

Long-lasting hippocampal potentiation and contextual memory consolidation

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Keywords: CA1 hippocampal slices, contextual fear conditioning memorization, exploration, input–output curves, nociceptive stimulation, paired-pulse facilitation curves

Abstract

In order to ascertain whether there are hippocampal electrophysiological modifications specifically related to memory, exploratory activity and emotional stress, extracellular electrical activity was recorded in hippocampal slices prepared from the brains of male adult rats. Several groups of animals were employed: (i) rats which had freely explored the experimental apparatus (8 min exposure); (ii) rats which had been subjected, in the same apparatus, to a fear conditioning paradigm training entailing the administration of aversive electrical footshocks (8 min exposure); (iii) rats to which the same number of aversive shocks had been administered in the same apparatus, but temporally compressed so as to make difficult the association between painful stimuli and the apparatus (30 s exposure); (iv) naïve rats never placed in the apparatus. Half of the rats from each treatment group were used for retrieval testing and the other half for hippocampal excitability testing. The conditioned freezing response was exhibited for no less than 4 weeks. Hippocampal excitability was measured by means of input–output curves (IOC) and paired-pulse facilitation curves (PPF). Retrieval testing or brain slices preparation were performed at increasing delays after the training sessions: immediately afterwards or after 1, 7 or 28 days. Only the rats subjected to the fear conditioning training exhibited freezing when placed again in the apparatus (retrieval testing). It was found that IOCs, with respect to naïve rats, increased in the conditioned animals up to the 7-day delay. In free exploration animals the IOCs increased only immediately after the training session. In all other rats no modification of the curves was observed. IOC increases do not appear to imply presynaptic transmitter release modifications, because they were not accompanied by PPF modifications. In conclusion, a clear-cut correlation was found between the increase in excitability of the Schaffer collateral–CA1 dendrite synapses and freezing response consolidation.

Introduction

The hippocampus is known to be one of the major areas involved in learning and memory processing. It has been shown that in the hippocampus (mainly in the CA1 and CA3 regions) some peculiar electrophysiological modifications appear concurrently with learning (Buzsaki *et al.*, 1983; Skelton *et al.*, 1987; Matthies, 1989; Moyer *et al.*, 1996; Thompson *et al.*, 1996; Jeffery, 1997). Up to now these phenomena have been most frequently observed either during information acquisition or following subsequent stimulations. There are fewer studies on the hippocampal electrophysiological modifications which appear during the formation of the memory trace, after the initial phase of acquisition, when the conditioned (CS) and the unconditioned (US) stimuli are no longer present (Skelton *et al.*, 1987; Moyer *et al.*, 1996; Thompson *et al.*, 1996; Jeffery, 1997). Moreover, hippocampal electrophysiological changes have been observed during the exploration of new surroundings (Moser, 1995; Erickson *et al.*, 1993) and under conditions of severe stress (Kim *et al.*, 1996; Garcia *et al.*, 1997).

It is known that after undergoing training in a behavioural paradigm like contextual fear conditioning experimental subjects easily learn and retain for a long time the association between new surroundings (CS) and a given number of aversive stimuli (US) which are administered to them during their exploratory activity in the conditioning apparatus. In fact, the rats subjected to the conditioning procedure will exhibit long-lasting freezing when placed in the conditioning apparatus again (Fanselow, 1990; Kim *et al.*, 1993; LeDoux, 1995; Milanovic *et al.*, 1998; Sacchetti *et al.*, 1999a, 1999b). Freezing (immobility) is defined as the complete absence of somatic motility, respiratory movements excepted (Fanselow, 1990; Kim *et al.*, 1993; Sacchetti *et al.*, 1999a, 1999b). On the other hand, freezing will not be exhibited when the same experimental subject who underwent the training session is placed in surroundings different from those in which aversive stimuli were administered (Fanselow, 1990; Kim & Fanselow, 1992; Milanovic *et al.*, 1998; Sacchetti *et al.*, 1999a, 1999b). Thus the freezing response exhibited by the rats when placed again in the conditioning apparatus is a specific conditioned response due to the association of those surroundings and the aversive stimuli. In addition, freezing will not be elicited when the experimental subject is returned to the freely explored surroundings in which no adverse stimuli had been administered (Fanselow, 1990; Milanovic *et al.*, 1998), or to those

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in which the same number of aversive stimuli of equal intensity had been administered in such a short time as to make quite difficult the association of the temporally compressed aversive stimuli to the surroundings (conditioning apparatus) (Fanselow, 1990; Milanovic *et al.*, 1998; Taubenfeld *et al.*, 1999).

The aim of the present work has been to ascertain the presence and the time course of specific hippocampal electrophysiological changes after diverse contingencies. The several groups of experimental subjects (i) underwent training following a contextual fear paradigm, (ii) explored freely or (iii) were subjected to exclusively painful stimulations. Training always consisted of a single training session (one-trial paradigm). The single session procedure makes it possible to know exactly when the mnemonic process begins, which is impossible when multitrial paradigms are employed (Bures & Buresova, 1990; Ambrogio Lorenzini *et al.*, 1997). Electrophysiological activity was recorded from hippocampal slices prepared at increasing delays after the acquisitive, exploratory or aversive training session (immediately afterwards, 1, 7 and 28 days later) and from naïve control rats.

Materials and methods

Animals

Seventy-day-old male albino Wistar rats (average body weight 290 g when the experiments began) were used. The animals were obtained from the breeding colony of the Department of Physiology and Biochemistry of Pisa University. The animals (4–5 in each cage) were kept in a room with natural light/dark cycle and constant temperature of 20 ± 1 °C. The rats had access to food and water *ad libitum* throughout the experiment. All animal care and experimental procedures were conducted in accordance with Italian law and European Communities Council recommendations on the use of laboratory animals (Directive of November 24, 1986, 86/609/ECC).

Behavioural procedures

Apparatus

A basic Skinner box module (Modular Operant Cage, Coulbourn Instruments L.L.C., Allentown, PA, USA) was used to induce freezing. Box dimensions were $29 \times 31 \times 26$ cm. The top and two opposite sides were made of aluminium panels. The other two sides were made of transparent plastic. The floor was made of stainless steel rods connected to a shock scrambler delivery apparatus (Grid Floor Shocker, model E13-08, Coulbourn). The apparatus was connected to a stimulus programming device (Scatola di comando Arco 2340, Ugo Basile, Comerio, Varese, Italy) to predetermine the number and duration of the electric shocks and the duration of the intervals between them. The apparatus was placed in an acoustically insulated room kept at a constant temperature of 20 ± 1 °C. Illumination inside the room was 60 lux.

Contextual fear conditioning procedure

The rat was gently taken manually from the home cage, placed in a bucket and carried from the housing room to the soundproofed room. Once there, it was placed inside the conditioning apparatus. The rat was left undisturbed for 3 min. After this time a series of seven single electric footshocks (1 s duration, 1 mA intensity) were administered at 30-s intervals. Two minutes after the end of the stimulation, the rats were brought back to the home cage, thus spending a total time of 8 min inside the conditioning apparatus.

Shock-only procedure

The rat was gently taken manually from the home cage, placed in a bucket and carried from the housing room to the soundproofed room. Once there, it was placed inside the conditioning apparatus. Immediately afterwards the rats were subjected to seven footshocks (1 s, 1 mA) at 3-s intervals. At the end of the stimulation, the rats were brought back to the home cage, thus spending a total time of ≈ 30 s inside the conditioning apparatus.

Exploration procedure

The rat was gently taken manually from the home cage, placed in a bucket and carried from the housing room to soundproofed room. Once there, it was placed inside the conditioning apparatus. The rat was left undisturbed in the apparatus for a total duration of 8 min before being brought back to the home cage.

Freezing measurement

To measure freezing, the animals were again placed inside the conditioning apparatus and left there for 3 min. After that time, they were brought back to the home cage. Behaviour of the rats was recorded by means of a closed-circuit television system. The total freezing time (i.e. the total amount of freezing (in seconds) during the 3-min period) was measured manually with a stop-watch. All behavioural testing was performed between 10.00 and 13.00 h to minimize circadian influences.

Experimental groups

A total of 242 rats were employed. The animals were randomly divided into 16 groups, ranging from 14 to 16 rats each, subjected to four different procedures, and subsequently tested (behaviourally or electrophysiologically) at four postacquisition delays. The procedures were: (i) subjected to contextual fear conditioning: 'conditioned'; (ii) subjected to shocks but not conditioned: 'shock-only'; (iii) free to explore the apparatus: 'exploration'; (iv) never having left the home cage: 'naïve'. After the session in the apparatus, or an analogous time lapse in the home cage for 'naïve' groups, the subjects were tested, either behaviourally or electrophysiologically, at one of the four increasing delays: 10 min (0 delay), 1, 7 or 28 days later. The stimuli of the training sessions were never presented again to the subjects from which the slices were prepared. This means that the electrophysiological modifications which were observed were related only to the consolidation and/or to the storage of engrams, if any, without the interferences which may be consequent to the repeated presentation of the stimuli received during acquisition. One half of each group (seven or eight rats taken randomly) was tested behaviourally (freezing measurement). Hippocampal slices (a single slice prepared from each animal for electrophysiological measurements) were obtained from the other half (seven or eight rats). Both behavioural and electrophysiological measurements were performed by operators blind to the group from which rats or brain slices were taken.

Slice preparation

Transverse hippocampal slices from the dorsal half of the hippocampus were prepared as described previously (Berretta *et al.*, 1990) (Fig. 1). Briefly, the rats were anaesthetized with ether and decapitated. Brains were rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF; in mM: NaCl, 130; KCl, 3.5; NaH_2PO_4 , 1.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2; NaHCO_3 , 24; and glucose, 10; gassed with 95% O_2 , 5% CO_2 and kept constantly at pH 7.4). Transverse hippocampal slices of 400 μm thickness were cut on a vibroslicer (Campden Instruments LTD, Leics, UK).

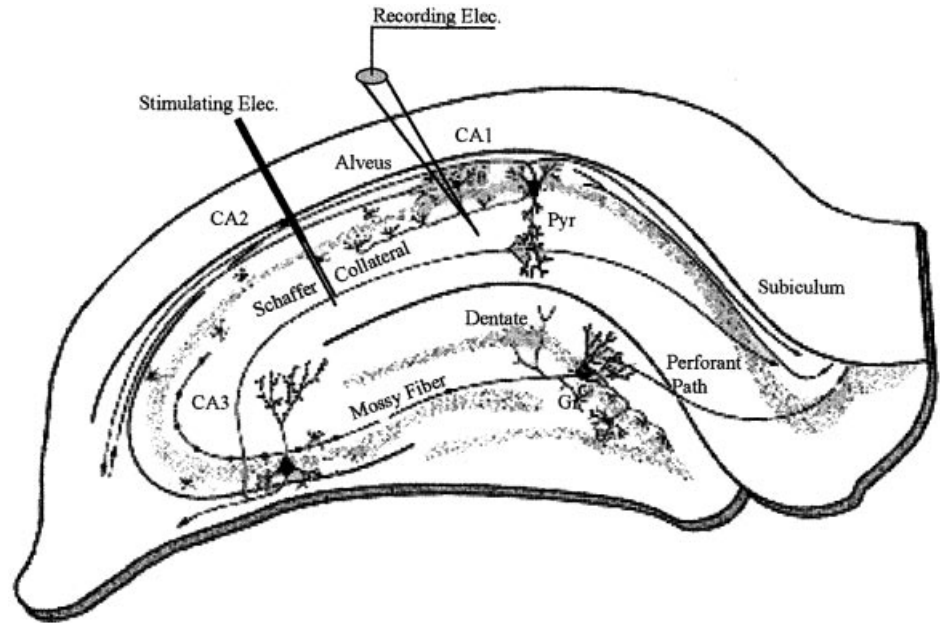


FIG. 1. Coronal section of the hippocampus showing its major areas and excitatory pathways. The dentate gyrus layer is composed of granular cells (Gr) which send their axons (mossy fibers) to CA3 pyramidal dendrites. CA3 pyramidal cells send axon collaterals called Schaffer collaterals to CA1 pyramidal apical dendrites. The stimulation is applied to Schaffer collaterals and the excitatory postsynaptic response (fEPSP) is recorded extracellularly in the layer of CA1 pyramidal dendrites.

Hippocampal slices were collected in oxygenated ACSF and preincubated for at least one hour at room temperature. For recording, slices were transferred to a submerged recording chamber and continuously perfused with ACSF at a constant rate (2 mL/min) and maintained at 33 °C. All slices were prepared between 10.00 and 12.00 h to minimize circadian influence. All experiments were performed between 1.5 and 4 h following slice preparation, i.e. during the period of maximum slice viability and response stability.

Extracellular recording

Micropipettes, pulled from 1.5-mm-OD glass tubing with the use of a Flaming/Brown micropipette puller (Sutter Instruments, San Rafael, CA, USA), were filled with ACSF (resistance 2–5 M Ω). Bipolar electrodes (SNEX 200, Rhodes Med. Institute, Woodland Hills, CA, USA) consisted of two 0.1-mm insulated stainless steel wires separated by a 0.25-mm shaft; the entire electrode was insulated with epoxyite except for the contacts. They were used to stimulate Schaffer collateral–commissural fibers (Fig. 1). The excitatory postsynaptic field potentials (fEPSPs) were recorded from the apical dendritic layer of CA1 (*stratum radiatum*). Rectangular pulses of 0.08-ms duration and 0–70 μ A intensity (adjusted for each slice) were delivered at 0.05 Hz. An average of five stimulus-evoked responses were collected. The signals were amplified and filtered (DC–3KHz) using an amplifier (Extracellular Amplifier MN622, Biomedica Mangoni, Pisa, Italy) and sampled with a personal computer (PCI-MIO-16E4 for the acquisition, PC-TIO-10 for the stimulation, Software with LABVIEW: National Instruments, Milano, Italy). The amplitude (in mV) of the fEPSPs was measured for the downward peak. The intensity of the test stimulus, delivered at 0.033 Hz, was adjusted for each slice so that the fEPSPs were equal to \approx 50% of the maximal amplitude of the fEPSPs.

Hippocampal activity was measured by means of input/output curves (IOC) over a range of input stimulus intensities, in order to simulate the physiologically varying incoming neural traffic (Buzsaki *et al.*, 1983; Leung & Ambrose, 1994). After 20–25 min of stable recording, IOCs were analysed. Stimuli of increasing intensity were administered in ascending order over a range of 0–70 μ A. PPF was analysed by means of paired responses elicited by twin pulses (40 ms

apart) in CA1 pyramidal neurons. In order to investigate a possible presynaptic involvement in synaptic plasticity modifications, paired-pulse stimuli were administered, and the relative paired-pulse facilitation curves (PPF) were analysed (Creager *et al.*, 1980; Green *et al.*, 1990; Shulz *et al.*, 1994). The PPF is expressed as the ratio of the second to the first EPSPs amplitude. For each stimulus intensity, three responses were collected at 30-s intervals. The interval between data collection at the different stimulus intensities was between 30 and 60 s. The magnitudes of the response to each of the three stimuli at each intensity were measured and averaged. Statistical analysis was performed on the surface areas defined by IOC and PPF ratio curves.

Statistical analysis

Two-way ANOVA and the Newman–Keuls multiple comparisons test were used.

Results

Freezing duration

The rats of the ‘conditioned’ and ‘exploration’ groups exhibited a very similar behaviour when placed in the apparatus for the first time. As a rule, no initial freezing was observed, and only very short immobility durations were recorded during the initial free-exploration 3-min period common to the two paradigms. Initial freezing duration in these groups was quite similar to that observed in ‘exploration’ and ‘naïve’ rats during the subsequent retrieval testing, i.e. no more than 10% of total time (data not shown). On the other hand ‘conditioned’ rats, after having received during the same first training session a series of seven nociceptive shocks, exhibited during the following 120 s an almost continuous freezing, lasting \approx 80% of the total duration (data not shown).

Figure 2 shows that ‘conditioned’ rats exhibited long-lasting freezing time when placed in the conditioning apparatus again at all the four postacquisition delays (no delay, 1, 7 or 28 days). All other experimental subjects when placed again in the apparatus always exhibited only short durations of immobility. Two-way

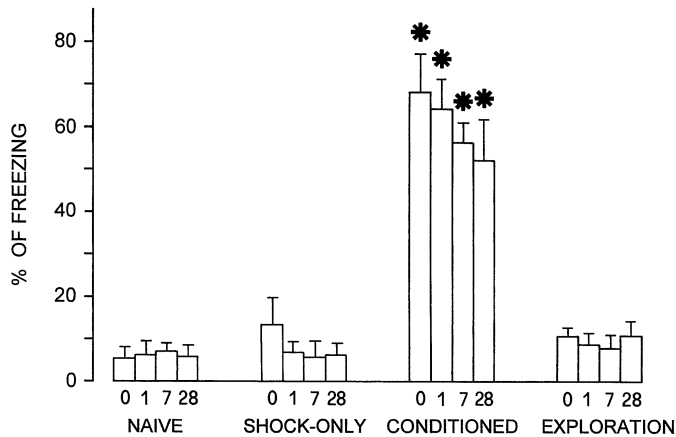


FIG. 2. Behavioural paradigms and freezing duration during retrieval testing. For retrieval testing all rats were placed for 3 min in the apparatus and cumulated total freezing duration was measured. Freezing (immobility) was defined as the complete absence of somatic motility, respiratory movements excepted. Retrieval testing was performed immediately after (0), or 1, 7 or 28 days after the training session. 'Conditioned' rats, which had received the electrical footshocks during the 8-min period spent inside the apparatus, exhibited long-lasting freezing at all postacquisition retrieval delays. Rats that had freely explored the apparatus (8 min) ('exploration') did not exhibit conditioned freezing. Rats that had received the same number of electrical footshocks, but temporally condensed (30 s) ('shock-only'), did not exhibit conditioned freezing. 'Naïve' rats (never before placed in the apparatus) did not exhibit freezing. * $P < 0.01$ between the four conditioned groups and all other groups (see Results).

ANOVA (4 procedures \times 4 delays) showed significant differences between procedures ($F_{3,103} = 23.24$, $P < 0.001$), but not for exposure delays ($F_{3,103} = 0.42$, N.S.). There were no significant interactions ($F_{9,103} = 0.25$, N.S.). The Newman-Keuls *post hoc* multiple comparisons test showed significant differences between the four conditioned groups and all other groups ($P < 0.01$ in all instances). There were never significant differences between groups of rats having undergone the same procedure.

Input-output curves

Figure 3a shows the time course of the IOCs obtained from hippocampal slices. In the slices prepared immediately after training, the highest curves are those of the 'conditioned' and 'exploration' groups. No such change was observed in the 'shock-only' group compared with the 'naïve' group. In slices prepared at increased after-training delays of 1 and 7 days the curves of the 'conditioned' group were higher than those of all the other groups. At the 28-day delay no more differences were observed among curves.

Figure 3b shows statistical analysis results of the findings of Fig. 2. Two-way ANOVA (4 procedures \times 4 delays) shows that there are significant differences between the areas of IOC, as regards both procedures ($F_{3,107} = 4.81$, $P < 0.001$) and delays ($F_{3,107} = 8.48$, $P < 0.001$). There are significant interactions ($F_{9,107} = 3.64$, $P < 0.001$). The Newman-Keuls *post hoc* multiple comparisons test showed that there are significant differences for the 'conditioned' groups at delays 0, 1 and 7 days against the respective 'naïve' and 'shock-only' groups ($P < 0.01$ in all instances). Only at the 0 delay is there a significant difference between the 'exploration' subjects and 'naïve' and 'shock-only' subjects ($P < 0.01$ in both instances). At the same delay there is no significant difference between 'exploration' and 'conditioned' groups. At all other delays (1, 7 and 28 days) there are no significant differences between 'exploration' groups and 'naïve' and 'shock-only' groups.

PPF stimulation

Figure 4a shows PPF curves of the four treatments at the four increasing delays. There are no differences between groups or between delays. Again, there are no differences in Fig. 4b (curve areas). Statistical analysis failed to show any significant between-group difference. Two-way ANOVA (4 procedures \times 4 delays) shows no significant difference between PPF curve areas, as regards either procedures ($F_{3,107} = 0.33$, N.S.) or delays ($F_{3,107} = 0.50$, N.S.). There are no significant interactions ($F_{9,107} = 0.80$, N.S.).

Discussion

Methodological considerations

Hippocampal excitability modifications were measured in brain slices prepared at increasing delays after the acquisition session, and never *in vivo*. The *in vitro* preparation excludes the possible effects of muscular activity and emotionality which can influence *in vivo* results (Moser *et al.*, 1993; Moser, 1995; Moyer *et al.*, 1996; Thompson *et al.*, 1996; Jeffery, 1997). In fact, *in vivo* an increase of hippocampal excitability was reported in animals having explored new surroundings (similar to the present 'exploration' groups) (Sharp *et al.*, 1985; Green *et al.*, 1990; Erickson *et al.*, 1993), although these results were later criticized because of the behavioural artefact due to a brain temperature increase (Moser *et al.*, 1993; Moser, 1995).

In order to measure excitability modifications, single low-intensity stimuli were applied. In fact, low-intensity stimuli do not greatly influence neuronal excitability, as is the case when stronger and more frequent stimuli (tetanization) are administered (Jeffery, 1997). Tetanization by itself may induce neuronal excitability modifications (even LTP), thus making it more difficult to ascertain the existence of a correlation between learning and excitability modifications (Barnes, 1995; Jeffery, 1997).

PPF was recorded and analyzed in order to study a synaptic facilitation thought to be based on presynaptic mechanisms (Creager *et al.*, 1980; Green *et al.*, 1990; Shulz *et al.*, 1994).

Effects of fear conditioning and exploration on CA1 hippocampal neuronal excitability

The present findings show that immediately after the training session (0 latency), i.e. at the very beginning of the postacquisition period of fear conditioning, there is an increase in synaptic excitability. The synaptic excitability increase is also present 1 day and 7 days later. However, 28 days later no synaptic excitability increase can be measured. After the free exploration of new surroundings there is also an increase in neuronal excitability but this is measurable only immediately after the exploration sessions. In both instances, it can be concluded that the observed increase of hippocampal neuronal excitability is not due mainly to presynaptic transmitter release changes, given the absence of measurable simultaneous PPF variations (Creager *et al.*, 1980; Green *et al.*, 1990; Shulz *et al.*, 1994). Animals of 'shock-only' groups, which received the same aversive stimulation as the 'conditioned' groups but in such a short time as to make the association of the temporally compressed aversive stimuli to the surroundings quite difficult (Fanselow, 1990; Milanovic *et al.*, 1998; Taubenfeld *et al.*, 1999), did not show any measurable modification of hippocampal excitability as against 'naïve' control rats. From a behavioural point of view only the animals trained according to the fear conditioning paradigm, 'conditioned' ones, exhibited long-lasting freezing. As stated above, the conditioned response was still present one month after the acquisition session. Without doubt this response is sustained by the

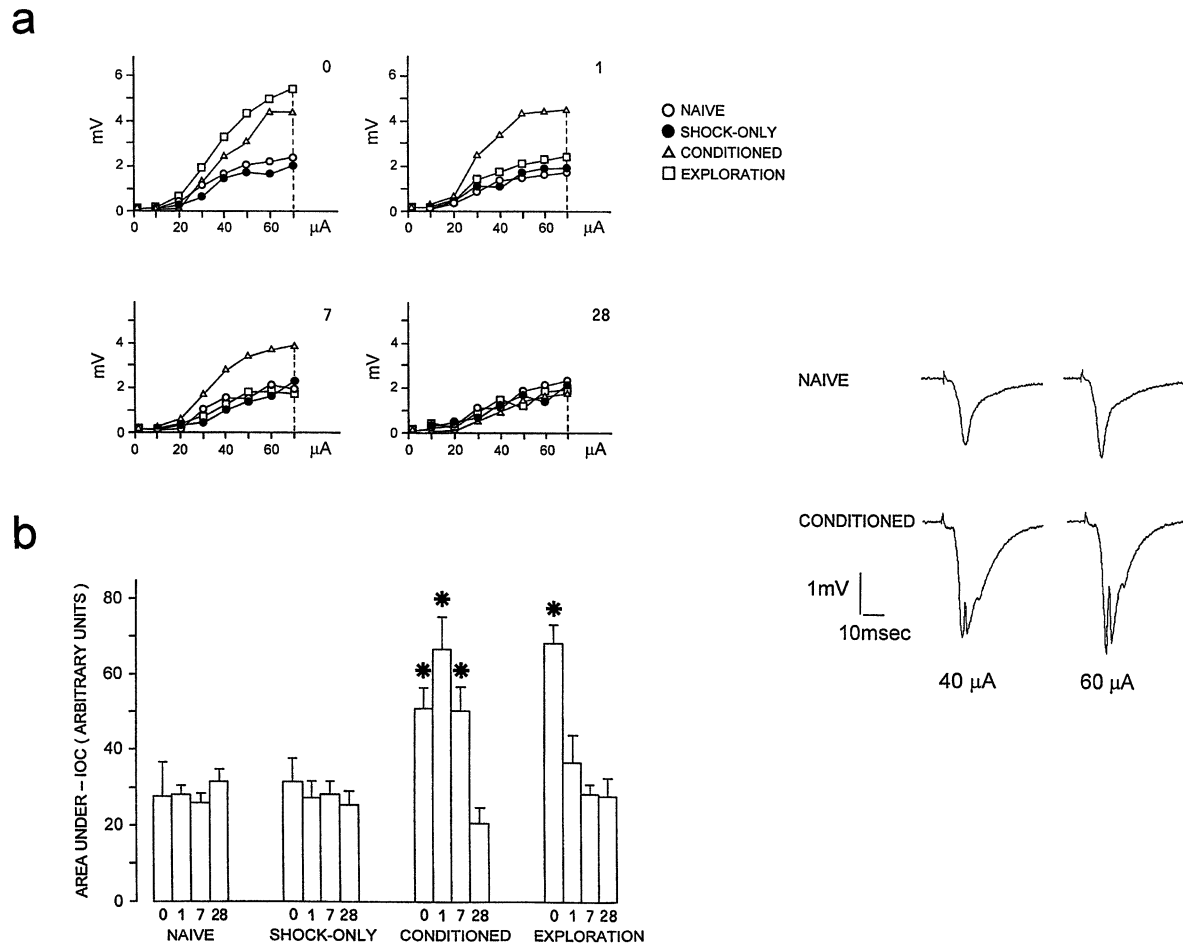


FIG. 3. Behavioural paradigms and hippocampal input-output curves (IOC). (a) The curves show mean values in mV of each group at increasing stimulation intensities (0–70 μ A). Recordings obtained from hippocampal slices prepared immediately after (0), or 1, 7 or 28 days after the training session for all paradigms: ‘conditioned’, ‘exploration’, ‘shock-only’, ‘naive’ (see Fig. 1 and Materials and Methods). ‘Conditioned’ animals exhibited increased IOCs up to the 7-day delay. ‘Exploration’ animals showed an increase only immediately after the training session. Neither ‘shock-only’ nor ‘naive’ rats ever exhibited any modification. (b) Histograms show under-IOC areas, i.e. the sums of the average amplitudes of fEPSPs obtained for the eight stimulus intensities employed. * $P < 0.01$ vs. the respective control groups (see Results).

neural process of associative learning (Fanselow, 1990; LeDoux, 1995; Milanovic *et al.*, 1998; Sacchetti *et al.*, 1999a, 1999b; Fanselow 2000). In fact, the freezing response is exhibited only by those experimental subjects which associated context and nociceptive stimuli, when they are placed again in the same surroundings where the association took place (retrieval testing), but not in a different one (Fanselow, 1990; Kim & Fanselow, 1992; Milanovic *et al.*, 1998; Sacchetti *et al.*, 1999a, 1999b).

There appears to be a clear-cut correlation between increased excitability of the Schaffer collateral–CA1 dendrite synapses and both contextual fear conditioning and exploration activity. In fact, there is a difference in excitability modification duration between the two groups of subjects. On the other hand, emotionality, muscular activity and arousing (nociceptive) stimulation, as such, do not appear to be sufficient to induce statistically significant hippocampal excitability modifications, as shown by the results from ‘shock-only’ groups. On this point, although in the present work no hormonal or vegetative findings are reported, it may be underlined that both ‘conditioned’ and ‘shock-only’ animals received total aversive stimulations quite lower than those reported as affecting hippocampal plasticity (Kim *et al.*, 1996; Garcia *et al.*, 1997). Indeed, the presently reported immediate postexploration increase in neuronal excitability

of ‘exploration’ groups could be a facet of the process of hippocampal activation induced by the exploratory activity. In fact, it is known that during exploration there are changes in the rat hippocampal electrical activity (theta rhythm, ‘place cells’ firing) which disappear when exploration ends (O’Keefe & Nadel, 1978; Thompson & Best, 1989). These phenomena are thought to be related to the acquisition of hippocampus-mediated spatial memories (O’Keefe & Nadel, 1978; Bland, 1986; O’Keefe, 1993). In the present study after an exploration followed by a learnable aversive stimulation (‘conditioned’), a long-lasting (no less than 7 days) increase of hippocampal excitability was measured. These long-lasting modifications were measured only in ‘conditioned’ animal slices. The modifications can be considered as associated to the neural processing that takes place only in these animals (association between surroundings and aversive stimuli). Conversely when the two types of stimuli were presented together, but in such a way as to make quite difficult the association between them (‘shock-only’) or when only one was presented (‘exploration’) no modifications of similar duration were measured. The whole of this experimental evidence makes it difficult to conclude that the reported long-lasting hippocampal functional changes could be not at all memory related. Further investigations will be needed to ascertain whether these

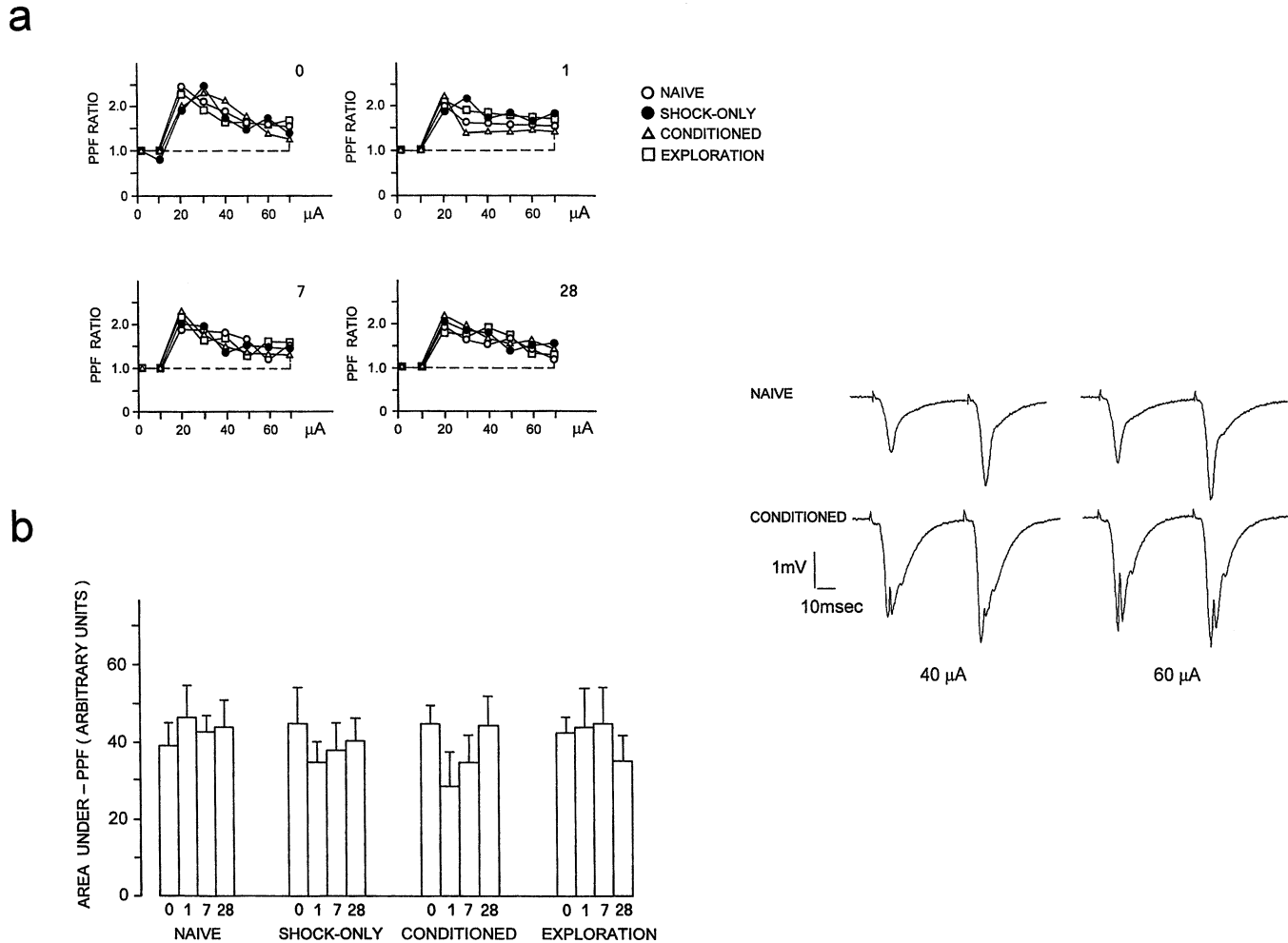


FIG. 4. Behavioural paradigms and paired pulse facilitation curves (PPF). (a) The curves show mean values of the rate between response amplitude of the first and second stimulus, at increasing stimulation intensities (0–70 μA). Values measured from hippocampal slices prepared as for IOC testing (see Fig. 2 and Materials and Methods). There were no between-group PPF differences ('conditioned', 'exploration', 'shock-only', 'naive') at any of the increasing post-training delays: immediately (0) or 1, 7 or 28 days after. (b) All histograms show very similar under-PPF areas.

modifications are due only to the known increase of hippocampal activity during cognitive processing, or are by themselves a prerequisite of learning.

It is to be underlined that the postacquisition hippocampal excitability modifications are quite similar, as to percent in increase, in 'exploration' and 'conditioned' subject slices, only the respective duration being different. We can surmise that contextual stimuli are sufficient, *per se*, to induce the full activation of the mechanisms controlling neuronal excitability. When the aversive stimuli are associated with the contextual (CS) ones, the larger amount of ingoing information can be processed only by increasing the duration of the enhanced excitability. The hippocampus is surely involved both in the exploration of new surroundings (CS) (Moser, 1995; Erikson *et al.*, 1993) and in its association with the US (nociceptive stimulation) (Kim & Fanselow, 1992; Kim *et al.*, 1993; LeDoux, 1995; Milanovic *et al.*, 1998; Fanselow, 2000). On the other hand, previously reported investigations employed sensory inputs (CS) known not to be processed in the hippocampus (e.g. acoustic stimuli) (Moyer *et al.*, 1996; Thompson *et al.*, 1996). Such paradigms allow the investigation of the effects of CS–US association on hippocampal excitability, but do not make it possible to study CS effects on it. The

presently employed experimental design makes it possible to investigate both contingencies.

IOC modifications, when present, appear to be due mainly to postsynaptic events because no PPF modification was ever found in all the present experiments. Indeed, the present results expand and confirm previous ones, which had shown that in subjects placed in enriched surroundings hippocampal excitability, as shown by IOCs, increased. The increase appears to be due to postsynaptic events, because no concurrent PPF modifications were found (Foster *et al.*, 1996). Similar results were obtained by means of intracellular recordings after trace–eye blink conditioning in the rabbit (Moyer *et al.*, 1996; Thompson *et al.*, 1996).

Concerning memorization phases, as far as we know, excitability modifications of the CA1 hippocampal region during postacquisition have been reported only employing a multitrial eye-blink paradigm. Electrical modifications were intracellularly recorded from ≈ 200 neurons in brain slices from conditioned rabbits. The modifications were well present a few hours after the last training session, becoming much less evident five days later and disappearing after a week (Moyer *et al.*, 1996; Thompson *et al.*, 1996). Instead the present results were obtained using a one-trial paradigm. This means that in

the present case the starting time of the learning process, if any, is precisely defined, making it possible to know when acquisition ends and consolidation begins, something which is not possible when animals are subjected to multitrial paradigms (Bures & Buresova, 1990; Ambrogi Lorenzini *et al.*, 1997). Given the recording technique employed (extracellular field recording) it appears that quite a large number of neurons exhibit increased excitability during the learning process (Jeffery, 1997).

Dissociation between the time course of IOC potentiation and memory

Concerning the time course of the presently reported modifications of neuronal excitability in 'conditioned' animals, excitability increases were measured up to the 7-day delay, but not at the 28-day one, when the freezing response was still quite well exhibited. The dissociation between IOC modifications and behavioural responses indicates that the former are not directly related to long-term retention and memorization of that conditioned response. It may be of interest to compare the presently reported durations and those of the hippocampal involvement in aversive conditioning mnemonic elaboration, as shown by means of the irreversible lesion technique. It appears that hippocampal involvement in aversive conditioning mnemonic elaboration is temporally circumscribed. In rats, permanent hippocampal lesions performed 10–14 days before the acquisition session did not impair contextual avoidance learning. In contrast, the same postoperative recovery duration was not sufficient to prevent learning impairment of a more complex spatial discrimination task (Gisquet-Verrier & Schenk, 1994). On the other hand, hippocampal lesions performed immediately after the acquisition session or one week later were followed by amnesia in a contextual fear conditioning task. The same damage, imposed one month later, was not followed by amnesia, i.e. at that time the hippocampus was no longer necessary (Kim & Fanselow, 1992). Because the freezing response was still well exhibited one month after acquisition, it was concluded that the hippocampus is not the single final long-term storage site of these engrams (Kim & Fanselow, 1992; Kim *et al.*, 1993; LeDoux, 1995). The present results expand and confirm on purely electrophysiological grounds (increased CA1 hippocampal neurons excitability) those previous findings. From a temporal standpoint, electrophysiological changes appear to be related to the consolidation of the contextual fear engram. The decrease in IOC potentiation that occurs over time may be due to already consolidated engram being expressed in some other form. In particular this potentiation should be related both to short- and long-term consolidation because it is present during both the phases during which these events appear to take place. In fact, hippocampal early postacquisition reversible inactivations induced amnesia (Matthies, 1989; Olds *et al.*, 1989; Ambrogi Lorenzini *et al.*, 1996; Bernabeu *et al.*, 1997; Izquierdo *et al.*, 1997; Sacchetti *et al.*, 1999b; Walz *et al.*, 2000), interfering with the short-term consolidation processes necessary for the subsequent encoding in long-term memory (Riedel *et al.*, 1999). On the other hand, it has been reported that in the hippocampus, a few days after the acquisition trial, there are ongoing processes possibly associated with long-term consolidation (Riedel *et al.*, 1999; Gewirtz *et al.*, 2000; Shimizu *et al.*, 2000) or with temporary storage (Kim & Fanselow, 1992; Moyer *et al.*, 1996; Thompson *et al.*, 1996). These later processes differ from those related to short-term memory because they are less easily disrupted. In fact, at these postacquisition delays only repeated and protracted reversible inactivations (Riedel *et al.*, 1999; Shimizu *et al.*, 2000) or irreversible lesions (Kim & Fanselow, 1992) are followed by amnesia. It can be concluded that the presently reported increase of hippocampal excitability (lasting up

to 7 days) is temporally related to both the short and long-term consolidation of contextual fear engrams, but not to their long-term storage. Further investigation may address the question of whether also the recall and reactivation of mnemonic traces (retrieval testing) may induce similar excitability modifications (Bucherelli & Tassoni, 1992).

In conclusion, there appears to be a clear-cut correlation between the increased excitability of the Schaffer collateral–CA1 dendrite synapses and either the consolidation of the freezing response induced by contextual fear conditioning or the exploratory activity. The effects of the two behavioural conditions differ only in the respective duration of the electrophysiological changes and do not appear to be supported by presynaptic transmitter release modifications.

Abbreviations

ACSF, artificial cerebrospinal fluid; CS, conditioned stimulus; fEPSP, excitatory postsynaptic field potential; IOC, input/output curve; PPF, paired-pulse facilitation curve; US, unconditioned stimulus.

References

- Ambrogi Lorenzini, C., Baldi, E., Bucherelli, C., Sacchetti, B. & Tassoni, G. (1996) Role of dorsal hippocampus in acquisition, consolidation and retrieval of rat's passive avoidance response: a tetrodotoxin functional inactivation study. *Brain Res.*, **730**, 32–39.
- Ambrogi Lorenzini, C.G., Baldi, E., Bucherelli, C., Sacchetti, B. & Tassoni, G. (1997) Analysis of mnemonic processing by means of totally reversible neural inactivations. *Brain Res. Protoc.*, **1**, 391–398.
- Barnes, C.A. (1995) Involvement of LTP in memory: are we 'searching under the street light'? *Neuron*, **15**, 751–754.
- Bernabeu, R., Bevilacqua, L., Ardenghi, P., Bromberg, E., Schmitz, P., Bianchin, M., Izquierdo, I. & Medina, J.H. (1997) Involvement of hippocampal cAMP/cAMP-dependent protein kinase signaling pathways in a late memory consolidation phase of aversively motivated learning in rats. *Proc. Natl. Acad. Sci. USA*, **94**, 7041–7046.
- Berretta, N., Berton, F., Bianchi, R., Capogna, M., Francesconi, W. & Brunelli, M. (1990) Effects of dopamine, D-1 and D-2 dopaminergic agonists on the excitability of hippocampal CA1 pyramidal cells in guinea pig. *Exp. Brain Res.*, **83**, 124–130.
- Bland, B.H. (1986) The physiology and pharmacology of hippocampal formation theta rhythms. *Prog. Neurobiol.*, **26**, 1–54.
- Bucherelli, C. & Tassoni, G. (1992) Engram activation reinstates the susceptibility of consolidated memory traces to retrograde amnesia by functional blockade of parabrachial nuclei. *Behav. Brain Res.*, **51**, 61–65.
- Bures, J. & Buresova, O. (1990) Reversible lesions allow reinterpretation of system level studies of brain mechanisms of behavior. *Concepts Neurosci.*, **1**, 69–89.
- Buzsaki, G., Leung, L.S. & Vanderwolf, C.H. (1983) Cellular basis of hippocampal EEG in the behaving rat. *Brain Res. Rev.*, **6**, 139–171.
- Creager, R., Dunwiddie, T. & Lynch, G. (1980) Paired-pulse and frequency facilitation in the CA1 region of the *in vitro* rat hippocampus. *J. Physiol. (Lond.)*, **299**, 409–424.
- Erickson, C.A., McNaughton, B.L. & Barnes, C.A. (1993) Comparison of long-term enhancement and short-term exploratory modulation of perforant path synaptic transmission. *Brain Res.*, **615**, 275–280.
- Fanselow, M.S. (1990) Factors governing one-trial contextual conditioning. *Anim. Learn. Behav.*, **18**, 264–270.
- Fanselow, M.S. (2000) Contextual fear, gestalt memories, and the hippocampus. *Behav. Brain Res.*, **110**, 73–81.
- Foster, T.C., Gagne, J. & Massicotte, G. (1996) Mechanism of altered synaptic strength due to experience: relation to long-term potentiation. *Brain Res.*, **736**, 243–250.
- Garcia, R., Musleh, W., Tocco, G., Thompson, R.F. & Baudry, M. (1997) Time-dependent blockade of STP and LTP in hippocampal slices following acute stress in mice. *Neurosci. Lett.*, **233**, 41–44.
- Gewirtz, J.C., McNish, K.A. & Davis, M. (2000) Is the hippocampus necessary for contextual fear conditioning? *Behav. Brain Res.*, **110**, 83–95.
- Gisquet-Verrier, P. & Schenk, F. (1994) Selective hippocampal lesions in rats

- do not affect retrieval processes promoted by prior cuing with the conditioned stimulus or the context. *Psychobiology*, **22**, 289–303.
- Green, E.J., McNaughton, B.L. & Barnes, C.A. (1990) Exploration-dependent modulation of evoked responses in fascia dentata: dissociation of motor, EEG, and sensory factors and evidence for a synaptic efficacy change. *J. Neurosci.*, **10**, 1455–1471.
- Izquierdo, I., Quirrelfeldt, J.A., Zanatta, M.S., Quevedo, J., Schaffer, E., Schmitz, P.K. & Medina, J.H. (1997) Sequential role of hippocampus and amygdala, entorhinal cortex and parietal cortex in formation and retrieval of memory for inhibitory avoidance in rats. *Eur. J. Neurosci.*, **9**, 786–793.
- Jeffery, K.J. (1997) LTP and spatial learning – where to next? *Hippocampus*, **7**, 95–110.
- Kim, J.J. & Fanselow, M.S. (1992) Modality-specific retrograde amnesia of fear. *Science*, **256**, 675–677.
- Kim, J.J., Foy, M.R. & Thompson, R.F. (1996) Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. *Proc. Natl. Acad. Sci. USA*, **93**, 4750–4753.
- Kim, J.J., Rison, R.A. & Fanselow, M.S. (1993) Effects of amygdala, hippocampus and periaqueductal gray lesions on short- and long-term contextual fear. *Behav. Neurosci.*, **107**, 1093–1098.
- LeDoux, J.E. (1995) Emotion: clues from the brain. *Annu. Rev. Psychol.*, **46**, 209–235.
- Leung, L.S. & Ambrose, S.A. (1994) Long-term potentiation as a function of test pulse intensity: a study using input/output profiles. *Brain Res. Bull.*, **33**, 453–460.
- Mathies, H. (1989) In search of cellular mechanisms of memory. *Prog. Neurobiol.*, **32**, 277–349.
- Milanovic, S., Radulovic, J., Laban, O., Stiedl, O., Henn, F. & Spiess, J. (1998) Production of the Fos protein after contextual fear conditioning of C57BL/6N mice. *Brain Res.*, **784**, 37–47.
- Moser, E.I. (1995) Learning-related changes in hippocampal field potentials. *Behav. Brain Res.*, **71**, 11–18.
- Moser, E.I., Mathiesen, I. & Andersen, P. (1993) Association between brain temperature and dentate field potentials in exploring and swimming rats. *Science*, **259**, 1324–1326.
- Moyer, J.R., Jr, Thompson, L.T. & Disterhoft, J.F. (1996) Trace eyeblink conditioning increases CA1 excitability in a transient and learning-specific manner. *J. Neurosci.*, **16**, 5536–5546.
- O'Keefe, J. (1993) Hippocampus, theta, and spatial memory. *Curr. Opin. Neurobiol.*, **3**, 917–924.
- O'Keefe, J. & Nadel, L. (1978). *The Hippocampus as a Cognitive Map*. Oxford University Press, New York.
- Olds, J.L., Anderson, M.L., McPhie, D.L., Staten, L.D. & Alkon, D.L. (1989) Imaging of memory-specific changes in the distribution of protein kinase C in the hippocampus. *Science*, **245**, 866–869.
- Riedel, G., Micheau, J., Lam, A.G.M., Roloff, E.V.L., Martin, S.J., Bridge, H., de Hoz, L., Poeschel, B., McCulloch, J. & Morris, R.G.M. (1999) Reversible neural inactivation reveals hippocampal participation in several memory processes. *Nature Neurosci.*, **2**, 898–905.
- Sacchetti, B., Ambrogio Lorenzini, C., Baldi, E., Tassoni, G. & Bucherelli, C. (1999a) Memorization of contextual and CS conditioned fear response (freezing) in a one-trial acquisition paradigm. *Arch. It. Biol.*, **137**, 235–248.
- Sacchetti, B., Ambrogio Lorenzini, C., Baldi, E., Tassoni, G. & Bucherelli, C. (1999b) Auditory thalamus, dorsal hippocampus, basolateral amygdala, and perirhinal cortex role in the consolidation of conditioned freezing to context and to acoustic conditioned stimulus in the rat. *J. Neurosci.*, **19**, 9570–9578.
- Sharp, P.E., McNaughton, B.L. & Barnes, C.A. (1985) Enhancement of hippocampal field potentials in rats exposed to a novel, complex environment. *Brain Res.*, **339**, 361–365.
- Shimizu, E., Tang, Y.-P., Rampon, C. & Tsien, J.Z. (2000) NMDA receptor-dependent synaptic reinforcement as a crucial process for memory consolidation. *Science*, **290**, 1170–1174.
- Shulz, P., Cook, E. & Johnston, D. (1994) Changes in paired-pulse facilitation suggest presynaptic involvement in long-term potentiation. *J. Neurosci.*, **14**, 5323–5337.
- Skelton, R.W., Scarth, A.S., Wilkie, D.M. & Phillips, A.G. (1987) Long-term increases in dentate granule cell responsiveness accompany operant conditioning. *J. Neurosci.*, **7**, 3081–3087.
- Taubenfeld, S.M., Wiig, K.A., Bear, M.F. & Alberini, C.M. (1999) A molecular correlate of memory and amnesia in the hippocampus. *Nature Neurosci.*, **2**, 309–310.
- Thompson, L.T. & Best, P.J. (1989) Place cells and silent cells in the hippocampus of freely-behaving rats. *J. Neurosci.*, **9**, 2382–2390.
- Thompson, L.T., Moyer, J.R. Jr & Disterhoft, J.F. (1996) Transient changes in excitability of rabbit CA3 neurons with a time course appropriate to support memory consolidation. *J. Neurophysiol.*, **76**, 1836–1849.
- Walz, R., Roesler, R., Quevedo, J., Sant'Anna, M.K., Madruga, M., Rodrigues, C., Gottfried, C., Medina, J.H. & Izquierdo, I. (2000) Time-dependent impairment of inhibitory avoidance retention in rats by posttraining infusion of a mitogen-activated protein kinase inhibitor into cortical and limbic structures. *Neurobiol. Learn. Mem.*, **73**, 11–20.