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Immunocytochemical Localization of the 26S Proteasome Complex in Neurodegenerative Disorders and Controls

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Abstract: ***Background:*** ***A functional ubiquitin proteasome system (UPS) is essential for all eukaryotic cells and therefore any alteration to its components has potential pathological consequences. Aging and neurodegeneration are often accompanied by a functionally impaired ubiquitin–proteasome system (UPS). The UPS orquestrates the turnover of innumerable proteins. Deregulation of the UPS has been implicated in the pathogenesis of many human diseases, cancer and neurodegenerative disorders. Methods: Immunocyto chemistry with Peroxydase-anti-Peroxydase.We used postmortem material coming from patients suffering of tauo-and synucleinopathies. Young and old controls were included in all the experiments. Results: In the present study we tested a series of 59 antibodies. These were raised against different parts of the 26S proteasome complex.17 antibodies against the beta-subunitsand show cytoplasmic localisation. 12 antibodies against the alpha subunits. They gave nuclear staining. 20 antibodies against 19S regulator ATPase-dependent subunits and 7 antibodies against 19S regulator non-ATPase-dependent subunits. Only 2 subunits show strong immunoreactivity. From the 11S regulator 3 antibodies were used. We didn't find siginificant results from these antibodies. Young controlsshow no immunoreactivity, but old controls show some staining. Conclusions: Only some subunits have shown net differences with controls. These are the ATPase subunit S6b, the 20S α5, α6 and the 20S β6 –subunits. As we see different cellular localization have been found in our investigations.***

Keywords: Ubiquitin-Proteasome-System, Tauopathies, Synucleinopathies

Abbreviations: **AD**, Alzheimer's disease; **AGD**, argyrophylic grain disease; **DS**, Down syndrome; FTD, frontotemporal dementia; LB, Levy bodies; LBD, Lewy body disease; **MC1**, antibody against aberrant Tau; **MSA**, multiple system atrophy; **PiD**, Pick's disease; **PSP**, progressive supranuclear palsy; **UPS**, ubiquitin-proteasome system; **S6b**, 19S regulator ATPase subunit 6b.

1. Introduction

The accumulation of aberrant proteins is a common pathogenic mechanism in several neurodegenerative disorders. The degradation of aberrant or misfolded proteins contributes to a healthy intracellular environment [1]. Degradation of the misfolded proteins can be executed by several proteolytic systems, like lysosomal degradation, chaperone-mediated autophagy, and the ubiquitin-proteasome system (UPS), the focus of this study. The ubiquitin-proteasomal system (UPS) is very essential in the degradation of misfolded proteins and its failing contributes to protein accumulation. Therefore, in the present study we screened an array of antibodies against the different proteasomal subunits (e.g.alpha, beta of the 20S core,19S regulator, and 11S regulator). The UPS removes damaged or misfolded proteins by ubiquitinating them via ubiquitin ligases and then targeting these ubiquitinated proteins to the proteasome for degradation [2; 3]. Normal UPS function is particularly important for preventing diseases that are caused by misfolded proteins. The majority of cellular proteins are degraded by the UPS, which consists of both substrate-recruiting and substrate-degrading machinery. The first one is composed of three enzymes, the first of which (E1) activates the polypeptide ubiquitin in an ATP-dependent manner, enabling its transfer onto a ubiquitin carrier enzyme (E2). Activated ubiquitin is further transferred by a ubiquitin protein ligase (E3) to a substrate protein. The substrate-recruiting machinery then catalyses the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin and the γ-amino group of a substrate protein lysine residue. Repeated addition of ubiquitin moieties onto the first results in a polyubiquitinated substrate protein that is recognized by the proteolytic machinery of the 26S proteasome. The 26S proteasome is a large, multisubunit complex containing a common proteolytic core, the 20S proteasome, which is composed of 28 subunits arranged in four, heptameric rings. The two outer rings are each composed of alpha-type subunits (alpha1-alpha7), while the two inner rings each contains seven beta-type subunits (beta1-β7). The proteolytic activity is enclosed within the inner rings, with only the beta1, beta2, and beta5 subunits possessing caspase-like, tyrosine-like, and chymotrypsine-like cleavage specificity, respectively [4; 5]. The activity of the 20S proteasome is modilated by a variety of regulators, including the 19S/PA700, as well as PA28 alpha/beta and PA28gama [6; 7]. The most common regulator, the 19S/PA700 complex, contains six AAA-family ATPases and is capable of binding both each of the 20S proteasome in an ATP-dependent manner, forming the 26S proteasome, which is involved in the degradation of ubiquitinated proteins.

Therefore, in the present study we screened an array of antibodies against the different proteasomal subunits (e.g.alpha, beta of the 20S core, 19S regulator, and 11S regulator).It appeared that several antibodies result in a consistent staining. In particular an antibody against the 19S regulator ATPase subunit S6b appeared to label the neuropathological hallmarks of AD, Down syndrome and aged non-demented controls, whereas in young controls no

immunoreactivity was found. In addition two of the 20S alpha subunits (alpha5 and alpha6) were localized in the nucleus, whereas beta6 (20S beta subunits) has been found exclusively in the cytoplasm. In other forms of tauopathies (Pick's disease, frontotemporal dementia, progressive supranuclear palsy and argyrophilic grains disease), the specific hallmarks (e.g., Pick bodies) were labeled as well. In synucleinopathies (Lewy body disease and Multiple system atrophy), the degree of labeling was much less clear.

The availability of well-characterized antibodies reactive with many proteasomal subunits allows us to study the proteasome subunits distribution in human postmortem tissue coming from patients suffering of different type of neurodegenerative diseases. Different proteasome subunits localization are shown.

2. Materials and methods

2.1 Autopsy material

Postmortem material of the major types of tauopathies and synucleinopathies was obtained from various sources (laboratory of pathology Oost Nederland, Enschede, Netherland Brain bank, Amsterdam). We were able to identify pure forms of each type of neurological disorder by neurohistological prescreenings (e.g., silver stains, Abeta and tau immunostains) exluding cases that showed a combination of different neuropathological entities, such as LBD and AD (see ref. [8]).

2.2 Antibodies

The antibodies against the subunits of the 26S proteasome complex were obtained generously from Affiniti Research Products Ltd, Mamhead Castle, Mamhead, Exeter, EX6 8HD, United Kingdom (now BIOMOL International LP, UK; see Table 2). Their method of origination, analysis and determination of specificity has been reported (all antibodies have been screened by Western blot using human erythrocyte-derived purified 20S/26S proteasome and on a Hela S3 S1000 cytosolic preparation, for details see **www.biomol.com**).

2.3 Immunostaining

6µm thick sections were deparaffined by immersing them at room temperature in two changes of xylene for 15 min, and two changes of 100% ethanol for 15 min each, and once in 96% ethanol, 90% ethanol, 80% ethanol, 70% ethanol, 60% ethanol, and 50% ethanol, respectively, for 5 min each. Sections were treated for 30 min in 98-100% formic acid on a rocking table to enhance staining and washed in tap water for 30 min and TBS for 15 min. They were then exposed to monoclonal anti-proteasome subunits, or polyclonal anti-proteasome subunits. Normal mouse (aberrant hyperphosphorylated tau, MC1, 1:100), or rabbit antibody against wt-ubiquitin(DAKO, Z 0458, 1:1000) were used as positive controls.

Sections were incubated with the first antibodies for one hour at room temperature followed by incubation overnight at 4oC. Sections were subsequently washed two times (15 min each) in TBS pH7.6. They were incubated for one hour at room temperature with secondary antibodies (anti-mouse IgG coupled to peroxydase, 1:100,for monoclonal antibodies, or goat anti-rabbit, Akon, 1:100, for polyclonal antibodies).

In the case of polyclonal antibodies, the peroxydase-anti-peroxydase step (1:1000) was added. The sections were then washed three times (10 min each) and developed in TBS, pH7.6 containing 0.05% of 3,3`-diaminobenzidine (DAB) and 0.01% H2O2 and Nickel ammonium-sulfate. After 5 to 20 min, sections were washed in distilled water and dehydrated in graded ethanols, cleared in xylene and cover slipped.

3. Results

In the present study we tested a series of 59 antibodies (see table1). These were raised against different parts of the 26S proteasome complex.

In the present study 17 antibodies were raised against the beta-subunits (see table I; one should keep in mind that sometimes many antibodies are raised against the same subunit).A total of 12 antibodies were raised against these subunits (see table 1). We have raised 20 antibodies against 19S regulator ATPase-dependent subunits and 7 antibodies against 19S regulator non-ATPase-dependent subunits. Another kind of cap, that binds to the core (20S), is the 11S regulator (known as PA28). Its main function is to open the channel through the complex, but this process is ATP-independent, and mediates the degradation of non-ubiquitinated short peptides. Three antibodies were used in the present study (see table 1). Among the 59 antibodies investigated, only 16 showed immunoreactivity to specific proteasome subunits. The stained subunits were found to be localized in different cell compartments (table 2).20S beta-subunits were localized in the cytoplasm. From the beta-subunits of the 20S core we selected a set of 5 subunits that give strong immunoreactivity in CA3 and CA4 areas of the hippocampus. Beta6-subunit appears to be down-regulated in AD, DS (Fig. 1, a; c). The results observed within the controls show some immunoreactivities as is seen in (Fig. 1 b; d). However, 3 subunits (beta1i, beta5i and beta2i) give good immunoreactivity but without difference to controls (data not shown).From the 20S alpha-subunits, 2 have shown strong immunoreactivity within CA1 and CA3 of the hippoampus from AD and DS patients. These are alpha5, alpha6. Their loclization was found exclusively within the nucleus (Fig. 2, a; b for alpha5 and fig.3 a; b for alpha6). Non-demented controls showed no immunoreactivity (Fig. 2, c and fig.3, c).From the 19S (Cap) subunits, two were very immunoreactive within AD (fig. 4, a and DS patients (fig.4, b).Young and old controls did not show any positive reactions (Fig. 4, c). The reaction is within the cytoplasm as shown in table 2. AD hallmarks such as neurofibrillary tangles and neuropil threads were strongly stained.

4. Discussion

The UPS plays an important role in maintaining neuronal homeostasis by regulating a variety of processes and by serving as a protein quality control mechanism to rid the cell

of aberrant or misfolded proteins. A number of studies have demonstrated that impairment of proteasome function is associated with cellular senescence. However, the available data are fragmented and contradictory [9; 10; 11]. Previous studies [12] have reported ATPase S6b (MS73) association with neuropathological hallmarks (NFTs, Lewy bodies). Studies from Carrard et al. (2003)[13] have reported that in vivo age-related decreased proteasome activity in lymphocytes is not due to a lower proteasome content but may be related to specific modifications of the three categories of the 26S proteasome subunits. Based on Carrard investigations, 19S regulatory complexe subunits are only weakly modified. From our results, ATPase S6b was strongly immunoreactive in tauopathies but to a much lower degree in synucleinopathies. Young non-demented controls did not show any positive reaction. In contrast, older controls showed some neuropathological hallmarks (e.g. NFTs).

In this study we used paraffin material coming from patients suffering of tau- and synucleinopathies. Many brain areas have been investigated (see materials and methods). Using the improved, more sensitive, protocols, we showed that in AD and DS patients the pyramidal cells of the hippocampus (CA1 and CA3) were the most positive areas.We compared the staining properties of proteasomal subunit specific antisera. Whereas the 20S beta subunit, 11S regulator (subunit PA 28 or immunoproteasome) and 19S regulator subunit antisera produced cytoplasmic staining, the 20S alpha subunit antisera resulted only in nuclear labeling, a difference in localization for which no explanation is available thus far.

Our data showed for the first time a different localization of the 26S proteasome subunits within the cell compartment. Previous studies have reported an age-related reduction of proteasome activity that results in the attenuation of both the activity and the amount of the 26S proteasome, which is associated with impaired assembly of the 26S proteasome from the 19S RP and the 20S proteasome in vivo [14]. The assembly of the 26S proteasome is known to be ATP dependent. We therefore, suppose that an age-associated decline in ATP levels could be the reason of the disassembly of the 26S proteasome. The assembly of the proteasome depends also on extraproteasomal proteins. This holds for Hsp90, a chaperone that promotes the assembly of the 26S proteasome. Therefore, it is possible that these proteins are affected by age and cause the disassembly of the 26S proteasome and its impairment.

Our results demonstrate a different subcellular localization of the 26S proteasome subunits that could be the key to its dysfunction and the accumulation of misfolded proteins and the onset of the neurodegeneration.

Results of the present study support the idea that the UPS can be considered as the major system responsible for proteolysis in tauopathies. However, ubiquitin- independent proteolytic functions of the proteasome have been reported in tauopathies as well [15; 16]. Mildly oxidatively modified proteins, which are a relatively poor substrate for ubiquitin, are degraded by the proteasome. Also DJ-1 protein, suggested to be an anti-oxidant and protecting neurons from abnormally aggregated proteins, has been localized in a subset of tau inclusions of neuronal and glial cells in various forms of tauopathies [17]. The same holds for the synucleinopathies, where it has been suggested that in Parkinson's disease alpha-synuclein, present in Lewy bodies, is mono- and diubiquitinated [18]. As the proteasome needs a chain with a minimum of four ubiquitin molecules for efficient recognition, there must be an ubiquitin-independent pathway in synucleinopathies [19]. Oxidized proteins may also be degraded by this route, as has been suggested for a sporadic form of Parkinson's disease, where mutations in the DJ-1 gene were shown [20]. In agreement with this report is the finding that alpha-synuclein, as an unfolded protein, can be directed to the proteasome for proteolysis in a UPS-independent way [21].

Presently, it is not fully understood if malfunction of the UPS is a result of the neurodegeneration or it is an initial factor in disease onset. This issue is further complicated by the fact that many disease-related proteins are also ubiquitin-modified in a non diseased state to exert their normal function or to regulate their half-life.

5. Conclusions and Perspectives

The upregulation of proteasome subunits in different cellular compartments (nucleus and cytoplasm) can be an indicator for a dysfunctional activity of the proteasome. Further studies are needed to clarify whether 26S proteasome impairment is linked to disassembly of its subunits. Although our data suggest that proteasomal subunits are differently distributed within the cell compartments, there is still much more work to do to understand what determines the different distribution of proteasome subunits. It is tempting to speculate that the 26S proteasome is not inhibited in all categories. 19S complex(one of its subunits, the ATPase S6b) appears to be up-regulated mainly in the tauopathies. This regulatory complex is certainly required for ATP and ubiquitin-dependent protein degradation. Further analysis of the 26S categories (alpha- and beta-subunits) will be an interesting step of investigations towards an understanding of neuropathological-related proteasome dysfunction.

In the present study we found different localizations of the proteasome subunits within the cell compartments (cytoplasm or nucleus). Therefore, the proteasome complex is truly impaired in neurodegenerative diseases or is the primary cause present elsewhere? The current advancements towards resolving the fundamental mechanisms of proteasomal degradation in vivo, using the increasing diversity of UPS model systems, will contribute to understanding the role of the UPS in neurodegenerative disorders. Further investigations are needed to elucidate if there are means by which altering the UPS with inhibitors (chemical compounds) or by silencing specific components of the UPS machinery, might alter disease progression in a favorable way.

As we already knew there is another way to degrade misfolded proteins in the CNS, the autophagy pathway. It is a major cellular process by which cytplasmic components, including organelles, are catabolized [22;23]. Although

autophagy is a distinct degradation system from the UPS, recent study suggests that there is a cross- talk between the two systems [24]. When one system is impaired (e.g., the UPS), the other is activated. In this regard we address the question if both systems are impaired in the same time as the aberrant proteins aggregate? Given the importance of the two systems, future studies are required to understand their way they function.

6. Future Scope

Our future project is to study the major pathways implicated in the process that eliminate misfolded proteins. The UPS was partially studied (Zouambia et al., 2008). My proposal is to continue these studies by focusing on the UPS escort pathway proteins and the Autophagic Lysosomal Pathway (ALP). Autophagy is defined as the homeostatic delivery of macronutrients and organelles to the lysosome for pH-dependent degradation, and has been shown to play an important role in responding to intracellular energy demands and maintaining energy balance. To date, three major types of autophagy have been described: macroautophagy, whereby bulk cytoplasm and organelles are enclosed within vesicles and are delivered via a series of vesicular fusion events to the lysosome for degradation by lysosomal hydrolases that function optimally at low pH; chaperone mediated autophagy (CMA), whereby proteins with "KFERQ" motifs are selectively shuttled via molecular chaperones including hsc70 to lysosomes, where lysosomal membrane-bound receptors (Lamp2a) selectively internalize these proteins into the lumen of lysosomes for degradation; and microautophagy, a process of direct nutrient uptake by lysosomal membranes. Recent evidence suggests that the degradation of mitochondria by macroautophagy, termed "mitophagy", may be a more selective form of macroautophagy and has been implicated in neurodegenerative disease.

7. Competing Interests

The authors have declared that no competing interests exist.

8. Acknowledgments

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Figures Legend

Figure 1: Immunostaining of beta subunits (20S beta subunits).
(a) Hippocampus from AD patient (#96332) and (c) from DS patient (#25002), note the absence of staining with PW9000, an antibody against beta6 subunit of the 20S core, (b) and (d) Hippocampus from control (#90202), light cytoplasmic staining is shown (arrows).

Figure 2: Immunostaining of alpha5 subunits (20S alpha subunits).
(a) Hippocampus from AD patient (#88252) (b) fromDS patient (#94146), note the nuclear staining with PW8125, an antibody against alpha5 subunits of the 20S proteasome core. (c) Hippocampus from control (#89004). No staining was seen in the control.

Figure 3: Immunostaining of alpha6 subunits (20S alpha subunits).
(a) Hippocampus from AD patient (#89166), some nuclear stainings are visiblewith PW8100 (Arrows), antibody against alpha6 subunit of the 20S core. No staining was obtained in DS patient (#94146H (b) and control (#1126H) (c).
(c) With PW8100.

Figure 4: Immunostaining ATPase S6b subunit (19S regulator)
(a) Hippocampus from AD patient (#99139) and (b) DS patient (#95325) stained with PW8175 (anti-ATPase 6b of the 19S cap). Note the stained NTFs (Arrows). (b) Hippocampus from control (#89004). No staining was showed.
(c) Hippocampus from control (#89004). No staining is visible

Table 1: Antibodies

Antibodies to 19S Regulator ATPase subunits

Specificity	Type	Product Code
Subunit Rpt2	rabbit pAb	PW8160
Subunit Rpt1	rabbit pAb	PW8165
Subunit Rpt5	rabbit pAb	PW8170
Subunit Rpt3	rabbit pAb	PW8175
Subunit Rpt6	rabbit pAb	PW8215
Subunit Rpt4	rabbit pAb	PW8220
Subunit Rpt5	rabbit pAb	PW8245
Subunit Rpt3	rabbit pAb	PW8250
Subunit Rpt1	rabbit pAb	PW8255
Subunit Rpt2	rabbit pAb	PW8260
Subunit Rpt2	rabbit pAb	PW8305
Subunit Rpt5	rabbit pAb	PW8310
Subunit Rpt1	rabbit pAb	PW8315
Subunit Rpt6	rabbit pAb	PW8320
Subunit Rpt5	rabbit pAb	PW8375
Subunit Rpt3	mAb	PW8765
Subunit Rpt5	mAb	PW8770
Subunit Rpt1	mAb	PW8825
Subunit Rpt4	mAb	PW8830
Subunit Rpt6	mAb	PW9265

Antibodies to 19S Regulator non-ATPase subunits

Specificity	Type	Product Code
Subunit Rpn8	sheep pAb	PW8180
Subunit Rpn7	rabbit pAb	PW8225
Subunit Rpn6	rabbit pAb	PW8370
Subunit Rpn12	rabbit pAb	PW8815
Subunit Rpn10	mAb	PW9250
Subunit Rpn12	mAb	PW9260
Subunit Rpn1	mAb	PW9270

Antibodies to 11S Regulator subunits

Specificity	Type	Product Code
Subunit PA28a	rabbit pAb	PW8185
Subunit PA28g	rabbit pAb	PW8190
Subunit PA28b	rabbit pAb	PW8240

Antibodies to 20S Proteasome -subunits

Specificity	Type	Product Code
Subunit a	mAb	PW8100
Subunit a	mAb	PW8105
Subunit a7	mAb	PW8110
Subunit a3	mAb	PW8115
Subunit a4	mAb	PW8120
Subunit al5	mAb	PW8125
Subunit a5/a7, b1, b5, b5i, b7	rabbit pub	PW8155
Subunits a, 2, 3, 5, 6&7	mAb	PW8195
a-subunit (specificity unknown)	mAb	PW8265
Subunit a6	mAb	PW8270
a-subunit (specificity unknown)	mAb	PW8275
a-subunit (specificity unknown)	mAb	PW8280

Antibodies to 20S Proteasome –subunits

Specificity	Type	Product Code
Subunit b3	mAb	PW8130
Subunitb7	mAb	PW8135
Subunit b1	mAb	PW8140
Subunit b2	mAb	PW8145
Subunit b2i	rabbit pAb	PW8150
Subunit a5/a7, b1, b5, b5i, b7	rabbit pAb	PW8155
Subunit b5i	rabbit pAb	PW8200
Subunit b1i	mAb	PW8205
Subunit b2/b2i	rabbit pAb	PW8210
Subunit b1i	rabbit pAb	PW8345
Subunitb2i	rabbit pAb	PW8350
Subunit b5i	rabbit pAb	PW8355
Subunit b1i	mAb	PW8840
Subunit b5i	mAb	PW8845
Subunit b4	rabbit pAb	PW8890
Subunit b5	rabbit pAb	PW8895
Subunitb6	rabbit pAb	PW9000

Note: a= alpha, b = beta and g = gama.

Table 2: Specific localization of Proteasome subunits

Proteasome Complexes	Intracellular Localization	
	nucleus	cytoplasm
19S subunits		
ATPase S6b (Rpt3) [a,g]		+++
Non-ATPase (Rpn8) [a]		++
ATPase (Rpt3)		++
ATPase (Rpt5) [c]		+++
11S subunits		
PA28g [c]		+++
PA28b [c]		+++
20Salpha-subunits		
a6-subunit	++	
a5-subunit [b]	+++	
a5/a7,b1, b5, b5i,b7 [c]		+++
a1,2,3,5,6&7 [c]	+++	
20S beta-subunits		
b1-subunit [c]		+++
b2i-subunit [c]		+++
a5/a7,b1, b5,b5i, b7		++
b2/b2i-subunit		++
b6-subunit [c]		+++

++: moderate staining; +++: intense staining ;(a): neurofibrillary tangles, plaque neurites , neuropil threads, Pick bodies and Lewy bodies; (b): staining in patients and old controls; (c): stainings in patients and controls (including young controls).

Note: a = alpha, b = beta and g = gama

Figure 1

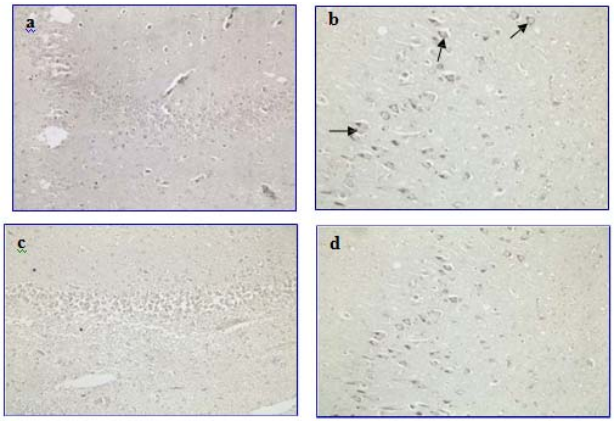


Figure 2

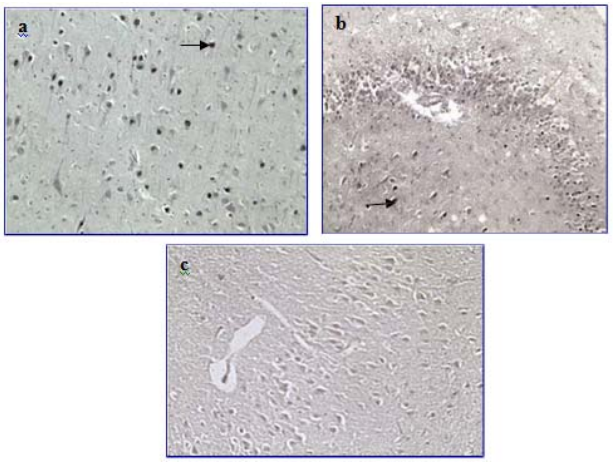


Figure 3

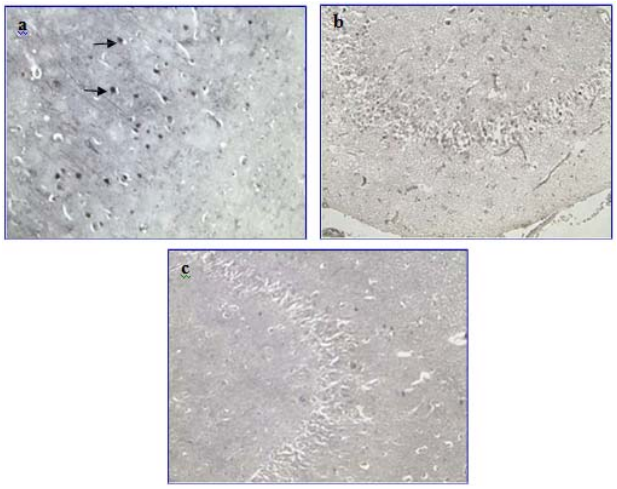
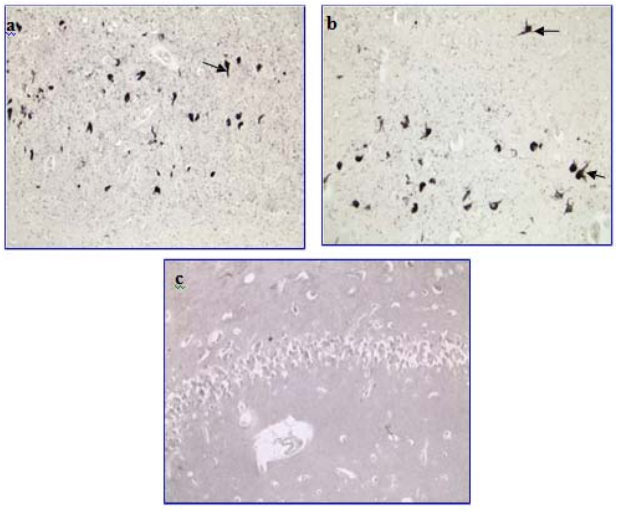


Figure 4



Author Profile

Dr Zouambia Mohamed is a teacher-researcher at the University of Science and technology of Algiers. I have been working in this University since 1981. I got my master degree in 1986 on the following project: Study of the cholinergic system, its implication in water and food intakes. This research work was done in collaboration with a French laboratory. In 2007 I defended my PhD thesis. The field of research was: Study of the neurodegenerative diseases. This research was undertaken in collaboration with "The Netherlands Institute of Neuroscience". At present I have developed a laboratory working on the same project.