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Upregulation of Myo6 expression after traumatic stress in mouse hippocampus

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Abstract

Traumatic stress has been believed to result in a variety of unusual alterations of the integrity and the functionality in the hippocampus. In this study, we searched for genes responsive to traumatic stress in the mouse hippocampus to elucidate the underlying mechanisms. Adult male mice were subjected to water-immersion restraint stress (WIRS) for 3 h as an extremely stressful experience, followed by dissection of the hippocampus and subsequent extraction of RNA for differential display polymerase chain reaction (PCR) analysis. The actin-based molecular motor protein myosin VI (Myo6) was identified as a gene markedly upregulated by traumatic stress in the mouse hippocampus 24 h after WIRS. Real-time PCR and Western blotting analyses clearly revealed a significant increase in the expression of both mRNA and corresponding protein for Myo6 in the hippocampus within 24 h after WIRS, while WIRS failed to significantly affect the expression of Myo6 protein in the cerebral cortex, cerebellum and olfactory bulb. Immunohistochemistry analysis revealed that Myo6 protein was ubiquitously expressed throughout the mouse brain, with an extremely high level in the olfactory bulb. These results suggest that Myo6 may be selectively and rapidly upregulated to play a hitherto unidentified role in the maintenance of the integrity and functionality in the hippocampus after traumatic stress.

Keywords: Traumatic stress; Hippocampus; Myosin VI; Differential display

Psychiatric disorders are a prevalent form of mental illness, while posttraumatic stress disorder (PTSD) is one of the common mental illnesses with a morbid risk of about 7% of the general population [4]. A considerably high percentage is reported for the rate of exposure to at least one serious traumatic stressful event in a lifetime, such as 61% for men and 51% for women [10]. On the basis of the aforementioned experience rate, the prevailing proposal is that 20–30% people will eventually develop PTSD within these populations exposed to traumatic stress once [2].

Exposure to an extremely deathly threat would result in abnormalities of the regulation mechanisms for the intricately integrated functional cascades required for maintaining homeostasis. For example, exposure to a stressful environment is highly associated with the occurrence of drastic structural changes in the hippocampus [13,17]. Stress is responsible for the deficits in working memory tasks and the inhibition of long-term potentiation relevant to the *N*-methyl-D-aspartate (NMDA) receptor activity in the hippocampus, which is believed to be a model of learning and memory at the molecular level [5,6]. These hippocampal damages are at least in part mediated through the disruption of cellular metabolism, leading to an increased release of endogenous excitatory amino acids [12,16]. However, no particular genes are conclusively identified as one of causative factors for the abnormalities of hippocampal structures after a traumatic stress to date.

Although water-immersion restraint stress (WIRS) is originally developed as a model of the acute gastric mucosal injury

Abbreviations: COX7a2l, cytochrome c oxidase subunit VIIa polypeptide 2-like; DD-PCR, differential display-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NMDA, *N*-methyl-D-aspartate; OB, olfactory bulb; PA, paraformaldehyde; PBS, phosphate-buffered saline; PTSD, posttraumatic stress disorder; WIRS, water-immersion restraint stress.

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[18], we have previously shown that WIRS leads to significant alterations of endogenous levels of both glutamate and GABA in particular discrete rat brain structures [21]. In this study, we used differential display-polymerase chain reaction (DD-PCR) to search for genes responsive to abnormalities in the hippocampus of mice exposed to WIRS as a traumatic stress.

Antibodies against β-tubulin, myosin VI (Myo6), biotinylated IgG and horseradish peroxidase-conjugated IgG were supplied by Sigma (St. Louis, MO, USA), Santa Cruz (Santa Cruz, CA, USA), Vector Labs (Burlingame, CA, USA) and GE Healthcare (Buckinghamshire, UK), respectively.

The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. Male Std-ddY mice (Japan SLC Inc., Shizuoka, Japan), weighing 30–35 g and aged 6 weeks at the beginning of the experiments, were bred under standard animal housing conditions at 23 ± 1 °C with a humidity of 55% and a light/dark cycle of 12 h, with free access to food and water. Animals starved overnight were subjected to restraint stress in a metallic cage immersed in water at 25 °C up to the individual clavicle for 3 h as described previously [21]. Control animals were similarly fasted, followed by removal from their home cages and subsequent placement in new breeding cages for 3 h. The experimental groups were chosen by means of a completely randomized design.

Total RNA was extracted from the mouse hippocampus 24 h after WIRS. After treating with DNase I to eliminate contaminated chromosomal DNA, DD-PCR was performed using fluorescence differential display kit with 24 upstream and 9 downstream random primers as described previously [9]. The DD-PCR products were electrophoresed on denaturing urea-6% polyacrylamide gel. Differentially expressed bands were excised and re-amplified by PCR using the corresponding primer sets. The PCR products from the second amplification were run on a 1% agarose gel. Differentially expressed cDNAs were again excised, and amplified by PCR using the corresponding primer sets. The third PCR product was purified, followed by the cloning with pT7 Blue vector (Novagen, Darmstadt, Germany). Differential DNA sequence similarity was searched with the BLAST algorithm using the public database.

Total RNA was extracted from the mouse hippocampus, followed by the synthesis of cDNA using ExScript RT reagent kit with 25 pmol Oligo-(dT) primers and subsequent real-time PCR analysis as described previously [9]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. Primers are designed for mouse cytochrome c oxidase subunit VIIa polypeptide 2-like (COX7a2l) (5′-AGAAGTTGGCTGGAGCTTGG-3′ and 5′-AGGTCAGTTTGGTTGGTGTGG-3′), Myo6 (5′-GATGGAGCTGCACCCTGACA-3′ and 5′-GCTCTCAATGGCGCTCTGAAG-3′) and GAPDH (5′-AAATGGTGAAGGTCGGTGTG-3′ and 5′-TGAAGGGGTCGTTGATGG-3′).

Mouse brains were homogenized in lysis buffer (20 mM Tris–HCl [pH 7.5], 1 mM EDTA, 1 mM EGTA, 1 μg/ml of various protease inhibitors [(*p*-amidinophenyl)methanesulfonyl fluoride, leupeptin, antipain and benzamidine]. After centrifugation at 12,000 × *g* for 5 min, supernatants were collected for Western blotting analysis. An aliquot of 20 μg protein was loaded on polyacrylamide gel, and then separated by sodium dodecylsulfate polyacrylamide gel electrophoresis. Immunoreactive signals for Myo6 and β-tubulin were individually visualized with the horseradish peroxidase-conjugated secondary antibody by the chemiluminescence method (ECL, Amersham Biosciences). Densitometric determination was carried out using the Scion Image 4.0 software.

Mice were deeply anesthetized and intracardially perfused with saline and 4% paraformaldehyde (PA). Brains were removed, post-fixed overnight and cryoprotected in 30% sucrose. Free-floating sections were cut at 40 μm using a Leica cryostat and stored at −20 °C in cryoprotective solution (30% sucrose, 30% ethylenglycol and 1% polyvinylpyrrolidone in phosphate-buffered saline (PBS) until processing [11]. Sections were washed in PBS and incubated for 10 min in 0.1% H_2O_2 to block endogenous peroxidase activity. After washing in PBS, sections were incubated for 2 h in blocking solution (3% normal serum and 0.1% Triton X-100 in PBS), followed by incubation at 4 °C overnight in blocking solution containing the primary antibody against Myo6. Sections were then incubated in the blocking solution containing biotinylated anti-goat antibody at 4 °C overnight, followed by rinsing in PBS and subsequent incubation for 1 h in the ABC solution. Following the incubation in the avidin-peroxidase complex solution for 1 h at room temperature, immunoreactive cells were visualized by the incubation in the diaminobenzidine/H_2O_2 solution. Sections were mounted onto slides, air-dried, dehydrated through graded alcohol followed by xylene, and then mounted for viewing.

The data are invariably shown as the mean ± S.E. The statistical significance of experimental observations was determined by the Student's *t*-test, Tukey–Kramer test, or one- or two-way ANOVA followed by Scheffe's *post hoc* test with the level of significance set at $P < 0.05$.

Animals exposed to WIRS for 3 h exhibited a variety of long-lasting bidirectional behavioral abnormalities similar to the symptoms seen in the patients with PTSD, such as freezing behavior and hyper locomotion, 14 days after the exposure to WIRS (data not shown). To search for genes responsive to traumatic stress, DD-PCR screening was performed using total RNA extracted from the mouse hippocampus obtained 24 h after WIRS for 3 h. The first DD-PCR analysis revealed the upregulation of 16 cDNAs and the downregulation of 7 cDNAs in the hippocampus of mice exposed to WIRS (Fig. 1A). The second DD-PCR analysis confirmed the upregulation of only 2 cDNAs (#19 and #22) (Fig. 1B), while both PCR products yielded a single band in the third DD-PCR analysis, respectively (Fig. 1C). No changes were seen in the expression profile of other 21 genes in the hippocampus of control and stressed mice by second DD-PCR analysis.

The fragments were excised from the gel, purified, cloned and subjected to automated sequencing analysis. Homology analysis revealed that #19 had an 86% homology to *Mus musculus* myosin VI (Myo6), with #22 being 94% homologous to *M. musculus* cytochrome c oxidase subunit VIIa polypep-