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# Chronic immobilization stress induces anxiety- and depression-like behaviors and decreases transthyretin in the mouse cortex

Yeon Joo, Kyung Mi Choi, Young Hyurk Lee, Gyeongwha Kim, Dong Hoon Lee, Gu Seob Roh, Sang Soo Kang, Gyeong Jae Cho, Wan Sung Choi, Hyun Joon Kim*

*Department of Anatomy and Neurobiology, Institute of Health Sciences, School of Medicine, Gyeongsang National University, 92 Chilam-dong, Jinju 660-751, Republic of Korea*

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ABSTRACT

In this study, we examined the changes in gene expression in the mouse cortex following chronic stress and behavioral tests. Mice were subjected to immobilization stress for 2 h per day for 15 consecutive days and the behavior of the mice was examined. The mice in the experimental group were more anxious and depressive than the control mice. The expression of mRNA in the cortex was analyzed by microarray analysis and 63 genes were found to show a greater than twofold change in expression between the control and experimental groups. Transthyretin was further investigated because its expression showed the greatest fold change. Transthyretin mRNA expression decreased in a chronic stress-specific manner, and protein levels were reduced in the cortex but not in the choroid plexus.



Modern humans cannot escape from stress and stressors during their normal activities. Stressors trigger physiological, behavioral and metabolic responses that are aimed at reinstating homeostasis. However, excessive, prolonged or inadequate responses, termed an "allostatic load", can lead to pathological outcomes [23]. Additionally, chronic stress may be a risk factor for psychiatric illnesses such as anxiety and depression [15].

The cortex plays an important role in the organization of neuronal signals and in decision making, cognition, and a variety of behaviors, both in healthy and illness-related physiological conditions [6]. Most depressive disorder patients have symptoms of impairment associated with cortical functions, including learning and cognition [16]. Therefore, it is important to determine the specific changes in the cortex that occur with psychiatric illnesses induced by chronic stress in order to understand the etiological mechanisms of such diseases.

Although the mechanisms of stress-induced psychiatric illnesses are complex, these conditions may be associated with changes in the expression of genes in the stressed brain. Therefore, the aim of this study was to uncover changes in gene expression in the cortex that are correlated with chronic stress-induced anxiety and depression-like behaviors. We used repeated immobilization as a chronic stressor, because this restraint stressor is known to induce both psychological and physical stress, which results in a wide range of behavioral and physiological alterations [10,14]. After confirming stress-induced behavioral changes, we performed microarray analyses using cortical tissue to provide a genome-wide, nonbiased analysis of gene expression and identify changes in cortical gene expression.

Male 7-week-old ICR mice (Samtako, Co. Ltd., Korea) were housed in a temperature-controlled (22 °C) environment under a 12-h light/dark cycle (lights on at 6 AM), with free access to laboratory chow and water. The animals were habituated for 1 week before experiments were initiated. The animals were subjected to chronic restraint stress daily from 14:00 to 16:00 h, for 15 days under 200 lux light at 22 °C. The mice were treated in accordance with the standard guidelines for laboratory animal care at the animal facility of the Gyeongsang National University School of Medicine.

Behavioral analyses were performed as depicted in Fig. 1A. The behavioral assessments were conducted using a computerized video-tracking system (EthoVision, Ver. 3.0; Noldus Information Technology, the Netherlands). Mice were placed in the test environment 1 h prior to behavioral testing. All tests were performed on 2 consecutive days at the same time of day, and the data presented are the mean of the 2 trials for each individual mouse.

The open field test (OFT) was performed in a rectangular chamber (60 cm × 60 cm × 20 cm). The bottom of the chamber was divided into a center area (38 cm × 38 cm) and a peripheral area using the arena definition tools in the video-tracking program. Each mouse was placed at the center of the open field, and movement was quantified. Movement was defined as the total time

* Corresponding author. Tel.: +82 55 751 8734; fax: +82 55 759 0779.
*E-mail address:* kimhj@gnu.ac.kr (H.J. Kim).

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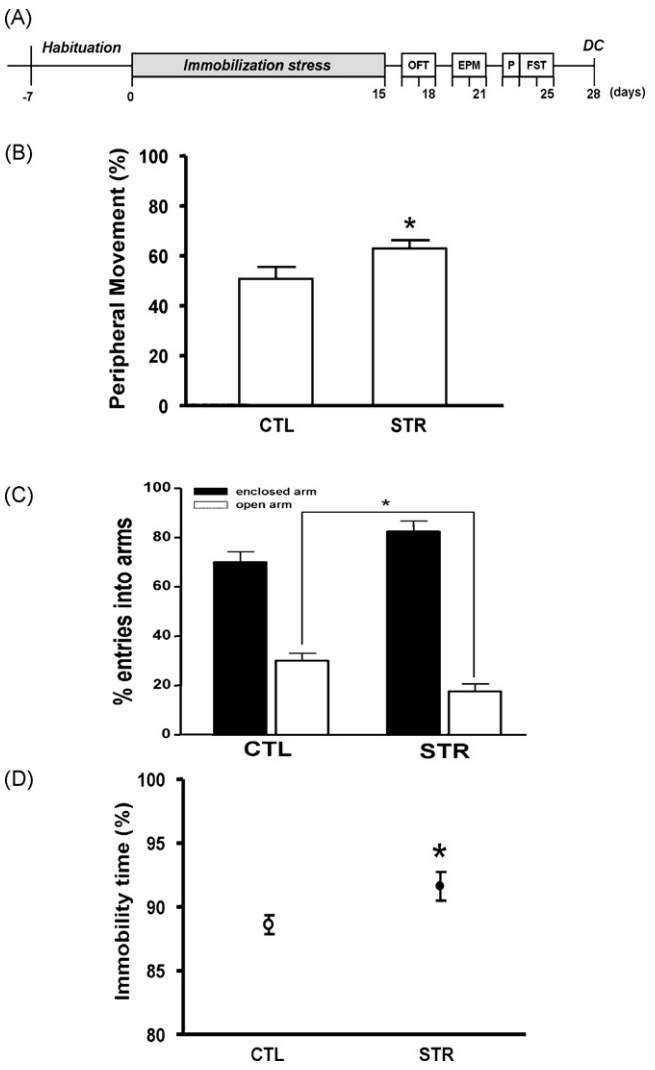


**Fig. 1.** Chronic immobilization stress changes physiology and behavior. (A) A schematic drawing of the administration of a 2-h immobilization stress for 15 consecutive days and subsequent behavioral assessments. OFT, open field test; EPM, elevated plus maze; FST, forced swim test; DC, decapitation; P, pre-swimming session. (B) Stressed (STR) mice showed significantly increased peripheral movement in the OFT than the control group (CTL) mice. (C) STR mice showed a significant reduction in the entries made into an open arm in the EPM. (D) STR mice spent a greater amount of time immobile in the FST. Data are presented as means ± SEM ($n = 10$). *$P < 0.05$ between CTL and STR.

during which a mouse moved at least 4 cm per second. Activity was recorded for 5 min.

The elevated plus maze (EPM) test was adapted from Lister [12] with the following modifications. The EPM consisted of two open arms and two enclosed arms, each 30 cm × 7 cm, and a connecting central platform (7 cm × 7 cm). The maze was elevated 50 cm above the floor. Each mouse was placed in the center of the maze facing one of the open arms and observed for 5 min. An entry into any arm was scored if the animal placed all paws in the arm.

The forced swim test (FST) was adapted from Porsolt et al. [21] and Schramm et al. [24] with the following modifications. The swimming chamber consisted of a Plexiglas cylinder (height: 25 cm, diameter: 12 cm) filled with water to a depth of 17 cm. The water temperature was 25 °C and the water was changed after each trial. Mice were subjected to a 5-min pre-swim habituation the day before the experiment. On the day of the experiment, the mice were forced to swim for 6 min and mobility was recorded for 5 min after 1 min pre-swimming without recording. Mobility was defined as a change of 15% in the recorded pixel threshold by the video-tracking software.

Mice were anesthetized with $CO_2$, decapitated, and the brains removed and quickly frozen in isopentane pre-chilled in liquid nitrogen. The frozen tissue was kept at −70 °C until sectioned. Brains were mounted on a cryostat and sectioned from the caudal end forward to −0.58 mm from the bregma [20]. After confirming the region of the brain by examining a section stained with cresyl violet, we collected tissues using a micro-punch (diameter 1.21 mm, Stoelting, IL, USA) with a 0.8-mm depth. Tissue punch samples were kept at −70 °C until use. Total RNA for DNA microarray and RT-PCR analyses was extracted from the tissue samples using the Trizol reagent and further purified using an RNeasy kit (Qiagen Inc., Valencia, CA).

The integrity of the total RNA was verified by visualizing intact and distinct 28S and 18S rRNA bands stained with ethidium bromide in a 1.5% agarose gel. DNA microarray analyses were performed at the DNA Microarray Core Facility (University of Kentucky, Lexington, KY) using Affymetrix equipment, protocols, and the GeneChip® Mouse Expression Set 430 2.0. The total RNA used for microarray analysis of each brain region was pooled from 10 mice. For RT-PCR and real time-PCR (qPCR) of transthyretin, the first strand cDNA was synthesized using 1 μg of total RNA. PCR primers for transthyretin were: forward, 5′-TGCTGGAGAATCCAAATGTC-3′ and reverse, 5′-GAAATGCCAAGTGTCTTCCA-3′ (NCBI accession number: X03351). GAPDH was used as an internal control (forward, 5′-TGCCGCCTGGAGAAACCTGC-3′ and reverse, 5′-TGAGAGCAATGCCAGCCCCA-3′). The end product of transthyretin (TTR) was confirmed by sequencing analysis. Real time PCR amplification and relative quantification were achieved using a LightCycler 480 (Roche) with LightCycler 480 SYBR Green 1 master mix. Advanced relative quantification was performed using automatically calculated ratio of target (TTR) to reference (GAPDH) Cp values in LC480 (version 4.0, Roche) software [8].

For immunohistochemistry, fixation and tissue processing were performed as previously described [9]. After drying, tissue sections were washed with tap water for 24 h and then submerged in 0.05 M Tris–HCl buffered saline (TBS, pH 7.4) containing 10% methanol and 3% hydrogen peroxide for 10 min at room temperature to quench endogenous peroxidase activity. After washing with 0.05 M TBS, sections were incubated with anti-transthyretin [Santacruz, sc-13098 (FL-147), sc-8105 (N-19), 1:50 dilution] at 4 °C for 24 h. Sections were incubated with biotinylated secondary antibodies for 2 h and color detection was performed using the ABC method, according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). After color development, sections were cleared and mounted. Equivalent results were obtained using sections from at least three different animals.

Data were evaluated using one-way ANOVA and a *post hoc* test. The *t*-test was used for comparisons of two groups (SigmaStat Ver. 10.0). Statistical significance was set at $P < 0.05$.

To create a reproducible animal model of stress-induced anxiety- and depression-like behaviors, we administrated a 15-day (2 h/day) immobilization stress to mice and subsequently performed behavioral assessments (Fig. 1A). The OFT and EPM tests were performed to determine the anxiety level of the mice. Stressed mice showed higher percentages of peripheral movement in the OFT (Fig. 1B) and lower percentages of entries into open arms in the EPM (Fig. 1C) than did control mice. These results indicate that the stressed mice were more anxious than the controls. To test whether our experimental paradigm evoked depression-like behaviors in the stressed animals, the FST was performed (Fig. 1A). Stressed animals spent a significantly greater amount of time immobile compared to control animals (Fig. 1D), indicating a higher level of depression in the stressed animals than in the controls [21,24]. These results indicate that our stress paradigm is sufficient to cause

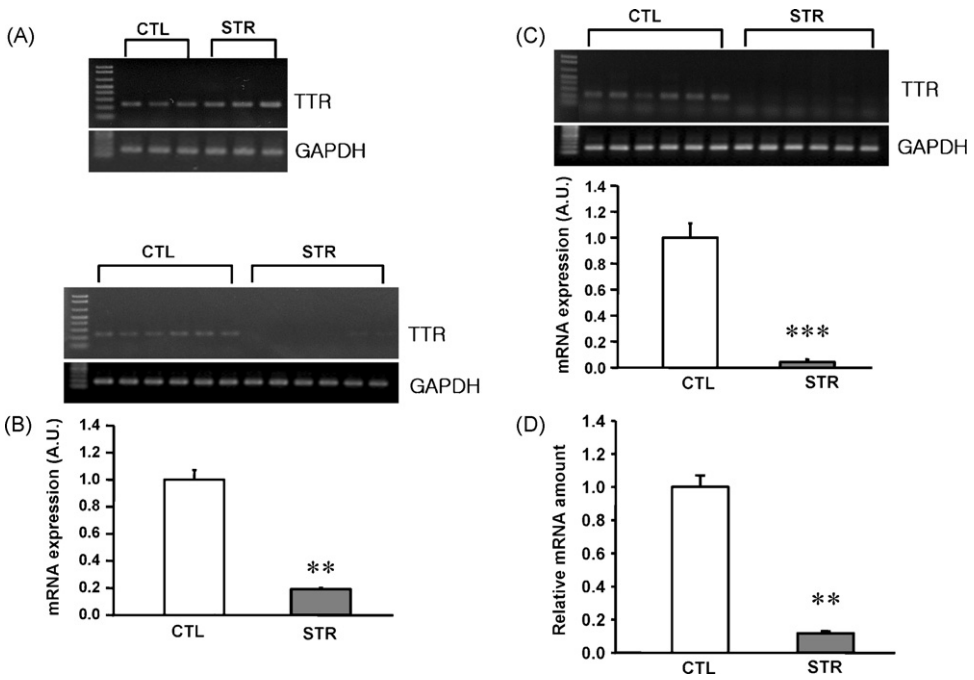


**Fig. 2.** Change in expression of transthyretin (TTR) after stress. (A) The level of TTR mRNA did not change after an acute 2 h immobilization stress. However, TTR mRNA decreased significantly after 15 days of a daily 2 h immobilization stress (B) and the decreased expression continued for at least the subsequent 14 days of behavioral tests (C). (D) Real time PCR analysis of the same samples used for (C). Data presented are mean + SEM ($n = 6$) from normalized expression measured by optical density or relative quantification. $^{**}P < 0.01$, $^{***}P < 0.001$ between CTL and STR.

stress-related psychiatric disorders such as anxiety and depression.

To examine changes in gene expression in the cortex upon psychiatric illness induced by chronic stress, a microarray analysis was performed. Although the DNA chip used for the microarray analysis contained over 30,000 transcripts, only 63 genes showed changes of greater than twofold between the control and stressed groups. Among these genes, 24 were genes of known function and 39 were novel (Supplementary Data). The change in a relative small number of genes may reflect the time of tissue collection for the microarray analysis (Fig. 1A). If the tissue had been collected immediately following the stress administration period, the microarray results may have revealed many more stress-induced changes in gene expression. However, we wanted to find genes whose changes in

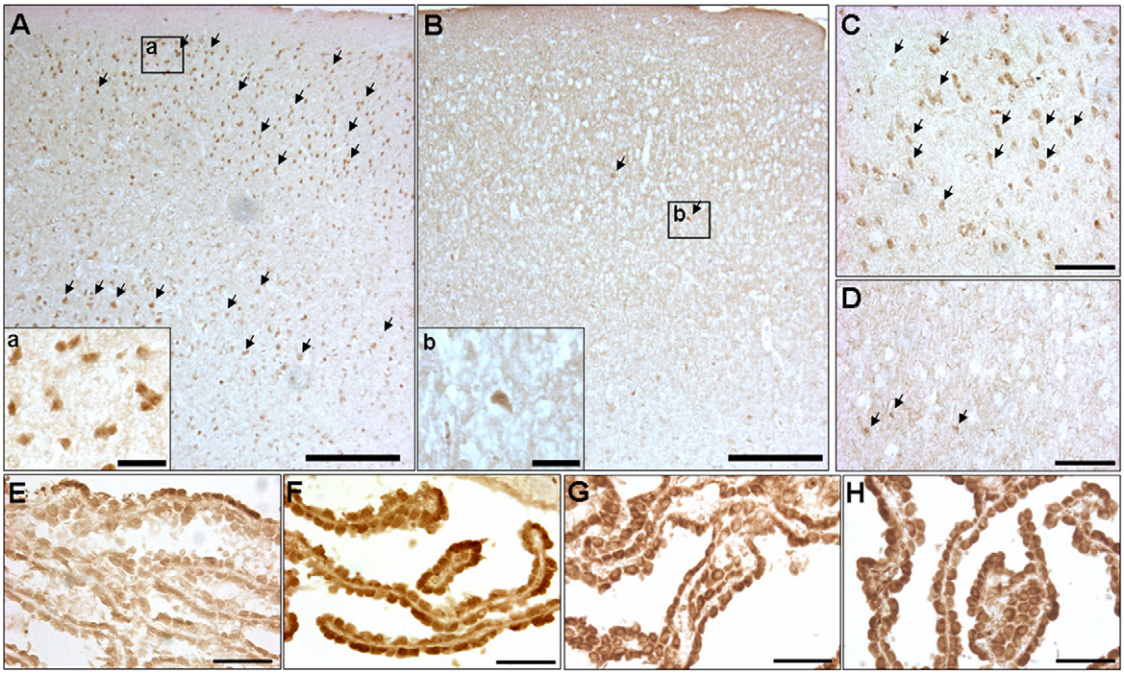


**Fig. 3.** Transthyretin protein levels were reduced in the cortex but not in the choroid plexus. Two anti-transthyretin antibodies detected TTR in the cortex (arrows, A with sc-13098, C with sc-8105) of control mice, as well as in the choroid plexus (E with sc-13098, G with sc-8105) of both control and stressed mice (F with sc-13098, H with sc-8105). TTR expression was significantly reduced in the cortex (B, b with sc-13098, D with sc-8105) of stressed mice. (a and b) Are magnified views of the boxed areas in (A) and (B). Scale bars in (A) and (B) 200 μm; in (a) and (b) 25 μm; in (C)–(H) 50 μm.

expression were correlated with long-lasting stress-induced behavioral changes, because such changes would be responsible for the long-term effects of chronic stress.

Among the known genes, transthyretin expression showed the greatest decrease in expression in response to chronic stress and all three TTR spots on the chip showed similar decreases (Supplementary Data). Therefore, TTR expression was further examined by RT-PCR using three different experimental groups. In the first group of animals, an acute immobilization stress was conducted by immobilization for 2 h. TTR expression did not change in the acute-stressed cortex (Fig. 2A). Two chronic immobilization stress groups were compared: mice subjected to a 15-day immobilization only (Fig. 2B) and mice subjected to a 15-day immobilization period followed by the OFT, EPM and FST behavioral tests, administered sequentially (Fig. 2C). The two chronically stressed groups exhibited a significant decrease in TTR expression. TTR mRNA was significantly reduced after the 15-day immobilization period and remained significantly lower than controls for at least 14 days after the last immobilization stress. To examine whether TTR protein levels decreased as well, immunohistochemistry (IHC) was performed on brains from control mice and stressed mice subjected to the 15-day immobilization stress and the behavioral tests (Fig. 3). Since TTR is synthesized in the liver and epithelium of the choroid plexus [4], we used the choroid plexus as a positive control for IHC. Two TTR antibodies that recognized different TTR epitopes were used, one antibody was made from full length TTR (Fig. 3A, B, E, and F) and the other from an N-terminal fragment (Fig. 3C, D, G, and H). Both antibodies gave similar results and indicated that TTR protein levels were markedly decreased in the cortex of chronic immobilization stressed mice. In contrast, the expression in the choroid plexus did not change after chronic immobilization stress.

TTR was originally characterized as a blood plasma and cerebrospinal fluid (CSF) transporter of thyroxine (T4) and vitamin A [7]. However, TTR-null mice have normal levels of T4 within the brain and exhibit thyroid hormone homeostasis [17,18], suggesting that TTR may have other biologically relevant ligands [19] and other biological functions. Although TTR-null mice exhibited decreased levels of depression and no differences in anxiety-like behavior [26], decreased levels of TTR in the CSF have been measured in patients with depression [30]. Additionally, TTR expression increased in the amygdala in response to conditioned fear stress [29], but decreased in the hippocampus in response to maternal separation stress [11]. Recently, TTR expression in the rat hippocampus was shown to increase in response to chronic administration of anti-psychotic drugs, including clozapine and olanzapine [1]. Additionally, TTR expression in the amygdala increased in response to chronic MK-801 treatment [13]. In the present study, TTR expression was reduced in the cortex but not in the choroid plexus. These findings suggest that TTR expression outside the choroid plexus may be affected by stressful conditions and may have a role in certain psychiatric conditions.

In addition to transport of T4 and vitamin A, TTR protects against β-amyloid aggregation in the nervous system [2,3,25,28]. Recent studies investigating additional TTR functions in the nervous system suggest that TTR plays a role in several aspects of basic neuronal cell physiology, including neurite differentiation and regulation of the cell cycle [5,22,27]. In the present study, it was speculated that the decreased cortical TTR after chronic stress would result in disturbance of the neuronal homeostatic mechanisms including efficient neurotransmission and clearance of accumulated oxidants. However, it should be investigated further in detail. Thus, further studies should examine whether cortical TTR has distinct properties that control the function of cortical neurons or influence cellular physiology to protect from stress.

Although an enormous number of studies have been performed to reveal the molecular mechanisms and biomarkers underlying chronic stress-induced psychiatric illnesses, the exact causes and potential therapeutic targets remain elusive, primarily because the stress response is very complex [14,23]. However, the present study suggests that the decrease in TTR in the cortex after chronic stress could be an etiological marker of stress-induced diseases.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2009.06.025.

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