

Regulation of hippocampal cell adhesion molecules NCAM and L1 by contextual fear conditioning is dependent upon time and stressor intensity

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Abstract

Cell adhesion molecules (CAMs) of the immunoglobulin superfamily, NCAM and L1, as well as the post-translational addition of α -2,8-linked polysialic acid (PSA) homopolymers to NCAM (PSA–NCAM), have been implicated in the neural mechanisms underlying memory formation. Given that the degree of stress elicited by the training situation is one of the key factors that influence consolidation processes, this study questioned whether training rats under different stressor intensities (0.2, 0.4, or 1 mA shock intensity) in a contextual fear conditioning task might regulate subsequent expression of NCAM, PSA–NCAM and L1 in the hippocampus, as evaluated immediately after testing rats for conditioning at 12 and 24 h after training. Behavioural inhibition (evaluated as a ‘freezing’ index) at testing and post-testing plasma corticosterone levels were also assessed. The results showed that 12 h post-training, conditioned animals displayed reduced NCAM, but increased L1, expression. At this time point, the group trained at the highest shock intensity (1 mA) also presented decreased PSA–NCAM expression. Analyses performed 24 h post-training indicated that the 1 mA group exhibited increased NCAM and L1 expression, but decreased expression of PSA–NCAM levels. In addition, L1 values that presented a shock intensity-dependent U-shaped pattern were also increased in the group trained at the lowest shock condition (0.2 mA) and remained unchanged in the intermediate shock condition (0.4 mA). Freezing and corticosterone values at both testing times were positively related with shock intensity experienced at training. Therefore, our results show a complex regulation of CAMs of the immunoglobulin superfamily in the hippocampus that depends upon stressor intensity and time factors. In addition, the pattern of CAMs expression found in the 1 mA group (which is the one that shows higher post-training corticosterone levels and develops the stronger and longer-lasting levels of fear conditioning) supports the view that, after a first phase of synaptic de-adherence during consolidation, NCAM and L1 might participate in the stabilization of selected synapses underlying the establishment of long-term memory for contextual fear conditioning, and suggests that glucocorticoids might play a role in the observed regulation of CAMs.

Introduction

Long-term memory formation is believed to involve the remodelling of specific neuronal circuitries, resulting in modifications of synaptic efficacy (Bailey & Kandel, 1993; O’Malley *et al.*, 1998; Skrebitsky & Chepkova, 1998). Increasing evidence indicates that cell adhesion molecules (CAMs), which have been largely implicated in cell–cell interactions during development of the nervous system (Edelman & Jones, 1998), also play a critical role in activity-dependent synaptic plasticity in adulthood (Fields & Itoh, 1996; Schachner, 1997; Murase & Schuman, 1999). CAMs are cell surface macromolecules that participate in target recognition and synapse stabilization (Murase & Schuman, 1999). Among them, the members of the immunoglobulin superfamily, neural cell adhesion molecule (NCAM) and L1, have been reported to participate in the synaptic changes underlying learning and memory processes (Bailey *et al.*, 1992; Doyle *et al.*, 1992a; Scholey *et al.*, 1993, 1995; Cremer *et al.*, 1994; Arami *et al.*, 1996; Skibo *et al.*, 1998; Wolfer *et al.*, 1998) and synaptic plasticity (Lüthi *et al.*, 1994; Ronn *et al.*, 1995; Schuster *et al.*, 1998) in

different animal species. Furthermore, the post-translational modification of NCAM that consists in the addition of α -2,8-linked polysialic acid (PSA) homopolymers to its fifth immunoglobulin-like domain, by attenuating interactions mediated by NCAM and other related molecules (Sadoul *et al.*, 1983; Zhang *et al.*, 1992; Rougon, 1993), provides another mechanism for structural plasticity. Interestingly, PSA–NCAM has also been implicated in memory formation (Doyle *et al.*, 1992b; Fox *et al.*, 1995; Murphy *et al.*, 1996; O’Connell *et al.*, 1997) and synaptic plasticity (Becker *et al.*, 1996; Muller *et al.*, 1996).

One of the key factors that influence consolidation processes that determine the storage of information into long-term memory is the stress reaction elicited by learning situations (for reviews see Gold & McCarty, 1995; Sandi, 1998). Using different animal and learning tasks, we have shown that the strength at which a new behavioural response is acquired and retained into a long-term memory is related to the intensity of the aversive unconditioned stimuli involved in each particular task (Sandi & Rose, 1994a; Sandi *et al.*, 1997; Cordero *et al.*, 1998). Among the possible mechanisms involved, evidence indicated that the release of adrenal glucocorticoid hormones induced by training exerts a facilitating effect on memory storage (Sandi & Rose, 1994a; Sandi *et al.*, 1997; Cordero *et al.*, 1998) through the

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activation of specific brain corticosteroid receptors (Oitzl & de Kloet, 1992; Sandi & Rose, 1994b; Roozendaal & McGaugh, 1997; Cordero & Sandi, 1998). Although the exact mechanisms by which stress affects consolidation remain largely unknown, different findings support the view that glucocorticoid actions might be dependent upon a modulation of CAMs (Sandi *et al.*, 1995; Venero *et al.*, 1996; Sandi & Rose, 1997; Sandi & Loscertales, 1999).

The possible differential regulation of CAMs expression by learning situations involving different stress levels has not as yet been addressed. We have recently characterized the contextual fear conditioning task as a model in which the strength of memory is related to stressor intensity and training-induced corticosterone activation (Cordero *et al.*, 1998). In this task, rats develop a characteristic immobility, or 'freezing' response, when re-exposed to the context in which they had previously experienced brief, inescapable shocks. We showed that the extent of conditioned fear and the levels of plasma corticosterone, following context exposure at training and at different times post-training (1 and 7 days), were dependent upon the intensity of the stressor (footshock; 0.2, 0.4 and 1 mA); i.e. the higher the intensity of the shock delivered at training, the higher the freezing levels and corticosterone values at all testing times. Even though there is some controversy in the literature, the hippocampus seems to play a pivotal role within the different neural processes involved in learning and performance of this task (Holland & Bouton, 1999). The experiments reported here were designed to: (i) evaluate whether the expression of NCAM, PSA-NCAM and L1 might be differentially regulated in the hippocampus, at different times (12 and 24 h) after training rats under different stressor intensities in the contextual fear conditioning task; and (ii) whether any relationship could be established between CAMs expression and plasma corticosterone values. Part of this work has been previously published in abstract form (Sandi *et al.*, 1998).

Materials and methods

Animals

Male Wistar rats (Harlan Iberica, Spain), weighing 120–150 g on arrival, were housed in groups of three per cage, under temperature- $(22 \pm 2^\circ\text{C})$ and light- (12:12 h light:dark cycle; lights on at 07.00 h) controlled conditions, and had free access to food and water in a colony room. Approximately 2 weeks after arrival, they were weighed and handled daily for 3–4 days for habituation to the experimental manipulation. Experiments started subsequently on the third week after arrival (rats weighing 250–300 g, in their 9–10th week of life), and were always conducted between 09.00 and 14.30 h. Animal care procedures were conducted in accordance with the guidelines set by the European Community Council Directives (86/609/EEC).

Contextual fear conditioning

Training and testing took place in a rodent observation cage ($30 \times 37 \times 25$ cm) that was positioned inside a sound-attenuating chamber. The side walls of the observation cage were constructed of stainless steel, and the back walls and doors were constructed of clear Plexiglas. The floor consisted of 20 steel rods through which a scrambled shock from a LETICA I.C. (Spain) shock generator (Model LI100-26 Shocker) could be delivered. Each observation cage was cleaned with a 1% acetic acid solution before and after each session. The sound-attenuating chambers were illuminated by a 20 W white light bulb. Ventilation fans provided background noise at 68 dB.

On the training day, each rat was transported from the colony room to the laboratory (situated in adjacent rooms) and placed into the

conditioning chamber. After 3 min, the rats received three 1-s shocks (unconditioned stimuli), intensity varying (0.2, 0.4 or 1 mA) for each rat depending on the experimental group to which it had previously been assigned. The intertrial interval was 60 s, and the rats were removed from the conditioning chambers 30 s after the final shock presentation, and returned to their home cages. Thus, a conditioning session lasted ~ 330 s. The experiments involved two additional groups that received the same handling manipulation as the shocked groups: one of them was also exposed to the conditioning chamber at training and testing, but did not receive any shock ('0-mA group'), whereas the other one was not exposed to the conditioning chamber ('undisturbed group'). Therefore, only endocrine and molecular, but not behavioural, measures were obtained for this latter group. Testing for contextual fear conditioning was performed either 12 or 24 h after conditioning, depending on the experiment. At testing, rats were placed back into the same chamber as used in conditioning in the absence of shock for a 8-min context test. A video-camera was used to record the behaviour of rats both during training and testing. Subsequently, the time spent by each rat either freezing or active was scored blind assisted by a computer programme. Freezing was defined as behavioural immobility except for movement needed for respiration. Behaviour was evaluated on each experimental session. At training, behavioural scores were carried out for the 3-min period prior to shock (pre-shock period) and for the 2.5-min period starting immediately after the first shock presentation (post-shock period). Scores for each of these periods were analysed separately (data not shown; however, freezing values during the post-shock period at training follow the same pattern as that found at testing). We established the criteria that if a rat showed freezing values above 15% during the pre-shock period, it would be discarded for further analyses. However, in this study, no rat had to be discarded for this reason. Therefore, behavioural data presented are related to the testing sessions, in which behaviour was scored during the 8-min re-exposure to the training context.

Two experiments were carried out to assess for CAMs modulation after fear conditioning. They only differed on the length of time (12 or 24 h) after training at which the rats were tested. Rats were decapitated immediately after the 8-min testing period.

Corticosterone assessment

After decapitation, trunk blood was collected and samples were centrifuged (3000 r.p.m. for 20 min, at 4°C). Plasma was stored at -35°C . Corticosterone was measured using a radioimmunoassay kit (Coat-A-Count, Diagnostics Products Corporation, CA, USA).

CAMS quantification

Immediately after decapitation, the brain was removed and the hippocampus dissected out on ice. Tissue samples were coded and stored at -80°C until use. Crude synaptosomal pellets (P_2) were obtained according to a modified protocol from Lynch & Voss (1991). Briefly, tissue was homogenized in 10 volumes of ice-cold sucrose (0.32 M) and *N*-2-[hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid] (HEPES, 5 mM) buffer that contained a cocktail of protease inhibitors (Complete TM, Boehringer Mannheim, UK) with 16 strokes and centrifuged at 1000 g for 5 min. The supernatant was then centrifuged at 15000 g for 15 min, and the pellet was resuspended in Krebs buffer, containing protease inhibitors, for use. Protein concentration for each sample was estimated by the method of Bradford (1976).

All CAMs were quantified by enzyme-linked immunoabsorbent assays (ELISAs). Briefly, flat-bottom 96-well microplates were allowed to adsorb a coating solution (0.1 M $\text{Na}_2\text{CO}_3/0.1$ M NaHCO_3)

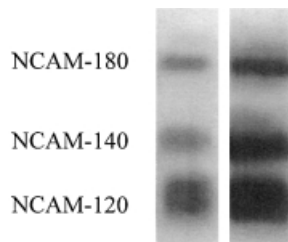


FIG. 1. NCAM polypeptide pattern in tissue samples from the rat hippocampus, as shown by SDS-PAGE and immunoblotting. Left lane, whole homogenates; right lane, crude synaptosomal preparations. Positions of the three major NCAM isoforms are indicated at the margin.

for 2 h at room temperature. The solution was removed and 50 μ L of pellet samples added at a concentration of 5 μ g/mL (NCAM assays) or 10 μ g/mL (PSA-NCAM and L1 assays) for 20–24 h at 4 $^{\circ}$ C. Additional binding sites were blocked with bovine serum albumin (BSA, 3%) for 1.5 h at room temperature. The wells were rinsed three times and incubated with 50- μ L aliquots of the corresponding first antibody for 20–24 h at 4 $^{\circ}$ C. The wells were washed and 50- μ L aliquots of peroxidase-conjugated second antibody were added for a 2-h incubation period. Afterwards, 50 μ L of citrate buffer (in mM: Na_2HPO_4 , 50; citric acid, 25; pH 4.5) containing 1 mg/mL *o*-phenylene-diamine and 0.06% H_2O_2 , added just before use, was placed in each well, and the peroxidase allowed to react for 10 min at room temperature. The reaction was terminated by the addition of 50 μ L of 5 N H_2SO_4 to each well. The optic density was determined by measuring absorbency at 492 nm with a Microplate Reader (DigiScan Reader V3.0 and DigiWIN software Program; ASYS Hitech GmbH, Austria).

For NCAM assays, we used a polyclonal rabbit antirat NCAM immunoserum (diluted 1:300; generous gift from Prof. Elisabeth Bock, University of Copenhagen, Denmark, Ibsen *et al.*, 1983) as the primary antibody, and an antirabbit immunoglobulin (Ig) peroxidase conjugate (whole molecule conjugate; diluted 1:500; Sigma, UK) as the secondary antibody. For PSA-NCAM assays, a monoclonal antibody was used (Men B, clone B1.2; generous gift from Prof. Genevieve Rougon, CNRS Marseille, France, Rougon *et al.*, 1986). This is a mouse IgM antibody (1:2 dilution of ascites fluid; diluted 1:300 for the ELISA) that recognizes specifically α -2,8-linked PSA with chain length superior to 12 residues, and binds with high specificity to PSA on NCAM (Kiss *et al.*, 1994). An IgM antimouse peroxidase (μ chain) conjugate (Sigma-Aldrich, Spain) was used, at a 1:1000 dilution, as the second antibody. For L1 assays, a monoclonal antirat IgG antibody was used (concentration 100 μ g/mL; Boehringer Mannheim, Spain) at a 1:10 dilution. The second antibody employed in L1 assays was an antirat Ig-POD Fab fragments (from sheep immunoglobulin) to IgG-rat peroxidase conjugated (Boehringer Mannheim), used at a 1:500 dilution.

Because NCAM consists of several isoforms (including three major ones of molecular weights 120, 140 and 180 kDa) that result from alternative splicing of a single gene (Goridis & Brunet, 1992), we also performed Western blots to check whether our experimental conditions using crude synaptosomal preparations would detect all major isoforms. Whole homogenates and crude synaptosomal samples from rat hippocampus were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted and stained with the polyclonal NCAM antibody used in ELISAs (see above). Equal amounts of protein (15 μ g) were applied in each lane. As can be seen in Fig. 1, the polyclonal antibody to total NCAM identified the three major NCAM isoforms of molecular weights around 180, 140 and 120 kDa both in whole homogenates

and in the crude synaptosomal preparations. As can be seen, crude synaptosomal preparations were enriched in NCAM content in comparison with whole homogenates of hippocampal tissue.

Statistics

The behavioural data regarding the time each rat spent freezing per session were transformed to a percentage of total time. All results were expressed as mean \pm SEM. Results were analysed by performing one-way analysis of variance (ANOVA). Differences in treatment levels were further evaluated for significance with Student's *t*-test *post hoc* comparisons. Significance was accepted at $P < 0.05$.

Results

Contextual fear conditioning and plasma corticosterone

Freezing values and plasma corticosterone levels displayed by rats at the different testing times (12 and 24 h) selected in our study are displayed in Fig. 2. ANOVAs indicated that, both at 12 h ($F_{3,37} = 27.25$, $P < 0.0001$) and 24 h ($F_{2,45} = 29.38$, $P < 0.0001$) post-training, freezing values showed a significant group effect. At both time points, *post hoc* Student's *t*-test analyses revealed that freezing levels were significantly higher in all conditioned groups as compared with the unshocked, 0 mA group (all $P < 0.05$). In addition, the group trained at the 1 mA shock intensity had higher freezing levels than in the 0.4 mA ($P < 0.05$) and 0.2 mA ($P < 0.005$) groups, and the 0.4 mA group displayed significantly more freezing than 0.2 mA trained rats ($P < 0.005$).

As for plasma corticosterone levels, ANOVAs corresponding to values obtained from rats tested at 12 h ($F_{4,59} = 7.88$, $P < 0.0001$) and 24 h ($F_{4,57} = 13.12$, $P < 0.0001$) post-training indicated a significant effect. At both time points, all conditioned groups showed increased plasma corticosterone levels as a result of training, as compared with the undisturbed group (all $P < 0.005$, except for the 12 h time point in which statistical significance was $P < 0.05$ for the 0.2 mA group). In addition, the 0 mA group also showed significantly higher levels than the undisturbed group ($P < 0.05$) when corticosterone was analysed 24 h post-training. On the other hand, when compared with the values from the 0 mA group, both the 0.4 and 1 mA groups displayed significantly higher hormones levels ($P < 0.005$) in the 12 h time point experiment, whereas only the 1 mA group displayed significantly higher hormone levels than the 0 mA group ($P < 0.05$) in the 24 h time point experiment.

Hippocampal expression of CAMs 12 h after contextual fear conditioning

Then, we explored whether training in the contextual fear conditioning task, at different shock intensities (0.2, 0.4 or 1 mA), might differentially modulate the expression of NCAM, PSA-NCAM and L1 at the level of the hippocampus when analysed at 12 h post-training. Results are represented in Fig. 3.

As can be seen, all trained groups showed a significant decrease in NCAM expression ($F_{4,59} = 29.76$, $P < 0.00001$; Student's *t*-tests: all conditioned groups $P < 0.005$ versus both the 0 mA group and the undisturbed group). ANOVA of PSA-NCAM also showed statistical significance ($F_{4,59} = 3.91$, $P < 0.008$). Student's *t*-tests indicated that PSA-NCAM values from all conditioned groups were reduced when compared with the undisturbed group (all $P < 0.05$). However, when compared with the 0 mA group, the only group that showed significantly lower values was the one trained at the highest shock intensity (1 mA, $P < 0.05$). As for L1, ANOVA indicated a significant treatment effect ($F_{4,59} = 8.75$, $P < 0.0001$). *Post hoc* Student's *t*-tests indicated that the groups trained at the lower (0.2 mA) and higher

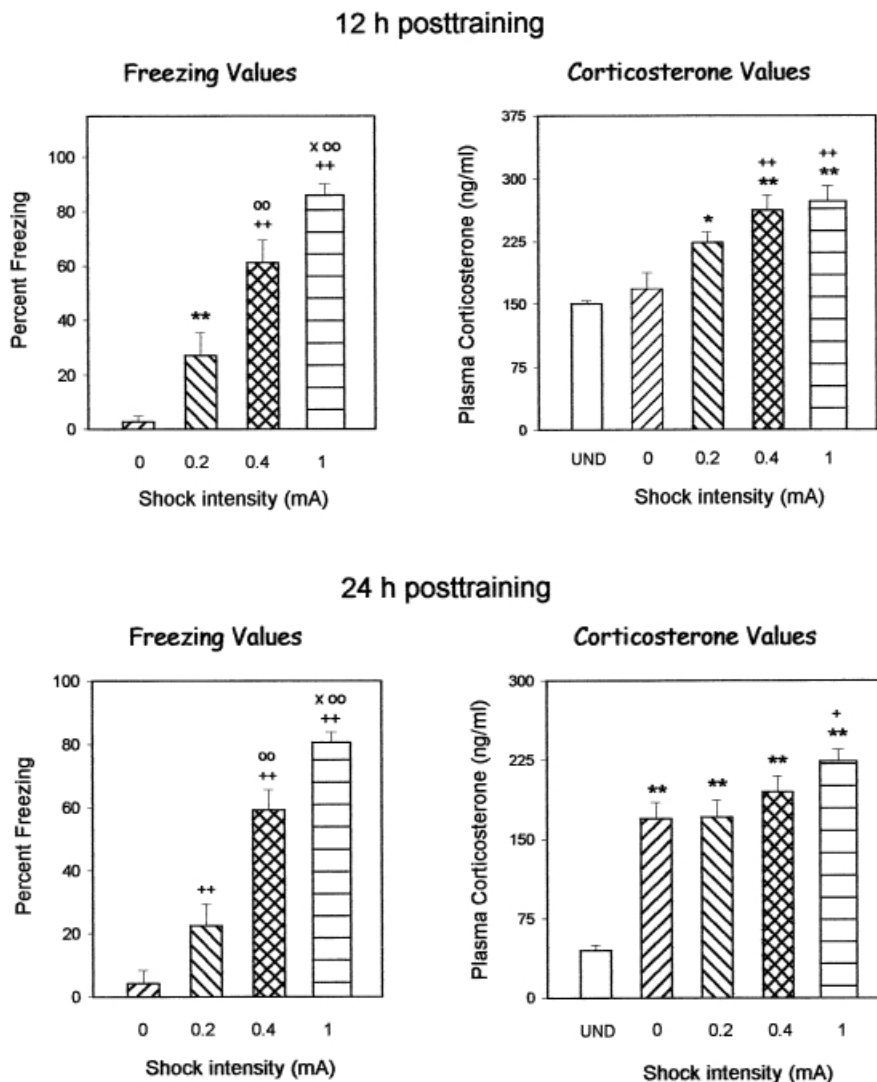


FIG. 2. Percentage of time spent freezing at testing (left panels) and post-testing plasma corticosterone values (right panels) evaluated at 12 and 24 h after training rats in a contextual fear conditioning task at different shock intensities. Results are the mean \pm SEM from 10 to 20 rats per group. * $P < 0.05$ and ** $P < 0.005$ versus the undisturbed (UND) group; + $P < 0.05$ and ++ $P < 0.005$ versus 0 mA group; oo $P < 0.005$ versus the 0.2 mA group; x $P < 0.05$ versus the 0.4 mA group.

(1 mA) shock intensity conditions displayed increased hippocampal L1 levels as compared to the 0 mA ($P < 0.05$) and undisturbed control groups ($P < 0.05$).

Hippocampal expression of CAMs 24 h after contextual fear conditioning

Then, we examined CAMs expression in the hippocampus 24 h after training rats in the contextual fear conditioning paradigm using the same shock intensities (0.2, 0.4 or 1 mA) as above. Results are represented in Fig. 4.

At this time point after training, NCAM expression was increased in the 1 mA group ($F_{4,57} = 4.78$, $P < 0.002$; Student's *t*-tests: $P < 0.005$ 1 mA group versus 'undisturbed' and '0 mA' groups). As before, values of PSA-NCAM were only significantly altered in the group trained at the highest shock intensity (1 mA), which showed decreased expression as compared to controls ($F_{4,57} = 2.48$, $P < 0.05$; Student's *t*-tests: $P < 0.05$ 1 mA group versus undisturbed group, and a trend towards significance ($P < 0.09$) between 1 mA group and 0 mA group). In addition, ANOVA for L1 data indicated a significant treatment effect ($F_{4,57} = 10.04$, $P < 0.0001$). *Post hoc* tests indicated that contextual fear conditioning modulated L1 according to a shock intensity-related U-shaped function, with the levels of this CAM being elevated, as compared to both control groups in the

groups trained at the lower (0.2 mA; $P < 0.02$ versus undisturbed group, and a trend of $P < 0.06$ versus 0 mA group) and higher (1 mA; $P < 0.0001$ versus undisturbed, and $P < 0.0001$ versus 0 mA group) shock intensity conditions, and remaining unchanged in the group trained at the intermediate shock intensity (0.4 mA; NS).

Discussion

In this study, we explored the possible modulation of hippocampal cell adhesion molecules of the immunoglobulin superfamily at different times (12 and 24 h) after exposing rats to a contextual fear conditioning experience. We had previously reported that the degree of fear conditioning displayed by rats, both during the acquisition session and at subsequent testing times, is dependent upon both the shock intensity delivered at training and the degree of post-training corticosterone secretion (Cordero & Sandi, 1998; Cordero *et al.*, 1998). The current study further supports that view and, by using a synaptosomal preparation, shows a complex regulation of NCAM, PSA-NCAM and L1 as a function of shock intensity and time factors. By 12 h post-conditioning, whereas NCAM expression was reduced in all conditioned groups, L1 was increased in the 0.2 and 1 mA groups. In addition, all conditioned animals displayed a reduction in PSA-NCAM expression when compared with undisturbed controls,

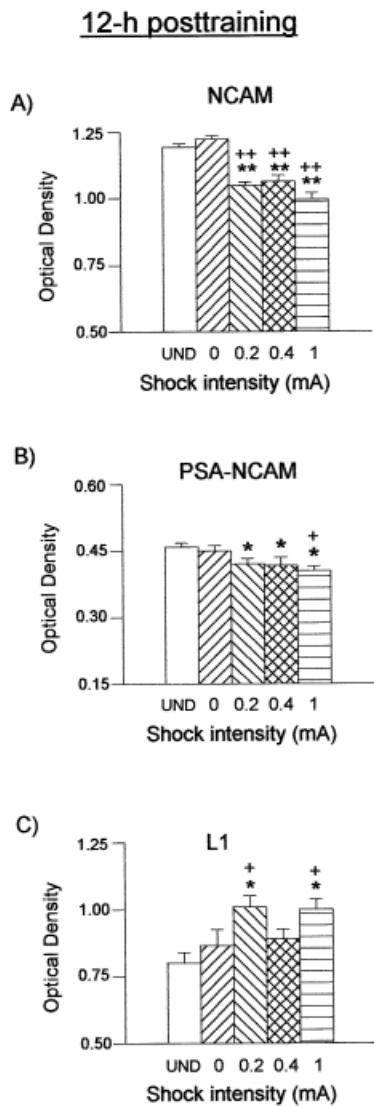


FIG. 3. Expression of NCAM, PSA-NCAM and L1 in crude synaptosomal preparations from the hippocampus of rats 12h after being trained in a contextual fear conditioning using different shock intensities at training. Results are determined by ELISAs and expressed as the optical density obtained by measuring absorption at 492 nm. Data are the mean \pm SEM from 10 to 20 rats per group. * P <0.05 and ** P <0.005 versus the undisturbed (UND) group; + P <0.05 and ++ P <0.005 versus the 0 mA group.

but this reduction only reached statistical significance, when compared with the 0 mA control group, in the group trained at the highest shock intensity (1 mA). At 24 h post-conditioning, only the 1 mA shocked group exhibited changes in NCAM expression, which were increased with regards to both control groups (undisturbed and 0 mA). This group also showed reduced levels of PSA-NCAM. As for L1, levels were increased in a shock-related U-shaped manner, with the groups in the lowest and highest shock conditions displaying enhanced expression, and levels from the group trained at the intermediate shock intensity remaining unchanged. There were no differences in the expression of the different CAMs between the undisturbed group and the 0 mA group at any of the time points studied.

The current view is that learning and memory processes involve the remodelling of neural circuits which, in addition to the possible formation of new synaptic connections, would require the modifica-

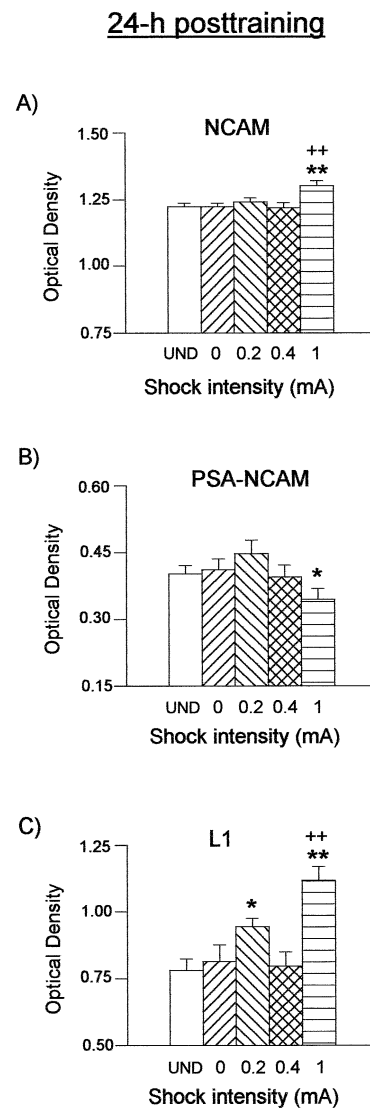


FIG. 4. Expression of NCAM, PSA-NCAM and L1 in crude synaptosomal preparations from the hippocampus of rats 24h after being trained in a contextual fear conditioning using different shock intensities at training. Results are determined by ELISAs and expressed as the optical density obtained by measuring absorption at 492 nm. Data are the mean \pm SEM from 10 to 15 rats per group. * P <0.05 and ** P <0.005 versus the undisturbed (UND) group; ++ P <0.005 versus the 0 mA group.

tion of pre-existing ones (Bailey & Kandel, 1993). Increasing evidence indicates that this structural rearrangement involves a preliminary step of synaptic de-adherence to allow for either the growth, or the alteration, of synaptic connections that accompany long-term memory (Bailey & Kandel, 1993; Rose, 1995; Abel & Kandel, 1998). Both NCAM and L1 are involved in the stabilization of cell contacts through homophilic and heterophilic binding between the extracellular domains of pre- and postsynaptic CAMs (Horstkorte *et al.*, 1993; Kiselyov *et al.*, 1997). In addition, CAMs can also undergo post-translational modifications that can transiently serve to facilitate synaptic de-adherence and therefore stimulate plasticity (Fields & Itoh, 1996). In this respect, most of the present knowledge is related to mechanisms pertained to the downregulation of stabilizing forms of NCAM (Doherty *et al.*, 1994), e.g.: (i) the reduction of synaptic NCAM; or (ii) the increase of NCAM polysialylation state.

In our study, we obtained evidence to account for the former possibility of NCAM-induced plasticity (i.e. the reduction of synaptic NCAM). By 12 h post-conditioning, all trained groups exhibited a decrease on hippocampal NCAM expression. Studies performed in the LTP model showed an increase in the extracellular levels of soluble NCAM (115 kDa), which was proposed to result from the removal of synaptic NCAM as a consequence of increased proteolytic activity (Fazeli *et al.*, 1994). In the marine mollusc *Aplysia*, the learning model of long-term sensitization of the gill-withdrawal reflex (which involves the formation of new synaptic connections) was found to be associated with a downregulation of apCAM, a close homologue of NCAM (Mayford *et al.*, 1992). This reduction was confined to presynaptic sensory neurons and appeared to be due, not only to a reduction of newly synthesized apCAM, but also to a decrease of pre-existing apCAM presumably due to endocytotic internalization (Bailey *et al.*, 1992, 1997; Mayford *et al.*, 1992). Although our experimental conditions do not allow us to determine whether the decreases in NCAM observed at 12 h post-training were due to any of the above mentioned mechanisms (i.e. extracellular secretion, intracellular endocytosis or decreased synthesis), our results further support the transient downregulation of NCAM as a molecular change involved in synaptic remodelling during a post-training period preceding the subsequent stabilization of selected synapses involved in long-term memory. In fact, by 24 h post-training, hippocampal levels of NCAM were normalized in the groups trained at 0.2 and 0.4 mA shock intensity, and appeared increased in the 1 mA trained group. This enhancement of hippocampal NCAM expression in the group trained at the highest shock intensity is in agreement with previous studies involving this molecule in the transition from labile to enduring memories (Doley *et al.*, 1992a; Scholey *et al.*, 1993; Sandi *et al.*, 1995; Murphy & Regan, 1998), because animals trained at 1 mA shock not only show the highest levels of fear conditioning, but also retain the conditioned response for longer periods than those trained at lower shock intensities (Cordero *et al.*, 1998).

We also explored the possibility that memory consolidation was associated to alteration in NCAM polysialylation state. Previous studies found a transient, time-dependent increase in hippocampal NCAM polysialylation occurring ~ 12 h after training rats in a number of learning tasks (Doyle *et al.*, 1992b; Fox *et al.*, 1995; Murphy *et al.*, 1996; O'Connell *et al.*, 1997). This increase in NCAM polysialylation appears to be required for morphological plasticity eventually leading to long-term memory (Doyle & Regan, 1993; Becker *et al.*, 1996; Muller *et al.*, 1996). However, we failed to find increased expression of PSA-NCAM at the two selected time points, 12 and 24 h, after contextual fear conditioning. In fact, the opposite result (i.e. a decrease in PSA-NCAM) was found for the group trained at the highest shock intensity. How could these data be reconciled with the consistent evidence implicating increased polysialylation among the mechanisms subserving long-term memory formation? Different possibilities might account for this apparent discrepancy. Firstly, the nature of the learning task used in our study is remarkably different to those tasks in which PSA was found to be increased. Tasks, e.g. the passive avoidance or the water maze, although involving stressful factors, allow the animals to acquire a learned response to cope with the adverse experimental conditions. On the contrary, contextual fear conditioning is an emotional learning model in which animals exhibit an inhibitory response when exposed to a fear-associated context and therefore do not learn to develop any behavioural response to operate over the stressful circumstances. We believe that distinct neural mechanisms might be involved in the processing of information concerned with these different task-related behavioural demands and

stress factors. A second possibility could be related to the decrease in NCAM expression observed at 12 h post-training. Given that one of the main mechanisms by which PSA has been proposed to influence plasticity is by decreasing membrane adhesion at the synapse (Rutishauser, 1993; Rutishauser & Landmesser, 1996), in contextual fear-conditioned animals, these de-adhesive functions might be undertaken by the downregulation of NCAM. Alternative possibilities are referred to methodological aspects. Whereas our experiments were performed in crude synaptosomal preparations from the whole hippocampus, most of the studies reporting increased PSA-NCAM after training were performed using immunohistochemistry techniques, and the reported changes in polysialylation were generally confined to a defined population of PSA-positive granule-like cells located at the granule cell layer/hilar border within the dentate gyrus (Fox *et al.*, 1995; Murphy *et al.*, 1996; O'Connell *et al.*, 1997). Therefore, the possibility exists that our study did not detect potential increases in polysialylation predominantly occurring in restricted cell subpopulations within the hippocampus. Further studies are in progress to address these issues.

In addition, our study showed an intriguing pattern of regulation of L1 after contextual fear conditioning. Both at 12 and 24 h after training, hippocampal L1 levels showed a shock intensity-dependent U-shaped pattern, with higher increases observed in rats in the low and high shock intensity conditions, and values not different from controls in those included in the intermediate stress condition. Previous studies in which antibodies against L1 were used indicated a role for this molecule in synaptic plasticity underlying memory storage (Scholey *et al.*, 1995; Arami *et al.*, 1996) and long-term potentiation (LTP, Lüthi *et al.*, 1994). Therefore, the increased expression of this CAM after contextual fear conditioning might be related to neural changes underlying memory storage. This hypothesis appears particularly reasonable with regard to the increase observed in the 1 mA group, as it is the group that forms the stronger and longer-lasting memory for the task, and it also shows enhanced NCAM expression by 24 h post-training. However, such a view does not hold for the relationship between freezing and L1 values observed in the other two groups of rats trained under lower stressor intensities. In fact, given the low levels of fear conditioning displayed by the 0.2 mA group, as well as the lack of modulation of L1 expression in the 0.4 mA group, it is difficult to connect the enhancement of hippocampal L1 to contextual fear conditioning memory mechanisms. Further studies will be required to try to ascertain the role of this molecule on the establishment of the different components involved in this contextual fear-related memory.

As for the potential mechanisms involved in the pattern of CAMs modulation, different possibilities could be considered with regard to the physiological stress response induced by the conditioning experience. Given that the level of conditioning developed is related to post-training levels of corticosterone (see Cordero *et al.*, 1998), it is tempting to consider a possible role of this corticosteroid on CAMs regulation. Corticosteroid actions on neural plasticity have consistently been found to follow an inverted U-shape function (for reviews, see Joëls & de Kloet, 1992, 1994; McEwen & Sapolsky, 1995) that seems to be explained by the differential occupation of the two classical intracellular receptors to which corticosterone binds with a 10-fold difference in affinity, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR, de Kloet, 1991; Lupien & McEwen, 1997). Given that L1 expression showed a biphasic modulation in our study, the possibility exists that it was related to biphasic actions of corticosterone in neural plasticity (Joëls & de Kloet, 1992, 1994). In fact, we have previously shown that NCAM antibodies injected 5.5 h after training prevented a memory-facilitat-

ing effect induced by corticosterone in weakly trained chicks (Sandi *et al.*, 1995). In addition, a corticosterone injection that facilitates contextual fear conditioning in rats trained at 0.2 mA shock (Cordero & Sandi, 1998) was shown to increase NCAM expression at the level of the frontal cortex (Sandi & Loscertales, 1999) and to decrease glycoprotein fucosylation in the hippocampus (Venero *et al.*, 1996). Corticosterone levels have also been shown to downregulate PSA-NCAM expression in the dentate gyrus of the hippocampus, as its expression was enhanced by adrenalectomy while a corticosterone replacement treatment reversed such effect (Rodriguez *et al.*, 1998), which suggests that the decrease in PSA-NCAM observed in our study in the 1 mA group might be related to the high corticosterone levels found in these animals immediately after training (Cordero *et al.*, 1998). In fact, we also show in this study that rats trained at 1 mA shock are those that also display the highest corticosterone values at testing. Ongoing studies in our laboratory, using the corticosteroid synthesis inhibitor metyrapone, are currently addressing to what extent post-training corticosterone levels might be related to CAMs' regulation associated to contextual fear conditioning.

In summary, training rats in the contextual fear conditioning task at different shock intensities results in a complex modulation of hippocampal expression of CAMs. Interestingly, the group exposed to the more stressful conditions at training, which is the one that develops the stronger and longer-lasting levels of fear conditioning, presents a pattern of CAMs modulation consistent with the idea that, after a period of synaptic de-adherence during the consolidation phase, NCAM and L1 (either independently or in a cooperative way) might participate in the stabilization of selected synapses underlying the establishment of long-term memory for this task. In addition, our results demonstrate the importance of including learning conditions with different levels of stress in studies aimed to understand the cellular and molecular mechanisms involved in memory storage.

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Abbreviations

BSA, bovine serum albumin; CAMs, cell adhesion molecules; ELISA, enzyme-linked immunosorbent assay; GR, glucocorticoid receptor; HEPES, *N*-2-[hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid]; Ig, immunoglobulin; LTP, long-term potentiation; MR, mineralocorticoid receptor; NCAM, neural cell adhesion molecule; PSA, α -2,8-linked polysialic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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