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Alterations in Heavy and Light Neurofilament Proteins in Hippocampus Following Chronic ECS Administration

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ABSTRACT Chronic administration of electroconvulsive seizures (ECS), one of the most effective treatments for depression, induces sprouting of the mossy fibers in the hippocampus. This sprouting requires chronic ECS administration and appears to occur in the absence of hilar neuronal loss. Dynamic regulation of cytoarchitecture plays a vital role in such profound alterations of neuronal morphology. In particular, alterations in the neurofilament protein subunits have been implicated in neurite sprouting, neuronal regeneration, and growth. The present study was carried out to determine the influence of chronic ECS administration on the neurofilament subunits and other molecular markers of neuronal plasticity. Chronic ECS administration decreases the level of phosphorylated heavy neurofilament subunit (NF-H). In addition, the total level of the light neurofilament subunit (NF-L) but not the medium neurofilament subunit (NF-M) is decreased following chronic ECS treatment. Other cytoskeletal proteins, including actin, microtubule-associated protein (MAP-2), and tau, are not influenced by chronic ECS administration. Expression of the growth-associated protein (F1/GAP-43) also remains unchanged following chronic ECS treatment. The changes observed in neurofilaments may be part of the cytoskeletal remodeling that contributes to the mossy fiber sprouting induced by chronic ECS treatment. **Synapse 35:137–143, 2000.**

INTRODUCTION

Recent evidence demonstrates that chronic administration of electroconvulsive seizures (ECS), one of the most effective treatments for depression, leads to mossy fiber sprouting in the hippocampus (Vaidya et al., 1999). Seizure-induced sprouting is likely to involve synthesis of structural or growth-associated proteins, or reorganization of existing proteins. The focus of this study is to examine the influence of chronic ECS on such structural and growth-associated proteins, in particular the neurofilament proteins and the growth-associated protein F1/GAP-43.

The neurofilament (NF) proteins are critical components in maintaining the structural integrity of neurons (Lazarides, 1980; Nixon and Sihag, 1991). The NFs are composed of three proteins: the light subunit (NF-L; 68 kDa), medium subunit (NF-M; 160 kDa), and the heavy subunit (NF-H; 200 kDa). The NF proteins play an important role in determining neuronal shape and axonal caliber (Hoffmann et al., 1984). NF-H and NF-M are extensively phosphorylated and their phosphorylation state is thought to influence their rate of axonal transport, sensitivity to proteolysis, interactions with other structural proteins, and also axon caliber and stability (Goldstein et al., 1987; Nixon and Sihag, 1991; Greenwood et al., 1993; Archer et al., 1994). Loss of immunoreactivity for phosphorylated NF-H has been demonstrated in regenerating peripheral nervous system (PNS) neurons (Hoffman et al., 1985; Bignami et al., 1986; Dahl and Bignami, 1986) and decreased phosphorylation also renders NF-H more susceptible to calpain-mediated proteolysis (Greenwood et al., 1993).

The protein F1/GAP-43 is also thought to play an important role in axonal growth (Benowitz and Perrone-Bizzozero, 1991; Strittmatter et. al., 1992; Benowitz and Routtenberg, 1997). Sprouting in the CNS has also been associated with increased expression of F1/GAP-43 (Benowitz and Schimdt, 1987; Aigner et al., 1995; McNamara and Routtenberg, 1995). Unlike pyramidal neurons of the hippocampus in adult rats, which

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express F1/GAP-43 at high levels, DG granule cells appear to lack this protein (Meberg and Routtenberg, 1991; Meberg et al., 1996). Kindling-induced seizures have been shown to induce the expression of F1/GAP-43 in granule cells, suggesting that expression of this protein may play a role in sprouting of the mossy fibers of granule cells (Meberg et al., 1993; McNamara and Routtenberg, 1995; Elmér et al., 1996).

The results of the current study demonstrate that chronic ECS treatment leads to alterations in the neurofilament proteins, but not in levels of the other cytoskeletal proteins examined or F1/GAP-43. The dynamic alterations observed in NF proteins may contribute to the structural plasticity of the hippocampus following chronic ECS treatment. The alterations in the neurofilaments induced by chronic ECS treatment are similar to those that result from kainate administration. However, unlike chronic ECS treatment, kainate also alters MAP-2 and F1/GAP-43 expression (McNamara and Routtenberg, 1995; Elmér et al., 1996). These differences suggest that different forms of mossy fiber sprouting occurring in response to kainate, kindling, and chronic ECS may involve distinct patterns of plasticity of cytoskeletal and growth-associated proteins.

MATERIALS AND METHODS

Animal treatment paradigms

Male Sprague-Dawley rats (180–220 gm) (CAMM, Wayne, NJ) were group housed and maintained on a 12-h light/dark cycle with freely available access to food and water. Animals were administered ECS once daily via earclip electrodes (50 mA, 0.3 sec) or sham treatment (earclips were placed on animals but no shock was administered) for 10 consecutive days and were sacrificed 18 h after the last treatment (n = 6/Western blotting; n = 4/in situ hybridization). In addition, animals administered 10 daily ECS or sham treatments were also sacrificed 6 and 12 days following the last treatment (n = 3/group). The effects of an acute ECS were studied by administering a single ECS or sham treatment as described above, and animals were sacrificed 2, 12, and 24 h later (n = 3/group). All animal use procedures were in strict accordance with the guidelines of the National Institutes for the Care and Use of Laboratory Animals and were approved by the Yale Animal Care and Use Committee.

Western blot analysis

Following decapitation, brains were removed rapidly and the hippocampus was dissected and frozen on dry ice. The hippocampus was then homogenized (10 mg wet weight/ml) in 1% SDS and protein levels were determined using the Lowry method (Lowry et al., 1951). Samples (10–40 µg protein) were then subjected to SDS-polyacrylamide gel electrophoresis as described previously (Beitner-Johnson et al., 1992). Final concentrations of samples were adjusted to contain: 50 mM Tris pH 6.7, 4% glycerol, 4% SDS, 2% β-mercaptoethanol, and bromophenol blue as a marker. Samples were boiled for 3 min and loaded onto 6–8% acrylamide / 0.24% bisacrylamide resolving gels. Proteins in gels were then electrophoretically transferred to nitrocellulose; for transferring NF-H and NF-M, 0.25% SDS was used in the transfer buffer to improve transfer efficiency. Following electrophoretic transfer, the nitrocellulose filters were incubated with 2% milk in a buffer containing 10 mM sodium phosphate pH 7.2, 140 mM NaCl and 0.05% Tween-20 (Sigma. St. Louis, MO). The filters were probed with the following antibodies: monoclonal NF-H antibody which recognizes phosphorylation-dependent epitopes on the neurofilament heavy chain (clone NE14; 1:2,000; Sigma; this antibody was one of several clones isolated following immunization of Balb/c mice with a crude neurofilament preparation of porcine spinal cord); monoclonal NF-H antibody which does not distinguish between the phosphorylated and nonphosphorylated forms of NF-H (clone N52; 1:2,000; Sigma); monoclonal NF-M antibody (clone NN18; 1:2,000; Sigma); monoclonal phosphorylated NF-M (SMI 31; 1:2,000; Sternberger Monoclonals, Baltimore, MD); monoclonal NF-L antibody (clone NR4; 1:2,000; Sigma); monoclonal MAP-2 antibody (clone AP-20; 1:5,000; Sigma); polyclonal actin antibody (1:3,000; Sigma). The monoclonal antibody against tau (5E2; 1:1,000), was kindly provided by Dr. Kosik (Harvard, MA). The filters were then incubated with goat antimouse antibody (1:2,000) (for NFs, MAP-2, and Tau blots) or goat antirabbit antibody (1:2,000) (for actin blots) conjugated with horseradish peroxidase (Vector Laboratories, Burlingame, CA). The nitrocellulose filters were developed using the enhanced chemiluminescence system and exposed to Hyperfilm (Amersham, Arlington Hts., IL).

In situ hybridization

In situ hybridization for F1/GAP-43 mRNA was carried out according to a previously described protocol (see Nibuya et al., 1995, 1996; Vaidya et al., 1997). In brief, coronal sections of 14 µm thickness were cut on the cryostat and thaw-mounted onto RNase free Probe-on (+) slides (Fisher, Fair Lawn, NJ). Tissue sections were fixed, acetylated, and dried. Levels of F1/GAP-43 mRNA were examined by probing with a ^{35}S-labeled antisense rat F1/GAP-43 riboprobe, kindly provided by Dr. Routtenberg (Northwestern University, IL). The sections were hybridized with 2×10^6 cpm/section for 18 h at 55°C in hybridization buffer (50% formamide, 0.6 M NaCl, 10 mM Tris, 1× Denhardts solution, 2 mM EDTA, 10 mM DTT, 10% dextran sulfate, 50 µg/ml salmon sperm DNA, and 250 mg/ml tRNA). Following hybridization, sections were washed in 2× SSC (0.15 M NaCl, 0.015 M sodium citrate at pH 7.0) at 25°C and then treated with 20 µg/ml RNase A for

30 min in RNase buffer (0.5 M NaCl, 10 mM Tris, and 1 mM EDTA). The sections were then washed for 10 min in 2× SSC at room temperature and twice for 20 min in 0.2× SSC at 55°C. The sections were dried and exposed to Hyperfilm (Amersham) for 7–14 days. ^{35}S-labeled sense riboprobe for F1/GAP-43 did not yield any significant hybridization (not shown), indicating that the signal observed with F1/GAP-43 antisense riboprobe is specific.

Quantitation and data analysis

Levels of immunoreactivity for various proteins were quantified using the Macintosh-based NIH-Image program, version 1.57. The protein bands were outlined and the optical density measurements were obtained for sham as well as treated animals. Light step standards (Amersham) were used for calibration. Levels of F1/GAP-43 mRNA were also analyzed using the NIH-Image 1.57 program. The regions that were analyzed for in situ hybridization were the DG granule cell layer, hilar region, and the CA3 pyramidal cell layers. These regions were analyzed by outlining the area of interest; an equivalent area was outlined for each sample. For each animal, the optical density measurements from both sides of three individual sections were analyzed from which the mean was calculated. To correct for nonlinearity ^{14}C step standards were used for calibration. Results were then subjected to statistical analysis. Experiments were analyzed for differences using the unpaired Student's *t*-test, with significance determined at $P < 0.05$.

RESULTS

Chronic ECS-induced alterations in NF-H and NF-L

Administration of ten daily ECS treatments decreased immunoreactivity for the phosphorylated NF-H in the hippocampus by approximately 30–40% (Fig. 1). Phosphorylated NF-H was determined using a monoclonal antibody (NE-14) generated against phosphorylation-dependent epitopes. NF-H has multiple potential phosphorylation sites, most of which are located in the carboxy terminus. The exact phosphorylation epitopes recognized by NE-14 have not been determined. In contrast, another monoclonal antibody (N52, which was generated against phosphorylation-independent epitopes and therefore recognizes both phosphorylated and nonphosphorylated NF-H), indicated that levels of immunoreactivity for the total amount of NF-H were unchanged (Fig. 1). This suggests that the decrease in immunoreactivity detected by the NE-14 antibody is due to dephosphorylation of NF-H in response to chronic ECS treatment and not a result of proteolytic breakdown.

Chronic ECS administration also resulted in a reduction in total levels of NF-L (Fig. 2). Levels of NF-L in the hippocampus were reduced significantly by about 20% as compared to sham. In contrast, chronic ECS administration did not significantly influence either total or phosphorylated levels of the medium subunit, NF-M (Fig. 2).

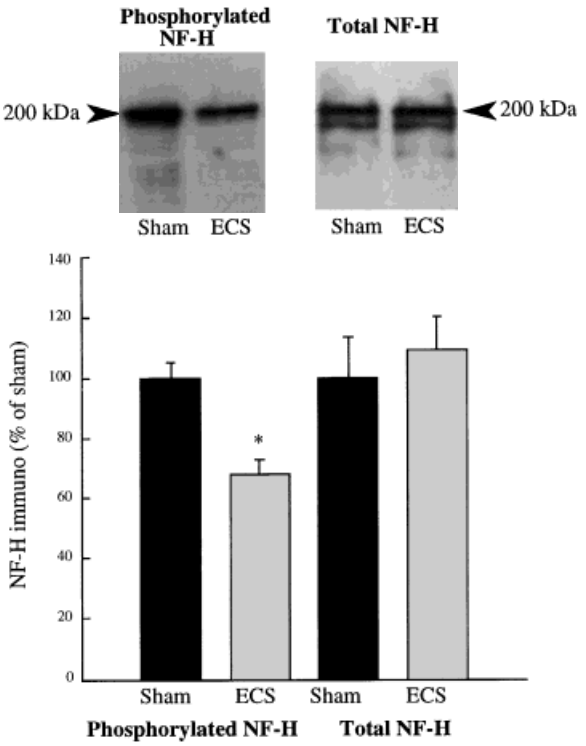

Fig. 1. Influence of chronic ECS treatment on the heavy neurofilament subunit (NF-H). Chronic ECS treatment decreased levels of immunoreactivity for the phosphorylated form of the heavy neurofilament subunit (NF-H) in the hippocampus. The NE14 antibody that recognizes a phosphorylation-dependent epitope was used to detect levels of phosphorylated NF-H. There appears to be no change in total NF-H as detected by the antibody, N52, that recognizes phosphorylation-independent epitopes. Representative immunoblots labeled with NE14 and N52 are shown. Results are expressed as percent of sham and are the mean ± SEM (n = 6). *$P < 0.05$ as compared to sham (Student's *t*-test).

The time course for ECS regulation of phosphorylated NF-H and total NF-L was also examined. The downregulation of both phosphorylated NF-H and NF-L was not observed after either 1 or 5 daily ECS treatments (Fig. 3), demonstrating that this effect is dependent on chronic treatment. In addition, these effects were relatively short-lived in that they were not observed at either 6 or 12 days after the last ECS treatment (Fig. 3).

Influence of chronic ECS treatment on other cytoskeletal proteins: actin, MAP-2, and tau

Immunoblots labeled with antibodies against actin and the microtubule-associated proteins MAP-2 and tau indicated that chronic ECS administration did not significantly influence the levels of these cytoskeletal

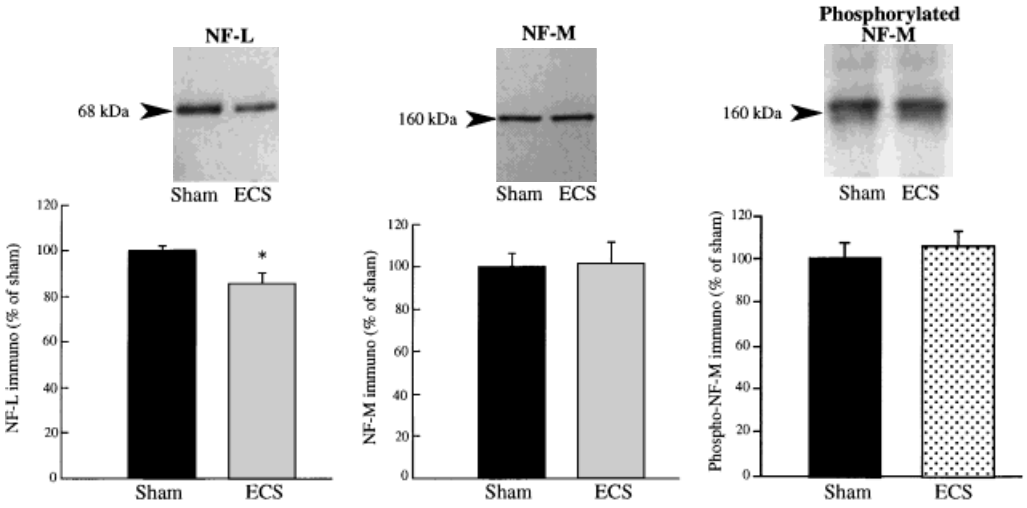

Fig. 2. Influence of chronic ECS treatment on levels of NF-L and NF-M. Chronic ECS treatment causes a decrease in levels of immunoreactivity of the light subunit of the neurofilament proteins (NF-L) in the hippocampus, but does not significantly influence the medium subunit (NF-M) of the neurofilaments. Levels of phosphorylated NF-M were also not influenced by chronic ECS treatment. The results are expressed as percent of sham and are the mean ± SEM (n = 6). *$P <$ 0.05 as compared to sham (Student's *t*-test).

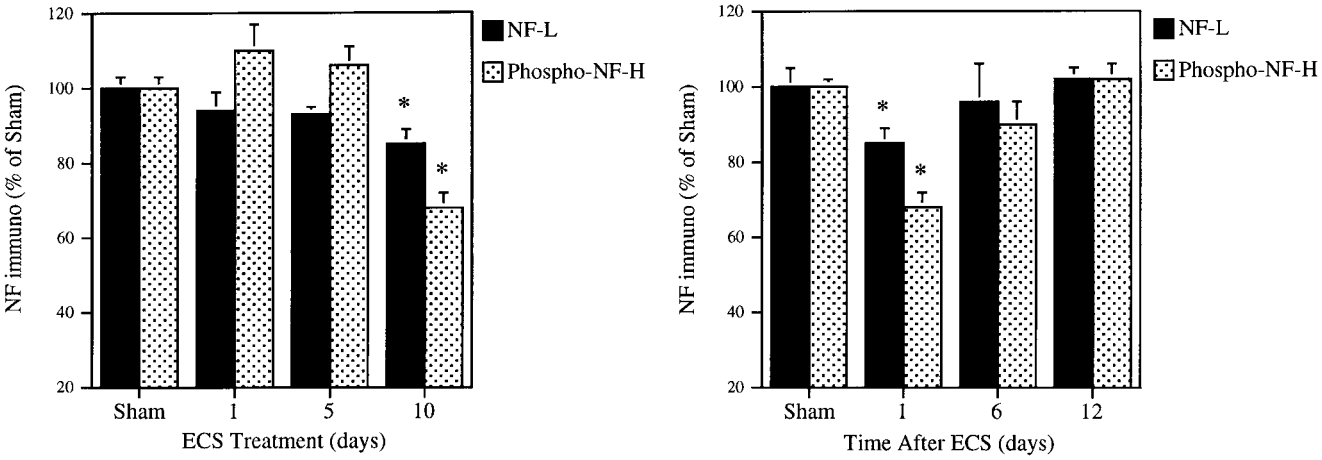

Fig. 3. Time course for ECS regulation of phosphorylated NF-H and total NF-L. Rats received sham or ECS treatment for the time indicated and levels of phosphorylated NF-H and total NF-L were determined by immunoblot analysis. The time course after the last of daily ECS treatments was also examined, including 1, 6, and 12 days. The results are presented as percent of control and are the mean ± SEM (n = 5). *$P <$ 0.05 compared to control (ANOVA and Fisher's test).

proteins (Fig. 4). The actin and MAP-2 antibodies detect the total levels of proteins and do not distinguish between phosphorylated and dephosphorylated forms. Levels of tau were determined with the monoclonal antibody 5-E2. This antibody was raised against fetal tau, which is similar to the aberrant form of tau in paired helical filaments seen in Alzheimer's disease. This form of tau is also increased in response to kainate treatment. Our rationale here was to determine if chronic ECS induces this aberrant form of tau. The antibody recognizes several different bands, none of which were significantly influenced by chronic ECS treatment.

Influence of chronic ECS treatment on expression of F1/GAP-43

Chronic ECS administration for 10 days did not significantly alter the levels of F1/GAP-43 protein, as detected by immunoblotting, within the hippocampus (Fig. 5). To determine if there was a regional regulation of F1/GAP-43 mRNA, in situ hybridization analysis was utilized. The level of F1/GAP-43 mRNA in the CA3 pyramidal cell layer was relatively high in sham animals. In contrast, the expression of F1/GAP-43 mRNA within dentate granule cells was very low, in agreement with previous reports (Meberg and Routtenberg, 1991; Meberg et al., 1996). The influence of chronic ECS on

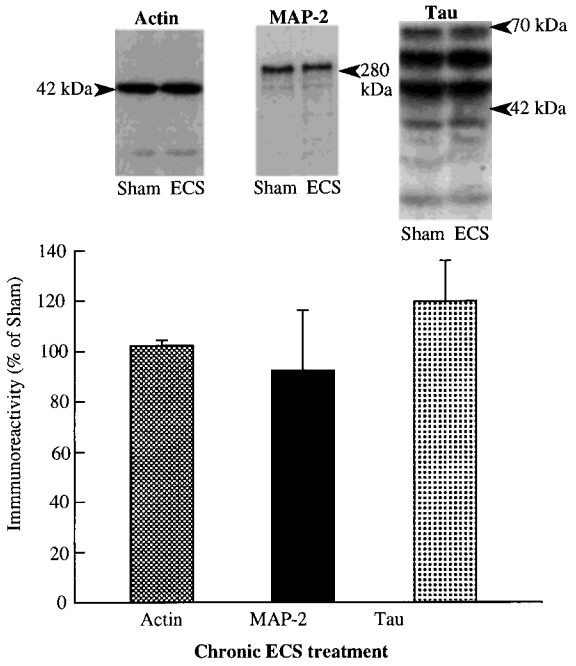

Fig. 4. Influence of chronic ECS treatment on the cytoskeletal proteins actin, MAP-2, and tau. Chronic ECS treatment did not significantly influence the levels of these proteins. Representative immunoblots labeled with these antibodies are shown. The antibody used to detect tau, 5-E2, preferentially recognizes phosphorylated tau. The several bands detected by this antibody may represent phosphorylated and hyperphosphorylated tau. Results are expressed as percent of sham and are the mean ± SEM (n = 6).

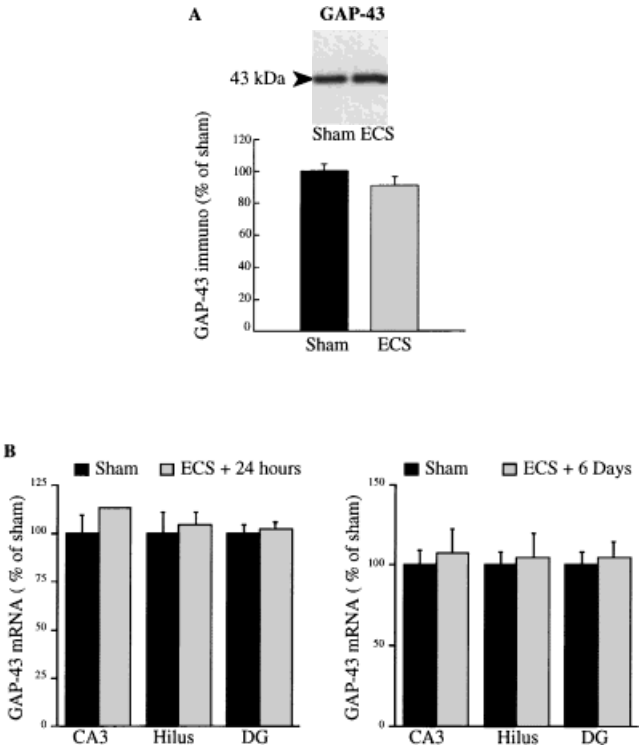

Fig. 5. Influence of chronic ECS treatment on expression of F1/GAP-43 protein and mRNA. **A**: Chronic ECS treatment does not alter levels of F1/GAP-43 immunoreactivity in the hippocampus as determined by immunoblotting. Results are expressed as percent of sham and are the mean ± SEM (n = 6). A representative immunoblot labeled with monoclonal antibody against F1/GAP-43 is shown. **B**: Levels of F1/GAP-43 mRNA 24 h and 6 days following the last of 10 daily ECS treatments did not differ significantly from sham-treated animals at the same time points. Levels of F1/GAP-43 mRNA were analyzed in the CA3 pyramidal layer, hilar region, and dentate granule cell layer of the hippocampus. Results are expressed as percent of sham and are the mean ± SEM (immunoblots: n = 6; in situ hybridization, n = 3).

levels of F1/GAP-43 mRNA was determined 24 h following the administration of the last of 10 daily ECS treatments. In addition, levels of F1/GAP-43 mRNA were also determined 6 days following the last ECS administration. This time point correlates with the beginning of ECS-induced mossy fiber sprouting. The levels of F1/GAP-43 mRNA of ECS-treated animals were not significantly different from those of sham-treated animals at either of the time points (Fig. 5). The influence of a single, acute ECS on levels of F1/GAP-43 was also determined. Levels of F1/GAP-43 mRNA in ECS-treated animals did not differ significantly from sham-treated animals at either 12 or 24 h following the seizure (ECS + 12 h: CA3, 100 ± 13; hilus, 106 ± 10; DG, 104 ± 7; ECS + 24 h: CA, 109 ± 18; hilus, 117 ± 25; DG, 111 ± 8; results are expressed as percent of sham and are the mean ± SEM, n = 3). The time points chosen in this study were based on previous studies that demonstrated regulation of F1/GAP-43 mRNA at these time points following kainate and kindling administration (Meberg et al., 1993, 1996).

DISCUSSION

The results of the present study demonstrate that chronic ECS treatment leads to alterations in hippocampus of neurofilament proteins, which serve as critical components of neuronal cytoarchitecture. Chronic ECS administration leads to a decrease in levels of phosphorylated NF-H, without regulation of the total amount of NF-H protein. This indicates that the reduced immunoreactivity for the phosphorylated NF-H results primarily from dephosphorylation rather than a proteolytic breakdown of the heavy subunit of neurofilaments. However, decreases in phosphorylated NF-H immunoreactivity through proteolytic degradation of phosphorylation-dependent epitopes cannot be completely ruled out. Neuronal depolarization has also been shown to induce dephosphorylation of NF-H (Mata et al., 1997), and it is possible that the ECS-induced depolarization could also induce dephosphorylation via activation of phosphatases. However, a single ECS treatment did not decrease phosphorylated NF-H immunoreactivity, suggesting that a long-term adaptive mechanism may be involved. In addition to altering the heavy neurofilament subunit, chronic ECS administration also leads to a decrease in the levels of the light neurofilament subunit, NF-L. This decrease is probably a result of

proteolytic breakdown of this subunit. NF-L is the most vulnerable of the neurofilament subunits to calcium-dependent proteases (Zimmerman and Schaepfer, 1982).

Neurofilaments are composed of NF-H, NF-M and NF-L subunits each of which have a conserved α-helical "rod" domain, an amino terminal "head" domain, and a carboxy-terminal "tail" domain. The rod domains contribute to the formation of the neurofilament core, the carboxy terminal tail domains project outward from the filament core. NF-H and NF-M have particularly long carboxy terminal tail domains which undergo extensive phosphorylation (Jones and Williams, 1982; Lee et al., 1988; Nixon and Sihag, 1991). In addition, the NF-H and NF-M subunits are phosphorylated within the amino terminal head domain (Sihag and Nixon, 1990; Nixon and Sihag, 1991). Phosphorylation of the carboxy terminal tail domain leads to extension of the tail further away from the filament core, contributing to the axonal caliber (Nixon and Sihag, 1991).

Developmental and regeneration studies indicate that NF-H, especially the phosphorylated form, stabilizes mature neuronal structure, enhances interactions with other cytoskeletal proteins, and retards axonal transport of the neurofilaments (Nixon and Sihag, 1991; Archer et al., 1994). The appearance of phosphorylated NF-H is considered a marker of mature neurons (Dahl and Bignami, 1986). Neurofilament proteins in developing neurons are composed primarily of NF-L and NF-M (Carden et al., 1987). NF-H subunits appear later in development and phosphorylation occurs even later (Carden et al., 1987; Dahl et al., 1986; Foster et al., 1987). Levels of phosphorylated NF-H and total NF-H have been shown to be decreased in regenerating PNS neurons appearing to recapitulate the developmental pattern of neurofilament protein expression (Hoffmann et al., 1985; Bignami et al., 1986; Dahl et al., 1989). Kainate administration also leads to a similar decrease in phosphorylation of the NF-H subunit (Wang et al., 1992, 1994; Yang et al., 1995, 1996).

Based on these findings, it is possible that downregulation of phosphorylated NF-H could contribute to the structural rearrangements that are necessary for mossy fiber sprouting, as well as other forms of synaptic remodeling that have been observed in the hippocampus following chronic ECS treatment (Vaidya et al., 1999). The time course for the reappearance of phosphorylated NF-H after the last ECS treatment is similar to that observed after kainate-induce sprouting; that is, levels of phosphorylated NF-H return to control levels at time points correlated with sprouting of the dentate gyrus granule cells (Yang et al., 1996; Vaidya et al., 1999). These changes in the neurofilament proteins may be critical components that contribute to the structural reorganization of the granule cell mossy fibers following both kainate and chronic ECS administration. Alterations of NF-L could also play a role in sprouting, although the functional consequences of altered levels of this neurofilament are less clear.

Chronic ECS treatment does not appear to influence the levels of other cytoskeletal proteins, such as actin, MAP-2, and tau. This indicates that chronic ECS exerts specific influences on the neurofilaments subunits while not altering other major components of neuronal cytoskeleton. The antibody used for analysis of tau was raised against fetal tau, which is similar to the aberrant form of tau in paired helical filaments seen in Alzheimer's disease. This form of tau is also increased in response to kainate treatment in vivo (Elliot et al., 1993) and in response to glutamate and calcium ionophore exposure in cultured cells (Mattson, 1990). Because increased levels of this form of tau are associated with these conditions, it is notable that a similar increase of this form is not observed in response to a therapeutic intervention used for the treatment of refractory depression.

In addition, chronic ECS treatment did not influence the expression of F1/GAP-43. F1/GAP-43 is an important component of neuronal growth cones during development and is induced during regeneration in the PNS (Hoffmann, 1989; Benowitz and Perrone-Bizzozero, 1991; Strittmatter et. al., 1992; Benowitz and Routtenberg, 1997). Certain regions of the CNS associated with plasticity continue to express high levels of the protein even in the adult animal (Meberg and Routtenberg, 1991; Kruger et al., 1993). Kainate and kindling administration, both of which induce mossy fiber sprouting in the hippocampus, have been shown to induce F1/GAP-43 mRNA expression in DG granule cells (McNamara and Routtenberg, 1995; Elmér et al., 1996; Bendotti et al., 1997). DG granule cells normally express very low levels of F1/GAP-43 (Meberg and Routtenberg, 1991). Overexpression of F1/GAP-43 has been associated with mossy fiber sprouting in transgenic mice (Aigner et al., 1995) and mice lacking this protein show abnormal neuronal pathfinding (Strittmatter et al., 1995). Based on these findings, it is somewhat surprising that ECS treatment does not influence the expression of F1/GAP-43. The reason for this difference is not clear, but may be related to the severity of the seizure paradigm used: kainate and kindling-induced seizure models produce a much more profound level of sprouting than observed with ECS treatment.

The changes observed in neurofilaments may be part of the dynamic cytoskeletal remodeling that is involved in mossy fiber sprouting caused by chronic ECS administration. The alterations of cytoskeletal and growth associated proteins induced by chronic ECS are distinct from those resulting due to kainate administration. The sprouting that results due to excitotoxin-induced seizures is also different from that caused by chronic ECS treatment. Chronic ECS-induced mossy fiber sprouting occurs in the absence of the hilar neuronal loss which is seen following kainate treatment. It is

possible that the mossy fiber sprouting which results from these different treatments is distinct and may thereby involve unique changes in cytoskeletal and growth-associated proteins.

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