendritic segments were randomly selected. In some cases, segments were from the same branch. Segments were sampled 50 μm away from the soma in order to exclude the spine-depleted zone which arises from the cell body. Only protuberances with a clear connection of the head of the spine to the shaft of the dendrite were counted as spines.

2.7. Remodeling coherence

To analyze structural changes such that a relationship between homogeneity of single neuron remodeling and mean number of spines counted at each retrieval time point could be established, we first calculated the mean number of spines per neuron for each

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