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Effect of treatment by hemorphin on mouse brain proteome pattern

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Hemorphins, a family of endogenous nonclassical opioid peptides derived from haemoglobin (Hb), are presented in the CNS [11, 30], peripheral tissues [54] and body fluids [22]. These peptides have been shown to demonstrate a wide spectrum of biological activities by affecting different receptors function (e.g. □-, □-, □- opioid receptors [55], angiotensin IV (AT_4) receptor [36], bombesin receptor subtype 3 (hBRS-3) [31] and corticotrophin releasing hormone receptors) [4]. All hemorphins, whatever their source, originate from the same region of the □-chain of Hb (residue 31-40 of bovine and residue 32-41 of human Hb); a peptide with this sequence is named LVV-hemorphin-7 (LVVYPWTQRF, LVV-H7) [22]. Earlier we have proposed that hemorphins could be formed in the organism during physiological or pathophysiological conditions as a result of limited proteolysis of Hb or Hb-like protein of nervous tissue [10]. Indeed, brain high molecular weight (HMW) aspartic proteinase has been shown to generate LVV-hemorphin-7 from the □-chain of Hb by cleavage of Leu^{30}-Leu^{31} and Phe^{40}-Phe^{41} bonds [10]. The same enzyme presented in erythrocytes membrane was identified as a cathepsin E [29]. It should be also noted that □- and □-globin mRNAs have been identified in mouse brain, implying the synthesis of globin in the CNS [39]. It has been shown that brain cathepsins E, D and B [3, 18, 29] participate in the cascade mechanism of hemorphins generation in vitro and it is proposed that the same mechanism of hemorphin generation takes place in vivo. Indeed, by using in vivo microdialysis in combination with electrospray mass spectrometry in vivo processing of LVV-

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hemorphin-7 in rat brain and blood was studied. Several hemorphins were formed, including LVVYPW in both brain and blood [47].

LVVYPW, also named mielopeptide-2 (MP-2), isolated for the first time from the supernatant of bone marrow cell culture, displays anti-tumor effect by restoring the activity of CD4+ T cells suppressed in tumor-bearing organisms. It has been demonstrated that MP-2 induces the restoration of interleukin-2 (IL-2) synthesis and IL-2 receptor (IL-2R) expression, depressed by tumor cell metabolites □35, 43□.

It is of particular interest our finding that hemorphins *in vitro* modulate calcineurin activity in the brain and immune system by binding with calmodulin (CaM) □8, 15□ both in Ca^{2+}-dependent and Ca^{2+}-independent manner [24], exhibiting a concentration-dependent biphasic response to enzyme activity. It should be underscored that hemorphins are able to modulate calcineurin activity both in vitro and in vivo [15, 8, 9, 13].

Calcineurin is Ca^{2+}/calmodulin (CaM)-dependent protein phosphatase, also known as a phosphatase 2B. This heterodimeric enzyme consists of catalytic, CaM binding A subunit (CNA) and regulatory, Ca^{2+}-binding B subunit (CNB) □49□. Earlier we have shown that hemorphins are able to regulate calcineurin basal activity in the absence of Ca^{2+} and CaM. We have proposed that in this case hemorphins may regulate the enzyme activity by binding with CNB [15], which demonstrates 35% of sequence homology with CaM □1□.

Calcineurin is known as a key enzyme in the signal transduction cascade leading to T cell activation. This enzyme controls gene expression of several cytokines, including IL-2, tumor necrosis factor □ (TNF□) and others via dephosphorylation and nuclear translocation of NFATc (nuclear factor of activated T cell) family members □49□. Earlier we have shown that calcineurin activity is down regulated in both brain and plasma of rats, inoculated with sarcoma-45 (S-45), and administration of LVVYPW has shown to recover calcineurin activity reduced in pathophysiology of S-45 [12]. The results obtained have indicated that calcineurin is involved in the molecular mechanisms of anticancer effect of LVVYPW [12] and other hemorphins [9]. Because calcineurin participates in gene expression and production of IL-2 via CN/NFAT pathway, it is clear why the anti-cancer effect of LVVYPW is bound with restoration of IL-2 synthesis suppressed in tumor bearing organism □35□. LVVYPW induces the increase of calcineurin [12] activity, and calcineurin, in turn, stimulates IL-2 synthesis on gene transcription level. It is of great interest that CNB itself has been reported to exert anti-cancer effect [28].

The immunosuppressive drugs cyclosporin A (CsA) and FK506 are inhibitors of calcineurin [34]. They are natural, but not endogenous compounds. Both these compounds exert their inhibitory action on calcineurin activity by binding to immunophilins (cyclophilin A and FKBP12, respectively) □34□. It

is proposed that hemorphins, being modulators of calcineurin activity, may have a functional link with immunophilins.

Earlier we have shown that hemorphins change the sensitivity of CaM to its antagonists (trifluoperazine, chlorpromazine, W-7, vinblastine and vincristine) □5□. All of these compounds are drugs that are implicated in the treatment of different diseases, including cancer □32, 51, 53□. Therefore in 1992 it was predicted that hemorphins had good prospects for applied medicine as a drug without side effects □5□.

Interestingly, CaM antagonists vinblastin and vincristine exhibit anti-cancer properties by binding with tubulin. They block microtubule polymerization, and thereby disrupt mitotic spindle formation during mitosis. Cell death results from an inability to segregate chromosomes properly. It is worth mentioning that calcineurin has been reported to affect assembly of tubulin into microtubules in pathophysiology [32]. This is additional confirmation that molecular mechanisms underlying anti-tumor effect of MP-2 involve calcineurin.

The involvement of hemorphins in pathophysiology of cancer [9,12,16], diabetes [13, 21] and Alzheimer´s disease [44] has also been reported. It should be emphasized that during the mentioned pathologies an alteration of hemorphins concentrations in the organism takes place [16, 21]. These findings give us reason to propose that hemorphins may be used as drugs for the treatment of these diseases. Indeed, studying the role of hemorphins in pathophysiology of diabetes, stress and cancer, we have discovered the homeostatic effect of hemorphins in pathophysiology of the mentioned diseases [9, 12-14].

Very recently by using differential scanning microcalorimetry, we have demonstrated that hemorphins (LVVYPW, LVV-hemorphin-7 and hemorphin-7) induce in vivo changes in the value of enthalpy of DNA, isolated from S-45, treated with hemorphin in comparison with S-45 DNA. It is necessary to underscore that hemorphins, depending on their structure, differently affect the value of enthalpy during in vivo treatment of sarcoma-45 [9, 12]. This fact indicates to some differences in their function. Furthermore, the formation of DNA-hemorphin complex has been detected in vitro by differential absorbance spectra measurement [9, 12]. The pleiotropic nature of hemorphins allows them to act based on several mechanisms and by implication of different signalling pathways. It has been shown [8-9,13,15, 57, 58] that molecular mechanisms, underlying hemorphins actions in the brain and immune system, involve integrated effects of Ca2+/calmodulin, calcineurin,3□5□-cyclic AMP, redox-pathway, and further signalling molecules.

Today proteomics, quantitative analysis technology is routinely used in clinical diagnosis, and it has been applied in the investigation of different diseases, including cancer, diabetes, neurological diseases etc.

Since 2005, by getting DAAD research grant, we have investigated together with Dr. F. Lottspeich and Dr. J. Kellermann (Max-Plank Institute of Biochemistry, Martinsried) the effect of treatment by hemorphin on mouse brain proteome pattern.

As a result of our collaboration some parts of our data obtained have been published and reported on international conferences [6-7, 14].

In the present paper we will introduce our data obtained by using two independent quantitative proteomic technologies: ICPL (isotope-coded protein label) and 2-D DIGE (2-dimentional fluorescence difference gel electrophoresis) for identification of regulated by ip injection of LVVYPW proteins in mouse brain [2, 48].

Materials and Methods

Animals and hemorphin treatment. In the experiments were used male mice of C57BL/6 strain, age 14 weeks (25-30 g). For each experiment the mice were injected with saline or hemorphin (LVVYPW) at doses of 280μg/kg. In order to reinforce the effect of hemorphin, ip administration of hemorphin was done twice (the second injection was done 1h after the first one) and the animals were sacrificed 2 h after the second injection.

Brain tissue protein samples preparation. Brain proteins were isolated as described elsewhere [2, 48] with some modifications. In order to prepare the samples for 2-DE, corresponding parts of the brain of 20 mice were homogenized with lysis buffer, containing 8M urea, 2M thiourea, 4% CHAPS and protease inhibitors (0.5 mM PMSF, 1μM pepstatin, 100 μM leupeptin, 1μg/ml aprotinin). The homogenate was sonicated 3 times for 10 sec with 30 sec intervals. After 1 h incubation at room temperature the homogenate was centrifugated for 35 min at 100 000 x g and the supernatant was frozen at -70° C until use.

For further ICPL labelling the brains of 20 mice were homogenized with 6M Guanidine-HCI and 0.1 M HEPES containing buffer, pH 8.5. All other conditions and procedures were the same as mentioned above.

Isotopic labelling of proteins with ICPL was done according to the method of Schmidt et al. □48□. The brain samples from 2 experimental mice treated with the same hemorphin were labeled with the ^{13}C and deuterium version of ICPL-label respectively, and compared to the control mice brain samples, tagged with ^{12}C ICPL-label. These three samples were mixed together and Triplex-ICPL aliquots were separated by 2-D gel electrophoresis.

For DIGE analysis 50 μg protein per sample were labeled with 400 pmol N-hydroxy succinimidyl ester derivates of the cyanine dyes (Cy3 and Cy5) according to the manufacturer's guidelines (GE Healthcare Bio-Sciences, Amersham plc, UK). The internal pooled standard was prepared from equal protein amounts of each biological sample and labeled with the Cy2

fluorophore. After labelling, Cy2, Cy3 and Cy5 labeled proteins were combined and dissolved in 400 µl rehydration lysis buffer, containing 8 M urea, 2 M Thiourea, 4 % CHAPS, 40 mM DTT and 2 % Pharmalyte (pH 3-10).

Enzymatic digestion of 2D-gel spots, MALDI target preparation and MALDI-TOF-TOF analysis

The protein spots were in-gel digested with trypsin (12ng/µl in 25 mM NH_4HCO_3). The tryptic digests were concentrated and desalted using ZipTip C-18 reversed-phase tips (Millipore, MA, USA). Peptides were eluted with α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) in 50% Acetonitrile/0.1%TFA and then spotted on MALDI steel target plates (Applied Biosystems, CA, USA). Mass spectra were acquired using a Proteomics Analyzer 4700 (MALDI-TOF-TOF) mass spectrometer (Applied Biosystems, CA, USA). A total of 2500 shots were carried out for both the MS- and MS/MS spectra. The modified proteins were identified by searching against the Swiss-Prot database using an in house version of MASCOT. Quantitative nano-LC-MALDI-MS/MS was also used for 2-D DIGE analysis.

It should be mentioned that for identification of some proteins (e.g. ferritin heavy chain, published for the first time in the materials of international conferences in 2008, see Ref. 6-7), tubulin alpha-1B chain, and isoform 2 of serine-treonine protein phosphatase 2B catalytic subunit 1 were used standard 2-D PADE approach. Gel images were analyzed using the Progenesis SameSpots (Nonlinear) software which performs gel alignment, spot detection and inter-gel normalization.

DIGE gels were analyzed in the "DIGE with internal standard" workflow.

For control of the technical and biological variations within samples and gels, five biological replicates per group were analyzed in both approaches.

Results and Discussion

As it has been mentioned above, hemorphins level is down regulated in plasma of patients with diabetes and breast cancer [16, 21]. The changes in the level of hemorphins have been observed in the Alzheimer´s disease brain post-mortem (44). These findings give us reason to propose that hemorphins may be used as drugs for the treatment of these diseases. Indeed, studying the role of hemorphins in pathophysiology of diabetes, stress and cancer, we have discovered the homeostatic effect of hemorphins in pathophysiology of the mentioned diseases. It is suggested that in pathophysiological conditions the generation of hemorphins in the organism is disturbed. In the present work by using proteome analysis technology we have revealed proteins identified in healthy mouse brain as regulated in response to treatment with LVVYPW.

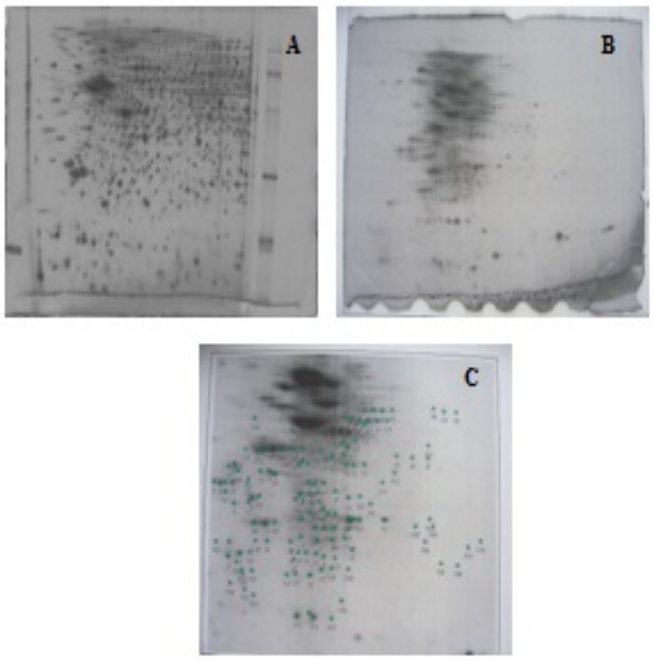

Fig. 1. 2-D gel electrophoresis of 1mg mouse brain sample (A); 2-D gel electrophoresis of 1, 25 mg Triplex-ICPL aliquots (B); Proteins were visualized by fluorescent staining with Sypro Ruby. Spots were picked by automatic spot picking (C). Proteins were in-gel digested by trypsin, eluted and analyzed by MALDI-MS-MS.

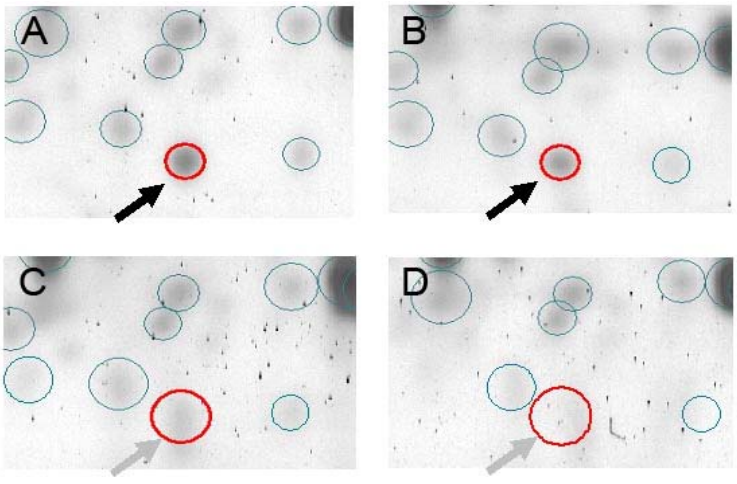

Fig. 2. Selected 2-D gel region of mouse brain protein samples. A and B are experimental mice N16 and N17 treated by the same hemorphin (LVVYPW) and showing up regulation of ferritin expression. C and D are control mice N8 and N15, treated by saline (the data were introduced for the first time in International Conference "Biotechnology and Health"-2 & DAAD Alumni seminar, Yerevan, Armenia, April 21-25, 2008).

Table

Identification of mouse brain proteins regulated by intraperitoneal (ip) LVVYPW injection

N	Anova (p)	Fold	Gene name	Protein
1	0,025	2,1	Ncald	neurocalcin-delta
2	0,017	1,9	Cplx1	complexin 1
3	0,009	1,9	Cplx2	complexin 2
4	0,001	1,9	Pea-15	isoform 1 of astrocytic phosphoprotein PEA-1
5	0.003	1.8	Ppia	peptidyl-prolyl cis-trance isomerase
6	0,002	1,8	Ndufs3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondria
7	0,005	1,5	Ndufs8	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondria
8	0,004	1,7	SOD1	superoxide dismutase, [Cu-Zn] SOD 1
9	0,002	1,6	Calb 1	calbindin, Ca^{2+}-binding EF hand protein
10	0,004	1,6	Park7	Parkinson's disease associated DJ-1 protein (autosomal recessive early onset)
11	0,012	1,5	Fabp7	fatty acid binding brain protein
12	0,03	1,5	Ift 74	intraflagellar transport protein 74 (IFT 74)
13	0,005	1,5	ApoE	apolipoprotein E
14	0,007	1,6	Hebp1	hem-binding protein-1
15	0,002	2,6	Txn1	thioredoxin
16	0,002	2,6	Glrx5	glutaredoxin-related protein 5
17	0,017	1,9	Tuba1b	tubulin alpha-1B chain
18	0,01	1,5	Ppp3r1	protein phosphatase 3 Regulatory subunit B alpha isoform, type 1
19	0,03	2,2	Ppp3ca	isoform2 serine-treonine protein phosphatase 2B catalytic subunit 1

Functional characteristics of the identified proteins

Neurocalcin-delta (also known as visinin-like protein -3, VILIP-3) is EF hand Ca^{2+}-binding protein, which is the most abundantly expressed in Purkinje cells of the cerebellum. The specific interaction of this protein with clatrin heavy chain, □- and -□ tubulin and actin has been reported [25]. The interaction of neurocalcin-delta with the mentioned proteins indicates to its participation in Ca^{2+}-dependent changes in cytoskeletal organization and in the regulation of endocytic events. The interaction between VILIP-3 and microsomal cytochrom

b_5 was also reported [40]. It is of special interest that neorocalcins are direct targets of insulinotropic agent repaglinide [41]

Complexins (CPLXs) are SNARE (soluble N-ethyl-maleimide-sensitive fusion attachment protein receptors) complex-associated proteins that modulate synaptic exocytosis and regulate late step in Ca^{2+}-dependent neurotransmitters release [46]. CPLX II expression is altered in different neurological diseases, including Huntigton's disease [37].

Isoform 1 of astrocytic phosphoprotein PEA-15

Phosphoprotein enriched in astrocites- 15 kDa (PEA-15), a major small cytoplasmic astrocytic phsphoprotein, is a PKC substrate. PEA-15 induced insulin resistance in type 2 diabetes [52] results from a dysregulation of the balance between the activities of PKC and PKC . PEA-15 demonstrate both tumor promotion (e.g. skin tumor) and tumors suppressor function (e.g. breast and ovary cancer) [20].

Peptidyl-prolyl cis-trans isomerase

Immunophilins (Iphs) possess peptidyl-prolyl cis-trans-isomerase activity. They form complexes with immunosupressant drugs cyclosporine A and FK506, which inhibit the activity of calcineurin [34]. It has been reported that immunophilins can be used for the treatment of neurodegenerative diseases [23]. The involvement of Iphs in pathophysiology of cancer was also shown [38].

NADH dehydrogenase [ubiquinone] iron-sulfur protein 8 mitochondria (Ndufs8)

represent Complex I of mitochondrial respiratory system. Defect in Ndufs8 causes Leigh syndrome (LS). LS is a severe neurological disorder characterized by bilaterally symmetrical necrotic lesion in subcortical brain region [19].

NADH dehydrogenase [ubiquinone] iron-sulfur protein3 (Ndufs3) mitochondria

Complex I (NADH ubiquinone oxidoreductase), often called NADH dehydrogenase (oxidized NADH) transfers two electrons to ubiquinone. Significant loss of complex I activity was found in frontal cortex of Parkinson's disease [42].

Supeoxide dismutase [Cu-Zn] (SOD1)

SOD1 as other types of SOD catalyses the dismutation of the superoxide anions and is the most important antioxidant enzyme. SOD1 is involved in pathophysiology of neurodegenerative diseases, stress, inflammation. It is necessary to emphasize that overexpression of SOD1 in mice causes axonal degeneration, mitochondrial vacuolization and premature motoneuron death [26]. The functional link between SOD1 and immunophilins in familiar amyotrophic lateral sclerosis was reported [33].

Thioredoxin and Glutaredoxin

Oxidative stress, which is associated with an increased concentration of reactive oxygen species (ROS), is involved in the pathogenesis of numerous diseases including cancer. In response to increased ROS levels, cellular antioxidant molecules such as thioredoxin, peroxiredoxins glutaredoxins, DJ-1, and supeoxidere dismutases are up regulated to counteract the detrimental effect of ROS. Thioredoxin, DJ-1 and glutaredoxins, which are up regulating in many human cancer types, correlating with tumor proliferation, survival, and chemo-resistance. These molecules serve as potential targets to treat cancer [45].

Calbindin

Ca^{2+}-binding EF hand protein calbindin has neuroprotective properties via regulation of intracellular calcium levels and suppresses the signaling phase of apoptosis. It should be underscored that calbindin is involved in pathophysiology of both diabetes and cancer [50,51].

Parkinson's diseases associated DJ-1 gene protein (autosomal recessive, early onset)

Mutation in the DJ-1 gene causes autosomal recessive Parkinson's disease. The implication of DJ-1 in pathophysiology of tumor as well as diabetes was reported [27].

Fatty acid-binding protein of mouse brain

The protein encoded by FABP7 is a brain fatty acid binding protein. FABPs are a family of small, highly conserved, cytoplasmic proteins that bind long chain fatty acids and other hydrophobic ligands. It has been reported that FABPs are involed in fatty acid uptake, transport, and metabolism.

Intraflagellar transport protein 74 (IFT74)

IFT74 is a component of intraflagellar transport system responsible for vesicular transport of material synthesized within the cell body into and along the dendritic and axonal processes of neurons.

Apolipoprotein E

The apolipoprotein E, a genetic risk factor for sporadic Alzheimer's disease (AD), is also involved in pathophysiology of type 2 diabetes and cancer [17] . In the CNS Apo E is implicated in repair, synaptogenesis, nerve growth and development.

Heme-binding protein-1

One of the main roles of the cytoplasmic heme-binding protein-1 (PhuS) of *Pseudomonas aeruginosa* is participation in intracellular heme trafficing and iron homeostasis.

The proteins mentioned in the Table were identified as regulated by LVVYPW treatment in mouse brain proteome pattern. Among these proteins there were proteins involved in the regulation of calcium homeostasis, as well as redox pathway. It should be noted that most of mentioned proteins have functional interaction with hemorphins and it has been reported their involvement in pathophisyology of cancer, diabetes and neurodegenerative diseases. This fact is confirmed by our previous functional data obtained during more than 25 years, which testify to the possibility to use hemorphins for creation of new effective complex drugs for the treatment of severe diseases, including cancer and diabetes.

The authors have declared no conflict of interests.

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Մկների գլխուղեղի պրոտեոմիկի նմուշներում հեմորֆինի ներորովայնային ներարկմամբ առաջացած փոփոխությունները

Ն. Հ. Բարխուդարյան, Ֆ. Պ. Սարուխանյան, Հ. Հ. Զաքարյան, Ֆ. Լոտշպայխ, Յ. Կելերման

Կիրառելով քանակական պրոտեոմիկի այնպիսի մեթոդներ, ինչպես ICPL (կոդավորված իզոտոպերով նշադրված սպիտակուցների կիրառմամբ) և DIGE (ֆլյուորեսցենցիայով տարբերվող սպիտակուցների երկչափ գել-էլեկտրոֆորեզ), C57BL/6 գծի մկների գլխուղեղում (արու, 14 ամսական) բացահայտվել են սպիտակուցներ, որոնց էքսպրեսիան կարգավորվում է հեմորֆինի (LVVYPW) ներորովայնային ներարկմամբ: Սպիտակուցների բացահայտումն իրականացվել է MALDI-MS-MS մաս-սպեկտրոմետրիայի կիրառմամբ: Դրանց թվին են պատկանում այն սպիտակուցները, որոնք ներգրավված են այնպիսի ծանր հիվանդությունների պաթոֆիզիոլոգիայում, ինչպես քաղցկեղը, շաքարախտը, նեյրոդեգեներատիվ հիվանդությունները (Ալցհեյմերի, Պարկինսոնի հիվանդություններ և այլն): Հոդվածում ներկայացված են միայն այն սպիտակուցները, որոնց էքսպրեսիան կարգավորվում է հեմորֆինով ոչ պակաս, քան 1.5 անգամով: Ստացված տվյալները ունեն կիրառական նշանակություն, քանի որ նպաստում են հեմորֆինների կիրառմամբ նոր արդյունավետ դեղամիջոցների ստեղծմանը:

Изменения в образцах протеомика головного мозга мышей, вызванные внутрибрюшинной инъекцией геморфина

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Используя такие методы количественного протеомика, как ICPL (с использованием белков, меченных кодированными изотопами) и DIGE (двумерный гель-электрофорез белков, различающихся по флюоресценции), в головном мозге мышей линии C57BL/6 (самцы, возраст 14 недель) были идентифицированы белки, экспрессия которых регулировалась внутрибрюшинной инъекцией геморфина (LVVYPW). Идентификация белков проводилась с использованием MALDI-MS-MS масс-спектрометрического анализа. К числу этих белков относятся белки, вовлеченные в патофизиологию таких тяжелых заболеваний, как рак, диабет, нейродегенеративные заболевания (болезнь Альцгеймера, Паркинсона и др.). В статье приведены лишь те белки, экспрессия которых регулируется геморфином не менее чем в 1.5 раза. Полученные данные имеют прикладное значение, поскольку открывают новые перспективы для дальнейшего грамотного их использования при создании эффективных комплексных лекарственных препаратов.

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