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NEURONAL CYTOSKELETAL ALTERATIONS IN AN EXPERIMENTAL MODEL OF DEPRESSION

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Abstract—It has been proposed that depression is associated with hippocampal morphological changes. The apical dendrite atrophy of hippocampal CA3 pyramidal neurons has been described in experimental models of depression. The aim of the present study was to determine which cytoskeletal components are involved in the morphological changes previously described in the hippocampus of depressed animals. The expression of different neuronal cytoskeletal markers was analyzed by immunohistochemistry in rats exposed to a learned helplessness paradigm, an experimental model of depression. Rats were trained with 60 inescapable foot shocks (0.6 mA/15 s) and escape latencies and failures were tested 4 days after training. Animals in which learned helplessness behavior persisted for 21 days were included in the depressed group. No foot shocks were delivered to control rats. Microtubule-associated protein 2 (MAP-2) and light (NFL; 68 kDa), medium (NFM; 160 kDa) and heavy (NFH; 200 kDa) neurofilament subunit immunostainings were analyzed employing morphometric parameters. In the depressed group, NFL immunostaining decreased 55% ($P<0.05$) and 60% ($P<0.001$) in CA3 and dentate gyrus, respectively. In the same areas, MAP-2, NFM and NFH immunostainings did not differ between depressed and control animals. Since NFL is present in the core of mature neurofilament, it is proposed that hippocampal depression-associated plastic alterations may be due to changes in the dynamics of the neurofilament assembly.

Key words: intermediate filaments, light neurofilament subunit, hippocampal plasticity, hippocampal atrophy, learned helplessness paradigm.

Major depression is a mood disorder affecting 8–12% of the population (Andrade et al., 2003). Several theories have been developed to explain the neurobiological substrate of this condition. One of the most consistently observed changes both in humans suffering depression and in animal models of this disorder is the atrophy and dysfunction of the hippocampus (Magariños and McEwen, 1995a; Sheline et al., 1996; Galea et al., 1997; Lambert et al., 1998). Considerable effort has been made in order to identify the mechanisms that produce these alterations. It has been demonstrated that high glucocorticoid and glutamate levels contribute to induce dendritic retraction in CA3 pyramidal neurons of hippocampus (Magariños and McEwen, 1995b; McEwen, 1999). In the long term, the high level of these neuroactive substances has been proposed to decrease hippocampal glutamate release (Ferrero et al., 2003) and described to impair the hippocampus-dependent behaviors, such as spatial or inhibitory avoidance learning (Bisagno et al., 2000). All these alterations should have structural correlation at subcellular level. However, reported attempts aimed to analyze microtubule-associated protein 2 (MAP-2), a neuronal phosphoprotein predominantly associated with dendritic microtubules, have failed to find significant changes in the hippocampus of chronically stressed rats (Kuroda and McEwen, 1998). These findings seem to have discouraged further investigation on this matter, and in fact no additional evidences are available on the cytoskeletal modifications observed in animal models of depression.

As cytoskeleton has a major role in neuronal cytoarchitecture and function (Lazarides, 1980; Nixon and Sihag, 1991; Julien, 1999), it is reasonable to predict that cytoskeletal components might be affected in depressed animals leading to hippocampal structural alteration and functional impairment. Neuronal cytoskeleton is composed of three types of filaments: actin microfilaments, microtubules and intermediate filaments. Neurofilaments (NF), the major type of intermediate filaments in adult neurons, comprise three subunits: light (NFL; 68 kDa), medium (NFM; 160 kDa) and heavy (NFH; 200 kDa) (Alberts, 1994). These subunits have been observed altered in several models of neurodegenerative diseases (Al-Chalabi and Miller, 2003; Norgren et al., 2003), and disruption of their expression has important consequences on neuronal function (Julien, 1999; Gotow, 2000).

Thus, the aim of this study was to determine which neuronal cytoskeletal components are involved in the structural changes described in the hippocampus of depressed animals. For this purpose, neuronal cytoskeletal markers were analyzed in the hippocampus of rats exposed to an experimental model of depression: the learned helplessness paradigm. In this behavioral procedure animals exposed to inescapable foot shocks fail to escape from similar stressful stimuli in a subsequent test session. Learned helplessness has been used for screening the

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Abbreviations: ANOVA, analysis of variance; DG, dentate gyrus; MAP-2, microtubule-associated protein 2; mf, mossy fibers; NF, neurofilament; NFH, heavy neurofilament subunit; NFL, light neurofilament subunit; NFM, medium neurofilament subunit; PBS, phosphate buffer saline; pyr, pyramidal cell layer.

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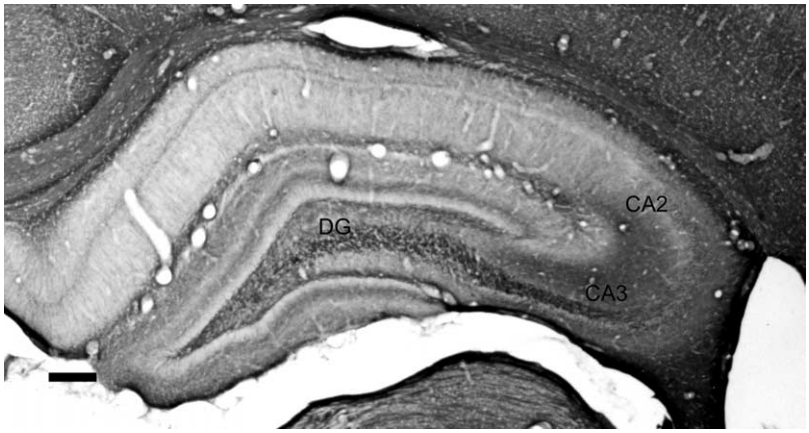

Fig. 1. Anatomical localization of the hippocampal areas used for the immunohistochemical studies. The bright field photograph shows a 60-μm-thick section of the hippocampus immunostained for NFM, corresponding to plates 32–34 (from Bregma −3.14 mm to −3.60 mm) of the atlas of Paxinos and Watson (1986). The staining allows observing the CA3, CA2 and DG areas employed for the immunohistochemical analysis. Scale bar=1.28 mm.

antidepressant activity of drugs and, as mentioned above, is also considered to be an experimental model of depression (Thiébot et al., 1992). The presence of neuronal intermediate filaments NFL, NFM, NFH and of MAP-2 was evaluated in different hippocampal areas by immunohistochemistry.

EXPERIMENTAL PROCEDURES

Animals and drugs

Male Wistar rats weighing 200–230 g at the beginning of the experiment were used. Animals were purchased at the Bioterio Central, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina, and housed in groups of five in an air- and light-controlled room (temperature: 22 °C, light phase: 08:00–20:00 h). Food and water were given *ad libitum*. Experiments were all carried out in accordance with the local guidelines and with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health, USA. Special care was taken to minimize the number of animals used and their suffering.

All chemical substances were of analytical grade. Mouse monoclonal anti-MAP-2, mouse monoclonal anti-NFL, mouse monoclonal anti-NFM, mouse monoclonal anti-NFH, secondary biotinylated antibodies and streptavidin complex used for immunohistochemistry studies were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Learned helplessness paradigm in rats

Learned helplessness induction. The learned helplessness paradigm was performed according to Nakagawa et al. (1999), with modifications. Rats were randomly assigned to shocked and control groups. Animals were individually placed in the treatment chamber that consisted in a 28×21×25 cm^3 box with black Plexiglas walls and equipped with a stainless-steel grid floor. A constant-current shocking device was used to deliver 60 inescapable foot shocks (0.6 mA) for 15 s every min (i.e. for 1 h; shocked group). Control rats were placed on the grid in identical chambers for 1 h, without receiving the uncontrollable electric shocks. After this session, animals were replaced in their own cages. All the helplessness induction trials were performed during the morning.

Learned helplessness behavior test. To evaluate escape deficits, testing sessions took place 4 days after the learned

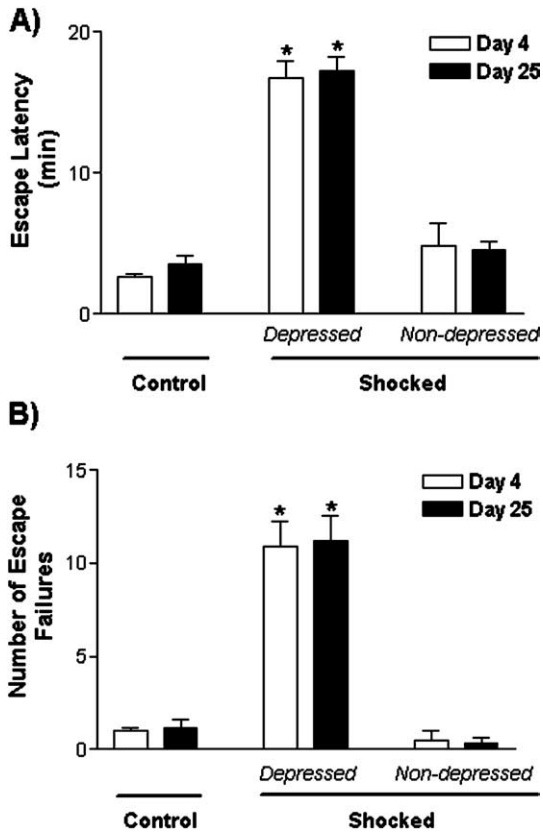

Fig. 2. Escape deficit in control and shocked animals. Rats were given inescapable foot shocks (shocked group) or placed on the grid in identical conditions without receiving uncontrollable electric shocks (control group). To evaluate the acquisition and persistence of learned helplessness behavior, escape deficit was measured respectively in animals subjected to an avoidance task at 4 and 25 days after learned helplessness induction. Results are mean values (±S.D.) of four to 16 animals and are expressed as (A) escape latency in minutes and (B) number of escape failures. Acquisition (day 4) and persistence (day 25) of learned helplessness behavior are shown as increased escape latency and number of escape failures in depressed animals. * $P<0.05$ versus control and shocked-non-depressed groups, by non-parametric Kruskal-Wallis test.

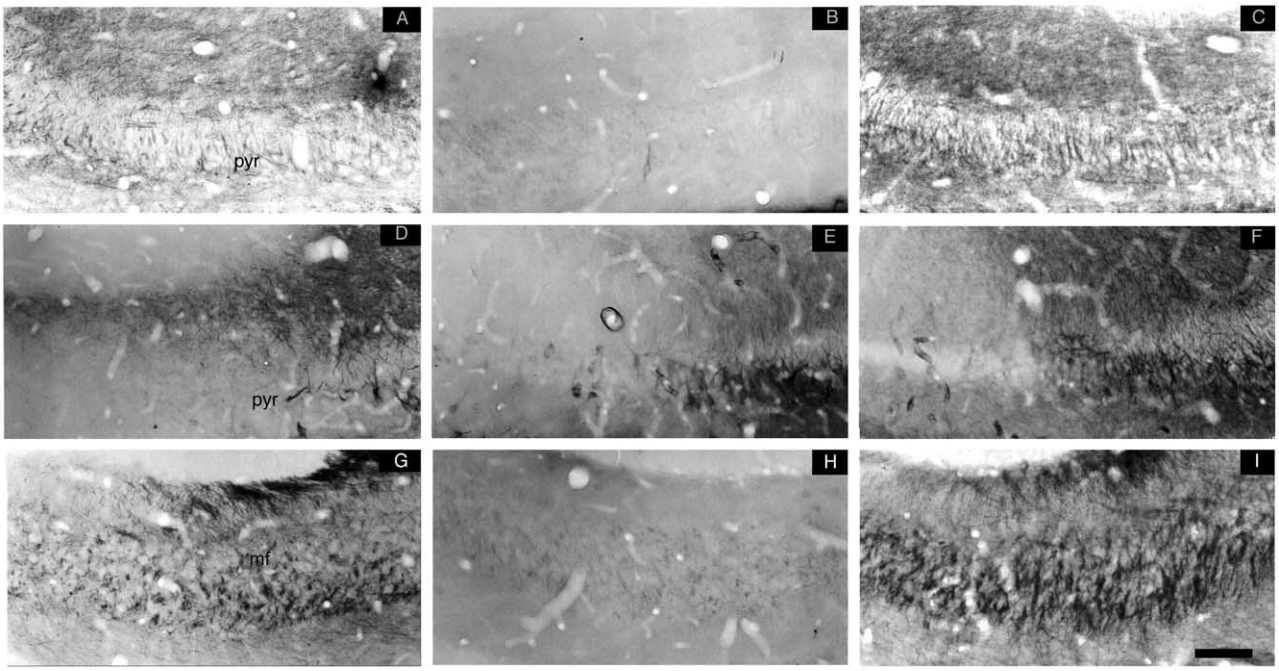

Fig. 3. Photographs show the NFL-immunolabeled structures of CA3 (A–C), CA2 (D–F) and DG (G–I) of hippocampus from shocked (depressed and non-depressed) and control animals. First panel: (A) control group in CA3; (B) shocked–depressed group in CA3; (C) shocked–non-depressed group in CA3. Middle panel: (D) control group in CA2; (E) shocked–depressed group in CA2; (F) shocked–non-depressed group in CA2. Last panel: (G) control group in DG; (H) shocked–depressed group in DG; (I) shocked–non-depressed group in DG. Scale bar=59 μm. mf, mossy fibers; pyr, pyramidal cell layer.

helplessness induction. Animals were subjected to an avoidance task in a shuttle box which consisted of two equal-sized compartments divided by a Plexiglas partition fitted out with an opening (7×7 cm) and with a grid floor consisting of stainless-steel rods placed 1 cm apart. Animals were placed individually into the shuttle box and allowed to habituate to the environment for 5 min. Following this exploration time, 15 stimulus-shock trials (0.6 mA) were presented for a period of 15 min, i.e. one trial per min. In each trial, the shock duration was 20 s with an intertrial time of 40 s. In the first five trials, the door was opened immediately after starting the shock and in the rest 10 trials there was a 4-s delay period after starting the shock. The number of failures to change from the shuttled side to the safe side (the failure to respond during the 20-s shock on) and the escape latency were recorded as the way to evaluate the learned helplessness behavior acquisition. Persistence of this behavior was re-evaluated 21 days later. Only animals in which learned helplessness behavior persisted for 21 days were included in the depressed group. In order to avoid unnecessary shock to control animals, they were exposed to the test at day 4 or 25.

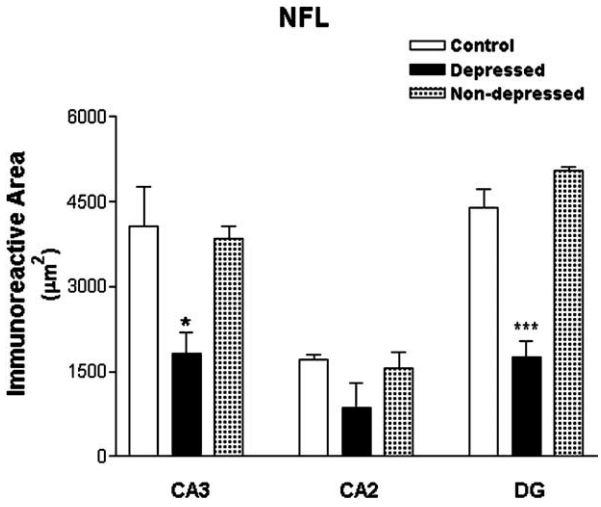

Fig. 4. Area of NFL immunoreactive fibers in shocked and control animals. Twenty-five days after the training session, shocked (depressed and non-depressed) and control animals were killed, brains immediately removed and processed by immunohistochemistry. Data are expressed as the area of NFL immunolabeled fibers (μm^2) and represent the mean values (±S.D.) of three to four individual experiments for each hippocampal region. Depressed animals showed a significant NFL immunoreactive area decrease in CA3 and DG. Animals that received the uncontrollable foot shock but failed to develop learned helplessness behavior presented no changes in hippocampal NFL-immunolabeled structures. * $P<0.05$, *** $P<0.001$ between bars, by one-way ANOVA followed by Bonferroni test.

Tissue fixation

After being tested in the learned helplessness paradigm at day 25, animals were deeply anesthetized (50 mg/kg ketamine hydrochloride and 2 mg/kg xylazine, i.p.) and perfused through the left ventricle, initially with a saline solution containing 50 IU of heparin and subsequently with a fixative solution containing 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were quickly removed and kept in the same cold fixative solution for 3 h. After that, brains were washed three times in cold 0.1 M phosphate buffer, pH 7.4, containing 5% (w/v) sucrose, and left in this washing solution for 18 h at 4 °C. Coronal 60 μm thick brain sections were cut using a vibratome. Sections were stored at −20 °C in 0.1 M phosphate buffer, pH 7.4, containing 25% (w/v) sucrose.

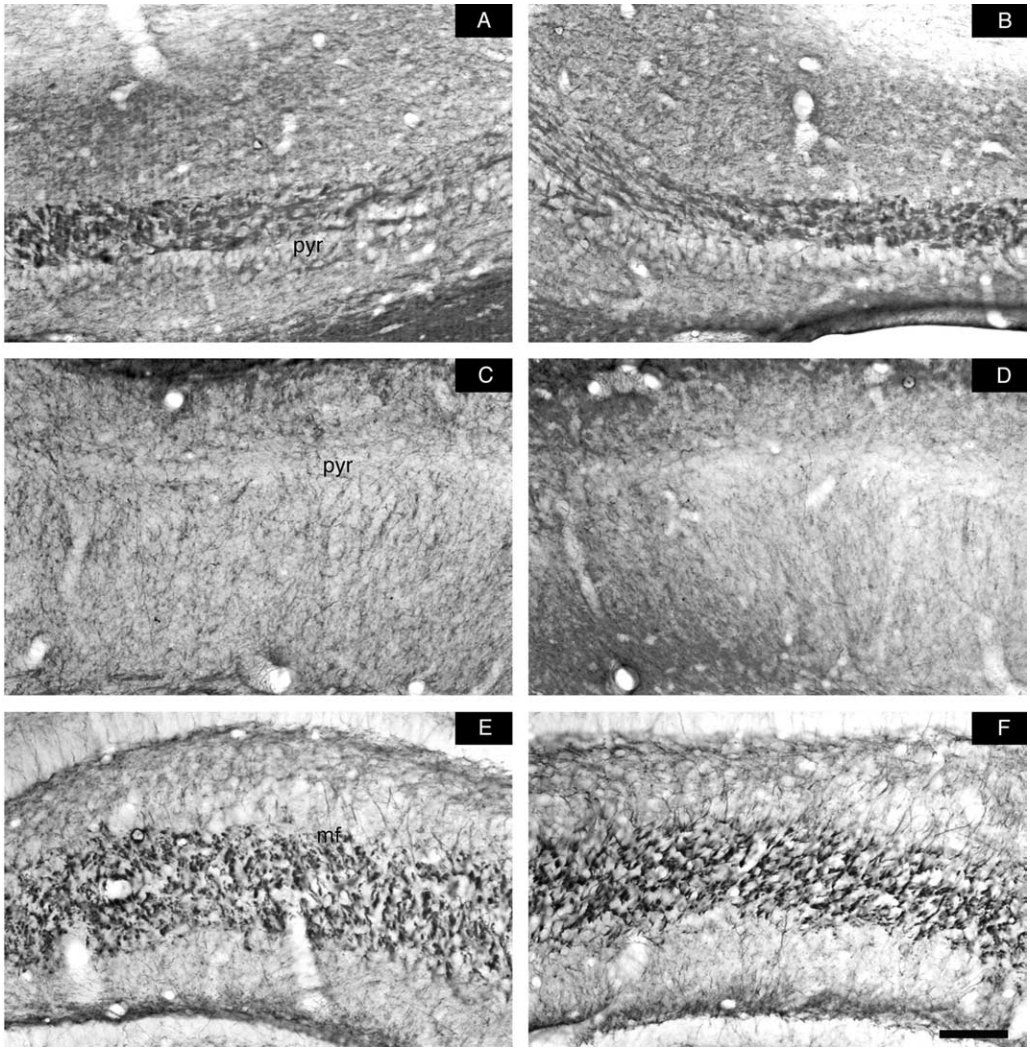

Fig. 5. Photographs show the NFM-immunolabeled structures of CA3 (A, B), CA2 (C, D) and DG (E, F) of hippocampus from depressed and control animals. First panel: (A) control group in CA3; (B) depressed group in CA3. Middle panel: (C) control group in CA2; (D) depressed group in CA2. Last panel: (E) control group in DG; (F) depressed group in DG. Scale bar=87 μm. mf, mossy fibers; pyr, pyramidal cell layer.

Immunohistochemistry

Brain sections of both shocked and control groups were simultaneously processed in free-floating state as previously described by Ramos et al. (2000). In order to inhibit endogenous peroxidase activity, tissue sections were previously dehydrated, treated with 0.5% (v/v) H_2O_2 in methanol for 30 min at room temperature and rehydrated. Free-floating brain sections were blocked for 1 h at room temperature with 3% (v/v) normal ovine serum in phosphate buffer saline (PBS). After two rinses in PBS, sections were incubated for 48 h at 4 °C with one of the following primary antibodies against NFL, NFM, NFH or MAP-2 diluted 1:2000 or 1:1000 (v/v) for NF and MAP-2, respectively. After five rinses in PBS, sections were incubated 1 h at room temperature with biotinylated secondary antibodies diluted 1:100. After further washing in PBS, sections were incubated for 1 h at room temperature with streptavidin–peroxidase complex solution diluted 1:200. After washing again five times in PBS and twice in 0.1 M acetate buffer, pH 6, development of peroxidase activity was performed in the same buffer acetate containing 0.035% (w/v) 3,3′-diaminobenzidine plus 2.5% (w/v) nickel ammonium sulfate and 0.1% (v/v) H_2O_2. To stop the enzymatic reaction, tissue sections were washed three times in 0.1 M acetate buffer, pH 6, and once in distilled water. Sections were mounted on gelatin-coated slides, dehydrated and coverslipped using Permount for light microscopic observation. All antibodies as well as streptavidin complex were dissolved in PBS containing 1% (v/v) normal ovine serum and 0.3% (v/v) Triton X-100, pH 7.4. Specificity of immunohistochemical procedure was assessed by omitting primary antibodies. In all cases, no immunohistochemical labeling was observed.

Morphometric measurement

All measurements were performed on coded slides by different observers to ensure objectivity. NFL, NFM, NFH and MAP-2 immunolabeled fibers were measured in CA3, CA2 and dentate gyrus (DG) areas of hippocampus (Fig. 1). Hippocampal areas were delimited according to plates 32–34 (from Bregma −3.14 mm to −3.60 mm) of the atlas of Paxinos and Watson (1986). Only tissue sections corresponding to these coordinates were included in the quantification, ensuring that the regions of interest (CA2, CA3 and DG) were equivalent among animals and experiments. To ensure the evaluation of similar fields inside the hippocampal regions of the different tissue sections, dorso-ventral

and lateral references corresponding to the mentioned plates 32–34 of the atlas of Paxinos and Watson (1986) were employed. To evaluate NFL, NFM, NFH and MAP-2 positive fibers, total area of immunolabeled fibers per field was measured in an Axiophot Zeiss light microscope equipped with a video camera on line with a Zeiss-Kontron VIDAS image analyzer. In each section, immunoreactive area of fibers was measured in three contiguous fields (without overlapping the measured fields) of CA2 and CA3 regions whereas six fields were measured in the DG. Images of neuronal cell bodies were excluded from measurement to ensure the evaluation of neuronal projections. The analog images were digitized into an array of 512×512 pixels corresponding to an area of 140×140 μm of tissue (40× primary magnification). The resolution of each pixel was 256 gray levels. Threshold setting was performed according to previous reports (Ramos et al., 2000; Tagliaferro et al., 2002). For that purpose, a range of gray value (threshold value) was interactively selected to allow the segmentation of the specific signal (i.e. neuronal fibers) from the background and thereafter was kept constant along each experiment. Plates containing scanned microphotographs were prepared with Adobe Photoshop 6.0 software (Adobe Systems Inc.).

Data analysis

Escape latency and number of escape failures are mean values (±S.D.) of 15–16 control and shocked animals. Statistical significance of the differences between shocked and control groups were determined by non-parametric Kruskal-Wallis test.

For each primary antibody, three to four separate immunohistochemical experiments were run. Individual experiments consisted of three to five tissue sections of each of the four to five animals per group. In each section, immunoreactive area of fibers was measured in three fields of CA2 and CA3 regions whereas six fields were measured in the DG. After that, mean values of immunoreactive area were calculated in each animal group per experiment. Finally, for each marker, immunoreactive areas are expressed as the mean values (±S.D.) of the three to four experiments performed. Differences among the mean immunoreactive areas were statistically analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni test.

The probability level indicative of statistical significance was set at $P<0.05$.

RESULTS

Learned helplessness

To evaluate the learned helplessness behavior, escape latency and number of escape failures were recorded in control and shocked groups. Four days after training, it was observed that escape latency as well as number of escape failures were increased in some of the shocked animals compared with control ($P<0.05$; Fig. 2A, B). The increase in the number of escape failures in the animals that received the inescapable electric shocks was indicative of the behavioral deficiency caused by exposure to an uncontrollable aversive situation, which is considered to be the emergence of depression. The animals presenting this behavior represented 75% of those that received the shock, thereafter termed depressed group. Twenty five percent of the animals failed to show the behavioral effect of inescapable shocks. These animals (shocked but non-depressed) were used as a negative control to rule out that morphological alterations are produced by the inescapable shock itself.

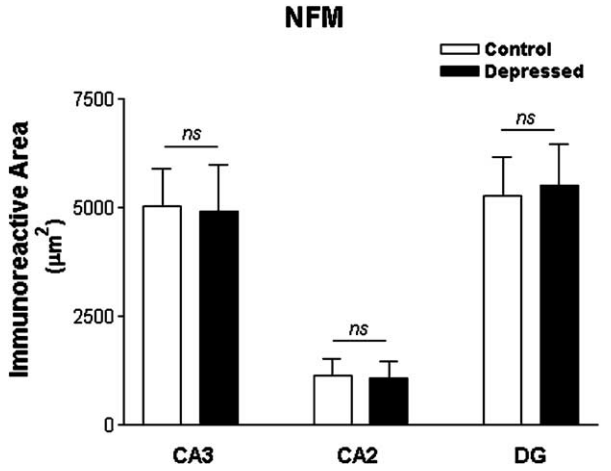

Fig. 6. Area of NFM immunoreactive fibers in depressed and control animals. Twenty-five days after the training session, depressed and control animals were killed, brains immediately removed and proceeded by immunohistochemistry. Data are expressed as area of NFM-immunolabeled fibers (μm^2) and represent the mean values (±S.D.) of four individual experiments for each hippocampal region. Depressed animals failed to show any significant change in NFM immunoreactive area of the hippocampus; ns, non-significant between bars, by Student's *t*-test.

Twenty-five days after training, escape latency as well as number of escape failures were still increased in the depressed group, indicating that the learned helplessness behavior persisted at least for that period (Fig. 2A, B).

NFL

NFL immunostaining was studied in hippocampal CA3, CA2 and DG areas from control and shocked (depressed and non-depressed) groups.

In CA3 area from control animals, NFL immunostaining allowed to observe some neuronal somata in the pyramidal cell layer as well as connecting fibers and projections of pyramidal neurons, including apical dendrites, in stratum lucidum and radiatum (Fig. 3A). In the depressed group, an important reduction of NFL fibers was observed, but some neuronal somata and proximal dendrites were still labeled (Fig. 3B). The CA2 and DG areas from control animals showed the typical hippocampal pattern for this marker, with projections as well as some neuronal somata labeled specially in the pyramidal cell layer from CA2 (Fig. 3D, G). Whereas no significant changes in the pattern of NFL immunostaining were observed in CA2, an important reduction of immunolabeled neuronal processes and somata was observed in DG from depressed animals (Fig. 3E, H). In all studied areas, shocked-non-depressed animals showed the same NFL immunostaining profile as controls (Fig. 3C, F, I).

NFL immunoreactive fibers were quantitatively evaluated in the hippocampus of control and shocked groups. The image analysis of CA3 area, including the stratum lucidum and radiatum, showed a significant reduction in NFL-labeled fibers in depressed animals (45.0% of control, $P<0.05$). In DG, the area of NFL immunoreactive fibers from the depressed group decreased 60% compared with control

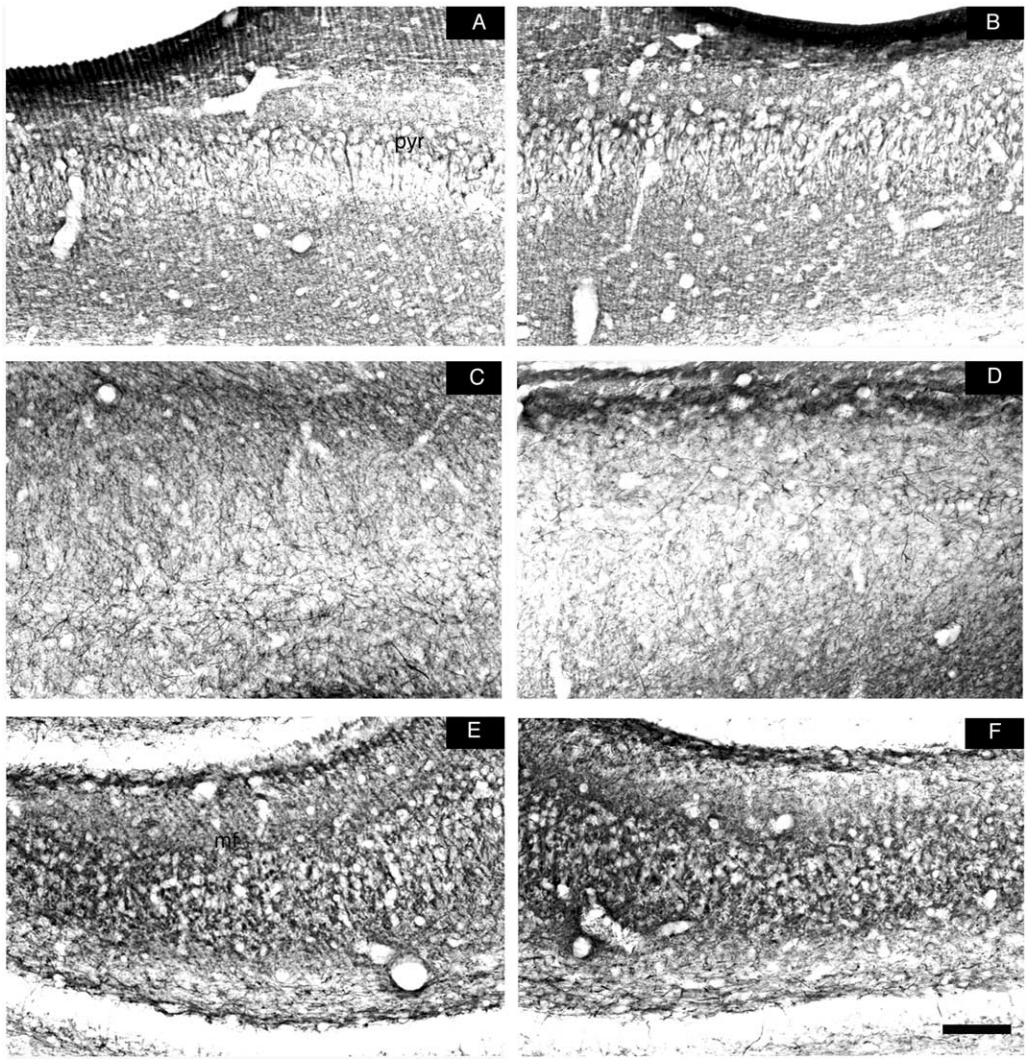

Fig. 7. Photographs show the NFH-immunolabeled structures of CA3 (A, B), CA2 (C, D) and DG (E, F) of hippocampus from depressed and control animals. First panel: (A) control group in CA3; (B) depressed group in CA3. Middle panel: (C) control group in CA2; (D) depressed group in CA2. Last panel: (E) control group in DG; (F) depressed group in DG. Scale bar=87 μm. mf, mossy fibers; pyr, pyramidal cell layer.

($P<0.001$). No differences were observed in the area of NFL labeled fibers from CA2 region between depressed and control groups. These morphological changes were not observed in any of studied areas from shocked-non-depressed animals, whose NFL immunoreactive area of fibers was not significantly different from control group (Fig. 4).

NFM and NFH

The immunostaining for NFM and NFH was analyzed in hippocampal CA3, CA2 and DG areas from control and depressed groups.

In control animals, the pattern for NFM immunostaining was somewhat different from that observed for NFL. In CA3, this marker showed the neurites of pyramidal neurons as well as the connecting fibers arriving to the area, but neuronal somata were not labeled. However, the perimeter of pyramidal neurons was easily observed due to the labeling of their contacting fibers (Fig. 5A). In CA2, NFM-labeled projections and connecting fibers as well as the perimeter of pyramidal neurons was also observed (Fig. 5C). NFM immunostaining in DG from control animals labeled mossy and connecting fibers (Fig. 4E). No changes were found in the NFM immunostaining pattern from any of the studied areas between control and depressed groups (Fig. 5B, D, F). Quantitative analysis of NFM immunostained area of fibers confirmed these observations, showing no significant changes in the area of fibers between control and depressed groups (Fig. 6).

In CA3 from control animals, NFH immunostained pyramidal neurons as well as apical dendrites and fibers arriving to the area (Fig. 7A). In CA2, pyramidal neurons were not easily observed but a great number of fibers crossing the area in different directions were immunolabeled (Fig. 7C). In DG, NFH labeled densely all fibers present in the area (Fig. 7E). No changes were observed in the NFH immunostaining pattern from any of the studied areas between control and

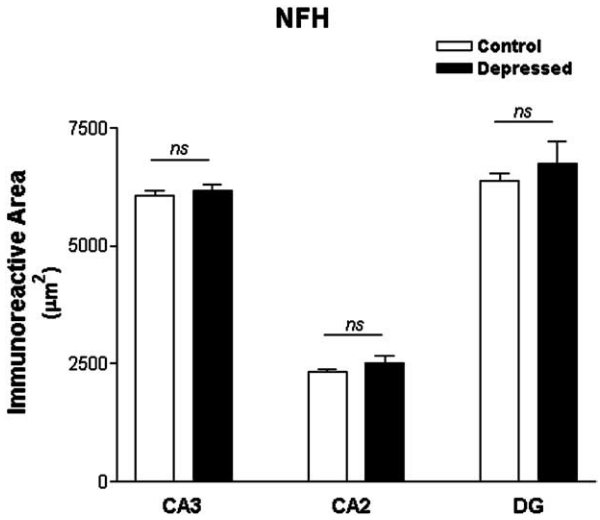

Fig. 8. Area of NFH immunoreactive fibers in depressed and control animals. Twenty-five days after the training session, depressed and control animals were killed, brains immediately removed and proceeded by immunohistochemistry. Data are expressed as area of NFH-immunolabeled fibers (μm^2) and represent the mean values ($\pm$S.D.) of three individual experiments for each hippocampal region. Depressed animals failed to show any significant change in NFH immunoreactive area of the hippocampus; ns, non-significant between bars, by Student's *t*-test.

depressed groups (Fig. 7B, D, F). Quantitative analysis of NFH immunostained area of fibers showed no significant changes in the area of fibers between both groups (Fig. 8).

MAP-2

MAP-2 immunostaining was studied in CA3, CA2 and DG of hippocampus from control and depressed animals.

MAP-2 is a dendritic marker, so the profile is different from the other cytoskeletal components evaluated. In CA3 and CA2 from the control group, neuronal somata and proximal dendrites were clearly observed, but also the dense dendro-dendritic connections were labeled (Fig. 9A, C). In DG, somata of granule cells were immunolabeled (Fig. 8E). No differences were found in the MAP-2 immunostaining pattern of any of the studied areas between control and depressed groups (Fig. 9B, D, F). Quantitative analysis of MAP-2 immunostained area of fibers showed no significant changes in the area of fibers between control and depressed groups (Fig. 10).

DISCUSSION

The hippocampal dysfunction observed in depressed patients and in animals subjected to experimental models of depression has been related to plastic morphological changes observed in this brain structure. Among the described alterations, dendritic atrophy of CA3 pyramidal neurons has been the most consistently reported (Magariños and McEwen, 1995a,b; Galea et al., 1997; Lambert et al., 1998; McEwen, 1999; Bisagno et al., 2000).

In this work we examined the effects of animal exposure to a learned helplessness paradigm (an animal model of depression) on four cytoskeletal markers: NFL, NFM, NFH and MAP-2. We observed that, in coincidence with the development of impaired escape behavior, CA3 and DG of the hippocampus showed a significant NFL immunoreactivity decrease, whereas no changes were observed either in the other two intermediate filaments or in MAP-2. The depression model that we used rendered two distinct groups: the *depressed* group (75% of shocked animals) and those in which the stressful experience did not induce the learned helplessness behavior, the *shocked non-depressed* group (25% of shocked animals). The percentage of depressed animals obtained with this paradigm is in accordance with previous studies (Drugan et al., 1989; Caldarone et al., 2000; Cryan and Mombereau, 2004).

Most of the reported studies characterizing the hippocampal structural alterations of animals subjected to an experimental model of depression have employed Golgi staining. However, this technique does not allow determining which cytoskeletal components may be involved in the observed changes. The immunocytochemical technique combined with morphometrical analysis presents the advantage of allowing the determination of the specific cytoskeletal proteins responsible for the alterations.

NFs, a major component of the neuronal cytoskeleton, are present in axons and dendrites and play a critical role in axonal and dendritic growth and transport. In dendrites, NFs have also been proposed to participate in plastic structural changes (Kong et al., 1998). Since alterations in the phosphorylation of dendritic NFL during long-term potentiation (Hashimoto et al., 2000a) as well as during long term depression (Hashimoto et al., 2000b) and in the total level of this subunit (Zhang et al., 2002) have been reported, NFL seems to be strongly involved in dendritic plasticity.

In depressed animals, the NFL immunoreactivity decrease occurred without changes either in the area of fibers or in the pattern of NFM and NFH immunostaining. Interestingly, in shocked non-depressed animals, no decrement in NFL immunoreactivity was found. NFL is considered to constitute the core of mature NF, as NFM and NFH need the expression of NFL to successfully assemble. Regarding dendritic structure, it has been observed that the absence or reduction of NFL, as well as the misbalance between NFL on one hand and NFM or NFH expression on the other, may lead to altered dendritic arborization (Kong et al., 1998; Zhang et al., 2002). Thus, it may be speculated that the NFL decrease we observed in the hippocampus of depressed animals is related to the diminution of dendrite shaping described by means of other techniques (McEwen, 1999). Since NFL is also present in axons, these results may as well involve axonal growth diminution due to an adaptive response of hippocampal neurons to the learned helplessness paradigm exposure. In fact, previous results reported by Kuroda and McEwen (1998) could be in accordance with this hypothesis, as they found in chronically stressed animals a decrement in GAP-43, a phosphoprotein associated with axonal growth cones.

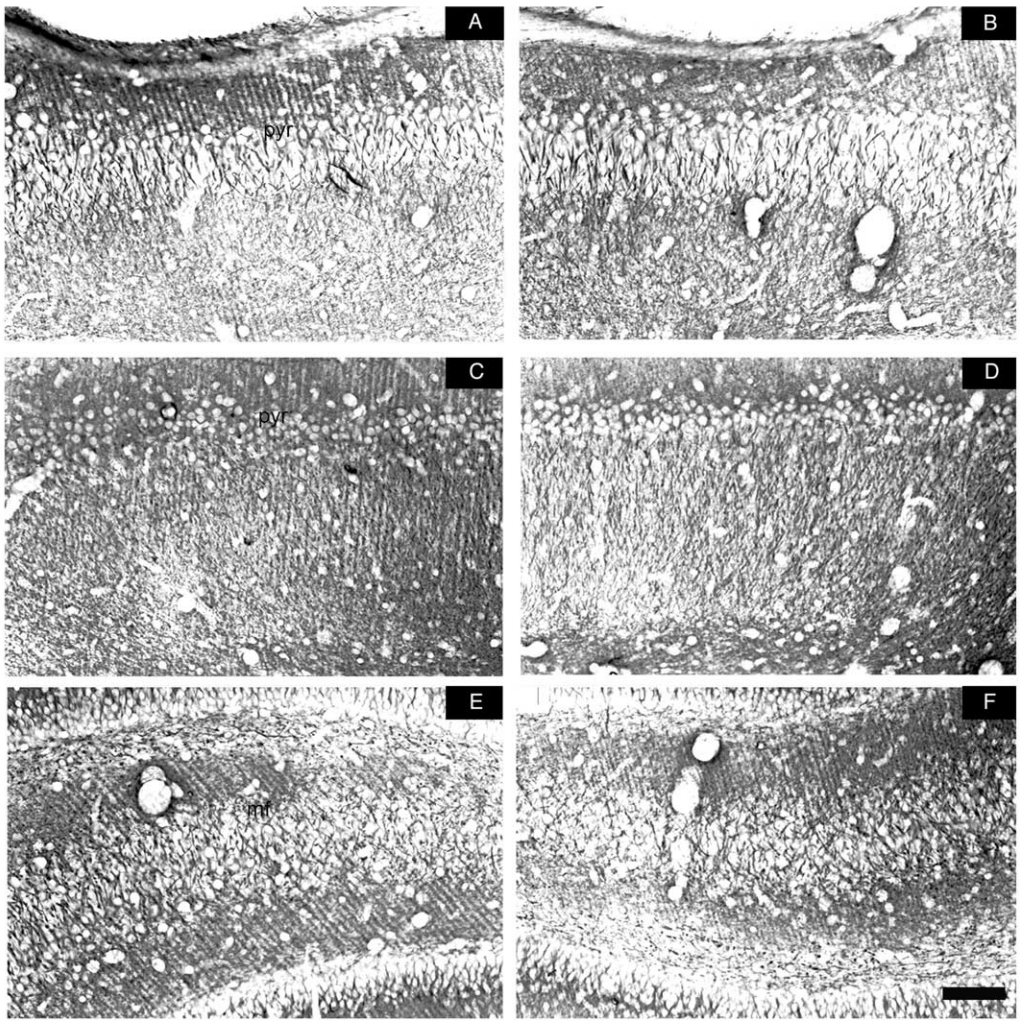

Fig. 9. Photographs show the MAP-2 immunolabeled structures of CA3 (A, B), CA2 (C, D) and DG (E, F) of hippocampus from depressed and control animals. First panel: (A) control group in CA3; (B) depressed group in CA3. Middle panel: (C) control group in CA2; (D) depressed group in CA2. Last panel: (E) control group in DG; (F) depressed group in DG. Scale bar=78 μm. mf, mossy fibers; pyr, pyramidal cell layer.

The underlying cause of the observed reduction in NFL is not known. Since changes in the NFM and NFH morphological structure were not observed, increased soluble or altered assembly forms of these subunits are hardly tenable. It has been previously proposed that a decrement in NFL along with no changes in the other two subunits could be due to the highest vulnerability of NFL to calcium-dependent proteases (Zimmerman and Schaepfer, 1982; Vaidya et al., 2000). Moreover, NFL has been reported to be disassembled by phosphorylation through several kinases which seem to differ from those involved in NFM and NFH phosphorylation (Hashimoto et al., 1998; Veeranna et al., 1998; Al-Chalabi and Miller, 2003). Since the phosphorylation of NFL has been described to occur during plastic events (Hashimoto et al., 2000a,b; Zhang et al., 2002), it may be speculated that the selective lost of NFL observed in depressed animals might be due to an increased phosphorylation of this subunit through a specific intracellular signaling cascade.

Regarding microtubules, in our model, the hippocampal NFL decrease occurs without modifications in the immunostaining for the dendritic marker MAP-2. In agreement with our results, when MAP-2 was analyzed in chronically stressed rats, no significant changes were found (Kuroda and McEwen, 1998). On the other hand, NF alterations have been reported along with (Saatman et al., 1998; Tagliaferro et al., 2002) or without (Vaidya et al., 2000; Zhang et al., 2002) changes in MAP-2. Whether morphological hippocampal alterations progress affecting the other two NF subunits or MAP-2 or whether NFL changes are reversible as it has been described for hippocampal atrophy in chronic stress employing Golgi technique (McEwen, 1999), remain to be explored. However, cautious analysis should be made when comparing chronic stress with other experimental models of depression, since it has been recently described that chronic immobilization stress but not chronic unpredictable stress affects dendritic morphology of CA3 pyramidal neurons (Vyas et al., 2002).

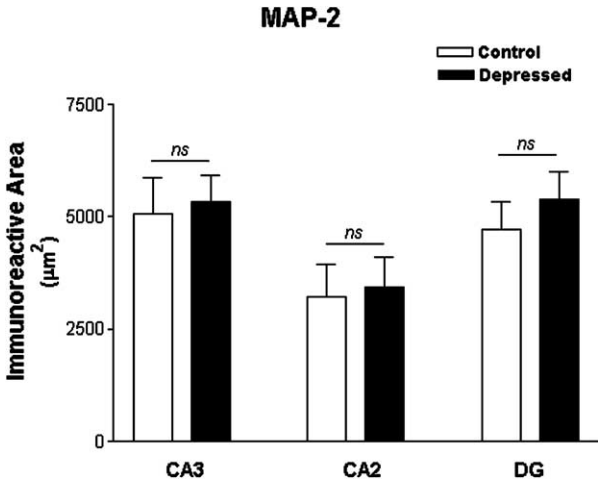

Fig. 10. Area of MAP-2 immunoreactive fibers in depressed and control animals. Twenty-five days after the training session, depressed and control animals were killed, brains immediately removed and proceeded by immunohistochemistry. Data are expressed as area of MAP-2-immunolabeled fibers (μm^2) and represent the mean values ($\pm$S.D.) of three individual experiments for each hippocampal region. Depressed animals failed to show any significant change in MAP-2 immunoreactive area of the hippocampus; ns, non-significant between bars, by Student's *t*-test.

Although the NFL decrement is not paralleled by similar changes in the other two NF subunits, it can be supposed that axonal and dendritic functions might be impaired in depressed animals due to the structural alterations. Interestingly, in a set of experiments separately performed, we observed a long term reduction in the *in vitro* potassium-evoked glutamate release in hippocampal slices of rats exposed to the learned helplessness paradigm (Ferrero et al., 2003). As hippocampus is a subregion of limbic system playing a crucial role in emotion processing, and glutamate is the main neurotransmitter in this region, it is possible to speculate that impaired release of this amino acid could have dramatic behavioral consequences in a model of depression. The application of inescapable shocks is a stressful experience, for which several neurochemical effects have been described. Among others, an increment in extracellular glutamate has been reported (Bagley and Moghaddam, 1997; Venero and Borrell, 1999), which in turn is well known to trigger excitotoxic mechanisms in hippocampal neurons leading to neuronal dysfunction. Mechanisms such as excitotoxicity or increased oxidative stress could likely underlie the NFL reduction.

To sum up, in the present work we demonstrated NFL alterations in the hippocampus of animals subjected to an experimental model of depression. Our results further support the hypothesis that plastic changes occur in the hippocampus of depressed animals and that those changes may be related to the hippocampal impaired function described in this pathology. Since NFL is present in the core of mature NF, it is proposed that hippocampal depression-associated plastic alterations may be due to changes in the dynamics of the NF assembly. A better understanding of the neurobiology of depression may shed light on the mechanisms underlying the action of antidepressant drugs, the recurrence of the symptoms and the resistance to treatment.

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