

## Proteomic Analyses of Exothermic Processes in Rat Brain Homogenate

Y Voynikov<sup>1</sup>, L Velkova<sup>1</sup>, L Tancheva<sup>2</sup>, P Mladenov<sup>3</sup>, A Dolashki<sup>1</sup>, L Alova<sup>2</sup>, W Voelter<sup>4</sup> and P Dolashka<sup>1\*</sup>

<sup>1</sup>Institute of Organic Chemistry with Centrum of Phytochemistry, Bulgarian Academy of Sciences, G. Bonchev 9, 1113 Sofia, Bulgaria

<sup>2</sup>Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

<sup>3</sup>Agrobiointitute, Abiotic stress, 1164 Sofia, Bulgaria

<sup>4</sup>Interfaculty Institute of Biochemistry, University of Tübingen, Hoppe-Seyler-Strasse 4, D-72076 Tübingen, Germany

### Abstract

Alzheimer's disease (AD) is the most widespread neurodegenerative disorder which can be induced by scopolamine, but the underlying molecular mechanism is poorly understood. Recently, differential scanning calorimetry (DSC) has been used to study healthy and scopolamine-treated mice. A well-expressed exothermic transition minimum in the range of 35 - 45°C was determined in the DSC profiles of healthy mice supernatants. To explain this process, using two-dimensional gel electrophoresis (2D-PAGE) coupled with MALDI-TOF-TOF, poorly soluble membrane proteins in hippocampal proteome of rat brain tissue were identified. The different behavior of the hippocampal proteome from the healthy rats before and after heating to 45°C was identified. Due to the demonstrated change in protein level of tau protein and tubulin in the rat hippocampus after heating to 45°C, it was suggested that the observed exothermic process at 35-45°C in rat may be due to the partial unfolding of tau protein, which leads to the release of tubulin. Both proteins together are involved in protein fibrillation and aggregation.

Another important result is the discovery of different profiles for the proteome of hippocampal rat homogenates with scopolamine-induced neurodegenerative disorder and its characteristics of healthy rats.

The reported results from this study can help clarify the molecular mechanisms of scopolamine-induced dementia and neurodegenerative processes in general.

**Keywords:** Protein solubility; Alzheimer's disease; Tau protein; Proteomic analyses; Rat brain homogenate; Mass spectrometry

### Introduction

Alzheimer's disease (AD) is the most widespread neurodegenerative disorder affecting more than 5 million people worldwide, mainly the elderly ones. At present time only symptomatic treatments are available. Histopathological hallmarks that accompany the diseased brain are extracellular amyloid plaques and intracellular neurofibrillary tangles. A number of multiple protocol studies and a combination of many analytical techniques such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), and nuclear magnetic resonance spectroscopy (NMR) allowed to identify different protein biomarkers [1-3]. From recent data it is suggested that for the development of AD, mainly  $\beta$ -amyloid peptides and neurofibrillary assemblies consisting of phosphorylated tau proteins are responsible for plaque formation. The structure, dynamic behavior and spatial organization of microtubules in neurons in the brain are regulated by their associated tau proteins. The microtubule tau protein is a member of a group of proteins that exist as heterogeneous isoforms that favor the formation and stabilization of microtubules.

For many years, the linkage between the structure and function of tau proteins has been intensively studied primarily through molecular biology and biochemical approaches. Microtubules were found to be composed of tubulin dimers which are subject to posttranslational modifications that affect the stability and function of microtubules [4-7]. In addition to the well-known function in microtubules, tau proteins play an important role in preserving the genomic integrity in stressful situations.

Applying 2D-electrophoresis and Western blot analyses, differential splicing and different phosphorylated tau proteins have been identified [8-11]. Specific phosphorylation is essential to modulate protein location and protein-protein interactions [12,13]. Tau hyperphosphorylation can be considered as one of the hallmarks

of Alzheimer's disease that can cause changes in the hydrophobicity thereby interacting with a large number of molecular structures.

Approximately 2756 proteins were identified in the hippocampus of Zucker diabetic fatty rats by 2D-PAGE electrophoresis and MALDI-TOF [3]. Tau proteins bind to specific microtubule residues that are essential for the pathological aggregation of tau, suggesting a competition between physiological interaction and pathogenic misfolding. The basis of interface binding between the tubulin heterodimers and the tau proteins is the conservative mechanism of microtubule polymerization, which is related to the regulation of axonal stability and cell morphology. The repeating domain of tau by transversal bonds to  $\alpha$ -tubulin is closely related to the flexibility of the structural elements forming the binding pocket of the  $\alpha$ - $\beta$ -tubulin heterodimer interface [14,15].

Recently, a transition from 3r to 4r tau in AD was proposed based on immunohistochemical studies with isoform-specific tau antibodies rD3 and rD4. The antibodies rD3 and rD4 are suitable for testing the expression and localization of the three isoforms of three tau replicates (3r) and four repeats (4r) [16]. Quantitative analysis of nitrophilic threads of AD type (NFT) and neurofibrillary nodes (NTs) show that disease progression leads to an increase in the 3R tau fraction in neurofibrillar brain stem cell changes [17]. Although tau's structure-

**\*Corresponding authors:** P Dolashka, Institute of Organic Chemistry with Centrum of Phytochemistry, Bulgarian Academy of Sciences, G. Bonchev 9, 1113 Sofia, Bulgaria, Tel: + 35929606163; Fax: +35928700225; E-mail: [pda54@abv.bg](mailto:pda54@abv.bg)

Received February 03, 2018; Accepted March 20, 2018; Published March 27, 2018

**Citation:** Voynikov Y, Velkova L, Tancheva L, Mladenov P, Dolashki A, et al. (2018) Proteomic Analyses of Exothermic Processes in Rat Brain Homogenate. J Proteomics Bioinform 11: 079-086. doi: [10.4172/jpb.1000470](https://doi.org/10.4172/jpb.1000470)

**Copyright:** © 2018 Voynikov Y, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

function dependence of tau proteins has been intensively studied for many years, little is known about the molecular mechanisms of interaction between tau proteins and microtubules and promotion of the assembly of microtubules. The thermal behavior of brain tissues of animal model (mouse) affected by drug induced neurodegenerative disorder has been studied by differential scanning calorimetry (DSC) [18-20]. Differential scanning calorimetry was also used to evaluate the efficacy of biologically active compounds of plant origin (myrtenal, elagic acid, lipoic acid and their combinations, including ascorbic acid) in mice with neurodegenerative disorders. After application of these drugs a delay or block progression of dementia has been identified [21]. Also, the significant differences in heat capacity curves on brain homogenate supernatants isolated from healthy animals in comparison to animals with scopolamine-induced dementia were observed by DSC measurements [22,23]. Herein, we present results of changes occurred at 45-50°C in the brain proteome of an experimental animal model (rat) with scopolamine-induced neurodegenerative disorder, type AD. The aim is to explain the observed extremes in DSC measurements on brain tissue homogenates isolated from healthy animals and from animals treated with scopolamine.

## Methodology

### Animals and scopolamine treatment

An experimental animal (rat) model of scopolamine-induced dementia of AD type was used for experimental induction of cognitive deficits in doses of 0.3–0.5 mg x kg<sup>-1</sup> intraperitoneally [24,25]. Two groups of 5-7 Male Wistar rats were analyzed in this study. First group were rats without treatment (control), in the second group rats were treated daily for 11 days with scopolamine (1 mg x kg<sup>-1</sup> body weight (b.w.) administered intraperitoneally (i.p.). All animals were treated in accordance with the European Commission standards concerning the care and use of laboratory animals, and the experiments with animals were conducted in accordance with the ethical and professional guidelines established by the Ethics Commission at the Institute of Neurobiology, Bulgarian Academy of Sciences.

### Extraction of proteins from the rat brain

Rat brains were rapidly removed after the animals were sacrificed, and three fractions were dissected: hippocampus, striatum and cortex. The samples (3-4 g) were washed with 10 ml of ice cold PBS buffer (0.2 g KCl, 8 g NaCl, 1.44 Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g KH<sub>2</sub>PO<sub>4</sub>), and then the samples were homogenized in 0.1 M phosphate buffer, pH 7.8, containing protease inhibitors (Calbiochem Protease Inhibitor Cocktail Set 111). Completely homogenized samples were centrifuged at 10,000 rpm for 15 min at 4°C to sediment large organelles and the obtained supernatants were centrifuged again at 10,000 rpm for 30 min at 4°C. From the clear supernatants protein concentrations were assessed using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA 94547 USA). The supernatants were used for examination by 1D- and 2D-PAGE.

### 2D-PAGE electrophoresis

Brain homogenates from control and experimental groups were treated with 2D protein clean-up Kit (GE Healthcare, Sweden) according to the manufacturer's instructions and the resulting purified protein pellet was dissolved first in IPG buffer (7M urea, 2M thiourea, 30 mM Tris, 4% CHAPS, 15mM DTT and IPG buffer, pH 3-10 PlusOne, GE Healthcare, Sweden) and then in DeStreak Rehydration Solution (GE Healthcare, Sweden). This mixture was used to rehydrate the immobilized pH gradient (IPG) strips (7 cm DryStrip pH 3-10; GE Healthcare, Sweden) overnight, and IEF electrophoresis was conducted

on Ettan IPGphor 3 IEF System (GE Healthcare, Sweden) according to the manufacturer's instructions. Isoelectric focusing was conducted at 20°C for 5750 Vh at a maximum of 300 V. For the second dimension, the IPG strips were equilibrated consecutively in 65 mM dithiothreitol (DTT) for 15min and then in 190 mM iodoacetamide (IAA), respectively. The IPG strips were then placed on top of a 10% SDS-PAGE (10 cm gels). Gels were run for approx. 2 h, fixed, stained with Coomassie blue R-250 (1-2 h), destained overnight and then scanned.

### SDS-PAGE electrophoresis

For SDS and 2D-PAGE electrophoreses, D,L-dithiothreitol, acrylamide/bis-acrylamide (30% solution) and bromophenol blue sodium salt (Sigma-Aldrich, Germany), N,N,N,N-tetramethylethylenediamine (TEMED) and ammonium persulphate (APS) (GE Healthcare, Sweden) were used. Equal volumes containing approximately 25 µg/lane of the samples, dissolved in Laemmli sample buffer (Tris/HCl pH 6.8, glycerin 20%, SDS 4%, and bromophenol blue 0.02%) and protein standard mixture (SERVA, Germany) were separated by 10% and 12% SDS-PAGE (precast gels SERVAGel™ TG PRiME™) and visualized by staining with Coomassie Brilliant Blue G-250.

### Analyses of proteins after tryptic digestion of spots

A volume of 10 µl digestion buffer (50 mM ammonium bicarbonate, pH 7.8) containing modified trypsin *per* microliter (Promega) was added to the dried gel spots and the tubes were kept on ice for 45 min to allow the gel pieces to be completely soaked with the protease solution. Digestion was performed overnight at 37°C, the supernatants were recovered and the resulting peptides were extracted twice with 35 µl of 60% ACN / 0.1% DIEA. The extracts were pooled and dried in the speedvac. The extracted peptides were redissolved in 10 µl of 0.1% formic acid and matrix solution (a saturated solution of alpha-cyano-4-hydroxycinnamic acid in acetonitrile/water 50:50 with 0.1% TFA, for peptide measurements of <10 kDa; a saturated solution of sinapinic acid in acetonitrile/water 50:50 with 0.1% TFA, for protein measurements >10kDa) and spotted on the MALDI plate.

### Protein identification and validation

Extracted peptides from the gel were analyzed by MALDI-TOF-TOF mass spectrometry on Autoflex™ III, High Performance MALDI-TOF & TOF/TOF Systems (Bruker Daltonics) which use a 200 Hz frequency-tripled Nd-YAG laser operating at a wavelength of 355 nm. Samples were prepared by mixing 5.0 µl of the sample with 5.0 µl matrix solution (7 mg/ml alpha-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN containing 0.1% TFA) and 1 µl of the mixture was spotted on a stainless steel target plate. These were dried at room temperature and analyzed by MS mode and collision energy of 4200 after a total of 3500 shots. A solution of protein standards was used to calibrate the mass scale. The mass spectrometer was externally calibrated with a mixture of angiotensin I, glu-fibrinopeptide B, ACTH (1–17), and ACTH.

### Database searches

Mass spectral data were searched against different protein databases using an in-house MASCOT server (Matrixscience, London, UK). Database SwissProt and NCBIport were used to carry out the Mascot searches. The generally used parameters were: Type of search: Peptide Mass Fingerprint; Enzyme: Trypsin; Taxonomy: Rattus (8036 sequences); Mass values: Monoisotopic Peptide Charge State: 1; Max Missed Cleavages: 1; Peptide Mass Tolerance: ± 2.5 Da; Variable modifications: Phosphorylation on serine (Phospho, ST), threonine

(Phospho, T) and tyrosine (Phospho, Y); Report top: 5 hits. On-line submissions were performed using MS-BLAST at the Heidelberg server (<http://dove.embl-heidelberg.de/Blast2/msblast.html>). FAST ([http://fasta.bioch.virginia.edu/fasta\\_www/cgi/](http://fasta.bioch.virginia.edu/fasta_www/cgi/)) were carried out using standard settings, and searches were performed against the NCBI/BLAST with BLOSUM 50 as search matrix.

## Results and Discussion

Various methodologies have been used to elucidate the causes for the development of Alzheimer's disease (AD). After application of a number of thermodynamic analyzes, the characteristics of purified tau protein from human brain supernatants were investigated. From defined thermodynamic characteristics, such as the equilibrium constant, changes in free energy, enthalpy and entropy that reflect the formation of the tau-DNA complex, it has been shown that binding of unphosphorylated tau protein to DNA is a reversible process [8]. Differential scanning calorimetry is a very sensitive technique by which human plasma is analyzed in patients with breast cancer [21]. Recently, DSC has also been found to be useful in the study of neurodegenerative disorders in an experimental mouse model of scopolamine-induced dementia, type AD. Brain homogenates, isolated from healthy and scopolamine-treated mice, were analyzed, and a well-expressed exothermic transition minimum in the range of 35 - 45°C was determined in the DSC profiles of healthy mice supernatants [18,22,23].

The main objective of the present work was to determine the protein profile and related proteins with exothermic transitions observed at 35°C - 45°C. For this purpose, the changes in the proteomic profile at 45°C of healthy rat brain homogenates and those of the experimental animal model of scopolamine-induced dementia, type AD, were analyzed. There are many published data on tau proteins responsible for neurodegenerative disorders and AD [26-28], and the role of the microtubule tau proteins involved in promoting the assembly and stabilization of microtubules is well known. Predominantly two forms of tau proteins, phosphorylated and non-phosphorylated forms, are mainly found in the cytosol of the cells. In a recent study, significantly increased tau phosphorylation and a number of neurofibrillary tangles within the hippocampus in mice with adeno-associated virus serotype 6 [29] were described. Six tau isoforms were identified in the human brain originating from a combination of 0, 1 or 2 amino-terminal inserts and 3- or 4-microtubule-binding repeats (3Rtau or 4R-tau) in neurotransmitter threads and neurofibrillary units. A gradual increase in 3R-tau percentage in the brain hippocampus has been established, and this preferential accumulation of 3R-tau in the brain stem can be fundamentally associated with the progression of AD [16]. Also, an increase in 3R-tau during the progression of neurofibrillary disease was reported by Uematsu et al., which may be fundamental to the pathogenesis of Alzheimer's disease [17].

This information was used as a basis for the assays of the proteome in healthy rats in comparison to that from an experimental model of scopolamine-induced dementia, type AD. The investigations of extracted proteins from three different parts (hippocampus, striatum and cortex) of rat brain were carried out after washing with ice cold PBS buffer. After homogenization of the samples in medium containing protease inhibitors and centrifugation at 10,000 g for 15 min at 4°C, the supernatants were analyzed. The protein concentrations in the extracted membrane fractions were determined by the Bradford method and the pure supernatants were subjected to various analyses.

Recently published data show great differences between the heat

capacity profiles of water soluble proteins in the brain of healthy animals and animals with scopolamine-induced dementia [22,23]. A well-expressed exothermic process with an extremum at 40-45°C was observed in the brain proteome profile of healthy animals, not observable in the brain proteome of mice after their treatment with scopolamine. In the present study, the behavior of a healthy animal model (rat) and animals with a scopolamine-induced deficiency was investigated to explain the proteomic profile of this low-temperature exothermic process. The experimental animals (rats) were divided into 2 groups: Group 1 - rats treated with scopolamine-induced dementia, type AD; Group 2 - 6 rats without treatment (controls).

## Comparative 2D-PAGE analyzes of rat brain homogenates

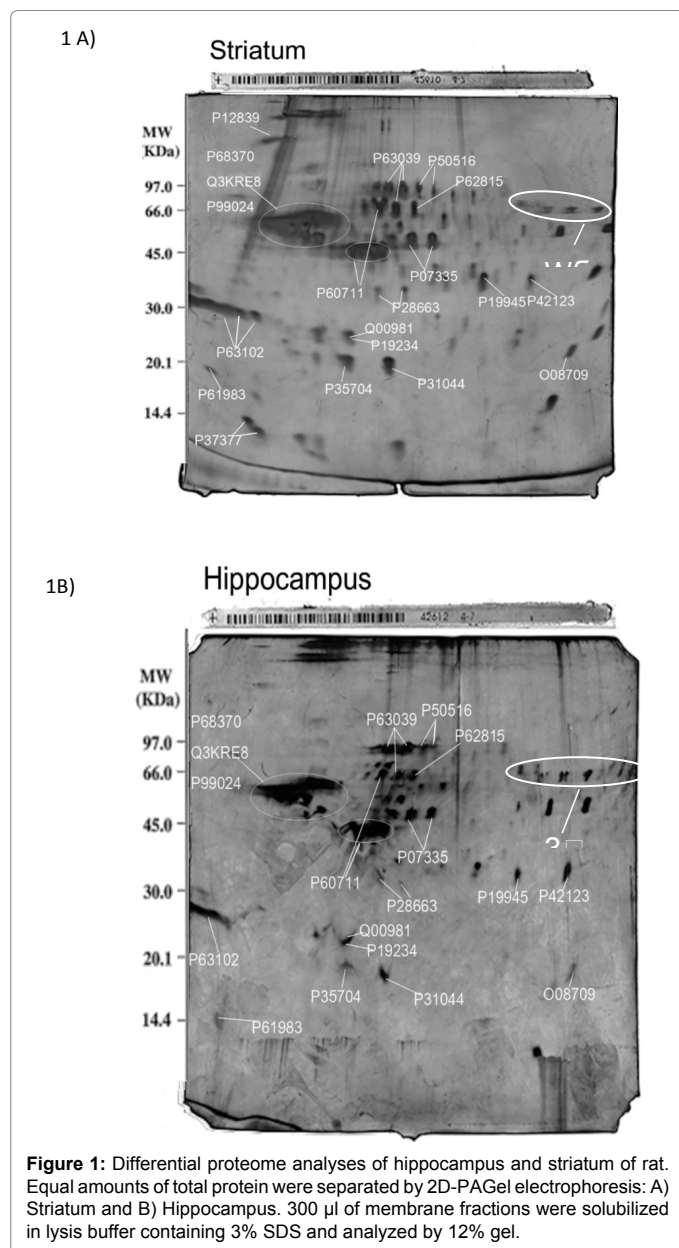
Proteomic analysis and 2D-PAGE are the most widely used techniques for protein determination, available at different concentrations in mixtures. These techniques were also used in the analysis of poorly soluble membrane proteins in rat brain tissue and compared with published results on specific structures of expressed proteins in rat's brain including their function in the central nervous system [2]. As demonstrated from Figure 1, approximately 20 protein stains were detected in the 2D-PAGEs of striatal (Figure 1A and Table 1A) and 18 proteins of hippocampal (Figure 1B and Table 1B) homogenates from healthy rat brains. The observed spots of the two gels after comparative analyses to the presented proteins in the rat brain by Nam et al. [3], 18 proteins in the rat hippocampus were identified. The most important proteins associated with the experimental dementia model were identified as P68370, Q3KRE8, P99024 and P60711, corresponding respectively to tubulin alpha-1A chain, tubulin beta-2B chain, tubulin beta-5 chain and cytoplasmic 1 actin. Two-dimensional analysis demonstrated the presence of Tau proteins in striatal and hippocampal tissues of rat brain. We detected much heterogeneity in isoelectric points and molecular masses of those proteins. Previously experiments indicate that much of the heterogeneity in isoelectric points of the 7 variants of isoforms of MAPs-tau is due to different degrees of phosphorylation among the variants and the more acidic variants are the more phosphorylated ones [30,31].

## Comparative analyses of native and heated rat brain homogenates by 1D-PAGE

After homogenization of brain tissue from hippocampus, striatum and cortex, a comparative 1D-PAGE analysis was performed on samples with and without heating to 45°C. The reason for this study is our assumption that the exothermic process observed by Abarova et al. [22] is associated with a change in the behavior of soluble proteins as a result of the scopolamine-induced dementia. While no such analysis has been conducted on human brain tissue, these data could be correlated to neurodegeneration in general. For the purpose of clarifying the abovementioned reasons, homogenates from three rat brain parts (hippocampus, striatum and nucleus) were analyzed by comparative analysis. After homogenization of the brain tissue, the supernatants were separated into two fractions by 10 kDa membranes. Fraction 1, containing components with Mw <10 kDa, was subjected to additional analysis by MALDI-MS, whereas Fraction 2 (Mw > 10 kDa) was investigated by 1D-PAGE.

The scheme applied for this analysis is shown in Figure 2 and includes: centrifugation of the homogenate at 10,000 rpm and dividing each extract into two samples: one sample was analyzed at 20°C (N) while the other one after heating for 15 min at 45°C(H). Samples N and H were subsequently subjected to proteomic comparison by 1D-PAGE, trypsinolysis of the proteins, mass spectrometric analysis of the





**Figure 1:** Differential proteome analyses of hippocampus and striatum of rat. Equal amounts of total protein were separated by 2D-PAGE electrophoresis: A) Striatum and B) Hippocampus. 300 µl of membrane fractions were solubilized in lysis buffer containing 3% SDS and analyzed by 12% gel.

extracted peptides and identification of the proteins. Figure 3 represent SDS-PAGE electrophoregram of homogenates of hippocampus, striatum and cortex from healthy rat at ambient temperature (N) and heated for 15 min to 45°C (H).

The different behavior of heat capacity profiles of the water soluble protein fractions from three parts of the brain of healthy animals and the observed proteome changes for the hippocampus samples are in coincidence with the published exothermic transition data in mice [18,22,23]. The established exothermic transition may be an evidence of protein aggregation in solution. Therefore, the exothermic transition observed at 40-45°C in the calorimetric thermogram of the mouse brain homogenate can also reflect the aggregation of soluble proteins.

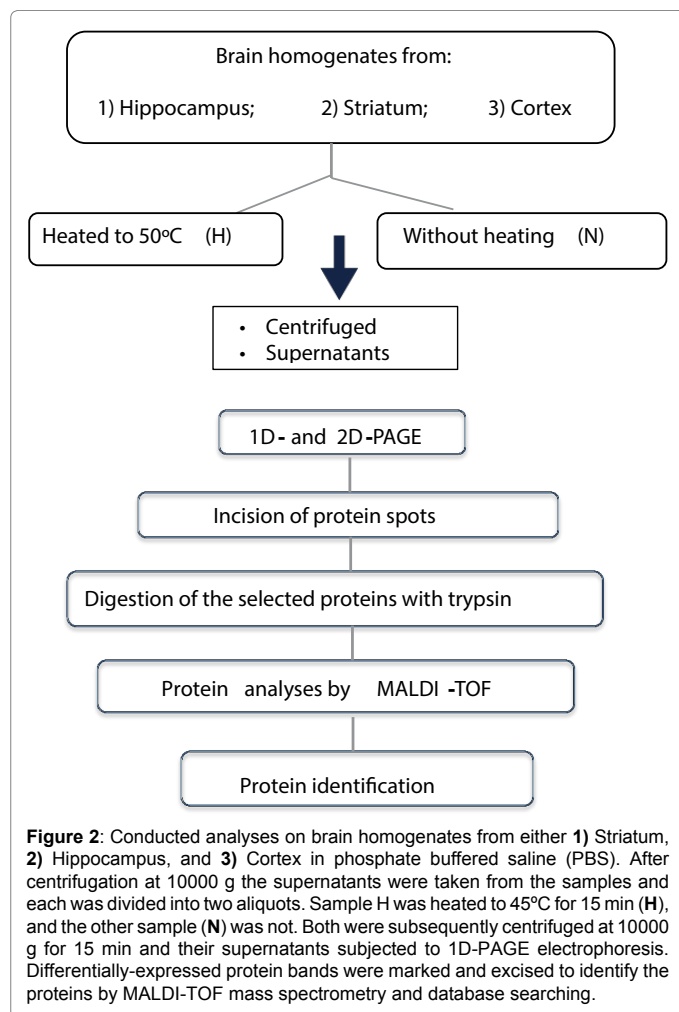
The comparative analysis in Figure 3 shows significant proteome changes in the hippocampal rat homogenate after heating the sample to 45°C. The changes are expressed in the different behavior of the proteins applied to lane 2 (bands 2, 6 and 7) of 1D-PAGE. It is worthwhile

Striatum				
No	Uniprot	Protein name	MW	pI
1	P12839	Neurofilament medium polypeptide (NF-M)	95659	4.77
2	P50516	V-type proton ATPase catalytic subunit A	68326	5.41
3	P63039	60 kDa heat shock protein, mitochondrial precursor	57926	5.35
4	P62815	V-type proton ATPase subunit B, brain isoform	56551	5.57
5	<b>P68370</b>	<b>Tubulin alpha-1A chain</b>	<b>50136</b>	<b>4.94</b>
6	<b>Q3KRE8</b>	<b>Tubulin beta-2B chain</b>	<b>49953</b>	<b>4.78</b>
7	<b>P99024</b>	<b>Tubulin beta-5 chain</b>	<b>49671</b>	<b>4.78</b>
8	P07335	Creatine kinase B-type	42725	5.39
9	<b>P60711</b>	<b>Actin, cytoplasmic 1 (Beta-actin)</b>	<b>41737</b>	<b>5.29</b>
10	P42123	L-lactate dehydrogenase B chain	36481	5.7
11	P19945	60S acidic ribosomal protein P0	34215	5.9
12	P28663	Beta-soluble NSF attachment protein	33557	5.32
13	P63102	14-3-3 protein zeta/delta	27771	4.73
14	O08709	Peroxiredoxin-6	24739	5.72
15	Q00981	Ubiquitin carboxyl-terminal hydrolase isozyme L1 precursor	24568	5.05
16	P19234	NADH dehydrogenase [ubiquinone] flavoprotein 2	23933	5.07
17	P35704	Peroxiredoxin-2	21652	5.34
18	P31044	Phosphatidylethanolamine-binding protein 1	20670	5.48
19	P37377	Alpha-synuclein	14515	4.74
20	P19332*	Microtubule-associated protein tau (heterogeneity)	5500--6600	6.5--8.5

**Table 1A:** Differential proteome analyses of striatum of rat.

Hippocampus				
No	Uniprot	Protein name	MW	pI
1	P50516	V-type proton ATPase catalytic subunit A	68326	5.41
2	P63039	60 kDa heat shock protein, mitochondrial precursor	57926	5.35
3	P62815	V-type proton ATPase subunit B, brain isoform	56551	5.57
4	<b>P68370</b>	<b>Tubulin alpha-1A chain</b>	<b>50136</b>	<b>4.94</b>
5	<b>Q3KRE8</b>	<b>Tubulin beta-2B chain</b>	<b>49953</b>	<b>4.78</b>
6	<b>P99024</b>	<b>Tubulin beta-5 chain</b>	<b>49671</b>	<b>4.78</b>
7	P07335	Creatine kinase B-type	42725	5.39
8	<b>P60711</b>	<b>Actin, cytoplasmic 1 (Beta-actin)</b>	<b>41737</b>	<b>5.29</b>
9	P42123	L-lactate dehydrogenase B chain	36481	5.7
10	P19945	60S acidic ribosomal protein P0	34215	5.9
11	P28663	Beta-soluble NSF attachment protein	33557	5.32
12	P63102	14-3-3 protein zeta/delta	27771	4.73
13	O08709	Peroxiredoxin-6	24739	5.72
14	Q00981	Ubiquitin carboxyl-terminal hydrolase isozyme L1 precursor	24568	5.05
15	P19234	NADH dehydrogenase [ubiquinone] flavoprotein 2	23933	5.07
16	P35704	Peroxiredoxin-2	21652	5.34
17	P31044	Phosphatidylethanolamine-binding protein 1	20670	5.48
18	P19332*	Microtubule-associated protein tau (heterogeneity)	550-6600	6.5--8.5

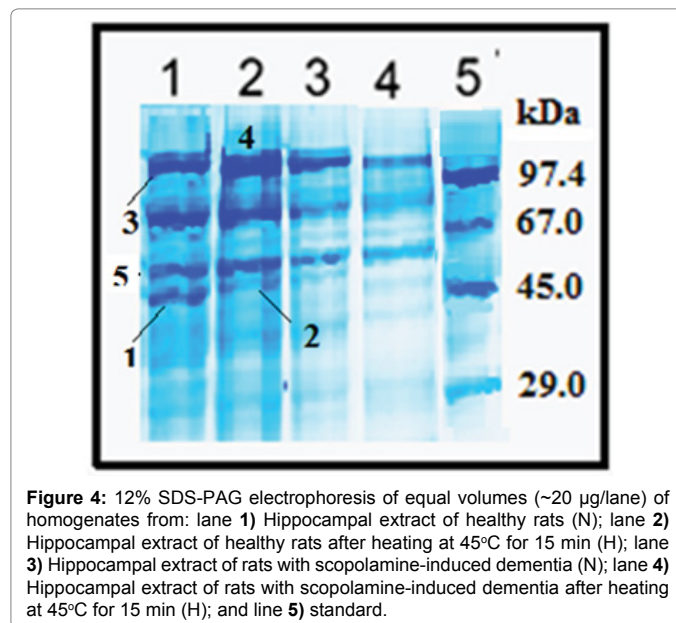
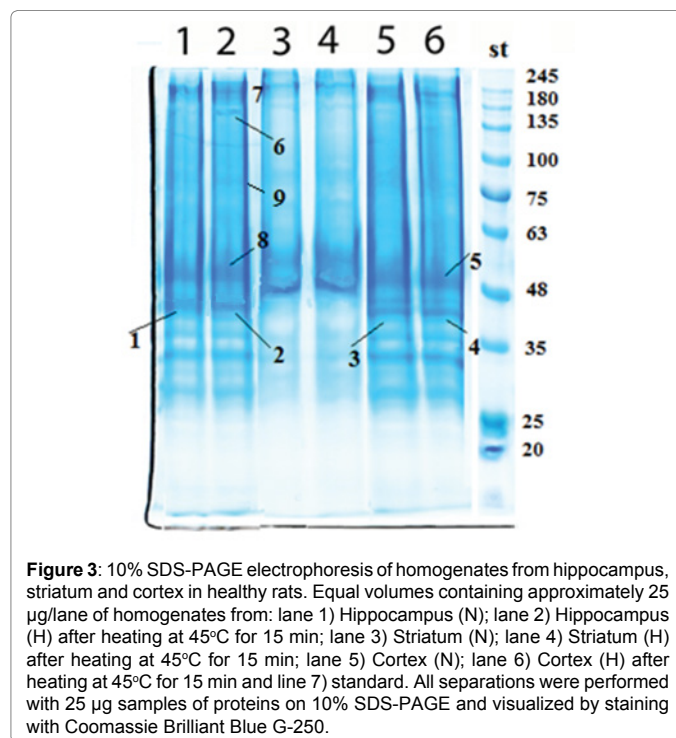
**Table 1B:** Differential proteome analyses of Hippocampus of rat.



mentioning that these studies did not show any changes in the proteome profile of the cortex (lanes 5 and 6; bands 3, 4 and 5). Moreover, the comparative analysis shows a different protein content in the rat brain cortex, compared to striatum and hippocampus (Figure 3).

It is important to note that these results represent the effect of heating of homogenates from three parts of healthy rat brains. The most pronounced effect was observed in the energy profiles of the brain in scopolamine-induced neurodegenerative disorder. The shown differences in the separated proteins on lane 2 (band 2) versus lane 1 (band 1) may correspond to the expression of partially unfolded tau protein after heating to 45°C. These partial conformation changes of tau lead to a breakdown of the tubules and release of tubulin. As a result of these processes, the observed more intensive bands 6 and 7 may be explained with aggregation of the partially-expanded tau protein and the released tubulin into oligomers. The protein fibrillation is reflected by the weaker intensity of band 2 from line 2 (Figure 3).

This hypothesis was additionally confirmed by the comparative analysis of the hippocampal proteome from healthy animals with the hippocampal proteome from animals with scopolamine-induced dementia, type AD. Approximately 20 µg samples of hippocampal extracts were applied on 1D-PAGE, line 1: electrophoregrams of hippocampal extract of healthy rats (N) in comparison to line 3: hippocampal extract of rats with scopolamine-induced dementia (N) (Figure 4). We would like to point out that band 2 in lane 1 and



2 is drastically decreased after heating to 45°C, possibly reflecting a decrease in tau protein due to heating. This reduction in tau protein could be associated with the increased intensity of band 4 compared to band 3, eventually arising from formed oligomers.

To verify this hypothesis, selected bands from the gels were identified after tryptic digestion and mass spectrometry analyses. The excised bands from the gel were washed and after reduction and amidation, the proteins were digested with trypsin. The obtained peptide mixtures were extracted from the gels with 60% acetonitrile and after drying and dissolving in 10 µl of MiliQ water several peptides were identified by MALDI-TOF-TOF. The resulting tryptic peptides

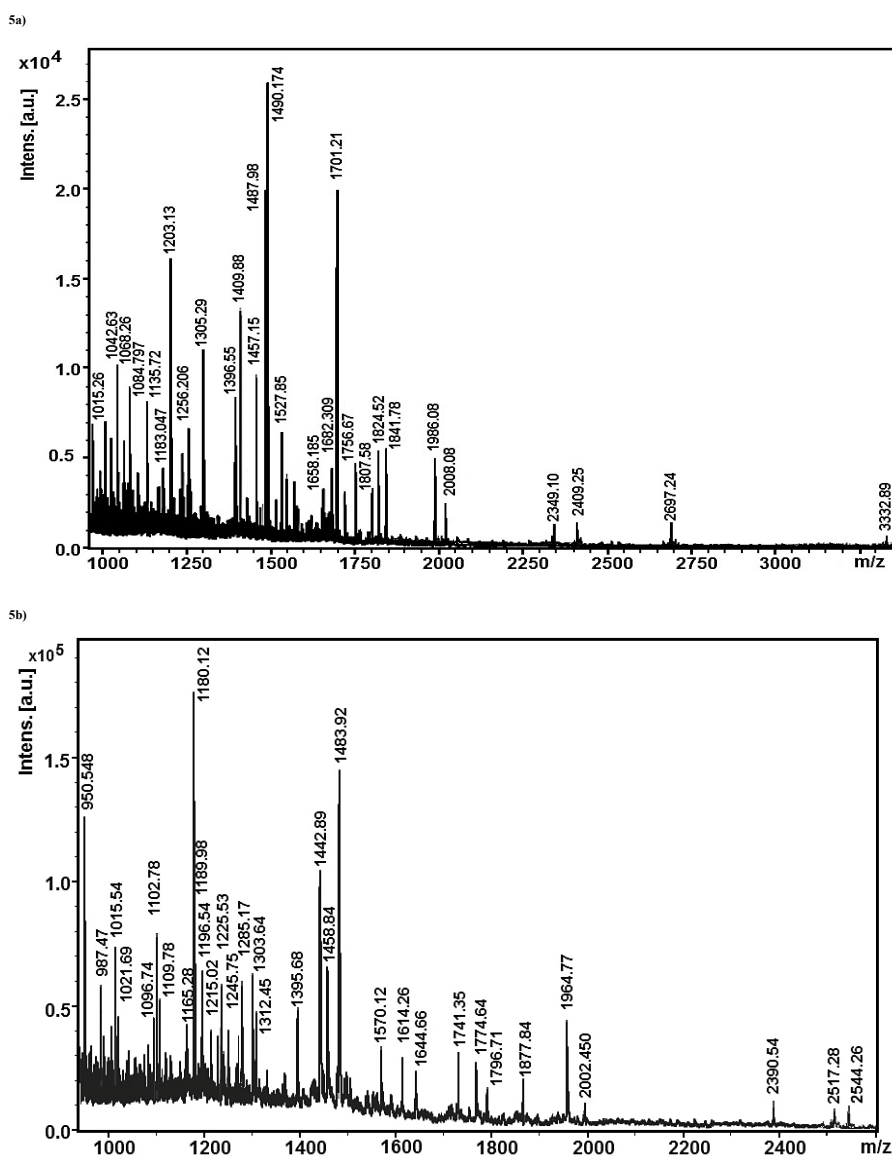
of three proteins are presented by their MS spectra (Figure 5A and B). Following the ions in MALDI-MS spectrum of the hydrolyzed peptides from lane 1, band 1, the microtubulin associated protein tau (MAP) (Figure 5A) was identified. It is involved in microtubule formation with tubulin subunits and also regulates their stability.

Additionally, the MALDI-MS spectrum of peptides from proteins of band 4 and lane 2 correspond to the resulting ions after tryptic digestion of tubulin and some tau protein. These tests indicate the existence of a tau-tubulin complex, identified by MASCOT analysis against protein databases.

The rat striatal analysis performed by 2D-PAGE also represents two spots (1 and 2) corresponding to the tau protein and the tubulin (Figure 1). Mainly tau-C isoform was detected in lane 1, band 1 (Figure 4) and the tau-tubulin complex (isoform tau-B) in lane 1, band 4.

Two groups of 3R tau species were published in the hippocampus of healthy rats with Mr 48–53 kDa (low molecular weight; LMR 3R tau) which predominates on the second with Mr 57–63 kDa (high molecular weight 3R tau) [32]. The total amount of 4R tau with Mr 52–68kDa was significantly higher in the hippocampus. Furthermore, the ratio 7: 1 of the 4R/3R tau isoforms was found in the hippocampus. It was also found that 4R tau isoforms interact more efficiently than 3R tau isoforms with microtubules [32]. Therefore, based on the obtained results we suppose that the same tau isoforms probably participate in the formation a complex with tubulin in scopolamine-induced AD rat.

Published results of mice brain homogenate presented great differences between the heat capacity profiles of water-soluble brain proteins from healthy animals and from animals with scopolamine-induced dementia analyzed by DSC [18,22,23]. The brain profiles in healthy mice show a well-exothermic process reaching a peak at 40-



**Figure 5:** Identification of differentially expressed proteins by MALDI-MS spectra of extracted peptide fractions after trypsinolyses of: A) line 1 / band 1; B) line 2 / band 4.

45°C, but this low-temperature exotherm was completely eliminated in scopolamine-treated mice.

The role of scopolamine generating neurodegenerative disorder in human and mice was presented by several authors [24,25,31]. The effect of scopolamine on the hippocampal proteome profile in healthy rats and after induction of neurodegenerative disorder was also investigated. Significant difference can be noticed on Figure 4 of proteomic profile of hippocampal brain homogenate from healthy animals after scopolamine-induced dementia. These results on 1D-PAGE with equal amounts (~ 20 µg) of hippocampal extracts show the lack of protein on position 3 and 4, which is shown on line 1 (Figure 4).

Another interesting result is that after heating to 45°C changes were observed in the proteomic profile of hippocampal brain with scopolamine-induced dementia in Figure 4 lane 3 with lane 4. This important result corresponds to the published data of DSC analyses on mouse brain homogenate where no low temperature exotherm was observed in scopolamine-treated mice.

## Conclusion

The main observation of this study is the different behavior of the hippocampal proteome from the healthy rats before and after heating to 45°C. Due to the demonstrated change in protein level of tau and tubulin in the rat hippocampus after heating to 45°C, it was suggested that the observed exothermic process at 35–45°C in mice may be due to the partial unfolding of tau protein which leads to the release of tubulin. Both proteins together are involved in protein fibrillation and aggregation.

Another important result is the discovery of different profiles for the proteome of hippocampal rat homogenates with scopolamine-induced neurodegenerative disorder and its characteristics from healthy rats.

The reported results from this study can help clarify the molecular mechanisms of scopolamine-induced dementia and neurodegenerative processes in general.

## Acknowledgements

This research was carried out with the support of a project No. DN 03/13 /2016, funded by the Scientific Research Fund of the Ministry of Education and Science in the Republic of Bulgaria.

## References

1. Van Chi P, Dung NT (2012) 2D-NanoLC-ESI-MS/MS for Separation and Identification of Mouse Brain Membrane Proteins. Book: *Chromatography - The Most Versatile Method of Chemical Analysis*, Chapter 3 published October 24, 2012.
2. Carboni L, Piubelli C, Righetti PG, Jansson B, Domenici E, et al. (2002) Proteomic analysis of rat brain tissue: Comparison of protocols for two-dimensional gel electrophoresis analysis based on different solubilizing agents. *Electrophoresis* 23: 4132-4141.
3. Nam SM, Yoo DY, Kwon HJ, Kim JW, Jung HY, et al. (2017) Proteomic approach to detect changes in hippocampal protein levels in an animal model of type 2 diabetes. *Neurochem Int* 108: 246-253.
4. Zhang F, Su B, Wang C, Siedlak S, Mondragon-Rodriguez S, et al. (2015) Posttranslational modifications of  $\alpha$ -tubulin in Alzheimer disease. *Transl Neurodegener* 4: 9.
5. Vu HT, Akatsu H, Hashizume Y, Setou M, Ikegami K, et al. (2017) Increase in  $\alpha$ -tubulin modifications in the neuronal processes of hippocampal neurons in both kainic acid-induced epileptic seizure and Alzheimer's disease. *Sci Rep* 7: 40205.
6. Jamasbi E, Hossain MA, Tan M, Separovic F, Ciccotosto GD, et al. (2018) Fluorescence imaging of the interaction of amyloid beta 40 peptides with live cells and model membrane. *Biochim Biophys Acta S0005-2736(18)30039-7*.
7. Liebnier S, Dijkhuizen RM, Reiss Y, Plate KH, Agalliu D, et al. (2018) Functional morphology of the blood-brain barrier in health and disease. *Acta Neuropathol* 311-336.
8. Camero S, Benítez MJ, Cuadros R, Hernández F, Avila J, et al. (2014) Thermodynamics of the Interaction between Alzheimer's Disease Related Tau Protein and DNA. *PLoS One* 9: e104690.
9. Janke C, Beck M, Holzer M, Bigl V, Arendt T, et al. (2000) Analysis of the molecular heterogeneity of the microtubule-associated protein tau by two-dimensional electrophoresis and RT-PCR. *Brain Res Protocols* 5: 231-242.
10. Bretteville A, Ando K, Ghestem A, Loyens A, Bégard S, et al. (2009) Two-dimensional electrophoresis of tau mutants reveals specific phosphorylation pattern likely linked to early tau conformational changes. *PLoS One* 4: e4843.
11. Birnbaum JH, Wanner D, Gietl AF, Saake A, Kündig TM, et al. (2018) Oxidative stress and altered mitochondrial protein expression in the absence of amyloid- $\beta$  and tau pathology in iPSC-derived neurons from sporadic Alzheimer's disease patients. *Stem Cell Res* 26 27:121-130.
12. Taylor LM, McMillan PJ, Liachko NF, Strovas TJ, Ghetti B, et al. (2018) Pathological phosphorylation of tau and TDP-43 by TTBK1 and TTBK2 drives neurodegeneration. *Mol Neurodegener* 13: 7.
13. Uversky VN (2018) Intrinsic Disorder, Protein-Protein Interactions, and Disease. *Adv Protein Chem Struct Biol* 110: 85-121.
14. Kadavath H, Hofele RV, Biernat J, Kumar S, Tepper K, et al. (2015) Tau stabilizes microtubules by binding at the interface between tubulin heterodimers. *PNAS* 112: 7501-7506.
15. Janke C, Holzer M, Klose J, Arendt T (1996) Distribution of isoforms of the microtubule-associated protein tau in grey and white matter areas of human brain: a two-dimensional gelelectrophoretic analysis. *FEBS Lett* 379: 222-226.
16. Hasegawa M, Watanabe S, Kondo H, Akiyama H, Mann DMA, et al. (2014) 3R and 4R tau isoforms in paired helical filaments in Alzheimer's disease. *Acta Neuropathol* 127: 303-305.
17. Uematsu M, Nakamura A, Ebashi M, Hirokawa K, Takahashi R, et al. (2018) Brainstem tau pathology in Alzheimer's disease is characterized by increase of three repeat tau and independent of amyloid  $\beta$ . *Acta Neuropathol Commun* 6: 1.
18. Tenchov B, Abarova S, Koynova R, Traikov L, Dragomanova S, et al. (2017) A new approach for investigating neurodegenerative disorders in mice based on DSC. *J Therm Anal Calorim* 127: 483-486.
19. Garbett NC, Miller JJ, Jenson AB, Chaires JB (2008) Calorimetry outside the box: a new window into the plasma proteome. *Biophys J* 94: 1377-1383.
20. Fekecs T, Zapf I, Ferencz A, Lorinczy D (2012) Differential scanning calorimetry (DSC) analysis of human plasma in melanoma patients with or without regional lymphnode metastases. *J Therm Anal Calorim* 108: 149-152.
21. Zapf I, Fekecs T, Ferencz A, Tizedes G, Pavlovics G, et al. (2011) DSC analysis of human plasma in breast cancer patients. *Thermochim Acta* 2: 88-91.
22. Abarova S, Koynova R, Tancheva L, Tenchov B (2017) A novel DSC approach for evaluating protectant drug's efficacy against dementia. *BBA* 1863: 2934-2941.
23. Tenchov B, Abarova S, Koynova R, Traikov L, Tancheva L, et al. (2017) Low-temperature exothermic transitions in brain proteome of mice, effect of scopolamine. *Thermochimica Acta* 650: 26-32.
24. Blokland A (2005) Scopolamine-induced deficits in cognitive performance: A review of animal studies. *Scopolamine Rev* 1-76.
25. Riedel G, Kang SH, Choi DY, Platt B (2009) Scopolamine-induced deficits in social memory in mice: reversal by donepezil. *Behav Brain Res* 204: 217-225.
26. Ciavardelli D, Silvestri E, Del Viscovo A, Bomba M, De Gregorio D, et al. (2010) Alterations of brain and cerebellar proteomes linked to A $\beta$  and tau pathology in a female triple-transgenic murine model of Alzheimer's disease. *Cell Death Dis* 1: e90.
27. Gibbons GS, Banks RA, Kim B, Changolkar L, Riddle DM, et al. (2018) Detection of Alzheimer Disease (AD)-Specific Tau Pathology in AD and NonAD Tauopathies by Immunohistochemistry With Novel Conformation-Selective Tau Antibodies. *J Neuropathol Exp Neurol* 77: 216-218.
28. de Leon MJ, Pirraglia E, Osorio RS, Glodzik L, Saint-Louis L, et al. (2018) The nonlinear relationship between cerebrospinal fluid A $\beta$ 42 and tau in preclinical Alzheimer's disease. *PLoS One* 13: e0191240.



29. Platt TL, Reeves V L, Murphy MP (2013) Transgenic models of Alzheimer's disease: Better utilization of existing models through viral transgenesis. *Biochim Biophys Acta* 9: 1437-1448.
30. Butler M, Shelanski ML (1986) Microheterogeneity of microtubule-associated tau proteins is due to differences in phosphorylation. *J Neurochem.* 47: 1517-1522.
31. Butterfield DA, Boyd-Kimball D, Castegna A (2003) Proteomics in Alzheimer's disease: insights into potential mechanisms of neurodegeneration. *J Neurochem* 6: 1313-1327.
32. Hanes J, Zilka N, Bartkova M, Caletkova M, Dobrota D, et al. (2009) Rat tau proteome consists of six tau isoforms: implication for animal models of human tauopathies. *J Neurochem* 108: 1167-1176.