THE ROLE OF CALPAIN IN SPINAL CORD DEGENERATION IN EXPERIMENTAL PARKINSON'S DISEASE

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In this review the critical role of Ca^{2+}-dependent protease calpain in the cascade of intracellular processes, leading to degeneration and death of spinal cord neurons in experimental models of Parkinson's disease (PD) is discussed. In experimental conditions parkinsonian neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone cause the activation of calpain and caspase-3, which subsequently cleave cytoskeletal protein spectrin and axonal neurofilament light protein (NF-L). In the absence of the protease inhibitors, these processes can lead to structural and functional alterations in neurons and axons. It has been demonstrated that calpain inhibition provides neuroprotection in the spinal cord in experimental PD.

Parkinson's disease – spinal cord – neurodegeneration – calpain

Ամփոփում քննարկվում է Ca^{2+}-կախյալ պրոտեազի` կալպաինի առանցքային դերը ներբջջային պրոցեսների կասկադում, որոնք կործանարար են ազդում ողնուղեղի նեյրոնների վրա փորձարարական Պարկինսոնի հիվանդության (ՊՀ) ժամանակ: Պարկինսոնիզմ առաջացնող նեյրոտոքսինների` 1-մեթիլ-4-ֆենիլ-1,2,3,6-տետրահիդրո-պիրիդինի (ՄՖՏՊ) և ռոտենոնի ազդեցության արդյունքում ակտիվանում են կալպաինը և կասպազ-3-ը, որոնք քայքայում են բջջի կառուցվածքային սպիտակուցը` սպեկտրինը և աքսոնների նեյրոֆիլամենտի թեթև սպիտակուցը: Պրոտեազների ինհիբիտորների բացակայության դեպքում այս պրոցեսները կարող են նեյրոնների և աքսոնների կառուցվածքային փոփոխությունների և ֆունկցիոնալ խանգարումների պատճառ դառնալ: Ցույց է տրվել, որ կալպաինի արգելակումը պաշտպանիչ դեր ունի ողնուղեղում:

Պարկինսոնի հիվանդություն – ողնուղեղ – նեյրոդեգեներացիա – կալպաին

В обзоре обсуждается ключевая роль Ca^{2+}-зависимой протеазы кальпаина в каскаде внутриклеточных процессов, ведущих к вырождению нейронов спинного мозга при болезни Паркинсона (БП). В экспериментальных условиях нейротоксины 1-метил-4-фенил-1,2,3,6-тетрагидропиридин (МФТП) и ротенон, вызывающие паркинсонический синдром, запускают активацию кальпаина и каспазы-3, которые расщепляют структурный белок цитоскелета спектрин и аксональный белок нейрофиламента легкого (NF-L). В отсутствие ингибиторов протеаз эти процессы могут привести к нарушениям структурной целостности и функциональной активности нейронов и аксонов в спинном мозге. Показано, что ингибирование кальпаина оказывает нейропротекторное действие в спинном мозге.

Болезнь Паркинсона – спинной мозг – нейродегенерация – кальпаин

Parkinson's disease

Parkinson' disease (PD) is a complex movement disorder, characterized by resting tremor, muscle rigidity, bradykinesia and/or akinesia, postural instability. The disturbances in motor functions are predominantly ascribed to degeneration and death of

neuromelanin-containing dopaminergic neurons situated in the A9 region of substantia nigra pars compacta (SNpc), and reduction of dopaminergic neurotransmission in caudate nucleus of corpus striatum [14]. Intracellular inclusions, called Lewy bodies and Lewy neurites, comprised of abnormally aggregated filaments of α-synuclein fibrillar protein, serve as histopathological hallmarks of damaged dopaminergic neurons [13]. It is well documented that sporadic PD-associated Lewy bodies are distributed over the central, peripheral, and enteric nervous systems; this indicates widespread distribution of neurodegenerative processes, including multiple extranigral nuclei of the nervous system [17]. Consequently, topographic distribution of the Lewy pathology can help in explaining complex functional disturbances, differentiated as non-motor autonomic, vegetative, sensory, and psychiatric symptoms, which are developed before or accompany motor dysfunctions at PD.

Despite substantial evidence of nigrostriatal degeneration, spinal cord, which is the final coordinator of motor functions, has also been considered to be implicated in the progression of PD. It has been discussed earlier that neurodegenerative processes related to PD and α-synuclein inclusions occur in the spinal cord as well [1, 2, 17]. In our studies, neurodegenerative changes have been detected in the spinal cord of PD patients (*postmortem*), as well as in experimental *in vivo* and *in vitro* models of PD [2, 18, 28-32]. It has been shown that calpain plays critical role in the development of intracellular processes leading to neurodegeneration and death of spinal neurons and motoneurons.

Calpain and its role in neurodegeneration

Calpain is a Ca^{2+}dependent cytosolic cysteine protease, which is widely expressed in the central nervous system (CNS) [12, 25]. Two ubiquitously distributed mammalian calpains have been identified: μ-calpain (calpain I, CAPN1) and m-calpain (calpain II, CAPN2), activated *in vitro* by low (2-80 μM) and high (0.2-0.8 мM) intracellular Ca^{2+} concentrations, respectively. Therefore, calpain is getting involved in signaling pathways regulated by increased concentrations of Ca^{2+}. The molecules of μ- and m-calpain are comprised of large catalytic (78-80 kDa) and regulatory (30 kDa) subunits, C-terminals of which contain calmodulin-like Ca^{2+}-binding domains. Glycin-containing N-terminal domain of the regulatory subunit plays role in calpain activation, facilitating binding of the proenzyme to the membranes. At relatively low intracellular Ca^{2+} concentrations (50-100 nM) calpain exists as a proenzyme, which is activated at increasing *in vivo* levels of free intracellular Ca^{2+} and dissociated from inactive into active subunits (78 kDa and 18 kDa) that have enhanced access to cytosolic and membrane-associated substrates.

The proteolytic activity of μ- и m-calpain in mammalian cells is regulated by its specific endogenous inhibitor, ubiquitously expressed protein, calpastatin [9]. Calpastatin prevents the calpain overactivation, providing physiologically normal environment in the cells. On the other hand, calpastatin is a substrate for calpain, therefore uncontrolled prolonged overactivation of the calpain leads to calpastatin proteolysis as well; thus, imbalance in calpain-calpastatin ratio enhances the level of intracellular proteolytic reactions. Neuroprotective role of calpastatin has been shown in calpastatin-expressing transgenic mice, which resist experimental brain injury, demonstrating significantly less behavioral alterations and lower level of proteolysis of calpain substrates [36]. However, having a large molecular size of 110 kDa, calpastatin is not permeable for cellular membranes, therefore is not considered as a potential therapeutic agent for inhibition of increasing calpain activity in CNS disorders, including PD.

Calpain usually modifies but does not fully degrade substrates. Under physiological conditions calpain causes limited proteolysis of proteins (break of specific peptide bonds depending on the amino acid sequence of a protein), modifying their

biological role and activities; the modified proteins are involved in the processes of cell migration and differentiation, rearrangement of the cytoskeleton [24]. There are many intracellular protein substrates for calpain, such as, receptors, kinases, phosphatases, synaptosomal and cytoskeletal proteins. Calpain-mediated cleavage of these substrates is essential for regulation of neurochemical processes, synthesis, release and uptake of neurotransmitters, and also for maintenance of integrity of neuronal cytoskeleton [15]. In some cases, proteolytic activity of calpain combined with other protein degradation machinery (proteosomes, lysosomes, etc.) leads to the breakdown of a complete peptide into amino acids, which is essential in detoxification of harmful proteins.

At pathological conditions, uncontrolled calpain activation along with other proteases causes breakdown of essential cellular proteins (e.g., spectrin), DNA fragmentation, leading to programmed cell death. Calpain is involved in activation and migration of inflammatory mediators, including astrocytes, microglia, T cells, and macrophages [4]. In neurodegenerative processes, associated with mitochondrial dysfunction and imbalance of intracellular Ca^{2+} levels, calpain activation triggers cascade of apoptotic reactions, leading to neuronal cell death [41]. Calpain activation is regulated by its intracellular protein substrates, particularly those, directly associated with cell degradation mechanisms. These are mitochondrial membrane proteins (Bax, Bcl-2), receptor inositol triphosphate protein (IP_3), calmodulin-binding G proteins and calcineurin), myelin basic protein (MBP) and myelin proteolipid protein (PLP), cytoskeletal proteins (α-spectrin, actin, mictotubule-associated protein 2, neurofilamentous proteins, tau protein, α-synuclein), apoptosis-inducing factor (AIF), signal transduction enzymes (phospholipase C, proteinkinase C, proteinphosphatase IIb), transcription factors (c-Fos, c-Jun), calpastatin and numerous structural signaling and cytosolic proteins [41].

Calpain upregulation has been implicated in neurological disorders, such as cerebral ischemia and traumatic brain injury [3, 8, 36], spinal cord injury [34], demyelinating disorders, amyotrophic lateral sclerosis and multiple sclerosis [26, 35, 38], chronic neurodegenerative disorders, including Alzheimer's disease, PD and Huntington's disease [41], experimental optic neuritis [37], and several non-neuronal disorders (muscular dystrophy, cataracts, arthritis, and others) [44]. Increased calpain expression has been demonstrated for the midbrain of PD patients *postmortem* [23]. Importantly, α-synuclein, the component of Lewy bodies, is the essential substrate for calpain in the brain of PD patients [21]. Through a multiple step process, calpain mediates a cleavage of α-synuclein, generating high molecular protein chains, aggregates of which are accumulated in Lewy boodies. This calpain-mediated mechanism of Lewy body formation plays crucial role in PD neuropathology. Moreover, detection of calpain-cleaved fragments of α-synuclein in the brain neurons and their colocalization with active calpain suggested direct involvement of calpain I in α-synuclein aggregation process at α-synucleinopaties [10].

Overactivation of calpain and subsequent cleavage of its intracellular substrates in CNS has detrimental effects, which destroy structural and functional integrity of neurons, axons, and myelin. Particularly, calpain I cleaves cytoskeletal protein α-II-spectrin, which plays important role in maintenance of plasma membrane integrity and cytoskeletal structure, producing spectrin breakdown products (SBDP); the latest are considered as biomarkers of the degenerating neurons damaged through proteolysis [20, 43].

Calpain-mediated mechanism for structural protein degradation of neurons and axons has been implicated in experimental cerebral ischemia and traumatic brain injury [3, 27], spinal cord injury [34], and demyelinating diseases [35]. In experimental optic neuritis early calpain activation and calpain-mediated cleavage of main downstream

target collapsing response mediator protein-2 (CRMP2) resulted in acute axonal degeneration of the optic nerve; however, calpain inhibition and/or CRMP2 overexpression rescued impairment of axonal mitochondrial transport, preventing fragmentation of proximal axons [45].

Calpain is involved in apoptotic pathway of cell death in association with initiator and effector caspases; cross activation of proteases at early stages of apoptosis may occur. According to this scenario, active calpain cleaves pro-caspase-3 with formation of active caspase-3, which in turn breaks calpain inhibitor, calpastatin. Imbalance of the intracellular calpain and calpastatin ratio results in uncontrolled calpain overactivation [42]. Since caspase-3 is also involved in regulation of calpain activity, we have simultaneously studied the active subunits of these two proteases in our work.

Calpain and its role pathogenesis of PD

There is a growing amount of evidence that PD pathology is associated with consistent systemic mitochondrial dysfunction and aberrant Ca^{2+} homeostasis, which lead to activation of cell signaling cascades and generation of the environment that is highly conducive to upregulate Ca^{2+} dependent proteases, i.e. calpain and caspase-3 [16, 33]. The impairment of mitochondrial function and unregulated elevation of intracellular Ca^{2+} can be induced by mitochondrial toxins, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone. *In vivo* MPTP is converted to active metabolite 1-methyl-4-phenylpyridinium ion (MPP^+); rotenone is a naturally occurring substance, known as a pesticide. These compounds are accepted parkinsonian neurotoxins, which affect mitochondrial function of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and therefore are widely used to generate experimental *in vivo* and *in vitro* PD models, where mitochondria play key role in cell death mechanisms.

The mechanisms of MPTP/MPP^+ and rotenone toxicity in CNS have been thoroughly investigated. Both, MPP^+ and rotenone are accumulated in dopaminergic neurons and bind to NADH dehydrogenase and coenzyme Q, inhibiting NADH:quinone reductase (complex I) activity in the mitochondrial respiratory chain. Subsequent chain reactions - inhibition of oxidative phosphorylation, loss of mitochondrial membrane potential, release of mitochondrial cytochrome *c*, ROS generation, resulting in sustainable mitochondrial dysfunction and oxidative stress, are characteristic for nigrostriatal degeneration at idiopathic PD as well in experimental PD models [22, 39]. The critical event is elevation and accumulation of intracellular free Ca^{2+}, inducing activation of proteases [16, 19]. It has been demonstrated that MPTP-induced caspase-3 activation mediates apoptosis of dopaminergic neurons in SNpc in mice [40]. Perhaps, activated caspase-3 can sustain calpain activation in PD [11]. However, timing for active protease induction is different, depending on certain experimental conditions. For example, rotenone-induced early activation of calpain in primary cortical neurons in vitro precedes caspase-3 activation, culminating in neuronal apotosis [5]. Thus, cross-communication between calpain and caspases has been suggested as a possible mechanism of apoptotic neuronal cell death [30].

The role of calpain and caspase-3 in the spinal cord degeneration in experimental models of PD was studied. The results of the study are briefly presented in this review. The expression of proteases and their active subunits were determined by Western blotting (immunoblotting) of spinal cord samples, using antibodies against inactive and active calpain (80 kDa and 76 kDa, respectively), and caspase-3 (proenzyme 32 kDa and active subunits). Neuronal localization was assessed by *in situ* double immunofluorescent staining of spinal cord slices with antibodies against calpain or caspase-3 in combination with NeuN (neuronal nuclei immunomarker). Neuronal cell damage and DNA fragmentation was evaluated with TUNEL (terminal deoxynucleotidyl

transferase (TdT) recombinant-mediated dUTP nick-end labeling) immunofluorescent assay.
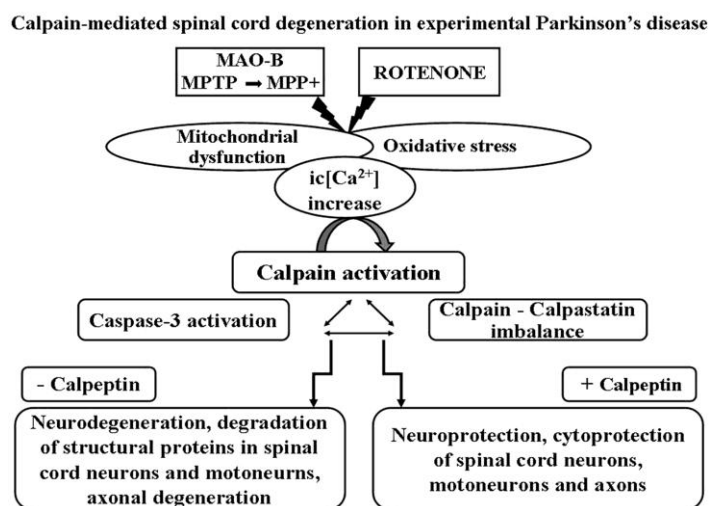

Fig.1. Schematic presentation of the calpain-mediated pathway of spinal cord degeneration at PD. Exposure to parkinsonian toxins MPTP/MPP$^+$ and rotenone (*in vivo, in vitro*) causes mitochondrial dysfunction, oxidative stress and consequent increase in intracellular Ca^{2+} level, leading to calpain and caspase-3 activation. In absence of calpeptin (-), a calpain inhibitor, activation of proteases converges to cleavage of intracellular substrates, culminating in neurodegeneration and axonal degeneration. Inhibition of calpain by calpeptin (+) provides neuroprotection in the spinal cord.

Rotenone–induced calpain activation in the spinal cord

We have explored the rotenone-induced *in vivo* model of PD and studied the activation of calpain and caspase-3 as possible intrinsic mechanism, mediating cell death and neurodegeneration in spinal cord [29]. Rotenone was injected to adult male Lewis rats (total 25 mg/kg over 21 days, s.c.). Sections from upper (cervical) and lower (lumbar) segments of spinal cord from rotenone-injected and vehicle-treated Lewis rats were isolated, sliced (5 μM), and then used for *in situ* double immunofluorescent staining of TUNEL combined with NeuN (neuronal nuclei immunomarker) or ChAT (choline acetyltransferase, cholinergic motoneuron immunomarker). Microscopic observation of immunofluorescent samples revealed TUNEL-positive damaged neurons and motoneurons in cervical and lumbar spinal cord slices of rotenone-injected rats compared to controls. Damaged neurons (NeuN) and motoneurons (ChAT) were identified in dorsal and ventral horn areas of the spinal cord, respectively. Western blotting of protein samples showed significant (p≤0.05; n≥4) elevation of the active calpain (76 kDa) levels in cervical (by 86%) and lumbar (90%) spinal cord tissues from rotenone-treated rats compared to controls. Meanwhile, the level of inactive calpain (80 kDa) in the spinal cord had not changed either in control, or rotenone-injected rats. These findings have been additionally confirmed by double immunofluorescent staining of cervical and lumbar spinal cord slices by antibodies against proteases. Colocalization of positive immunoreactivity for active calpain and caspase-3 along with NeuN-immunoreactive neurons in rotenone-treated rats, but not in controls, demonstrated neuronal localization of the active proteases. Besides, presence of two active proteases in neurons disclosed their parallel participation in proteolytic pathways leading to neuronal damage.

Rotenone-induced activation of calpain and caspase-3 in spinal cord caused proteolysis of α-II-spectrin (270 kDa) into calpain-specific 145 kDa SBDP and caspase-3-specific 120 kDa SBDP, which were assessed by Western blotting. The levels of calpain- and caspase-3-specific SBDP significantly ($p \leq 0.05$; $n \geq 4$) increased in cervical spinal cord by 55% and 60%, and in lumbar spinal cord – by 35% and 30%, respectively. These results substantiated the calpain- and caspase-3-mediated damage of the cytoskeleton and degeneration of the neurons in response to rotenone neurtoxicity in the spinal cord [29].

MPTP neurotoxicity in spinal cord and neuropotection via calpain inhibition

The calpain-mediated pathway of spinal cord neurodegeneration has been studied in MPTP-induced *in vivo* model of PD [2, 28, 32]. MPTP was injected to linear C57BL/6N mice (2 x 25 mg/kg, i.p., 6 h interval). Degenerated neurons in the spinal cord were assessed through double TUNEL and NeuN immunofluorescent assay, mentioned above. The results of microscopic observation of slices showed that MPTP induced neurodegenerative processes in both, midbrain SNpc and spinal cord. Numerous positive TUNEL-NeuN neurons in the spinal cord slices of MPTP-injected mice, but not in controls, indicated degenerating neurons in the spinal cord. Obtained proof of the *in vivo* metabolic conversion of MPTP to the active MPP^+ ion in the spinal cord additionally demonstrated the direct impact of $MPTP/MPP^+$ on the development of neurodegenerative processes in the spinal cord [28]. It has been suggested that mitochondrial dysfunction, oxidative stress and imbalance of intracellular Ca^{2+} lead to calpain upregulation with concomitant activation of signaling pathways, leading to neuroapoptosis. The earlier reports from the same laboratory had shown MPTP-induced increase in expression of calpain in the neurons, as well as in microglia and astrocytes, suggesting calpain-mediated activation of inflammatory reactions [6].

The critical role of activated calpain in PD pathogenesis impelled investigations toward discovery of calpain inhibitors, which could be considered as potential therapeutic agents to slowdown and/or to prevent neurodegenerative processes [24]. For example, inhibition of calpain by MDL-28170 or increased calpastatin expression had protective effects on dopaminergic neurons in the midbrain and prevented behavioral deficits in an MPTP mouse model of PD [7].

To elucidate the calpain-mediated neurodegeneration of spinal cord in MPTP-induced mouse model of PD, we studied the neuroprotective efficiency of a calpain inhibitor, calpeptin [32]. Calpeptin is a synthetic cell-permeable peptide aldehyde, cytoprotective effects of which have been explored *in vivo* and *in vivo*. We found significant decrease of TUNEL-NeuN colocalization sites in the spinal cord of MPTP-injected mice pretreated with calpeptin (25 µg/kg), then in merely MPTP-injected mice; these data signified that neuroprotection in the spinal cord occurred via calpain inhibition by calpeptin, and additionally confirmed calpain involvement in spinal cord neurodegeneration induced by MPTP. Immunofluorescent assay with antibody detecting dephosphorylayted neurofilament protein (deNFP) showed increased level of deNFP immunoreactivity, indicating that effects of MPTP are not limited, but extended beyond neuronal bodies, targeting and damaging axons in the mice spinal cord. Nevertheless, pretreatment of mice with calpeptin significantly reduced deNFP immunoreactivity about to the control level. Neuroprotective efficiency, provided by calpeptin via calpain inhibition, extended all through ventral and dorsal areas of the cervical and lumbar spinal cord regions. In these studies we also demonstrated that inhibition of calpain mitigates MPTP-induced alterations in symptomatic for PD gait parameters - stride length and stride frequency, measured with DigiGait videography. Thus, calpain inhibition by calpeptin protected spinal neurons and axons against MPTP-induced neurotoxicity *in vivo*, whereas in absence of calpeptin neurodegenerative processes steadily developed,

resulting in neuronal and axonal damage, and significant impairment in gait dynamics [32]. Importantly, in PD *postmortem* studies immunofluorescent staining showed that immunoreactivity of axonal 62 kDa neurofilament light protein (NF-L) in the cervical spinal cord was significantly lower (by 29%; $p \leq 0.05$) than in normal cases [31]. Axonal degeneration was further confirmed by increased deNFP immunoreactivity in the spinal cord of PD patients than in normal cases (by 53% and 55%, $p \leq 0.05$, higher in cervical and thoracic sections, respectively). These findings indicated the involvement of calpain in axonal degeneration at PD. These data suggest calpain as a therapeutic target to prevent processes of neurodegeneration and axonal degeneration in the spinal cord at PD.

MPP^+ *and rotenone toxicity in motoneurons and cytoprotection by calpeptin*

We have studied *in vitro* the mechanisms of MPP^+- and rotenone-induced calpain activation using hybrid ventral spinal cells (VSC 4.1) differentiated into spinal motoneurons and possible cytoprotection via calpain inhibition [30]. Incubation of motoneurons in the presence of MPP^+ (25 and 100 μM) and rotenone (10 and 50 nM) dramatically reduced viability of these cells. A cascade of intracellular events had been triggered by neurotoxin exposure, including DNA fragmentation, rise in TUNEL immunofluorescence, and increase in the ratio of mitochondrial membrane pro- and anti-apototic Bax:Bcl-2 proteins, respectively. Such intracellular processes are characteristic of intrinsic mitochondrial signaling pathway activation, culminating in apoptotic cell death. The critical step in this machinery is increasing Ca^{2+} level, which drives activation of proteases.

Calpain and caspase-3 were diversely upregulated by MPP^+ and rotenone depending on the neurotoxin dose and time of exposure, with concomitant formation of active subunits of calpain (76 kDa) and caspase-3 (12 and 20 kDa). Induction of active proteases promoted proteolytic cleavage of spectrin with formation of calpain-specific (145 kDa) and caspase-3 specific (120 kDa) SBDP, respectively. However, preincubation of motoneurons with calpeptin before exposure to neurotoxins markedly suppressed neurotoxic effects of MPP^+ and rotenone. The positive outcomes of calpain inhibition included increased cell viability, decreased levels of active proteases and hence, reduced SBDP formation; the latter is essential to preserve structural and functional integrity of motoneurons. Thus, neuroprotective efficacy of calpain inhibition by calpeptin *in vitro* confirmed the role of calpain in degeneration of spinal motoneurons [30].

Taking together, the results of our studies suggest calpain inhibition as a prospective tool to preserve structural and functional integrity of spinal neurons and motoneurons, and to prevent and/or slow down development of neurodegenerative processes in the spinal cord at PD.

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