

The role of GluN1 activated nitric oxide synthase in a rat model of post-traumatic stress disorder

Post travmatik stres bozukluğu sıçan modelinde GluN1 ile aktiflenmiş nitrik oksit sentetazın rolü

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ABSTRACT

Objectives: Activation of neuronal nitric oxide synthase (nNOS) and interrelated alterations of calmodulin and ionotropic glutamate receptor (GluN1) levels are unknown in post traumatic stress disorder (PTSD).

Materials and Methods: Sprague-Dawley rats of both sexes were exposed to dirty cat litter, and then placed on an elevated plus maze. An anxiety index was calculated and tissue samples from hippocampus and amygdala were prepared in order to detect calmodulin, NOS and GluN1 by immunoblotting.

Results: The anxiety indices of the traumatized rats were markedly higher than those of the controls ($p < 0.05$). GluN1 and calmodulin levels were decreased in the dorsal hippocampus and amygdaloid complex of the traumatized rats. NOS expression increased significantly in both the amygdaloid complex and dorsal hippocampus where the increase was statistically more prominent in the amygdaloid complex ($p < 0.001$) than in the dorsal hippocampus of the traumatized rats ($p < 0.05$).

Conclusion: Predator exposure in rats causes long-lasting anxiogenic effects associated with increases in NOS and decreases in GluN1 expressions in brain areas related to PTSD symptoms and excitotoxicity. The results suggest that excitotoxicity occurs through other mechanisms rather than GluN1 receptors.

Keywords: Predator scent test, nNOS, Glutamate, Calmodulin, Amygdala, Hippocampus

ÖZ

Amaç: Post travmatik stres bozukluğunda (PTSD) iyonotropik glutamat reseptör (GluN1)'lerinin (GluN1) ve kalmodyülinin birlikte etki ederek nitrik asit sentaz (NOS) aktivasyonu yaptığı bilinmemektedir.

Gereç ve Yöntem: Her iki cinsiyetten Sprague-Dawley sıçanları kirdi kedi kumuyla saldırgan hayvan kokusuna maruz bırakıldıktan sonra, sıçanlara yükseltilmiş t labirenti deneyleri uygulanmıştır. Deney sonrası sıçanların anksiyete endeksleri hesaplanmış ve kalmodyülin, NOS ve GluN1 ölçümleri için hipokampus ve amigdala doku örnekleri Western Blot yöntemi için hazırlanmıştır.

Bulgular: Travmatize edilen sıçanların anksiyete indeksleri kontrol grubuna göre anlamlı olarak yüksek bulunmuştur ($p < 0.05$). Dorsal hipokampus ve amygdaloid komplekste travmatize sıçanların GluN1 ve kalmodyülin düzeyleri azalmıştır. NOS ekspresyonu her iki bölgede artış göstermesine karşın, amigdaloid kompleksteki artış ($p < 0.001$) dorsal hipokampüse göre ($p < 0.05$) istatistiksel olarak daha anlamlı bulunmuştur.

Sonuç: Saldırgan hayvan kokusu maruziyeti sıçanlarda, PTSD semptomları ve eksitotoksosite ile ilişkili beyin alanlarında nNOS ekspresyonunda artma ve GluN1 reseptörlerinde azalma ile birlikte uzun süre devam eden anksiyete ilişkili etkilere neden olmaktadır. Sonuçlarımız, NOS aktivitesinin GluN1 dışındaki nedenlerle arttığını göstermektedir.

Anahtar kelimeler: Saldırgan hayvan kokusu testi, nNOS, Glutamat, Kalmodyülin, Amigdala, Hipokampus

Introduction

Post-traumatic stress disorder (PTSD) is a condition that may arise immediately or many years after exposure to a serious traumatic event or injury. It can occur by a revisit of the initial trauma and may be accompanied by one or more of the following: anxiety, insomnia, nightmares, memory loss, behavioral changes, hyper-vigilance and hyper-arousal, crowd or social avoidance, cognitive changes or losses, increased susceptibility to infections, immune suppression, autoimmune diseases, depression and potentially, violent acts [1, 2].

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A satisfactory understanding of the etiology and neurobiological basis of PTSD [3] is not currently available, a lack that is significant since PTSD is the 4th most common psychiatric disorder, with a 6.8% life-time prevalence in the US [4]. In addition, PTSD is associated with increased rates of psychiatric and neurologic disorders (e.g., substance abuse, depression, anxiety, suicide, psychosis, traumatic brain injury, pain) as well as cardiovascular, musculoskeletal, respiratory diseases [5]. Many patients are resistant to having their symptoms improved by treatment and never achieve complete remission. Therefore, animal models of PTSD are of a great value for understanding the pathophysiology of PTSD and may assist in the development of novel and potentially more effective treatments or prevention strategies [6, 7]. The most commonly used stressors in animal models of PTSD include escapable/inescapable electric shocks, predator (e.g., cat) exposure, predator scent (e.g., cat litter or cat/fox urine) exposure or prolonged-stress (e.g., serial exposure to multiple intense stressors [8-10]. An alternative model of PTSD addresses the issue of how animal models of PTSD should recreate the variability in human individual differences in behavioral responses to traumatic stress.

Although, the precise biological mechanisms involved in PTSD remain unclear, recent studies suggest that an interaction between the ventromedial prefrontal cortex (vmPFC), the hippocampus and amygdala are critically involved in the pathogenesis of PTSD. Nitric oxide (NO) is an atypical neurotransmitter produced by a family of enzymes called NO synthases (NOS) [11,12]. There are three different isoforms of NOS which account for NO production. They include neuronal nitric oxide synthase nNOS, Type I, NOS-I), inducible nitric oxide synthase (iNOS, Type II, NOS-II) and endothelial nitric oxide synthase (eNOS, Type III, NOS-III) [13,14]. The nNOS isoform is constitutive in the central nervous system (CNS) and located in specific brain regions [15], where it is intimately associated with glutamate N-Methyl-D-aspartate (NMDA) receptors by the post-synaptic density protein-95 [16]. NO has been proposed as playing a role in several brain functions and dysfunctions such as neuronal excitability, synaptic plasticity, neurotoxicity and neuroprotection [17]. It can also modulate anxiety-like behavior. The nNOS enzyme is located in key brain regions associated with defense responses, including the amygdala, hippocampus, ventromedial prefrontal cortex (vmPFC) and the dorsolateral periaqueductal grey region [15]. The number of nNOS expressing neurons increases

in anxiety-related brain areas after restraint stress or anxiogenic stimulation [18]. Many studies suggest that calmodulin functions as an allosteric activator of nNOS [19-21].

NMDA receptors (NMDAR) are glutamate-gated calcium channels that play crucial roles in many neuronal functions. The interaction between nNOS and NMDARs is well known. NMDARs gate flux of calcium across the plasma membrane and relentless activation of the receptor results in substantially increased intracellular concentrations of ions in neurons [22]. Neuronal NOS is activated by calcium/calmodulin complex.

The present study examined the hypothesis that alterations induced by predator exposure are associated with activation of nNOS and related increases in the levels of calcium-calmodulin in brain areas that have previously been shown to be related to PTSD like vmPFC, dorsal hippocampus and amygdala [23,24].

Materials and Methods

1. Animals and experimental conditions

An approval from the institutional local ethical committee was obtained before the experiments were started (Marmara University Ethics Committee approval no: 13.2009.mar). Sprague-Dawley rats of both sexes weighing 200–250 g supplied from Marmara University School of Medicine Animal Center were used in the study. The rats were habituated to the housing conditions for 10 days with a reversed 12 h light/dark cycle at $21 \pm 3^\circ\text{C}$ and $50 \pm 5\%$ humidity with unlimited access to standard rat chow and water. All experiments were performed in the dark phase at 10:00 a.m. using a dim light source. Each experimental group contains 6 rats.

2. Predator scent test

The stress paradigm was produced by placing the rats on 125 ml of dirty cat litter for 10 min in a plexiglass cage (30 cm × 30 cm × 40 cm). The control animals were exposed to identical fresh, unused litter for the same amount of time. The cat litter had been used for 2 days by the same cat and had been sifted for stools as described previously [12–14]. The rats were subjected to clean cat litter as a situational reminder 1 week after the onset of the stress. The behavioral experiments were recorded using an overhead video camera and behavioral parameters were scored from the recordings later.

3. Elevated plus maze experiments

The rats were placed on an elevated plus maze for 5 min immediately after they had been subjected to the situational reminder. The elevated plus maze had two open (50 cm × 10 cm) and two closed (50 cm × 10 cm) arms. The closed arms were surrounded by 40 cm long walls. The height of the maze was 50 cm from the ground. The labyrinth was cleaned with a 5% alcohol solution before the rats were placed on it. Each rat was placed in the central square of the plus maze facing the open arms. An arm entry was defined as an animal entering the arm with all four feet and the number of entries into open and enclosed arms was scored as described previously [15]. The anxiety index (N_{anxiety}) was calculated by using the following parameters and the formula:

a = cumulative time spent in open arms (s), b = open arm entries, c = total arm entries

$$N_{\text{anxiety}} = 1 - \frac{1}{2} \times [(a(s) / 300(s)) + (b/c)]$$

The cumulative freezing time, a fear parameter, was also recorded and evaluated. Upon completion of the experiments the most affected rats were sacrificed with a high dose of pentobarbital and the selected brain regions were dissected and kept at -80 ° C for immunoblotting.

4. Tissue preparation and immunoblotting

After rapid decapitation, the hippocampus and the amygdaloid complex were removed in accordance with the Rat Brain Atlas [16] and were frozen at -80 ° C until further preparation. Anteroposterior planes used for removing the dorsal hippocampus and the amygdaloid complex were located between 6.70–4.70 mm and 7.20–5.70 mm anterior to the interaural line, respectively. The interaural line was accepted as 9.00 mm posterior to the tip of the brain as indicated [16]. The frozen tissues were weighed and homogenized in ice-cold 10 mM Tris-HCl (pH 7.2) buffer containing 1 mM EDTA and protease inhibitors (0.2 mM PMSF, 1 µg/ml leupeptin, 1 µM pepstatin, 10 µg/ml soybean trypsin inhibitors) with Ultraturrax homogenizer. The samples were centrifuged at 300g for 5 min at 4°C. The resulting supernatant was centrifuged at 13200 g for 85 min. The pellets were re-suspended and washed twice in the same buffer and stored at -80°C. The protein content of the crude membrane fraction was determined as indicated [17]. Fifty micrograms of protein was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels

(SDS-PAGE) and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell, 0.45µm, Germany) for 120min at 80 V. The membranes were blocked with tris buffered saline containing 1% bovine serum albumin and 0.05% Tween-20 at room temperature for 60 min and incubated overnight at 4°C with antibodies against calmodulin and NOS proteins. The calmodulin and NOS specific antibodies were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All chemicals were obtained from Sigma, unless stated otherwise.

The blots were washed three times with theta burst stimulation (TBS) containing 0.05% Tween-20 (TBS-T) and incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at room temperature (20°C). The antibody-antigen complex was detected with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP). The apparent molecular weights of calmodulin and NOS are 17 kDa, ≈155 kDa, respectively. The densitometric analyses were carried out with Bio-Rad Molecular Analyst software (free edition, Image Studio Lite). Immunoblots were standardised according to expression of β-actin.

5. Statistical analysis

All data are expressed as mean ± SEM. The calmodulin and NOS expressions in the amygdaloid complex/dorsal hippocampus of the stress and control groups were calculated and compared between the two groups with the two-tailed Student's t test. A p value < 0.05 was considered to be statistically significant.

Results

1. The effects of agents on behavioral parameters

When the rats were subjected to the trauma reminder one week after the predator scent test, the anxiety indices of the rats treated with physiological saline were found to be markedly higher than those of the control rats (subjected to only clean cat litter during trauma and trauma reminding sessions) treated with saline (p < 0.05).

2. Immunoblotting analyses

We performed immunoblotting experiments in rats of whose anxiety index was calculated as 1 individually. The dorsal hippocampus and the amygdaloid complex of the rats were homogenized and representative immunoblots are shown in Fig 1.

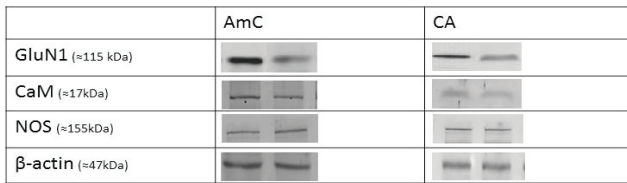


Fig.1. The representative photographs of nitrocellulose membranes collected from immunoblotting experiments of rats subjected to clean or dirty cat litter.

GluN1 expression decreased significantly both in the amygdaloid complex and the dorsal hippocampus, where the level of GluN1 decreased from 2.01 ± 0.02 to 1.60 ± 0.03 in the amygdaloid complex (Fig.2b; $p < 0.0001$) and GluN1 expression decreased from 1.47 ± 0.02 to 1.18 ± 0.003 in the dorsal hippocampus homogenates of the traumatized rats (Fig.2a; $p < 0.0001$).

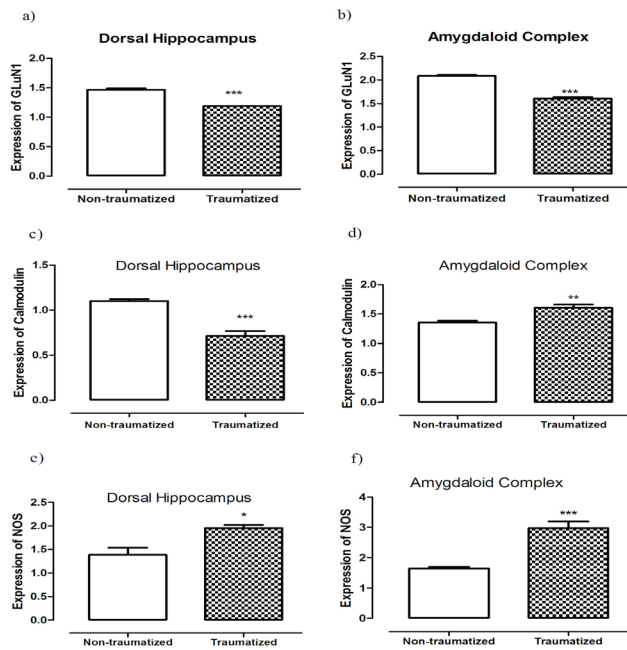


Fig.2. Expression of NOS, GluN1 and calmodulin in the dorsal hippocampus and amygdaloid complex homogenates of rats. NOS expression increased both in the amygdaloid complex and the dorsal hippocampus significantly where the increase was found to be more prominent in the amygdaloid complex (Fig.2f; $p < 0.001$) from that of the dorsal hippocampus homogenates of the traumatized rats (Fig.2e; $p < 0.05$). GluN1 expression decreased both in the amygdaloid complex and the dorsal hippocampus significantly, where the level of GluN1 decreased from 2.01 ± 0.02 to 1.60 ± 0.03 in the amygdaloid complex (Fig.2b; $p < 0.0001$) and GluN1 expression decreased from 1.47 ± 0.02 to 1.18 ± 0.003 in the dorsal hippocampus homogenates of traumatized rats (Fig.2a; $p < 0.0001$). The calmodulin levels decreased from 1.10 ± 0.02 to 0.71 ± 0.05 in the hippocampus (Fig.2c; $p < 0.005$), however the expression of calmodulin increased from 1.35 ± 0.03 to 1.61 ± 0.05 in the amygdaloid complex of traumatized rats (Fig.2d; $p < 0.007$).

The calmodulin levels decreased from 1.10 ± 0.02 to 0.71 ± 0.05 in the hippocampus (Fig.2c; $p < 0.005$), however the expression of GluN1 increased from 1.35 ± 0.03 to 1.61 ± 0.05 in the amygdaloid complex of traumatized rats (Fig.2d; $p < 0.007$).

For the homogenates of the traumatized rats NOS expression increased both in the amygdaloid complex and the dorsal hippocampus significantly where the increase was statistically found to be more prominent in the amygdaloid complex (Fig.2f; $p < 0.001$) than the dorsal hippocampus (Fig.2e; $p < 0.05$).

Discussion

Predator exposure produces long lasting (one week) angiogenic responses in the elevated plus maze which mimic the PTSD symptoms. The present work confirmed earlier findings showing similar structural and functional changes in the amygdaloid complex and the dorsal hippocampus.

Our results show that although the GluN1 expression and the calmodulin levels decreased in the dorsal hippocampus with trauma, the NOS expression was found to be elevated. However, in the amygdaloid complex, both calmodulin and the NOS levels were found to be elevated in the traumatized rats.

It can be suggested that behavioural changes may be associated to increased production of NO, a critical molecule which is produced by NOS. Although NO is highly liposoluble and has a short life which makes determination of its roles in the CNS difficult, detection of expression of NOS in brain areas related to anxiety and stress strongly suggests its contribution to the pathophysiology of the disorder. NOS activity in regions such as the amygdala, dorsal hippocampus and the prefrontal cortex (PFC) increases in stress-related conditions like an exposure to aversive stimuli [25-28]. Our results also demonstrated the increase in NOS expression in the amygdaloid complex relating to other findings in the literature [29].

It has been reported that calmodulin acts as an activator of NOS. NOS is inactive at basal intracellular Ca^{2+} levels and with the aid of stimulating factors that increase intracellular Ca^{2+} levels, NOS is activated by calmodulin. Calmodulin disassociates from NOS when Ca^{2+} levels decrease to basal levels [19, 20]. Thus, increases in nNOS and calmodulin expressions can be expected to occur together. In present study, NOS and calmodulin levels in the amygdala were increased. Our results, contrary to expectations, results showed decrease in calmodulin in the dorsal hippocampus. This suggests that

in the dorsal hippocampus, a once there may be a possible mechanism other than calmodulin producing NOS activation that can be involved in the NO effects that are seen after the predator exposure. The decrease in the GluN1 expression may be a consequence of excitotoxicity mediated by the NO.

Glutamate is the main excitatory neurotransmitter in the CNS which has been involved in many processes such as neurodevelopment, learning and memory formation. It has been suggested that glutamate receptors play an important role in fear-mediated learning, affecting both hippocampus and amygdala after a stressful stimuli. Two different types of glutamate receptors are responsible for glutamate activities: ligand-gated ion channel receptors (ionotropic) and G-protein-coupled metabotropic receptors. One of the ligand-gated ion channel receptor subtypes is the N-methyl-d-aspartate receptors (NMDARs). The well known interaction between NMDARs and NO suggests that NMDAR signalling through nNOS contributes the changes related to stress and anxiety. Although our findings show an increase in nNOS in the amygdala and dorsal hippocampus, they also reveal a decrease in NMDAR expression in the same areas.

Conclusion

In conclusion, the results of the present study indicate that predator exposure in rats causes long-lasting (one week) anxiogenic and that these are associated with changes in nNOS, calmodulin and NMDARs expression in brain areas related to PTSD symptoms and excitotoxicity. The decrease in the GluN1 type glutamate receptors may be a consequence of the trauma. Another finding may be that NOS can be activated by mechanisms other than NMDARs and calmodulin in the dorsal hippocampus. Clearly, more studies are required to understand the effects of glutamate receptors and NO in the stress-relevant disorders.

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