

Dysfunction of Ca^{2+} /CaM kinase II α cascades in the amygdala in post-traumatic stress disorder

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Received July 21, 2009; Accepted September 7, 2009

DOI: 10.3892/ijmm_00000294

Abstract. Single-prolonged stress (SPS) is an established animal model for post-traumatic stress disorder (PTSD). The calcium (Ca^{2+})-calmodulin (CaM)-CaM kinase (K)II α signal passage plays an important role in the plasticity of central nervous, learning and memory, mind and behavior and other types of cognitive activities. The amygdala is known to play an important role in fear, rage and emotional memory. In this study, we investigated changes in Ca^{2+} -CaM-CaMKII α in the basolateral amygdala of rats after SPS which may reveal part of the pathogenesis of PTSD. The intracellular free calcium level in the basolateral amygdala was examined by fluorescence spectrophotometry. CaM and CaMKII α expression in basolateral amygdala was examined using immunohistochemistry, western blotting and reverse transcription-polymerase chain reaction. The intracellular free calcium level in the basolateral amygdala was increased when compared to that in the control group 1 day after SPS exposure ($P < 0.05$). CaM expression significantly increased, and CaMKII α expression significantly decreased ($P < 0.05$) in the basolateral amygdala after SPS. These findings suggest dysfunction of Ca^{2+} -CaM-CaMKII α in the basolateral amygdala of SPS rats, which may play important roles in the pathogenesis of PTSD rats.

Introduction

Post-traumatic stress disorder (PTSD) is an anxiety disorder that develops after exposure to a life-threatening traumatic experience. It is characterized by symptoms that often endure for years including the continuous re-experience of the traumatic event, avoidance of stimuli associated with the trauma, numbing of general responsiveness, and increased

arousal (1-3). The pathophysiology of PTSD has been widely studied in neuroscience (4). Several clinical neuroendocrinological studies have significantly improved our understanding of PTSD. One of the core neuroendocrine abnormalities related to the disorder is the dysfunction of the hypothalamo-pituitary-adrenal (HPA) axis, characterized by low levels of adrenocorticotrophic hormone (ACTH), plasma cortisol and urinary cortisol and enhanced suppression of cortisol in response to low-dose dexamethasone administration (5,6). These neuroendocrine findings specific to PTSD have served as the basis for animal models that are useful for elucidating the pathophysiology of PTSD. Single-prolonged stress (SPS) is a reliable animal model of PTSD based on the time-dependent dysregulation of the HPA axis and has been developed and employed for PTSD research (7-9).

Calcium (Ca^{2+}) is an important intracellular second messenger. Elevated [Ca^{2+}]_i binds to numerous proteins, from low-affinity/high-capacity buffer proteins. The influx of calcium ions results in calmodulin (CaM) activation. Many Ca^{2+} /CaM targets modulate cellular signaling pathways. CaM kinase(K) II is a major mediator of calcium signaling and is of particular importance in the brain, playing a key role in the regulation of nerve functions (10).

The amygdala, one of the key regions in the limbic system of the brain, is known to have an important role in fear, rage and emotional memory (11,12). The amygdala modulates memory consolidation with the storage of emotionally relevant information and is critical for the formation of long-term aversive memory, especially for the modulation of anxiety, fear and aggression (13,14). In addition, the amygdala plays a pivotal role in the neural circuitry of stress (15). The amygdala is usually divided into three distinct nuclear subgroups: the central nucleus, corticomedial nucleus and basolateral nucleus (16). Of these nuclear subgroups, the basolateral nucleus is the largest of the amygdala (17,18) and is one of the key regions of fear formation. This study focused on the observation of changes in Ca^{2+} -CaM-CaMKII α in the basolateral amygdala to ascertain how Ca^{2+} -CaM-CaMKII α cascades participate in PTSD.

Materials and methods

Experimental animals. Ninety male Wistar rats, weighing 150-180 g, aged 8 weeks at the start of the study, were supplied by

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Key words: single-prolonged stress, post-traumatic stress disorder, calcium, calmodulin, CaM kinase II α , basolateral amygdala

the Animal Experimental Center, China Medical University. The protocol was carried out in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, the Ministry of Science and Technology of the People's Republic of China (19).

Model establishment and grouping. Animals were divided randomly into three groups: i) the control group; ii) SPS 1d (1-day) group; and iii) SPS 7d (7-day) group. Control animals remained in their home cages with no handling for 7 days and were sacrificed at the same time as the SPS groups. SPS rats underwent the SPS procedure on the first day. The SPS protocol was based on a combined plural stress paradigm (7,20): immobilization (compression with plastic bags) for 2 h, forced swimming for 20 min (24 \pm 1°C), rest for 15 min, followed by drying and ether anesthesia (until consciousness was lost).

Perfusion-based sections. Rats of both the normal control and SPS groups were prepared by left ventricle perfusion and fixation (21), and post-fixed in the same fixative at 4°C for 6–10 h. They were then immersed in a 20% sucrose solution in 0.01 M phosphate buffer (PB, pH 7.4) at 4°C. Samples were snap-frozen in liquid nitrogen and sectioned. Coronal sections (12- μ m) were prepared for the morphological studies.

Determination of free calcium content in basolateral amygdala cells. Rats were rapidly decapitated, and the brains were removed and immediately placed in a dish standing on crushed ice. The basolateral amygdala was then dissected out according to the atlas of rats (22) using a stereomicroscope and snap-frozen in liquid nitrogen and washed twice with cold 200 mM Tris-buffered saline (TBS, pH 7.5). A cell suspension (10⁹–10¹⁰/l) was prepared by a routine method and loaded with 1 mmol/l Fura-2/AM (Beyotime, China) for 35 min, and then detected with a spectrofluorometer (23).

Immunohistochemical analysis of CaM and CaMKII α . The sections were treated with 5% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS for 30 min at room temperature (RT) to block non-specific staining. The sections were then incubated with mouse monoclonal antibody against CaM (Sigma, USA; 1:100) or CaMKII α (Santa Cruz, USA; 1:200) in 2% BSA-PBS overnight at 4°C. After being washed with PBS, the sections were incubated with goat anti-mouse IgG (Boster, China; 1:100) for 2 h and then with the streptomyacin-avidin-biotin-peroxidase complex (SABC) for 1 h. The sections were washed three times with PBS after each incubation and subsequently incubated with 3,3'-diaminobenzidine (DAB) and H₂O₂. To assess nonspecific staining, a few sections in every experiment were incubated in buffer without primary antibody.

Five slides were randomly selected from each group. Five visual fields of the basolateral amygdala were randomly selected in each slide (x400). We recorded the optical density (OD) of positive cells in each field to evaluate the average OD. The ODs of immunoreactivity of CaM- and CaMKII α -immunopositive cells were analyzed using the MetaMorph/DPIO/BX41 morphology image analysis system.

Table I. The primer sequences of CaM, CaMKII α and β -actin.

Name	Upstream primer	Downstream primer	Product size (bp)
CaM	5'-ggcctcctgctt tagcctgag-3'	5'-acatgctatccc tctcgtgtga-3'	328
CaMKII α	5'-catcctcaccac tatgctg-3'	5'-atcgatgaaagt ccaggccg-3'	284
β -actin	5'-atcaccacact gtgcccatc-3'	5'-acagagtacttg cgctcagga-3'	542

Western blotting to detect CaM and CaMKII α . Materials were obtained as above. Samples of normal control rats and SPS rats were respectively homogenized with a sample buffer containing 200 mM TBS, pH 7.5, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and denatured by boiling for 3 min. The protein fraction (30 μ g/lane) prepared from each sample was separated by 12% (w/v) gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electroblotted to a PVDF membrane (Millipore, Bedford, MA, USA) from the gel by a semi-dry blotting apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The membrane was blocked with 5% dried skim milk, 0.05% Tween-20 in TBST at RT for 2 h and incubated with mouse monoclonal antibody against CaM (1:1,000) or CaMKII α (1:5,000) overnight at 4°C.

Blots were washed three times with TBST, and then incubated with anti-mouse IgG-HRP (Santa Cruz; 1:5000) for 2 h at RT and washed with TBST. After the incubation, the PVDF membrane was washed three times with TBST before visualization using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). To confirm equal protein loading, the same blots were reincubated with antibodies specific for β -actin (Abcam, UK; 1:1,000). Immunoreaction for β -actin was detected with ECL. The OD was analyzed on the Gel Image Analysis System. The levels of CaM and CaMKII α were determined by calculating the OD ratio of CaM/ β -actin and CaMKII α / β -actin.

Reverse transcription-polymerase chain reaction to detect CaM and CaMKII α . Total mRNA was extracted from the basolateral amygdala using the Trizol Kit according to the manufacturer's instructions. The primers were designed by Shenggong Biotech Co. (Shanghai, China) according to the serial number from Genbank and are shown in Table I. The reaction was started at 94°C for 4 min and amplified for CaM for 32 cycles of 30 sec at 94°C, 30 sec at 58°C, 40 sec at 72°C and ended with a 7-min extension at 72°C. It was started at 95°C for 2 min and amplified for CaMKII α for 33 cycles of 30 sec at 95°C, 30 sec at 55°C, 40 sec at 72°C and ended with a 5-min extension at 95°C. β -actin mRNA used as an internal control was co-amplified with CaM mRNA and CaMKII α . The products were observed after electrophoresis on a 1.2% agarose gel, and the density of each band was analyzed on the Gel Image Analysis System. The levels of

CaM mRNA and CaMKII α mRNA were determined by calculating the density ratio of CaM mRNA/ β -actin mRNA and CaMKII α mRNA/ β -actin mRNA.

Statistical analysis. All data were expressed as the mean \pm SD. Data among groups were analyzed by one-way analysis of variance (ANOVA) using SPSS 13.0 software. A value of $P < 0.05$ was considered statistically significant.

Results

Analysis of fluorescence of free calcium content in basolateral amygdala cells. Intracellular free calcium in the basolateral amygdala neurons was notably higher at day 1 after SPS stimulation than that in the control group and gradually reached a normal level at day 7 after SPS (Fig. 1).

Immunohistochemical analysis of CaM and CaMKII α . CaM and CaMKII α widely distributed throughout the basolateral amygdala region, mainly in the cytoplasm, appeared as buffy particles (Fig. 2A and B). Evaluation of the CaM content by immunohistochemistry indicated a significant increase in the SPS model groups compared with the normal control group; the CaMKII α content was most significantly decreased in the amygdala of the SPS group compared to that in the normal control rats (Fig. 2C).

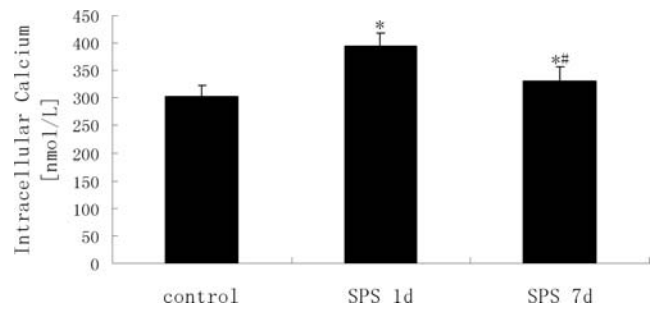


Figure 1. Alterations of intracellular Ca²⁺ (nmol/L) (mean \pm SD). * $P < 0.05$ vs control group, ** $P < 0.05$ vs SPS 1d (1-day) group.

Western blotting of CaM and CaMKII α . Immunoreactive signals for CaM, CaMKII α and β -actin appeared at 16.7, 50 and 42 kDa, respectively (data not shown), and the mean value of band densities of the control group was set as 100%. Data were expressed as normalized optical density. Changes in CaM and CaMKII α expression in the basolateral amygdala region between the control and SPS groups are presented in Fig. 3. CaM protein expression of the basolateral amygdala showed a significant up-regulation at day 1 after SPS stimulation in comparison with the control rats. In contrast,

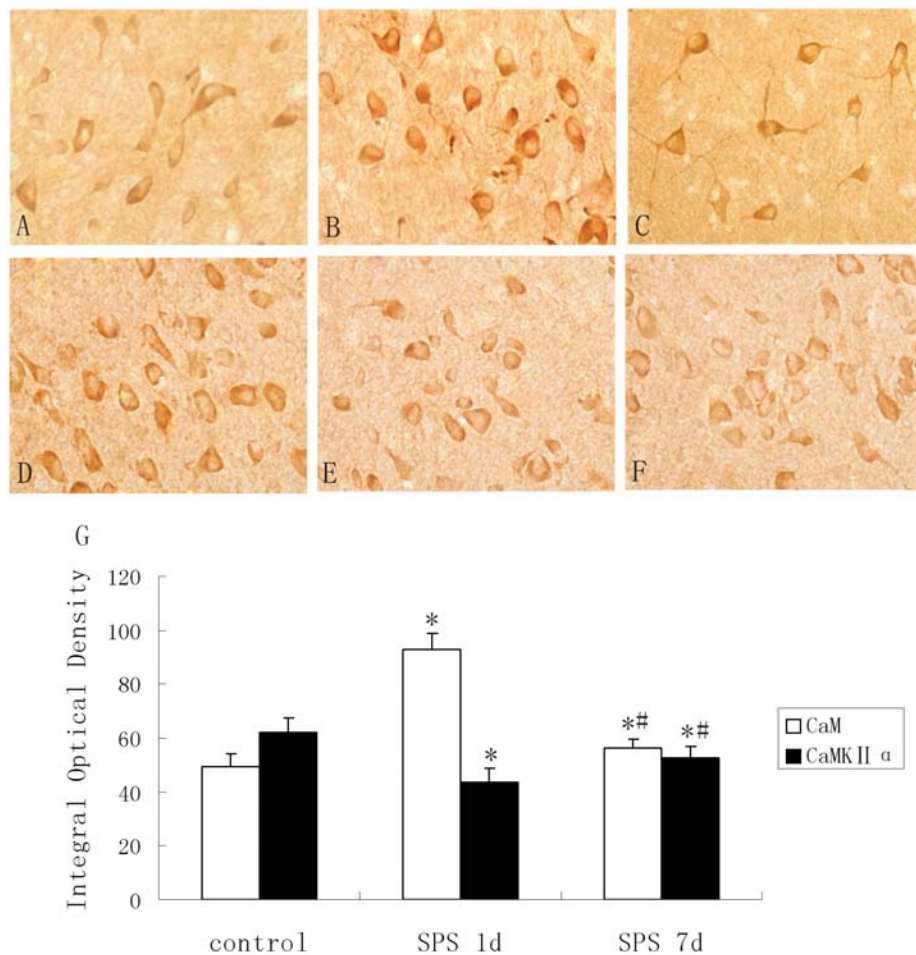


Figure 2. CaM and CaMKII α expression in the basolateral amygdala in each group (A-E, magnification $\times 400$). The quantity of CaM-immunoreactive cells in the SPS 1d rats (B) exceeds that of the control (A) and SPS 7d (C) rats. The quantity of CaMKII α -immunoreactive cells in the SPS 1d rats (E) was decreased compared with that of the control (D) and SPS 7d (F) rats. Quantitative analysis of the optical density (G). * $P < 0.05$ vs control group, ** $P < 0.05$ vs SPS 1d group.

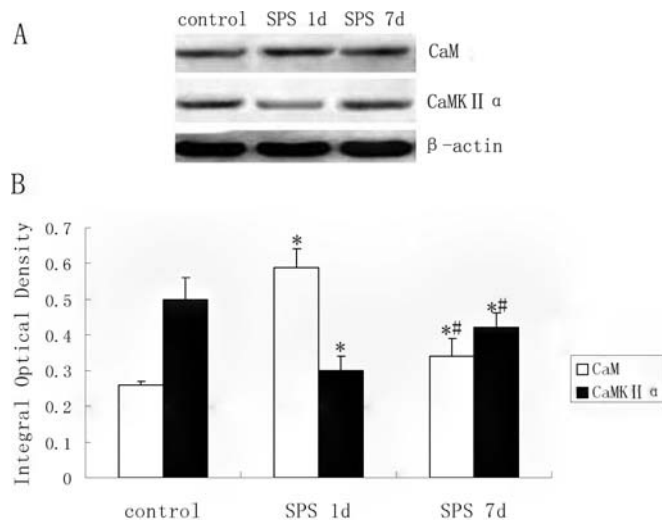


Figure 3. CaM and CaMKII α expression in basolateral amygdala by Western blotting. Bands show CaM and CaMKII α protein levels (A). Relative quantitative levels of CaM and CaMKII α (B). * $P < 0.05$ vs the control group, # $P < 0.05$ vs the SPS 1d (1-day) group.

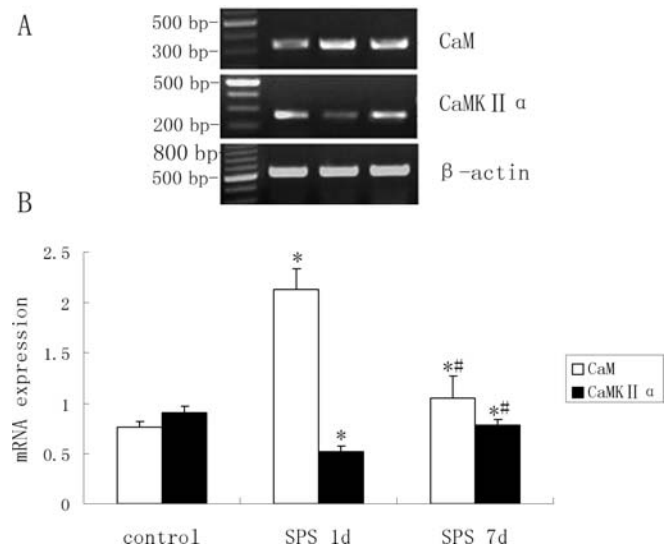


Figure 4. Representative gel pattern of CaM, CaMKII α , β -actin cDNA bands (Lane 0, marker; lane 1, control; lane 2, SPS 1d; lane 3, SPS 7d) (A) and relative CaM and CaMKII α mRNA expression (B). * $P < 0.05$ as compared to the control group, # $P < 0.05$ as compared to the SPS 1d group.

the basolateral amygdala CaMKII α expression showed a significant down-regulation throughout.

CaM and CaMKII α mRNA expression. The levels of CaM mRNA and CaMKII α mRNA were normalized with β -actin mRNA level. The levels of CaM mRNA increased more significantly in the SPS group than in the control group. In contrast, CaMKII α mRNA expression decreased throughout (Fig. 4).

Discussion

SPS is one of the animal models proposed for PTSD (20). The SPS rats show enhanced inhibition of the hypothalamo-pituitary-adrenal (HPA) axis, which has been frequently demonstrated in patients with PTSD.

The special role of the amygdala in the processing of threat-related stimuli, in particular anger and fear, is well documented (14). Abundant evidence from both animal and human investigations strongly suggests that the amygdala, one of the regions in the limbic system of the brain, is responsible for enhancement of explicit memory associated with emotional arousal (24-26). In addition, many lines of evidence have implicated the basolateral amygdala as a substrate for stress-related modulation of memory (27).

Calmodulin (CaM), a ubiquitous calcium sensor protein, is involved in almost all intracellular events. CaMKII is the molecular basis of learning and memory (28), but in the absence of bound calcium/calmodulin, CaM kinase II is in an inactive conformation. The influx of calcium ions results in CaM kinase II activation (29). Ca^{2+} /CaM kinase II is a major mediator of calcium signaling and is of particular importance in the brain (30), playing a key role in the regulation of nerve functions, including learning and memory (31,32). CaMKII α and CaMKII β are thought to have different roles in regulating neuronal functions. It is speculated that CaM kinase II α

responds to a strong and/or repeated stimulus in which cellular Ca^{2+} concentration is relatively high. CaM kinase II α is more effective in synaptic plasticity than CaM kinase II β , and is thought as one of the best candidates of memory molecule (33).

Evidence that the amygdala is part of a neural mechanism that can modulate a differential susceptibility of multiple memory systems to emotional arousal during acquisition (34), memory consolidation (35) and/or memory retrieval (present study), may provide a novel approach to elucidating the range of mnemonic dysfunction in PTSD.

In this study, detection of free calcium content in the basolateral amygdala neurons revealed calcium overload 1 day after SPS stimulation. Further analysis of CaM, the main calcium conjugated protein in the CNS, showed that expression of total CaM in the basolateral amygdala markedly increased 1 day after SPS stimulation suggesting that the CaM content changed synchronously with changes in the Ca^{2+} concentration. This occurred because SPS increased the intracellular free calcium level in the basolateral amygdala neurons inducing overexpression of the CaM protein. Changes in CaMKII α from inactive to pCaMKII α lead to decreased content of CaMKII α in the basolateral amygdala after SPS exposure. Due to the important roles of the Ca^{2+} -CaM-CaMKII α signal passage in the plasticity of central nervous, learning and memory, mind and behavior and other types of cognitive activities (36), the dysfunction of Ca^{2+} -CaM-CaMKII α of the basolateral amygdala might be the important pathobiological basis for abnormality of affect and behavior induced by PTSD.

At present, the pathogenesis of PTSD is not yet entirely clear. PTSD may result in a series of biochemical and physiological abnormalities in the brain, which leads to dysfunction of the amygdala. Thus, the pathogenesis of PTSD requires further study.

Acknowledgements

The authors thank all of the staff members of the China Medical University Experiment Center for their technical support. In addition, this research was supported by a grant from the National Natural Science Foundation of China (no. 30600341).

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