Effects of chronic noise on mRNA and protein expression of CRF family molecules and its relationship with p-tau in the rat prefrontal cortex

Zhihui Gai, Kang Li, Huanrui Sun, Xiaojun She, Bo Cui, Rui Wang

Abstract

Chronic noise exposure has been associated with Alzheimer’s disease (AD)-like pathological changes, such as tau hyperphosphorylation and β-amyloid peptide accumulation in the prefrontal cortex (PFC). Corticotropin-releasing factor (CRF) is the central driving force in the stress response and a regulator of tau phosphorylation via binding to CRF receptors (CRFR). Little is known about the CRF system in relation to noise-induced AD-like changes in the PFC. The aim of this study was to explore the effects of chronic noise exposure on the CRF system in the PFC of rats and its relationship to tau phosphorylation. Male Wistar rats were randomly divided into control and noise exposure groups. The CRF system was evaluated following chronic noise exposure (95 dB sound pressure level white noise, 4 h/day × 30 days). Chronic noise significantly accelerated the progressive overproduction of corticosterone and upregulated CRF and CRFR1 mRNA and protein, both of which persisted 7–14 days after noise exposure. In contrast, CRFR2 was elevated 3–7 days following the last stimulus. Double-labeling immunofluorescence co-localized p-tau with CRF in PFC neurons. The results suggest that chronic noise exposure elevates the expression of the CRF system, which may contribute to AD-like changes.

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1. Introduction

Noise is becoming a widespread and daily source of stress in the living environments of modern societies. People are increasingly exposed to hazardous noise levels coming from many sources, including their work environment, urban traffic, the media, and household appliances. The World Health Organization documented that noise affects mental health, decreases work capacity, induces sleep disturbances, and may be a risk factor for cardiovascular diseases. Long-term noise exposure, in particular, is a health hazard, increasing the risk of physical damage. Such exposure can have physiological or even pathological effects on the classical auditory system, as well as on non-lemniscal brain regions such as the hippocampus and cerebral cortex. Such exposure has been associated with the persistent tau and β-amyloid peptide (Aβ) pathology in the hippocampus and prefrontal cortex that is observed in Alzheimer’s disease (AD), suggesting that chronic noise exposure might result in an increased risk of developing AD. However, the molecular mechanisms responsible for such noise-induced modifications in brain structures have not been established yet.

The physiological impact of stress is mediated by the hypothalamic-pituitary-adrenal (HPA) axis, a self-regulated pathway that utilizes its end product, corticosterone, to control its own activation through a negative feedback mechanism. In response to stress, neurons of the hypothalamic paraventricular nucleus secrete corticotropin-releasing factor (CRF), which binds to its type 1 receptor (CRFR1) in the pituitary gland to activate the HPA axis. While physical stressors can directly activate the HPA axis, psychological stressors indirectly regulate the HPA axis, requiring higher-order sensory processing via specific brain structures, such as the hippocampus and the amygdala. Noise is a physical and psychological stressor, exerting its effects on the HPA axis through these structures by connections with the auditory system.

The CRF system plays a prominent role in the coordination of neuroendocrine and neuropsychiatric responses to stress. The CRF system comprises CRF and its receptors, CRFR1 and CRFR2. CRF and its receptors are found in the hippocampus and PFC, and the dysregulation of the CRF system in these regions cause alterations in AD-like pathology in animals. Supporting a role for CRF in AD neuropathology, work from many laboratories has demonstrated that both CRF overexpression and acute or repeated exposure to stressors induce phosphorylated tau (p-tau) and accumulation of Aβ within the hippocampus, a process that

Abbreviations: AD, Alzheimer disease; BSA, bovine serum albumin; CRF, corticotropin-releasing factor; CRFR1/2, corticotropin-releasing factor receptor 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPA, hypothalamic-pituitary-adrenal; p-tau, phosphorylated tau; PFC, prefrontal cortex; TBS, Tris-buffered saline.

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is dependent on CRFR1 [9,11,13,14]. However, only limited information is available on the control of this system in the PFC.

CRFR1 and CRFR2 are widely but heterogeneously distributed in the central nervous system [15,16], suggesting distinctive functional roles for each receptor subtype. A prominent role for CRFR2 has been suggested in the regulation of anxiety-like behaviors after stress [17–20], but whether CRFR2 participates in AD-like responses to stress remains unclear.

Although the involvement of the CRF signaling system in AD-like changes induced by restraint or other stressors has been widely documented [9,11,13], the molecular mechanisms responsible for changes in this system following noise exposure have not been previously studied. In this study, we aimed to investigate the effects of noise stress on CRF system molecules and to explore the relationship between the CRF system and long-term noise-induced AD-like changes in rat PFC. For this purpose, mRNA and protein expression levels of CRF, CRFR1, and CRFR2 and the co-localization of CRF and p-tau in the PFC were analyzed after chronic white noise exposure.

2. Materials and methods

2.1. Animal use and experimental grouping

In total, 64 male 8-week-old Wistar rats (Lab Animal Center, Institute of Health and Environmental Medicine, Tianjin, P.R. China), weighing 200–220 g, were used in this study. The rats were kept in a room with a 12-hour light/dark cycle (with lights on from 06:00 to 18:00) and controlled ambient temperature (23 ± 2 °C) and humidity (50–70%). The rats had free access to water and food in their cages. The rats were habituated to the laboratory environment for 5 days prior to the start of the experiment, in which they were randomly assigned to either the noise-exposed or control group. Animals in the noise-exposed group were exposed to noise in a reverberation chamber with 95 dB sound pressure level white noise (4 h per day for 30 days, from 8:00 to 12:00). The animals were in wire-mesh cages placed in the center of the sound field, with one animal per cage. The loudspeaker was suspended directly above the cages. Rats in the control group were housed in similar cages but were exposed to background noise (below 40 dB sound pressure level) from another chamber. At different time points (days 0, 3, 7, or 14) after the final exposure, rats in the noise-exposed and control groups were sacrificed under chloral hydrate anesthesia (10%, 0.3 mL/100 g) for subsequent biochemical analyses (n = 8 rats per group and time point). Two rats from each time point and group were used for immunofluorescence, and 6 rats were used for ELISA, RT-PCR, and western blot analyses. Furthermore, two cortical tissue samples were isolated from the same part of the prefrontal cortex in each animal for RT-PCR and western blot analyses, respectively. All experiments were performed in accordance with approved guidelines specified by the Animal and Human Use in Research Committee of the Tianjin Institute of Health and Environmental Medicine.

2.2. Noise exposure apparatus

White noise was generated using a noise generator (BK 3560C, B&K Instruments, Denmark), amplified with a power amplifier (YONG-SHENG AUDIO-P-150D, The Third Institute of China Electronic Technology Group, China), and delivered through a loudspeaker (ZM-16 S, Tianjin Zennay Electroacoustic Equipment Co., Ltd., China). The center of the sound spectrum of the noise emitted from the speaker was in the range of 400–6300 Hz (1/3 octave). All exposures were performed as described in our previous study [5].

2.3. Determination of plasma corticosterone by ELISA

Rats were sacrificed at the time points indicated under Section 2.1, and blood samples were collected in tubes containing heparin sodium as an anticoagulant. Samples were centrifuged at 4 °C, and after separation, the plasma was stored at −80 °C until assayed using a corticosterone ELISA kit (BlueGene Biotech, Shanghai, China), according to the manufacturer’s instructions.

2.4. Determination of gene expression by real-time PCR

PFCs from noise-exposed and control rats were removed after the animals were sacrificed under chloral hydrate anesthesia, at the time points indicated in Section 2.1, and homogenized using a rapidly oscillating masher. Total RNA was extracted using an RNasy Mini kit (TakaRa Bio, Dalian, China), according to the manufacturer’s protocol. Total RNA was converted to cDNA by reverse transcription using a Transcriptor First Strand cDNA Synthesis Kit (TakaRa Bio, Dalian, China). Specific primers and probes designed for rat glyceraldehyde phosphate dehydrogenase (GAPDH; internal control), CRF, CRFR1, and CRFR2 sequences were used, as described in Table 1. Gene expression levels for CRF and CRFR1/2 were assessed by quantitative real-time PCR performed under the following thermal cycling conditions: 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 95 °C for 5 s followed by 57 °C for 30 s. Real-time PCR was performed using gene expression assays-on-demand and a Takara PCR Thermal Cycler Dice Real Time system (TakaRa Bio, Dalian, China). Target gene transcript levels were calculated after normalizing cycle thresholds (Ct) to GAPDH expression and are presented as fold-induction values (\(2^{-\Delta\Delta Ct}}\), \(\Delta Ct = Ct_{\text{GAPDH}} - Ct_{\text{Control group}}\) relative to those of control rats.

2.5. Western blot analysis

The PFC was dissected immediately and homogenized in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris–hydrochloric acid, pH 7.4, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM ethylenediaminetetraacetic acid). Homogenates were centrifuged at 14,000 × g for 15 min at 4 °C, and the supernatants were collected. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin (BSA) (Sigma, USA) as a standard. Proteins were then denatured in boiling water for 10 min. Samples (20 μg protein/lane) were run on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to microporous polyvinylidene difluoride membranes (0.45 μm, F. Hoffmann-La Roche Ltd., Germany) for 25 min at 12 V in a semi-dry blotting apparatus (DYPC-40C, Beijing Liuyi Instrument, China). Membranes were blocked with Tris-buffered saline (TBS) containing 2% (w/v) BSA and 0.5% Tween 20 (Sigma) for 1 h, incubated with primary antibodies for 12 h at 4 °C, washed in TBS with 0.1% Tween 20, and then incubated with peroxidase-conjugated Affinipure goat anti-rabbit IgG secondary antibodies (1:10,000, ZSGB-BIO, Beijing, China) for 1 h at room temperature. After visualizing with an enhanced chemiluminescence system (EMD Millipore Co., USA), the integrated intensity values of the immunoreactive signals were analyzed using Gel-Pro 3.1 software (Media Cybernetics Inc., USA).

#### Table 1

<table>
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<tr>
<th>Rat primers used for real-time RT-PCR</th>
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<td>Gene</td>
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CRF, corticotropin-releasing factor; CRFR1, corticotropin-releasing factor receptor 1; CRFR2, corticotropin-releasing factor receptor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Rats were deeply anaesthetized with chloral hydrate and perfused through the ascending aorta with 200 mL of saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. After perfusion, brains were removed and post-fixed by immersion in the same fixative overnight. Brains were dehydrated through a graded ethanol series and embedded in paraffin. Serial 6 mm coronal sections were cut on a Leica microtome (Leica RM2235). Cutting was done with reference to The Rat Brain in Stereotaxic Coordinates. Coronal sections were then mounted onto polylysine coated glass slides. The sections were deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in distilled water. Following TBS (0.05 M Tris buffer pH 7.4; 0.15 M NaCl) washes, the sections were microwaved (700 W) in 0.01 M EDTA/Tris buffered saline (pH 9.0) twice for 10 min for antigen retrieval and then cooled at room temperature for 30 min.

After washing in TBS, sections were incubated for 30 min in 0.5% triton X-100 to permeabilize the tissue. Sections were blocked with 10% goat serum in TBS for 1 h at 37 °C to reduce nonspecific binding, and then were incubated for 1 h at 37 °C with anti-CRF and anti-p-tau (T205) antibodies diluted in TBST with 10% goat serum before incubating overnight at 4 °C. The next day, the sections were washed and incubated with FITC-labelled anti-goat antibody (1:200, ZSGB-BIO, Beijing, China) and rhodamine-conjugated anti-rabbit antibody (1:200, ZSGB-BIO, Beijing, China), diluted in TBS with 10% goat serum, for 1 h at 37 °C. After washing, the sections were coverslipped with Antifade Mounting Medium and examined using a fluorescence microscope (Olympus DP71, Japan).

All data were analyzed using SPSS 17.0 software (SPSS, Inc., USA). Student’s t-test was used to determine statistical significance. Statistical significance levels were set to p < 0.01 for all tests. The data presented in the graphs indicate group means ± SEM unless otherwise noted.

3. Results

3.1. Plasma corticosterone levels

To study the effects of chronic noise exposure on the PFC CRF system expression, a chronic noise model of stress was established in rats. Firstly, we detected the level of plasma corticosterone, which was used as a marker of stress, in the exposed rats and control subjects. In our study, the level of plasma corticosterone was significantly increased after 30 days of exposure to noise, with an increasing trend that persisted up to 7 days after the cessation of exposure (Fig. 1).

3.2. Chronic noise exposure increases CRF, CRFR1, and CRFR2 expression in the PFC

In order to explore the effect of chronic noise exposure on the expression of the CRF system, we determined the mRNA and protein levels of CRF, CRFR1, and CRFR2 by RT-PCR and western blot analysis, respectively. The results showed that the expression of CRF and CRFR1 mRNA and protein were significantly increased after 30 days of exposure to noise, with an increasing trend that persisted up to 7 days after the cessation of exposure (Fig. 1).

3.3. Co-localization of CRF and p-tau in the rat PFC

To explore the relationship between CRF and p-tau, double labeling of CRF and p-tau was performed on sections through the rat PFC. The results show that a high proportion of CRF cells co-localized with p-tau in the PFC. CRF immunoreactivity within cell bodies showed green cytoplasmic staining (Fig. 3, A1–D1), and red p-tau staining was also observed in the cytoplasm (Fig. 3, A2–D2), yellow is the double staining of CRF and p-tau (Fig. 3, A3–D3).

4. Discussion

In this study, we explore the effects of noise stress on the expression of the CRF system in the PFC. Firstly, our results showed that chronic noise exposure resulted in sustained abnormal changes in plasma corticosterone levels and CRF system expression in the PFC. The HPA axis is viewed as the primary regulator of circulating corticosterone and is critical for its elevation in response to a stressor. Repeated exposure to stressors elevates basal corticosterone levels during the circadian nadir [21], and in our study, we found significant elevations in plasma corticosterone levels, which persisted for 7 to 14 days after the cessation of noise exposure. It has been reported that noise stress increases stress hormone levels after 1-hour long continuous noise stress and after chronic intermittent noise exposure [22,23]. Similarly, repeated or chronic exposure to other stressors also triggers the elevation of plasma corticosterone, such as intermittent hypoxia, social isolation, and restraint stress [21,24,25]. These studies are in broad agreement with our results. However, there are also reports that corticosterone responses have habituated to stress after a long-term exposure, such as chronic noise, restraint, and other stressors [26–30]. It appears, therefore, that a feature of corticosterone responsiveness to stress is not only dependent on the stress-type but also on the stimulating-intensity and time-course. Therefore, considering corticosterone as a primary indicator of HPA axis activity, our results suggest that long duration noise exposure leads to stimulation of the stress response.

Following noise stress, significantly higher levels of CRF and CRFR1 mRNA and protein expression were detected in the PFC and a delayed
A decrease was detected in CRF2 gene and protein expression. The variation of CRF and CRFR1 are similar to the changes of tau phosphorylation in our previous study, which persisted up to 7 days after the cessation of noise exposure [5]. Because this is a reversible process, the CRF system expression can be recovered to normal levels after the end of the stimulus. However, if the exposure time is extended, the levels of CRF, CRFR1, and p-tau would be expected to be maintained in an abnormally elevated state. The effects of noise stress on stress hormones, neurotransmitters, oxidative status markers, and neuronal activity in different brain regions has been studied previously [31–36]. In addition, exposure to different physical, physiologic, and psychologic stressors is known to increase CRF mRNA in the hypothalamic paraventricular nucleus and the amygdala [37–42]. However, to our knowledge, our study is the first to find that chronic noise stress significantly elevates the levels of expression of CRF and its receptors in the PFC.

The impact of these changes in the CRF system is not yet clear. However, previous studies showed that following chronic stress, tau phosphorylation and Aβ accumulation levels in the hippocampus and PFC are significantly elevated, and CRF may play a key regulatory role in these changes [11,12,14]. The phosphorylation levels of tau were also significantly elevated in CRF overexpressing transgenic rats, which express a high level of CRF [9]. In addition, the CRFR1-specific antagonist, R121919, blocks hyperphosphorylation of tau in this model and reduces phosphorylation of c-Jun N Terminal Kinase [9]. Furthermore, previous studies have also reported that compared to wild type mice, CRFR1-deficient mice, but not CRFR2-deficient mice, show a significantly lower level of p-tau and Aβ accumulation in the hippocampus after chronic stress exposure [11,13,43]. Collectively, the literature suggests that CRF acts via CRFR1 to play a leading role in mediating AD-like changes in selected brain regions although a role for CRFR2 in AD-like changes remains unclear.

In accordance with this literature, our previous studies have demonstrated that chronic noise exposure in experimental animals can cause significant and persistent tau hyperphosphorylation in the PFC [5]. Furthermore, our current results show sustained abnormal alterations of CRF and CRFR1 gene and protein expression after long-term noise exposure and co-localization of CRF and p-tau within PFC neurons. These data support the existence of interactions between the CRF signaling system and tau phosphorylation. In summary, in accordance with the regulatory role of the CRF system on tau phosphorylation in many stress exposure experiments [9–13], as well as the relationship between CRF signaling and p-tau discovered in this study, we infer the CRF signaling system is likely to be involved in the process of tau phosphorylation induced by chronic noise exposure. It should be noted that previous studies have shown increased numbers of CRF-immunoreactive (CRF-ir) cells in the rat PFC, and CRF secretion from CRF-ir neurons in the

Fig. 2. Chronic noise exposure increases the expression of CRF, CRFR1, and CRFR2 in the rat PFC. (A, D, G) Comparison of CRF, CRFR1 and CRFR2 mRNA expression levels in control and noise-exposed rats by quantitative real-time PCR at various time points following the cessation of noise exposure. (B, E, H) Western blot analysis of PFC CRF, CRFR1 and CRFR2 expression under C (control) and N (chronic noise exposure) conditions. GAPDH was used as a loading control. (C, F, I) Quantification of immunoreactive band density measured in Panels B, E, and H, normalized against GAPDH. Data are presented as the percent changes relative to control samples. Bars represent means ± SEM. *p < 0.01, compared with respective controls, by Student’s t-test (n = 6 per group).
hypothalamus occurs within minutes after the onset of a stressful stimulus [44–48]. For this reason, the animals in this study were kept free of disturbance and handling stress before perfusion to avoid diminishing the intensity of the CRF-ir signals.

In the central nervous system, the distribution of CRFR2 is not as widespread as that of CRFR1, and the two receptors seem to perform different functions under physiological conditions [49,50]. Of relevance to the current study, neurodegenerative diseases often trigger neuroinflammation [51]. CRFR1 has a key role in the regulation of the pro-inflammatory response during neural inflammation in the brain [52,53], whereas CRFR2 has anti-inflammatory and anti-cytotoxic actions [54]. We have demonstrated in a previous study that long-term noise stress can upregulate hippocampal pro-inflammatory molecules, such as tumor necrosis factor-α and the advanced glycation end products receptor, and markers of glial activity, such as glial fibrillary acidic protein and ionized calcium binding adapter molecule-1 [1]. This suggests that long-term noise exposure can lead to inflammatory changes in the brain. The hippocampus and cortex are two closely related brain regions; therefore, we hypothesize that noise stress can also induce an inflammatory reaction in the PFC. The sustained upregulation of CRFR1 suggests that this receptor may directly mediate pro-inflammatory responses in response to chronic noise stress, whereas the delayed upregulation of CRFR2 may be a compensatory mechanism against inflammatory lesions. Valentino et al. [55] proposed that CRFR1 and CRFR2 receptor subtypes have opposing effects, and the opposing regulation of biological responses by multiple CRF receptors may serve to facilitate different coping strategies in response to stress [55]. For example, the influence of CRFR1 may be greater in the early stages of stress when CRF is first released, whereas CRFR2 activation may require more or longer duration of CRF exposure. Therefore, further studies focusing on the availability and functionality of these receptors are required to reveal the mechanisms responsible for the observed AD-like and inflammatory alterations.

Fig. 3. Co-localization of CRF and p-tau in rat PFC. (A1–D1) CRF fluorescence (green); (A2–D2) p-tau fluorescence (red); (A3–D3) Merged images of CRF and p-tau (yellow) in the PFC. Representative images of rat PFCs at days 0 (C.A1–A3, N.B1–B3) and 3 (C.C1–C3, N.D1–D3) after cessation of noise exposure. C (control) and N (chronic noise exposure). Scale bar = 10 μm.
The chronic noise stress-induced upregulation of the CRF signaling system in our study may represent a sensitization process that could provide subsequent responses to future stressors. Changes in the mRNA levels of CRFR1 and CRFR2 may be associated with observed alterations in tau phosphorylation and potential inflammatory responses in this model. We acknowledge that the findings in our current and previous studies of noise-induced CRF/p-tau increases do not yet show a causal link to the p-tau-related pathology in AD. However, with this work, we establish a new model system in which to explore more fully the contribution of noise stress to neurodegenerative diseases such as AD. Further detailed studies are required to clarify the molecular mechanisms underlying the regulation of the CRF signaling system in AD-like neuropathological changes. Understanding the neurobiology of altered stress responses will contribute to our understanding of the relationship between chronic stress and brain health and may improve maintenance strategies for mental and cognitive health.

Conflicts of interest

The authors declare they have no competing financial interests.

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