Original Article

Noise Stress-Induced Changes in mRNA Levels of Corticotropin-Releasing Hormone Family Molecules and Glucocorticoid Receptors in the Rat Brain

(noise stress / CRH family molecules / glucocorticoid receptor / hypothalamus / hippocampus)

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Abstract. Noise is a widespread stress resource that may lead to detrimental effects on the health. However, the molecular basis of the stress response caused by noise remains elusive. We have studied the effects of acute and chronic noise stress on stress-related molecules in the hypothalamus and hippocampus and also corticosterone responses. Sprague Dawley rats were randomized into control, acute and chronic noise stress groups. While the chronic noise stress group animals were exposed to 100 dB white noise for 4 h/a day during 30 days, the acute noise stress group of animals was exposed to the same level of stress once for 4 h. The expression profiles of corticotropin-releasing hormone (CRH), CRH1, CRH2 receptors and glucocorticoid receptor (GR) mRNAs were analysed by RT-PCR. Chronic noise stress upregulated CRH mRNA levels in the hypothalamus. Both acute and chronic noise increased CRH-R1 mRNA in the hypothalamus but decreased it in the hippocampus. GR mRNA levels were decreased by chronic noise stress in the hippocampus. The present results suggest that while corticosterone responses have habituated to continuous noise stress, the involvement of CRH family molecules and glucocorticoid receptors in the noise stress responses are different and structure specific.

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Abbrevations: ANS – acute noise stress, CNS – chronic noise stress, CRH – corticotropin-releasing hormone, CRH-R1 – CRH type 1 receptor, CRH-R2 – CRH type 2 receptor; Ct – threshold cycle, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, GR – glucocorticoid receptor, HPA – hypothalamic-pituitary-adrenocortical axis, qPCR – quantitative real-time PCR, SEM – standard error of the mean, SPL – sound pressure level.

Introduction

Noise is a widespread source of stress in modern societies: people are exposed to noise stress related to their work environment, urban traffic and household appliances (Suter, 2002; Eggermont, 2014). Furthermore, considerable noise is unavoidable in animal facilities arousing from the direct activity of personnel using the facility and the increased activity of animals in response to the presence and actions of people (Turner et al., 2005). Noise, a psychosocial stressor, can affect physiological functions (Babisch, 2003). It induces systemic alterations in the organism directly or indirectly (Turner et al., 2005) and may lead to detrimental effects on the health including abnormal cardiovascular function, increased blood pressure, hypertension and sleep disturbances (Lenzi et al., 2002; Gitanjali and Ananth, 2003; Turner et al., 2005; Eggermont, 2014).

Exposure to stressors triggers activation of the nervous, endocrine and behavioural systems to promote physiological adaptations and maintain homeostasis (Figueiredo et al., 2003). The principal endocrine component of the stress response involves activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, a self-regulatory pathway that utilizes its end products (cortisol and corticosterone) to control its own activation through a negative feedback mechanism (Aguilera, 1998). While the physical component of stressors directly activates the HPA axis, psychological stressors, requiring higher-order sensory processing via limbic brain structures, such as the hippocampus and the amygdala, lead to indirect HPA regulation (Herman and Cullinan, 1997). Noise stress, being a psychological stressor, exerts its effects on the HPA axis through limbic structures by connections of the auditory system (Turner et al., 2005).

Corticotropin-releasing hormone (CRH) family molecules in the brain, CRH and its receptors CRH1 and CRH2, play a prominent role in the stress response. CRH is produced by the hypothalamus and regulates the stress response by activating the HPA axis and eventually causes release of glucocorticoids (Aguilera, 1998; Bale and Vale, 2004). CRH is also produced in other

central brain regions including the hippocampus, where it acts as a neurotransmitter and participates in behavioural and autonomic responses to the stress (Aguilera, 1998; Chen et al., 2004). CRH exerts its effects by plasma membrane receptors CRH1 and CRH2, which are differentially expressed on neurons located in neocortical and limbic regions, and both CRH receptors are effective in the maintenance and regulation of homeostasis in response to stress activation (Hauger and Dautzenberg, 2000; Bale and Vale, 2004). Although the role of the CRHergic system in the hypothalamus in response to stress has been well documented, only limited information is available on the regulation of this system in the hippocampus (Brunson et al., 2002).

Glucocorticoid (GR) receptors also mediate regulation of the HPA axis during the stress response and are found in the hypothalamus and hippocampus (Kloet et al., 1998). Corticosteroid hormones bind to these receptors. GRs have lower affinity to corticosterone and can be activated when corticosterone levels are high during the stress (Reul and Kloet, 1985). However, regulation of these receptors is not merely controlled by corticosteroids (Gądek-Michalska et al., 2013); other factors such as neural inputs, neurotransmitters and other steroids may affect their regulation as well (Herman, 1993). While GRs located in the hypothalamus mediate feedback inhibition of the HPA axis in response to stress, GRs in the hippocampus generally facilitate disinhibition of HPA (Sapolsky et al., 1984).

Most studies of the effects of noise exposure on the stress-related structures in the brain have been performed by measuring behavioural, endocrine, and biochemical variables (Armario et al., 1984; Uran et al., 2010; Akyazi and Eraslan, 2014), whereas the molecular mechanisms responsible for modifications in these variables in the related brain structures have not been studied so far. We aimed to investigate the effect of noise stress on the role of CRH family molecules and GR in the hypothalamus and hippocampus. For this purpose, mRNA levels of CRH, CRH-R1, R2 and GR in related brain regions were analysed after acute and chronic white noise exposure.

Material and Methods

Animals and grouping

A total of 24 adult (weighing 250 ± 10 g) Sprague Dawley male rats (purchased from the Institute of Experimental Medicine of Istanbul University, Istanbul, Turkey) were randomized into control (CON), acute noise stress (ANS) and chronic noise stress (CNS) groups, each consisting of eight animals.

The animals were kept in polycarbonate cages in groups of 4/cage with wood chip bedding in standard lighting (12h/12h light/dark cycle) and temperature conditions (22 ± 3 °C). Food and water were provided *ad libitum*. All experimental procedures were approved by the local ethics committee of the Istanbul University.

Noise stress induction

The white noise was produced by a general radio random noise generator (Type 1390, General Radio Company, Cambridge, MA). The output of the noise generator was amplified and emitted by loudspeakers installed into a sound-isolated cabinet. Loudspeakers (one speaker per each cage) were fixed directly above the cages at the shelves of the cabinet. Noise levels were adjusted to a 100 dB (± 1 dB) sound pressure level (SPL) that was measured with a sound level meter (CEM DT–8820, Shenzhen Everbest Machinery Industry Co., Ltd, Shenzhen, China) at the bottom of the cages. The background noise level in the cabinets was 50 (±5) dB SPL. A control cabinet with the same specifications with unplugged loudspeakers was also used.

Continuous white noise stress was applied to stress group animals during the same time of the day between 08:00 and 12:00 in the stress cabinet. While the CNS group animals were exposed to white noise for 4 h/day during 30 days, the ANS group of animals was exposed to the same level of stress only once for 4 h. Animals from the CON group were kept in the control cabinet during the stress sessions.

All animals were sacrificed by rapid decapitation, always between 12:30 and 13:00. Animals from the CON group were sacrificed without stress application. Chronically stressed animals were sacrificed 24 h following the end of the stress procedure to avoid the acute influence of the last stress session (Mamalaki et al., 1992; Kitraki et al., 1999).

Corticosterone assay

Trunk blood was collected into tubes containing EDTA, centrifuged at 1040 *g* (Sigma 3-16K, Sigma Laborzentrifugen GmbH, Osterade am Harz, Germany) for 10 min at 4 °C. The plasma was separated into microcentrifuge tubes and stored at –80 °C until assayed. Plasma corticosterone concentrations were quantified by using a corticosterone EIA kit (Assay Designs, Inc. Ann Arbor, MI).

Tissue sampling and RNA preparation

Upon sacrifice, brains were immediately removed and kept at –80 °C. The brain was dissected to separate the hippocampus and the hypothalamus. Frozen brains were placed into a 1-mm rodent brain matrix (Electron Microscopy Sciences, Hatfield, PA, catalogue No. 69026-C) and cut into 2 mm thick slices using razor blades (Electron Microscopy Sciences, catalogue No. 70933-70) on ice. The hippocampus and the hypothalamus were removed from these slices with a 2-mm diameter punch tool according to the brain atlas of Paxinos and Watson (1998).

Total RNA was isolated using a PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA) in accordance with manufacturer's instructions. The concentration of total RNA was measured fluorimetrically (Qubit fluorimeter, Invitrogen, Carlsbad) using the Quant-iT™ RNA Assay Kit (Invitrogen, Eugene, OR).

Quantitative real-time RT-PCR

Total RNA (1 µg) was reverse-transcribed into cDNA using random primers (High capacity RNA to cDNA Master Mix Kit, Applied Biosystems, Foster City, CA). The concentration of the resulting cDNA was measured using the Quant-iT™ DNA Br Assay Kit (Invitrogen, Eugene). One ng of cDNA was used for quantitative real-time PCR (qPCR). TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays reagents (Applied Biosystems) for CRH (Rn01462137_m1), CRHR1 (Rn00578611_m1), CRHR2 (Rn00575617_m1) and GR (Rn00561369_m1) were used to detect and quantify the PCR products. Housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (4352338E_0906011) was amplified in the same experiment. The real-time reaction was carried out in ABI 7500 Real-Time PCR Systems (Applied Biosystems).

The threshold cycle (Ct) values represent the results of real-time reactions. The housekeeping gene, *GAPDH* Ct values across the different groups were compared statistically to control whether the procedure used in the study affected the housekeeping gene expression. There were no statistical differences between these values, indicating that *GAPDH* expression was not affected by the procedure. The mRNA expression levels of the tested genes were normalized to those of *GAPDH*. Analyses of the data were performed using the ΔΔ Ct method (Livak and Schmittgen, 2001). Fold changes of genes were calculated using the expression $2^{-\Delta\Delta Ct}$ with respect to the mean value of Δ Ct in the control group.

Statistical analyses

The SPSS-software package (ver. 11.5.2.1, SPSS Inc., Chicago, IL) was used for statistical analysis. First, Shapiro-Wilk test was used to test for the normality of data. All results are expressed as means ± SEM. ANOVA was used for normally distributed gene expression data. Non-parametric Kruskal-Wallis test was applied when the normality assumption was violated. Duncan and Mann-Whitney U tests were used for pairwise comparisons following parametric and nonparametric tests, respectively.

Results

Plasma corticosterone levels

The analyses of variance showed that corticosterone levels of animals did not differ significantly between the groups (Table 1).

Effects of noise stress on the levels of mRNA expression of stress-related genes in the hypothalamus

Chronic noise stress significantly increased mRNA expression of the *CRH* gene (post-hoc, $P < 0.05$ after ANOVA, $F(2, 21) = 50.46$, $P < 0.001$). Both acute and chronic noise exposure increased CRH-R1 mRNA levels (Man Whitney U, $z = –2.63$, $P = 0.009$, $z = –3.36$, $P = 0.001$ after Kruskal-Wallis, $\chi 2 = (2) = 18.48$, $P < 0.001$). The differences between the ANS and CNS groups were found to be significant as well, $z = –3.36$, $P = 0.001$ (Fig. 1).

Table 1. Effects of noise stress on plasma corticosterone levels

Groups	Corticosterone, ng/ml
CON	223 ± 28
ANS	182 ± 24
CNS	140 ± 13

Data are the means ± SEM.
CON = control, ANS = acute noise stress, CNS = chronic noise stress

Effects of noise stress on the levels of mRNA expression of stress-related genes in the hippocampus

The acute and chronic noise exposure decreased mRNA expression of the *CRHR1* gene (post-hoc, $P < 0.05$ after ANOVA, $F(2, 21) = 7.76$, $P = 0.003$). Chronic noise exposure also decreased GR mRNA levels (post hoc, $P < 0.001$ after ANOVA, $F(2, 21) = 7.08$, $P < 0.001$) (Fig. 2).

Discussion

In this study, the effects of noise stress on mRNA levels of the genes involved in stress response in the hypothalamus and hippocampus and corticosterone responses of rats were investigated. Our results show that while corticosterone responses have habituated to stress, brain structure and molecule-specific changes occurred in the mRNA expressions of the CRH system genes and glucocorticoid receptors by the noise exposure.

In the present study, corticosterone levels did not significantly differ between the control and noise-exposed groups, although there was a numerical decrease in the stressed groups. We have previously reported that noise stress increased stress hormone levels after 1-hour long continuous noise stress and after chronic intermittent noise exposures (Uygur and Arslan, 2010; Akyazi and Eraslan, 2014). However, in the present study, we applied 4-h noise exposure to ANS group animals. Therefore, it is likely that acute hormone increase has ceased because of habituation of the HPA axis hormone response to 4-h continuous noise exposure. Similarly, considering the CNS group, the corticosterone response might have habituated to continuous, repeated stress. In the same way, it has been previously reported that 98 dB noise stress for 30 min increased corticosterone levels but had no effect when applied repeatedly for 11 days (Sasse and Greenwood, 2008). In addition, habituation to the noise stress has been previously reported (Campeau and Dolan, 2002; Masini et al., 2008; Nyhuis

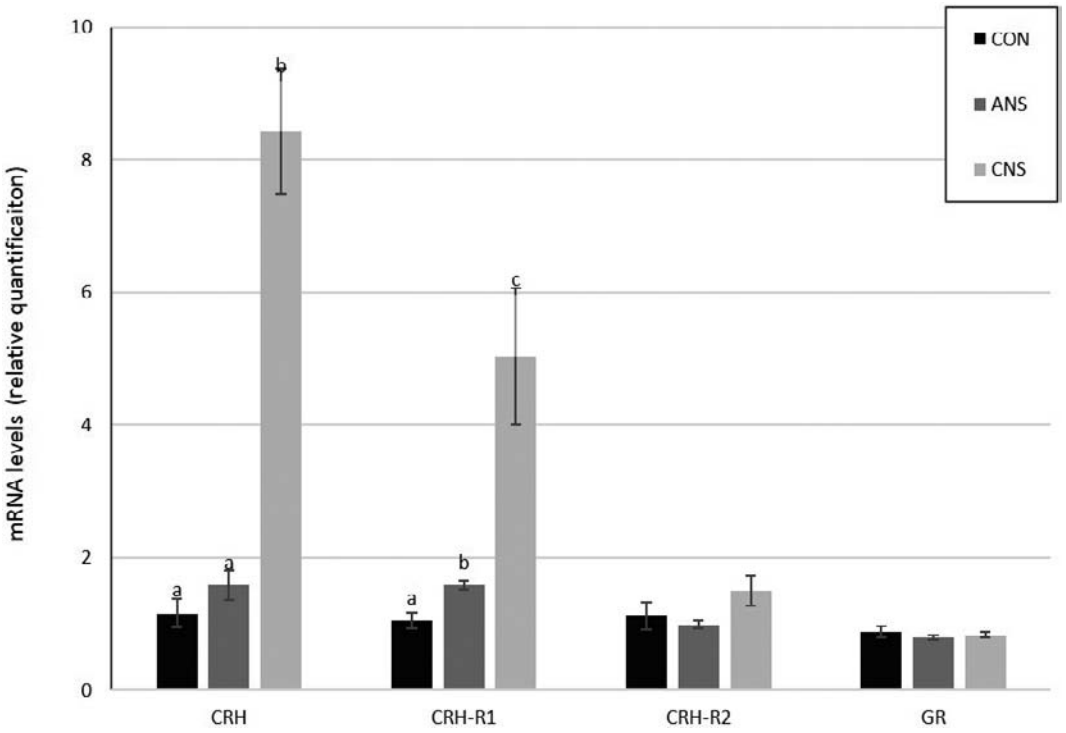

Fig 1. Changes in expression of mRNA for CRH, CRH-R1, CRH-R2 and GR in the hypothalamus after noise stress exposure. Each bar represents the mean ± SEM.
CON = control, ANS = acute noise stress, CNS = chronic noise stress, a, b, c = columns not sharing a letter differ significantly.

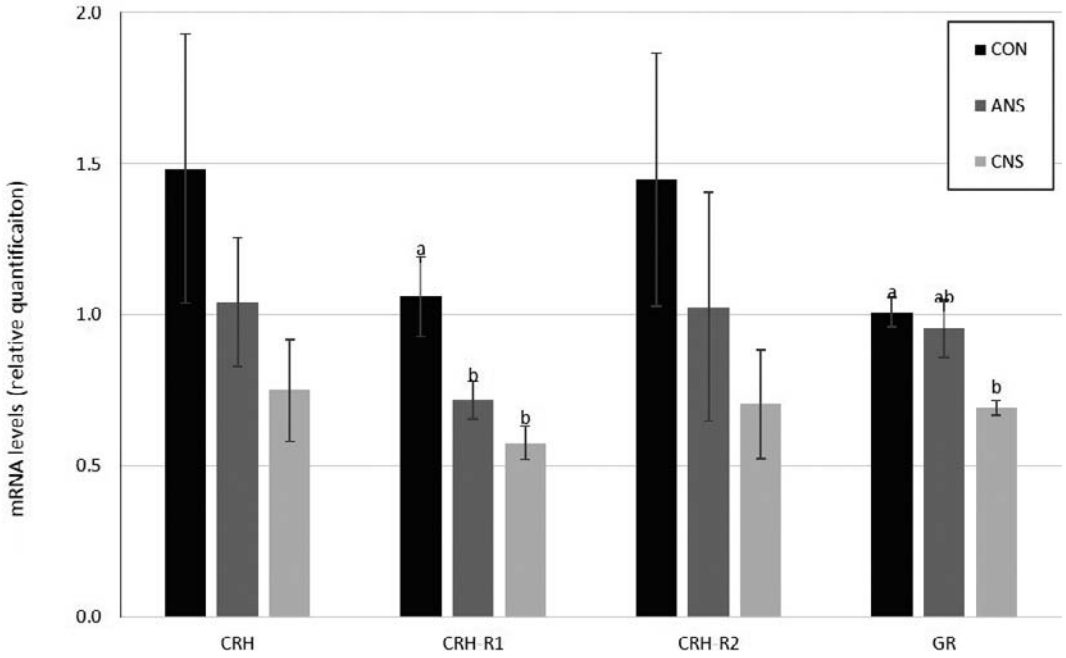

Fig 2. Changes in expression of mRNA for CRH, CRH-R1, CRH-R2 and GR in the hippocampus after noise stress exposure. Each bar represents the mean ± SEM.
CON = control, ANS = acute noise stress, CNS = chronic noise stress, a, b = columns not sharing a letter differ significantly.

et al., 2010). For instance, insignificant corticosterone levels were found after 6th exposure to 30 min of 95 dB white noise stress (Masini et al., 2008). Habituation of HPA activity to repeated homotypic or same stress and diminution of HPA axis responses have been suggested, supporting our results (Martí and Armario, 1998; Ostrander et al., 2006; Grissom and Bhatnagar, 2009). A numerical but not significant decrease observed in corticosterone levels of the stressed groups in our study may also be explained by partial habituation. It has been

previously suggested that the habituation may progress to responses lower than baseline (Grissom and Bhatnagar, 2009). There are also supporting studies finding lower corticosterone levels in stressed animals compared to the non-stressed ones (Campeau and Dolan, 2002; Ostrander et al., 2006). Therefore, considering corticosterone as a primary indicator of HPA axis activity, our results again strongly suggest that long duration noise exposure leads to habituation of the stress response.

We found that while single-session acute noise stress did not have any effect, chronic noise stress up-regulated mRNA expression of CRH in the hypothalamus. Different types of acute stressors were reported to up-regulate mRNA levels of this gene (Imaki et al., 1995; Makino et al., 1995b; Figueiredo et al., 2003). On the other hand, no changes in expression of CRH mRNA after acute foot shock stress exposure were reported in accordance with our results (Imaki et al., 1991). It has been suggested that mRNA expression of CRH is regulated in a stress-specific manner (Tanimura et al., 1998; Vázquez et al., 2003). Moreover, because of a large pool of mRNA in the cell, gene transcription was suggested to be a delayed and adaptive response to stress, which prepares neurons for further responses (Imaki et al., 1991; Watts, 2005). Therefore, the lack of increase in mRNA expression levels of CRH after ANS in our study could be explained by these suggestions.

Supporting our results obtained from the CNS group, chronic or repeated stress exposures were reported to increase CRH mRNA levels in the hypothalamus (Sawchenko et al., 1993; Makino et al., 1995b; Figueiredo et al., 2003). It has been reported that chronic psychogenic stress induced morphological plasticity in CRH synthesizing neurons of the hypothalamus and this may be the cause of increased excitability of CRH neurons during chronic stress (Miklós and Kovács, 2012). It has also been previously reported that the effects of chronic stress are mediated by synaptic inputs to CRH neurons in the hypothalamus (Sawchenko et al., 1993) and interactions between sensory stimuli and corticosterone determine how afferent inputs regulate the *CRH* gene expression (Watts, 1996). Furthermore, the mean daily corticosterone levels were reported to act as a facilitator agent to support *CRH* gene transcription during sustained stress (Tanimura and Watts, 1998; Watts, 2005). Therefore, considering that corticosterone levels were not different in the stress condition compared to the control condition in our study, neural inputs could cause up-regulation of CRH mRNA during the chronic noise exposure.

In the present study, both acute and chronic noise stress exposures up-regulated CRH-R1 mRNA in the hypothalamus. Supporting our results, different types of chronic (Makino et al., 1995a; Harris et al., 2006) and acute (Aguilar-Valles et al., 2005; Harris et al., 2006) stressors were reported to cause similar up-regulation in the CRH1 receptor expression in the hypothalamus. This is the first time we demonstrated that a psychological stressor, namely noise, induced up-regulation of CRH and CRH-R1 mRNA expression. It had been suggested that hypothalamic CRH may up-regulate its own receptor expression (Imaki et al., 1996; Makino et al., 2002). Therefore, hypothalamus CRH-R1 mRNA expression occurring concomitantly with CRH mRNA may indicate a positive feedback loop in which CRH induced up-regulation of its own receptors. This may be a critical mechanism that prepares CRH neurons for subsequent challenges in novel and unexpected conditions.

Both a decrease and no change in CRH-R2 mRNA levels after different stressors were reported in the hypothalamus and it has been suggested that the regulation of *CRHR2* gene expression is different from *CRHR1* (Vázquez et al., 2003; Harris et al., 2006). Similarly, both a decrease and increase, and no change in the expression levels of GR mRNA in the hypothalamus were reported by different stress treatments (Karandrea et al., 2002; Mizoguchi et al., 2003; Aguilar-Valles et al., 2005; Raone et al., 2007). These studies revealed that the changes in expression levels of the receptors are stressor-specific and our results showed that noise stress did not significantly affect the expression levels of CRH-R2 and GR mRNA in the hypothalamus. Also GRs were reported to have a lower affinity for corticosterone and are activated by increased hormone concentrations after stress exposure (Reul and Kloet, 1985). Since the corticosterone concentrations were not significantly higher than normal after stress exposure in this study, no change in GR mRNAs may be expected.

In the present study, mRNA expression of GR was down-regulated by chronic noise stress in the hippocampus. Different types of chronic physical stressors have been reported to down-regulate GR expression in the hippocampus (Kitraki et al., 1999; Paskitti et al., 2000; Raone et al., 2007). Although decreased GR expression by chronic stress has been ascribed to the increased ligand corticosterone levels (Mizoguchi et al., 2003; Raone et al., 2007), high corticosterone levels may not be the exclusive regulator of GR mRNA down-regulation. In the literature, there are several studies showing a disassociation between circulating corticosterone and GR mRNA levels (Kitraki et al., 1999; Makino et al., 2001, 2002; Aguilar-Valles et al., 2005). It has been suggested that synaptic inputs play a prominent role in the regulation of GR mRNAs. Monoaminergic, catecholaminergic systems and NMDA or GABA-A receptors are involved in the neural regulation of GR mRNA expression (Herman, 1993; Tritos and Kitraki, 1999). Since corticosterone levels did not significantly increase in our study, down-regulation of GR mRNA expression in CNS animals may be neuronally modulated, indicating that the regulation of GR mRNA expression is not a simple function of circulating glucocorticoid levels. An inhibitory role has been suggested for the hippocampus in HPA axis regulation, and it has been reported that loss of glucocorticoid receptors in the hippocampus may be associated with HPA up-regulation (Sapolsky et al., 1984; Issa et al., 1990; Herman and Cullinan, 1997).

Furthermore, CRH mRNA up-regulation in the hypothalamus was reported in rats bearing hippocampal lesions (Herman et al., 1989). Therefore, our results of decreased GR mRNA in the hippocampus and increased CRH, CRH-R1 mRNAs in the hypothalamus are compatible with each other.

The hippocampus CRH-R1 mRNA levels decreased by acute and chronic noise exposure, while the expression levels of CRH and CRH-R2 mRNAs did not change in this study. The data from previous stress studies yielded mixed results regarding changes in the expression of these genes in the hippocampus (Iredale et al., 1996; Aguilar-Valles et al., 2005; Marini et al., 2006; Veenit et al., 2014). It may be suggested that the effect of stress on CRH and receptors in the hippocampus is complex and influenced by the duration and type of the stress. In line with our findings, decreased CRH-R1 mRNA expression in the hippocampus was also found in mice subjected to restraint stress (Chen et al., 2004). Likely, ligands CRH and urocortins are released in the brain during stress (Hauger and Dautzenberg, 2000; Bale and Vale, 2004); therefore, increased ligand binding may be the possible underlying mechanism for stress-induced down-regulation of CRH-R1 mRNA, and this may be an adaptive response to stress.

In conclusion, we show white noise-induced changes in mRNA levels of stress-related genes in the rat brain with a focus on hypothalamic and hippocampal structures. The noise stress-induced changes are specific both for the molecules and within the structures. The changes in the receptor mRNA levels are not correlated with corticosterone responses. Further studies are required to clarify the molecular mechanisms underlying the regulation of stress-related genes in the brain and the functional relevance of these findings.

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