EFFECTS OF CHRONIC STRESS ON CONTEXTUAL FEAR CONDITIONING AND THE HIPPOCAMPAL EXPRESSION OF THE NEURAL CELL ADHESION MOLECULE, ITS POLYSIALYLATION, AND L1

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Abstract—Chronic stress has been shown to induce time-dependent neurodegeneration in the hippocampus, ranging from a reversible damage to a permanent neuronal loss. This damage has been proposed to impair cognitive function in hippocampus-dependent learning tasks. In this study, we have used a 21-day restraint stress procedure in rats, previously reported to induce reversible atrophy of apical dendrites of CA3 pyramidal cells, to assess whether it may influence subsequent performance in the contextual fear conditioning task under experimental conditions involving high stress levels (1 mA shock intensity as the unconditioned stimulus). In addition, we were interested in the study of the possible cellular and molecular mechanisms involved in the reversible phase of neural damage. Cell adhesion molecules of the immunoglobulin superfamily, such as the neural cell adhesion molecule and L1, are cell-surface macromolecules that, through their recognition and adhesion properties, regulate cell–cell interactions and have been reported to play a key role in cognitive functioning. A second aim of this study was to evaluate whether chronic stress would modulate the expression of the neural cell adhesion molecule, its polysialylation, and L1 in the hippocampus. The results showed that chronic stress facilitated subsequent contextual fear conditioning. They also showed that chronically stressed rats displayed reduced hippocampal neural cell adhesion molecule, but increased polysialylated expression as well as a trend towards exhibiting increased L1 expression.

In summary, these results support the view that a 21-day chronic stress regimen predisposes individuals to develop enhanced contextual fear conditioning responses. They also indicate that cell adhesion molecules might play a role in the structural remodelling that occurs in the hippocampus as a consequence of chronic stress exposure. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: corticosterone, learning, memory, learned helplessness, neural plasticity.

Prolonged exposure to stress or elevated levels of glucocorticoids (adrenal steroids whose release is increased in stressful situations) can induce time-dependent neuronal damage in the hippocampus, ranging from a reversible atrophy of pyramidal CA3 apical dendrites induced by 21-day stress treatments,[50,51,93,95] to a permanent neuronal loss observed with longer treatments in all pyramidal cell areas.[65] The implication of glucocorticoids in both types of damage (reversible and enduring) was further supported by studies in which morphological damage induced by stress was prevented, or reduced, by treatments that blocked glucocorticoid synthesis.[45,50] In addition, high levels of glucocorticoids have been hypothesized to accelerate brain ageing,[45,86] a condition which is frequently associated with learning and memory

impairments.[59] This stress-induced neural damage has attracted considerable attention for the critical role of the hippocampus in cognitive function,[58] and recent evidence in humans supports the same type of interactions between chronic stress or glucocorticoid exposure and hippocampal alterations.[48,85]

We are interested in the study of the cellular mechanisms involved in the reversible phase of neural damage, since such an understanding could contribute to the elucidation of the processes that lead to stress-induced neurodegeneration, as well as to eventual development of possible therapeutical interventions. Consistent evidence implicates excitatory amino acid transmission, involving the activation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors, and nitric oxide in the deleterious effects of chronic stress in CA3 hippocampal cells.[50,53,74] Since chronic stress seems to potentiate the glutamatergic input from the mossy fibre terminals to CA3, dendritic atrophy of these neurons has been interpreted as a possible compensatory reaction to limit the enhanced excitatory input.[52]

Which mechanisms might be involved in these structural alterations? Over the past years, we have focused our attention on the possible involvement of cell adhesion molecules (CAMs) in the actions of stress

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Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; BSA, bovine serum albumin; CAMs, cell adhesion molecules; ELISA, enzyme-linked immunoabsorbent assay; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid); Ig, immunoglobulin; NCAM, neural cell adhesion molecule; NMDA, *N*-methyl-D-aspartate; PSA, α-2,8-linked polysialic acid; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with 0.05% Tween 20.

and/or glucocorticoids in cognitive and neural function (for a review see Ref. 82). CAMs of the immunoglobulin superfamily, such as the neural CAM (NCAM) and L1, are cell-surface macromolecules that, through their recognition and adhesion properties, regulate cell–cell interactions during both development and activity-dependent plasticity in the adult.[28,87] These molecules have been implicated in neuritic outgrowth during nervous system development, as well as in neural regeneration after injury in the adult nervous system.[4,22,38] In particular, NCAM participation in these functions is critically regulated by the post-translational attachment of α-2-8-linked polysialic acid (PSA) chains.[21,66] PSA–NCAM is highly expressed during development,[75,76,89] and has been implicated in learning-[11,30,68,69] and activity-dependent[11,67] plasticity in the adult rodent. The presence of PSA on NCAM appears to interfere with NCAM-[80,81,96] and L1-mediated adhesion,[1,97] eventually leading to decreased membrane–membrane contacts and attenuation of cell interactions.[76,80] Since CAMs play a pivotal role in the formation and stabilization of neural circuits ensuring appropriate neural and cognitive functioning, a breakdown in the organization and interactions of key CAMs has been hypothesized as one of the possible key events underlying the generation of brain pathology.[20] Recent studies in humans have shown alterations in the regulation of these CAMs in several neuropsychiatric and neurodegenerative disorders associated, to certain extent, with stress factors.[9,61,62,72,73] In addition, we have previously found evidence indicating a role for glucocorticoids in NCAM regulation.[83,84,92]

At the behavioural level, chronic stress might also influence the way individuals react to further stressful or traumatic experiences. We have recently characterized the contextual fear conditioning task in rats, as a learning model that is dependent upon the degree of stress activation.[18,19] 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.[12] Despite recent controversy in the literature, the hippocampus has been implicated in certain functions involved in this type of contextual learning (for a review, see Ref. 36). Given that the hippocampus has been reported to be markedly altered in a number of cellular and molecular parameters as a consequence of chronic stress, it might be reasonable to hypothesize that chronic stress impairs subsequent contextual fear conditioning. However, Conrad *et al.*[17] have recently reported that a 21-day stress procedure, instead of impairing, potentiates contextual fear conditioning. Since their experimental conditions included moderate stress levels (0.4 mA shock intensity as the unconditioned stimulus), and the role of the hippocampus in this learning task has been related to the intensity of the unconditioned stimulus,[31] the first aim of the present study was to investigate whether a 21-day restraint stress would influence performance in the contextual fear conditioning task under experimental conditions involving high stress levels (1 mA shock intensity as the unconditioned stimulus). The second aim of this study was to evaluate whether chronic restraint and/or contextual fear

conditioning would modulate the expression of NCAM, its polysialylated form PSA–NCAM, and L1, in the hippocampus. In order to address these issues, we performed a factorial design with "stress" and "contextual fear conditioning" as the factors.

EXPERIMENTAL PROCEDURES

Experimental animals

Male Wistar rats (Harlan Iberica, Spain), weighing 150–175 g on arrival, were housed in groups of three per cage, under temperature- (22 ± 2°C) and light- (12-h light/dark cycle; lights on at 07.00) controlled conditions and had free access to food and water in a colony room. Approximately five weeks after arrival, they were handled daily for around four days before being weighed. In order to address the two main objectives of the study, we followed a 2×2 factorial design, involving four groups, with "stress" and "contextual fear conditioning" as the factors. Rats were matched by groups of four according to their weight, and each of the four matched animals assigned randomly into one of the four experimental groups. On the fifth week after arrival (rats weighing around 350 ± 25 g, in their 13th week of life), the chronic stress procedure started for those animals assigned to the stress groups. Body weights were recorded periodically. Behavioural experiments were always conducted between 08.00 and 14.30. All efforts were made to minimize both the suffering and the number of animals used. Animal care procedures were conducted in accordance with the guidelines set by the European Community Council Directives (86/609/EEC).

Chronic stress procedure

Twenty rats were subjected to chronic restraint stress for 21 days. The sessions consisted of 6 h/day (08.30–14.30) restraint of the rats in plastic restrainers secured at the head and tail ends with clips. During the restraint sessions, the rats were placed in a room adjacent to their colony room and every day, at the termination of the stress session, they were placed again in their home cages. During this period, control animals ($n = 22$) were left undisturbed in their home cages.

Contextual fear conditioning

Training and tests took place in a rodent observation cage (30×37×25 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 lightbulb. Ventilation fans provided background noise at 68 dB.

One day after the termination of the stress procedure (day 23), a subgroup of rats from each "stress" ($n = 10$) or "undisturbed" ($n = 12$) condition was submitted to contextual fear conditioning. Each of these rats was transported from the colony room to the laboratory (situated in an adjacent room) and placed into the conditioning chamber. After 3 min, the rats received three 1 mA 1-s shocks (unconditioned stimuli). The intershock 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 approximately 330 s. Testing for contextual fear conditioning was performed 24 h after conditioning. At testing, rats were placed back into the same chamber as used in conditioning in the absence of shock for a 6-min context test. A video camera recorded the behaviour of rats both during training and testing. Subsequently, the time spent by each rat either freezing or active was scored blindly assisted by a computer program (Ethovision 1.9, Noldus, NL). Freezing was defined as behavioural immobility except for
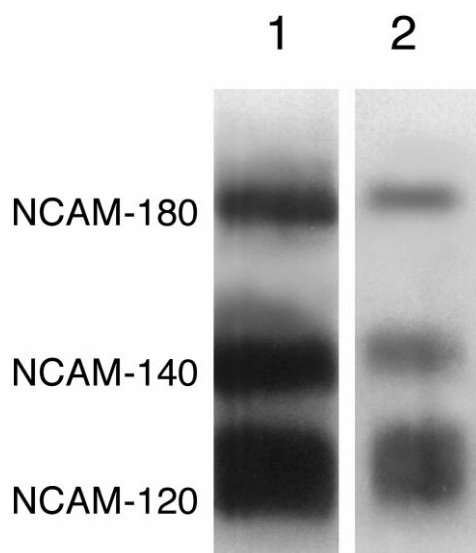
Fig. 1. NCAM polypeptide pattern in tissue samples from the rat hippocampus, as shown by SDS–PAGE and immunoblotting. Left lane: crude synaptosomal preparations; right lane: whole homogenates. Positions of the three major NCAM isoforms are indicated in the margin (in kDa).

movement needed for respiration. Behaviour was evaluated in each experimental session. At training, behavioural scores were noted 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. At the testing session, behaviour was scored during the 6-min re-exposure to the training context. Eight to 10 minutes after the start of the testing session, rats were decapitated. Rats not assigned to the groups that included behavioural testing (undisturbed–control, $n = 10$, and stressed–control, $n = 10$) were also decapitated at the same time.

Corticosterone and thymus weight measurements

After decapitation, trunk blood was collected and samples were centrifuged (3000 rpm 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). In addition, thymus glands were removed, cleaned and weighed.

Quantification of cell adhesion molecules

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 (P2) were obtained according to a modified protocol from Lynch and Voss.[49] In brief, tissue was homogenized in 10 volumes of ice-cold sucrose (0.32 M) and 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 $15,000\,g$ for 15 min, and the pellet was resuspended in Krebs buffer, containing protease inhibitors and 1% Nonidet P-40 (Sigma, Spain), for use. Protein concentration for each sample was estimated by the method of Bradford.[14]

All CAMs were quantified by enzyme-linked immuno-absorbent assays (ELISAs) according to a previously described protocol.[23,88] In brief, flat-bottom 96-well microplates were allowed to adsorb a coating solution (0.1 M Na_2CO_3/0.1 M $NaHCO_3$) for 2 h at room temperature. The solution was removed and 50 μl of pellet samples were added at a concentration of 5 μg/ml (NCAM assays) or 10 μg/ml (PSA–NCAM and L1 assays) for 20–24 h at 4°C. Non-specific 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-μl aliquots of the corresponding primary antibody for 20–24 h at 4°C. The wells were washed and 50-μl aliquots of peroxidase-conjugated secondary antibody were added for a 2 h incubation period. Afterwards, 50 μl of citrate buffer (50 mM Na_2HPO_4, 25 mM citric acid, 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 μl 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). Previous tritration experiments, using different concentrations of the corresponding primary and secondary antibodies for each CAM (see below) as well as different antigen concentrations (1–50 μg range), had been carried out in order to select the appropriate experimental conditions to accurately discriminate changes >10% in the expression of the different CAMs in the tissue samples.

For NCAM assays, we used a polyclonal rabbit anti-rat NCAM immunoserum (diluted 1:300; generous gift from Professor Elisabeth Bock, University of Copenhagen, Denmark)[40] as the primary antibody, and an anti-rabbit Ig peroxidase conjugate (whole molecule conjugate; diluted 1:500; Sigma, UK and Spain) as the secondary antibody. For PSA–NCAM assays, a monoclonal antibody was used (Men B, clone B1.2; generous gift from Professor Genevieve Rougon, CNRS Marseille, France).[77] 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.[44] An IgM anti-mouse peroxidase (μ chain) conjugate (Sigma-Aldrich, Spain) was used, at a 1:1000 dilution, as the second antibody. For L1 assays, the primary antibody was a monoclonal anti-rat IgG antibody (concentration 100 μg/ml; dilution 1:10; Boeringher Mannhein, Spain). The secondary antibody employed in L1 assays was an anti-rat Ig-POD Fab fragments (from sheep immunoglobulin) to IgG-rat peroxidase conjugated (Boeringher Mannhein, Spain), used at a 1:500 dilution.

Since NCAM consists of several isoforms (including three major ones of 120,000, 140,000 and 180,000 mol. wt) that result from alternative splicing of a single gene,[33] 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 dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted and stained with the polyclonal NCAM antibody used in ELISAs (see above). Equal amounts of protein (15 μg) 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 180,000, 140,000 and 120,000 both in whole homogenates and in the crude synaptosomal preparations. Crude synaptosomal preparations were enriched in NCAM content in comparison with whole homogenates of hippocampal tissue.

In addition, western blot analyses were also performed to further assess the expression of PSA–NCAM in control and stressed animals. In brief, hippocampal synaptosomal samples obtained from chronically stressed and control rats were denatured at 100°C for 2 min in 30 mM Tris–HCl buffer (pH 7.4) containing 0.05% SDS and 3% β-mercaptoethanol. Twenty micrograms of each sample were separated on 7% (w/v) SDS–PAGE and transferred (1 mA/cm², 1 h) to an Immobilon-P membrane (Amersham). After saturation of the non-specific sites with 5% (w/v) skimmed milk in 50 mM Tris–HCl, pH 8, 138 mM NaCl, 0.05% Tween 20 (TBST) the blots were incubated for 1.5 h at room temperature with the same anti-PSA monoclonal antibody used in ELISAs (diluted 1:500). The blots were washed with TBST, incubated for 1 h with the same secondary antibody as in the ELISAs (diluted 1:4000) and finally developed using the enhanced chemiluminescence (ECL) system (Amersham).

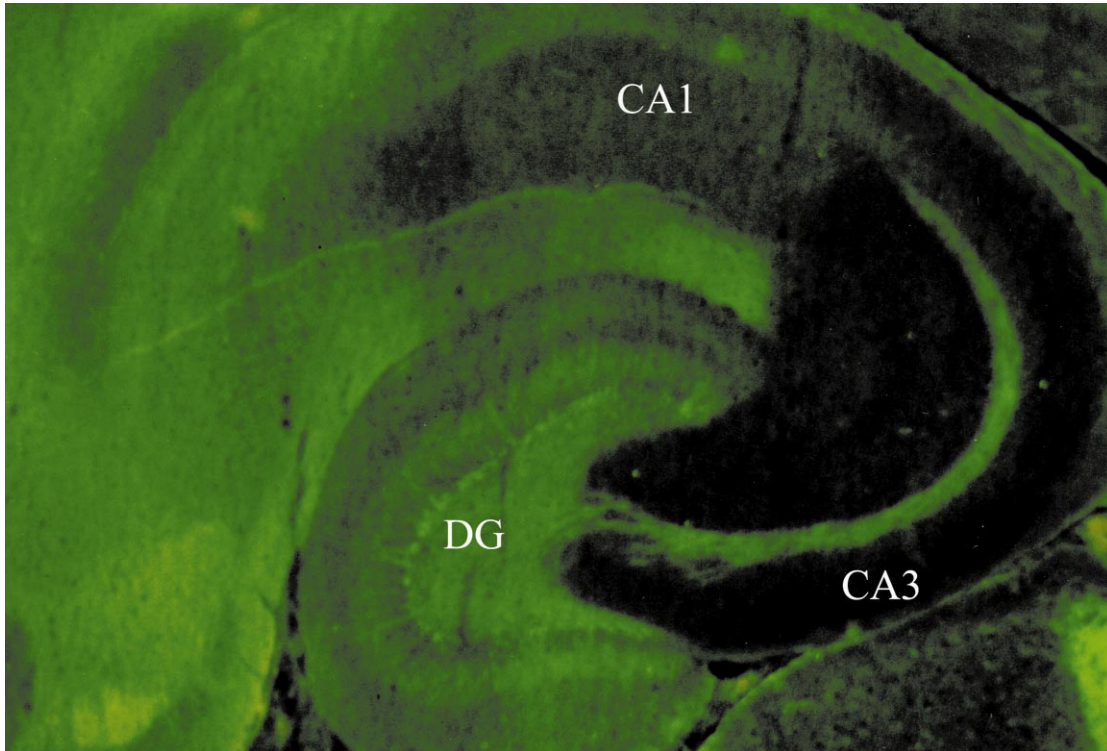Furthermore, the actual localization of PSA–NCAM in the

Fig. 2. Distribution of PSA immunoreactivity in the adult rat hippocampus. The most intense immunoreactivity is apparent in the dentate gyrus, particularly in the granule cell layer at the hilus border, and mossy fibre axons extending to the CA3 region. A diffuse staining was also observed in the CA1 area.

hippocampus was determined by immunohistochemistry by using a previously described protocol.[30] In brief, cryostat-cut axial sections of 12 μm thickness were fixed in 70% (v/v) ethanol and incubated overnight with the anti-PSA antibody used in ELISAs (diluted 1:500). The sections were exposed for 3 h to fluorescein-conjugated goat anti-mouse IgM diluted 1:100 (Calbiochem, UK) and mounted in Citifluor (Agar, UK). Figure 2 shows the distribution of PSA immunoreactivity in the rat hippocampus. As can be seen, the most intense immuno-reactivity is apparent in the dentate gyrus, particularly in the granule cell layer at the hilus border, and mossy fibre axons extending to the CA3 region. A weak diffuse staining was also observed in the CA1 area.

Statistics

The behavioural data regarding the time each rat spent freezing were transformed to a percentage of freezing per minute. All results were expressed as mean ± S.E.M. and analysed using two-way analysis of variance (ANOVA), either factorial (with "stress" and "fear conditioning" as factors) or with repeated measures (with time as the repeated measure in the analyses of the behavioural data). Differences between treatments were further evaluated for significance with Student's *t*-test post hoc comparisons. Significance was accepted at $P < 0.05$.

RESULTS

Effects of chronic stress on contextual fear conditioning and corticosterone levels

We firstly addressed the effect of chronic restraint stress on contextual fear conditioning by using a shock intensity (1 mA) that leads to high and long-lasting levels of conditioning.[19] As shown in Fig. 3, chronically stressed rats displayed increased levels of freezing both during the

post-shock period at training ($F_{1,21} = 5.42$, $P < 0.03$) and at testing ($F_{1,21} = 4.30$, $P < 0.05$), compared to undisturbed, non-stressed rats. However, it should be noted that no freezing was observed in any of these two groups during the pre-shock period at training (undisturbed: 0% freezing; stressed: 0% freezing; n.s.).

We also evaluated whether corticosterone levels might be differentially affected by prior stress history both under basal conditions and after testing in contextual fear-conditioned animals. Results are shown in Table 1. A two-way ANOVA indicated a lack of significant effect of the "stress" factor ($F_{1,38} = 1.90$, $P = 0.17$, n.s.), a highly significant effect of testing in the "contextual fear condition-ing" task ($F_{1,38} = 62.61$, $P < 0.0001$), and a lack of interac-tion of "stress" and "conditioning" factors ($F_{1,1} = 0.01$, n.s.). Therefore, the slight trend observed in chronically stressed rats to show increased plasma corticosterone values did not reach statistical significance ($P = 0.17$).

Effects of chronic stress and/or contextual fear conditioning on the expression of neural cell adhesion molecule, polysialylated-neural cell adhesion molecule and L1 in the hippocampus

Since alterations on hippocampal structure in studies that used the same chronic stress procedure were pre-viously reported,[50–52,93] a major aim of our experiments was to analyse the expression of NCAM, PSA–NCAM and L1 at the level of the hippocampus.

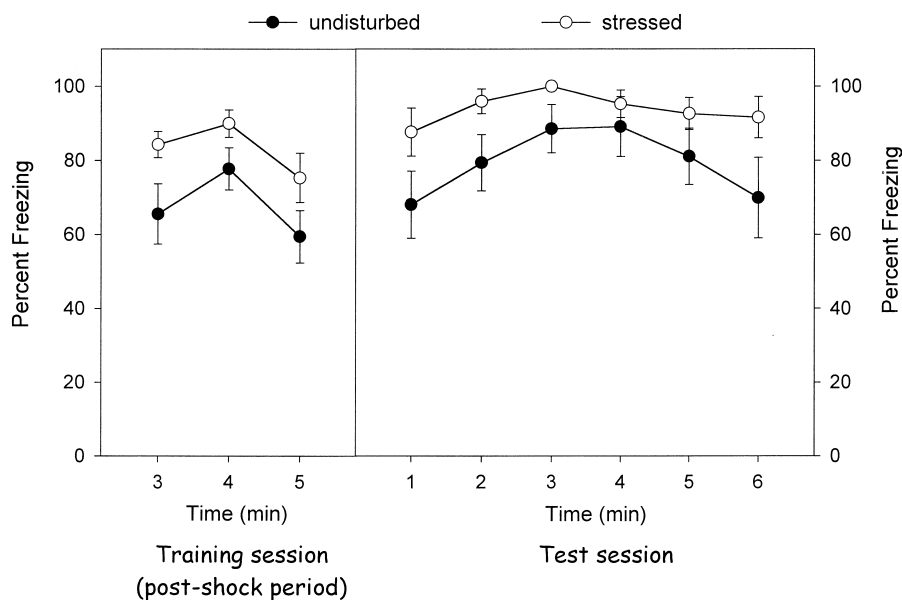With regard to NCAM data (see Table 2), a two-way ANOVA indicated a significant effect of the "stress"

Fig. 3. Percentage of time rats spent freezing per minute during the post-shock period at training in the contextual fear conditioning task (left panel) and at testing (right panels). Results are the mean ± S.E.M. from 10–12 rats per group. Stressed rats displayed significantly higher freezing levels than controls.

Table 1. Effects of chronic restraint stress and/or contextual fear conditioning on post-testing plasma corticosterone values

	Plasma corticosterone (ng/ml)
Undisturbed–control	54.1 ± 9.1
Undisturbed–CFC	163.5 ± 13.6*
Stressed–control	73.1 ± 22.6
Stressed–CFC	186.0 ± 8.5*

CFC: contextual fear conditioning. Results are the mean ± S.E.M from 10–12 rats per group.
*$P < 0.001$ vs corresponding control group (without CFC).

Table 2. Effects of chronic stress and/or contextual fear conditioning on neuronal cell adhesion molecule expression in the hippocampus

	NCAM
Undisturbed–control	1.203 ± 0.047
Undisturbed–CFC	1.138 ± 0.058
Stressed–control	0.973 ± 0.021*
Stressed–CFC	1.009 ± 0.033†

CFC: contextual fear conditioning. Results are determined by ELISAs and expressed as the optical density obtained by measuring absorption at 492 nm. Data are the mean ± S.E.M. from 10–12 rats per group.
*$P < 0.004$ vs Undisturbed–control group.
†$P < 0.05$ vs Undisturbed–CFC group.

factor ($F_{1,38} = 12.87$, $P < 0.0011$), a lack of effect of the "conditioning" factor ($F_{1,38} = 0.077$, n.s.), and a lack of effect of the interaction between the "stress" and "conditioning" factors ($F_{1,1} = 0.903$, n.s.). Post-hoc Student's t-test analyses further revealed that the two groups submitted to chronic restraint stress, both the control ($P < 0.004$) and the one trained in the contextual fear conditioning task ($P < 0.05$), displayed significantly lower NCAM levels than their respective undisturbed control groups.

Moreover, a two-way ANOVA performed on PSA–NCAM data also showed a significant effect of the "stress" factor ($F_{1,38} = 4.78$, $P < 0.036$), a lack of effect of the "conditioning" factor ($F_{1,38} = 0.713$, n.s.), and a lack of effect of the interaction between the "stress" and "conditioning" factors ($F_{1,1} = 0.353$, n.s.). Chronic stress induced an increase in PSA–NCAM expression in both stressed groups ($P < 0.05$) compared to their respective "undisturbed" control groups (Table 3). In addition, immunoblot analysis of PSA–NCAM expression revealed a more intense band for PSA–NCAM (200,000 to 220,000 mol. wt) in the hippocampus from "stressed" rats as compared to "undisturbed" controls (Fig. 4).

Results for L1 expression are shown in Table 4. A two-way ANOVA indicated a slight trend for the "stress" factor towards significantly increased L1 levels ($F_{1,38} = 2.44$, $P < 0.13$), a lack of effect of the "conditioning" factor ($F_{1,38} = 0.00$, n.s.), and a lack of effect of the interaction between the "stress" and "conditioning" factors ($F_{1,1} = 0.00$, n.s.).

Effects of chronic stress on body and thymus weights

As shown in Table 5, rats exposed to restraint stress showed significantly reduced body weight by the end of the chronic stress procedure, compared to undisturbed controls. This conclusion is supported by a two-way ANOVA that indicated a highly significant effect for the "stress" factor ($F_{1,38} = 78.01$, $P < 0.0001$). As expected, contextual fear conditioning did not influence body weight ($F_{1,38} = 0.945$, n.s.), and there was no interaction to influence this parameter between "stress" and "contextual fear conditioning" factors ($F_{1,1} = 0.66$, n.s.).

ANOVA on relative thymus weight (mg/100 g body weight) data (Table 5) indicated a lack of effect of
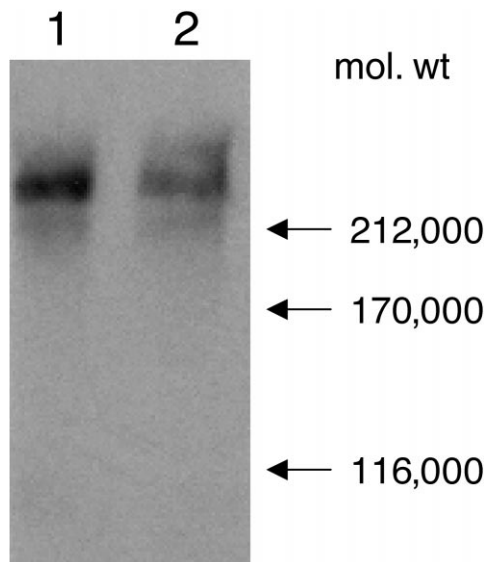
Fig. 4. Immunoblotting analysis of PSA–NCAM within hippocampus of chronically stressed (lane 1) and control (lane 2) rats. Protein samples (20 µg) of hippocampal synaptosomes were separated using SDS–PAGE, transferred to immobilon-P and immunostained with anti-PSA antibodies. The migration of molecular weight standards (212,000 170,000 and 116,000) is indicated. Note that the amount of PSA is the highest for the chronically stressed rats.

Table 3. Effects of chronic stress and/or contextual fear conditioning on polysialylated-neural cell adhesion molecule expression in the hippocampus

	PSA–NCAM
Undisturbed–control	0.409 ± 0.018
Undisturbed–CFC	0.434 ± 0.020
Stressed–control	$0.502 \pm 0.016*$
Stressed–CFC	$0.512 \pm 0.013†$

CFC: contextual fear conditioning. Results are determined by ELISAs and expressed as the optical density obtained by measuring absorption at 492 nm. Data are the mean $\pm$ S.E.M. from 10–12 rats per group.
$*P < 0.005$ vs Undisturbed–control group.
$†P < 0.05$ vs Undisturbed–CFC group.

Table 4. Effects of chronic stress and/or contextual fear conditioning on L1 expression in the hippocampus

	L1
Undisturbed–control	0.895 ± 0.055
Undisturbed–CFC	0.885 ± 0.034
Stressed–control	0.963 ± 0.037
Stressed–CFC	0.949 ± 0.042

CFC: contextual fear conditioning. Results are determined by ELISAs and expressed as the optical density obtained by measuring absorption at 492 nm. Data are the mean $\pm$ S.E.M. from 10–12 rats per group.

"stress" factor ($F_{1,38} = 0.01$, n.s.), "contextual fear conditioning" factor ($F_{1,38} = 1.04$, n.s.), as well as a lack of interaction of "stress" and "conditioning" factors ($F_{1,38} = 0.04$, n.s.).

DISCUSSION

The findings of the present study show that 21 days of restraint stress facilitated subsequent contextual fear conditioning in rats. They also show that chronic stress profoundly modulated the expression of cell adhesion molecules in the hippocampus. Thus, stressed rats displayed reduced NCAM, but increased PSA–NCAM, expression, as well as a trend for increased L1 expression.

Effects of chronic stress on contextual fear conditioning

The same stress procedure as the one used in our study has been repeatedly found to produce an atrophy of apical dendrites of CA3 hippocampal pyramidal neurons[51,52,93] as well as learning impairments in hippocampus-dependent tasks.[16,47] Given that the hippocampus has been implicated to play—at least to a certain extent—a significant role in contextual fear conditioning[32,43,71] (but see Ref. 31 for the opposite view; and Ref. 36 for a review on this discussion), a decreased conditioning could have been hypothesized as the expected outcome of chronic stress. However, we found increased conditioning even using experimental conditions (1 mA shock intensity as the unconditioned stimulus) that lead to considerably high levels of freezing; a result which is in agreement with a previous study[17] in which stressed rats also showed enhanced contextual fear conditioning under experimental condi-

tions (0.4 mA shock intensity as the unconditioned stimulus) that lead to intermediate freezing levels.

Previous findings suggest that extensive hippocampus damage is required to interfere with contextual fear conditioning.[32,56] However, current knowledge indicates that hippocampal damage induced by the 21-day restraint protocol used in our study induces a selective atrophy in apical dendrites of CA3 pyramidal neurons[93] and ultrastructural changes in mossy fibre terminals.[52] From our results, showing that chronic stress does not impair but potentiates contextual freezing, it seems reasonable to propose that the specific structural alterations induced in the hippocampus by 21 days of restraint are insufficient to cause a disruption of contextual fear conditioning which can be found when the hippocampus is largely damaged.[32,43,71]

The striking finding was that chronic stress not only did not impair contextual fear conditioning, but enhanced it. Conrad *et al.*[17] found that the potentiation of fear conditioning was also observed in stressed rats treated with daily injections of tianeptine to prevent the development of hippocampal atrophy, which suggested that the morphological alterations induced by chronic stress in the hippocampus might not be involved in the conditioning enhancement. It might be possible that other brain regions, known to be neurochemically affected by this chronic stress regimen (such as the amygdala, the prefrontal cortex[10] or the cortex[70]) are implicated in such a behavioural effect. Further studies in our laboratory are currently addressing the possibility that chronic stress affects CAMs expression in other brain regions.

However, before addressing the issue of the molecular changes observed in this study, the question remains as to whether or not the potentiation of contextual fear

Table 5. Effects of chronic restraint stress and/or contextual fear conditioning on body and relative thymus weight

	Body weight (g)	Thymus weight (mg/100 g bw)
Undisturbed–control	411.9 ± 9.9	163.9 ± 7.6
Undisturbed–CFC	426.3 ± 8.8	169.5 ± 7.2
Stressed–control	346.5 ± 6.7*	160.9 ± 6.0
Stressed–CFC	347.3 ± 6.0†	167.4 ± 7.5

CFC: contextual fear conditioning. Results are the mean ± S.E.M from 10–12 rats per group.
*$P < 0.001$ vs Undisturbed–control group.
†$P < 0.001$ vs Undisturbed–CFC group.

conditioning could be interpreted as an effect on memory processes. Firstly, it is important to note that stressed rats showed increased freezing not only at testing, but also during the post-shock period at training. This observation is in agreement with studies in which rats previously submitted to inescapable stressors showed enhanced freezing when experienced shock in a new environment,[54] an effect which has been related to "learned helplessness"[2,24,55] and "behavioural sensitization".[3] Therefore, the increased freezing observed in the post-shock period in our study could be interpreted as the facilitation of a fear behaviour by prior restraint history. As a result, the conditioning of fear in a new environment would have been facilitated and, therefore, stressed subjects would have more conditioned fear to transfer into a long-term memory. To summarize, we believe that the potentiated freezing response observed in the chronically stressed animals at testing, instead of being due to a facilitation of memory, reflects their altered reaction to the conditioning procedure. We are currently exploring the possible role of other brain regions (i.e. the amygdala or the prefrontal cortex) in the potentiation of fear conditioning induced by chronic stress.

Effects of chronic stress and/or contextual fear conditioning on the regulation of hippocampal cell adhesion molecules

This study also provides evidence, for the first time, for a marked regulation of hippocampal cell adhesion molecules as a consequence of chronic stress exposure. In particular, NCAM was substantially modulated in stressed rats, which showed reduced levels of NCAM polypeptide, but increased polysialylation levels. The observed reduction in NCAM levels could be due to different, though not mutually exclusive, mechanisms such as (i) the extracellular release of the molecule;[27] (ii) an increased degradation rate of NCAM; (iii) its internalization from the cellular membrane;[57] and/or (iv) a decreased rate of its synthesis.[41] Although our experimental conditions do not allow us to determine the exact mechanism/s involved in our study, different lines of evidence give support to hypothesize a role for the extracellular secretion of NCAM. Thus, recent studies have reported an NCAM-associated ecto-adenosine triphosphatase (ecto-ATPase) activity supposedly localized in the fibronectin type-III region of the protein.[26] The extracellular binding and hydrolysis of adenosine triphosphate (ATP) have been shown to prevent shedding of NCAM from synaptic membranes[26] and to modulate neurite outgrowth.[90] ATP is know to act as a neurotransmitter and to be released by exocytosis from the synaptic vesicles in which ATP is co-packaged with conventional neurotransmitters.[25] Interestingly, chronic stress, through the induction of elevated levels of glucocorticoids, has been reported to induce an energetic deficit at the level of the hippocampus, including a decline in ATP levels in response to certain insults.[91] It could, therefore, be speculated that a reduction in ATP content induced by chronic stress might lead to diminished extracellular ATP levels, which in turn could result in increased NCAM proteolytic activity and reduced membrane-bound NCAM levels. In support of this view are the observations obtained by Poltorak *et al.*[72] in human patients with mood disorder. These bipolar type I and major depression patients, who normally present abnormally high amounts of glucocorticoids,[37] showed increased amounts of NCAM content in the cerebrospinal fluid,[72] which the authors interpreted as possibly deriving from CNS cells as a secreted soluble NCAM isoform. Nevertheless, the possibility that NCAM reduction is due to an increase in the release from the cellular membrane is not incompatible with the possibility of a concomitant or alternative reduction on NCAM synthesis. Enhanced levels of glucocorticoids have been proposed to mediate to a great extent the morphological alterations induced by chronic stress in pyramidal CA3 neurons,[50,95] and glucocorticoids are known to modulate, either stimulating or inhibiting, the transcription of a number of genes.[8] In fact, our previous studies support a negative regulation of NCAM by sustained high levels of glucocorticoids, as indicated by reduced NCAM expression in the frontal cortex and the hypothalamus as a consequence of a 21-day regimen of corticosterone injections.[83]

Since NCAM is involved in the stabilization of synaptic contacts, its observed reduction might be implicated in the structural alterations induced by chronic stress.[50–53] The two main features of these structural alterations that have been described so far in the hippocampus are (i) a reduction in the number of branch points and length of the apical dendrites of CA3 pyramidal neurons,[50,51] and (ii) ultrastructural changes in mossy fibre terminals,[52] the major excitatory afferent input to CA3 pyramidal cells.[15] Further morphological studies need to be conducted to establish whether the down-regulation of NCAM is widely distributed in the hippocampus or might be confined to either the degenerative processes that occur in CA3 neurons or the structural remodelling that takes place in associated areas. However, current knowledge supports the view that the increase in PSA–NCAM and the trend for an increase in L1 observed in our study might be related to compensatory responses to the degeneration process reported for CA3 pyramidal cells.

Thus, PSA–NCAM has been found to be consistently associated with morphological rearrangements,[13,64,79] including the induction of synaptic plasticity, neurite outgrowth, neuronal migration, and events requiring

remodelling or repair of tissue. In the adult hippocampus, the expression of PSA was observed to increase under situations associated with structural remodelling, such as learning and memory processes[30,68,69] and lesions of specific neural pathways.[64] Therefore, in chronically stressed rats, the loss of natural contacts from input fibres induced by the shrinkage of CA3 dendrites might be hypothesized to trigger a plastic response in the remaining neuronal structures. Interestingly, mossy fibres (which are a major projection to CA3 pyramidal cells) are densely labelled with PSA–NCAM,[63] and hence a possible substrate for the PSA increases observed in our study. Since negatively charged PSA epitopes alter the adhesive properties of the membranes allowing structural changes to occur, including the outgrowth of neurites,[28] the up-regulation of polysialylation found in stressed rats could be regarded as a compensatory reaction to provide an opportunity for reinnervation to take place. Interestingly, in Alzheimer's disease patients, PSA–NCAM was found to be massively re-expressed in hippocampal areas where neurodegeneration processes are occurring.[62] Similarly, in patients with temporal lobe epilepsy, polysialylation was also shown to be increased in the hippocampus, as well as to correlate with the density of mossy fibre sprouting.[61]

A similar interpretation could be proposed for the trend towards an up-regulation of L1 expression observed in chronically stressed rats, i.e. a role for this molecule in the cellular reactions to the reversible neurodegenerative process induced by 21 days of restraint stress. In fact, this CAM, which is involved in neurite outgrowth, axon fasciculation and cell migration,[78,87] has been suggested to play a role in the repair process of the lesioned adult CNS.[42] Thus, in the deafferented dentate gyrus of adult rats, L1 was shown to be expressed on reinnervating fibres once they have made synaptic contacts with their targets.[42] In addition, in cell culture studies, L1 promoted the survival of dopaminergic neurons.[39]

Previous studies have suggested that CAMs are involved in long-term memory formation.[5,7,30,68,69,94] Even though contextual fear conditioning was previously shown to result in increased NCAM and L1, but decreased PSA–NCAM, expression in the hippocampus, when evaluated 24 h post-training,[60] the present study did not find any change in the content of these molecules as a consequence of fear conditioning. Given that the role and regulation of these molecules have been found to be closely dependent upon the age of the animals,[6,29,46] a possible explanation for this discrepancy might be the fact that the rats used in this study were older (adults) than in the previous one (young adults or adolescents).

Effects of chronic stress on corticosterone levels and body on thymus weight

Circulating corticosterone levels showed a tendency, though not statistically significant, to increase in stressed rats. These results are in agreement with previous studies in which chronic stress did not produce major changes in basal corticosterone secretion.[34,35] As for post-testing corticosterone levels, since they were measured at the beginning of corticosterone release (8–10 min from the start of the testing session), we cannot discard possible differences between stressed and control rats at later time points, such as the peak or the recovery of the corticosterone response. What also remains to be elucidated is whether corticosterone increases during the 21-day stress procedure intervened in the observed regulation of the hippocampal content of CAMs. Although we did not find evidence for the modulation of these molecules in the hippocampus after a 21-day regimen of corticosterone injections,[83] the possibility that this glucocorticoid acted synergistically with other hormonal or neuronal factors induced by chronic stress, to regulate the expression of hippocampal CAMs cannot be excluded. This question should be addressed by studying CAM content in rats in which the release of corticosterone is inhibited during exposure to restraint stress.

Finally, we found that body weight was reduced by chronic stress, whereas relative thymus weight was not significantly altered, which is in agreement with previous studies in which the same stress protocol was used.[50,51]

CONCLUSIONS

The results of this study support the view that chronic restraint stress predispose individuals to develop enhanced contextual fear conditioning responses, even when fear conditioning is induced by highly stressful conditions. They also suggest that the cell adhesion molecule NCAM, and its polysialylated form, might be involved in the structural remodelling that occurs in the hippocampus as a consequence of chronic stress exposure. Although further work is needed to elucidate the functional implications of these regulatory changes, our findings highlight CAMs as a possible target to prevent neurodegenerative processes induced or accompanied by stress.

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