Chronic Restraint Stress Induces an Isoform-Specific Regulation on the Neural Cell Adhesion Molecule in the Hippocampus

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SUMMARY

Existing evidence indicates that 21-days exposure of rats to restraint stress induces dendritic atrophy in pyramidal cells of the hippocampus. This phenomenon has been related to altered performance in hippocampal-dependent learning tasks. Prior studies have shown that hippocampal expression of cell adhesion molecules is modified by such stress treatment, with the neural cell adhesion molecule (NCAM) decreasing and L1 increasing, their expression, at both the mRNA and protein levels. Given that NCAM comprises several isoforms, we investigated here whether chronic stress might differentially affect the expression of the three major isoforms (NCAM-120, NCAM-140, NCAM-180) in the hippocampus. In addition, as glucocorticoids have been implicated in the deleterious effects induced by chronic stress, we also evaluated plasma corticosterone levels and the hippocampal expression of the corticosteroid mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). The results showed that the protein concentration of the NCAM-140 isoform decreased in the hippocampus of stressed rats. This effect was isoform-specific, because NCAM-120 and NCAM-180 levels were not significantly modified. In addition, whereas basal levels of plasma corticosterone tended to be increased, MR and GR concentrations were not significantly altered. Although possible changes in NCAM-120, NCAM-180 and corticosteroid receptors at earlier time points of the stress period cannot be ignored, this study suggests that a down-regulation of NCAM-140 might be implicated in the structural alterations consistently shown to be induced in the hippocampus by chronic stress exposure. As NCAM-140 is involved in cell-cell adhesion and neurite outgrowth, these findings suggest that this molecule might be one of the molecular mechanisms involved in the complex interactions among neurodegeneration-related events.

KEYWORDS

neurodegeneration, mineralocorticoid receptor, glucocorticoid receptor, corticosterone, neural plasticity, learning

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Abbreviations: ANOVA: analysis of variance; AMPA: α-amino-3-hydroxy-5methyl-4- isoxazole propionate; BSA: bovine serum albumin; CAMs: cell adhesion molecules; ELISAs: enzyme linked immunosorbent assay; GR: glucocorticoid receptor; HEPES: N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MR: mineralocorticoid receptor; NCAM: neural cell adhesion molecule; NMDA: N-methyl-D-aspartate; PSA: α-2,8-linked polysialic acid; SDS: sodium dodecyl sulphate; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis.

INTRODUCTION

Chronic exposure to stress or to glucocorticoid hormones has been shown to result in brain and cognitive disturbances (McEwen, 1999; Sapolsky, 1999). Available evidence obtained in rats indicates that sustained stress induces a time-dependent neurodegeneration in the hippocampus, which goes from an initial reversible damage (McEwen, 1999) to a more enduring vulnerability to metabolic insults (Stein-Behrens et al., 1994), which under certain circumstances could lead to overt neuron loss (Sapolsky, 2000). The reversible phase of hippocampal damage occurs after 3 to 4 wk of exposure to either high levels of corticosterone (Watanabe et al., 1992b; Wooley et al., 1990) or to repeated stressful situations (Magariños & McEwen, 1995a, 1995b; Sousa et al., 2000; Watanabe et al., 1992a). Widespread studies have consistently reported that this phase is characterized by an atrophy of the apical dendrites of the CA3 pyramidal neurons (Magariños and McEwen, 1995a, 1995b; Sousa et al., 2000; Watanabe et al., 1992a, 1992b; Wooley et al., 1990), which some authors have detected in other hippocampal subregions as well (Sousa et al., 2000; Wooley et al., 1990). In addition, presynaptic mossy fiber terminals also show profound ultrastructural alterations (Magariños et al., 1997). Although a general consensus has not been reached about whether such structural changes result in impaired hippocampal function (Bodnoff et al., 1995), several studies have shown that such chronic treatments result in altered performance in hippocampus-dependent learning tasks, including spatial learning impairments on a number of mazes (Bardgett et al., 1994; Conrad et al., 1996; Luine et al., 1994) and the potentiation of contextual fear conditioning (Conrad et al., 1999; Sandi et al., 2001).

Several studies have implicated the cell adhesion molecules (CAMs) of the immunoglobulin super-family—particularly the neural cell adhesion molecule (NCAM) and L1—in the impact of chronic stress and glucocorticoid treatments on memory and plasticity (Gootendorst et al., 2001; Sandi and Loscertales, 1999; Sandi et al., 2001; Venero et al., 1996; 2002). These cell surface glycoproteins play key roles in neural development, synaptic plasticity and stabilization, neurogenesis, regeneration of the nervous system, and learning and memory mechanisms (Fields & Itoh, 1996; Ronn et al., 2000; Schachner, 1997; Sandi et al. 1995; Sandi, 1998; Murphy & Reagan, 1998). Using the 21-d restraint stress protocol that reportedly induces the described neuronal dendritic atrophy in rat hippocampus (see above), our group found that whereas L1 mRNA was increased, the expression of NCAM mRNA was decreased in the hippocampus of stressed rats (Venero et al., 2002).

An important feature of NCAM is that it comprises several isoforms, derived by alternative splicing from a single gene (Cunningham et al., 1987). The three major isoforms NCAM-120, NCAM-140, and NCAM-180, having respective molecular weights of 120, 140, and 180 kDa, share similar extracellular parts, but differ in their length of their cytoplasmic domain and their attachment to the cell membrane. Whereas NCAM-140 and NCAM-180 are integral membrane glycoproteins, NCAM-120 is anchored to the cell membrane through a phosphatidylinositol linkage (Jorgensen, 1995). Using the enzyme-linked immunoabsorbent assay (ELISA) technique (with a first antibody for NCAM assays that recognizes all three major NCAM isoforms), we confirmed that stress-induced modifications in CAMs mRNA expression are paralleled by the corresponding changes at the protein level, with L1 showing a tendency to increase, whereas NCAM markedly decreases in the hippocampus of stressed rats (Sandi et al., 2001).

Given the different localization and role played by the various NCAM isoforms (Jorgensen, 1995; Schuster et al., 2001), we designed the present study to assess whether chronic stress

might differentially affect the expression of the three major NCAM isoforms (NCAM-120, NCAM-140 and NCAM-180) in the hippocampus. Thus, rats were either left undisturbed or submitted to a 21-day restraint stress protocol, and NCAM was subsequently measured in the hippocampus by Western blotting. In addition, given that glucocorticoids have been implicated in the deleterious effects induced by stress on hippocampal structure (Magariños et al., 1995a), we also evaluated the hippocampal expression of corticosteroid receptors—the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR)—at the protein level.

EXPERIMENTAL

Animals

Male Wistar rats (Faculty of Medicine, Complutense University, Madrid, Spain), weighing 150 to 175 g on arrival, were housed in groups of 3 per cage, under controlled conditions of temperature (22 ± 2 °C) and light (12:12 light-dark cycle; lights on at 7 a.m.). The animals had free access to food and water in a colony room. Approximately 5 wk after arrival, the rats were handled daily for around 4 d before being weighed. Rats were then matched by groups of four according to their body weight, and each of the four matched animals was assigned randomly into one of four experimental groups. On the 5th wk after arrival (rats weighing around 290 ± 45 g, in their 13th wk of life), the chronic stress procedure started for those animals assigned to the stress groups. Body weights were recorded periodically. All behavioral experiments were conducted between 08:00 and 14:30 h. 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).

The Chronic Stress Procedure

A group of rats (n = 9) was subjected to chronic restraint stress for 21 d. The sessions consisted of 6 h/d (8:30 a.m. to 2:30 p.m.) restraining 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, untreated control animals (n = 9) remained undisturbed in their home cages.

Hippocampal and synaptosomal preparations

One day after the termination of the stress procedure, rats from 'stressed' or 'undisturbed' condition were decapitated, the brain was removed, and the hippocampus dissected out on ice. Tissue samples were coded and stored at –80 °C until use. Then, the tissue was further processed to obtain homogenate and synaptosomal preparations for the subsequent corticosteroid receptor and NCAM isoform assessments, respectively. Homogenates were obtained by homogenizing the hippocampus 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. Crude synaptosomal pellets were obtained according to a modified protocol from Lynch and Voss (1991). Briefly, the homogenized tissue was centrifuged for 5 min at 1,000 g. The supernatant was then centrifuged for 15 min at 15,000 g and the pellet resuspended in Krebs buffer containing protease inhibitors for use. The protein concentration for each sample was estimated by the method of Bradford (1976).

Quantification of NCAM isoforms

Since NCAM consists of several isoforms (including 3 major ones of molecular weights 120, 140, and 180 kDa) resulting from the alternative splicing of a single gene, we prepared Western blots to compare the expression of the NCAM major isoforms in crude synaptosomal preparations of control and stressed animals. In brief, hippocampal synaptosomal samples obtained from chronically stressed and control rats were denatured for 2 min at 100 °C in 30 mM Tris-HCl buffer (pH 7.4) containing 0.05% SDS and β-mercaptoethanol. Equal amounts of protein (15 μg) were applied in each lane, separated on 7% (w/v) SDS-PAGE (1 mA/cm^2, 1 h), and then transferred 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 polyclonal rabbit-anti-rat NCAM antibodies diluted 1:15000 (a generous gift from Prof. Elisabeth Bock, University of Copenhagen, Denmark). The immunoblots were washed with TBST, incubated for 1 h with a secondary antibody, anti-rabbit Ig peroxidase whole molecule conjugate; diluted 1:20000 (Sigma, UK and Spain), and finally developed using the enhanced chemiluminescence (ECL) system (Amersham).

For the quantification of autoradiographic films, images were captured by high-resolution (600 x 600 dpi), 8 bit (256 grey level) microdensitometry with a falt-bed scanner (AGFA Arcus II). Video images of the autoradiograhs were converted to grey values and analyzed for optical density measurements using image-analysis software (LeicaQwin). The integrated measures of band optical density multiplied by the area in number of pixels were recorded.

As can be seen in Fig. 1, the polyclonal antibody directed to total NCAM identified the three major NCAM isoforms of molecular weights around 120,000, 140,000, and 180,000 in crude synaptosomal preparations obtained from control and stressed animals.

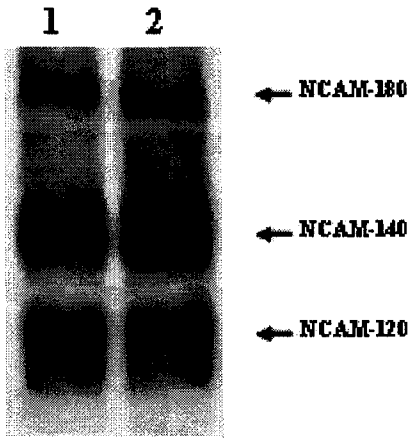

Fig. 1: Immunoblotting of NCAM polypeptide within the hippocampus of chronically stressed (lane 1) and control (lane 2) rats. Protein samples (15 μg) of hippocampal synaptosomes were separated using SDS-PAGE transferred to immobilon-P and reacted with anti NCAM antibodies followed by incubation with labeled anti-Ig. Positions of the three major isoforms are indicated in the margin (in kDa). Note that the amount of NCAM-140 was lower in chronically stressed rats.

Quantification of receptors

The MRs and GRs were quantified at the protein level using an enzyme-linked immunosorbent assay (ELISA). Briefly, flat-bottomed 96-well microplates were allowed to adsorb a coating solution (Na_2CO_3 0.1 M/$NaHCO_3$, 0.1 M) for 2 h at

room temperature. The solution was removed and 50 µL of homogenate (10 µg/mL) was added for 20 to 24 h at 4 °C. Additional binding sites were blocked with BSA (3%) for 90 min at room temperature. The wells were rinsed three times and incubated for 20 to 24 h at 4°C with 50 µL aliquots of the first antibody, polyclonal goat IgG anti-MR (N-17; 1:150) or rabbit anti-GR (M-18; diluted 1:250 (both from Santa Cruz Biotechnology; CA, USA), which detect MRs and GRs in rat brain (Gesing et al., 2001; Moutsatsou et al., 2001). The wells were washed and 50 µL aliquots of anti-goat (for MR assays) or anti-rabbit (for GR assays) peroxidase- Ig whole molecule conjugates; 1:500; Sigma, UK/Spain) second antibodies were added for 2 h. Citrate buffer (50 µL of 50 mM Na_2HPO_4, 25 mM citric acid, pH 4.5) containing 1 mg/mL o-phenylenediamine and 0.06% hydrogen peroxide added just before use was placed into each well and allowed to react for 10 min at room temperature. The reaction was terminated by the addition of 50 µL of 5N H_2SO_4 to each well. Optical density was determined at 492 nm using a Microplate Reader (DigiScan Reader V3.0 and DigiWIN software Program; ASYS Hitech GmbH, Austria).

Plasma corticosterone

For the evaluation of basal levels of corticosterone, samples of trunk blood were centrifuged (20 min at 3000 rpm, 4°C), and the plasma was stored at –35°C. Corticosterone was measured using a radioimmunoassay kit (Coat-A-Count, Diagnostics Products Corporation; CA, USA). The intra-assay variability of the RIA ranged between 3.1% and 4.5%. The sensitivity (minimal detectable concentration) was ca. 5.7 ng/mL.

Statistics

All results were expressed as mean ± SEM and analyzed using analysis of variance (ANOVA) or the Student-t test, as appropriate. Significance was accepted at $p \leq 0.05$.

RESULTS

Effects of chronic stress in the hippocampus

Expression of three major NCAM isoforms. As shown in Fig. 2, stressed and undisturbed rats did not differ in their protein content of NCAM-120 ($t = 0.84$, n.s.) and NCAM-180 ($t = 0.28$, n.s.) isoforms, but stressed rats showed significantly lower protein levels of NCAM-140 than controls ($t = 2.10$, $p = 0.05$).

MR and GR expression. As shown in Fig. 3, chronic stress did not elicit significant changes in MR ($t = 1.56$, $p = 0.14$) or in GR ($t = 0.59$, n.s.) protein levels in the hippocampus. However, it should be noted that a slight tendency toward significance was found for the reduction in MR levels observed in the stress group, as compared with control animals.

Corticosterone levels. Basal corticosterone levels were evaluated in control and stressed rats. There was a tendency for chronically stressed rats to display higher corticosterone levels (44.61 ± 9.14 ng/mL) than did controls (24.85 ± 5.29 ng/mL), which only approached significance ($t = 1.9$, $p = 0.08$).

Body weight. In both groups, body weight gradually increased throughout the restraint stress procedure (Fig. 4; $F = 237.82$, $p<0.0001$). The percent body weight gained along the whole restraint period was significantly lower in stressed than in undisturbed control rats ($F = 93.7$, $p<0.0001$).

DISCUSSION

We report here that exposure to a 21-d restraint stress regime reduced the concentration of NCAM-

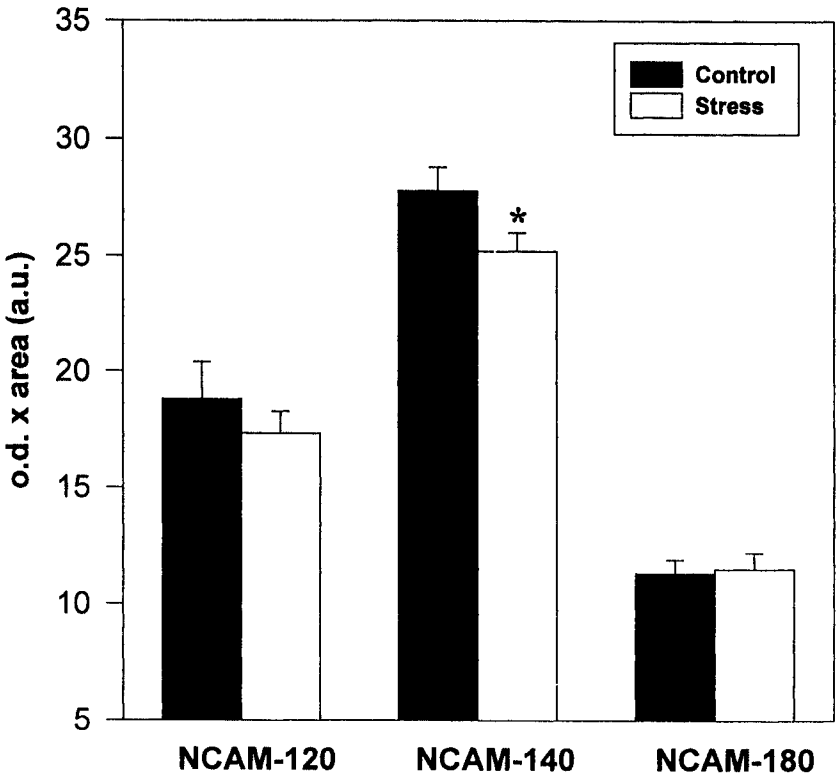

Fig. 2: Effects of chronic stress on the immunolabeling of NCAM isoforms in the hippocampus. Image analysis was used to determine the change in NCAM immunoreactivity in stressed animals as compared to control animals. The ordinate represents the integrated measures of band optical density multiplied by the area in number of pixels shown for each NCAM isoforms (NCAM-120, -140, -180). Data are the mean ± S.E.M from 9 rats per group. * $p < 0.05$ vs. corresponding control group.

140 protein in the rat hippocampus, as assessed by Western blot analyses in crude synaptosomal preparations. This effect was isoform-specific because levels of NCAM-120 and NCAM-180 were not significantly modified. These observations confirm and extend previous findings, in which NCAM-total mRNA (Venero et al., 2002) and protein (Sandi et al., 2001) diminished by the same stress procedure. In addition, the present results showing a lack of regulation of NCAM-180 protein are also consistent with previous evidence indicating that NCAM-180 might not be a candidate isoform to be regulated under our experimental conditions as mRNA for this species has been found to remain unchanged after chronic restraint (Venero et al., 2002).

In the adult rodent brain, the transmembrane isoforms, NCAM-140 and NCAM-180, are mainly expressed in neurons (Gegelashvili et al., 1993), whereas NCAM-120 (and to a lesser extent NCAM-140) seems to be the predominant species in glial cells (Noble et al., 1985). In neurons,

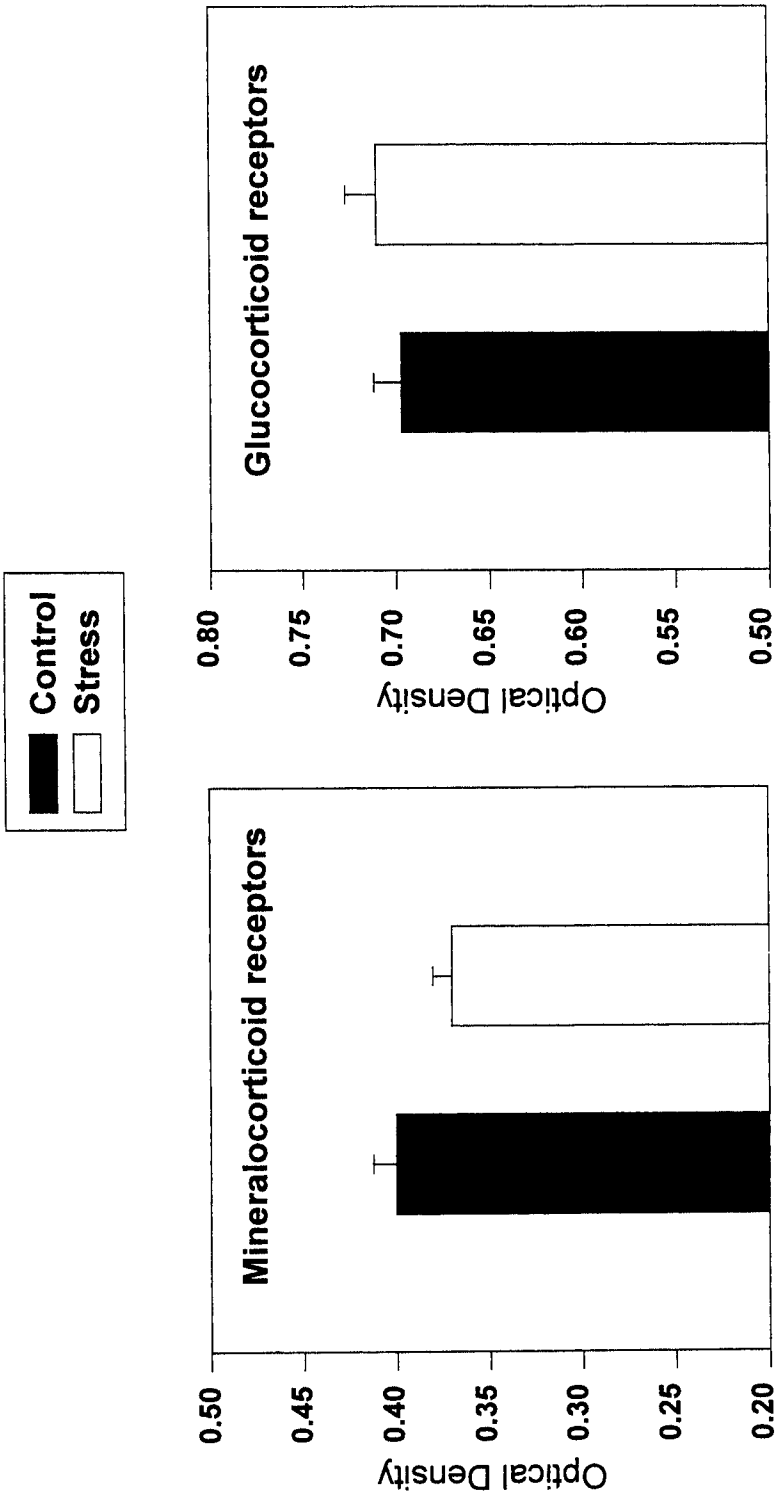

Fig. 3: Effects of chronic stress on the expression of mineralocorticoid and glucocorticoid receptors, as determined by ELISA. Results are expressed as the optical density obtained by measuring absorption at 492 nm. Data are the mean ± S.E.M from 9 rats per group.

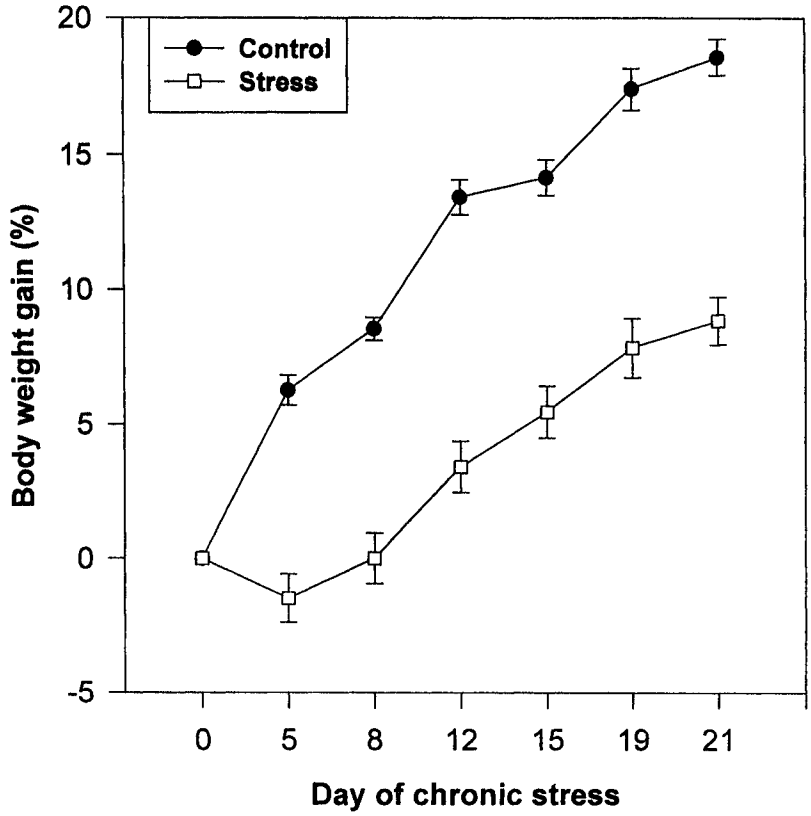

Fig. 4: Comparaison of weight gain between stressed and control rats. Animals' weights were monitored during the stress period (21 consecutive days). Data are the mean ± S.E.M from 9 rats per group.

NCAM-140 and NCAM-180 are principally plasma membrane proteins (DiFiglia et al., 1989). At the synapses, whereas NCAM-140 can be localized on both pre- and post-synaptic membranes, NCAM-180 appears to be predominantly detected only in post-synaptic membranes and densities (Persohn et al., 1989; Schuster et al., 2001).

In this study, therefore, we identified NCAM-140 as the NCAM isoform showing reduced hippocampal expression after exposure to chronic stress. To what extent this effect might be related to the alterations in hippocampal morphology reportedly induced by chronic stress (Magariños & McEwen, 1995a, b) remains unclear. As other investigators also observed a reduction on NCAM expression concomitant to a process of neuronal atrophy occurring in CA3 after injection of kainic acid—a glutamate analog—into the lateral cerebral ventricles (Endo et al., 1999), it is tempting to speculate that down-regulation of NCAM-140 is one of the molecular mechanisms involved in the complex interaction of neurodegeneration-related events (Brodkey et al., 1993; Sapolsky, 2000). As already mentioned, NCAM-140 can be expressed on pre- and post-synaptic membranes and exerts its adhesive properties through homophilic and heterophilic binding (Schachner, 1997). Therefore, a decrease in the concentration of NCAM-140 might reduce cell-cell adhesion, and the resulting elimination of synaptic contacts eventually might lead to a loss of spines (McKinney et al., 1999) and dendritic shrinkage.

In addition, NCAM-140 has been shown to be critically involved in neurite outgrowth through the activation of different transduction pathways (Doherty et al., 1995; Kolkova et al., 2000). Therefore, it seems plausible to propose that a reduction in the concentration of this isoform could result in the converse structural phenomenon; namely, dendritic retraction—as found after chronic stress—instead of neurite outgrowth. Although biosynthesis-independent pathways cannot be discarded (Endo et al., 1999), previous data from our laboratory support the view that chronic stress impinges on the mechanisms involved either in the synthesis of NCAM and/or on a feedback system that governs mRNA splicing in response to different modulatory actions (Venero et al., 2002). Available evidence supports the view that glutamatergic transmission, which has been implicated in stress-induced neurodegenerative processes (McEwen, 1999; Sapolsky, 2000), might be involved in the down-regulation of NCAM-140 observed in our study. Glutamate levels are known to be increased by stress and glucocorticoids in the hippocampus (Moghaddam et al., 1994; Venero and Borrell, 1999) and glutamate receptors to be subsequently regulated. Particularly relevant for the purpose of this discussion is that AMPA glutamate receptors, whose activation was shown to stimulate the NCAM promoter (Holst et al., 1998), appear to be diminished by exposure to stressful situations (Bartanusz et al., 1995).

Although, based on the results of our earlier *in situ* hybridization studies (Venero et al., 2002), we did not expect to observe changes in NCAM-180 concentration in the current study, the lack of modulation of this molecule after chronic stress is, somehow, an unexpected finding. Given that, as opposed to NCAM-140, the lateral mobility of NCAM-180 within the surface membrane is markedly reduced because of its interaction with the membrane-cytoskeleton linker protein spectrin, NCAM-180 has been proposed to be critical for the stabilization of cell contacts at synaptic sites and to play a role in modulating synaptic efficacy (Pollerberg et al., 1986) and remodeling (Schachner, 1997; Schuster et al., 1998). Because chronic stress affects hippocampal structure (Magariños & McEwen, 1995a, 1995b; Magariños et al., 1997; Sousa et al., 2000; Watanabe et al., 1992a) and cognitive processes (Bardgett et al., 1994; Conrad et al., 1996, 1999; Luine et al., 1994; Sandi et al., 2001), a modulation of NCAM-180 levels by stress could have been expected. The failure, however, to find changes in the concentration of this isoform in our study should not be interpreted as a lack of involvement of this NCAM species on the neuronal modifications induced by chronic stress. This isoform has been proposed as the major carrier of α2-8 linked polysialic acid (PSA) homopolymers (Doyle et al., 1992), and PSA-NCAM is increased in the hippocampus of rats submitted to the same chronic restraint procedure (Sandi et al., 2001). Polysialylation is a posttranslational modification of NCAM that interferes with NCAM- and L1-mediated adhesion, eventually leading to decreased membrane-membrane contacts and attenuation of cell interactions (Regan, 1991; Rougon, 1993; Rustihauser & Landsmesser, 1996). Therefore, changes in the polysialylation state of NCAM-180 might participate in the structural alterations impinged by stress.

In addition, it should be noted that the time point selected in our study for the evaluation of NCAM expression was designed to assess changes in steady-state of the different isoforms after stress exposure. Possible changes in the prevalence of NCAM-180 polypeptide occurring dynamically at earlier time points of the stress period, and eventually returning to baseline concentrations with repeated exposure, cannot be detected with our experimental design. Although further experiments are needed to specifically address the question of whether a time-dependent regulation of NCAM-180 expression occurs at an earlier time in

the repeated stress procedure, the available evidence suggests that this could be the case. For example, whereas brief stimulation of the N-methyl-D-aspartate (NMDA) glutamate receptor, which has been implicated in the dendritic atrophy induced in the hippocampus by chronic stress (Magariños & McEwen, 1995a), increased NCAM-180 levels, prolonged infusions of NMDA were followed by a substantial decrease in the concentration of this isoform (Hoffman et al., 2001).

Similarly, our findings indicate that hippocampal expression of the corticosteroid receptors MR and GR was not modulated by chronic stress. The lack of regulation of MR levels agrees with the results of previous studies, including different lengths of exposure to stress (Herman et al., 1999; Paskitti et al., 2000). The failure of a down-regulation of GRs observed here contrasts with previous reports (Sapolsky et al., 1984; Gomez et al., 1996) but agrees with data from other studies showing unmodified levels of GRs after exposure to stress procedures of similar duration (Herman & Spencer, 1998; Mamalaki et al., 1993). It has been suggested that the corticosterone levels induced by chronic stress procedures might not be high enough to induce the down-regulation of GRs (Herman & Spencer, 1998). Nevertheless, given that our study was performed on homogenates of the whole hippocampus, the possibility that local changes occurring in specific hippocampal sub-regions like the decrease found in GR mRNA in CA3 after chronic stress (Paskitti et al., 2000) were neglected cannot be discarded. Furthermore, we should also mention that, as argued above, our experimental design did not allow the assessment of possible dynamic changes in the expression of corticosteroid receptors that, according to previous data (Paskitti et al., 2000), might have occurred earlier in the stress procedure.

It should also be noted that the validity of the restraint procedure to induce chronic stress in our study was supported by the substantial reduction in body weight, and a tendency of basal corticosterone levels to increase, which agree with results from previous studies using the same stress protocol (Magariños & McEwen, 1995a, b; Sandi et al., 2001; Venero et al., 2002).

CONCLUSIONS

The protein concentration of the NCAM-140 isoform was reduced in the hippocampus of rats submitted to a 21-d restraint stress protocol. Given that NCAM-140 is involved in cell-cell adhesion and neurite outgrowth, these findings suggest a role of this molecule on the dendritic atrophy that has been consistently shown to be induced in the hippocampus by chronic stress exposure. Although no changes were found in the expression of the two other major NCAM isoforms, NCAM-120 and NCAM-180, or in the protein content of the two corticosteroid receptors MR and GR, the possibility of dynamic regulations occurring in these molecules, either at an earlier time of the stress procedure or in specific hippocampal subfields, cannot be discarded.

ACKNOWLEDGEMENT

This work was supported by grants from the Ministry of Science and Technology (MCYT, PM99 027, Spain), and from the EU 5th framework programme (QLK6-CT-1999-02187). The authors wish to thank Prof. E. Bock (Denmark) for the kind gift of the NCAM antibody, and Dr. Cesar Venero for experimental help.

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