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Short-term restraint and emotional-painful stressors increase DNA instability in different brain areas of rats with contrast excitability

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Abstract
Stress-reaction developed after exposure to stressors of different natures, increases the level of DNA damage in cells of target organs, including the central nervous system. However, the time of stressing exposure needed to induce genome destabilization in different brain areas and individual differences in animals defining their brain cell genome response to stressors is unclear. In this research, we show that acute stressors (2-h immobilization or 13-min emotional-painful stressor) increase the level of DNA damage in at least one of the brain regions studied: the prefrontal cortex, hippocampus, and amygdala in rat strains with the high or low threshold of nerve tibialis excitability, and non-selected Wistar rats. The results reveal the inter-strain differences in the genome response to acute stressors of each brain area, different from the repeated emotional-painful stressor effects shown earlier.

Keywords Stress · Nervous system excitability · DNA damage · Prefrontal cortex · Hippocampus · Amygdala

Introduction

Since the formulation of the 'general adaptation syndrome' concept [21], many models, mostly rodents, have been created to study the stress reaction at different levels: from behavioral to molecular [3, 17, 20]. The regulatory changes (gene expression and epigenomics) are described in various models [2], 15, however, the structural changes in the genome of cells are underexplored.

In the central nervous system (CNS), genome flexibility is important for neuronal plasticity needed for physiological brain activity, learning, and memory [11, 13]. Despite some potential adaptive role of DNA breaks in neurons and glia [1, 28], the increase in genome rearrangement frequency is associated with different neuropathologies [10, 19]. However, the mechanisms of developing them in mature brains with no hereditary deficiency in DNA repair are still unclear. Chronic stress is one of the risk factors for psychiatric, neurological, and neurodegenerative disorders [12, 14, 27], though, genome destabilization in CNS cells is rarely considered as a mechanism involved in stress-induced pathologies formation.

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Furthermore, even acute exposure to stressors of different natures can increase the level of DNA damage in brain cells [6], 23, but its mechanisms and long-term effects are insufficiently studied.
The studies of individual differences (and their causes) in sensitivity to any factors and the dynamics of the stress realization are important to predict the outcome of exposure to stressors in different individuals. Animal models with hereditary different levels of nervous system excitability could help to understand the mechanisms underlying post-stress pathologies formation. In Pavlov Institute of Physiology RAS two rat strains were created by selection for the high or low threshold of n.tibialis excitability. Originated from the Wistar strain, two contrast strains – HT (with High Threshold of excitability, i.e. low-excitable strain) and LT (with Low Threshold of excitability, i.e. high-excitable strain) – demonstrate a variety of interstrain differences in physiological and behavioral tests, both in non-stress and post-stress conditions [29].
The most used stressor exposure to rat strains with contrast excitability was repeated emotional-painful stressor (PEPS, 15 days according to K. Hecht's scheme [9] because it was shown that after PEPS the HT strain develops depressive-like behavior, and the LT strain – increases in compulsive behavior. These post-stress behavioral changes (some of which stay up to 6 months after the last episode of PEPS) allowed us to consider one strain as a model of post-traumatic stress disorder, and another as a model of compulsive disorder in human [29]. Previously, we showed that PEPS induces DNA instability and even disintegrity in cells of the hippocampus, amygdala, and prefrontal cortex (PFC) in some post-stress periods (1 or 2 h, 2 weeks, and 2 months) in a strain-specific manner [24]. However, it was unclear if the prolonged (15 days) repeated exposure to the emotional-painful stressor was needed to destabilize the genome of CNS cells or if the effects we found were induced only by the last episode of the stress procedure. In addition, we have never measured the level of DNA damage after other types of stress treatments in rats with contrast excitability.
In this research, we studied the genome destabilization in cells of three brain areas involved in stress response – the PFC, hippocampus, and amygdala – after two types of acute exposure – 13 min of emotional-painful stressor (EPS) or 2-h immobilization – in two rat strains with contrast excitability (HT and LT) and non-selected Wistar rats. The ability of short-term stressors to induce DNA damage in CNS, its potential role in normal physiology, and the formation of stress-induced pathologies are discussed.

Materials and methods

Materials

Male rats of strains with high and low thresholds of sensitivity to electrical stimuli (HT and LT, respectively), originating from the Wistar strain, were used for the experiments. The strains were created in the Laboratory of Higher Nervous Activity Genetics of the I. P. Pavlov Institute of Physiology, RAS, and included in the Biocollection of the I. P. Pavlov Institute of Physiology, RAS (No. GZ 0134–2018-0003, patents for selection invention No. 10769 and 10768 issued by the State Commission of the Russian Federation for Testing and Protection of Selection Inventions, registered in the State Register of Protected Selection Inventions on 15 January 2020). The selection was carried out according to the value of the voltage at which a motor reaction appeared in a test of electric shock irritation (rectangular electrical impulses with a duration of 2 ms) of the tibial nerve, n. tibialis. The strains were selected for over 80 generations, and from the 10th generation, four-fold differences in threshold of n. tibialis excitability significantly exceeded intrastrain variability [29].
All animals were kept under standard environmental conditions (23 ± 2 °C; 12 h/12 h dark/light cycle) with ad libitum water and food in the animal care facility at I. P. Pavlov Institute of Physiology, RAS. For each experiment, we took eighteen males from each strain: HT, LT, and Wistar, all at the age of five months. The animals (weighing 430.4 g ± 43.7 apiece) of each strain were separated into three groups of six animals. Three groups (HT, LT, and Wistar) were stressed by 2-h immobilization, three other groups (HT, LT, and Wistar) were stressed by EPS, while another three, respectively, served as controls. In total, 54 animals (18 of each strain) were used.

Exposure to stressors

The experimental males were exposed to one of the stressors: emotional painful stressor (EPS) or immobilization. For EPS each animal was placed in a special transparent box and exposed to 12 neutral light stimuli per 10 s and 6 of these stimuli were randomly reinforced by a current (2.5 mA, 4 s) according to the first day of K. Hecht's scheme [9]. The

interval between stimuli was 1 min,the total exposure time was 13 min. After the end of the EPS procedure animals were undisturbed till decapitation. For immobilization, rats were restrained in a plastic cylinder restrainer for 2 h. For a matching control, 6 animals of each strain were served undisturbed. All animals were slaughtered two hours after the start of the stressing procedure (1 h 47 min after the end of EPS exposure or immediately after the end of immobilization). Guillotine was used for immediate decapitation to avoid the effects of anesthesia on DNA damage. The PFC, hippocampus and whole amygdala were extracted by the same highly experienced experimenter according to the coordinates of the standard rat brain stereotactic atlas [18] and resuspended in standard phosphate-buffered saline (pH = 7.4).

Comet assay

The alkaline comet assay was performed according to the standard procedure with small modifications (Shcherbinina et al. 2024). All cell suspensions were diluted to a final concentration of ~ 10^5 cells/mL. 150 µL of suspension per specimen was mixed with an equal volume of a 37 ◦C 1% solution of low-melting agarose, and the mixture obtained was applied to microscope slides (pre-coated in advance with 500 µL of 1% universal agarose solution). Then slides were cooled in a refrigerator (t = 4 ◦C) for 10 min to harden a gel and 150 µL of cold lysing solution (10 mM Tris–HCl, 2.5 M NaCl, 100 mM EDTA-Na_2 to pH = 10 and 1% Triton X-100) was applied on each slide for 1 h at 4 ◦C. After the lysis, the slides were placed into the chamber for electrophoresis (COMPAC-50, Cleaver Scientific, Rugby, UK) containing 500 ml of alkaline solution containing 300 mM NaOH, 1 mM EDTA-Na_2 (pH > 13) for 20 min. Electrophoresis was conducted at 20 V (1 V/cm, 300 mA) for 30 min. After the procedure, the slides were fixed for 5 min in 70% ethyl alcohol-water solution and then dried at room temperature for 12 h. For each brain area sample (PFC, hippocampus, and amygdala of each animal), two slides were prepared. The slides were encoded and stained with 0.01% SYBR Green I (Sigma-Aldrich, St. Louis, MO, USA) for 20 min. The cell nuclei of the PFC, hippocampus, and amygdala (not less than 200 per specimen) were imaged using Axio Scope.A1 (Carl Zeiss, Oberkochen, Germany) and QIClick digital CCD-camera with QCapturePro 7 software (QImaging, Tucson, AZ, USA) and analyzed (not less than 400 nuclei from each brain sample) with TriTek CometScore™ Freeware v1.5 software (TriTek Corp., Sumerduck, VA, USA).

Statistics

The DNA content in comet tails (% of tDNA) was considered a DNA damage indicator for our study. Further statistical analysis was performed using the GraphPad PRISM v. 9.1.0 software package for Windows. After decoding, individual data sets were merged within each experimental group and summarized data were checked for the normality of their distribution (Kolmogorov–Smirnov test). Due to extremely low medians of tDNA content (less than 1% for each experimental group) in the comet assay data, all completely undamaged cells (containing less than 0,1% tDNA) were extracted from the analysis. Since the data did not pass the normality test ($p > 0.1$), the Kruskal–Wallis test (with the Dunn multiple comparison test) was used for the next analysis. For the visualization of DNA destabilization, the data for each structure are shown as medians with 95% CI. Only significant p-values (< 0.05) are shown on graphs.

Results and discussion

In our previous studies, we used PEPS (15 repeats of EPS, one EPS per day) and showed different changes in DNA damage levels in the PFC, hippocampus, and amygdala of the HT, LT, and Wistar strains [24]. Here we exposed rats of the same strains to single EPS or 2-h immobilization and showed the interstrain differences in the effects of short-term immobilization on the DNA damage of cells in three brain areas (PFC, hippocampus, and amygdala) cells.

In PFC, both stressors – immobilization and EPS increase the median level of tDNA in only one strain – HT (in 1.32 and 1.29 folds, correspondingly) (Fig. 1). On the contrary, two hours after PEPS the HT strain was the only strain, where the decreased level of DNA damage in PFC was demonstrated [24]. In other research in Sprague–Dawley rats was shown that "acute" (6 h) restraint stressor increases the expression of *Ercc1* and *Nudt1* genes in the PFC, however, no effects of acute stressor were shown on the transcription (of 7 genes involved in DNA reparation studied) in the hippocampus nor on oxidatively damaged DNA level [8]. In addition, in the same research, "subchronic" (7 days) and "chronic" (21 days) repeated restraint stressor decreased expression of some repair genes both in the PFC and hippocampus after 7 repeats

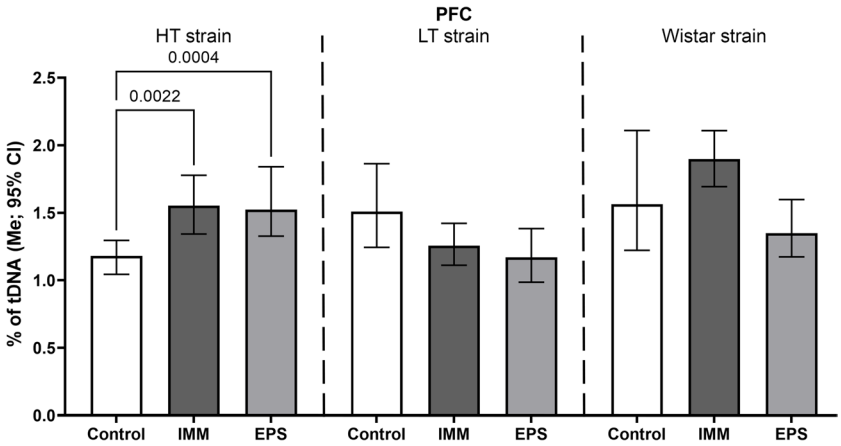

Fig. 1 Changes in PFC cells tDNA content of stressed (by immobilization or EPS) vs. corresponding control groups of different rat strains (HT, LT, and Wistar). IMM – 2 h immobilization (dark grey bars), EPS – 13 min of emotional-painful stressor (light grey bars)

(not 21) of stressing procedure [8]. Therefore, there is a possible negative correlation in PFC between increased DNA damage (and expression of genes involved in its reparation) in the case of a single action of the stressor and a decreased level of genome instability in the case of repeated stressing procedures. This observation (if confirmed in other studies in different models) could be used to predict the dynamics of DNA destabilization in experiments with different timing and other varying stressing conditions.

In the hippocampus, only immobilization and only for two strains (HT and Wistar) increases the median level of DNA damage in 1.72 and 1.44 folds, correspondingly. EPS had no effect (Fig. 2).

In amygdala cells, only immobilization increases the tDNA level (in 1.45 folds) and only in the LT strain (Fig. 3). As in the hippocampus, EPS had no genotoxic effect (Fig. 3).

In different rodent models, the individual differences in behavioral, physiological, and genetic responses to factors of a variety of nature were shown [4, 5, 16], and the reasons for it are being studied. For example, neuronal activity during exploratory behavior or fear learning causes double-strand breaks (DSBs) in the brain of mice, which are involved in memory formation by regulating gene transcription and chromatin remodeling. The mechanisms for neuronal DSBs generation have been actively studied and to the present day include aberrant activation of endonucleases, dephosphorylation of Top2B, convergent transcription, oxidative stress, and inhibition of DNA repair machinery [7, 25, 26]. The stress response adds DNA damage induced by a physiological activity because stress hormones also generate DSBs in glial cells [25], and the expression of DNA repair genes decreases after stress [8]. As a result, stress reaction increases the chances of structural changes in brain cells genome and the formation of neuropathologies in sensitive to stressors individuals.

Our findings in rat strains with contrast levels of excitability contribute to understanding the role of the hereditary state of the nervous system in individual differences in stress response and its consequences. Here we showed that immobilization increases the level of DNA damage in 2 h, however, it could also have long-term effects. In previous

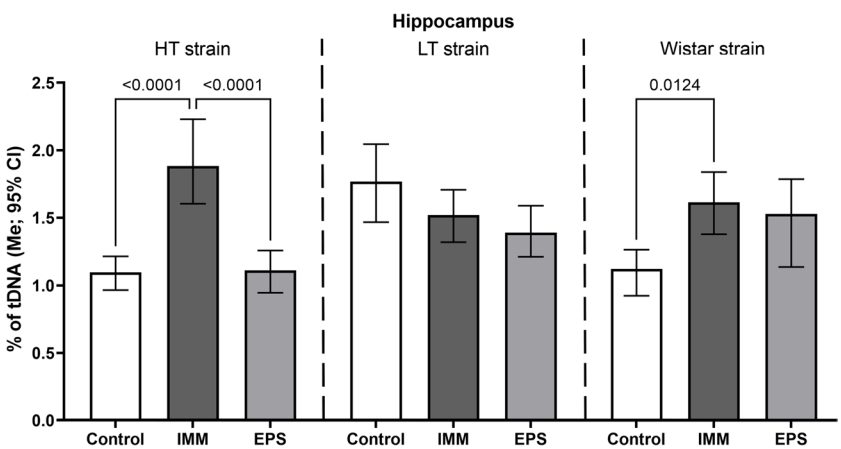

Fig. 2 Changes in hippocampal cells tDNA content of stressed (by immobilization or EPS) vs. corresponding control groups of different rat strains (HT, LT, and Wistar). IMM – 2 h immobilization (dark grey bars), EPS – 13 min of emotional-painful stressor (light grey bars)

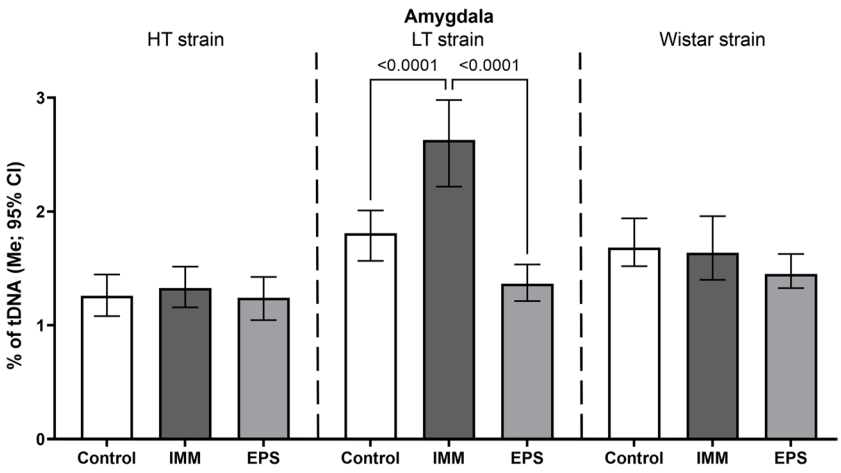

Fig. 3 Changes in amygdala cells tDNA content of stressed (by immobilization or EPS) vs. corresponding control groups of different rat strains (HT, LT, and Wistar). IMM – 2 h immobilization (dark grey bars), EPS – 13 min of emotional-painful stressor (light grey bars)

research with PEPS, stress-induced genome instability was found up to two months after the end of the stress procedure [24]. It is in good agreement with the data that the 30-min restraint and forced swimming induce genome instability in the brain cells of Wistar rats. The increase in the level of damage is significant in the hippocampus 7 days after the stressing procedure [6], which demonstrates that a single stressful event can lead to genome instability in CNS. It could then be involved in the formation and maintenance of the neuropathologies.
In summary, our data show that short-term restraint for two hours increases genome instability:

- for the HT strain in both the hippocampus and the PFC (Figs. 1 and 2);
- for non-selected Wistar rats – only in the hippocampus (Fig. 2);
- for the LT strain – in the amygdala only (Fig. 3).

These differences in genome response can reflect the hereditary peculiarities in the CNS functioning of those strains, not only in the reaction of different brain regions to the stressors' exposure but also in the speed of DNA damage and repair. From the research of the neural circuit activated by processive stressors like immobilization, it is known that the three structures we studied are affected in a sequence of PFC → hippocampus → amygdala, and c-fos mRNA expression also induced by immobilization in the same order [22]. Thus, if the DNA destabilization develops in brain areas in the same sequence, there is a possibility that the high-excitable strain (LT) cell genome reacts to immobilization faster, and after 2 h of stressor exposure we did not observe the changes in the level of DNA instability in PFC and hippocampus of this strain, because the reparation had already finished. The low-excitable strain (HT) cell genome reacts slowly and in 2 h, the increased DNA damage level is still found in the PFC and hippocampus. In contrast, in the LT strain, which reacts faster, the genome destabilization, is shown in the amygdala, and the HT strain, this process has not yet developed at the end of the experiment.
Taken together, these findings highlight the importance of considering the dynamics (and its complexity) of DNA damage and repair in the research of genome effects of stress and studying different post-stress periods.

Author contributions Veronika Shcherbinina: Formal analysis, Investigation, Data Curation, Writing—Original Draft, Visualization. Eugene Daev: Conceptualization, Writing—Review & Editing, Supervision. Marina Pavlova: Methodology, Investigation, Writing—Review & Editing. Natalia Dyuzhikova: Conceptualization, Methodology, Resources, Writing—Review & Editing, Supervision, Project administration, Funding acquisition.

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Data availability Data reported in this study will be made available upon request from the corresponding author.

Declarations

Ethics All animal experiments were conducted in accordance with the Council of the European community directives (86/609/EEC) on the use of animals for experimental research. The experimental protocol was approved by the Animal Care and Use Committee at the Pavlov Institute of Physiology of RAS (protocol No. 01/16 of 16 January 2023).

Competing interest The authors have no competing interests to declare that are relevant to the content of this article.

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