Research article
Region-specific changes in expression and activity of calpains in the CNS of native rats

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ABSTRACT

Introduction and Aim: Active calpains change the activity and structure of their substrates by limited proteolysis, which regulates various cellular processes. In recent research it has been proposed, that µ-calpain is responsible for the neuronal survival, while m-calpain - for the degeneration and processes leading to apoptosis. Therefore, it can be assumed that the "susceptibility" to the damage factor for neurons in different CNS regions will depend on the content/activity of calpain isoforms. We analyzed the mRNA levels and the activity of µ- and m-calpain in the different CNS structures of native rats.

Materials and Methods: The experiment was carried out on intact male Wistar rats (n=40). After decapitation the prerontal cortex, striatum, hippocampus, midbrain, brainstem, cerebellum, and spinal cord were removed. Each structure was divided into two parts: casein zymography was performed on one part of the material to determine the activity of µ- and m-calpains; real-time RT–PCR was performed on the other part of the material to determine the level of expression of mRNA of µ- and m-calpains.

Results: We have shown that mRNA of µ-, m-calpain and calpastatin is presented in the prefrontal cortex and cervical spinal cord in comparable amounts. In the striatum, midbrain, and brainstem mRNA of m-calpain predominates, while the µ-calpain mRNA enrichment was noticed for the hippocampus and cerebellum. The highest µ-calpain activity was in cervical spinal cord, the lowest - in striatum. The m-calpain activity was relatively high in the midbrain, striatum, hippocampus, and brainstem, while in the cervical spinal cord and cerebellum it was moderate.

Conclusion: The selective neuronal death observed during neurodegeneration can be partially determined by the initial level of calpains expression and/or activity.

Keywords: Calpain; c-DNA; CNS; calpastatin.

INTRODUCTION

The calpain family consists of 15 calcium-dependent neutral proteases and their highly specific endogenous inhibitor calpastatin. All calpains are tissue- and substrate-specific. µ-calpain and m-calpain are most represented in the nervous system cells; they are highly expressed in neurons and glial cells. µ-Calpain is more abundant in neurons, while m-calpain is mostly presented in glial cells (1). Calpain 1 and calpain 2 have ~60% homology, and are activated by interacting with a small (30 kDa) regulatory subunit (CAPNS1), resulting in the formation of heterodimers, µ and m-calpain, respectively (2,3). The prefix (µ and m-) reflects the Ca²⁺ concentrations required for calpain activation: half of the maximum in vitro activity for µ-calpain is achieved at 3-50 μM Ca²⁺, while 0.4-0.8 mM is necessary for the activation of m-calpain (2). The higher µ-calpain sensitivity to Ca²⁺ relative to m-calpain is due to the presence of the PEF (Peflin – Ca²⁺ - binding protein) (4,5). Functions of calpains, including those in the CNS, are determined by the calpain isoform, the contribution of each to the total calpain-like proteolytic activity, and the availability of substrates, which, among other things, depends on the region of the central nervous system. Hence, it is necessary to investigate calpain representation / activity in different CNS regions.

The study of calpain-calpastatin system member’s expression in rat brain showed that the expression of calpain 2 and calpastatin in adult rats is small in the forebrain and moderate in almost all pyramidal and granular cells (6). In another study, competitive reverse transcription PCR was used to analyze mRNA levels of calpastatin and µ, m-calpain in the brain and spinal cord of mice. It was established, that the expression of calpastatin and m-calpain is 3 and 15 times higher compared to µ-calpain (7). It was confirmed by qPCR, that m-calpain is highly expressed isoform in CNS, followed by µ-calpain. The exploration of rat brain slices by in situ hybridization method showed a heterogeneous distribution of m-calpain transcripts in different neuron populations. In the hippocampus CA pyramidal neurons also showed intensive expression of m-calpain (3 times higher) relative to glial cells from the hippocampal cells. On the other hand, µ-calpain and calpastatin mRNAs have been found in neurons and glia and are uniformly expressed throughout the brain (7,8). The expression protein

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level of μ- and m-calpains have been identified in different regions of the rat brain, including the granular and pyramidal neurons, cerebral cortex of the hippocampus and cerebellum, and the spinal cord. An immunohistochemical study on the distribution of calpains in the rabbit hippocampus showed that μ-calpain is found in almost all granular and pyramidal cells, while the immunoreactivity of m-calpain was mainly detected in various populations of interneurons (9). The activities of μ- and m-calpain are frequently assessed in pathological conditions, due to the absence of the methodology approaches for vital enzymatic activity measurement. There are analysis of the mRNA levels and activities of μ- and m-calpain in the cells of: cortex, striatum, hippocampus, brainstem, cerebellum, cervical spinal cord, and midbrain of rats. Here, we analyzed the mRNA levels and activities of calpains in the cortex, striatum, hippocampus, brainstem, cerebellum, cervical spinal cord, and midbrain of rats as brain regions that are selectively damaged in various neurodegenerative diseases.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats (180 ± 20 g) were housed in one-room cages with controlled standard conditions (T° = 24 ± 1 °C, p = 45–65 % cycle: 12 h light/12 h dark). The rats were given rat pellets and water ad libitum. All animal experiments were performed in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and national laws (Russian Federation, Ministry of Health N 267, June 19, 2003; Guidelines for the Use of Laboratory Animals, Moscow, 2005) and certified by the local ethics committee of the Institute of Experimental Medicine (Ethics Issue No. 1/20, 2020). The rats were decapitated using a guillotine (OpenScience AE1601, OOO SPC OpenScience, Russia). Manipulations with animals were carried out under the control of the internal quality control department. The brain was quickly taken out and divided according to an earlier protocol (10). The cortex, striatum, hippocampus, brainstem, cerebellum, cervical spinal cord, and midbrain were isolated using brain slice accumulation according to the brain atlas (11). Structures were immediately frozen and stored at -80°C until analysis.

RNA isolation

We isolated total RNA from different regions of CNS with TRIzol reagent (Invitrogen, UK). We measured the RNA concentration on a spectrophotometer (NanoDrop 2000 Thermo Scientific, USA) according to the standard method. The purity of the RNA samples was checked at A260/A280 optical density and confirmed for ratio > 1.8., the 18S/28S RNA ratio was analyzed after electrophoresis in 1.4% agarose gel and then analyzed the 18S/28S RNA ratio to verify the integrity of the samples.

**cDNA synthesis and real-time RT–PCR**

cDNA synthesis was performed using cDNA reverse transcription kit (Applied Biosystems). For RT-PCR, Evrogen 5q qPCR mix-HS SYBR reagents were used. Primers used in RT-PCR were as follows:

μ-Calpain (12)

F 5′ – ACCCAGGACTAGATGACCA – 3′

R 5′ – TACCGTCTGATCCATGAGG – 3′

m-Calpain (13)

F 5′ – AGCCAATGAGGAGACATTG – 3′

R 5′ – CTCCCATCTTATCCAGCAT – 3′

Calpastatin (14)

F 5′ – TGGCACTGAGGAGAGACA – 3′

R 5′ – TGCATCTTATCCACCTTTGGC – 3′

GAPDH (15)

F 5′ – AAACCCATACCACATCTTCCA – 3′

R 5′ – GGTGTCACACACACACAA – 3′

GAPDH mRNA was used as internal control. The calculation was carried out using the delta-delta CT method, in contrast to the control group. The results are presented as histograms. Three independent PCR repeats were performed for each cDNA obtained from 7 animals, the results of which were then averaged.

**Casein zymography**

The structures of interest were brought to a homogeneous state in Tissue Lysis Buffer pH 7.4 (Promega) and centrifuged for 30 min at 4°C and 12,000 g. The concentration of protein was measured using a Bio-Rad DC protein assay, with bovine serum albumin as control. The 9% acrylamide release gel was copolymerized with casein (0.1%), the concentration gel was 4% acrylamide. The preliminary cycle lasted 45 minutes, after which each sample in the amount of 50 μg of protein was loaded and subjected to electrophoresis at 125 V for 2.5 hours at 4°C in working standard buffer. The gel was stained with Coomassie Brilliant Blue (Hi Media). The upper unstained zone is μ-calpain, and the lower light zone is m-calpain, the protein activity is proportional to the area of unstained zones. Zone areas were quantified on a Gel Doc XR (Bio Rad) with backlight where the gels were scanned. The MultiSpec program (https://mygeohub.org/resources/multispec) was used to obtain band data (from band brightness and area transition) and the activity of calpain evaluated as described previously (16,17).

**Statistical analysis**

Statistica-10.0 program (StatSoft, USA) was used for statistical analysis. The work was performed by a member of the team who did not participate in the experiments (DST). The data was given to him blind. G*Power software 3.1.9.2 with an effect size of 0.5, at
a 5% significance level, a power of 0.90 was used to calculate the sample size. The Shapiro-Wilk test was used to test the data distribution for normality. For the analysis of outliers, the criterion of the mean absolute deviation was used. Results are presented as mean ± CI. Differences between the cortex and other structures were detected by t-test for individual mean values, p < 0.05 was considered as statistically significant.

RESULTS

It was seen mRNA of µ-, m-calpain and calpastatin is presented in the cells of the prefrontal cortex and cervical spinal cord in comparable amounts. There was predominance of m-calpain in the striatum, midbrain, and thalamus, and of µ-calpain in the hippocampus and cerebellum. The highest activity of µ-calpain was characteristic for the cervical spinal cells, moderate activity was in the brainstem, midbrain, and cerebellum, and the lowest one was revealed in striatal cells. The different picture was observed for the m-calpain activity: in cells of the midbrain, striatum, hippocampus, and thalamus it was relatively high, while in the cells of the cervical spinal cord and cerebellum it was moderate (Fig. 1).

![Fig. 1: Representative casein zymogram, reflecting the activity of µ- (upper zone) and m-calpain (lower zone) in different regions of Wistar rat CNS. Lane1: prefrontal cortex, 2: striatum, 3: hippocampus, 4: midbrain, 5: trunk, 6: cerebellum, 7: cervical spinal cord.](https://example.com/fig1)

DISCUSSION

In this study we analyzed the mRNA levels and the activity of µ- and m-calpain in the different CNS structures of native rats, and our results partially coincide with the data available in literature. In 1994, the distribution of m-calpain mRNA in different brain regions of Wistar rats was studied by in situ hybridization method (5). It was shown that the cortex was characterized by a weak production of m-calpain, and a weak to moderate signal intensity noticed for the hippocampus, locus ceruleus, and Purkinje cells. In the midbrain and hindbrain m-calpain mRNA was detected mainly in the red nucleus and in most of the cranial nerve nuclei. The level of mRNA was low or moderate in the gray matter of the spinal cord, and it was high in the neurons of the posterior horns (6). Our data do not contradict the results described in this work (6). In another study, mRNA level of µ- and m-calpain was determined by RT-PCR in the brainstem, cerebellum, cortex, and spinal cord of Sprague-Dawley rats. Maximum value was found in the spinal cord, what is also consistent with our data (18). Controversial results were obtained in mice by Li et al., (7). They demonstrated maximum content of µ- and m-calpain in the spinal cord, a relatively high level of m-calpain in the Purkinje cells (about 50% of the spinal cord content), and approximately the same production level of both proteases was detected in the cortex and hippocampus. Thus, there are different representations of the calpain system components for identical parts of rats and mice rat and mice CNS.

The discrepancy between the production level of calpains and the degree of protease activity can be explained by the participation of activator proteins, calpastatin, cell plasma membrane phospholipids etc., but the identification of such regulation mechanism in each of the analyzed regions is the subject of a separate study. In the study by Kenessey et al., m-calpain activity was determined in various rat brain regions (19). Using [methyl-3H] ~ casein as a substrate, they showed that the lowest activity was in the cerebral cortex. Activity in other tested regions was 10-50% higher, for instance, in the pons it was more than twice higher. The specific activity of m-calpain was distributed as follows: cortex < hippocampus < striatum < hypothalamus < cerebellum < cervical spinal < pons. When endogenous proteins (200, 150 NF and 70 [NFT] neurofilament proteins, glial fibrillar acidic protein [GFAP], desmin and actin) were used as substrates, it turned out that not only a region-specific change in the activity of m-calpain was observed, but also the substrate-specific. For example, regardless of the substrate used, m-calpain activity in the pons was always the highest. Activity was minimum in the cerebral cortex when using actin, it was intermediate when desmin was used, and was comparable to the pons when using NF 200 (19). Our data on the region-specific change of m-calpain activity using casein as a substrate almost completely coincide with the results of the study described above, but our work additionally showed that the activity of µ-calpain is also characterized by regional specificity, which is different from m-calpain regional specificity: the minimum activity was also in the cortex and the maximum in the cervical spinal cord.

In a 1993 the total activity of calpains was analyzed in study on human brain samples without significant postmortem pathologies. The distribution of the total calpain activity was not equable. The highest activity was found in the spinal cord and in the amygdala, and high levels of calpain activity were detected in the mesencephallic regions and the cerebellar gray matter. The activity in the cerebellar white matter, VTA, pons, shell and cortical areas was low (20). This data also does not conflict with our results.
CONCLUSION

Thus, the selective neuronal death which was observed during neurodegeneration can be at least partially determined by the initial level of expression and/or activity of calpains.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES