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13. ABSTRACT (Maximum 200 Words) The cause of Parkinson's disease (PD) is not known but the pattern of neurodegeneration can be replicated by the systemic administration of the neurotoxin 1-methyl-4-phenyl-tetrahydropyridine (MPTP). MPTP inhibits mitochondrial oxidative phosphorylation and causes oxidative injury leading to cell death. Neurons that degenerate in PD develop inclusions called Lewy bodies that are composed of aggregates of a synaptic protein, alpha synuclein. The purpose of this study is to determine how MPTP affects cytoskeletal and synaptic proteins and to study the relationship between oxidative damage and the formation of synuclein aggregates within neurons. We have shown that primates and mice develop alpha synuclein and ubiquitin immunoreactive aggregates in degenerating neurons 7-10 days after MPTP administration. Degenerating neurons are identified using dopamine transporter, and calbindin immunocytochemistry and glial reaction is identified with glial acidic fibrillary protein. Synaptophysin and neurofilament immunoreactivity are not strikingly altered. The neurodegenerative process is associated with increased levels of oxidative markers for DNA, protein and lipids as indicated by immunocytochemistry for 8-hydroxydeoxyguanosine, 3-nitrotyrosine and malondialdehyde respectively. Over the next year we plan to complete time course studies and complete studies to define the cytoskeletal, synaptic and oxidative changes associated with synuclein aggregation.				
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Introduction

The cause of Parkinson's disease (PD) is not known but the pattern of neurodegeneration found in PD can be replicated in some animal species, including primates and mice, by the systemic administration of the neurotoxin 1-methyl-4-phenyl-tetrahydropyridine (MPTP). MPTP inhibits mitochondrial oxidative phosphorylation and causes oxidative injury leading to cell death. Neurons that degenerate in PD develop characteristic inclusions called Lewy bodies that are composed of aggregates of a synaptic protein, alpha synuclein. The purpose of this study is to determine how MPTP affects cytoskeletal and synaptic proteins and to study the relationship between oxidative damage and the formation of synuclein aggregates within neurons. Two important studies showed that overexpression of mutant forms of synuclein associated with familial PD can lead to aggregate formation in both transgenic mice (1) and fruit flies (2). New research reported in the last 18 months suggests that overexpression of alpha synuclein in nigral neurons may not be sufficient to cause neurodegeneration (3). Other research, however, suggests that protein aggregation is associated with oxidative injury (4) and that alpha synuclein can induce neuronal death (5). Indeed oxidative stress may be a key factor causing synuclein aggregation (6). This new data supports the continued relevance of our proposed studies. This annual report updates our progress in the second year of this three-year funding cycle. In the first year of our study we showed that both acute and chronic MPTP treatment, which cause nigral dopaminergic neurons to degenerate, are associated with the displacement of alpha synuclein from its normal synaptic location into neuronal cell bodies. Neuronal degeneration was evident with DAT and calbindin immunocytochemistry and glial reaction was evident with GFAP immunocytochemistry. We also found that the redistribution of synuclein is associated with increased ubiquitin immunoreactivity and increased levels of oxidative markers in the substantia nigra and that the redistribution of synuclein does not

appear to be associated with changes in distribution of synaptophysin or neurofilament proteins. In the second year of the study we continued to make progress in accomplishing the experiments outlined in the Statement of Work as described below.

Body

We proposed two series of experiments in the approved Statement of Work. The first series of experiments were designed to define changes in the distribution and morphology of alpha synuclein immunoreactivity produced by systemic treatment of MPTP in mice. Both the time course of these changes and their relationship to synaptic (synaptophysin) and neurofilament proteins (NF-M) are being studied. The second series of experiments focuses on spatial and temporal relationships between synuclein aggregation and oxidative injury at the cellular level. Patterns of cell death and apoptosis associated with MPTP toxicity are to be determined and related to the changes in synuclein and oxidative damage. Changes in the regional and cellular distribution of oxidative markers (3-nitrotyrosine, 8-hydroxydeoxyguanosine (8OHdG), and malondialdehyde (MDA)), apoptosis (activated caspase 3 (Idun)), and stress response (ubiquitin) are being examined with respect to synuclein in the substantia nigra and striatum of mice treated with MPTP. In the first year of funding we completed the first series of experiments (series 1) on 72 adult male C57BL mice treated with intraperitoneal MPTP followed by sacrifice after a 7-10 day survival period. In the second year we further explored the process of synuclein aggregation by testing four unique synuclein antibodies in different mouse strains and we completed the second set of experiments (series 2) on mice treated with intraperitoneal MPTP followed by sacrifice after a 7-10 day survival period. In year three we will complete the time course and double labeling studies proposed in the approved Statement of Work.

As in year 1, two different MPTP toxicity models were tested. In addition three different mouse strains (C57BL/6, B6CBA and B6SJL) were used and the pattern of synuclein immunoreactivity was studied using four unique alpha-synuclein antibodies. Studies using oxidative markers (3-nitrotyrosine, 8-hydroxydeoxyguanosine (8OHdG), nitrotyrosine (NT) and malondialdehyde (MDA) and stress response (ubiquitin) were studied. Histological results are summarized in the color photographs in the appendix. For the "acute" model 88 day old mice were given a single IP injection of 20 mg/kg MPTP every 2 hours until symptoms appeared. Animals were given five injections of MPTP on day one and four injections on day two, a total of 9 injections of MPTP. Control mice were given an equivalent volume of PBS according to the same schedule. The mice were perfused with paraformaldehyde 11 days after the last injection at a final age of 100 days. Serial sections of the brains were cut @ 50um into 8 wells. With this acute regimen, the mice were mildly symptomatic after the first day of five injections. It was not until the second day, at the time of the 8th injection that they were very symptomatic. For the "chronic" model, 88-day-old mice were given daily IP injections of 30 mg/kg MPTP. Control mice were given an equivalent volume of PBS at the same time (12PM daily). This regimen continued for 10 days. The mice were perfused 11 days after the last injection at the age of 108 days. Serial sections of their brains were also cut @ 50um into 8 wells. With this chronic regimen mice show little or no symptomatology for the first three days. On the fourth day, one hour after the injection, they became lethargic for 90 to 120 minutes. This behavioral response recurred daily after each injection. By the sixth day, the animals developed quickened respirations and hyperactivity immediately after the injection that lasted 15-20 min. This pattern of behavior also continued until the final injection.

The extent of MPTP-induced neurodegeneration is defined immunocytochemically using a monoclonal antibody against the dopamine transporter (DAT). In our hands this is a very

reliable method to define dopaminergic neurons and their projections. In both the acute and chronic MPTP treated animal, there is a clear reduction in the intensity of immunoreactivity in the striatum that is more severe in the caudal and dorsal aspects of the striatum. There is also depletion of neurons in the substantia nigra, especially in the middle third of the nigra (A8 field) with relative sparing of the medial ventral tegmental area (A10). Individual DAT positive neurons show dendritic and axonal pruning and fragmentation and distortion of immunoreactive processes.

The extent of the lesion produced by MPTP varies from animal to animal and differed between strains. The C57BL/6 was most resistant, B6CBA was intermediate and B6SJL was most sensitive. In year one we quantitated groups of 5 B6CBA animals treated with either chronic or acute MPTP versus controls. In year two we increased sample size and confirmed that our MPTP protocol results in a 15% reduction in DAT-positive neuronal number but the variability within groups remained high. The C57BL6 animals were more resistant to the effects of MPTP toxicity than B6CBA mice and lesion extent was smaller and more variable. Quantitation of MPTP toxicity in B6SJL mice, however, showed a greater mean reduction in DAT-positive neurons and less variability within individual groups.

Many of the histological observations reported in year one have been confirmed on larger numbers of cases and with new antibodies. In the acute and chronic MPTP lesioned nigra the number and intensity of GFAP-positive astrocytes is clearly increased. In both the acute and chronic MPTP lesioned animals there is striking depletion of calbindin immunoreactivity. The loss of calbindin and DAT immunoreactive neurons is associated with increased synuclein immunoreactivity in both the acute and chronic MPTP models. Four anti-alpha synuclein antibodies were tested (Zymed, Chemicon, Affinity, and courtesy of D. Clayton). Quantitative analysis using all antibodies showed a striking increase in synuclein positive cell

bodies after MPTP treatment. Similar changes are seen with ubiquitin immunocytochemistry. A few ubiquitin positive cellular profiles are seen in the control substantia nigra. In the acute and chronic MPTP lesions there is a clear increase in the number of ubiquitin positive profiles. In contrast to the striking changes seen with alpha synuclein and ubiquitin staining, the staining pattern of synaptophysin, a synaptic protein, and neurofilament (medium chain), a marker of cell bodies and dendrites, changes minimally.

A series of 40 MPTP-treated mice were studied for evidence of oxidative injury. Markers of oxidative damage, such as 8-hydroxydeoxyguanosine, a marker of DNA oxidation, were clearly increased in neurons in the substantia nigra of MPTP-treated animals after 7-10 day survivals. Examples of histology are included in the appendix materials.

Because in situ methods to detect DNA fragmentation have been shown to not be specific for apoptosis, we used a novel immunocytochemical marker to detect apoptosis: activated caspase 3 antibody (Idun). As shown in the appendix, increased immunoreactivity is detected in the substantia nigra of MPTP treated mice, consistent with activation of apoptotic pathways.

Key Research Accomplishments:

- 1) The redistribution of alpha synuclein from its normal synaptic location into neuronal cell bodies caused by MPTP treatment was confirmed in three mouse strains (C57BL/6, B6CBA and B6SJL) using four different anti-alpha synuclein antibodies. Previous observation made in year one showing that neuronal degeneration is evident with DAT and calbindin immunocytochemistry and that glial reaction is evident with GFAP immunocytochemistry were confirmed in larger sample sizes.

- 2) The redistribution of alpha synuclein is associated with increased ubiquitin immunoreactivity and increased levels of malondialdehyde (MDA), 3-nitrotyrosine (NT) and 8-hydroxydeoxyguanosine immunoreactivity in the substantia nigra, but changes in distribution of synaptophysin or neurofilament protein do not occur. This suggests that a general disruption of neuronal polarity affecting synapses or dendrites is not produced by MPTP toxicity.
- 3) The localization of activated caspase 3 immunoreactivity to neurons in the substantia nigra suggests that apoptotic pathways are activated by MPTP.

Reportable Outcomes

- 1) An abstract was published in 2001 and a manuscript is in preparation for submission.
- 2) Presentations were made at the American Association of Neuropathologists in June 2001 and at the Workshop on DoD sponsored Parkinson's research in March 2001.
- 3) A database of histological materials has been enlarged and a large number of specimens have been added to our tissue bank and catalogued. These will be available for future research.
- 4) Two postdoctoral fellows and two technicians have been trained in surgical and histological procedures and have gained experience in the laboratory supported by this award

Conclusions

MPTP treated mice develop alpha synuclein aggregates in neurons that are degenerating in the substantia nigra 7-10 days after MPTP administration in both the acute and

chronic MPTP models in three different strains of mice using four different synuclein antibodies. Degenerating neurons are identified using dopamine transporter and calbindin immunocytochemistry and glial reaction is identified with glial acidic fibrillary protein. Synaptophysin and neurofilament immunoreactivity are not altered suggesting that MPTP toxicity may have a specific effect on alpha synuclein. The neurodegenerative process is associated with increased levels of oxidative markers for DNA, protein and lipids as indicated by immunocytochemistry for 8-hydroxydeoxyguanosine, 3-nitrotyrosine and malondialdehyde respectively. Ubiquitin immunoreactivity is also prominent in degenerating neurons. In addition activated caspase 3 is detected in degenerating neurons suggesting that apoptotic pathways are activated. Our observations validate the MPTP model of PD by demonstrating that MPTP causes synuclein aggregation in degenerating neurons even though classical Lewy bodies are not produced. In year three we will conclude our time course studies and perform double labeling to confirm the colocalization of oxidative markers and alpha synuclein in degenerating neurons. Our findings suggest that therapeutic strategies targeted at interfering with the process of synuclein aggregation that we have demonstrated may lead to novel therapeutic approaches to the treatment of PD.

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- 2) Feany MB, Bender WW. A *Drosophila* model of Parkinson's disease. *Nature* 2000;404:394-8
- 3) Matsuoka Y, Vila M, Lincoln S, McCormack A, Picciano M, LaFrancois J, Yu X, Dickson D, Langston WJ, McGowan E, Farrer M, Hardy J, Duff K, Przedborski S, Di Monte DA. Lack of

nigral pathology in transgenic mice expressing human alpha-synuclein driven by the tyrosine hydroxylase promoter. *Neurobiol Dis.* 2001;8:535-9.

- 4) Butterfield DA, Kanski J. Brain protein oxidation in age-related neurodegenerative disorders that are associated with aggregated proteins. *Mech Ageing Dev.* 2001;122:945-62.
- 5) Saha AR, Ninkina NN, Hanger DP, Anderton BH, Davies AM, Buchman VL. Induction of neuronal death by alpha-synuclein. *Eur J Neurosci.* 2000;12:3073-7.
- 6) Souza JM, Giasson BI, Chen Q, Lee VM, Ischiropoulos H. Dityrosine cross-linking promotes formation of stable alpha -synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J Bio Chem.* 2000;275:18344-9.

Appendices

Abstracts:

- 1) Mahoney SC, Ferrante RJ, Dedeoglu A, McKee AC, Kowall NW. Alpha Synuclein is selectively redistributed in the substantia nigra of MPTP treated mice. *J Neuropathology Exptl Neurology* 2001; 60:549.
- 2) Kowall NW. Alpha Synuclein in a neurotoxic model of Parkinson's disease. Workshop on DoD-Sponsored Parkinson's Related Research. March 2001, page 73.

Color Photographs (set of 8):

- 1) Overview of histology of the substantia nigra
- 2) Dopamine transporter: control vs MPTP
- 3) Alpha synuclein: control vs MPTP
- 4) Ubiquitin: control vs MPTP
- 5) Neurofilament-M: control vs MPTP
- 6) Synaptophysin: control vs MPTP
- 7) Oxidative markers in MPTP treated substantia nigra
- 8) Apoptosis and stress markers in MPTP treated substantia nigra

Alpha Synuclein Aggregation in a Neurotoxic Model of Parkinson's Disease

Neil Kowall
Boston University School of Medicine

The central role that α -synuclein plays in the pathogenesis of Parkinson's disease (PD) is becoming clear. α -Synuclein mutations cause familial PD and α -synuclein is a major constituent of the Lewy body (LB), the pathological hallmark of PD. The cause of PD is not known but the neurotoxin 1-methyl-4-phenyl-tetrahydropyridine (MPTP) causes parkinsonism in animals and humans. The relationship between oxidative damage and α -synuclein aggregation is unknown. We hypothesize that processes causing oxidative stress lead to displacement and aggregation of α -synuclein, and interaction with neurofilaments. This "pre-Lewy body stage begins in the axons as α -synuclein is displaced from synapses and involves the cell body where accumulated α -synuclein and neurofilaments are ubiquitinated to form a true Lewy body. Cytoskeletal and synaptic dysfunction due to re-localization of α -synuclein and ubiquitination of α -synuclein/neurofilament aggregates triggers apoptotic cell death. This hypothesis will be tested by assessing the redistribution and morphological features of α -synuclein immunoreactivity produced by treatment of mice with MPTP and by defining the spatial and temporal relationship between α -synuclein aggregation and oxidative injury. Our preliminary in vivo data suggests that systemic administration of MPTP causes α -synuclein to accumulate within neuronal cell bodies in the substantia nigra of rodents and monkeys. This process is associated with evidence of oxidative injury and cell death in the substantia nigra. The experiment we propose will help define the relationship among key molecules relevant to the pathogenesis of PD and provide a basis of future studies to ameliorate the process of cell death in PD.

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CAUSES OF DEMENTIA IN MARACAIBO, VENEZUELA: A REAPPRAISAL. JJ Cardozo*, DP Cardozo, GD Lizardo, OM Molina. School of Medicine, University of Zulia, Maracaibo, Venezuela.

In a previous report communicated in 1991 we analyzed the causes of dementia in an autopsy population from several Hospitals of Maracaibo, Venezuela. Our results showed that vascular dementia (VD) accounted for 86.7% of all clinically diagnosed demented patients, whereas no cases of Alzheimer's disease (AD) were evidenced. Nine years later we undertook a similar study in order to re-evaluate the frequency of the different causes of dementia in our country. A total of 813 adult brains obtained from autopsies performed during the period 1992-2000 were studied. 55 of these came from clinically demented patients. VD was diagnosed in 46 cases (83.6%), 7 cases (12.7%) showed specific neuropathological features for dementing disorders other than VD or AD, and in 2 cases (3.6%) the neuropathological changes were specific for AD. The current results confirm the conclusion of our previous studies and contrast with the vast majority of reports in the medical literature regarding the frequency of the different causes of dementia, with the exception of studies originated in different Chinese, Japanese and Russian cities. At present further investigations are in progress in order to obtain possible explanations for these differences.

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PHYSIOLOGIC AND PATHOLOGIC EFFECTS OF WILD-TYPE AND MUTATED HUMAN ALPHA-SYNUCLEIN IN TRANSGENIC MICE. EK Richfield*, MJ Thiruchelvam, DA Cory-Slechta, C Wuertzer, RR Gainetdinov, MG Caron, and HJ Federoff, University of Rochester School of Medicine and Dentistry, Rochester, NY and Duke University Medical Center, Durham, NC.

The human alpha-synuclein (halpha-SYN) gene product is implicated in the Parkinson's disease phenotype (PDP), although its mechanism remains unknown. A role for halpha-SYN in the PDP is based on the presence of two mutations associated with familial forms of the disease, the presence of aggregates of the protein in vulnerable neurons, and the increased risk associated with a polymorphism in the 3' untranslated region of the gene. We generated transgenic mice expressing either wild-type (halpha-SYN) or a doubly-mutated (hm2alpha-SYN) form of halpha-SYN under control of the rat tyrosine hydroxylase promoter. Both transgenic lines had an increased density of the dopamine transporter (DAT) in the striatum, enhanced toxicity to low doses of the dopaminergic neurotoxin MPTP, and altered locomotor responses to amphetamine. Adult halpha-SYN-5 transgenic mice had morphologically unremarkable dopamine (DA) axons and terminals, normal age-related measures on two motor screens and normal measures of DA and its metabolites in striatum. Whereas, adult hm2alpha-SYN-39 transgenic mice had morphologically abnormal axons and terminals, age-related impairment on two motor screens and reductions in DA and its metabolites in striatum. These results suggest that halpha-SYN is functional in DA terminals, acts in part through the DAT, and that hm2alpha-SYN leads to a PDP by altering the integrity and homeostasis of DAergic terminals.

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ALPHA SYNUCLEIN IS SELECTIVELY REDISTRIBUTED IN THE SUBSTANTIA NIGRA OF MPTP-TREATED MICE. SC Mahoney, RJ Ferrante*, A Dedeoglu, AC McKee*, NW Kowall*. GRECC, Bedford VAMC and Boston University, Bedford MA.

The pattern of neurodegeneration in Parkinson's disease (PD) can be replicated by the systemic administration of 1-methyl-4-phenyl-tetrahydropyridine (MPTP). Neurons that degenerate in PD develop characteristic inclusions, Lewy bodies, composed of aggregates of alpha synuclein, a synaptic protein. We studied the relationship between MPTP neurotoxicity and the distribution of cytoskeletal and synaptic proteins using immunocytochemistry to localize alpha synuclein, ubiquitin (UBI), neurofilament (NF-M), and synaptophysin. Two MPTP protocols were used: an acute protocol (20 mg/kg q 2h for a total of 9 injections over 2 days) and a chronic protocol (30mg/kg qd x 10 days). Animals were sacrificed 11 days after their last injection. Quantitation of dopamine transporter (DAT) neurons showed that MPTP-induced neurodegeneration varied from animal to animal but was similar with both protocols. DAT positive striatal terminals were most severely depleted in the caudal striatum; the ventral striatum was relatively spared. In control animals, alpha synuclein was largely confined to terminal-like staining, much like synaptophysin. NF-M antibody primarily labeled dendrites and some cell bodies. In MPTP treated animals, alpha synuclein was found in neuronal perikarya, especially in animals with more severe neuronal loss. UBI positive neurons were increased to a lesser degree. Synaptophysin and NF-M staining were reduced in areas of neuronal loss but were otherwise unchanged from controls. Our findings show that MPTP neurotoxicity is accompanied by a selective redistribution of alpha synuclein from synapses to neuronal cell bodies reproducing some of the key features of PD.

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TWO NONGLYCOSYLATED PRION PROTEIN (PrP) ISOFORMS COEXIST WITHIN THE SAME MICROREGION IN THE BRAIN OF SOME INDIVIDUALS WITH SPORADIC CREUTZFELDT-JAKOB DISEASE (sCJD). P. Piccardo*, A. William*, S.R. Dlouhy*, M. Takao*, B. Ghetti*. Indiana University School of Medicine, Indianapolis, IN.

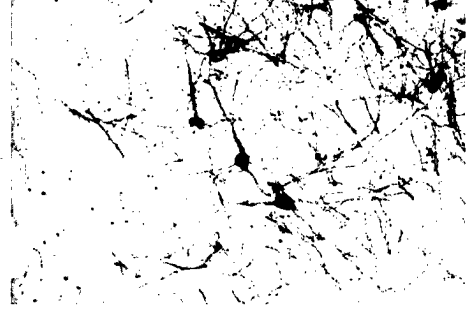
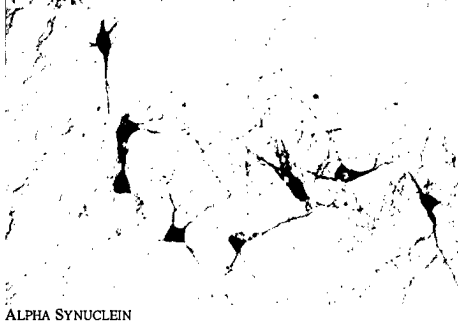
Previous studies have shown that patients with CJD present one of two types of proteinase K resistant prion protein (PrPres) (type 1 and type 2) in brain tissue. The nonglycosylated species of type 1 and type 2 PrPres have a size of ca. 21 and 19 kDa, respectively. Recent studies have shown that PrPres types 1 and 2 may coexist in the brain of patients with CJD. Both PrPres types have been seen in the temporal lobe, lateral geniculate nucleus, and hypothalamus. In order to carry out immunoblot analysis, we removed 50 mg samples from various brain regions of 31 deceased individuals affected with sCJD. The immunoblot analysis was carried out using the anti-PrP antibody 3F4. In three of the individuals, we found that both the ca. 21 and 19 kDa bands appear on immunoblots of a single 50 mg sample. It is important to note that these three patients were heterozygous (M/V) at residue 129 of PrP. A comparison of immunoblots obtained from various anatomical brain regions showed differences in the intensity of the ca. 21 and 19 kDa bands of PrPres. A prominent ca. 21 kDa and a weak 19 kDa band are seen in the caudate nucleus, while a weak ca. 21 kDa and a prominent 19 kDa band are seen in the neocortex, amygdala and cerebellar cortex. These results show that two different nonglycosylated prion protein isoforms may not only coexist in one individual with sCJD, but also may coexist within the same microregion of the cerebrum and cerebellum. This study was supported by PHS R01 NS29822, P30 AG10133.

Histology of the Substantia Nigra (High Power)

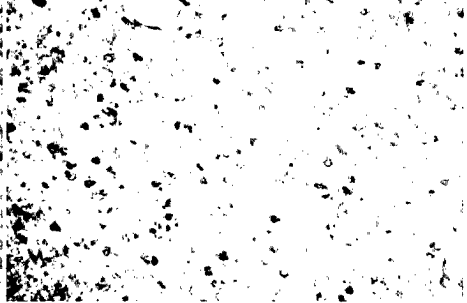
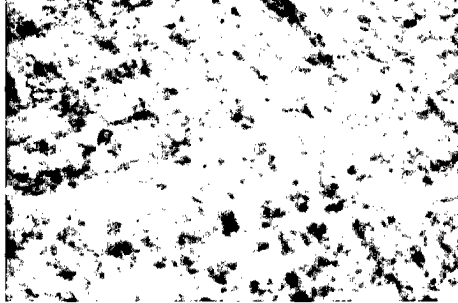
DOPAMINE TRANSPORTER Control

Acute MPTP

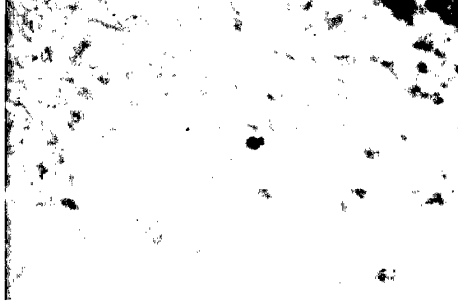
Chronic MPTP



ALPHA SYNUCLEIN



UBIQUITIN



SYNAPTOPHYSIN



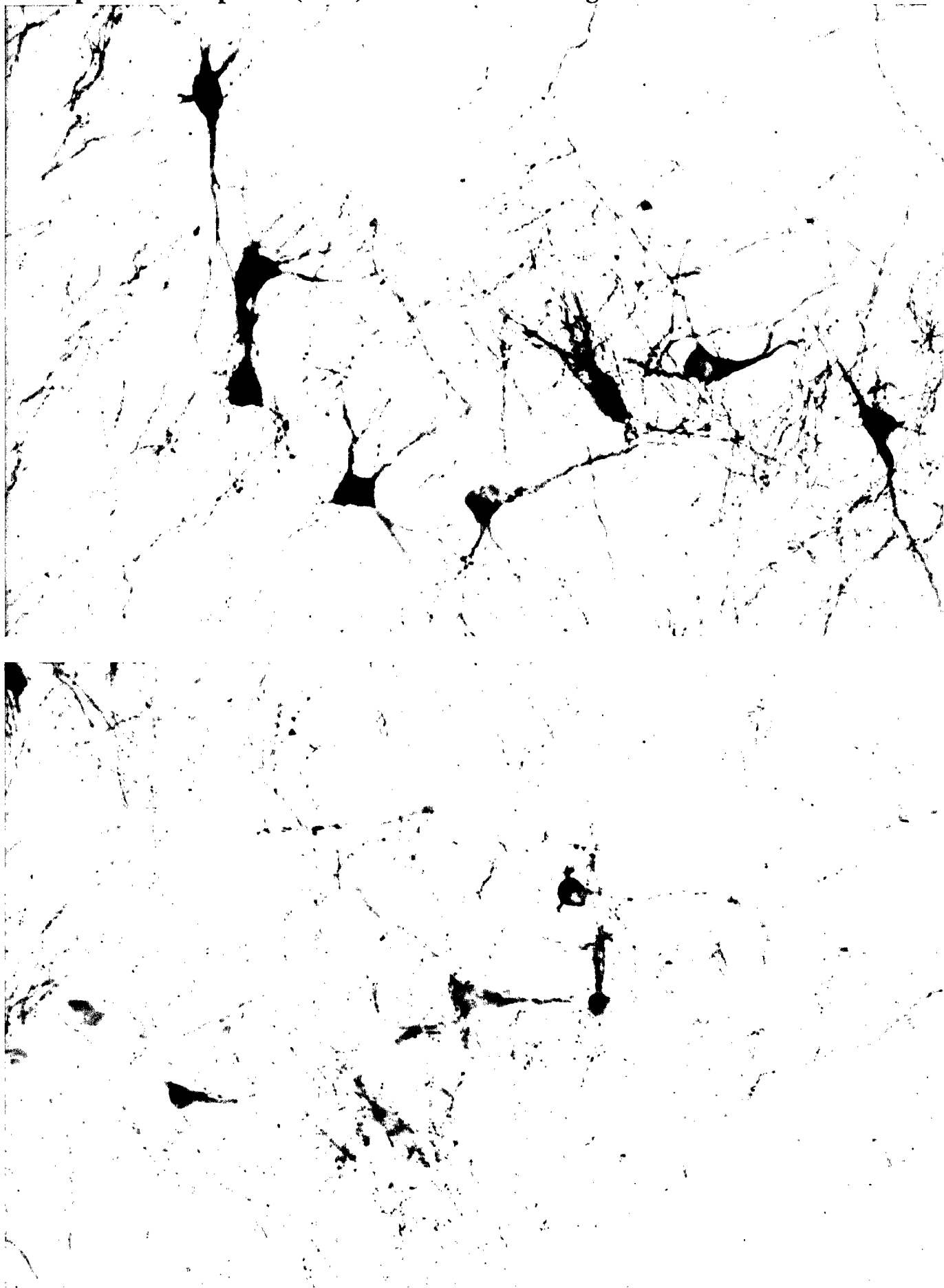
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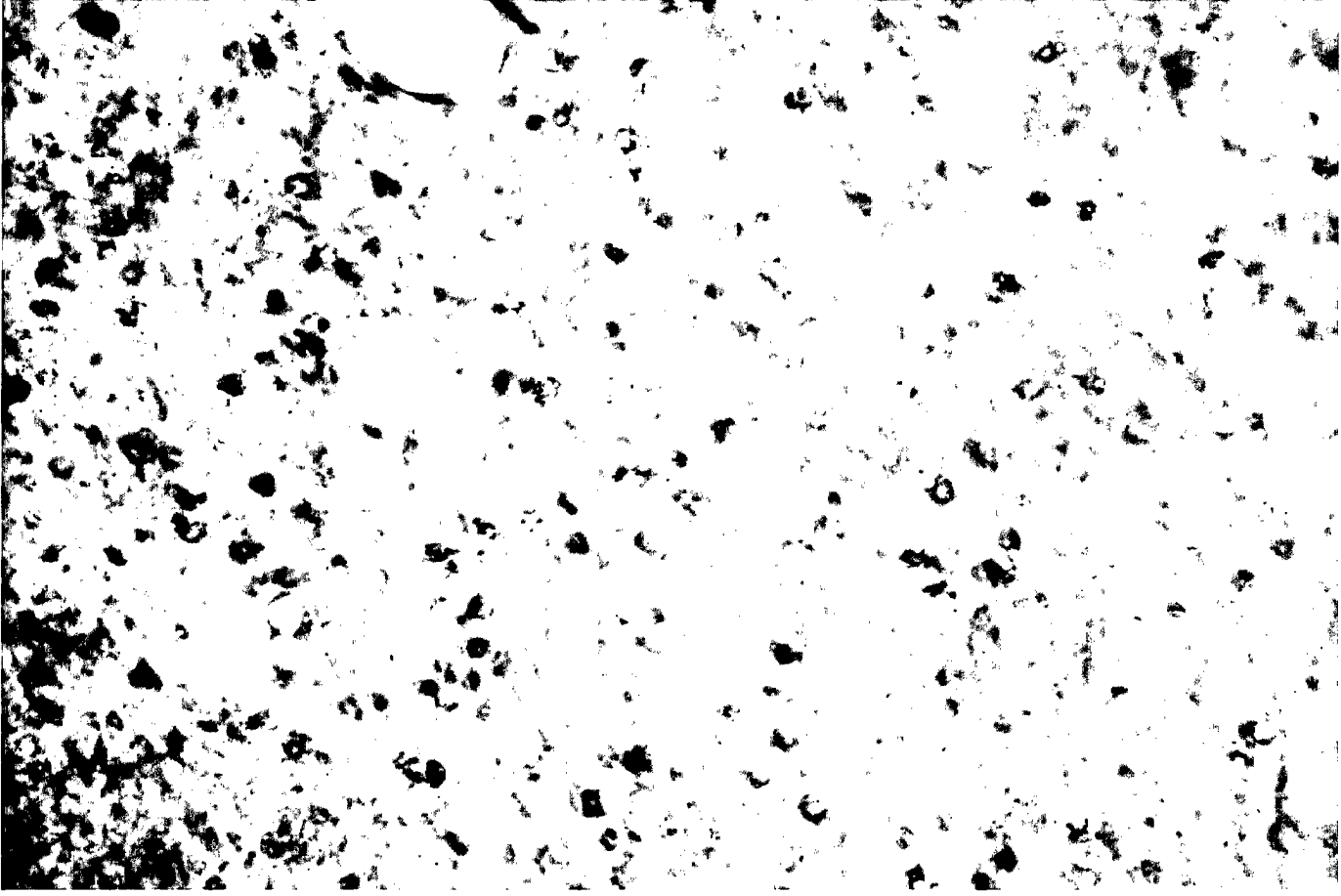
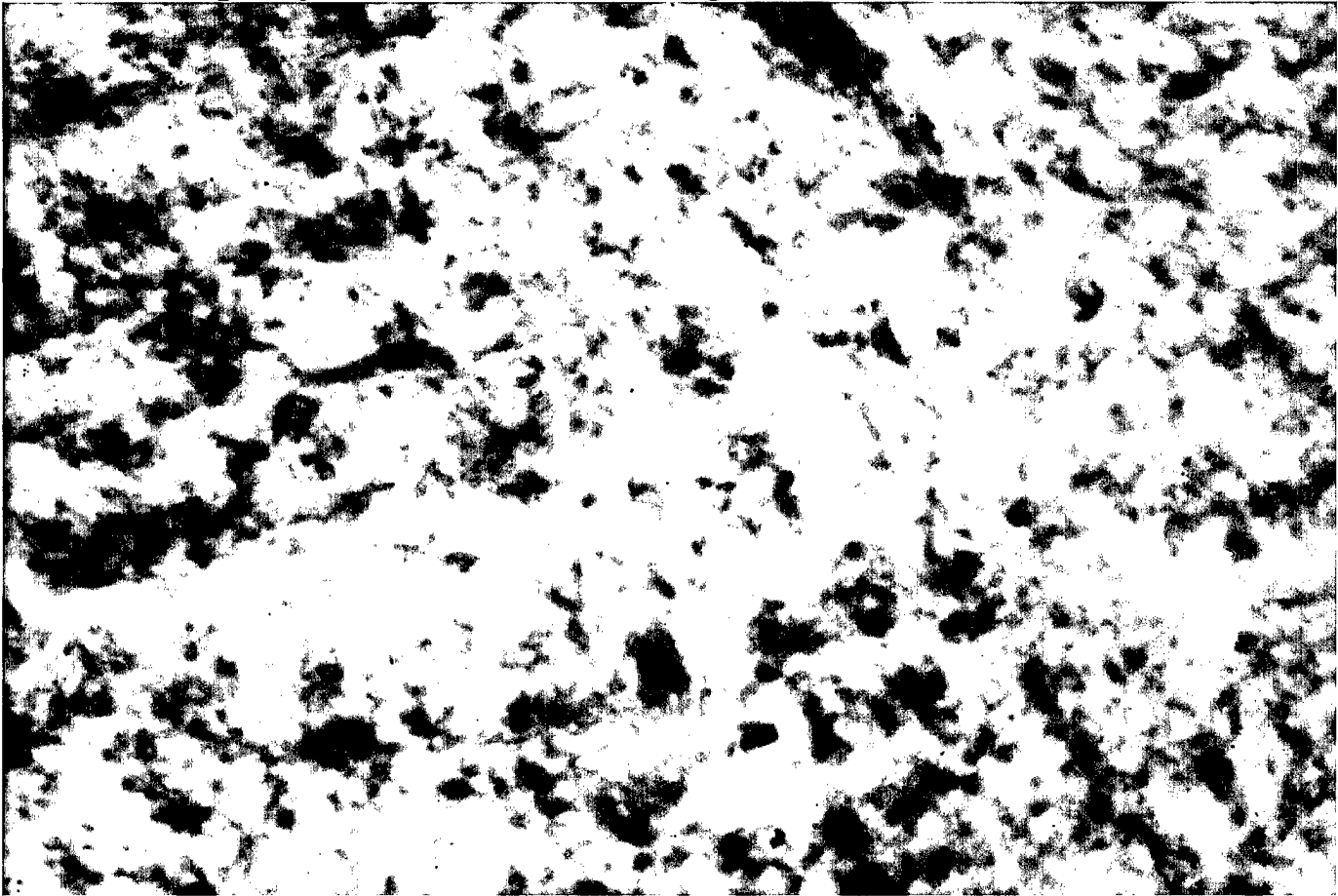
8-HYDROXY-DEOXYGUANOSINE (ACUTE MPTP)



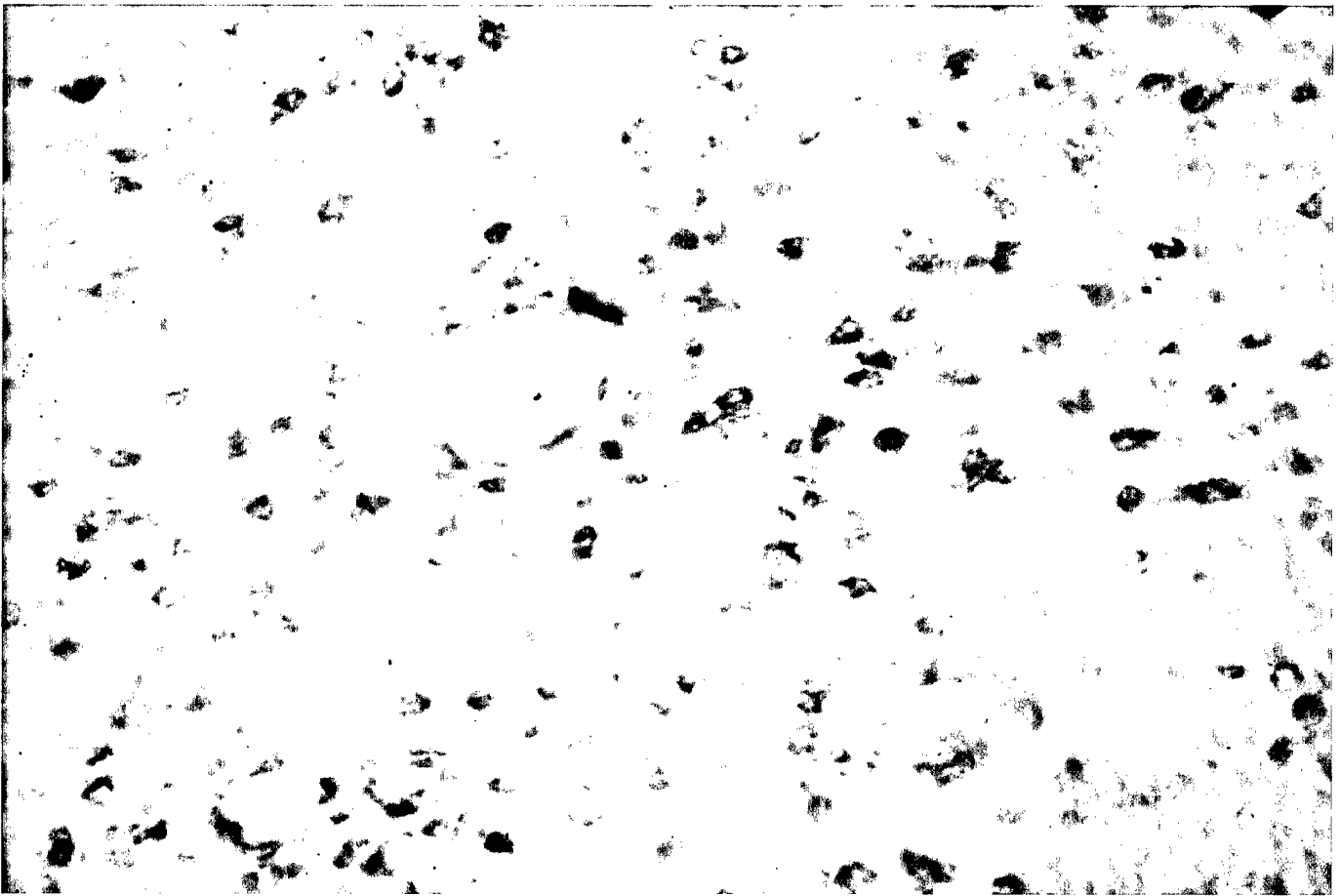
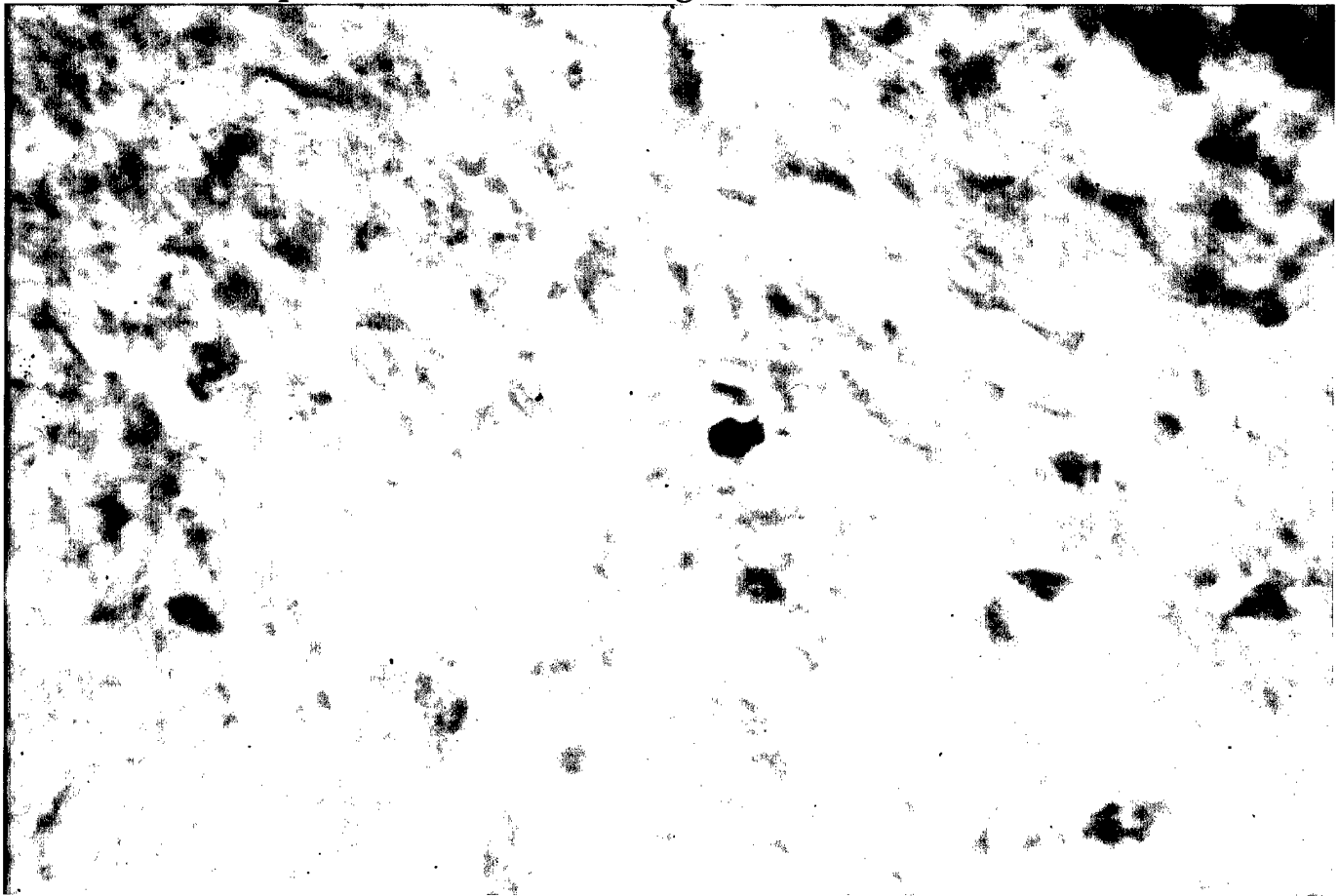
Dopamine transporter (DAT) in the Substantia Nigra: Control versus Acute MPTP



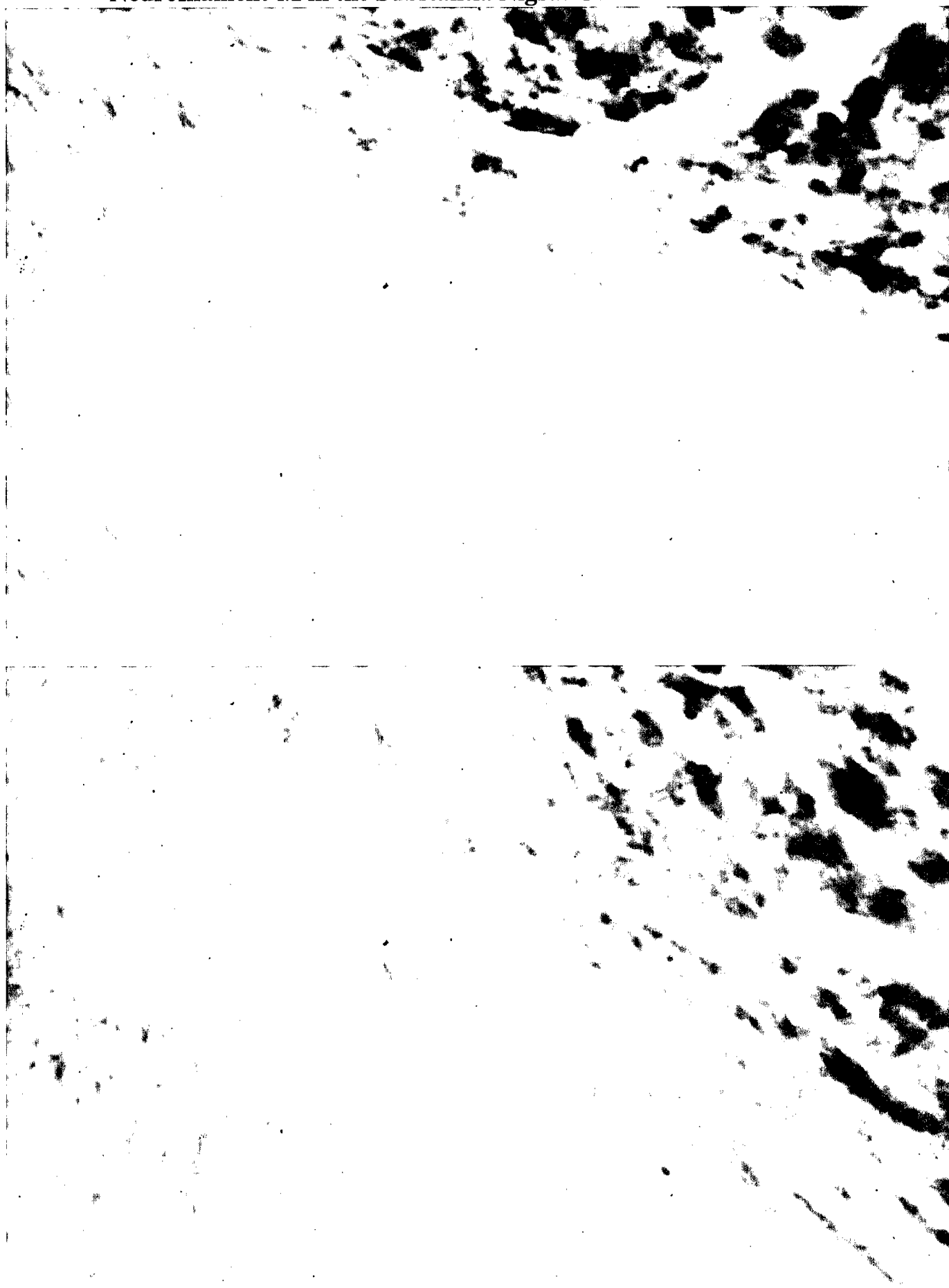
Alpha synuclein the Substantia Nigra: Control versus Acute MPTP



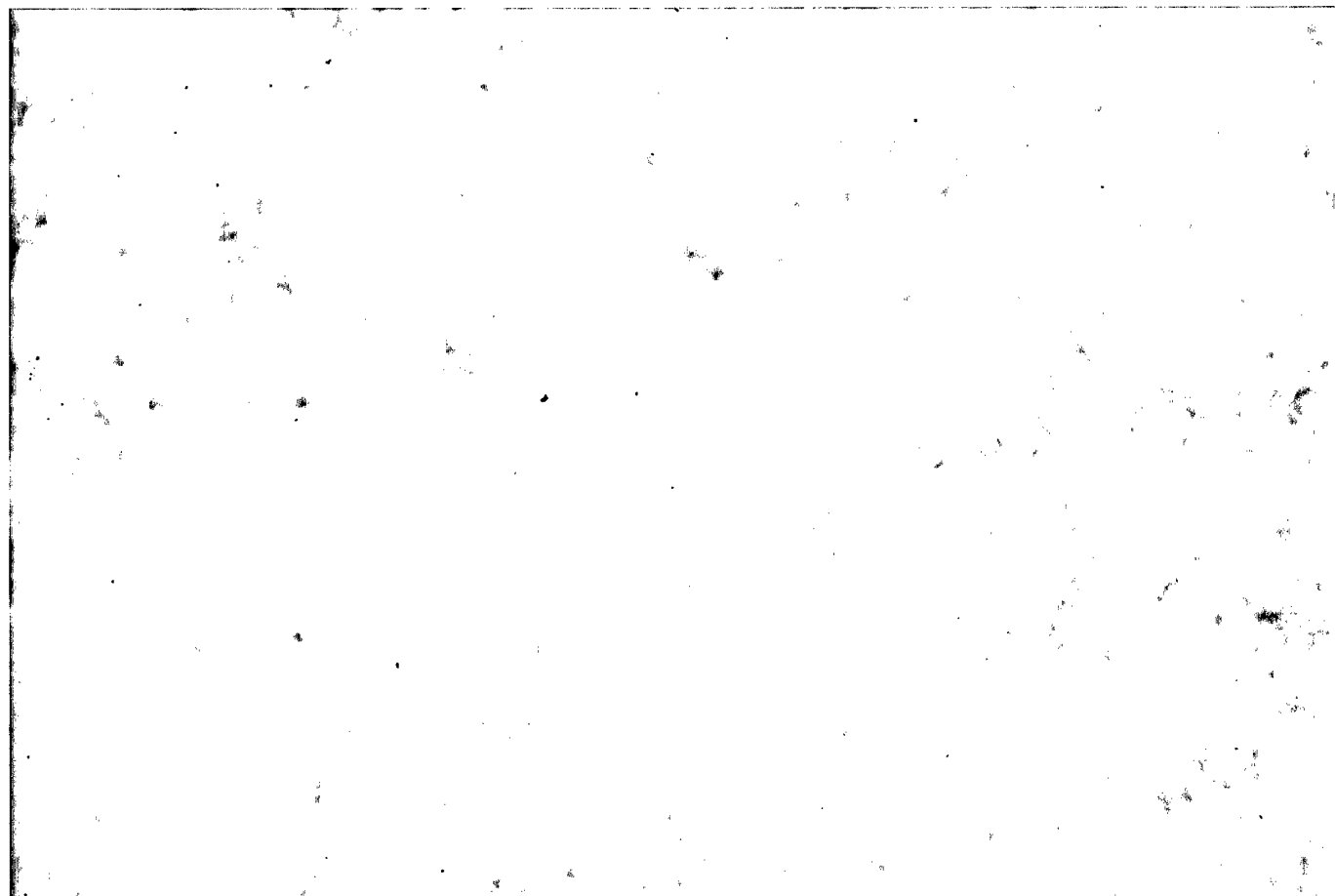
Ubiquitin in the Substantia Nigra: Control versus Acute MPTP



Neurofilament-M in the Substantia Nigra: Control versus Acute MPTP

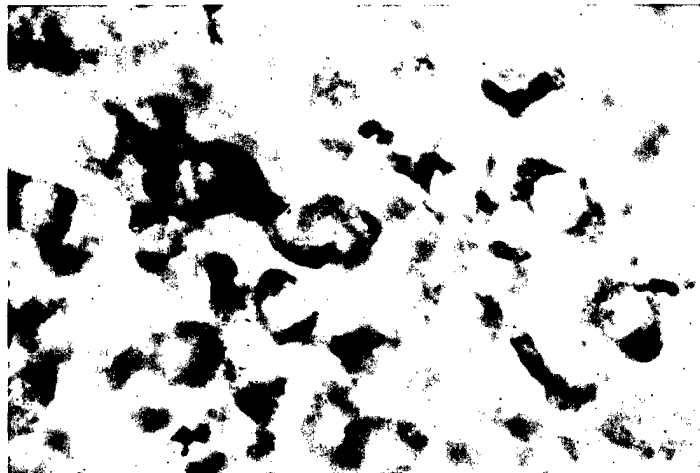
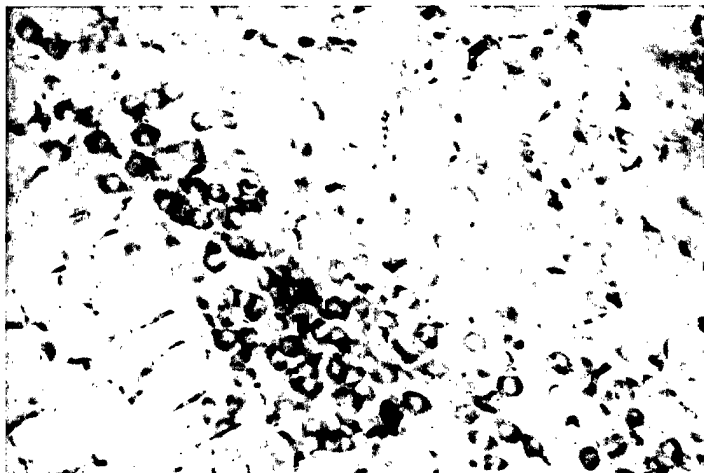


Synaptophysin in the Substantia Nigra: Control versus Acute MPTP

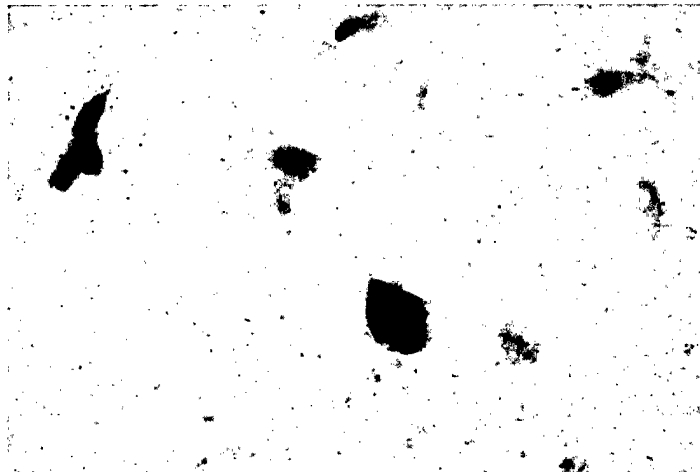


OXIDATIVE MARKERS IN THE MPTP-TREATED MOUSE SUBSTANTIA NIGRA (LOW AND HIGH POWER)

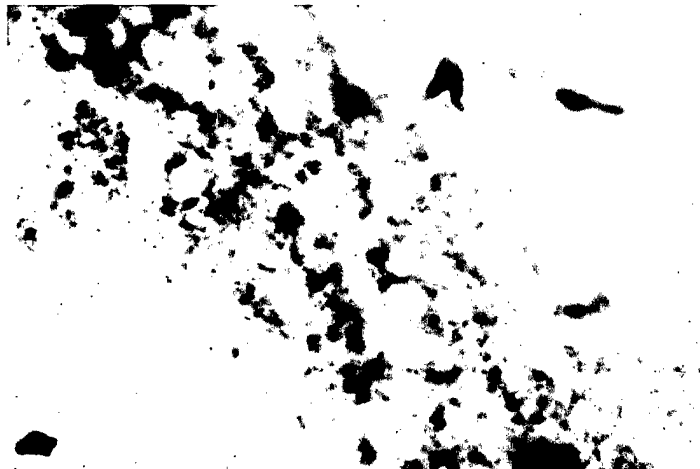
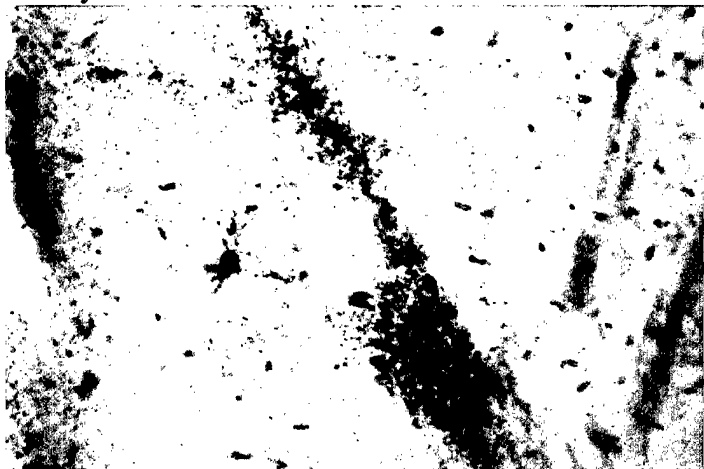
8-OHDG



Malondialdehyde

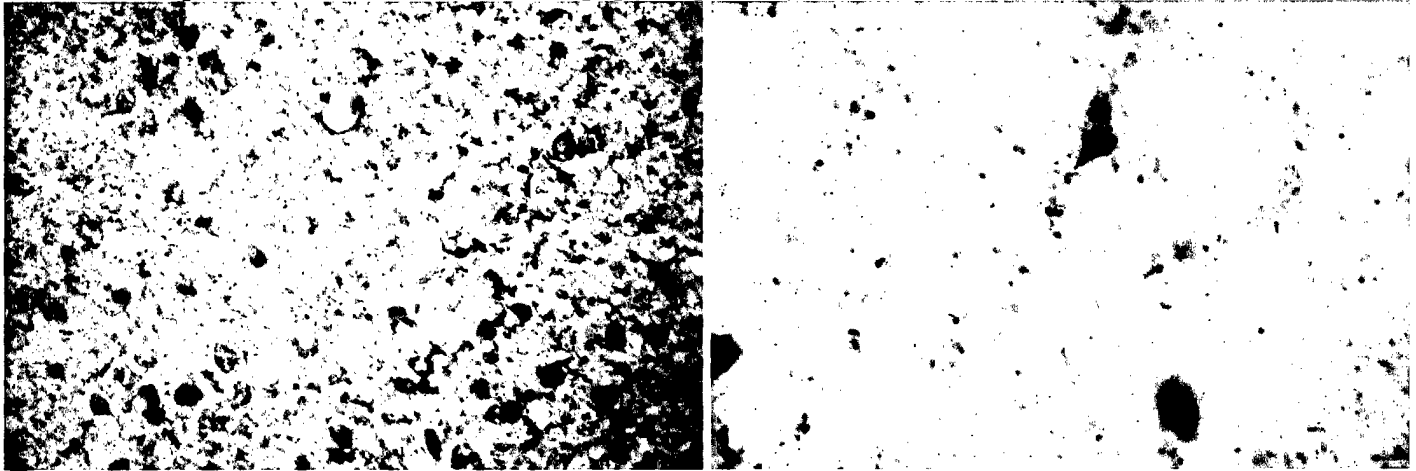


Nitrotyrosine



APOPTOSIS AND STRESS MARKERS IN MPTP-TREATED MOUSE SUBSTANTIA NIGRA (LOW AND HIGH POWER)

Activated Caspase 3 (indicates activation of apoptotic pathways)



Ubiquitin

