

Some Biochemical Changes of Rat Hippocampal Slices under the Long-term Creatine Intraperitoneal Supplementation

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Abstract For the last few decades, aggression has been linked to various modifications in cellular metabolic pathways. It has also been shown that patients undergoing extended creatine (Cr) treatment display increased aggressiveness. However, molecular mechanisms underlying a correlation between the two phenomena have not been sufficiently identified. To gain a deeper understanding of the changes resulting from supplementation of Cr into non-aggressive animals, we compared some behavioral and biochemical characteristics of Cr-treated and originally non-aggressive individuals with those of naturally non-aggressive and naturally aggressive groups. In this paper we show that extended supplementation of creatine into non-aggressive animals modifies the number of exploratory characteristics and fear-related reactions. In addition, it also changes several biochemical properties in the hippocampus among rats, by significantly reducing the content of Ca^{2+} ions. This could be the result of Cr's direct influence on the NMDA-receptor and its functional inhibition. In conclusion, behavioral and biochemical parameters of non-aggressive individuals display changes under extended supplementation of creatine, which could provide a significant insight into formation of aggression-related behaviors and processes.

Keywords Aggressive Behavior, Creatine, Ca^{2+} -ATPase, Na^+/K^+ -ATPase, Mg^{2+} -ATPase, Calcium

1. Introduction

Creatine (Cr) (α -N-methylguanidino acetic acid) is an organic compound, which, with phosphocreatine (PCr), plays an important role in energy metabolism in the cell. The reaction is catalyzed by the enzyme Creatine Kinase (CK), which can be found in almost all types of cells, with the most

active isoforms in tissues with high-energy demand, particularly in muscle and brain [1, 2].

Apart from the key role of Cr in energy metabolism, it also presumably performs a number of other functions too, such as transmission of nerve impulses in synapses, maintaining membrane potential and ion gradient, cellular homeostasis, axonal and dendritic transport and participation in other important processes in the central nervous system [3, 4]. There is data showing that Cr can also serve as a neuromodulator and activate or inhibit certain post-synaptic receptors [5].

At the same time, the phenotypic diversity of neurological diseases observed under Cr deficiency points to the importance of the compound in terms of psychomotor development and realization of cognitive functions [6]. It has been found that quantitative deficit of Cr affects functionality of the central nervous system and has external effects on neurodegenerative diseases [7, 8, 9]. On the other hand, increase in the Cr quantity has a neuroprotective effect [10]. Cr is characterized by similar features in a number of psychiatric diseases, such as schizophrenia, psychological stress, etc [11, 12, 13, 14].

Activity of the enzymes found in the central nervous system (L-arginine: glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) shows that the brain can endogenously synthesize Cr [15]. However, the Cr-specific transporter SLC6A8, which is present in endothelial cells of micro-capillaries can import Cr through the blood-brain barrier (BBB) [16]. Activity of AGAT and GAMT depends on a variety of conditions in the central nervous system, including oxidative stress. Oxidative stress is known to have a negative influence on the performance of mitochondria and, hence, on the energy homeostasis [15]. Use of exogenous Cr improves cognitive functionality of the brain, making it potentially possible to apply the compound as a pharmacological agent to treat a number of mental disorders [17]. However, it has also been claimed that

supplementing Cr over an extended period of time raises the level of aggressiveness in an individual and gives rise to aggressive behavior. On the other hand, there is no experimental data to confirm this point of view, which is mainly based on positive changes of behavioral parameters among depressed animals [14, 18].

The aim of our experiment was to study the role of intraperitoneally (i.p.) supplemented Cr in activation of certain processes that are known to be characteristic of aggression in general and the influence it has on biochemical mechanisms, which are closely linked to development of several biochemical processes.

2. Materials and Methods

2.1. Study Design

Experiments were conducted on 200-250 gr male Sprague-Dawley rats. As per the mouse-killing test (MKT) a single adult mouse was placed in the home cage with a single rat for 5 min. The time to each lethal attack was recorded. Dead mice and survivors were removed from the cage at the end of the behavioral test session. The killer behavior of the rat was taken as a sign of aggression [19]. According to the results of the test, the animals were divided into three main groups: G1 – non-aggressive, G2 – non-aggressive Cr-treated (30 days), and G3 – aggressive individuals, ten animals in each group. The experiment was repeated for three individual series.

The open-field test followed a modified Brittney and co-authors' method [20] and assessed the changes in fear- and anxiety-related reactions, as well as in the exploratory activity of the three groups.

The test chamber represented a black 50 cm x 50 cm x 50 cm Plexiglas cube, with a light bulb (60 W) placed at 40 cm from the floor. At the beginning of the test, each animal was introduced into the same left back corner of the arena and allowed to explore the arena freely for 5 min. The floor of the field was divided into 4 equal squares and the central zone. The number of entries into the center, ambulation (line-crossing), rearing, grooming, defecation and freezing was counted and recorded.

The open-field camera contained an additional dark box that was separated from the light compartment by a wall with an opening (13 × 5 cm) at the floor level. The number of entries into the dark box was also counted and the time spent there was measured.

The experiments were conducted in full accordance with the legal and statutory acts applicable in Georgia and the international agreements ratified by the country, such as the Law of Georgia on Health Care and European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

2.2. Creatine Supplementation

Cr was purchased from Sigma-Aldrich (St. Louis, MO, USA), and diluted in 5% dimethyl sulfoxide (DMSO). 140 mg/kg/day was i.p. injected into the non-aggressive animals during 30 days. The rest of the experimental animals were supplemented with 5% dimethyl sulfoxide, depending on the animal weight (1 ml/100 gr). The Cr dose to be injected was chosen based on the data taken from the research by various authors [21, 22].

2.3. Preparation of Plasma Membrane from Hippocampal Slices

Slices of the hippocampus were homogenized in a sucrose solution containing (in mM) 250 Sucrose, 10 Tris-HCl and 1 EDTA (pH 7.7). The homogenate was centrifuged at 1000 × g for 10 min at 4°C to remove excess debris. The supernatant was removed and centrifuged at 7700 × g for 20 min at 4°C, followed by centrifugation of the supernatant at 7700 × g for 5 min at 4°C. The supernatant was then centrifuged at 55,000 × g for 30 min at 4°C. The precipitate was re-suspended into Tris-HCl buffer (pH 7.7) and frozen at -20°C before the use [23].

2.4. Measurement of Total NO_x Level

The level of NO_x in hippocampal samples was determined by a modified method of Miranda et al. [24].

As the first step, sample deproteinization was achieved by adding equal volumes 0.3 M NaOH to 100 µl of tissue homogenate. It was mixed well and incubated for 5 min at room temperature (RT). Then 100 µl of 5% ZnSO₄ was added, mixed well and incubated for additional 5 min at RT. After the incubation the mixtures were centrifuged at 3000 rpm at 4°C for 15 min. An aliquot of 100 µl of the clear supernatant was then mixed with 200 µl of Griess Reagent. Griess Reagent was prepared just prior to the assay and contained 0.25% VCl₃, 0.1% sulfanilamide and 0.05% N-(1-Naphthyl)-ethylene diamine (NEDD) in 0.5 M HCl. Reagent blank was the same, but contained 100 µl of distilled water instead of the sample. The mixture was incubated for 30 min at 37°C and absorbance was measured at 540 nm with a microplate reader (Multiscan GO, Thermo Fischer Scientific, Finland).

The standard curve for NaNO₂ was used to calculate total NO concentration in the samples.

2.5 Quantitative Determination of Ca²⁺ Concentration

The calcium concentration was determined with a commercial colorimetric kit (Sigma-Aldrich, cat. # MAK022, St. Louis, MO, USA). The calcium ion concentration was determined by the chromogenic complex formed between Ca²⁺ and O-cresolphthalein. Absorbance of the Ca²⁺-O-cresolphthalein complex was measured at 575 nm and was proportional to the concentration of calcium ions present.

2.6 Assessment of the Ca²⁺-ATPase Activity

Ca²⁺-ATPase activity was determined by the amount of inorganic phosphate released after the reaction. The assay medium (1 ml) contained 50mM Tris-HCl buffer (pH 7.4), 0.4mM MgCl₂ or 0.4mM CaCl₂, 2mM ATP, and the protein fraction (50 µg proteins). After 15 min of incubation at 37°C the reaction was stopped by adding 1.2 ml of ice-cold 10% trichloroacetic acid. The Ca²⁺-ATPase activity was taken as the difference between the values of enzyme activity in the presence and absence of Ca²⁺ [18].

2.7 Determination of Na⁺/K⁺-ATPase and Mg²⁺-ATPase Activity

Na⁺/K⁺-ATPase activity was calculated as the difference between total ATPase activity (Na⁺/K⁺, Mg²⁺-dependent) and Mg²⁺-dependent ATPase activity. Total ATPase activity was assayed at 37 °C in an incubation medium consisting of 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 20 mM KCl, 4 mM MgCl₂, 3 mM disodium ATP and 80-100 µg protein of the plasma membrane, total 1 ml. Ouabain (1 mM) was added to the medium to determine the activity of the Mg²⁺-ATPase. The values of Mg²⁺-ATPase (1) with NaCl, KCl and MgCl₂ and (2) with MgCl₂ only were compared. The amount of inorganic phosphate (Pi) released was quantified calorimetrically, as described by Fiske and Subbarow (1925), using KH₂PO₄ as a reference standard. Specific Na⁺/K⁺-ATPase and Mg²⁺-ATPase activities were computed by subtracting the blank from the overall activity and expressed in µmol Pi/mg protein/min [23].

2.8 Statistical Analysis

All statistical analyses were conducted by using SPSS software (version 23, SPSS, Chicago, IL). One-way ANOVA was used to assess group differences in all physiological and biochemical values. Tukey HSD or Games-Howell post hoc test was performed to assess the differences between groups. The values are expressed as the mean ± SEM. P values less than 0.05 were considered as statistically significant.

3. Results

The results presented in Figure 1 indicate that the non-aggressive (G1) and the naturally aggressive or killer animals (G3) differ in certain behavioral parameters. Particularly, G3 shows a significant increase in the number of line-crossings in the big chamber of the experimental cage (F (2, 36) = 15.102; p<0.05), the number of entries into the dark box (F (2, 36) = 4.552; p<0.05), as well as the number of rearing (F (2, 36) = 16.270; p<0.001). Certain fear-related reactions showed a decline. This, in particular, concerns the number of defecations (F (2, 36) = 4.754; p<0.05) and freezing (F (2, 36) = 11.827; p<0.0001). The time spent in the dark box and number of grooming did not vary

significantly.

In order to establish the influence of Cr on the behavioral parameters we further studied the latter in the non-aggressive individuals that had been daily injected 140 mg/kg of Cr for 30 days (G2). The obtained data show that similar to G3 animals, they displayed an increase in locomotor activity compared to that among the control group (G1). Just as in the case described above, the number of ambulation (F (2, 36) = 15.102; p<0.001) and frequency of rearing (F (2, 36) = 16.270; p<0.001) increased. Likewise, the frequency of fear-related behaviors, such as freezing (F (2, 36) = 11.827; p<0.05) and defecation (F (2, 36) = 4.754; p<0.05), was reduced. The other behavioral parameters did not vary significantly.

In order to find how behavioral parameters among non-aggressive animals relate to those of aggressive individuals under the influence of i.p. injected creatine, we further compared these two groups. The difference was not statistically significant in any parameter, except in parameter a (F (2, 36) = 15.102; p<0.05).

Apart from behavioral parameters, we also studied certain biochemical markers. The experiments showed that Cr supplementation has a significant effect on the NO level in hippocampal samples, which was reduced in G2 compared to G1 individuals (F (2, 21) = 6.574; p<0.01). Similar to the behavioral data, no changes were found between the NO levels in G2 and G3 individuals (Fig.2a).

Repeated measures ANOVA showed a significant change in Ca²⁺ levels (F (2, 21) = 13.319; p<0.001) across the three groups. Results of a post hoc analysis (Games-Howell) to compare G1 and G3 animals was nonsignificant, while G2 members displayed a statistically significant decrease in the ion concentration in hippocampal samples compared to G1 (p<0.001) and G3 (p<0.05).

The next step was to study changes in the activity of some transport ATPases in the hippocampal slices of experimental rats.

The data presented in Figure 3a shows that Cr supplementation has no impact on non-aggressive animals' hippocampal plasma membrane Ca²⁺-ATPase (PMCA) activity, nor were any differences found between G1 and G3. Microsomal Ca²⁺-ATPase (MCA) activity did not change either (Fig. 3b). Considering the importance of mitochondrial Ca²⁺-ATPase (MitCA) in maintaining intracellular Ca²⁺ levels, change in the activity of MitCA was also monitored, but the results were similar to the above and the groups did not vary considerably (Fig. 3c).

Unlike the Ca²⁺-ATPase, Na⁺/K⁺-ATPase showed a statistically significant increase under Cr-supplementation (G2) compared to G1 individuals, F (2, 21) = 138.420; p<0.001, which was also true for G3 (Fig. 4a). Tukey HSD post hoc test detected a 37% increase in the Na⁺/K⁺-ATPase activity of G2 animals (p<0.001), the same indicator being approximately 11% for G3 individuals (p<0.01); and the increase in enzyme activity of G2 animals was equal to 27%, compared to G3 (p<0.001).

No statistically significant changes were found in the case of Mg^{2+} -ATPase, indicating that 30-day Cr supplementation

to aggressive or non-aggressive animals does not affect its enzymatic activity (Fig. 4b).

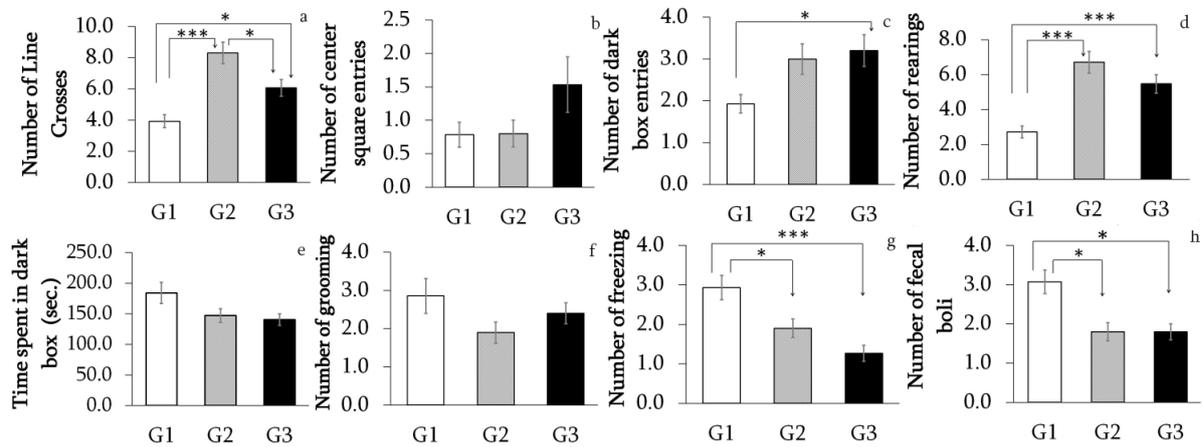


Figure 1. Behavioral characteristics of Non-aggressive (G1), Cr-treated non-aggressive (G2) and Aggressive (G3) animals in open field. a - number of line crossed; b - center square entries; c - entries in dark box; d - rearing frequency; e - time spent in the box camera (sec.); f - number of grooming; g - number of freezing; h - defecation. Data are mean \pm SEM for $n = 10 - 15$ in each group. * $p < 0.05$, *** $p < 0.001$

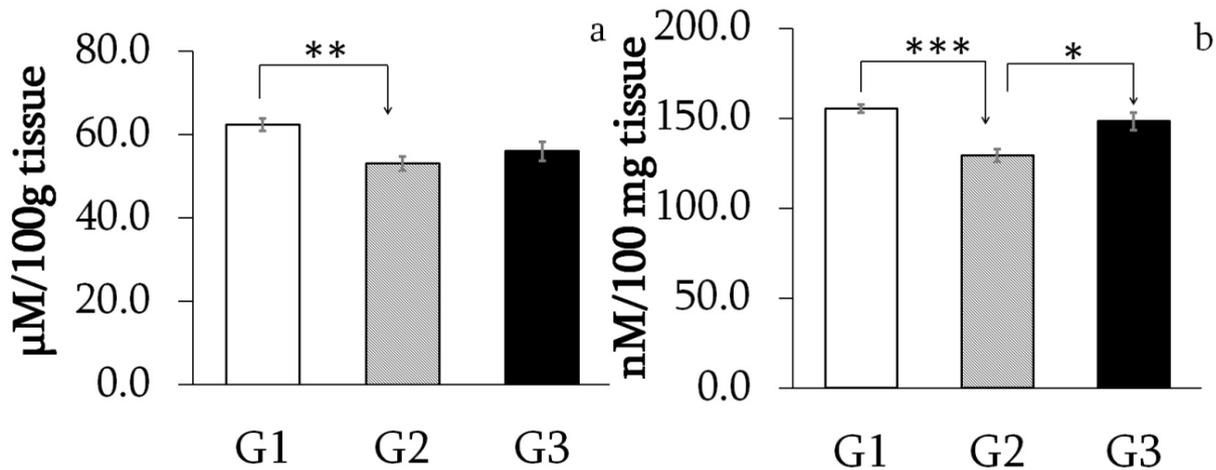


Figure 2. Alteration in NO (a) and Ca^{2+} (b) levels in Non-aggressive (G1), Cr-treated non-aggressive (G2) and Aggressive (G3) animals. Data are mean \pm SEM for $n = 8$ in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

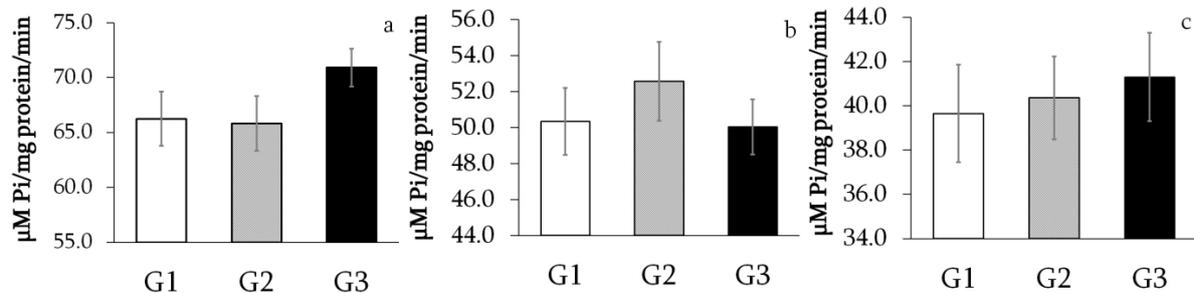


Figure 3. Alteration in plasma membrane (a), microsomal (b) and mitochondrial (c) Ca^{2+} -ATPase activity in Non-aggressive (G1), Cr-treated non-aggressive (G2) and Aggressive (G3) animals. Data are mean \pm SEM for $n = 8$ in each group

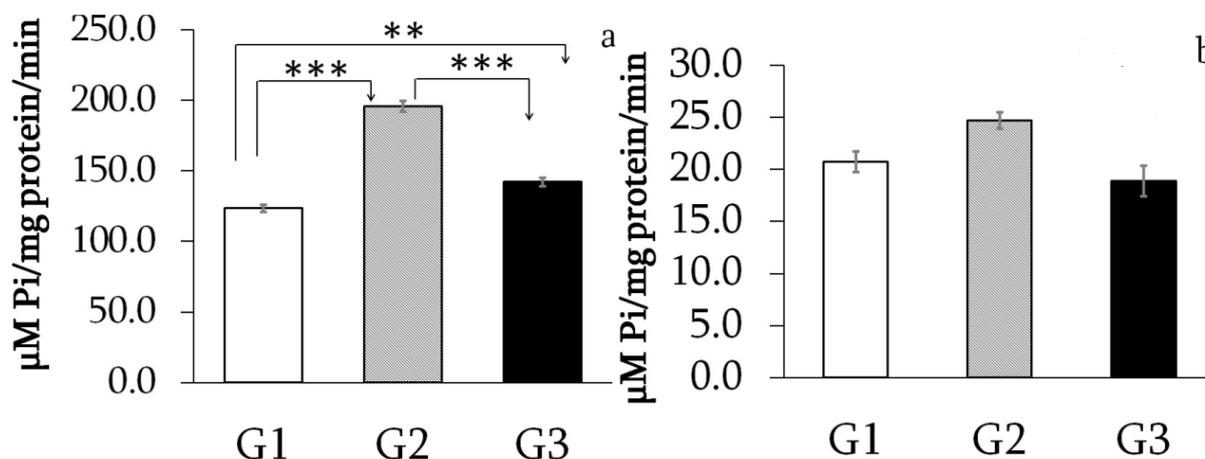


Figure 4. Alteration in hippocampal Na⁺/K⁺-ATPase (a) and Mg²⁺-ATPase (b) activity in Non-aggressive (G1), Cr-treated non-aggressive (G2) and Aggressive (G3) animals. Data are mean ± SEM for *n* = 8 in each group. ***p* < 0.01, ****p* < 0.001

4. Discussion

The experimental observations show a significant difference in behavioral parameters of non-aggressive (G1) and naturally aggressive (G3) laboratory rats (Fig. 1). G3 animals exhibited a higher rate of exploratory activity and lower fear-related reactions, which is generally characteristic of aggressive behavior [19]. We also observed G2 and found that an extended, 30-day i.p. supplementation of 140 mg/kg Cr modified their behavioral parameters, which approximated those prevalent among G3. Similar effect was reported when depressed animals were fed Cr [6]. This makes it possible to suggest that therapeutically Cr may be potentially used as an anti-depressant solution [25]. Thus, results of our experiments are clearly compliant with the historical data that describe improvement of physiological characteristics among laboratory animals subjected to Cr diet, notwithstanding the difference in the forms of supplementation.

Aggressive and non-aggressive rats also differ by some biochemical parameters, such nitric oxide (NO) among others. It is an important mediator closely involved in various metabolic processes within the cell. Recent publications prove the importance of variation in NO concentration in the way various biochemical processes in the brain are regulated. Aggressive animals are known to be characterized by NO deficiency resulting from a lowered activity of its synthesizing enzyme - neuronal NO-synthase (Nanos) [26]. Therefore, injection of a selective Nanos inhibitor, such as 3-bromo-7-nitroindazole (3BrN) into the rats increases the level of aggression [27]. Similar results were found in male rats with knockout nNOS gene [28]. It is worth noting that in our findings NO content in the hippocampus of G2 rats was significantly lower than that among G1 animals (Fig.2a). Values from G2 and G3 were identical, which serves as another argument in favor of Cr being a factor in development of aggression-related changes.

Under natural conditions, cellular NO is synthesized by NO-synthase (NOS) that is activated by Ca²⁺ and is, thus,

sensitive to the concentration of the ion [29]. So, as the next step, content of the Ca²⁺ was determined in hippocampal samples among the animals of all the three groups. The findings indicated that G3 individuals did not display a significant change in the Ca²⁺ concentration compared to G1 animals (Fig. 2b). Such results differ from the data reported by other authors in that the latter speak of an intensified locomotor activity under a higher Ca²⁺ concentration as a sign of higher aggression level. The disparity may be accounted for by the difference in the experimental conditions applied by our team and the other authors. In particular, we studied the intracellular content of Ca²⁺ in the hippocampus of G3 rats. Godinho et al. [30], on the other hand, considered correlation of behavioral parameters when the individuals were injected calcium exogenously, which, in turn, increased the ion content in brain cells. Therefore, under constant calcium quantity, NO reduction in aggressive animals might be caused not by the Ca²⁺-dependent regulation of NO-synthase, but rather, as hypothesized by some authors, by a lower translation rate for mRNA [31]. However, another reason may lie in a reduced antioxidant activity and activated oxidative processes, which is characteristic of aggressive animals. This could be another impact on the activity of various enzymes, including the NO-synthase [32, 33].

Meanwhile, the results were different in case of G2 animals. In particular, extensive intake of Cr by non-aggressive animals reduced the Ca²⁺ content in hippocampal cells, compared to both G1 and G3 (Fig. 2b). In addition, PMCA and mitochondrial Ca²⁺-ATPase, as well as microsomal Ca²⁺-ATPase did not reveal any meaningful changes in their activities (Fig. 3). Based on our and recent scientific data, it could be hypothesized that reduction in the level of Ca²⁺ accompanied by i.p. supplementation of exogenous Cr may be due to Cr's modulating effect on the glutamate reuptake by the pre-synaptic membrane, which allows NMDA-receptors of the post-synaptic membrane to restrict Ca²⁺ influx into the cell and reduce its intracellular content [34, 35].

As the functional and structural correlation between the NMDA-receptor and Na^+/K^+ -ATPase is well known [36], we also studied the Na^+/K^+ -ATPase activity across the three groups, namely in the hippocampal slices of the animals and established that aggressive individuals, as shown on figure 4a, did exhibit higher Na^+/K^+ -ATPase activity than non-aggressive rats, which is in accordance with scientific data [37, 38]. The results were the same for G2 animals.

Scientific data suggest that the cause of the observed changes has to be not just Cr impact on glutamate reuptake, but also its direct influence on NMDA-receptor and modulation of NMDA-calcineurine signaling pathway, which increases Na^+/K^+ -ATPase activity [24, 39]. Involvement of the NMDA-receptor in the Na^+/K^+ -ATPase and the correlation to Cr was also proved by a study that targeted the influence of Cr on Mg^{2+} -ATPase activity (Fig. 4b). Unlike Na^+/K^+ -ATPase, Mg^{2+} -ATPase is known not to be regulated by the NMDA-receptor [40]. Our findings indicate that hippocampal cells of G1 and G3 rats do not display any significant changes in Mg^{2+} -ATPase, unlike Na^+/K^+ -ATPase. Thus, i.p. supplementation of Cr in G3 does not affect its activity.

Therefore, it would be reasonable to assume that extended Cr supplementation alters various physiological and biochemical characteristics among laboratory animals and also increases aggressiveness in non-aggressive individuals, due to its active role in energy metabolism, as well as its neuro-modulating action. However, the results are still insufficient to make definitive conclusions. We anticipate that further research in Cr's influence on NMDA-receptor and oxidative processes as part of aggression-linked changes will lead to a bigger and clearer picture in understanding molecular mechanisms of aggression and finding possible ways leading to its prevention.

5. Conclusions

To sum up, it is clear that extended Cr supplementation induces certain types of aggressive behavior in naturally non-aggressive individuals. In addition, it also changes biochemical parameters, by e.g. causing a decrease in NO and Ca^{2+} levels and in Na^+/K^+ -ATPase activity.

Supposedly, such changes could be induced by the modulating effect of Cr on glutamate reuptake and alterations in NMDA receptor activity. Finally, it has to be pointed out that the results do not allow for any definitive conclusions and there is need for further and more detailed investigations.

Acknowledgments

This work was supported by the financial support of the Shota Rustaveli National Science Foundation of Georgia [grant number FR/226/7-220/13]. Any idea contained in this publication is the property of the authors and may not

represent the opinion of the Shota Rustaveli National Science Foundation of Georgia.

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