

Brain Mitochondrial Subproteome of Rpn10-Binding Proteins and Its Changes Induced by the Neurotoxin MPTP and the Neuroprotector Isatin

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Abstract—Mitochondria play an important role in molecular mechanisms of neuroplasticity, adaptive changes of the brain that occur in the structure and function of its cells in response to altered physiological conditions or development of pathological disorders. Mitochondria are a crucial target for actions of neurotoxins, causing symptoms of Parkinson's disease in various experimental animal models, and also neuroprotectors. Good evidence exists in the literature that mitochondrial dysfunction induced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) influences functioning of the ubiquitin-proteasomal system (UPS) responsible for selective proteolytic degradation of proteins from various intracellular compartments (including mitochondria), and neuroprotective effects of certain antiparkinsonian agents (monoamine oxidase inhibitors) may be associated with their effects on UPS. The 19S proteasomal Rpn10 subunit is considered as a ubiquitin receptor responsible for delivery of ubiquitinated proteins to the proteasome proteolytic machinery. In this study, we investigated proteomic profiles of mouse brain mitochondrial Rpn10-binding proteins, brain monoamine oxidase B (MAO B) activity, and their changes induced by a single-dose administration of the neurotoxin MPTP and the neuroprotector isatin. Administration of isatin to mice prevented MPTP-induced inactivation of MAO B and influenced the profile of brain mitochondrial Rpn10-binding proteins, in which two pools of proteins were clearly recognized. The constitutive pool was insensitive to neurotoxic/neuroprotective treatments, while the variable pool was specifically influenced by MPTP and the neuroprotector isatin. Taking into consideration that the neuroprotective dose of isatin used in this study can result in brain isatin concentrations that are proapoptotic for cells *in vitro*, the altered repertoire of mitochondrial Rpn10-binding proteins may thus represent a part of a switch mechanism from targeted elimination of individual (damaged) proteins to more efficient ("global") elimination of damaged organelles and whole damaged cells.

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Mitochondria play an important role in molecular mechanisms of neuroplasticity, adaptive changes of the brain that occur in the structure and function of its cells in response to altered physiological conditions or development of pathological disorders [1]. These intracellular organelles are a crucial target for actions of neurotoxins,

Abbreviations: a.u., arbitrary units; GO, Gene Ontology; i.p., intraperitoneally; MAO B, monoamine oxidase B; MAPK, mitogen activated protein kinase; MPP⁺, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; UPS, ubiquitin-proteasomal system.

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causing symptoms of Parkinson's disease [1-3]. In the context of parkinsonism induced by administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the protoxin undergoes catalytic conversion by monoamine oxidase B (MAO B), which is self-inactivated during this process. The resultant neurotoxin MPP⁺ (1-methyl-4-phenylpyridinium) inhibits complex I of the respiratory chain and causes development of symptoms typical for Parkinson's disease [3, 4]. Administration of MAO B inhibitors (e.g. deprenyl or isatin [5, 6]) or substrates competing for the active site of this enzyme (e.g. phenylethylamine) [7] prevented not only metabolic acti-

vation of MPTP, but also deficiency of the neurotransmitter dopamine and locomotor impairments typical for this disease.

Thus, brain mitochondria obviously represent not only the principle subcellular organelles where “the first act of the scenario” of MPTP-induced Parkinsonism is realized, but also crucial targets for endogenous and exogenous neuroprotectors. Good evidence exists in the literature that neuroprotector mechanisms of certain antiparkinsonian agents, monoamine oxidase inhibitors, may be associated with their effects on the ubiquitin-proteasomal system (UPS) [8] responsible for selective proteolytic degradation of proteins from various intracellular compartments including mitochondria. Mitochondrial dysfunction induced by MPTP has a significant impact on functioning of the UPS [9, 10].

Ubiquitin, a 76-residue protein, is widely distributed in all eukaryotic cells, where it targets proteins for subsequent degradation [11-14]. The ubiquitination process includes several sequential stages that involve several enzymes (Fig. 1): ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3)

[11-14]. The major function of ubiquitin is (poly)ubiquitination of proteins for their subsequent proteasomal degradation. In the context of UPS functioning, the 19S proteasome Rpn10 subunit (as well as extraproteasomal Rpn10) plays a role as the ubiquitin receptor [15] responsible for delivery of client proteins to the 20S proteolytically active proteasome, where their subsequent proteolytic degradation occurs (Fig. 1) [16].

Isatin (indole-2,3-dione) is an endogenous neuroprotector found in mammalian brain, peripheral tissues, and body fluids [17-19]. Besides reversible inhibition of MAO B and receptor guanylate cyclases [17-19], isatin interacts with numerous isatin-binding proteins located in various subcellular organelles including mitochondria [20, 21]. Proteomic profiling of brain isatin-binding proteins revealed several enzymes directly involved in UPS functioning [22]. Taking into consideration that some isatin derivatives act as proteasome inhibitors [23, 24], in this study we investigated the effect of a single-dose administration of MPTP and isatin to mice on MAO B activity and the subproteome of brain mitochondrial Rpn10-binding proteins.

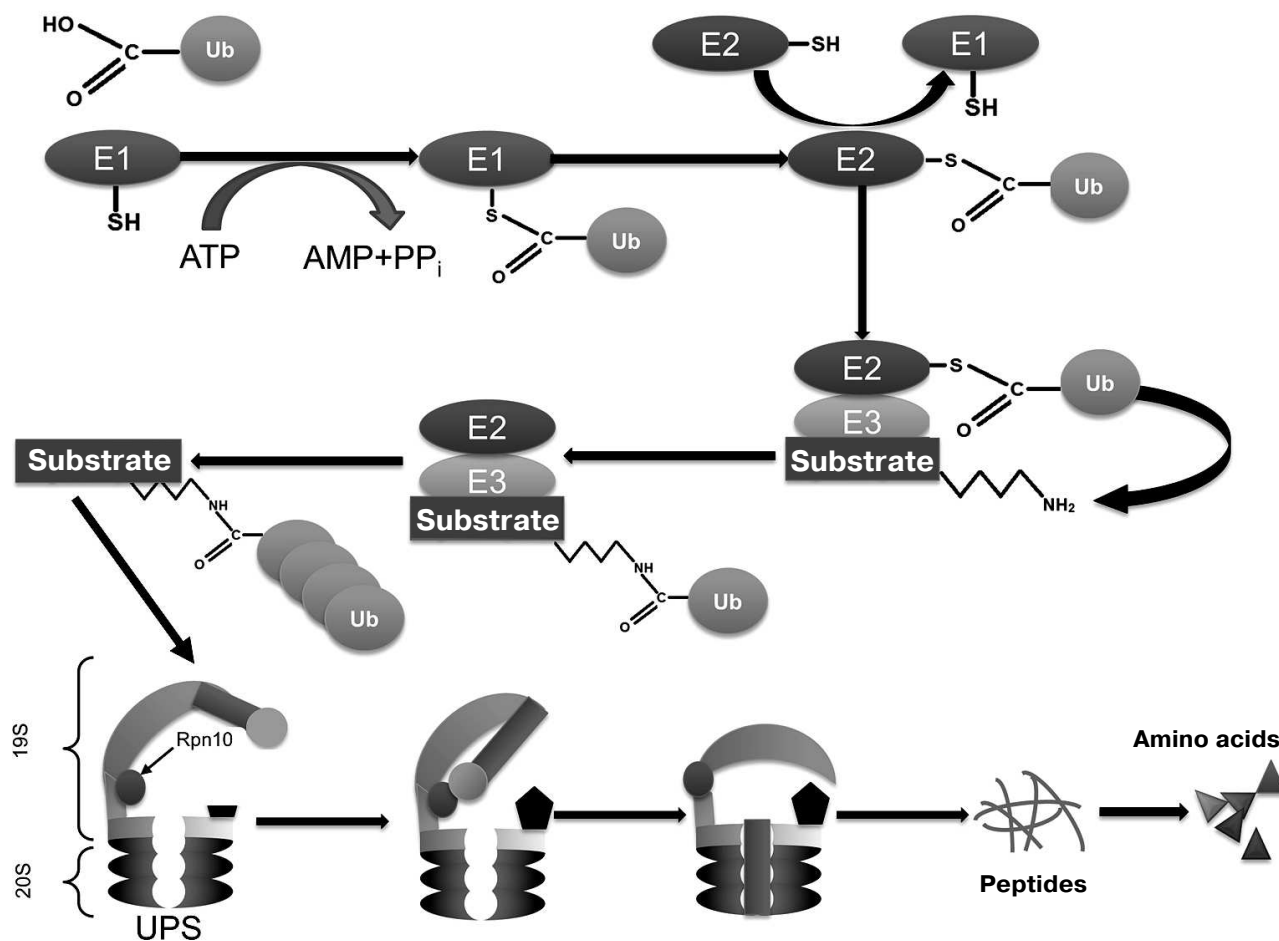


Fig. 1. Scheme of functioning of the ubiquitin-proteasomal system (UPS).

MATERIALS AND METHODS

Male C57BL/6 mice (20–25 g) obtained from the Stolbovaya nursery (Moscow Region) were used in experiments, which were performed at least one week after their arrival from the nursery. The animals were maintained under natural illumination and received standard laboratory chow (full ration extruded briquette feed, GOST 50258–92) and water *ad libitum*. MPTP was injected intraperitoneally (i.p., 30 mg/kg). Isatin (100 mg/kg, i.p.) was injected 30 min before MPTP. Control mice were treated with intraperitoneal injection of saline (0.1 ml/10 g of weight). Each group contained 6–8 mice. Behavioral changes induced by MPTP or isatin were analyzed 90 min after the last administration by means of the open field test [25] and the rotarod and static rod tests [26]. The exploratory reaction of mice in the open field test was defined as the sum of horizontal activity (units) and vertical activity (units). All procedures were approved by local authorities for animal research.

Isolation of the brain mitochondrial fraction and determination of MAO B activity were carried out as described previously [27, 28].

All procedures related to isolation of mouse brain Rpn-binding proteins, sample preparation for mass spectrometric analysis including liquid chromatography, high resolution mass spectrometry, and data analysis are given in the Supplementary materials (see Supplement to this report on the site of the journal <http://protein.bio.msu.ru/biokhimiya> and Springer site Link.springer.com).

Generation and prediction of an interactome/protein interaction network. Protein annotation in terms of gene ontology (GO) using categories “Biological process” and “Molecular function” was performed using UniProt/SwissProt IDs [29] (release 2016_08 updated on 7.09.2016) as input data and applied for interactive network processing. Interactions of proteins were predicted using the open access STRING version 10.0 software

platform [30]. A list of positive identifications obtained during proteomic profiling was used for generation and prediction of the protein interaction network. The network of physically and functionally interacting proteins was visualized using a high confidence level ($C \geq 0.7$). The visualization was performed using information about proteins from three sources: experimental data, databases, and analysis of available texts (e.g. joint references in annotations to PubMed publications).

RESULTS

Effect of MPTP and isatin on behavioral activity of mice and on brain MAO B activity. In accordance with previous studies [3, 31, 32], a single-dose administration of MPTP caused appearance of movement disorders typical for animal models of Parkinson’s disease (Table 1). Pretreatment of mice with isatin before MPTP attenuated the locomotor impairments induced by the neurotoxin. In the control mice, isatin administration also decreased locomotor activity, which may be attributed to its sedative effect known from the literature [17, 18]. Under these conditions, MAO B activity assayed in the brain mitochondrial fraction of MPTP-treated mice demonstrated a statistically significant decrease compared with the control group ($18.0 \pm 2.6\%$ versus control; $p < 0.01$; $n = 6$ in each group). After sequential treatment of mice with isatin and MPTP, brain mitochondrial MAO B activity was basically the same as in the control group. This suggests that the reversible MAO B inhibitor isatin effectively prevents interaction of this enzyme with MPTP and is then effectively washed out of brain mitochondria during their isolation.

Proteomic profiling of brain mitochondrial Rpn10-binding proteins. Affinity-based profiling of Rpn10-binding proteins of control brain mitochondrial samples resulted in confident identification of 48 individual pro-

Table 1. Effect of MPTP and isatin on behavioral activity of C57BL/6 mice

Treatments	Open field		Vertical rod		Rotarod, retention time, s
	horizontal activity, units	vertical activity, units	time of turn, s	time of descent, s	
Saline (control)	73.3 ± 3.6	14.6 ± 1.53	2.8 ± 0.4	7.4 ± 0.8	158.8 ± 20.8
MPTP (30 mg/kg)	32.4 ± 4.6 [#]	3.5 ± 1.77 [#]	15.6 ± 5.1 [#]	35.6 ± 11.8 [#]	44.6 ± 11.4 [#]
Isatin (100 mg/kg) + MPTP (30 mg/kg)	55.0 ± 7.7*	5.4 ± 0.84	5.5 ± 1.7*	14.0 ± 1.5*	89.6 ± 23.0*
Isatin (100 mg/kg)	52.9 ± 3.1*	7.5 ± 1.38	2.9 ± 0.5	8.0 ± 1.3*	123.3 ± 24.8

Note: Behavioral activity of mice was evaluated 90 min after MPTP administration. Isatin was injected 30 min before MPTP administration. Data represent mean ± SEM. Each group contained 6–8 animals. Statistical significance of differences: [#] $p < 0.05$ versus control; * $p < 0.05$ versus MPTP.

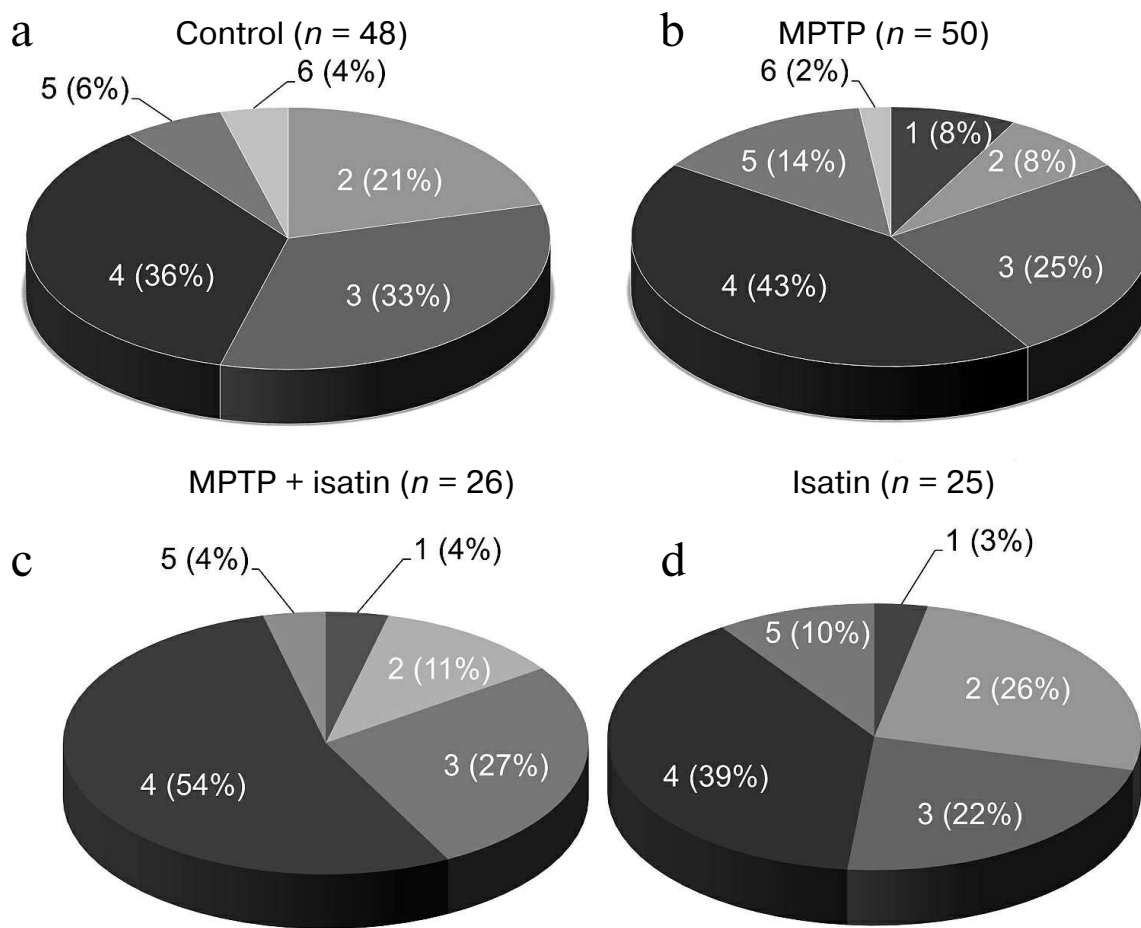


Fig. 2. Distribution of Rpn10-binding proteins of the brain mitochondrial fraction into functional groups: 1) proteins/enzymes involved in energy generation and carbohydrate metabolism; 2) proteins involved in cytoskeleton formation and exocytosis; 3) protein regulators of gene expression, cell division, and differentiation; 4) proteins involved in signal transduction and regulation of enzyme activity; 5) antioxidant and protective proteins/enzymes; 6) enzymes of lipid metabolism.

teins (Fig. 2a). Functionally, they can be subdivided into the following groups: (i) proteins involved in energy generation and carbohydrate metabolism; (ii) proteins involved in cytoskeleton formation and exocytosis/trafficking; (iii) proteins involved in regulation of gene expression, cell division, and differentiation; (iv) proteins involved in signal transduction and regulation of enzyme activity; (v) antioxidant and protection proteins/enzymes; (vi) enzymes involved in lipid metabolism. Most of these proteins (including those traditionally identified in cytosol or nucleus) are associated with internal or external mitochondrial compartments, and functional links have been already established for several groups of the identified proteins (Table S1 in Supplement). For example, histones identified in this study (Table S1 and Fig. S1 in Supplement) have been previously found in different mitochondrial sub-compartments [33]. In the internal mitochondrial compartment, histones H1, H2A, H2B, H3, and H4 were identified among so-called D-loop DNA-binding proteins

[33]. In the external mitochondrial compartment, they were identified in the outer mitochondrial membrane; it is even suggested that “H2A and H2B must be integral outer membrane proteins protruding towards the cytoplasm” [33]. *In vitro*, histones H2A, H2B, H3, and H4 bind to isolated mitochondria and increase outer membrane permeabilization followed by release of proapoptotic intermembrane space proteins [34].

Effect of neurotoxin MPTP and neuroprotector isatin on the subproteome of brain mitochondrial Rpn10-binding proteins. Development of the MPTP-induced parkinsonian disorder insignificantly influenced the total number of identified Rpn10-binding proteins (50 versus 48 control proteins), but it dramatically changed the profile of Rpn10-binding proteins (Figs. 2b and 3). In contrast to the control, the Rpn10-binding proteins of the MPTP-treated animals were characterized by almost complete disappearance of cytoskeletal proteins and a reciprocal increase in proteins referred to the antioxidation/protection group (Fig. 2). It is especially interesting that Rpn10-

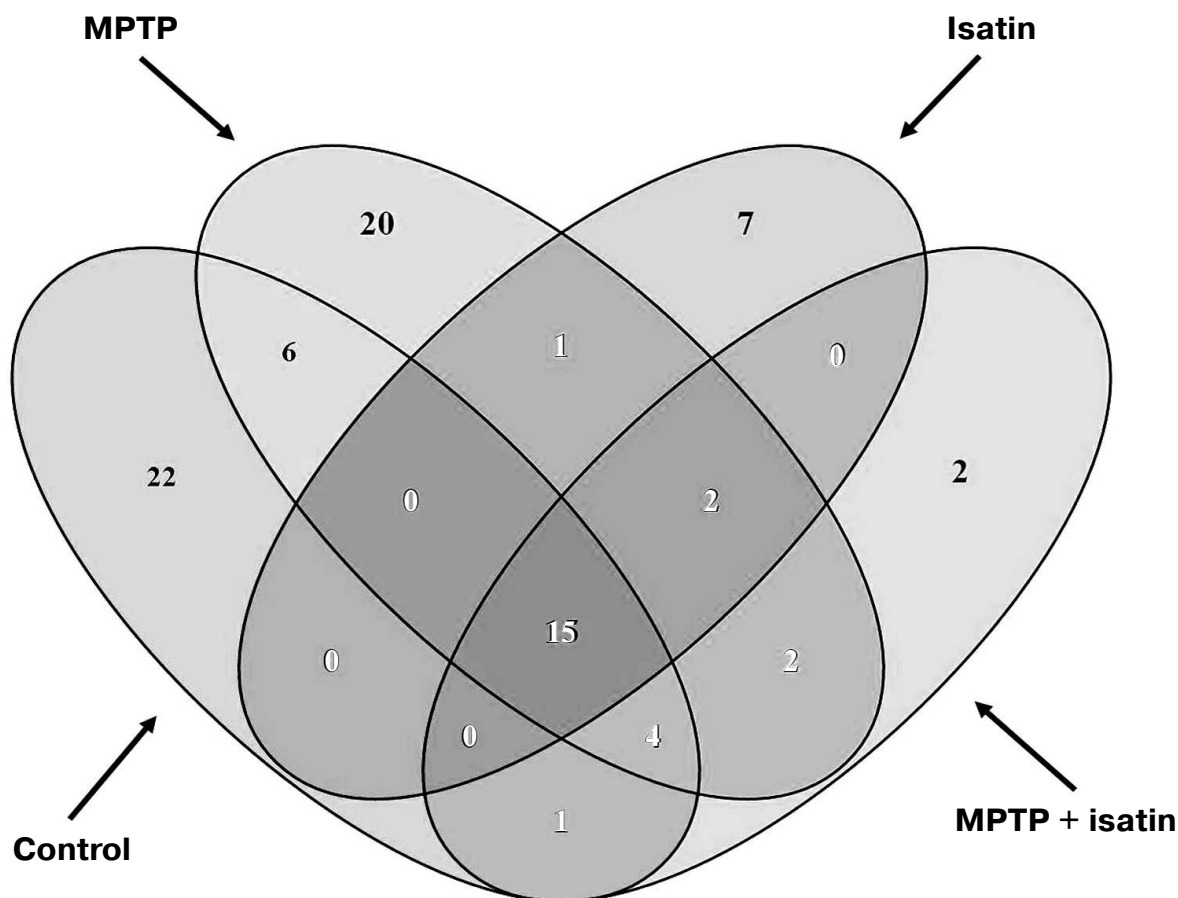


Fig. 3. Venn diagram showing the number of common Rpn10-binding proteins in brain mitochondria of investigated groups of mice.

binding proteins specific for MPTP-treated animals include Cul5-RING ubiquitin ligase complex (G3X914) directly involved in ubiquitin-dependent catabolic processes and cerebral cortex cell functioning [35], protein disulfide isomerase, mitochondrial heat shock proteins, and caspase 3, which is crucial for the development of MPTP-induced Parkinson's disease in mice [36, 37] (Table S2 in Supplement). Interestingly, heat shock proteins and caspase 3 are in the cluster of interacting proteins (Fig. S2 in Supplement).

Administration of isatin to intact mice significantly reduced the number of Rpn10-binding proteins to 25 and also altered the profile of Rpn10-binding proteins qualitatively (Figs. 2c and 3). Some of the Rpn10-binding proteins specific for isatin-treated mice (cytochrome P450 1A2, cytochrome P450 2D26) (Table S3 in Supplement) and destined to proteasomal degradation may be involved in metabolism of isatin itself [17, 18].

Pretreatment of mice with isatin before administration of MPTP also reduced the number of Rpn10-binding proteins to 26 (Fig. 2d; Table S4 in Supplement), i.e. to the level observed after a single administration of isatin, however, their profiles demonstrated clear differences

(Figs. 2 and 3). It is especially important that the sedative decrease in behavioral activity of intact mice treated with isatin and oligokinesia of MPTP-treated mice are associated with different proteomic profiles and therefore are realized by different mechanisms.

Thus, it appears that the repertoire of Rpn10-binding proteins is influenced not only by MPTP, but also by isatin, which significantly reduces the number of Rpn10-binding proteins and influences the proteomic profile of brain mitochondrial proteins interacting with the proteasomal Rpn10 subunit.

DISCUSSION

Previously it was found that isatin administration to rats at a dose 50-100 mg/kg resulted in a level of brain isatin of 9 $\mu\text{g/g}$ [38]. Taking into consideration brain water content of 0.78 $\mu\text{l/g}$ [39], this corresponds to 77 μM (provided the administered isatin uniformly distributed in the brain). Such concentration effectively protected MAO B against irreversible inhibition by specific mechanism-based inhibitors [40]. Earlier, it was also demon-

strated that administration of a MAO B substrate prevented MPTP-induced toxicity by competitively inhibiting MPTP conversion into MPP⁺ [41]. A smaller (but statistically significant) decrease in brain mitochondrial MAO B activity compared with values reported in the literature [41] may be attributed to the use of different brain preparations for assay of MAO B activity. We determined MAO B activity in the total fraction of brain mitochondria, while others assayed MAO B activity in homogenates of brain regions representing the main MPTP targets (striatum) [41]. Nevertheless, taken together all these results indicate that pretreatment of mice with a large dose of isatin (100 mg/kg) is sufficient for inhibition of MPTP

biotransformation into MPP⁺. In this context, it should be noted that isatin concentrations 50-100 μ M induce apoptosis in various cell cultures [42-45]. Moreover, for some cell lines it was demonstrated that apoptosis induced by isatin involved the mitochondrial pathway [45].

Besides MAO B, isatin can possibly interact with numerous isatin-binding proteins [21, 22]. Recent proteomic profiling of brain isatin-binding proteins revealed several UPS-related enzymes [22]. These included probable E3 ubiquitin protein ligase MYCBP2 (Q9TPH6), ubiquitin carboxyl terminal hydrolase 24 (B1AY13), E3 ubiquitin protein ligase MIB2 (Q8R516), E3 ubiquitin

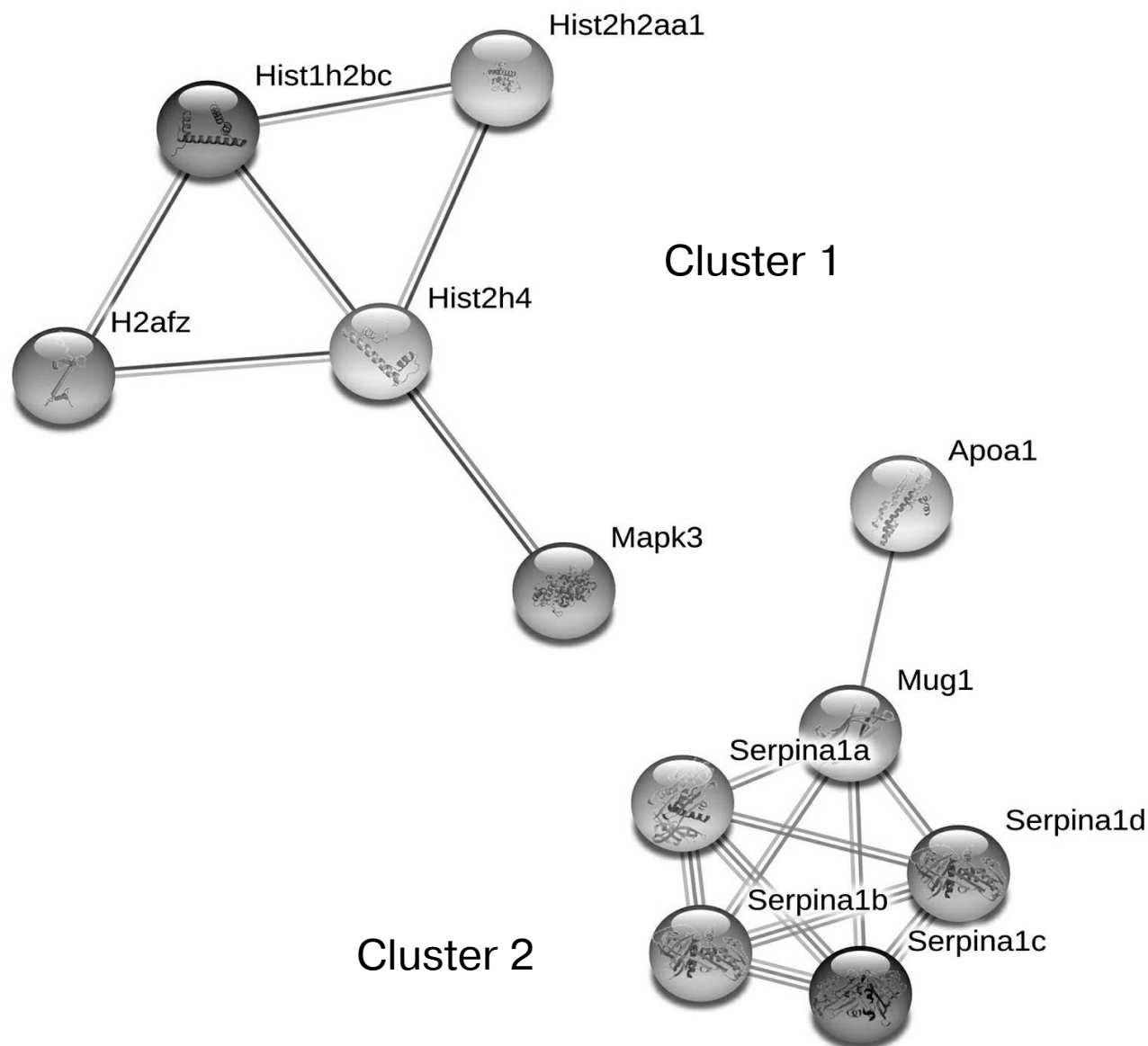


Fig. 4. Clusters of interactions of brain mitochondrial Rpn10-binding proteins (the constitutive pool). Clusters of physical and functional interactions of proteins were generated by results of identification obtained during proteomic profiling. Names of genes coding the protein products correspond to those listed Table 2.

Table 2. Brain mitochondrial Rpn10-binding common for all groups of proteins

No.	Number in UniProt database	Protein name	Gene	Protein function	Biological process	Cluster of interaction (C ≥ 0.7)
1	Q00623	apolipoprotein A-I	<i>Apoa1</i>	lipid binding	lipid transport, phospholipids removal	1
2	Q00896	alpha-1 antitrypsin 1-3 (serpina 1c)	<i>Serpina1c</i>	serine endopeptidase inhibitor	negative regulation of endopeptidase activity	1
3	P07758	alpha-1 antitrypsin 1-1 (serpina 1a)	<i>Serpina1a</i>	serine endopeptidase inhibitor	negative regulation of endopeptidase activity	1
4	P07759	serpin (serine protease inhibitor A3K)	<i>Serpina3k</i>	serine endopeptidase inhibitor	negative regulation of endopeptidase activity	1**
5	P22599	alpha-1 antitrypsin 1-2 (serpina 1b)	<i>Serpina1b</i>	serine endopeptidase inhibitor	negative regulation of endopeptidase activity	1
6	P28665	murinoglobulin-1	<i>Mug1</i>	serine endopeptidase inhibitor	negative regulation of endopeptidase activity	1
7	Q00897	alpha-1 antitrypsin 1-4 (serpina 1d)	<i>Serpina1d</i>	serine endopeptidase inhibitor	negative regulation of endopeptidase activity	1
8	Q03734	serpin (serine protease inhibitor A3M)	<i>Serpina3m</i>	serine endopeptidase inhibitor	negative regulation of endopeptidase activity	1**
9	Q9Z1R9	MCG124046 – serine protease 1 (trypsin 1)	<i>Prss1</i>	serine endopeptidase inhibitor	proteolysis	1**
10	D3Z3G6	MAPK 3	<i>Mapk3</i>	MAP kinase activity	MAPK cascade	2
11	P62806	histone H4	<i>Hist1h4a</i>	DNA binding	nucleosome assembly	2
12	Q6GSS7	histone H2A type 2-A	<i>Hist2h2aa1</i>	DNA binding	nucleosome assembly, suppression of gene expression	2
13	Q6ZWY9	histone 42B type 1-C/E/G	<i>Hist1h2bc</i>	DNA binding	nucleosome assembly	2
14	Q8BFU2	histone H2A type 3	<i>Hist3h2a</i>	DNA binding	nucleosome assembly, suppression of gene expression	2**
15	G3UWL7	histone H2A	<i>H2afz</i>	nucleic acid binding	*not shown	2

* Biological process not shown in available databases.

** Referred to this cluster at moderate confidence level (C ≥ 0.4).

protein ligase HUWE1 (Q7TMY8), ubiquitin conjugating enzyme E2 variant 1 (Q9CZY3), and polyubiquitin B (P0CG49). Mapping of identified isatin-binding proteins to known pathways suggests that some of them participate in the parkin (E3 ubiquitin-protein ligase)-associated pathway (Gene ontology reference CH000000947) [22]. Since the probability that hits in this pathway represent accidental events is as low as $2.5 \cdot 10^{-5}$ [22], it appears that isatin may interfere with various components constituting the UPS.

The 19S proteasome Rpn10 subunit plays an important role in recognition of substrates destined to proteaso-

mal degradation [15, 16]. Results of the present study indicate that the Rpn10 subunit can bind certain mitochondrial proteins and thus participate in their delivery to targeted proteasomal degradation.

Proteomic profiling of mitochondrial fractions from animals of all groups (control, MPTP-treated mice, isatin-treated mice, and mice treated with isatin and MPTP) revealed existence of a constitutive pool of mitochondrial Rpn10-binding proteins. This pool insensitive to either the neurotoxin MPTP or the neuroprotector isatin (Table 2 and Fig. 4) includes serpins/protease inhibitors, histones, MAPK (mitogen-activated protein

kinase), and apolipoprotein A-I. There is increasing evidence that serpins (serine protease inhibitors) not only control activity of certain proteases, but also regulate cell proliferation and apoptosis [46]. Mutant serpins are degraded by UPS enzymes [47]; a conjugate of serpin 2a with a ubiquitin homolog was found in activated macrophages [48]. Recently, it has been demonstrated that apolipoprotein A-I, the principal HDL (high-density lipoproteins) protein, is associated with mitochondria [49], and glycated HDL induced apoptosis of cells via mitochondrial dysfunction [50]. Taking into consideration MAPK association with mitochondria [51], submitochondrial localization of histones [34, 35], and an important role of MAPK-dependent phosphorylation of histones for development of apoptosis [52], functional links between proteins identified in this study (Fig. 4) suggest that the constitutive pool of mitochondrial Rpn10-binding proteins play a role in regulation of apoptosis.

The repertoire of the variable pool of brain mitochondrial Rpn10-binding proteins is sensitive to effects of both neurotoxin MPTP and neuroprotector isatin (Figs. 2 and 3; Figs. S3 and S4 in Supplement). Considering that isatin stimulates apoptosis in various cells [42-45] and its analogs act as apoptosis-inducing inhibitors of UPS [23, 24], there are reasons to believe that the altered repertoire of mitochondrial Rpn10-binding proteins induced by isatin may represent a part of a switch mechanism from targeted elimination of individual (damaged) proteins to more efficient ("global") elimination of damaged organelles and whole damaged cells.

Thus, in the context of molecular mechanisms of neuroplasticity that are associated with regulatory effects of isatin on brain mitochondria under conditions of MPTP-induced experimental parkinsonism, the neuroprotector effect of isatin may be associated with several interrelated actions:

- inhibition of MAO (B)-dependent conversion of MPTP to MPP⁺;
- interaction with isatin-binding proteins and modulation of their functions;
- altered repertoire of mitochondrial proteins interacting with the receptor, Rpn10, responsible for proteasomal delivery of (damaged) proteins.

Acknowledgements

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