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FATIGUE REVERSIBLY REDUCED CORTICAL AND HIPPOCAMPAL DENDRITIC SPINES CONCURRENT WITH COMPROMISE OF MOTOR ENDURANCE AND SPATIAL MEMORY

J.-R. CHEN,[a] T.-J. WANG,[b] H.-Y. HUANG,[a] L.-J. CHEN,[c] Y.-S. HUANG,[a] Y.-J. WANG[d]* AND G.-F. TSENG[d]*

[a]*Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan*

[b]*Department of Nursing, National Taichung Nursing College, Taichung, Taiwan*

[c]*Institute of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan*

[d]*Department of Anatomy, College of Medicine, Tzu Chi University, No. 701, Section 3, Jhongyang Road, Hualien, Taiwan*

Abstract—Fatigue could be induced following forced exercise, sickness, heat stroke or sleep disturbance and impaired brain-related functions such as concentration, attention and memory. Here we investigated whether fatigue altered the dendrites of central neurons. Central fatigue was induced by housing rats in cage with 1.5-cm deep water for 1–5 days. Three days of sleep deprivation seriously compromised rats' performance in weight-loaded forced swimming and spatial learning tests, and 5 days of treatment worsened it further. Combinations of intracellular dye injection and three-dimensional analysis revealed that dendritic spines on retrograde tracer-identified corticospinal neurons and Cornu Ammonis (CA)1 and CA3 pyramidal neurons were significantly reduced while the shape or length of the dendritic arbors was not altered. Three days of rest restored the spine loss and the degraded spatial learning and weight-loaded forced swimming performances to control levels. In conclusion, although we could not rule out additional non-hypothalamic–pituitary–adrenal stress, the apparent fatigue induced following a few days of sleep deprivation could change brain structurally and functionally and the effects were reversible with a few days of rest.

Key words: corticospinal, dendrite, fatigue, hippocampus, plasticity, PSD-95.

Modern life stresses many of us and results in fatigue. Fatigue could be induced by infectious, immunological, and neuroendocrine disorders, sleep disturbances and psychological conditions but the etiology remains unclear. Fatigue often results in symptoms related to CNS function (Afari and Buchwald, 2003) including reduced activities and muscle endurance and impaired coordination, concentration, attention, learning and memory. In human, chronically fatigued patients could display persistent or relapsing unexplainable fatigue lasted for over 6 months (Fukuda et al., 1994). There have been studies exploring the effect of central fatigue on brain structure (e.g. Buchwald et al., 1992; Natelson et al., 1993) and function (e.g. MacHale et al., 2000; Schmaling et al., 2003) and dramatic structural changes such as a substantial and consistent reduction of the gray matter volume of brain have been reported (Lange et al., 1999; Okada et al., 2004; de Lange et al., 2004, 2005). This suggests that fatigue could alter neurons structurally and is in line with the understanding that dendrites, the receiving part of neurons, are dynamic structures that can change in response to environment or stimuli (McEwen, 1994; Tseng and Prince, 1996; Woolley et al., 1997; Wang et al., 2002; Chen et al., 2003, 2004; Deller et al., 2006). In the rat, extended wakefulness reduced cortical concentration of a novel dendritic protein dendrin by 42% (Neuner-Jehle et al., 1996). This also suggests that dendrites could be modulated following the prolonged wakefulness associated with the induction of central fatigue and prompted us to investigate whether and how fatigue alters the dendritic structures of central neurons. To explore this, we chose the recently described rat sleep disturbance–induced central fatigue model in which the extent of the fatigue could be evaluated with weight-loaded forced swimming test (Tanaka et al., 2003). The dendritic structures, including dendritic arbors and spines of corticospinal and hippocampal Cornu Ammonis (CA)1 and CA3 pyramidal neurons were investigated for they are known to be associated with motor coordination and learning and recall of spatial memory, respectively. Neurons were filled with intracellular dye in partially fixed brain slices under visual guidance of a fluorescence microscope. Corticospinal neurons were prelabeled *in vivo* with retrograde tracer so that they could be filled selectively in isolation. The injected fluorescence dye was converted immunohistochemically into nonfading material following resection of the injected slice into sections. Neurons were then reconstructed three-dimensionally with PC-based software on a microscope fitted with a motorized stage and integrated focusing control and analyzed accordingly for further analysis.

EXPERIMENTAL PROCEDURES

Animals

Thirty male CD®3(SD) IGS rats (BioLasco, Ilan, Taiwan), 250–350 g body weight were tested. Twenty-five of them were divided into control (n=5), fatigue (n=15) and fatigue and rest groups (n=5). The fatigue group was further subdivided into three sets for different durations of fatigue induction, 1 day (n=3), 3 days (n=3)

*Corresponding author. Tel: +886-3-8564641; fax: +886-3-8564641.
E-mail addresses: guofang@mail.tcu.edu.tw (G.-F. Tseng), chris@mail.tcu.edu.tw (Y.-J. Wang).

Abbreviations: CA, cornu ammonis; CFS, chronic fatigue syndrome; DAB, 3,3′-diaminobenzidine tetrahydrochloride; LTP, long-term potentiation; LY, Lucifer Yellow; PB, phosphate buffer; PBS, phosphate-buffered saline; ROD, relative optical density; ROS, reactive oxygen species; TMS, transcranial magnetic stimulation.

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and 5 days (n=9). Six of the 5-day fatigue animals were processed for intracellular dye injection. Three rats each of the 1, 3, and 5 day fatigue-induction group were processed for Western blot analysis of protein expression in their sensorimotor cortex. The fatigue and rest groups were first subjected to 5 days of fatigue treatment followed by 3 days of rest. Since the above animals were sacrificed at separate time points, an additional five rats were subjected to fatigue induction and subsequent rest so that behavioral tests could be collected serially before fatigue induction, 3 and 5 days after fatigue induction and then following 3 days of rest. We followed the method of Tanaka et al. (2003) with slight modification to induce fatigue in rats. To induce fatigue, rats were individually housed in cage filled with water (24±1 °C) to a height of 1.5–2 cm. Animals were housed in a temperature (24±1 °C), humidity (60%±5%), and light (light on at 06:00 h and off at 18:00 h)-controlled environment. Food and water were available *ad libitum*. Animal care and experiments were approved and conformed to guidelines of the Animal Care and Use Committee of National Chung Hsing University and all efforts were taken to minimize the number of animals used and the suffering during experiments.

Retrograde labeling of corticospinal neurons

Animals were anesthetized with 7% chloral hydrate (0.45 ml/kg) injected i.p. and mounted on a stereotaxic device. A midline incision was made over the spinous processes of the C3–4 vertebrae. The lamina of the C4 vertebra was then removed and the spinal dura mater incised. A total of 1.5 μl of the retrograde tracer fluorogold (FG, 2% in distilled water; Fluorochrome, Denver, CO, USA) was injected into the dorsal column bilaterally with a 10-μl Hamilton microsyringe (Reno, NV, USA) connected through a cannula to an infusion pump (KD Scientific, Holliston, MA, USA). Each injection was performed over a 10-min period and the pipette was left in place for 2 min following each injection and then slowly withdrawn over 4 min. Animals were allowed to survive for at least two weeks before subjecting them to fatigue induction.

Behavioral test

We used weight-loaded forced swimming test (Tanaka et al., 2003) in which rats swam with a load of steel rings attached to their tails that weighed approximately 10% of their body weight to assess the extent of the fatigue and the Morris water maze test (Moser et al., 1995; Morris, 1984; Mizunoya et al., 2004; Gibertini et al., 1995) to evaluate the effect of fatigue on memory. For the weight-loaded forced swimming test, the swimming time from the beginning of swimming with weight until the point at which rats could not return to water surface 10 s after sinking was measured. The time, to a maximum of 10 min, was recorded in two trials, separated by at least 30 min, for each rat. The water maze was a circular pool (200 cm in diameter, 60 cm in height) located in a well lit room and filled with water (50 cm height, 23 °C) and contained a platform approximately 2–3 cm below water surface. Distal visual cues arrayed around the room were available for the rats to learn the location of the hidden platform. Rats received six training runs in the water maze before the fatigue experiment to confirm that all rats could find the platform within 30 s and the time and the swimming path for each rat to find the hidden platform were recorded. The performance of each rat following treatment was similarly recorded and normalized to that before fatigue induction and then analyzed. A 30-minute interval was allowed between weight-loaded forced swimming test and Morris water maze. For the 25 rats sacrificed at designated time points, weight-loaded endurance tests were conducted before the Morris water maze. For the five rats for serial behavioral tests, we ran the Morris water maze test before the weight-loaded endurance test at each time point to avoid the possibility that the weight-loaded endurance test might introduce additional strain to the animals to affect the outcome of spatial memory test.

Intracellular dye injection and subsequent immunoconversion of the injected dye

To prepare tissue for intracellular dye injection, rats were deeply anesthetized with chloral hydrate again and perfused with 50 ml of lukewarm saline, followed by fixative containing 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3, at room temperature for 30 min. The brain was then carefully removed and the area of interest sectioned with a vibratome into 300-μm-thick coronal slices immediately. In the present study, we used Lucifer Yellow (LY, Sigma, St. Louis, MO, USA) as the intracellular dye to reveal neuronal dendritic arbors. Slices were pre-treated with 0.1 M PB containing 10^{-7} M 4′,6-diamidino-2-phenyl-indole (DAPI; Sigma) for 30 min to make cell nuclei fluoresce blue under the same filter set that visualized LY as yellow. This enabled us to select individual cells of specific cortical layers for dye injection. To inject neurons, a slice was placed in a dish covered with a thin layer of 0.1 M PB on the stage of a fixed-stage, epifluorescence microscope (BX51, Olympus, Tokyo, Japan). An intracellular micropipette filled with 4% LY in water mounted on a three-axial hydraulic micromanipulator (Narishige, Tokyo, Japan) and a long-working distance objective lens (20×) was used to facilitate the selection of corticospinal and CA1 and CA3 hippocampal neurons for dye injection. Negative current generated by an intracellular amplifier (Axoclamp-IIB, Axon, Foster City, CA, USA) was used to inject the LY till all terminal dendrites fluoresced brightly under the scope. Several well-separated neurons could be injected in the area of interest in each slice. Following injection, the slice was removed, rinsed in 0.1 M PB and postfixed in 4% paraformaldehyde in 0.1 M PB for 3 days. They were then rinsed thoroughly in 0.1 M PB, cryoprotected, and carefully sectioned into 60-μm-thick serial sections with a Leica cryostat (Chen et al., 2003, 2004; Wang et al., 2009).

To convert the intracellular dye LY into nonfading material, sections were first preincubated in 1% H_2O_2 in PB for 30–60 min to remove endogenous peroxidase activity. They were then rinsed in phosphate-buffered saline (PBS) three times and incubated for an hour in PBS containing 2% bovine serum albumin and 1% Triton X-100. Sections were then treated in solution containing biotinylated rabbit anti-LY (1:200; Molecular Probes, Eugene, OR, USA) in PBS for 18 h at 4 °C. Following rinses in PBS, sections were incubated with standard avidin–biotin HRP reagent (Vector, Burlingame, CA, USA) for 3 hours at room temperature. They were then reacted at room temperature with a solution containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.01% H_2O_2 in 0.05 M Tris buffer. Reacted sections were mounted onto slides, processed and coverslipped with Permount (Fisher, Fair Lawn, NJ, USA).

Western blotting of PSD-95

To determine whether changes of the densities of dendritic spines represent alterations of functional excitatory connections, we measured and compared the amount of PSD-95, a glutamatergic postsynaptic marker (Furuyashiki et al., 1999) in the primary sensorimotor cortex of control and fatigue rats following protocol described earlier (Chen et al., 2009). Briefly, the cortex of interest was rapidly dissected and homogenized immediately at 4 °C in 25 mM Hepes (pH 7.4) containing 0.3 M sucrose, protease inhibitor cocktail, 50 mM sodium vanadate (to inhibit phosphatases) and 0.1 M EDTA. Following centrifugation at 600×g for 15 min to remove unbroken cells and nuclei, the postnuclear supernatant was centrifuged at 21,000×g to obtain the postmitochondrial supernatant fraction. Protein concentrations in this fraction were determined with Bio-Rad reagents (Hercules, CA, USA). Twenty micrograms of protein was separated on a 15% acrylamide gel