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SHORT COMMUNICATION
Disruption of hippocampal CA3 network: effects on episodic-like memory processing in C57BL/6J mice

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Abstract

Lesion studies have demonstrated the prominent role of the hippocampus in spatial and contextual learning. To better understand how contextual information is processed in the CA3 region during learning, we focused on the CA3 autoassociative network hypothesis. We took advantage of a particularity of the mossy fibre (MF) synapses, i.e. their high zinc concentration, to reversibly disrupt the afferent MF pathway by microinfusions of an intracellular (DEDTC) or an extracellular (CaEDTA) zinc chelator into the CA3 area of the dorsal hippocampus of mice. Disruption of the CA3 network significantly impaired the acquisition and the consolidation of contextual fear conditioning, whereas contextual retrieval was unaffected. These results also suggest a heterogeneity between the cognitive processes underlying spatial and contextual memory that might be linked to the specific involvement of free zinc in contextual information processing.

Introduction

In humans, certain forms of learning and memory which are dependent on the hippocampus, such as spatial and episodic memories, are particularly vulnerable to amnesia in neurodegenerative diseases. The contextual fear conditioning paradigm (Phillips & LeDoux, 1992) is a good model for studying episodic-like memory in rodents because the subject has to build up a representation of the context, assembling elements from the environment of the training place, and to pair it with an aversive unconditioned stimulus such as an electric foot shock. Fear conditioning is dependent on the amygdala complex, certainly because fear memory is formed and stored there (Maren *et al*., 1996), but the hippocampus, and in particular the dorsal hippocampus, is also necessary for contextual representation processing (Anagnostaras *et al*., 2001). The architecture of the hippocampal CA3 region (Fig. 1), defined as an autoassociative network (Rolls & Treves, 1998), displays all the characteristics required to support episodic memory formation. The response of the CA3 neuron is determined by the sparse and strong MF inputs and, much more finely, by the direct temporo-ammonic path inputs. The autoassociation occurs because the strength of the modifiable synapses of the recurrent collaterals is increased when both the presynaptic fibre and the postsynaptic dendrite are strongly activated. Therefore, this hippocampal CA3 network could process episodic memory, which can be formed during a single event, and be linked to the particular context in which it occurred (Eichenbaum, 2001).

To study the involvement of the MF pathway in episodic-like memory in mice, we took advantage of the high zinc concentration in MF synapses (Nicoll & Malenka, 1995) to reversibly inactivate this region. Zn^{2+} is the second most prevalent trace element encountered in the mammalian brain, where vesicular zinc is highly concentrated in the glutamatergic neurons of the hippocampus and the amygdala (300–350 μM in vesicles). The rodent hippocampal formation is one of the best described zinc-containing systems. Vesicular zinc can be detected in each component of the trisynaptic circuit, which includes (i) perforant path projections from the entorhinal cortex to the dentate gyrus, (ii) MF projections from the granule cells to the CA3 pyramidal cells and (iii) Schäffer collaterals from CA3 to CA1 neurons (Slomianka, 1992). To inactivate the MF pathway selectively, we proceeded with focal microinjections in the CA3 region of highly specific zinc chelators which can act either directly into the vesicles (DEDTC: diethyldithiocarbamate) or into the synaptic cleft (CaEDTA: Ca^{2+}-ethylenediamine tetraacetic acid). The reversible lesions produced in this way enabled us to study the specific involvement of the MF pathway in the different stages of episodic-like memory processing.

Materials and methods

Subjects and surgery

C57BL/6JIco male mice (Iffa Credo, France) aged ≈ 3 months at the time of testing were used in these experiments. They were housed in groups of 3–5 per cage in a temperature-controlled room (21 ± 1 °C) subjected to a 12-h light–dark cycle, with lights on at 08.00 h. Food and water were available *ad libitum*. This work was carried out in accordance with the Policies of the French Committee of Ethics. The mice were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotactic apparatus (David Kopf Instruments). Guide cannulae were implanted bilaterally into the dorsal hippocampus of 153 animals according to the atlas of Franklin & Paxinos (1997): AP −1.6 mm from bregma; Lat, ±2 mm; and DV, −1.5 mm from skull.

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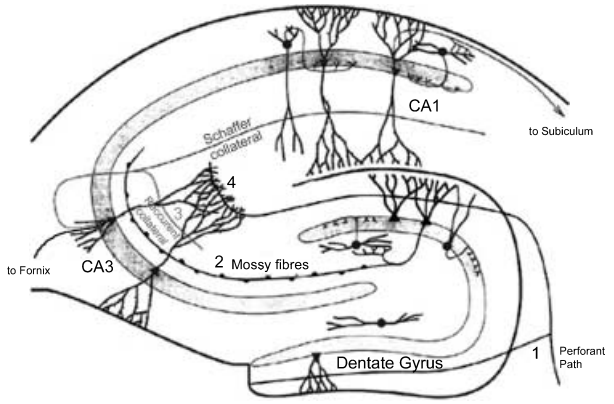

FIG. 1. Architecture of the hippocampus CA3 area. Inputs arrive to the CA3 area from the entorhinal cortex by the perforant path (1), both directly by the temporo-ammonic pathway (4) and indirectly by the mossy fibre pathway (2). The axons of the lateral perforant path synapse on the distal part of the apical dendrites of the CA3 pyramidal cells (4), whereas the axons of the dentate granule cells, the mossy fibers (MF), contact 'en passant' the proximal part of the apical dendrites and the basal dendrites of the CA3 cells, close to the cell body, through giant MF synapses (2). Collateral axons from the CA3 pyramids contact the median part of the apical dendrites of neighbouring CA3 neurons by modifiable synapses (3) and constitute the recurrent network.

Dental cement was used to fix the guide cannulae to the skull (polycarboxylate; Sigma, France). After surgery, the animals were allowed to recover for at least a week during which time they were gently handled daily by the experimenter to minimize the stress of experimental manipulation. At the end of the behavioural experiments, all mice were deeply anaesthetized with an overdose of chloral hydrate (800 mg/kg, i.p.) and their brains removed. After fixing, brains were cut into 40-μm coronal sections and slices were stained. They were then examined with a light microscope and every mouse who was not well implanted and injected was excluded from the analysis. Moreover, studies have been made to ensure that the diffusion of the infused volume (0.25 μL of a dye) is contained within the CA3 area and, consequently, that drugs operated specifically within this subregion.

Drugs and infusion

All drugs were purchased from Sigma Chemical Co. (Lyon, France). DEDTC was selected for its ability to permeate the membrane and thus to chelate free zinc in the MF synaptic vesicles. It has been successfully used to bind vesicular zinc in numerous *in vitro* and *in vivo* studies (Frederickson *et al*., 1990; Takeda *et al*., 1999; Lassalle *et al*., 2000; Lu *et al*., 2000). Calcium-saturated EDTA is used widely as an extracellular zinc chelator (Koh *et al*., 1996; Li *et al*., 2001a,b) and because of its membrane-impermeability can only chelate free zinc present in the synaptic cleft; as a consequence, it will only block the effect of ionic zinc on pre- and postsynaptic terminals (K_d for zinc $10^{-16.4}$ M). As a control we used zinc-saturated EDTA (ZnEDTA), for its extremely high affinity for zinc (K_d 10^{-15} M; Koh *et al*., 1996). The effects of EDTA chelators are governed by their affinities for divalent cations. As previously mentioned, their affinity for zinc is very high whereas the affinities for calcium and magnesium are lower (K_d values are, respectively, $10^{-7.3}$ and $10^{-5.4}$ M). Thus CaEDTA will remove zinc but not calcium or magnesium, and ZnEDTA will remove none of these ions, so the action of chelators will be only on zinc cations without modifying the calcium or magnesium concentration. Li *et al*. (2001a) have shown by direct fluorescence imaging that 10 mM CaEDTA is sufficient to chelate 85% of released zinc. Therefore, the most efficient way to chelate extracellular zinc is to choose a chelator concentration that provides enough free EDTA and MgEDTA to act rapidly (< 0.1msec) on released free zinc (Lu *et al*., 2000).

Chelators were dissolved in isotonic saline (pH 7) to a final concentration of 200 mM. Saline alone was used as a control for possible side-effects of the injections. The concentration of the chelators and the volume injected (0.5 μL per mouse) were based on previous studies (Frederickson *et al*., 1990; Lassalle *et al*., 2000). In Experiment 1 the infusion was made 15 min before training, in order to disrupt the acquisition phase. In Experiment 2, drugs were infused immediately after the training session to act on the consolidation stage. In Experiment 3, the mice were treated 15 min before the contextual test to study the retrieval. Preliminary experiments (S. Daumas, unpublished data) showed that the effects of the two zinc chelators, DEDTC and CaEDTA, were completely reversible when they were injected 24 h before contextual fear conditioning.

For infusion, injection cannulae were inserted into the guide cannulae and protruded 1.1 mm beyond the tip of the guide cannulae to reach the CA3 area. The injection cannulae were connected to a 1-μL Hamilton microsyringe with flexible polyethylene tubing. A pump delivered the infusion volume (0.25 μL per side) at a speed of 0.11 μL/min. At the end of the infusion, the injection cannula was maintained in place for 1 min to ensure absorption by tissue. Both sides were injected successively in this way.

Apparatus and behavioural testing

Conditioning was done in a rectangular conditioning chamber (length 35 cm, width 20 cm, height 25 cm) with a stainless steel rod floor. The shock (0.7 mA) was delivered through the grid floor. The tone (85 dB, 2 s) was emitted through the top of the conditioning chamber. The experimental device was lit by a 60-W white bulb. Two black-and-white patterns faced the conditioning chamber. The conditioning chamber was cleaned with 70% aqueous ethanol before each training session. Contextual learning was checked under the same experimental conditions as training, whereas tone learning was assessed in a modified context. The external patterns were then removed. The modified chamber was triangular, with white Plexiglas walls and floor. The apparatus was washed with 1% acetic acid and lit by a 40-W white bulb. All experiments were videotaped. Conditioning consisted of a single session with two trials. After a 120-s exploration period, the sound was emitted for 30 s and a foot shock was superposed on the tone during the last 2 s. This sequence was repeated twice in succession. Thirty seconds after the last foot shock, the mice were gently removed from the chamber and returned to their home cage. Twenty-four hours after learning, the mice were individually checked for freezing to the context in the conditioning chamber for 4 min. Two hours later, they were tested for freezing to the tone in the modified context. Two minutes after their introduction in the modified context, mice received a 2-min tone presentation. Freezing was scored every 5 s during conditioning and test sessions. Freezing is defined as the lack of all movement other than respiration and heart-beat. In order to ensure that the drugs did not act directly on motor functions, chamber crossings were measured during the first 2 min of training.

Data analysis

To satisfy the requirements for the use of the ANOVA, the mean percentage of freezing scores (P) was transformed in $Q = \arcsin(\sqrt{P}/100)$. Statistical analyses were performed on the Q variable,

using a one-way analysis of variance (ANOVA), or a repeated-measures ANOVA design for related samples (SYSTAT9 for Windows). *Post hoc* comparisons were conducted using Fisher's LSD test. Alpha levels were set at $P < 0.05$ for all tests.

Results

After examination of the coronal sections, a total of 23 mice were excluded because of bad cannulae placement and/or injection site. The number of animals retained in each group for statistical analyses was as follows: Experiment 1, NaCl, $n = 15$; ZnEDTA, $n = 12$; CaEDTA, $n = 14$; DEDTC, $n = 14$; Experiment 2, NaCl, $n = 15$; ZnEDTA, $n = 12$; CaEDTA, $n = 15$; DEDTC, $n = 14$; Experiment 3, NaCl, $n = 9$; DEDTC, $n = 10$.

Experiment 1 was designed to study the involvement of the CA3 area in contextual learning, by applying pretraining infusions of chemical agents capable of blocking the regular processing of information in this region. No significant overall variation among the different groups appeared ($F_{3,51} = 0.384$, $P = 0.76$), confirming the lack of side-effects of the drugs or of the infusion procedure itself on the mice's locomotor activity. A significant overall variation of the contextual freezing appeared between groups ($F_{3,50} = 6.76$, $P < 0.001$; Fig. 2A). *Post hoc* analysis revealed no significant difference, either between NaCl- and ZnEDTA-injected groups ($P = 0.201$) or between CaEDTA- and DEDTC-treated mice ($P = 0.34$). Comparison of zinc chelator groups (CaEDTA plus DEDTC) with control groups (NaCl plus ZnEDTA) revealed a significant impairment of the contextual fear acquisition due to zinc chelation ($F_{1,52} = 17.463$, $P < 0.001$). Hence, our results suggest a role for both intracellular and extracellular zinc in contextual fear conditioning.

The average percentage of time spent freezing during the tone presentation was 80% ± 2.94 for NaCl, 78.82% ± 3.71 for ZnEDTA, 78.53% ± 3.74 for CaEDTA and 71.73% ± 3.74 for DEDTC. The one-way ANOVA failed to demonstrate any significant effect of the drug infusion on cue conditioning ($F_{3,50} = 1.428$, $P = 0.246$).

Pre-training administration of zinc chelators has shown that the CA3 area is implicated in contextual fear acquisition. Given that the effect of the chelators lasts for more than an hour, their possible interference with the process of memory consolidation could not be discounted. Accordingly, to analyse the role of zinc chelators on CA3-dependent memory consolidation, in Experiment 2 chelators were infused immediately after the conditioning session (Fig. 2B). A one-way ANOVA showed a significant overall variation between groups ($F_{3,52} = 23.171$, $P < 0.001$). Although *post hoc* analysis failed to demonstrate any difference between the control groups (NaCl and ZnEDTA, $P = 0.323$), a significant difference appeared between CaEDTA and DEDTC groups ($P < 0.001$), which themselves differed from NaCl and ZnEDTA groups (respectively for CaEDTA, $P < 0.005$ and $P < 0.001$; and for DEDTC, $P < 0.001$ for both controls). The blockade of intracellular ionic zinc had an even greater effect on contextual memory consolidation than the chelation of extracellular zinc, suggesting a different role for vesicular and extracellular zinc blockade.

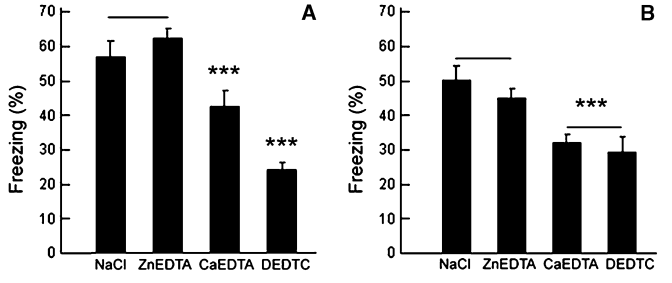

FIG. 2. Effects of (A) pre- and (B) post-training infusions on contextual fear conditioning. Freezing is expressed as the mean percentage of freezing bouts (± SEM) during the Context test session. (A) Mice injected with the zinc chelators before conditioning showed a significant deficit in contextual fear with respect to the two control groups ($P < 0.001$). (B) DEDTC infusion after conditioning resulted in a greater impairment of the contextual consolidation stage than did CaEDTA infusion ($P < 0.001$).

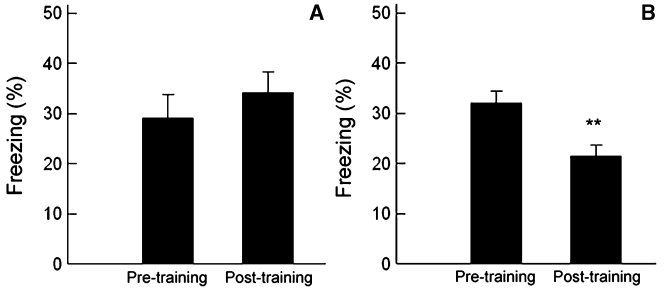

FIG. 3. Differential effects of (A) zinc chelators CaEDTA and (B) DEDTC on acquisition and consolidation of the contextual fear. CaEDTA-injected mice, before or after training, showed no conditional fear difference, whereas conditional levels of DEDTC-injected groups differed ($P < 0.005$).

The percentage of freezing displayed by the four groups during the presentation of the tone in the modified context was 75.83 ± 3.43% for NaCl, 84.03 ± 3.51% for ZnEDTA, 76.39 ± 2.78% for CaEDTA and 73.51 ± 3.59% for DEDTC. The one-way ANOVA demonstrated the absence of significant variation between groups ($F_{3,52} = 1.63$, $P = 0.194$).

As the two chelators proved efficient and showed differential effects on both contextual acquisition and consolidation, we compared directly their effects when they were administered either before or after the conditioning session. No effect of infusion appeared for CaEDTA ($F_{1,27} = 3.397$, $P = 0.076$; Fig. 3A) whereas contextual fear performances were more impaired when DEDTC was administered during memory consolidation rather than prior to learning ($F_{1,26} = 10.208$, $P < 0.005$; Fig. 3B).

To study whether the MF pathway is necessary for memory recall, in a third experiment MF synapses were blocked before the contextual recall using DEDTC, which had proved to be the most efficient chelator. Mice were injected with either DEDTC or NaCl 15 min before the contextual recall session. No effect of the DEDTC infusion appeared on contextual or on tone fear recall (context, $F_{1,17} = 2.54$, $P = 0.129$; tone, $F_{1,17} = 0.759$, $P = 0.396$; data not shown).

Discussion

The aim of the study was to use zinc chelators to cause reversible blockade of the MF pathway in the dorsal hippocampus, in order to analyse CA3 disruption on contextual memory processing in mice.

The general outcomes strengthen the data from the literature. The dorsal hippocampus, and more particularly the MF pathway, appears critical for forming a contextual memory and does not contribute to discrete CS learning, because all mice showing contextual freezing impairments displayed normal freezing to the tone. Relying on the accuracy of the injection site and the limited diffusion of the infused volume (demonstrated by systematized histological controls and studies on dye diffusion), it can be assumed that the CA3 region and the MF pathway play a prominent role in the acquisition and consolidation of contextual fear memory. Conversely, the MF pathway is not involved in long-term recall of contextual fear.

These results further demonstrate vesicular zinc release to be necessary for optimal acquisition and consolidation of contextual memory, which is a novel finding.

Post-training MF pathway blockade by DEDTC impaired fear conditioning to the context more than did pretraining blockade. These results are in good accordance with the theory of Maren *et al*. (1997), who propose that, in the case of hippocampal pretraining lesions, animals are forced to use alternative strategies or neuronal networks to learn the task and to recognize the aversive context whereas, in the case of post-training lesions, contextual information already encoded within the hippocampus during conditioning will be disrupted and become unavailable for retrieval. Consequently, we assume that the observed freezing deficit stems from a partial hippocampal dysfunction, resulting in a loss of the context representation. Such a dysfunction could be caused by the chelation of zinc directly within the synaptic vesicles by DEDTC and, to a lesser extent, by the chelation of the sole extracellular zinc, after its release into the synaptic cleft, by CaEDTA.

Unexpectedly, in the contextual fear conditioning paradigm, chelation of extracellular zinc by CaEDTA also resulted in partial learning impairment, contrary to what had been observed in spatial learning (Frederickson *et al*., 1990; Lassalle *et al*., 2000). This effect is specific to synaptically released zinc because in the same conditions ZnEDTA had no effect on performances. Indeed, CaEDTA infusions revealed that extracellular free zinc is specifically involved in both the acquisition and consolidation processes of contextual fear.

We provide, for the first time, behavioural evidence for a neuromodulatory role of MF-zinc in contextual memory processing, confirming the hypothesis that neural circuits of zinc-containing neurons are associated with episodic memory function.

All together, our results reinforce the prediction arising from the CA3 autoassociative network model that the hippocampal MF pathway is necessary for the encoding and consolidation of episodic-like memory whereas it is not necessary for retrieval. Moreover, they provide further evidence to support the idea that different forms of hippocampus-dependent memory may involve different patterns of neural processing involving zinc within the hippocampus.

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Abbreviations

CaEDTA, Ca^{2+}-ethylenediamine tetraacetic acid; DEDTC, diethyldithiocarbamate; ZnEDTA, zinc-saturated EDTA.

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