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**ORIGINAL ARTICLE**

# Investigation of Brain Creatine Levels Under the Mental Stress Conditions

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Alterations in brain creatine levels are considered to be associated with various pathological conditions. However, there is still no exact evidence on character of this changes and clear link between disorders and upstream and downstream direction of creatine changes.

Chronic mental stress conditions are thought to be connected with upstream regulation of cellular oxidative pathways, thus oxidizing various structural and active compounds. Oxidative stress also takes part in increase of permeability of blood brain barrier (BBB) that, in turn, makes it possible for a number of molecules to cross the BBB in both directions.

Observations on long-term social isolation and circadian rhythm violation show a rising trend in brain creatine amount, while there was remarkable down-regulation in creatine synthesizing system, as the key-enzymes' (AGAT and GAMT) activity was decreased.

Investigations of BBB permeability for creatine under the stress conditions by mass-spectrometric analyses revealed no changes in creatine transport in the stress group, compared to the control. However, the activity of mitochondrial CK was reduced for about 25% and $V_{max}$ had fallen down in the stressed group, the Km was not drastically changed.

To sum up, it could be supposed that the reason for the elevations of creatine levels in brain under the mental stress conditions could be stimulated by the activated oxidative stress that induces conformational changes in mitochondrial Creatine Kinase structure and decreasing the ability of enzyme to phosphorylate the creatine and as a result free creatine levels in brain are being arisen.

*Key words: Mental stress, Creatine, Creatine kinase, L-Arginine:glycine amidinotransferase, guanidinoacetate methyltransferase*

Creatine/Phosphocreatine/Creatine Kinase shuttle is one of the key player in maintaining energy homeostasis in tissues with high energy demand, such as nervous and muscular tissue (Almeida *et al*., 2006; Brosnan and Brosnan 2007; Andres *et al*., 2008). Functional state of the Cr shuttle is markedly dependent on the intracellular level of Cr and PCr and on the activity of CK that exists in various tissue specific and subcellular isoforms (Wallimann *et al*., 1998).

Cr is also believed to have some more functions, such as participation in signal transduction, maintaining membrane potential and taking part in a wide range metabolic pathways in the central nervous system (CNS) (Bothwell *et al*., 2001; Galbraith *et al*., 2006). Recent findings underline Cr as a possible neuromodulator agent that can have an impact on various post-synaptic receptors (Almeida *et al*., 2006). Recent observations show that Cr could be an important factor in psycho-motoric development and cognition, as well as in embryonic development (Beárd and Braissant 2010).

A wide range of neurodegenerative diseases are characterized with decrease in intracellular Cr levels, thus proving importance of Cr in normal cellular metabolism (Cagnon and Braissant 2007; Donnan *et al*., 2008; Beal 2000; Chaturvedi and Beal 2008). Cr deficiencies are connected with various pathological conditions of neural system such as epilepsy, shizophrenia and psychological stress (Duman and Monteggia 2006; Pittenger and Duman 2008; Allen 2012).

For a long period of time brain Cr was believed to be just transported via blood brain barrier (BBB) and originated just from kidney and liver (Wyss and Kaddurah-Daouk 2000), but findings about the presence of Cr synthesizing enzymes, L-Arginine: glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT) in CNS prove the ability of brain to produce some portion of Cr endogenously (Beárd and Braissant 2010). Although, specific Cr transporter (SLC6A8) transfers Cr across the BBB (Tachikawa *et al*., 2004), recent studies declare that about 95% of whole brain Cr is of brain origin (Beard and Braissant 2010).

In the past years many authors reported about the influence of various external and internal factors (oxidative stress, neurodegenerative diseases, as well as alterations in CK activity) on Cr levels in brain (Aksenov *et al*., 2000; Tachikawa *et al*., 2004; Beard and Braissant 2010; Kuchukashvili *et al*., 2011).

Following our previous findings that long-term social isolation and circadian rhythm disorders induce oxidative stress and down-regulation of energy metabolic pathways and antioxidant system (Koshoridze *et al*., 2010; Kuchukashvili *et al*., 2012), in this article we aimed to determine the brain Cr levels and changes in activity of AGAT and GAMT, as well as alterations in BBB permeability for Cr.

## MATERIALS AND METHODS

**Animals and social conditions.** The experiments were conducted on 20 adult male laboratory rats (120±10g) divided into two groups. Group 1 (10 rats) – control group (C-group) – were kept in a common cage under natural conditions (dark/light ratio = 10/14 h); Group 2 (10 rats) – stressed rats (S-group) were maintained in individual cages in the dark (dark/light ratio= 23.5/0.5 h) for 30 days.

During the experiments, the rats were given water and a standard laboratory chow *ad libitum*. The experiment was repeated for four independent series.

The investigations 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 (Directive 2010/63/EU).

**Creatine supplementation.** For monitoring creatine transport across BBB under the stress conditions $d_3$-creatine intraperitoneal injection was used (Perasso *et al*., 2003). Experimental animals were administered $d_3$-creatine (14 mg per 1 ml of 5% Dimethyl sulfoxide (DMSO); 140 mg/kg) six hours prior to decapitation, the control animals were injected 1 ml of solvent (5% DMSO).

**Intracellular Creatine (Cr) Concentration**. Intracellular Cr contents were measured by

colorimetrical assay kits (Catalog #K635-100, #K354-100, Biovision Inc., USA). The optical density was read on microplate reader (Multiscan GO, Thermo Fischer Scientific, Finland) at 570 nm. Creatine and ATP concentrations were calculated according to the standard curves.

**Concentration of Phosphocreatine (PCr)**. Assay depends on enzymatic conversion of PCr by which NADP+ is reduced. Reduced NADPH is then measured spectrophotometrically (Szasz *et al*., 1976). The optical density was read on UV spectrophotometer (CT-2200, Chrom Tech, Taiwan) at 570 nm wavelength. PCr concentration was calculated according to the standard curve.

**GAMT and AGAT activity assay.** Activity of AGAT was measured according to the method described by Da Silva *et al*. (2014) and the GAMT activity was monitored by the enzymatic assay described by Ogawa *et al*. (1983).

Creatine transporter amount was estimated by ELISA test kit obtained from MyBioSources Inc. (MBS086588, MyBioSource Inc., USA).

**Assessment of the CK activity.** The enzyme catalyzes incorporation of phosphate into creatine to form creatine phosphate. The amount of phosphates existing in a free form as a result of ATP hydrolysis in the mitochondrion was evaluated in the form of the phosphovanadiummolybdate complex and analyzed spectrophotometrically. The reaction medium contained 100 µl of suspension under study and 0.5 ml solution of creatine (10.0 mM) prepared in special buffer (3.0 mM glycine-NaOH + 5.0 mM $MgSO_4$, pH 9). The resulting mixture was suspended for 5 min at 37°C; then ATP (3.0 mM) was added and further incubation was performed at 37°C for 20 min. The enzyme reaction was stopped by adding a 5% solution of trichloroacetic acid. The resulting solution was then centrifuged for 10 min at 3000 g. About 0.5 ml of supernatant was mixed with 0.5 ml of an ammonia vanadate and ammonia molybdate mixture (1:1). The phosphate was assessed spectrophotometrically at λ = 400 nm wavelength (Nozadze *et al*., 2005).

**Estimation of the kinetic parameters of the CK by the Johansen and Lumry method.** We defined the nature of changes in kinetic parameters of CK (Km, Vmax) based on the affinity of the enzyme for the substrates (creatine, ATP) and the change in the maximum reaction rate. To this end, we studied the kinetic parameters under standard creatine concentrations and variable ATP concentrations, 0.5–5.0 mM, respectively. We also studied the same parameters under standard ATP concentrations and variable creatine concentrations, 1.0–10.0 mM, respectively. We used the results obtained to measure the kinetic parameters using the Johansen and Lumry equations (Johansen, Lumry 1961).

**HPLC-ESI-MS/MS.** Chromatographic analysis was performed according to modified methods of Jiang *et al.* (2012) and Bodamer *et al.* (2001).

Freshly obtained rat brain samples were quickly weighted and placed in a glass homogenizer with addition of pre-cooled 0.5 N $HClO_4$ (300 mg/10 ml) to precipitate proteins, and homogenized in ice-cold conditions. After centrifugation of the extract at 4°C for 10 min at 10 000 x g, the supernatant was neutralized by addition of 2 N KOH. Formed precipitate ($KClO_4$) was removed by centrifugation (4°C; 20 min at 3 000 x g) and remained supernatant was stored at −40°C prior to the HPLC-ESI-MS/MS analysis.

HPLC analysis was run on Varian ProStar liquid chromatographer (Varian, Denmark) and chromatographic separation was performed on SunFire™ C18 column (4.6x150 mm, 5µm; Waters, Irland). Prior to the HPLC procedure, the samples/standards were evaporated under nitrogen flow 45°C and the precipitate was re-suspended in 2 ml of HPLC grade acetonitrile. For isocratic separation of Sample/standard (20 µL) mobile phase consisting of acetonitrile was used: Trifluoroacetic acid (TFA):$H_2O$ (3.0:0.05:96.95 ml). The flow rate of the mobile phase was 400 µL/min and the column temperature was maintained at 30°C. The total run time was 10 min and the measurement was performed at 216nm wavelength. $D_3$-creatine was used as a standard (D-1972; CDN Isotopes, Quebec, Canada) with concentration 80 µg/ml, prepared according to the sample preparation procedure.

Mass spectrometry was performed using Varian

500MS IT Mass Spectrophotometer (Varian, Denmark). ESI ionization was performed in the positive ion mode with a spray voltage of 4500 V. The sample/standard introduction was via the HPLC system described above. Source spray temperature was 400°C. Nitrogen was used as both curtain and collision gas (40 psi) and heater gas pressure was 40 psi. The transition chosen for $d_3$-creatine and creatine was m/z 91-93 (DP, 20V; CE, 26V) and 88-91 (DP, 20V; CE, 26V) respectively. MS Workstation Version 6.8 software was applied for data acquisition and processing.

**Data analysis.** The statistical analysis was performed using software IBM SPSS Statistics 20.0 (IBM Inc, Armork, NY, USA). The data treatment was performed using One-Way ANOVA which revealed statistically significant differences between control and experimental groups at $P<0.05$.

## RESULTS

### *Mental stress conditions and brain Cr/PCr levels*

As a primary step we assessed changes in brain Cr levels under the 30 day social isolation and violation of circadian rhythm. The data obtained (Fig. 1A) clearly show that under the chronic stress conditions (S-group) the amount of Cr increases by about 36% ($Cr_{control}$=110±12.3; $Cr_{stress}$=180±27.1; $n_1=n_2=8$; $t_9$=-6.65; $P<0.001$) compared to the control individuals (C-group).

Apart from this, the experiments revealed that S-group animals have ≈22% ($PCr_{control}$=1.48±0.3; $PCr_{stress}$=0.9±0.1; $n_1=n_2=8$; $t_8$=5.18; $P<0.001$) decrease in brain PCr levels in contrast with C-group members (Fig. 1B).

Since the amount of brain Cr greatly depends on the endogenous synthesis (Béard and Braissant 2010), it was important to estimate changes in activity of Cr synthesizing enzymes AGAT and GAMT. Data presented on figure 2 emphasize that under chronic stress conditions have negative impact on Cr production pathways, thus decreasing activity of AGAT from 5.25±0.25 nmol/min*mg protein-1 (C-group) to 4.10±0.5 nmol/min*mg protein-1 (S-group) ($n_1=n_2=5$; $t_8$=11.64; $P<0.05$). The falling trend of enzymatic activity was observed in case of GAMT, where the control value was decreased by about 31% ($GAMT_{control}$=3.2±0.15; $GAMT_{stress}$=2.2±0.3; $n_1=n_2=5$; $t_8$=28.11; $P<0.05$).

As it was shown, Cr synthesizing pathways must be down-regulated due to the decrease in enzymatic activity of AGAT and GAMT. In this case it was important to evaluate the changes of sodium and chloride-dependent transporter protein (CrT) that are responsible for Cr blood to brain transport across BBB.

ELISA analyses show that there were no significant differences in CrT content between C- and S-group animals (Fig.3, $CrT_{control}$=36.7±1.7; $CrT_{stress}$=35.2±1.2; $n_1=n_2=7$; $t_{12}$=1.3; $P>0.05$), proving that the increase in brain Cr level is not due to activation of transport processes via CrT across the BBB.

### *Blood-to-Brain transport of Cr under the mental stress condition*

As the increase in brain Cr content was not caused by activation of synthesizing pathways nor by increase in CrT amount, which are the main players in maintaining brain Cr homeostasis, it was important to check the cause of Cr increase in the brain under the long-term social isolation and violation of circadian rhythm.

Cr blood-to-brain transport was assessed by d3-creatine monohydrate ($d_3$Cr) intraperitoneal injection (140 mg/kg) before 6 h prior to decapitation. C-group individuals were injected in the same manner with the equal amount of solvent (5% DMSO). After decapitation Cr content was measured in brain samples by HPLC-ESI-MS/MS analyses to monitor the transport of Cr via BBB.

High performance liquid chromatography separation was unable to reveal any kind of differences between endogenous Cr and $d_3$Cr as the retention time for both compounds was the same (Fig.4), so it was not clear whether the exogenous Cr could cross the BBB.

It was essential to use a more sensitive detection method to determine existence of $d_3$-Cr in brain samples and to distinguish it from endogenous Cr. The HPLC coupled ESI-MS/MS qualitative determination was used to estimate labelled Cr in the whole brain homogenates.

As it is clearly visible from fig.5 no $d_3$-Cr was detected in C-group individuals as well as in S-group animals' brain. The data confirm the idea that Cr increase was not due to the up-regulation of its peripheral transport, but via a different pathway and the

reason must be in the Cr/CK/PCr shuttle.

***Character of changes in activity of CK under the chronic stress conditions***

As the experiments were unable to prove the differences with the blood-to-brain transport of Cr under the stress conditions, it was ultimately important to find out whether any changes were present in Cr/CK/PCr cycle and mainly in the activity of CK.

Experiments revealed that under the chronic stress conditions activity of CK was decreased compared to C-group individuals (fig.6, $CK_{control}$=36.3±1.6; $CK_{stress}$=28.6±2.1; $n_1=n_2=10$; $t_{18}$=4.94; $P$<0.05), marking that long-term social isolation and violation of circadian rhythm has a negative influence on CK activity that in turn can down-regulate Cr/CK/PCr shuttle.

Changes that were observed in CK activity complement the findings that were obtained previously about the Cr content in brain under the mental stress conditions, but the reason for this changes was, at that time, known.

To investigate further the characteristic of down-regulation of CK the Johansen and Lumry weighted least squires method was used. The calculations allow determining exact values of $V_{max}$ and $K_m$, the results are presented in table 1.

The data presented in table show that under the long-term stress conditions $V_{max}$ value decreased under the changeable concentrations of Cr as well as variable ATP value. Unlike $V_{max}$, Michaelis–Menten's constant (Km) did not undergo any significant changes.

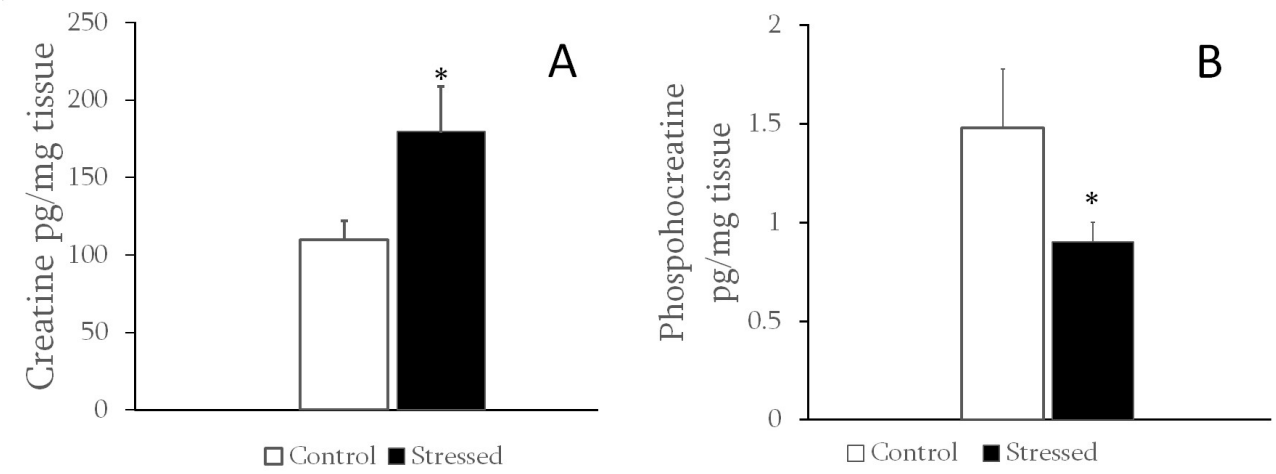


**Figure 1.** Levels of Cr (A) and PCr (B) in Brain under the long-term mental stress conditions.
*Data are expressed as mean ±SD from four individual experiments; *P<0.05*

**Table 1.** Kinetic parameters (Vmax, Km) of CK under the substrates changeable conditions. n=15; *p≤0.05

| **Kinetic Parameters** | Changeable concentration Cr (ATP=const) | | Changeable concentration ATP (Cr=const) | |
|---|---|---|---|---|
| | *Control* | *Stressed* | *Control* | *Stress* |
| **$V_{max}$** | 35.30 ± 1.45 | 29.05 ± 0.90* | 31.45 ± 1.97 | 25.37 ± 1.25* |
| **$K_m$** | 12.66 ± 1.56 | 11.57 ± 2.31 | 2.91 ± 0.62 | 3.13 ±0.36 |

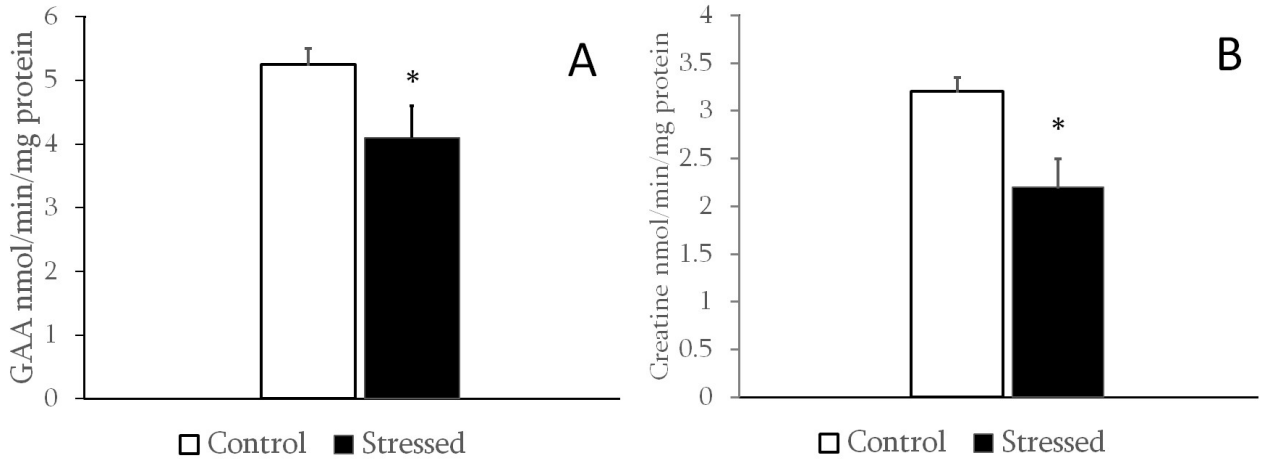


**Figure 2.** Differences in activity of AGAT (A) and GAMT (B) under the psycho-emotional stress.
*Data are expressed as mean ±SD (n=15) from four individual experiments; *P<0.05*

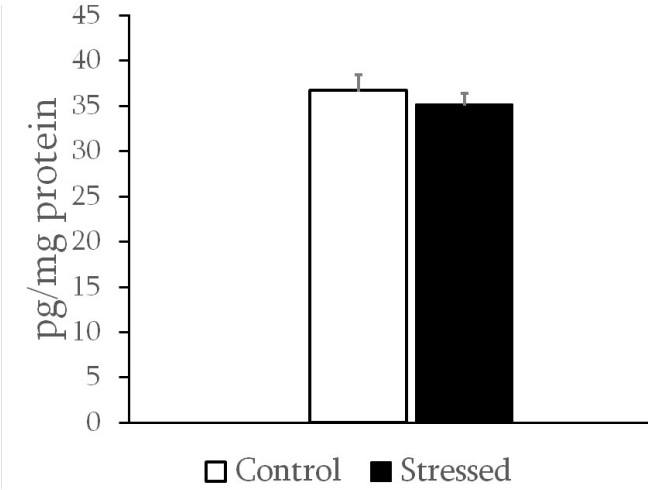


**Figure 3.** Amount of Cr transporter protein (CrT) in brain samples of stressed and control individuals
*Data are expressed as mean ±SD from four individual experiments; *P<0.05*

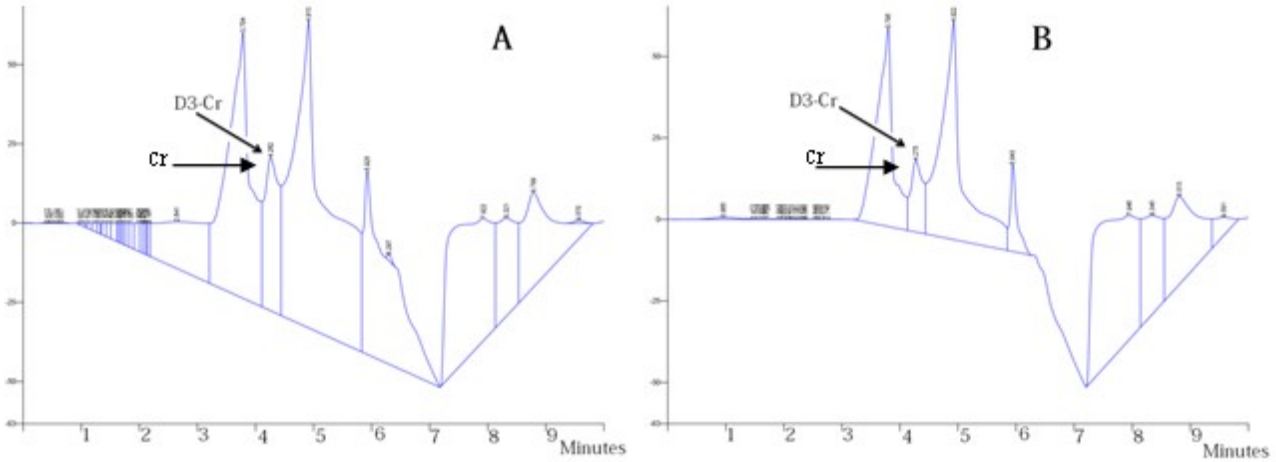


**Figure 4.** HPLC Chromatogram of C- and S-group individuals' brain samples, marked as A and B respectively. y axis - Absorbance; x axis – retention time (min)

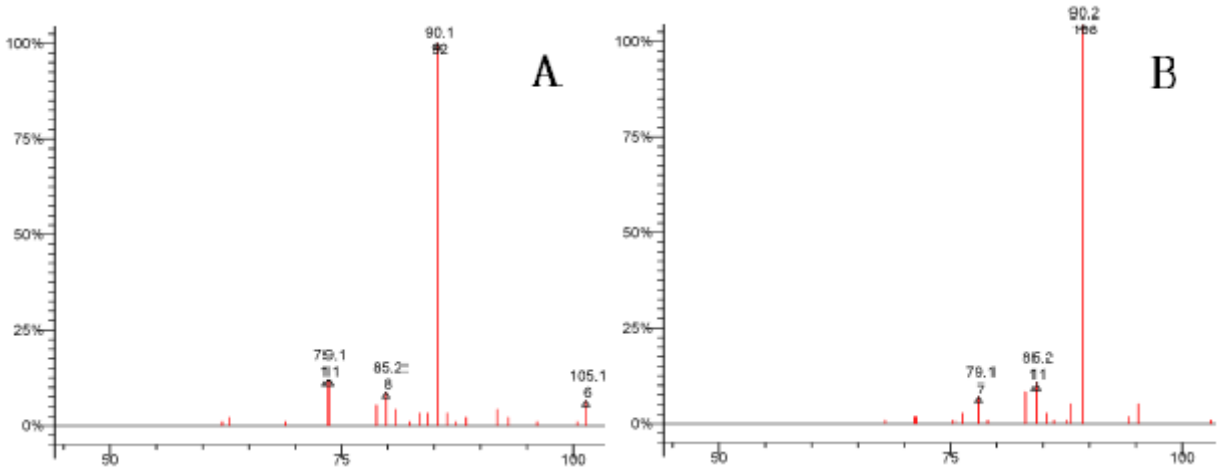


**Figure 5.** Mass ESI tandem mass spectra of Cr and d3-Cr with product ion scan (m/z 90.1 and 93.1 respectively) of C- (A) and S-group (B) individuals' brain samples. y axis -Intensity; x axis – m/z

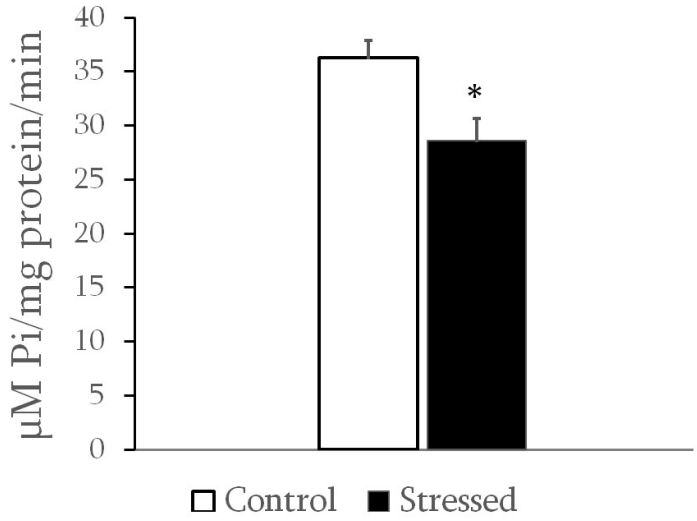


**Figure 6.** CK activity in brain under the stress conditions
*Data are expressed as mean ±SD (n=8) from four individual experiments;*P<0.05*

## DISCUSSION

Cr is a well known compound participating in high energy phosphate transfer from mitochondria (ATP synthesis) to cytoplasm (ATP use), thus contributing to cellular homeostasis. The main portion of Cr is taken from everyday diet and is also produced by means of endogenous synthesis. As it was established the main sites of Cr accumulation are muscle and neural tissues, where Cr is delivered from blood flow, via the plasma membrane sodium- and chloride-transporter protein family member SLC6A8, also known as CrT.

Although the Cr synthesis is distributed between kidney and liver, recent data suggests that brain demand on Cr is mostly balanced by its endogenous production. Simultaneously, existence of CrT in BBB proves that some portion of inner Cr is transported from blood stream.

The present study analyzes alterations of Cr levels in rat brain under mental stress conditions induced by 30 day social isolation and violation of diurnal cycle. The measurements of brain Cr level (Fig.1) revealed about 25% increase of Cr in S-group individuals compared to C-group animals' brain (Fig. 1A). On the other hand, the amount of PCr in brain under the extended social isolation, accompanied by the circadian rhythm disorders was decreased in stressed animals (Fig. 1B). Despite

the fact that the Cr/PCr levels are changed in stressed group members there is no significant alteration in total Cr pool. This could be due the difficulties in Cr phosphorylation process under the mental stress conditions.

Investigations about AGAT and GAMT activity was done to clarify more the reason of elevated Cr levels in the brain. Surprisingly, the rise in Cr levels was accompanied by a significant decrease in AGAT, as well as GAMT activity (Fig.2), thus suggesting that changes in Cr amount is not caused by activation of the producing pathways. Markedly, similar results were also reported by other authors (Loo *et al*., 1986).

It is assumed that under the various pathological conditions ongoing oxidative processes promote increase in permeability of BBB and in turn giving possibility to low molecular weight compounds to cross it (Barnham *et al*., 2004; Skowrońska and Albrecht 2013). Our previous findings declare the formation of oxidative stress under the mental stress conditions (Kuchukashvili *et al.,* 2012; Burjanadze *et al.,* 2014; Dachanidze *et al.,* 2015) so we aimed to check whether Cr increase was due to activation of oxidative pathways and increase in permeability of BBB intraperitoneal injection of high concentration of labeled, $d_3$-creatine (140 mg/kg) was used.

Investigations that were done by the HPLC-ESI-MS/MS detection of $d_3$-creatine in rat brain samples and therefore by observations on changes in the content of CrT protein give us possibility to have some view on the reasons of Cr increase in brain samples of stressed animals.

Measurement of CrT protein levels (fig.3) couldn't show any drastically changes between S- and C-group members, suggesting that under the stress conditions there is no significant alterations in Cr transport system. Surprisingly, the HPLC-ESI-MS/MS also, didn't reveal existence of d3-creatine in brain samples of stressed animals, as well as in control group (Fig.5). The results contribute to the findings that brain Cr content mainly depends on the endogenous synthesis and it is the true for the psycho-emotional stress conditions. Also the idea about the brain Cr increase due to its arisen transport from outside lacks the serious evidences. Therefore, to take into account the $d_3$-creatine detection by ESI-MS/MS gives an idea, that this kind of mental stress disorders aren't the reason for increase in BBB permeability.

One of the reasons of the changes in brain Cr content could be in alterations in Cr/PCr/CK shuttle that are mainly connected with different isozymes of CK (mitochondrial and cytoplasmic). Observations revealed about 25% decrease in CK activity (Fig.6) showing the influence of mental stress on Cr/PCr/CK.

As our previous experiments show formation of oxidative stress under the long-term social isolation and violation of diurnal cycle it has to be taken into account, that mitochondrial, as well as cytosolic CK is one of the targets for various free radicals that are mainly formed into mitochondria and can led to some conformational modifications of protein structure and negative changes in enzymatic activity (Bürklen *et al*., 2006).

Despite the fact that estimations of $V_{max}$ of S-group animals showed significant decrease in the value, $K_m$ wasn't changed (Tab. 1). The results are very similar to those taken by other authors (Aksenov 2000), were that was thought to be due to the oxidative changes in enzyme structure that in turn induces decrease in CK activity.

It is known that active site of CK is formed by about 12 amino acid residues: Pro143, Cys146, Arg151, Met207, Ala208, Arg209, Arg215, Asn230, Glu231, Glu232, Asp233 and His234. Although it has to be noted that structural cleft around the Cys146 is responsible for interaction with different compounds, including nitric oxide (NO), that in turn have influence on protein conformation and could have negative impact on enzymatic activity (Sheng *et al*., 2009).

Significant results that were taken about the long-term social isolation and circadian rhythm disorders prove that under this stressful conditions amount of NO in brain was arisen, that was thought to be one of the stimulators of oxidative and nitrosylation reaction and inducer of oxidative stress (Kuchukashvili *et al*., 2012). Supposedly, inducer of such changes in the content of NO in brain could be increase in brain calcium levels, which is activator of nitric oxide synthase (Menabde *et al*., 2011). Changes in brain NO could be one of the

reasons of down-regulation of CK via the oxidative pathways.

As the brain Cr increase via the arisen BBB transport wasn't declared, so the alterations in brain Cr content could be due to changes in Cr/PCr shuttle via decrease in CK activity induced by conformational changes in the enzyme molecule.

To sum up it could be proposed, that psycho-emotional stress that is induced by long-term social isolation and circadian rhythm disorders is accompanied by increase in brain free Cr levels, that is due to the down-regulation of phosphorylation pathways via Cr/PCr shuttle. Such changes must stimulated by the formation of oxidative stress and conformational changes in mitochondrial CK molecule. It has to be noted, that such kind of changes are very common for various neurodegenerative pathologies, such as Alzheimer's disease and etc (Bürklen *et al*., 2006).

## ACKNOWLEDGMENTS

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