GENETICS

Genome response of hippocampal cells to stress in male rats with different excitability of the nervous system

Veronika Shcherbinina[1,2], Alexander Vaido[†2], Diana Khlebaeva[2], Natalia Dyuzhikova[2], and Eugene Daev[1,2]

[1]Department of Genetics and Biotechnology, Faculty of Biology, Saint Petersburg State University, Universitetskaya nab., 7–9, Saint Petersburg, 199034, Russian Federation
[2]Laboratory of Higher Nervous Activity Genetics, Pavlov Institute of Physiology, Russian Academy of Sciences, nab. Makarova, 6, Saint Petersburg, 199034, Russian Federation

Address correspondence and requests for materials to Natalia Dyuzhikova, dyuzhikova@infran.ru

Abstract

Changes of genome stability in hippocampal cells of male rats with hereditary high and low thresholds of *nervus tibialis* response to electric stimuli (HT and LT strains, respectively) were studied in unstressed and stressed animals. HT and LT originated from Wistar strain, males of which were also used as a control. The comet assay was used after prolonged emotional painful stressor action. There were no interstrain differences in the spontaneous percentage of DNA in comet tails (tDNA). However, the prolonged emotional pain stressor induced genome instability differently in animals of different strains. The highest level of DNA damage in hippocampal cells was shown in highly sensitive animals of LT strain. Males of Wistar strain had intermediate levels of tDNA, while HT animals had the lowest stress reactivity.

Keywords: DNA damage, comet assay, stress, hippocampus, nervous sensitivity, selection, rats, genome instability

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Authors' information: Veronika Shcherbinina, Research Assistant, orcid.org/0000-0002-4813-736X; Alexander Vaido, Dr. of Sci. in Biology, Chief Researcher; Diana Khlebaeva, Junior Researcher, orcid.org/0000-0002-5157-486X; Natalia Dyuzhikova, Dr. of Sci. in Biology, Head of Laboratory, orcid.org/0000-0003-3617-5948; Eugene Daev, Dr. of Sci. in Biology, Professor, Leading Researcher, orcid.org/0000-0003-2036-6790

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Introduction

All living organisms exist and evolve because of their genome ability for non-directional changes in response to environmental challenges followed by natural selection of the best adapted beings among them. Therefore, there is a tight connection between any cell genome and the environment. The nervous system of a multicellular organism provides such interconnection between the surroundings and almost all of its cells. Responding to environmental changes, neuronal cells send signals to themselves as well as to the rest of cells inside the organism, causing them to modify their genome activity (or even structure) in an attempt to adapt. The results of genome lability appear in different tissues and organs of the multicellular organism (Horne, Chowdhury and Heng, 2014). Genome instability is especially important for cells of the central nervous system (CNS) of higher animals because of the brain's functional role in the regulation of all aspects of life (Suberbielle, Sanchez and Kravitz, 2013). At the same time, instability may be the cause of CNS pathologies (McKinnon, 2017). Accumulation of mutations, disturbances in DNA replication and reparation during neurogenesis, or abnormal proliferation all can be the causes of atypical CNS functioning (Iourov, Vorsanova and Yurov, 2012; Tiganov et al., 2012; McKinnon, 2013, 2017). However, the role of genome instability in brain cells for ensuring mental health and the development of neuropsychiatric diseases in humans, including under the influence of stress, has been poorly studied (Hare et al., 2018).

Data about the impact of instability of somatic genomes were obtained mainly for autism, schizophrenia, and Alzheimer's disease (Yurov, Vorsanova and Iourov,

2010; Van Leeuwen and Hoozemans, 2015). As for animal models, there have been only limited investigations with a clearly insufficient amount of neuropsychological and genetic information. This especially concerns individual genetically determined functional differences in the central nervous system of animals and genome reactivity of neural cells under a stress state.

A thorough investigation of DNA damaging mechanisms in brain cells — taking into account the hereditary functional state of the CNS — is theoretically and practically essential for the creation of approaches to reduce the risk of neuropathology appearance.

The alkaline comet assay is one of the methods for detecting genome instability of single cells. It demonstrates "fragile" sites of a genome where the risk of DNA breakages is high or where they already formed (Olive and Banath, 2006).

The aim of this research was to study the genome reactivity of hippocampal cells in rat strains with inherited high and low excitability thresholds of *n. tibialis* to prolonged emotional pain stressor (PEPS) action, using the alkaline comet test.

Materials and methods

Materials

Male rats of strains with high and low thresholds of *n. tibialis* sensitivity to electrical stimuli (HT and LT, respectively), originated from Wistar strain, were used for the experiment. The strains are 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 January 15, 2020).

Animals were selected at the Laboratory of Higher Nervous Activity Genetics at the I. P. Pavlov Institute of Physiology, RAS (Vaido et al., 2018). The source material was an outbred population of Wistar albino rats (breeding nursery Rappolovo, Leningrad Region). The selection was carried out according to the value of the threshold of neuromuscular excitability in a test of electric shock irritation (rectangular electrical impulses with a duration of 2 ms) of the tibial nerve — *n. tibialis*. The threshold was estimated as a value of the voltage at which a motor reaction appeared.

In the first two generations, full siblings were crossed. Starting from the third generation, intrastrain breeding was carried out in a random order. From the tenth generation on, breeding threshold values reached a plateau. At the same time, the four-fold threshold differences between the strains significantly exceeded the intra-strain variability (Vaido et al., 2018).

All animals were maintained in standard environmental conditions (23 ± 2 °C; 12 h/12 h dark/light cycle) with water and food *ad libitum* in the animal care facility at I. P. Pavlov Institute of Physiology, RAS.

For the experiment, we took ten males of HT and LT strains, as well as Wistar males, all at the age of five months. The animals of each strain were separated into two groups of five animals. Three groups (HT, LT and Wistar) were stressed, while another three, respectively, served as controls. In total, 30 animals were used.

Exposure to stressor

The experimental males were exposed daily to prolonged emotional pain stressors (PEPS) for 15 consecutive days (13 min/day). Each animal was placed into a special transparent box and exposed to 12 neutral light stimuli per 10 sec according to K. Hecht's scheme (only 6 of the stimuli were randomly reinforced by a current (2.5 mA, 4 sec) (Hecht et al., 1972). The interstimulus interval lasted for 1 min. Previous studies have shown that the exposure used contributes to the emergence of persistent behavioral disorders in animals that persist for up to 6 months after exposure (Vaido et al., 2018).

Three males (HT, LT, and Wistar) were exposed to the stressor. Three other undisturbed rats served as a matching control. In accordance with the timing of stressor action, all rats were slaughtered 2 h after the end of the stressing procedure. The hippocampus was extracted and resuspended in the standard phosphate buffered saline (pH 7.4). To use all 30 animals, the procedure was repeated 5 times consequently.

Comet assay

For the comet assay, the cell suspension was dissolved to the final concentration of ~10^5 cells/mL. The alkaline comet assay was performed in accordance with the standard procedure with small modifications (Daev et al., 2017). The cell suspension (150 μL per specimen) was mixed with an equal volume of 37 °C 1 % solution of low-melting agarose (t_m < 42 °C) in microcentrifuge tubes. The obtained 150 μL of the mixture was applied to microscope slides, prepared in advance using the standard method with 1 % universal agarose solution base (t_m < 65 °C). Then the mixture was covered with a coverslip (25 × 25 mm), and the microscope slides were placed for 10 min in a refrigerator (t = 4 °C) to harden the gel.

All further operations were conducted in the dark or under green light. On the cooled slides, 150 μL of cold lysing solution containing 10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA-Na2 (pH 10), 1 % Triton X100, and 10 % DMSO was applied. The slides, covered with parafilm strips, were kept in the refrigerator (4 °C) for

1 h; after that, the lysing solution was washed with phosphate buffer, and the slides were placed into the chamber for horizontal electrophoresis (Cleaver Scientific CSL-COM10), and electrophoretic buffer (300 mM NaOH, 1 mM EDTA-Na_2 (pH > 13)) was added. After 20 min, electrophoresis was started (C. B. S. Scientific, EPS-300 X Mini-Power Supply apparatus), which lasted for 30 min (1 V/cm). After the procedure had been finished, the preparations were fixed for 5 min in freshly prepared 70 % ethyl alcohol water solution and then were dried at room temperature for 12 h.

Two slides from each animal were prepared. The specimens were encoded and stained with 1 % SYBR Green I (Sigma) for 10 min. The cell nuclei of the hippocampus (not less than 200 per specimen) were imaged using Axio Scope.A1 and QIClick digital CCD-camera with QCapturePro 7 software. The obtained images (not less than 400 suitable for analysis of the nuclei from each animal) were analyzed using TriTek CometScore™ Freeware v1.5 software.

Statistics

The DNA content in comet tails (% of tDNA) was considered as a DNA damage indicator for our study. Overall, 13382 hippocampal cells were analyzed.

Further statistical analysis was conducted using the GraphPad PRISM v. 9.1.0 software package for Windows. After decoding, each individual data set was checked for the normality of its distribution (Kolmogorov — Smirnov test). Since the data did not pass the normality test ($p > 0.1$), the Mann — Whitney criterion was used for the next analysis. To make a pooled analysis of tDNA content, the individual and then grouped median values for all control and all stressed rats was calculated and compared. For analysis of the interstrain differences of the animals, which belonged to three strains (HT, LT, or Wistar), summarized data (all cells from 5 animals within each group of strains) were analyzed. Because the three control groups had no difference in tDNA content (Kruskal — Wallis test, $p = 0.4124$), their data were merged.

Results and discussion

First of all, individual data were examined for their type of distribution. The frequencies of hippocampal cells with different tDNA content for all stressed and unstressed males were distributed exponentially ($R^2 \geq 0.98$). Preliminary pooled analysis of tDNA content in hippocampal cells of unstressed (n = 15) and stressed (n = 15) male rats showed that prolonged exposure to an emotional pain stressor (PEPS) increased the damage of DNA (Fig. 1).

Further analysis took into consideration the interstrain differences of the animals, which belonged to high and low threshold (HT and LT, respectively) strains or Wistar strain (Fig. 2). Since no significant differences were found between unstressed animals of all strains used, the data were combined into a joint control group.

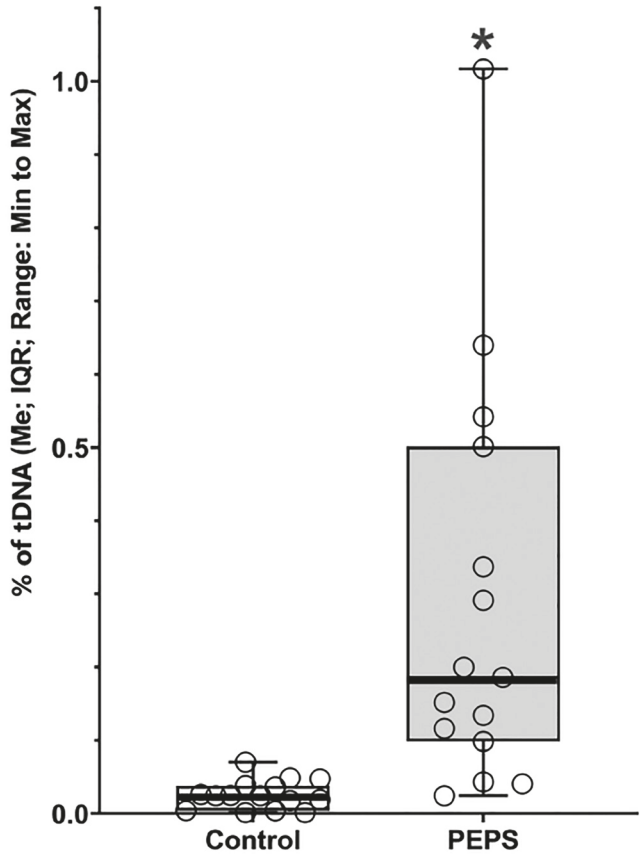

Fig. 1. tDNA content in hippocampal cells of unstressed (control) and stressed male rats (Mann — Whitney test). PEPS — prolonged emotional pain stressor. Each point corresponds to individual median for the animal; the median and IQR for groups of corresponding variants are also shown. * — stress vs. control ($p < 0.0001$).

However, stressed animals of all strains show distinctive differences in genome reactivity of hippocampal cells. All of them respond to PEPS action with an increased level of cell damage. The males of LT strain show the highest elevation in genome damage of cells in the hippocampus. Wistar strain has an intermediate genome reactivity, while HT males were less sensitive to the PEPS.

In spite of some heterogeneity, pooled data showed a general increase in hippocampal cells' tDNA content in stressed Wistar males (Fig. 3, A).

Stressed male rats of HT strain originated from Wistar showed no significant increase of tDNA content in hippocampal cells vs. the corresponding unstressed HT animals. In spite of that, an increasing tendency was evident: the median value of tDNA content in hippocampal cells amplified near 8 times (Fig. 3, B).

Stressed males of LT strain showed an increase of tDNA content in hippocampal cells. It was approximate-

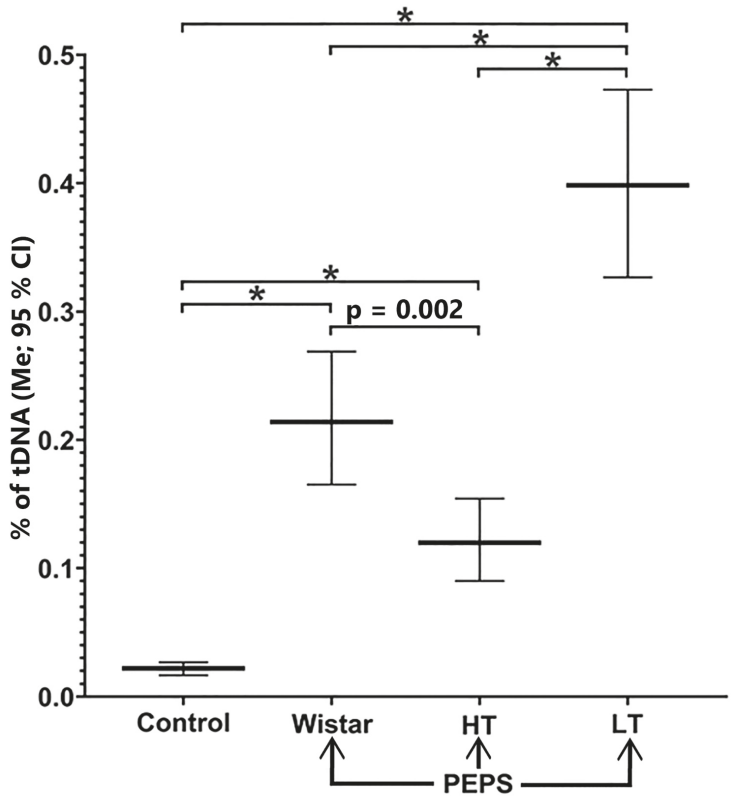

Fig. 2. Percentage of DNA damage in hippocampal cells of control unstressed males vs. stressed males of used strains. The data for all control animals were summarized as well as for all stressed animals inside each strain. * — differences between variants ($p < 0.0001$).

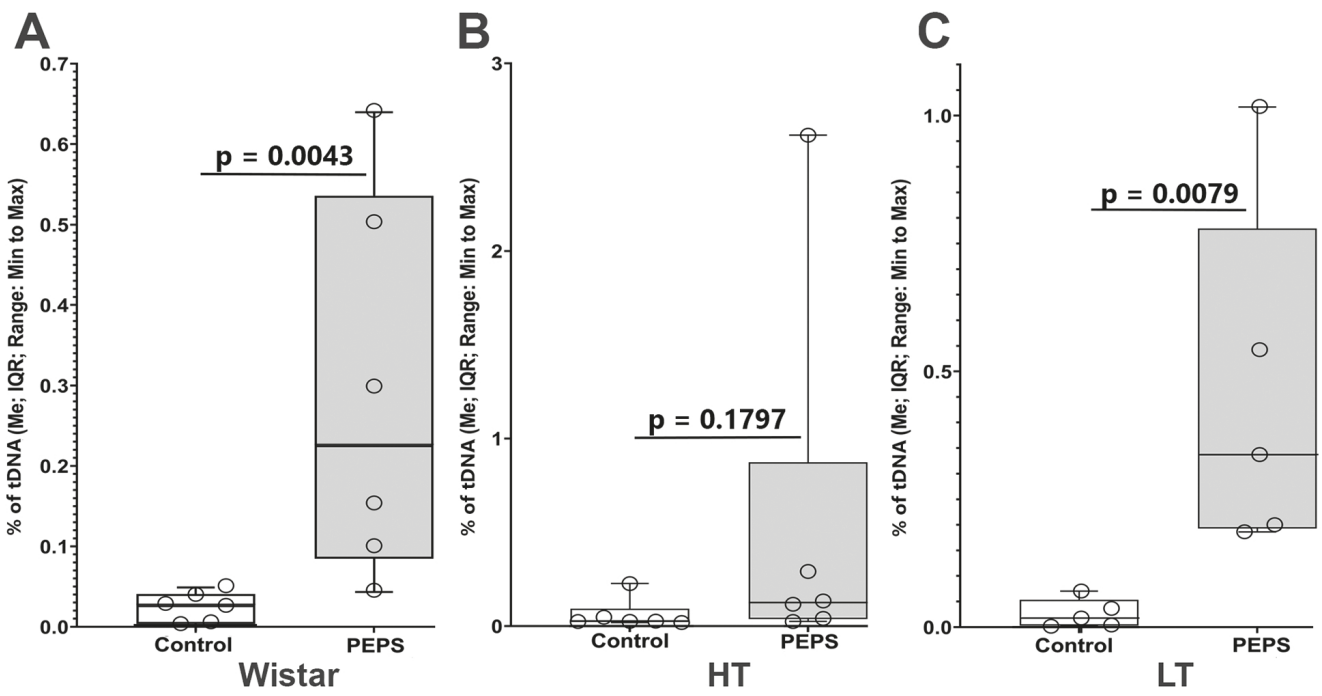

Fig. 3. tDNA content in hippocampal cells of unstressed (Control) and stressed (PEPS) rat males of Wistar (A), HT (B), and LT (C) strains.

ly 16 times higher after PEPS compared to unstressed animals (Fig. 3, C).

Therefore, the data obtained show interstrain differences between LT and HT males with respect to their reactivity to PEPS: males of LT strain are more sensitive to PEPS than HT and Wistar males (Fig. 2).

Discussing the data obtained, it should be said that the natural selection of multicellular organisms moves toward high sensitivity to any minute environmental changes. At the same time, an adequate response of the organism is necessary for adaptation. Artificial selection for the *n. tibialis* low threshold somehow simulates natural processes in favor of high sensitivity of the peripheral and central nervous system. We found here that high sensitivity of *n. tibialis* corresponds to high genome response to stress in hippocampal cells of rat males. Selection in the opposite direction led to a decrease in the stress reactivity of the genome of hippocampal cells, while the unselected Wistar males showed high nervous and intermediate genome response to stress.

Excitability is an important characteristic of nervous system functional state. Selection of rats for high and low thresholds of *n. tibialis* is really selection for their low and high sensitivity to the current stressor. However, such selection can modulate the excitability of the central nervous system. It is shown that spontaneous and induced neuronal activity in the amygdala of LT and HT rats differs just like the threshold value of peripheral *n. tibialis* (Sivachenko et al., 2021).

It was shown earlier that 2 hrs after PEPS, the cells of PFC of both HT and LT rats increase their reactivity to H2AX phosphoSer139, which is a marker of double-strand breaks. At the same time, a dentate gyrus's cells show a similar increase only in HT rats (Pavlova et al., 2020). It is possible that the varied genome response to the same stressor is based on specific functional interactions of different brain areas. The latter may be involved in the initiation and maintenance of pathological states. Moreover, this may or may not be related to inherited features of the animal's nervous system (Pavlova et al., 2020; Sivachenko et al., 2021).

It seems interesting that the bone marrow cells show a similar response to mutagen cyclophosphamide. The level of chromosomal aberrations is higher in LT than in HT male rats (Dyuzhikova et al., 1996). Presumably, LT rats are more sensitive to genotoxic action due to the selection in favor of a low nerve excitability threshold, which is somehow connected with the genome response to stress in cells of different brain structures. Previously, it was shown that LT rats are characterized by increased stress reactivity of the HPA, accelerated development of the hormonal response, and reduced sensitivity of the HPA to feedback signals (Ordyan et al., 1998).

It should be noted that the variability in the excitability level of the nervous system determines the characteristics of the response to stress. It has a number of physiological, biochemical, and neuroendocrine correlates, which affect genetic and epigenetic processes. In turn, such processes underlie the formation of long-term post-stress pathological conditions (Dyuzhikova, Skomorokhova and Vaido, 2015; Vaido et al., 2018).

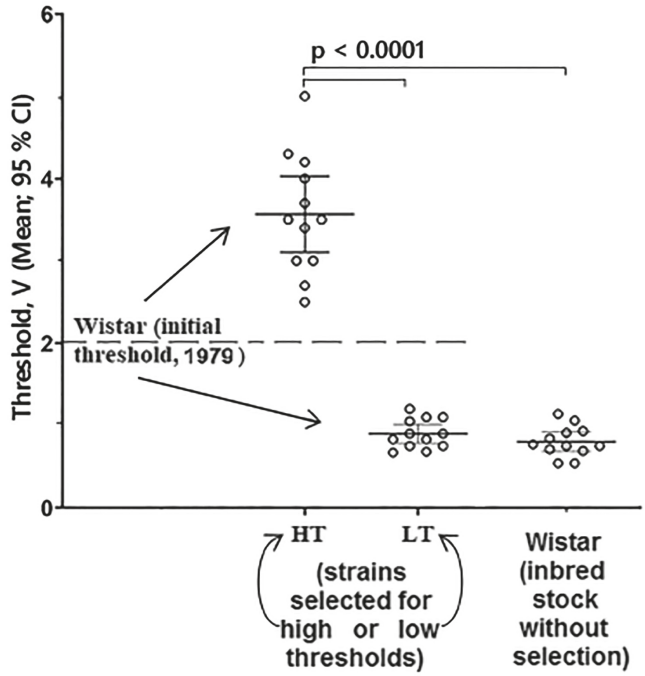

Fig. 4. The threshold means values of *n. tibialis* for original Wistar male rats and selected HT and LT strains. Data are also shown for Wistar males from current stock. Differences between variants are shown (ANOVA, Tukey's multiple comparison).

Genome integrity can be assessed at least in part by single-cell gel electrophoresis. The hippocampal cell genome reacts to PEPS by DNA fragmentation, which appears as comet tails after single cell electrophoresis. It seems there is an association between a low threshold of *n. tibialis* and a high degree of DNA damage of hippocampal cells in male rats of selected strains, while a high threshold corresponds to less severe DNA impairment. However, the increase in damaged cells in unselected Wistar males (while threshold values are the same as in HT strain, Fig. 4) means that there might be differences in genetically determined mechanisms of *n. tibialis* threshold and genome sensitivity of hippocampal cells. Opposite selection to PEPS affected both mechanisms, but without sorting, only the nerve high threshold was spontaneously changed. Thus, stressor-sensitivity selection to PEPS is connected with the genome reactivity of hippocampal cells to the same psycho-emotional stressor. It can also be assumed that the varied degree of DNA damage is connected with the different impact of psychic and emotional components, as well as direct pain action.

The alkaline comet test shows labile sites of DNA connected with its damage (Azqueta and Collins, 2013;

Afanasieva and Sivolob, 2018). Therefore, the increase in the number of such sites, as well as the amount of damaged hippocampal cells after PEPS, could reflect a disturbance in hippocampus functioning. It would be interesting to study how local the effect of the stress state is with respect to other brain areas.

It is known that earlier stress (maternal separation from pups) increases the DNA break index in the comet assay of hippocampal cells in adult mice. Additional stress (footshock) in adulthood increases the DNA damage in the hippocampus (Diehl et al., 2012). Stress (when induced in mice by 5-day exposure to a predator (rats)) decreased Ki-67+ cell density (40 % reduction) in the subgranular area of the dental gyrus of the mouse hippocampus. This means that stress suppresses proliferative activity and neurogenesis in the hippocampus. (Gudasheva, Povarnina and Seredenin, 2017). Restraint stress in rats specifically affects the activity (mRNA expression) of genes involved in oxidatively damaged DNA repair in PFC and HC (Forsberg et al., 2015). Increased levels of DNA damage may cause cell death and atrophy of the neurons in the hippocampus and cortex during stress state. It can decrease the grey matter density of the hippocampus, amygdala, and prefrontal cortex (Wigner et al., 2021).

Different stressors may trigger different physiological responses, including specific temporal changes in different brain areas. For example, restraint or forced swimming induced distinct changes in rat brain areas (PFC, Amygdala, hippocampus) as immediately as one week later after the stressor action (in the hippocampus) (Consiglio et al., 2010). *In toto*, any impacts that disrupt the processes of replication, transcription and intracellular allostasis can cause direct or indirect DNA damage (McKinnon, 2017; Wilhelm, Said and Naim, 2020). That could (if referring to neuronal brain cells) disturb the adequate interconnection of the organism with the environment.

Conclusions

The emergence of labile and fragile DNA sites in a stress state could mark the formation of neuropsychic pathologies. Severe genomic disintegration of brain cells may induce their death in some areas of the central nervous system and their abnormal functioning. It could help further to localize genes which are important for synapse functioning and neuronal vitality (Brazilai, 2007; Wei et al., 2016; McKinnon, 2017). Genomic disturbances can be associated with mechanisms of the formation of anxiety disorders and neurodegenerative diseases, the features of the manifestation of which depend on the hereditary level of excitability of the nervous system. This requires special approaches in the development of personalized methods of disease prevention and therapy.

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