

## Research paper

## Lateral Hypothalamus Corticotropin-releasing Hormone Receptor-1 Inhibition and Modulating Stress-induced Anxiety Behavior

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**ABSTRACT**

**Introduction:** Stress is a reaction to unwanted events disturbing body homeostasis and its pathways and target areas. Stress affects the brain through the lateral hypothalamic area (LHA), the orexinergic system that mediates the effect of corticotropin-releasing hormone (CRH) through CRH Receptor Type 1 (CRHr1). Therefore, this study explores the outcome of stress exposure on anxiety development and the involvement of the LHA through LHA-CRHr1.

**Methods:** Male Wistar rats (220-250 g) implanted with a cannula on either side of the LHA received acute or chronic stress. Subsequently, exploratory behavior was examined using the Open Field (OF), and anxiety was tested by Elevated Plus Maze (EPM). Before sacrifice, the cerebrospinal fluid (CSF) and the blood were sampled. Nissl stain was performed on fixed brain tissues.

**Results:** Acute stress reduced exploration in of and increased anxiety in EPM. LHA-CRHr1 inhibition reversed the variables to increase the exploration and decrease anxiety. In contrast, chronic stress did not show any effect on anxiety-related behaviors. Chronic stress decreased the cell population in the LHA, which was prevented by the CRHr1 inhibition. However, the CRHr1 inhibition could not reverse the chronic stress-induced increase in the CSF orexin level. Furthermore, plasma corticosterone levels increased through acute or chronic stress, impeded by the inhibition of CRHr1.

**Conclusion:** Our results recognize LHA-CRHr1 as a capable candidate that modulates acute stress-induced anxiety development and chronic stress-induced changes in the cellular population of the region.

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## Highlights

- Acute stress, increased immobility of the rat in open field and elevated plus maze.
- Chronic stress, increased orexin production while decreasing neuronal survival.
- The anxiety and immobility were not developed in presence of CRHr1.
- CRHr1 blocking reversed the chronic stress changes in corticosterone and orexin.

## Plain Language Summary

Lateral Hypothalamus (LH) is a region involved in sleep and appetite regulation and recently known to play role in stress patho-physiology. The stress mediating function of the LH is performed through Corticotropin Releasing Hormone Receptor type-1 (CRHr1). This study explored the role of LH- CRHr1 in anxiety development and orexin production. Acute and chronic stress affected the behavior and molecular changes, differently. The acute stress increased the anxiety condition, while the chronic stress could only change the molecular criteria. Although we assumed that the inability of the chronic stress to develop anxiety may be attributable to habituation, the chronic stress could increase the plasma corticosterone and orexin level. All of the stress mal-changes in behavior and molecular level prevented by antagonising CRHr1 in the LH, indicating a gating function of LH-CRHr1 for stress development.

### 1. Introduction

**S**tress, as an unpredictable and somehow devastating outcome (Lupien & McEwen, 1997; McEwen & Sapolsky, 1995), leads to physiologic imbalances, such as homeostatic challenges and functionality of the hypothalamic-pituitary-adrenal (HPA) axis (Bale & Vale, 2004). This activation is directed to over-activity of the axis structures beginning with the paraventricular nucleus (PVN), which stimulates the pituitary gland by its corticotropin-releasing hormone (CRH). Finally, it increases the production of adrenal corticosterone (CRT) (Smith & Vale, 2006; Tsigos & Chrousos, 2002). The CRH and CRT may change the stress-related structures (Kim, Pellman, & Kim, 2015).

The orexinergic neuronal population of the lateral hypothalamic area (LHA) receives CRH-containing fibers from hypothalamic PVN (Carolyn, Christine, & Tallie, 1998), affecting the neurons through CRH receptors (CRHr1) (Winsky-Sommerer, Boutrel, & Lecea, 2005; Winsky-Sommerer et al., 2004). The secreted orexin-A regulates the arousal/wakefulness and motivation due to activation (Sakurai et al., 2005; Tsujino & Sakurai, 2013) and thereby projects to various brain regions (Nambu et al., 1999; Peyron et al., 1998), including cortico-limbic, hypothalamic, and peripheral structures (Gunnar & Quevedo, 2007). Any increase in arousal may also result in

stress and anxiety. Thus, the orexin peptide may create stress-induced changes in the behavior and physiological aspects of stress (Carter, Borg, & de Lecea, 2009; Winsky-Sommerer et al., 2005). Accordingly, the perfusion of orexin increased neuronal activity of the BNST slice preparation and anxiety in the behavioral examination (Lungwitz et al., 2012). In addition, knocking out the mice orexin gene has shown blunted responses following either acute or chronic stress (Kayaba et al., 2003). Furthermore, acute stress influences orexin neurons and increases the secretion of orexin as a response to CRH release (Winsky-Sommerer et al., 2004).

Orexin is critical in orchestrating a panic state, including anxiety and depression (Johnson, Molosh, Fitz, Truitt, & Shekhar, 2012). Palotai et al. demonstrated that intracerebroventricular (ICV) injection of the neuropeptide orexin caused an increase in visiting the dark chamber in the Elevated Plus Maze (EPM) apparatus (Palotai, Telegdy, & Jaszberenyi, 2014). Furthermore, pharmacologic inhibition of Orexin Receptor 1 (OXr1) ameliorated the pentylenetetrazole-induced anxiety disorder (Kordi Jaz et al., 2017). Conversely, homozygous orexin-deficient mice revealed an increase in the anxiety measure in the light-dark maze and the EPM (Khalil & Fendt, 2017). On the other hand, the orexin neuronal population is susceptible to stress conditions induced morbidity, which mainly results in narcolepsy (Crocker et al., 2005). The LHA has a heterogeneous population of neurons with the two types of stress-related neurons,

orexin, and MCH (melanin-concentrating hormone) neurons, which are influenced mainly by homeostatic perturbations (Bonnayon, Mickelsen, Fujita, de Lecea, & Jackson, 2016).

Based on the survival sensitivity of LHA neurons to stress, exploring the role of CRHr1 on orexin neurons, their survival, and the consequent anxiety may yield a better recognition of the stress and anxiety interrelationship. Therefore, this study explores the consequence of the CRHr1 blockade on anxiety and neuronal survival modification due to acute or chronic stress.

## 2. Material and Methods

### Study animals

Male Wistar rats (weighing 200–250 g) were obtained from Razi Institute (Karaj City, Iran) and were kept five per cage with the standard condition. All experiments were performed based on the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 23-80, revised 1996) and adapted to the ethical standards for research, the care and use of animals in Damghan University. Rats were maintained on a standard 12-12 hour light/dark cycle, with lights on at 07:00 AM and food and water ad libitum.

### Study drugs

The non-peptide CRHr1 antagonist (NBI27914 hydrochloride; Tocris), injected intra-LHA, was prepared in 10 µg/µL doses in 0.2 µL. The antagonist was dissolved in diluted DMSO (dimethyl sulfoxide) that reached the concentration of 2% by adding normal saline. Ketamine (90 mg/kg, IP) and xylazine (6.0 mg/kg, IP) anesthetics were obtained from local markets in Iran.

### Surgery

Cannulae were surgically implanted into both LHAs under anesthesia using a mixture of ketamine and xylazine anesthetics, while rats were placed in a stereotaxic device (Stoelting Instruments, USA), and their body temperature was kept stable with a towel mat. Stainless steel guide cannulae (23 gauge) were bilaterally positioned above the LHA, including 1 mm longer tip infusion cannulae (30 gauge) based on coordinates (AP=-2.8 mm caudal to bregma, ML=±1.2 mm, and DV=-10 mm ventral from skull surface) from the atlas of Paxinos and Watson for the rat brain (Mokhtarpour, Salmani, Lashkarbolouki, Abrari, & Goudarzi, 2016). The guide cannulae were anchored using surgical screws

and cemented to the skull. The infusion cannulae were substituted with solid ones and cut to protrude 0.5 mm out of the guide cannulae to preclude obstruction between daily infusions. Surgery aimed to infuse CRHr1 antagonist (in drug injection groups) or saline/DMSO (in the remaining groups). Tetracycline antibiotic was used on the wounded area to inhibit impurities.

The antagonist, NBI27914 (NBI), or saline/DMSO, was infused bilaterally into the LHA of awake, freely moving rats on day 8 following the surgery. The drug infusion system was a cannula (30 gauge) attached to a polyethylene tubing (PE-20) and a 1-µL Hamilton syringe from the other end. The infusion took three minutes for each animal, with the cannula kept on the site for about 5 minutes to let the drug be infused thoroughly. Single intra-LHA infusions were performed once daily during 10 day-stress model, 20 min before stress application. The correct cannulae placement was certified during brain extraction and confirmed on the three brains used for Nissl staining per group (Figure 1).

### Stress paradigm

Acute stress comprised two consecutive; 0.7 mA intensity foot-shocks taking two seconds, set apart by 5.5 min intervals, on a grid floor, using the shocking device (Noldus, Netherlands). Plexiglas restrainer was used to apply chronic stress, six hours a day for 10 days, starting at 8 AM every day.

### Experimental design

The study was divided into two experiments. Each consists of three groups. Three brains were extracted and assigned for Nissl stain, the Cerebrospinal Fluid (CSF) was drained from five rats per group for orexin assay, and the plasma was separated from the blood samples of five animals. The group descriptions are as follows (Figure 2).

#### First experiment

**Control (CTRL; n=9):** the animals of this group tolerated neither the stress nor the antagonist injections; they received the saline/DMSO following recovery from surgery exclusively.

**Acute stress (A.STS; n=9):** the acute stress (see above), on day 8 (24 hours post-recovery) following surgery, was applied to animals that were evaluated 30 min later by the open field (OF), and 20 min later, the EPM test (Figure 3).

**Acute stress-NBI (A.STS.NBI; n=10):** the animals received the shock 20 min after the infusion of CRHr1 antagonist, and following 30 min, the OF and another 20 min, the EPM test were applied.

## Second experiment

**Control (CTRL; n=8-10):** the animals of this group were treated the same as in the first experiment; they received the saline/DMSO following recovery from surgery exclusively.

**Chronic stress (Ch. STS; n=10-12):** following the recovery, the rats experienced immobilization stress; six hours a day for 10 days. Twenty-four hours after the end of the last stress day, they were evaluated in the OF box. Twenty minutes later, the EPM test was performed (Figure 3).

**Chronic stress-NBI (Ch.STS.NBI; n=10-12):** the animals received an intra-LH injection of NBI followed by a daily six-hour restraining up to 10 days during the chronic treatment. Finally, the behavioral appraisal of OF and EPM was accomplished.

## OF test

Plexiglas square box (70×70×35) with 16 squares of the same size (4 centers, 12 externals on the floor side) was used to assess the exploratory behavior of the rat. The duration of staying immobile, as an inverse criterion for the exploratory behavior, was considered the variable (Bahramzadeh Zoeram, Elahdadi Salmani, Lashkarbolouki, & Goudarzi, 2018). The number of entering the central square (number in the center), the number of rearing (rearing number), and the number of crossing the squares (crossing number) were also evaluated.

## EPM test

A painted flat black plywood device was used as the EPM apparatus to assess anxiety behavior (Walf & Frye, 2007). Two open arms were facing oppositely and two closed arms facing oppositely, each 10 cm wide and 50 cm in length, including a 10×10 cm square in the middle, are the structural features of the apparatus standing on the height of 53 cm of a wooden base. The open arms had a small edge to protect the rats from gliding off the platform. Conversely, the closed arms' border height comprised a thin wooden 40 cm wall. Before beginning the experiments, the animals experienced a freely moving activity in a wooden box (60×60×40) for one minute to increase their exploratory behavior. Then, for the main experiment, the animal was put in the central square facing an open arm

and monitored visually for 5 minutes, moving or staying in the device. The percentage of the time the animal spent either in the Open Arm (OAT) or the Closed Arm (CAT) was calculated. Anxiety is expressed as an increased ratio of OAT to CAT, which means an increase in OAT and or a decrease in CAT (Walf & Frye, 2007).

## Orexin measurement

Orexin is the primary production of LHA orexinergic neurons, and its measurement in the CSF will help confirm the functionality of orexin neurons and its final secretion into target areas. The CSF was drained from cisterna magna at the end of the procedure and stored at -70°C for later assessing orexin level as a criterion of LHA orexin neuronal activity using an ELISA kit (MBS762821, MyBioSource, USA). Briefly, the extraction method (Nirogi et al., 2009) was as follows; after anesthesia with ketamine and xylazine, the rat was placed in a stereotaxic apparatus and positioned flexed (45°) angle using the ear bars. Then, the shaved back-head area was disinfected with 70% alcohol, and a 23-gauge needle connected to a polyethylene tube, in line with a small volume syringe, was inserted in a small, depressed surface between occipital protuberance and the atlas bone. Feeling a change in the movement of the needle, a gentle aspiration was applied, which drained the CSF into the tube. During the measurement and upon the kit protocol, optical densities were read at 450 nm. The orexin content of CSF was quantified in the order of pg/mL. The intra-assay coefficient of variation was <8%, and the inter-assay coefficient was <10%.

## Plasma CRT measurement

As a criterion for stress application, the potential effect of CRHr1 blocking on stress level, i.e., the plasma CRT, was measured. After the experiments, the blood was sampled from the trunk veins under anesthesia. Then, the centrifuge at 2320×g for 20 min was employed, and the supernatant was taken as the plasma, which was stored at -70°C until molecular measurement. During the assay, the amount of plasma CRT was evaluated by an immune Enzyme Assay (EIA) competitive kit (Cayman Chemical; cat, 500655). Briefly, 50 µL out of 100 µL of supernatant plasma was incubated with CRT Acetylcholine Esterase (AChE) tracer (50 µL) and CRT EIA antiserum (50 µL) for 2 hours at 22°C in each well. Subsequently, the plate was buffer washed using 200 µL of volume three times, and then the 200 µL of Ellman's buffer (The substrate for AChE) was added to the kit and incubated in the dark for 60-90 min on a horizontal slow-swing shaker. Finally, the absorbance of plate content was

measured at 412 nm using a spectrophotometer, and a standard curve was drawn to calculate the plasma CRT content. The kit's sensitivity was 150 pg with a signal-to-noise ratio of 2:1.

### Nissl stains

This method stains the nuclei of the neurons in the interested region to evaluate the changes in neuronal survival. Since the method does not differentiate between different neurons and even the glia, it was used as an indirect measure of structural changes concomitant to the behavioral and molecular alterations. For this purpose, deeply anesthetized animals with ketamine and xylazine were perfused with 50 mL of formalin 10% through ascending aorta. Following liver clearance, as insurance criteria for perfect perfusion, the extracted brains were submerged in formalin for 24-48 hours. On the testing day, brains were sectioned into 10  $\mu$ m slices and stained with cresyl violet (Sigma). After drying out, five consecutive serial sections were inspected through a grid (250  $\mu$ m)- installed light microscope, and the cells of LHA were counted at 40x magnification. The average cell numbers for five region sections were conducted as the final approximation.

### Data analysis

The mean values of multiple independent groups were compared by employing ANOVA statistics using SPSS v.23 software. Tukey test was employed as a post-hoc comparison when the ANOVA was statistically significant. The  $P < 0.05$  was considered the minimum level of significance. The data were presented as Mean  $\pm$  SEM.

## 3. Results

### CRHr1 antagonist preventing anxiety development induced by acute stress

Animals in the acute and chronic stress-treated experiments were subjected to OF and EPM tests. The acute-stress treated groups showed significant differences for the immobility time ( $F[2, 25]=38.64, P=0.0005$ ), number of crossed squares ( $F[2, 25]=25.39, P=0.0005$ ), number of crossing the central square ( $F[2, 25]=5.21, P=0.013$ ) and total rearing number ( $F[2, 25]=28.24, P=0.0005$ ) in OF apparatus. Comparing the behavioral results by the Tukey test for OF test in the acute-stress experiment showed a significant decrease in crossing number ( $P < 0.001$ ), number in the center ( $P < 0.05$ ), and rearing number ( $P < 0.001$ ); and an increase in the immobility time ( $P < 0.001$ ) in Acutely Stressed (A.STS)

animals. Blocking the CRHr1 in the LHA of Acutely Stressed Animals (A.STS.NBI) recovered the four criteria of crossing number ( $P < 0.01$ ), number in the center ( $P < 0.01$ ), rearing number ( $P < 0.001$ ), and immobility time ( $P < 0.001$ ) (Figure 3-A1-z4) in the acute-stress experiment. The ANOVA comparison of the four criteria in OF test in the chronic-stress experiment did not reveal any remarkable results (Figure 3-B1-4).

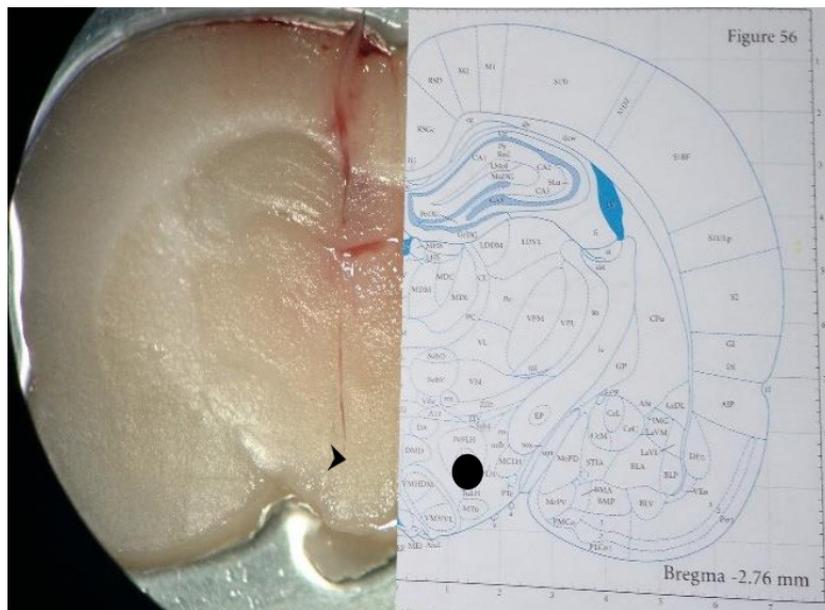
ANOVA comparison of the acute-stress treated experiment in EPM test revealed significant differences in OAT ( $F[2, 23]=18.03, P=0.0005$ ), center ( $F[2, 23]=2.71, P=0.08$ ) and CAT [ $F[2, 23]=12.34, P=0.0005$ ]. Between groups comparison of the acute experiment for the acutely stressed animals showed a decrease in OAT ( $P < 0.001$ ), which was reversed by CRHr1 blocking ( $P < 0.05$ ), and an increase of CAT ( $P < 0.001$ ) that was recovered due to NBI administration ( $P < 0.01$ ) (Figure 4-A). In contrast to the acute experiment, the chronic stress showed no significant difference either in the EPM test of the open arm and closed arms in the chronic experiment (Figure 4-B).

### CRHr1 antagonist restoring the effect of chronic stress on plasma CRT level

Analysis of variances for plasma CRT level in different groups of acute and chronic experiments showed a between-group significant difference for the first ( $F[2, 12]=24.13, P=0.0005$ ) and the second ( $F[2, 12]=39.71, P=0.0005$ ) experiments. The comparison using Tukey post-hoc revealed an increase of CRT in the acute-stress (A.STS;  $P < 0.01$ ) treated group and the inability of CRHr1 blocking to reverse the effect. The post-hoc comparison for the second experiment demonstrated an increase in corticosterone concentration in the chronically stressed animals (Ch. STS;  $P < 0.001$ ), which was compensated by the CRHr1 blockade ( $P < 0.01$ ). (Figure 5).

### CSF orexin level buildup following chronic stress

ANOVA of the acute treatment revealed no significant change in CSF orexin ( $F[2, 12]=3.06, P=0.08$ ). In contrast, in the chronic treatment of chronically stressed animals with (Ch.STS.NBI) or without (Ch. STS), the infusion of the CRHr1 antagonist revealed significant differences in the level of CSF orexin ( $F[2, 12]=6.21, P=0.014$ ). Between groups, Tukey's post-hoc comparison demonstrated that CSF orexin significantly increased due to either chronic stress (Ch. STS,  $P < 0.05$ ), as compared to the control group, or the infusion of NBI (Ch.STS.NBI;  $P < 0.01$ ) compared to the chronic group (Figure 6).



**Figure 1.** Placement of Cannula in Lateral Hypothalamus Area (LHA) confirmed by histology

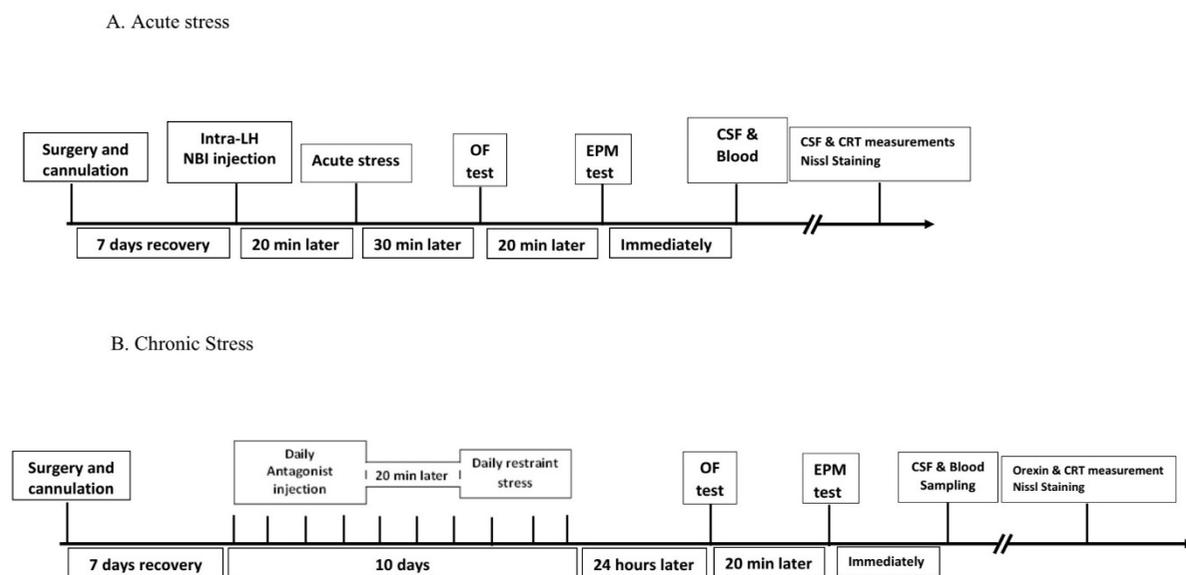
**NEURSCIENCE**

The image represents a coronal section based on the atlas of Paxinos and Watson at a 2.8-mm distance from bregma. The black circle and arrow show the injection site and the tip of the cannula on the left and right sides, respectively.

**CRHr1 antagonist preventing the reduction of neuronal survival in the LHA**

Hypothalamus sections were Nissl stained after the end of the treatment period, and neurons of the LHA region were counted in five slices and averaged for each brain to

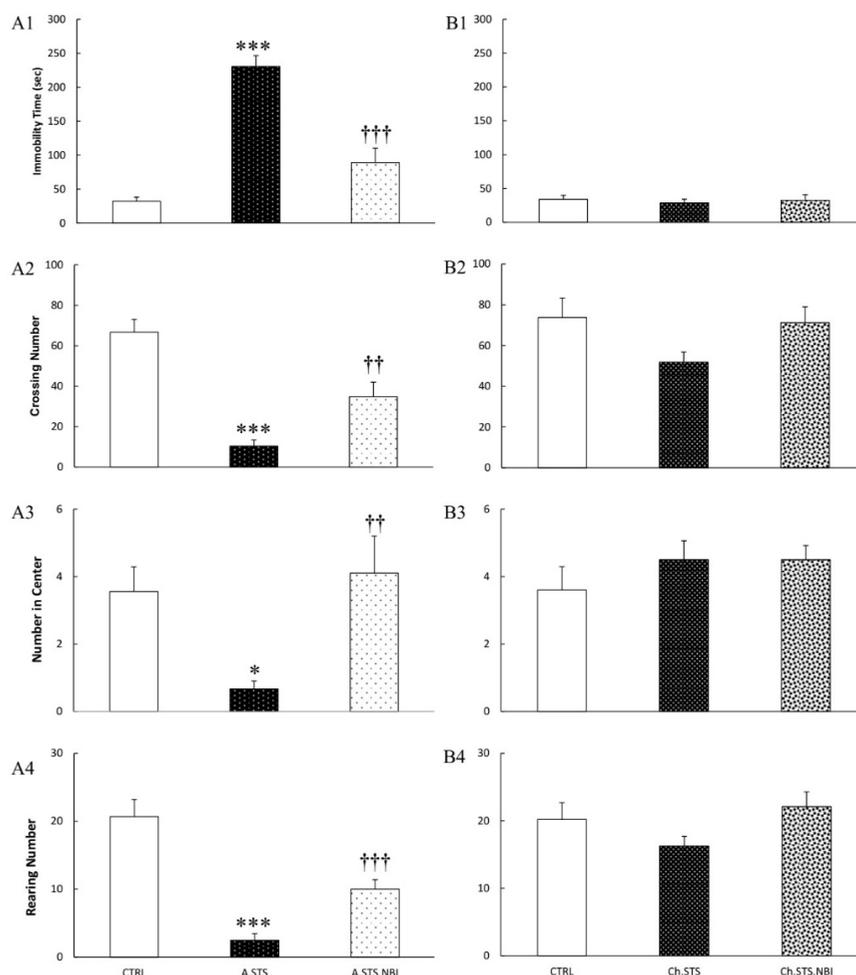
assess the surviving cell numbers. The comparison with 1-way ANOVA of the acute treatment revealed no significant difference between the control and acute stress groups regarding the effect of CRHr1 blocking groups ( $F[2, 12]=2.35, P=0.14$ ). On the other hand, the chronic treatment demonstrated a remarkable difference between



**Figure 2.** Experiment timeline

**NEURSCIENCE**

One week after surgery and cannulation, saline or Corticotropin Releasing Hormone (CRHr1) antagonists were infused, and 20 min later, the animals experienced foot-shock (acute) or restraint (chronic) stress. Following 30 min in acute stress and 24 hours in chronic one, the open field (OF) and then Elevated Plus Maze (EPM) tests were performed separated by 20 min intervals. Immediately after the EPM test, Cerebrospinal Fluid (CSF) was drained through cisterna magna, and plasma was separated from trunk blood; both samples were stored at -80°C until measurement.



**Figure 3.** CRHr1 Blocking of Lateral Hypothalamus Area (LHA) neurons precluding the change of immobility following stress

Acute stress increases immobility while decreasing crossing, center, and rearing numbers. Perfusion of NBI could reverse all of the variables mentioned above. The chronic stress demonstrated no effect.

\*\*\* $P < 0.001$  and \* $P < 0.05$  compared with the CTRL group, ††† $P < 0.001$  and †† $P < 0.01$  compared with the A.STS group.

CTRL (n=9-10): control; A.STS (n=9): acutely stressed; A.STS.NBI (n=10): acutely stressed with the corticotropin releasing hormone (CRHr1) antagonist infusion; Ch.STS (n=12): chronically stressed; Ch.STS.NBI (n=12): animals in chronic stress condition that infused with CRHr1 antagonist.

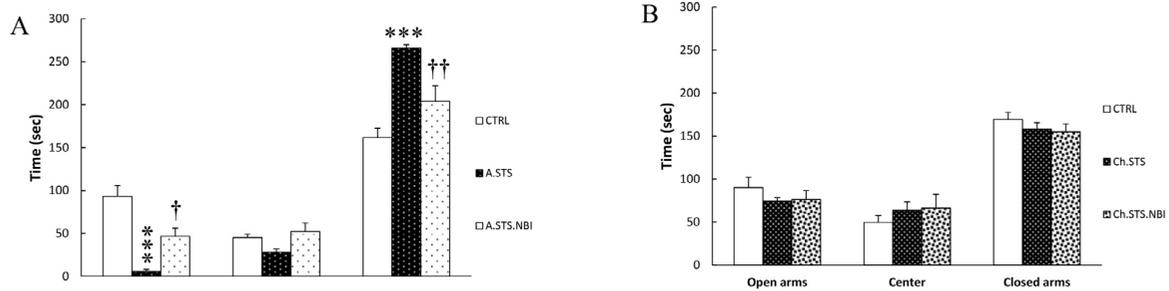
Data are presented as Mean  $\pm$  SEM.

different groups of control, chronic stress, and CRHr1 blockade ( $F[2, 12]=293.54, P=0.0005$ ). Tukey's post-hoc comparison of Nissl stained sections showed that the total cell count was reduced following the chronic stress ( $P < 0.001$ ), while the CRHr1 blocking of chronically induced animals restored the neuronal count (Ch.STS.NBI;  $P < 0.001$ , Figure 7).

#### 4. Discussion

This research explored the effect of CRHr1 blockade on anxiety development, CSF orexin level, plasma CRT level, and LHA neuronal survival following stress. The

results demonstrated that acute stress increased immobility and anxiety based on the exploration of the animals in the OF and the duration of closed arm visits in the EPM, which were deterred by the blockade of LHA-CRHr1. Furthermore, the plasma CRT level was heightened following the acute and chronic stresses, while it was recovered exclusively following the CRHr1 blocking in the chronic condition. Furthermore, chronic restraint stress augmented the orexin content without compensation following the blocking in the chronic conditions.



**NEURSCIENCE**

**Figure 4.** Corticotropin Releasing Hormone (CRHr1) Blocking of Lateral Hypothalamus Area (LHA) preventing the anxiety changes developed by acute stress

The decrease of open arm (OAT) and the increase of close arm (CAT) after the acute stress were reinstated to the control condition following the blockade of LH-CRHr1. The chronic stress and the following receptor blocking revealed no effect.

\*\*\*P<0.001 compared with CTRL, \*\*P<0.01 and †P<0.05 compared with A.STS.

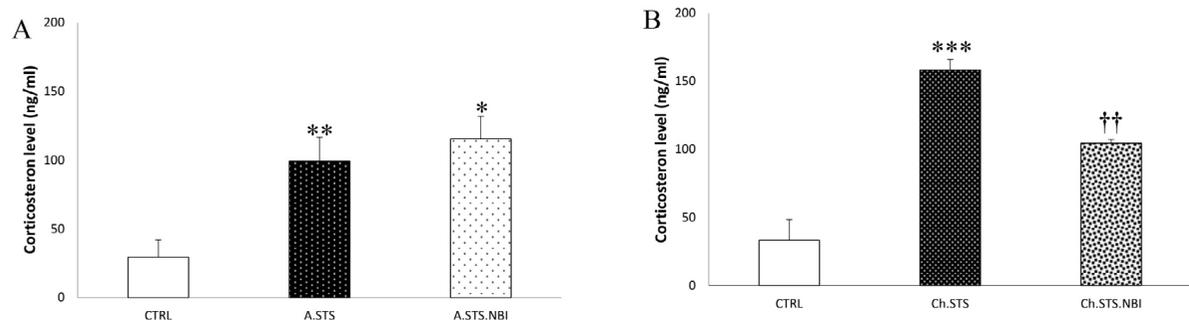
CTRL (n=9-11): control; A.STS (n=7): acutely stressed; A.STS.NBI (n=10): acutely stressed with the CRHr1 antagonist infusion; Ch.STS (n=12): chronically stressed; Ch.STS.NBI (n=12): animals in chronic stress condition that infused with CRHr1 antagonist.

Data are presented as mean±SEM.

Stress creates anxiety behaviors and certain emotional disorders (Johnson et al., 2012). Since LHA is involved in the orchestration of adaptive responses to undesirable challenges, it appears that stress may impact the LHA indirectly to form homeostasis (Mickelsen et al., 2017). The exposure to acute stress in our experiments confirmed the results of previous studies on stress-induced anxiety development (Atchley, 2010; Solomonow, 2015), probably through the demonstration of an increase in the anxiety behavior in the EPM and simultaneous reduction of the exploratory behavior in the OF. On the contrary, chronic restraint stress could not provoke anxiety and immobility in the animal behavior by testing in either the EPM or OF, in an apparent contradiction to the literature which has shown chronic stress-induced

deterioration in the behavior and molecular mechanisms (Chiba, Numakawa, Ninomiya, Richards, Wakabayashi, & Kunugi, 2012; Greenwood, Thompson, Opp, & Fleshner, 2014; Qin et al., 2015). Similarly, our previous study showed that the same chronic restraint stress could not produce a change in anxiety behavior which confirms our proposed hypothesis regarding the chronic stress-induced adaptation (Bahramzadeh Zoeram et al., 2018) and the potential reduction of HPA responsiveness (Grisson, Iyer, Vining, & Bhatnagar, 2007).

The orexin system, as the primary neurons of the LHA, connected functionally to the HPA origin, is involved in many panic and anxiety disorders by orchestrating adaptive panic/defense (anxiety, cardiorespiratory, and en-



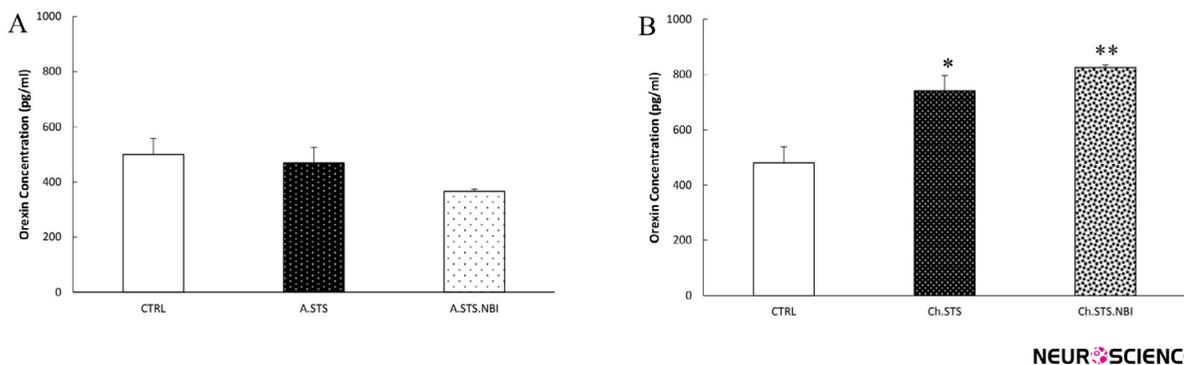
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**Figure 5.** LH-CRHr1 blocking counteracting the change of plasma corticosterone (CRT)

The acute or chronic stress and acute stress with NBI increased the level of CRT in plasma, while the level was only counteracted, through CRHr1 blocking, in the chronic treated group.

\*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 compared with CTRL, †P<0.01 compared with Ch.STS.

CTRL (n=5): control; A.STS (n=7): acutely stressed; A.STS.NBI (n=5): acutely stressed with the CRHr1 antagonist infusion; Ch.STS (n=5): chronically stressed; Ch.STS.NBI (n=5): animals in chronic stress condition that infused with CRHr1 antagonist. Data are presented as Mean±SEM.



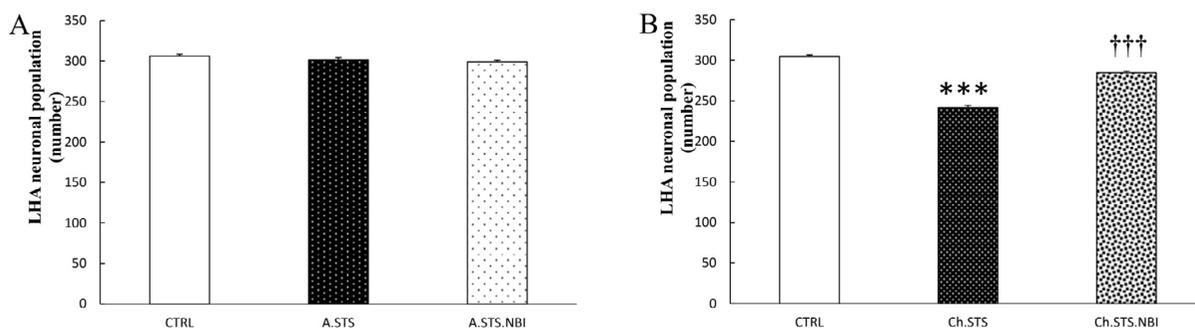
**Figure 6.** Chronic stress and Corticotropin Releasing Hormone (CRHr1) increasing Cerebrospinal Fluid (CSF) orexin levels

The antagonist infusion cannot recover the chronic stress-induced increase level in the chronic conditions. The acute stress treatment could not change the orexin level. \*P<0.05 and \*\*P<0.01 compared with CTRL.

Abbreviations: CTRL (n=5), control; A.STS (n=5), acutely stressed; A.STS.NBI (n=5), acutely stressed with the CRHr1 antagonist infusion; Ch.STS (n=5), chronically stressed; Ch.STS.NBI (n=5), animals in chronic stress condition that infused with CRHr1 antagonist. Data are presented as Mean±SEM.

doctrine) responses (Johnson et al., 2012). In this study, blocking the CRHr1 of LHA neurons revealed a recovery of acute stress-induced anxiety disorder and exploratory behavior. CRH, as the starter hormone of the HPA, affects LHA neurons not only through fibers originating from PVN (Winsky-Sommerer et al., 2004) but also via axons originating from the amygdala and BNST extra-hypothalamic regions involved in the stress responses (Keegan et al., 1994; Lungwitz et al., 2012). Accordingly, orexin neuronal activity reduced after the acute stress in knockout mice for CRHr1 (Winsky-Sommerer et al., 2004), which further confirms CRHr1 involvement in the acute stress behavioral responses. Thus, CRHr1 may play a gating function in controlling LHA neuronal activity and stress-induced behavioral responses.

The acutely applied electric shock stress has been shown to increase CRH production, affecting orexinergic neurons to increase their orexin secretion (Winsky-Sommerer et al., 2005) and expression (Mokhtarpour et al., 2016). The acute stress could not change the orexin production in this experiment, and even our previous study (Bahramzadeh Zoeram et al., 2018), ruled out the orexin involvement in acute stress affected anxiety and exploratory behaviors. However, the mentioned acute shock stress induced an increase in the expression of the orexin in our previous work (Mokhtarpour et al., 2016). Following acute stress, the expression-production discrepancy highlights the time needed for the hormone from expression to production and reaching the CSF compared with the synaptic-induced neurotransmitter



**Figure 7.** Chronic Stress-Induced Reduction of the Lateral Hypothalamus Area (LHA) Neuronal Population Prevented Through Corticotropin Releasing Hormone (CRHr1) blocking

The antagonist infusion recovered the chronic stress-induced decrease of neuronal population, while NBI infusion precluded the effect. The acute stress treatment could not change the neuronal population. \*\*\*P<0.001 compared with CTRL and †††P<0.001 compared with Ch.STS.

CTRL (n=3): control; A.STS (n=3): acutely stressed; A.STS.NBI (n=3): acutely stressed with the CRHr1 antagonist infusion; Ch.STS (n=3): chronically stressed; Ch.STS.NBI (n=3): animals in chronic stress condition that infused with CRHr1 antagonist. Data are presented as Mean±SEM.

release (Bahramzadeh Zoeram et al., 2018). Accordingly, Chen and Kirauc (2014) showed that the blockade of hypothalamic CRHr1 eradicated evoked fear induced by the acute shock stress but had no effect on the expression of pre-pro orexin (Chen, Li, & Kirouac, 2014), suggesting that the acute stress has differential effects on fear development and orexin production.

In contrast, the orexin level was shown to be increased following the chronic restraining stress in this study, which did not return to the control conditions after the CRHr1 blockade. This lack of effect following the receptor blockade confirms the mentioned hypothesis of the differential effect of stress on behavior and orexin production, an interesting subject for future directions. The chronic stress-induced orexin buildup in the CSF partly confirms the chronic condition time scale as the time for the expression, production, and secretion sequence.

Plasma CRT is a sensitive factor raised in response to acute or chronic stress (Bahramzadeh Zoeram et al., 2018; Modir, Elahdadi Salmani, Goudarzi, Lashkarbolouki, & Abrari, 2014). In this study, the amount of plasma CRT was heightened due to the acute stress, which may be due to the raised activity of HPA following stress (Garabadu et al., 2011). Similarly, the chronic consecutive stress treatment boosted the amount of CRT in plasma. The outcome of a chronic condition in plasma CRT may be a response of HPA to long-lasting stress (Lowrance, Ionadi, McKay, Douglas, & Johnson, 2016), which provokes CRT secretion through the axis as well as stimulation of other pathways terminating in LHA and produce orexin. The orexin, in turn, has been shown to increase HPA production. CRT (Johnson et al., 2012), which results from HPA activation following stress by stimulation of the PVN (Winsky-Sommerer et al., 2005), could be an alternative way of keeping the HPA in an active mode following chronic stress.

Orexin neurons are located in the perifornical lateral hypothalamus (Nambu et al., 1999), a strategic region on the stress pathway to the brain stem. These orexin neurons and, generally, LHA neurons are affected in narcoleptic conditions, and their population is decreased dramatically (Sakurai, Mieda, & Tsujino, 2010). Our data showed a noticeable reduction of the LHA neuronal population following chronic stress, similar to narcoleptic dogs and rats. Since the blockade of the CRHr1 in the LHA precluded the deteriorative effect of the chronic condition in the neuronal population, it is presumed that the effect may be activated through the PVN-LHA pathway impinging on the LHA neurons, which may make a loop of stress from PVN to orexin neurons and from

there back to PVN (Spinazzi, Andreis, Rossi, & Nussdorfer, 2006). This stress-likely positive feedback loop may over-stimulate the orexin neurons and pave the way to reducing neuronal viability. However, the remaining population may increase their activity following the chronic stress, which may justify the heightened production of orexin observed in this study. Another presumption may be explained by the heterogeneity of LHA neurons, in that the stress reduces the population of non-orexin LHA neurons, a direction for future research.

The results of this study demonstrate that acute stress increased anxiety and developed immobility, and CRHr1 inhibition reversed the effect. Neither chronic stress nor the receptor blockade could affect the behavior. On the other hand, chronic stress augmented the plasma CRT and CSF orexin while reducing LHA neuronal survival, which was reversed through the receptor blockade. The acute stress increased the plasma CRT, but not the orexin and survival of LHA neurons. The discrepancy in acute and chronic stress results may be due to the different activity thresholds for LHA neurons in developing a behavior and orexin production; these findings open new horizons for future investigations. The main conclusion of this research is that the LHA-CRHr1 blockade prevents the alteration in animal behavior following acute stress and the plasma CRT and CSF orexin level due to chronic stress.

## Ethical Considerations

### Compliance with ethical guidelines

All experiments were adapted to the ethical standards for research and the care and use of animals in Damghan University.

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### Authors' contributions

Experimental procedure and Data analysis: Masoumeh Eghtesad; Methodology, Data analysis and Writing-original draft: Mahmoud Elahdadi Salmani; Investigation and Resources: Taghi Lashkarbolouki; Conceptualization and Data analysis: Iran Goudarzi..

### Conflict of interest

The authors declared no conflict of interest.

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