STUDIES OF TYROSINE HYDROXYLASE ENZYME AND ALPHA-SYNUCLEIN PROTEIN IN NORMAL AND PARKINSONISM CONDITIONS IN COMMON TREE SHREW BRAIN (*Tupaia glis*)

A THESIS

BY

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Cheewan Sunthonwipat

The author

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Chapter 1

Introduction

1.1 The movement controls

Many areas in the brain are involved in a complex chain of decisions required for even the smallest muscular movement. A central area of the brain, called the striatum, which controls many aspects of body movement. The striatum works with other areas of the brain, including the substantia nigra, to send out the commands for balance and coordination (1).

The commands move from the brain to the spinal cord through nerve networks to the muscles. The entire nervous system is made up of individual units called nerve cells. Nerve cells actually serve as a communication network within the body (2). Nerve cells use a variety of chemical messengers called neurotransmitters to carry any messages between nerve cells (Figure 1).

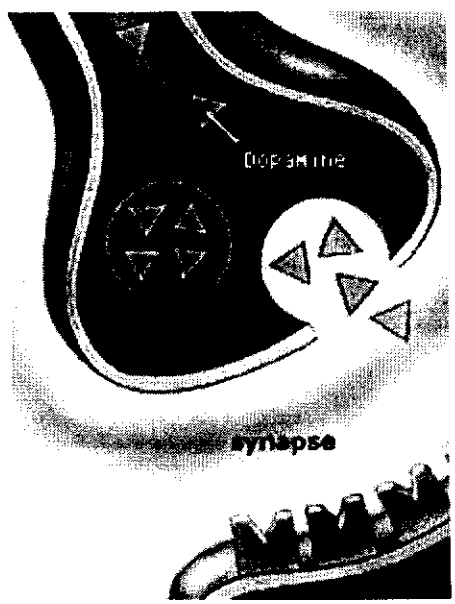

Figure 1 Cell synapse: The communication between nerve cells (2).

1.2 Neurotransmitter: dopamine

The communication between the nerve cells and the muscles use the chemicals, called neurotransmitters. One important neurotransmitter is dopamine, which is produced in the substantia nigra. Dopamine is crucial to human movement and is the neurotransmitter that helps transmit messages to the striatum that both initiate and control movement and balance (4). The dopamine messages make sure that muscles work smoothly, under precise control, and without unwanted movement.

Dopamine message is packing in the dopamine vesicles. The vesicles carrying the dopamine move to the end of the nerve cell and release the dopamine particles into the synapse (Figure 2).

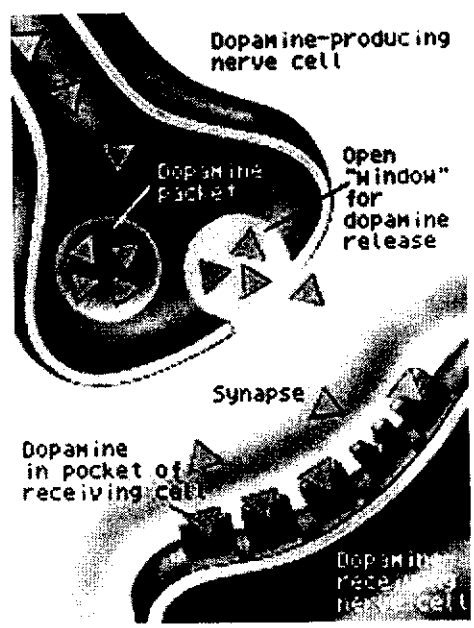

Figure 2 Dopamine releases, the dopamine particles flow across the synapse (2).

The dopamine-receiving cell is stimulated to send on the message and passes along the message to the next nerve cell in the same way.

After stimulated to pass along the message, the packing then release the dopamine back into the synapse. To fine-tune coordination of movement, the used dopamine particles, along with any excess dopamine that did not originally fit into a packing on the receiving cell, are broken down by a chemical in the synapse called MAO-B (5).

The important step in these control of muscle movement use more or less dopamine can disrupt the normal balance between the dopamine system and another neurotransmitter system. Acetylcholine is the other neurotransmitter that works in conjunction with the dopamine system to produce smooth movement.

1.3 Dopaminergic pathway

Dopamine is one type of neurotransmitter. It is synthesized from tyrosine, an amino acid which is part of a normal diet by use mechanism of tyrosine hydroxylase enzyme (Figure 3).

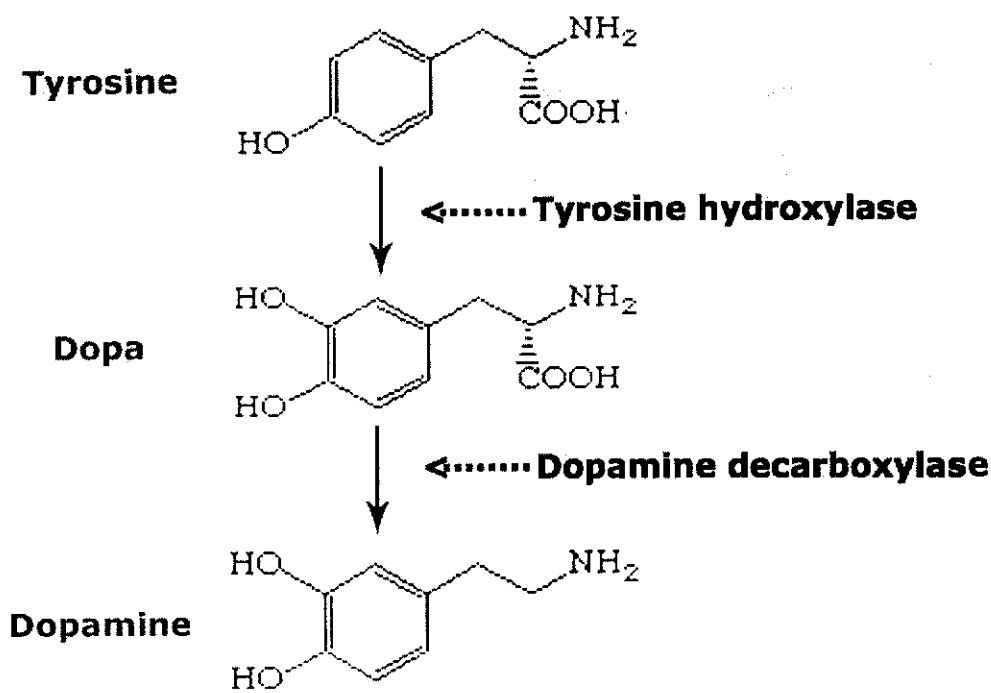

Figure 3 Dopamine synthetic pathways (6).

Dopamine, like many neurotransmitters, acts on both post- and pre-synaptic receptors. Post-synaptic receptors are involved in neurotransmission and pre-synaptic receptors provide feedback loops which regulate neurotransmission (7). Such mechanisms may control the amount of dopamine release; they may even cause changes in the number

or sensitivity of receptors. This allows the nervous system to adapt to changes in stimulation.

While receptors for some neurotransmitters are distributed widely in the brain, others are localized in discrete pathways. Neurons containing catecholamine neurotransmitters were first identified by use of a chemical technique which makes them glow when exposed to ultraviolet light (8). More is known about the location and functions of dopaminergic neurons in the central nervous system than is the case for neurons releasing acetylcholine (9).

The remaining pathways originate in the brainstem. The nigrostriatal pathway runs from the substantia nigra to produce dopamine in the brainstem to the basal ganglia, such as the caudate nucleus and putamen (Figure 4).

1.4 Parkinsonism

One of the most important CNS dopaminergic pathways projects from the substantia nigra pars compacta to the striatum (11). The nigrostriatal pathway degenerates in Parkinson's disease, which produces a loss of dopamine in the striatum, an area involved in the coordination of motor function (12,13). Tyrosine hydroxylase levels are lower in substaintia nigra of Parkinsonism than in normal conditions (Figure 5).

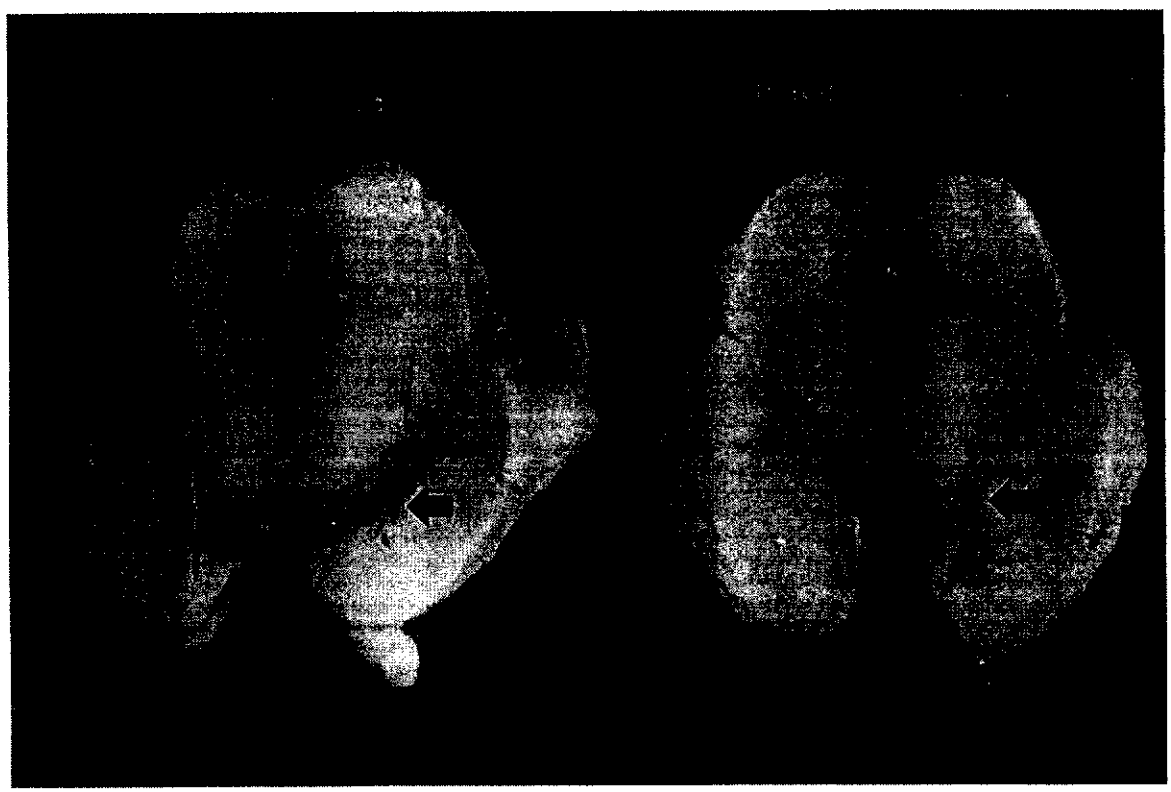

Figure 5 Tyrosine hydroxylase levels are lower in substaintia nigra of Parkinsonism than in normal conditions (14).

Parkinson disease is a common neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons, nerve cells in the part of the brain that produces neurotransmitter: dopamine (15, 16). Parkinson's disease was first described in 1817 by **James Parkinson**, the neuronal death is localized to dopaminergic neurons in the substantia nigra (17). The substantia nigra divided into two sections, substantia nigra pars

reticulata and substantia nigra pars compacta (18). The dopamine can be produced by using nigro-striatal pathway in substantia nigra and transfer to caudate and putamen.

Table 1 The causes of Parkinson disease is unknown, but a number of conditions can cause an akinetic-rigid syndrome in adults (15)

	Condition		
	Pure Parkinsonism	**Parkinsonism with other features**	**Pseudo Parkinsonism**
Cause 1	Parkinson's disease	Progressive supranuclear palsy	Essential tremor
Cause 2	Drug-induced Parkinsonism	Multiple system atrophy	Vascular (pseudo Parkinsonism)
Cause 3	Postencephalitic Parkinsonism	Basal ganglia calcification	
Cause 4	MPTP Parkinsonism	Repetitive head trauma	
Cause 5	Other toxins, e.g. Manganese	Cerebral anoxia	

activity of alpha motor neurons), akinesia/dyskinesia (impaired movement initiation and speed, abnormal movement velocity/amplitude impaired movement sequencing (e.g., walking)), tremor (low frequency; esp. resting, but also action tremor die to brain dysfunction), and other symptoms postural instability, gait disturbances (19, 20). Some symptoms of Parkinson's disease show that motor system is 'hyperactive", not "hypoactive" e.g., increased muscle tone, rigidity, tremor; this interferes with normal performance of movement sequences (20).

1.5 Tyrosine hydroxylase enzyme

Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of catecholamines (Figure 6). Since the discovery of this enzyme, the enzyme has been intensively studied in relation to both its physiological function in the brain and brain disorders. Especially the successful application of L-DOPA therapy for Parkinson's disease suggests that this enzyme should have a primary role in the progress of this disease (22).

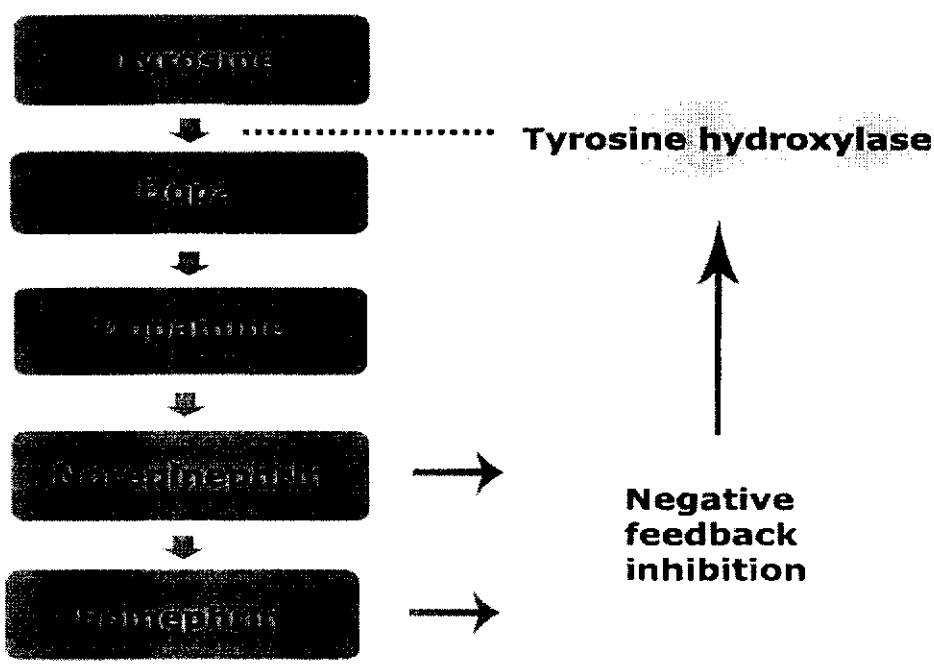

Figure 6 Activity of tyrosine hydroxylase enzyme (21).

Tyrosine hydroxylase is catalyses the conversion of L-tyrosine to L-dopa befor L-dopa changes to dopamine (23). This is the initiation step for all catecholamine biosynthesis. Tyrosine hydroxylase is, therefore, a useful marker of all CA neurons and allows their localization in different areas of the brain (24). The end product can be inhibiting the activity of tyrosine hydroxylase enzyme (25).

1.6 Lewy bodies and alpha-synuclein protein

Parkinson's disease is defined by nerve cell loss in the substantia nigra and the presence of Lewy bodies (Figure 7). Lewy bodies are found in other brain regions, such as the dorsal motor nucleus of the vagus, the nucleus basalis of Meynert, and the locus coeruleus (26-28). Lewy bodies, the fibrous portion of which contains the protein alpha-synuclein. This finding is consistent with the premise that alpha-synuclein fibrillization is pathogenic (29). The natively unfolded and monomeric alpha-synuclein, a normal neuronal protein, and the alpha-synuclein amyloid fibril, the appearance of which, in the form of Lewy bodies, correlates with Parkinson's disease (30).

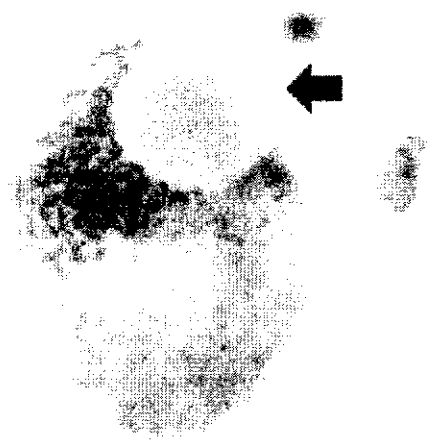

Figure 7 The Lewy bodies contain alpha-synuclein protein in Parkinson's disease (22).

The alpha-synuclein gene is implicated in the pathogenesis of Parkinson's disease (30). Although alpha-synuclein function is uncertain, the protein has homology to the chaperone molecule 14-3-3 (31). In addition, alpha-synuclein can bind to 14-3-3, and reduce activity of tyrosine hydroxylase (the rate-limiting enzyme in dopamine biosynthesis) (32, 33).

The overexpression of alpha-synuclein gene had significantly reduced tyrosine hydroxylase activity and a corresponding reduction in dopamine synthesis (34). The reduction in cellular dopamine levels was not caused by increased dopamine catabolism or dopamine efflux. Alpha-synuclein plays a role in the regulation of dopamine biosynthesis, acting to reduce the activity of tyrosine hydroxylase (35).

Parkinson's disease is linked to two autosomal dominant point mutations in the alpha-synuclein gene encoding alpha-synuclein protein (36). Recently, the discovery of point mutations in the alpha-synuclein gene (A30P and A53T) as a rare cause of familial Parkinson's disease has led us to the finding that alpha-synuclein is a component of Lewy bodies abnormal aggregates of proteins that include synuclein in brains (37, 38).

The possibility that protofibrils and/or protofibril rings are actually the pathogenic species and that fibrils are innocuous (or less toxic) is consistent with pathological studies that have suggested that Lewy bodies may be neuroprotective (39). This proposal has several important consequences. First, the most pathogenic mutations, which presumably would be selected against by evolution, would promote protofibril formation and prevent fibrillization (40, 41). This information could be useful in constructing animal models in which pathogenesis is accelerated. Second, animal models should be evaluated based on neuronal loss in the substantia nigra, rather than on the presence of Lewy bodies, because the two are correlated but not necessarily linked (42). Finally, compounds that inhibit alpha-synuclein fibrillization, but allow protofibril formation, may promote disease (43). This is an important consideration for high-throughput screening efforts in which compounds are selected for their ability to inhibit fibrillization (44). The experimental testing of this proposal is the subject of ongoing studies.

Mutations in the alpha-synuclein gene have been linked to the Parkinson's disease (45, 46). But in most Parkinson's cases, scientists cannot detect mutations in these genes (47). So a hallmark of Parkinson's disease is the presence of Lewy bodies—abnormal aggregates of proteins that include alpha synuclein protein in brains (48-50).

1.7 Common tree shrew (*Tupaia glis*)

Family: Tupaiidae, order: Scandentia (climbing mammals), subclass: Eutheria (true mammals), class: Mammalia (animals with milk glands) (51, 52).

The name "tree shrew": "shrew" is a Middle English word for "villain," which has attached to these animals probably because of their pugnacious manner. They live in trees, and are therefore called scandentia which is climbing mammals. Their locations are found in Southern China, India, and Southeast Asia. The habitats and terrestrial are in mountainous forest and shrub areas. In both appearance and size, these animals closely resemble squirrels. Their fur is soft and dense, with colors ranging from gray to green, and either whitish or dark chestnut-brown on the belly. The snout is pointed, the ears small and bare, and the toes are clawed. The big toe is not opposable. The tail is covered with long hairs. The tree shrew is about 5.5 to 6" long, and the tail is about the same. It weighs 80 to 150 grams (Figure 8).

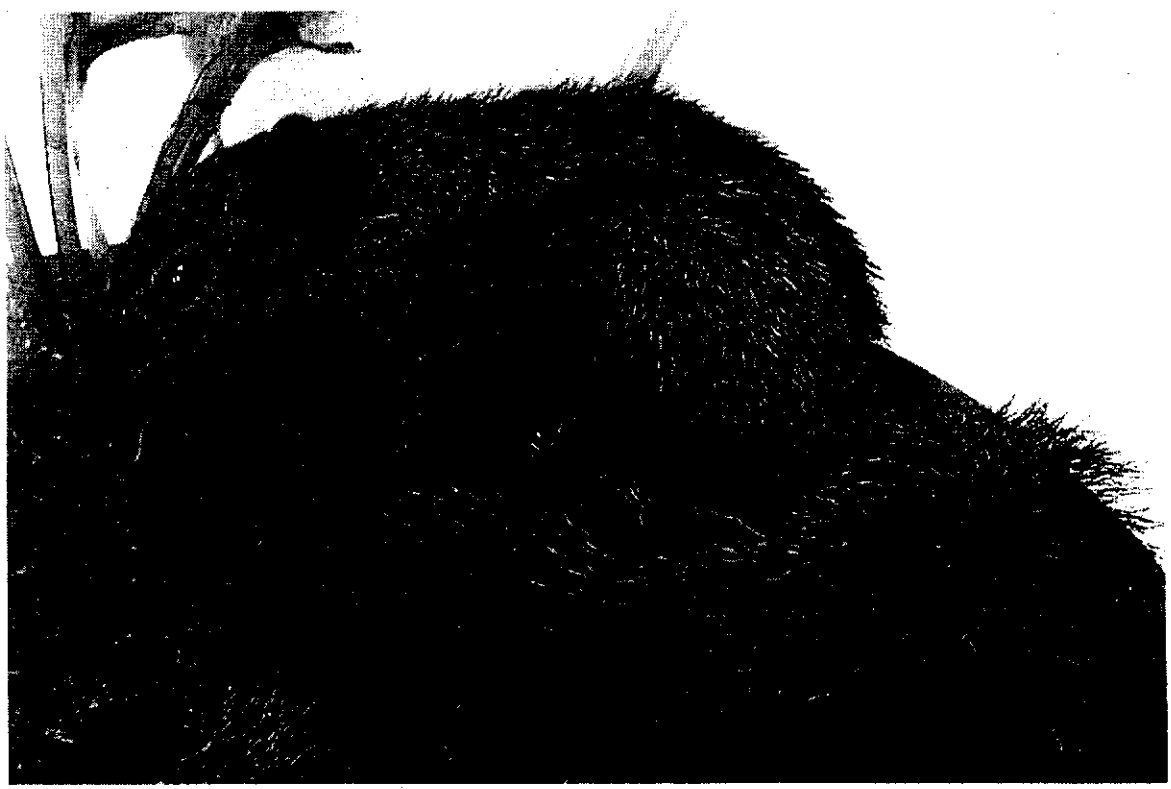

Figure 8 Common tree shrew (*Tupaia glis*).

The common tree shrew's diet consists of insects, fruit, seeds, and leaves. Like the squirrel, it sits on its hind legs and holds its food with its front feet while eating. It is active during the day, and appears to be constantly on the moving and searching for food. It lives mainly on the ground, building its nest among tree roots or in fallen trunks. Tree shrews live in pairs, with the male marking his territory with a strong smelling substance secreted from glands situated in the throat. Gestation requires 46 to 50 days, with the litter usually consisting of two young. Tree shrews become sexually mature at four months of age (52, 53).

1.8 MPTP induced Parkinsonism

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin (54). It has been known for more than 60 years that idiopathic Parkinson's disease is characterized pathologically by the death of dopaminergic neurons of the nigrostriatal pathway (55). This compound appears to be selectively toxic to the dopaminergic cells in the substantia nigra, and is capable of producing virtually all the signs Parkinson-like syndrome (56).

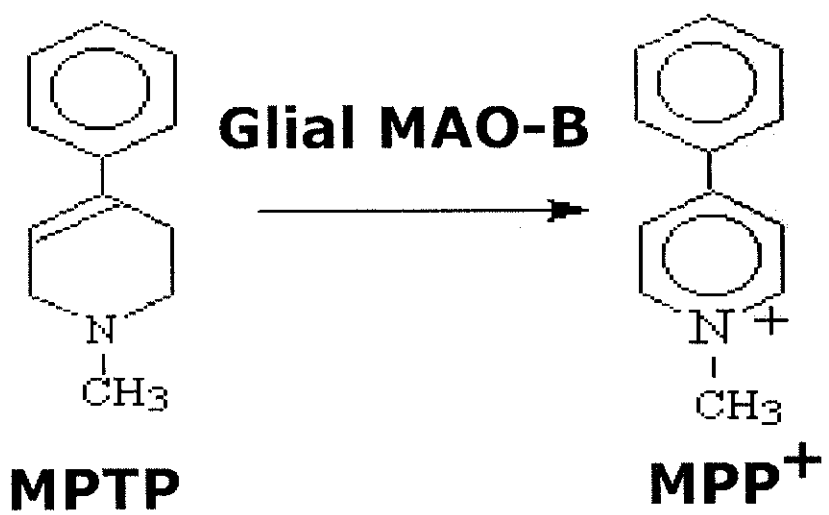

Figure 9 MPTP is metabolized to the active form of MPP+ in glial cell (56).

It is less specific and much less potent in mice and has only slight effects in rats. Differences in rates and sites of metabolism of MPTP to its active, toxic, highly polar

metabolite, MPP+ (1-methyl-4-phenylpyridine), appear to influence species specificity (Figure 9). MPP+ is a substrate for catecholamine uptake sites and is concentrated in these neurons (57). The molecular mechanism of MPP+ toxicity has not been established definitively, but conversion to a free radical or uptake by mitochondria and inhibition of mitochondrial respiratory enzymes (58, 59), leading to calcium release and cell death have been suggested (Figure 10). Discovery of MPTP toxin causes an animal model of Parkinson's disease.

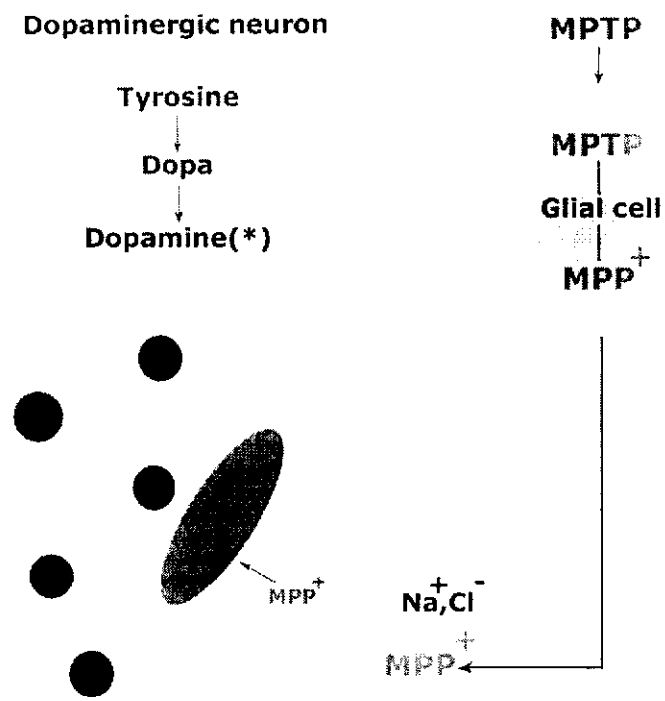

Figure 10 The molecular mechanism of MPP+ toxicity to inhibit of mitochondrial respiratory enzymes, leading to ATP depletion and cell death (60).

MPTP was first tested for its possible therapeutic use in 1947, but the primates that were given the drug became rigid and unable to move, eventually dying. The compound was being tested as a possible anti-Parkinson agent (61). After 6 humans were given the drug and developed Parkinsonian symptoms and 2 died, the drug was abandoned.

The first case of MPTP causing Parkinsonism occurred in 1976 when a young college student who manufactured and abused MPTP made a mistake in his synthesis and produced MPTP. Within 3 days he was severely Parkinson and his family thought he was schizophrenic (61). Two years later he committed suicide and the autopsy showed cell destruction in the substantia nigra, and the damage was, in fact, the same as that seen in Parkinson's disease patients (62).

In 1982 MPTP was again manufactured and sold in the street as synthetic heroin. It was not long before contaminated batches containing MPTP hit the streets, which dealt a devastating blow to the young users (63). The proposed biochemical mechanism of action of MPTP involves the rapid oxidation of MPTP to MPP+ after systemic administration (62). MPP+ is then taken up by neurons in the substantia nigra where it destroys dopaminergic neurons in this area (64, 65). Although the formation of MPP+ occurs in many parts of the brain, it remains unclear as to why it selectively accumulates in the substantia nigra and not in other dopaminergic areas of the brain such as the striatum.

Hospitals began admitting patients as young as 19 with severe end stage Parkinson symptoms. The source was tracked down to MPTP and its effect was found to be permanent damage to the dopaminergic neurones of the substantia nigra.

All the evidence to date suggests that patients with MPTP-induced Parkinsonism have lesions limited to the substantia nigra. MPTP-induced Parkinsonism is very selective, whereas with idiopathic Parkinson's disease other areas of the brain are affected (66).

MPTP-induced Parkinsonism is very rapid in its onset (as quick as a few days to full symptoms), whereas idiopathic Parkinson's disease has a slow progression and may take years to become evident. Another major difference is the presence of Lewy bodies. These are concentric inclusion bodies which can be identified by using a red dye called eosin. They are considered a hallmark of idiopathic Parkinson's disease but are yet to be demonstrated in MPTP-induced Parkinsonism (67).

MPTP was tested on animals with an amount of valuable information on the effects and consequences of this drug. One of the most important findings of the animal tests is that MPTP can produce severe Parkinsonism in many animals (68-70).

The most fascinating part of the discovery of MPTP is that it has caused scientists to take an interest in finding an environmental cause for the disease. Probably the most astounding case of possible environmental factors is the case of a chemist who contracted

Parkinson disease whilst working with MPTP (71) The only possible forms of infection were through the skin or by inhaling the vapors.

Three phases of MPTP toxicity have been identified. The first is an acute phase which occurs on initial exposure to MPTP. Symptoms include disorientation, hallucinations, blurred vision, "nodding off" (a slow downward drifting of the head, and drooping and closure of the eyelids), difficulties in speech and swallowing, intermittent jerking of the limbs, slow movement, and tremor at rest. The second phase is a subacute event which occurs after exposure to the drug (72).

Two to three days post-exposure there are reports of increased bradykinesia and rigidity of extremities, abrupt onset of "freezing up" and inability to move. Up to three weeks after exposure, awkward posture, progressive slowness of movement and "freezing up" have been reported. Finally, if there is no recovery from the above two phases, a chronic syndrome results. A permanent Parkinsonian syndrome evolves consisting of classical Parkinsonian symptoms such as bradykinesia, rigidity, resting tremor, fixed stare, and loss of postural reflexes. Recovery from the acute or subacute phase may occur, but it is unlikely once the chronic phase has been reached.

Several mechanisms have been proposed to explain the manifestations of each of the three phases. Possible mechanisms regarding the acute phase include an opiate receptor interaction with MPTP, serotonergic effects of the substance, and a slight dopaminergic deficiency caused by MPTP. Because MPTP is a meperidine analog, an opiate receptor interaction is probably responsible for the "nodding off" which takes place. This phenomenon is typical of exposure to heroin and is due to the same type of opiate receptor interaction. An initial suppression of serotonin in the central nervous system by MPTP is the suggested cause for the hallucinations and retropulsions which occur. Motor symptoms are attributed to MPTP's effect on the dopaminergic neurons in the substantia nigra, but the dopamine deficiency is not yet substantial.

The subacute phase is thought to occur once MPTP accumulation reaches a critical threshold before killing cells in the substantia nigra (73). This theory thus offers an explanation for the delayed onset of symptoms and for the continuation of symptoms after exposure. Metabolic damage, such as impaired dopamine synthesis, is also suggested as a cause of dopamine depletion (74, 75).

1.9 Drug therapy for Parkinsonism

Parkinson's disease can be treated with drugs that increase or enhance dopamine levels. Some time the drugs fail to alter disease progression and most produce undesirable side effects (76).

In the past several years, the development of newer medications and an understanding of how best to use them have significantly improved the outlook for people with Parkinson's disease (77). The medications outlined below form the mainstay of Parkinson's disease therapy.

The doctor may prescribe one or a combination of these medications to replace or simulate the effect of the dopamine or bring the level of acetylcholine turn into balance with the level of dopamine in the brain.

These medications can increase the level of dopamine and correct the imbalance between dopamine and acetylcholine (78). This may be achieved by using one medication alone in the earlier stages of Parkinson's disease, or combining two or more medications in various combinations later on in the disease process.

1.9.1 Dopamine replacements

Levodopa is the single most effective medication for controlling Parkinson's disease (79). Levodopa is transported to the brain and is picked up by the nerve cells that produce dopamine. Levodopa is converted into dopamine for dopaminergic neurons to use it as a neurotransmitter. Most patients can use Levodopa to replace their missing dopamine and control their symptoms for many years (80).

Moreover, as the loss of dopamine-producing nerve cells continues, Parkinson symptoms will continue, thus the dose of Levodopa will often have to be increased.

1.9.2 Mimic dopamine substitutions

Bromocriptine mesylate and Pergolide mesylate are medications that mimic the action of dopamine by fitting into the dopamine packing on the surface of the nerve cell that is received (81). One advantage of this substitution approach is that dyskinesias. This is because the amount of dopamine is not actually being increased, as with Levodopa/Carbidopa. Instead, Parlodel or Permax acts as a substitute for dopamine. This makes it less likely for dyskinesias to occur since they are caused by too much dopamine in the brain.

1.9.3 Release of stored dopamine

Amantadine hydrochloride appears to make it easier for dopamine-producing nerve cells to open a window and release their stored dopamine into the synapse (81). This approach is helpful in milder cases of Parkinson's disease, in which symmetry works to reduce disease symptoms.

1.9.4 Acetylcholine adjustment to restore the dopamine/acetylcholine balance

Trihexyphenidyl-HCl can be used to restore the dopamine/acetylcholine balance by reducing the activity of acetylcholine in the brain. This successfully reduces the tremor and muscle stiffness that result from having much more acetylcholine than dopamine (82). But, these medications on their own do not correct the basic problem of too little dopamine. Usually, these agents will be used in the early stages of Parkinson's disease or in combination with some of the other agents discussed here.

1.10 Western blot analysis

Western blot analysis, this is a powerful technique for determining levels of protein expression and for assaying proteins during purification (83). This method usually involves the following steps: A protein sample is subjected to polyacrylamide gel electrophoresis. The gel is placed over a sheet of nitrocellulose membrane and the protein in the gel is electrophoretically transferred to the nitrocellulose membrane (84). The nitrocellulose membrane is then soaked in blocking solution to block its ability to non-specifically bind proteins. The nitrocellulose membrane is then incubated with the specific antibody for the protein of interest and then incubated with a second antibody which is specific for the first antibody (85). For example, if the first antibody was raised in rabbits, the second antibody might be termed "goat anti-rabbit immunoglobulin". What this means is that rabbit immunoglobulin were used to elicit an antibody response in goats. The goat antibodies (polyclonal) will include those which recognize the conserved region in the rabbit antibodies. Since the Fc region is conserved, it will bind to any and all rabbit antibodies, including those on the nitrocellulose membrane paper. The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction (86). Thus the molecular weight and amount of the desired protein can be characterized from a complex mixture (e.g. crude cell extract) of other proteins.

In Western blot analysis, the first step is a denaturing polyacrylamide gel analysis (87). Denaturing gel electrophoresis separates proteins based on their size. Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique. Generally the sample is run in a support matrix such as paper, cellulose acetate, starch gel, agarose or polyacrylamide gel. The matrix inhibits convective mixing caused by heating and provides a record of the electrophoretic run: at the end of the run, the matrix can be stained and used for scanning, autoradiography or storage.

In addition, the most commonly used support matrices - agarose and polyacrylamide - provide a means of separating molecules by size, in that they are porous gels. A porous gel may act as a sieve by retarding, or in some cases completely obstructing, the movement of large macromolecules while allowing smaller molecules to migrate freely. Because dilute agarose gels are generally more rigid and easy to handle than polyacrylamide of the same concentration, agarose is used to separate larger macromolecules such as nucleic acids, large proteins and protein complexes. Polyacrylamide, which is easy to handle and to make at higher concentrations, is used to separate most proteins and small oligonucleotides that require a small gel pore size for retardation.

Proteins are amphoteric compounds; their net charge therefore is determined by the pH of the medium in which they are suspended. In a solution with a pH above its isoelectric point, a protein has a net negative charge and migrates towards the anode in an electrical field. Below its isoelectric point, the protein is positively charged and migrates towards the cathode. The nett charge carried by a protein is in addition independent of its size - ie: the charge carried per unit mass (or length, given proteins and nucleic acids are linear macromolecules) of molecule differs from protein to protein. At a given pH therefore, and under non-denaturing conditions, the electrophoretic separation of proteins is determined by both size and charge of the molecules.

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1 (88). In so doing, SDS confers a negative charge to the polypeptide in proportion to its length - ie: the denatured polypeptides become "rods" of negative charge cloud with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2- mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. SDS binding strongly to most proteins and causes those to unfold to a random, rod-like chain and also makes them net negative in charge. No covalent bonds are broken in this process. Therefore, the amino acid composition and sequence remains the same.

Since its specific three-dimensional shape is abolished, the protein no longer processes biological activity. Proteins that have lost their specific folding patterns and biological activity but have their polypeptide chains remaining intact are called denatured. Proteins which contain several polypeptide chains that are associated only by non-covalent forces will be dissociated by SDS into separate, denatured polypeptide chains. Protein can contain covalent cross links known as disulfide bonds. These bonds are formed between two cysteine amino acid residues that can be located in the same or different polypeptide chains. High concentrations of reducing agents, such as 2-mercaptoethanol, can break disulfide bonds. This allows the SDS to completely dissociate and denatured the protein. During electrophoresis, the SDS denatured proteins migrate through the gel towards the positive electrode at a rate that is inversely proportional to their molecular weight. In other words, the smaller the protein, the faster it migrates. The molecular weight of the unknown is obtained by the comparison of its position after electrophoresis to the positions of standard SDS denatured proteins electrophoresed in parallel.

After electrophoresis, total protein can detect by Coomassie blue staining. Coomassie blue is the most frequently used protein stain in SDS-PAGE because of its sensitivity and ease of use. It can stain protein band containing 0.1-1 ug of protein concentration (89).

Specific protein detection, Sufficiently separated proteins in an SDS-PAGE can be transferred to a solid membrane for western blot analysis The next step in Western Blot Analysis involves the direct transfer of the resolved protein bands from the gel to a membrane sheet of charged nylon, nitrocellulose membrane or polyvinylidene fluoride (PVDF) membrane. The membrane is subsequently probed for specific proteins by immunochemical methods. Proteins are absorbed to the membrane by hydrophobic associations. After electrophoresis is completed, the membrane is placed directly on the slab gel (90). The transfer of proteins can be done electrophoretically in a specially designed chamber. Electrical transfer requires a much higher current than the gel electrophoresis run. Transfer can also be accomplished by capillary flow or suction. Following transfer, an immobilized replica of the protein bands is now present on the membrane surface. The membranes are much stronger and more pliable than gels and can undergo many manipulations without tearing. Total protein can be visualized by staining the membrane with protein specific dye (91).

Usually, the proteins that are the antigens of interest cannot be detected by total protein staining in crude mixtures because the amount of protein is too low or the complex banding pattern of the numerous other proteins in the sample block it from view. For immunological detection the unstained membrane is placed in blocking solution which contains detergents and proteins that bind to all unoccupied site on the membrane. The membrane is then incubated in buffers that contain primary antibody to one or more of the blotted proteins. The primary antibody recognizes and binds to the target antigen bound to the membrane. Remove primary antibody excess by washed. Use the secondary antibody to detect the bound primary antibody.

The secondary antibody is purified from serum and covalently linked to enzymes such as alkaline phosphatase or horseradish peroxidase. The cross linking is done under conditions that do not appliciably affect the antigen binding specificity and affinity of the antibody or the catalytic activity of the enzyme. In the next step, the membrane is incubated in a solution containing phosphatase or peroxidase substrates that yield chromogenic products. Areas containing IgG conjugates will develop a color, depending on the type.

Chapter 2

Review literature

2.1 Visualization and isolation of live dopaminergic neurons developed for transplantation.

Visualization and isolation of live dopaminergic neurons in the embryonic ventral mesencephalon, generated transgenic mice expressing green fluorescent protein (GFP) under the control of the rat tyrosine hydroxylase gene promoter. In the transgenic mice, GFP expression was observed in the developing dopaminergic neurons containing tyrosine hydroxylase. The outgrowth of GFP-labeled axons was monitored in vitro with brain culture systems.

Isolation of dopaminergic neurons expressing GFP from brain tissue with GFP fluorescence were sorted by fluorescence-activated cell sorting. More than 60% of the sorted GFP+ cells were positive for tyrosine hydroxylase, confirming that the population had been successfully enriched with dopaminergic neurons. The sorted GFP+ cells were transplanted into a rat model of Parkinson's disease. Some of these cells survived and innervated the host striatum, resulting in a recovery from Parkinsonian behavioral defects. This strategy for isolating an enriched population of dopaminergic neurons should be useful for cellular and molecular studies of these neurons and for clinical applications in the treatment of Parkinson's disease (92).

Dopaminergic neurons, the cell bodies of which are located in the ventral midbrain, play a central role in a variety of brain functions, such as motor control, cognition, memory processing, and emotion. The development of midbrain dopaminergic neurons provides a good model system for the investigation of molecular mechanisms underlying the determination of specific neuronal fates and axonal guidance pathways. During embryonic development, the midbrain dopaminergic neurons appear in the intermediate zone of the ventral neural tube and extend their fibers en masse to target regions, including the striatum, through selective axonal pathways. Recent studies have identified several factors involved in the development of dopaminergic neurons. The axonal guidance of dopaminergic neurons toward the forebrain is regulated by chemorepulsive factors secreted from the mesencephalic floor plate and directional cues associated with the midbrain

substrate. However, the precise mechanisms controlling the development of dopaminergic neurons are not fully understood. The gradual loss of dopaminergic neurons in the substantia nigra is responsible for most of the symptoms of Parkinson's disease. Intrastriatal grafting of dopamine-secreting cells has been reported to result in the amelioration of the motor syndrome in Parkinsonian patients and in animal models of the disease (93).

Although transplantation is considered a promising therapy for Parkinson disease, its clinical use is still restricted to a very few cases. The major limiting factors of this therapy are the difficulty in obtaining sufficient viable embryonic mesencephalic tissue and the controversial ethical and legal issues raised by the use of human fetal allografts. In the reviewed literature, the study successfully obtained the expression of green fluorescent protein (GFP) in midbrain dopaminergic neurons under the control of a tissue-specific gene promoter in transgenic mice. The study shown the labeling of dopaminergic neurons with GFP is useful for their dynamic imaging in living tissue and cell culture systems.

2.2 Synuclein protein act as the Parkinsonism marker

Many neurodegenerative diseases are characterized by fibrous protein inclusions. Mutations in the genes encoding the fibrillar proteins are associated with early-onset familial forms of these diseases, suggesting that protein unfolding, oligomerization, and/or fibril formation could be promoted by the mutations.

Comparative in vitro biophysical studies of wild-type and mutant protein fibrillization processes can implicate discrete species in disease pathogenesis. The step(s) that are affected by mutations are likely to induce accumulation of the pathogenic species. Several caveats to this type of modeling must be emphasized. First, our interpretation is based on the likely possibility that a shared molecular mechanism accounts for both forms of alpha-synuclein-linked Parkinson disease. However, this matter may not be the dominant case. Second, the comparisons made herein are not relevant if the expression levels of each alpha-synuclein variant are not identical, for example, if the mutations affect mRNA stability. Third, the cytoplasmic concentration of free alpha-synuclein may vary with the mutation, if lipid or protein binding or ubiquitin-dependent degradopaminergiction are affected.

The rate of alpha-synuclein amyloid fibril formation is not accelerated by both Parkinson-linked mutations. The study currently is able to directly monitor two distinct

species along the alpha-synuclein fibrillization pathway (94): soluble, natively unfolded and monomeric alpha-synuclein, a normal neuronal protein, and the alpha-synuclein amyloid fibril, the appearance of which, in the form of Lewy bodies, correlates with Parkinson's disease. The time-dependent behavior of other putative species in vitro experiments currently must be inferred. Under controlled conditions, soluble A30P monomer is consumed at a comparable rate or slightly more rapidly than WT monomer; however, WT fibrils appear more rapidly than A30P fibrils. This is consistent with the previous observation that nonfibrillar, apparently spherical, species are formed by A30P before fibrils can be detected. In mixed incubations containing both A30P and WT [relevant to the heterozygous Parkinson disease patients], both loss of monomer and appearance of fibrils are slowed relative to the pure WT incubation. It may be that the latter effect is of greater magnitude, so a prefibrillar intermediate may be populated to a greater extent in the A30P/WT incubation than in the WT incubation (95).

The differences in kinetic behavior between the pure and mixed incubations are mildly surprising, considering that mutant and wild-type fibrils are not easily distinguished and that A53T fibrils seed polymerization of WT alpha-synuclein. This raises the questions of whether the preferred intermediates contain one or both variants and whether one variant can inhibit fibrillization of another (96).

Nonfibrillar alpha-synuclein "spheres," "chains," and "rings" may be sequential species in a pathway. The kinetic dopaminergicta discussed above could be reconciled with the genetic dopaminergicta by a mechanism in which a nonfibrillar pathogenic oligomer accumulates in both mixed incubations. To directly quantities such a species and to determine the effects of the Parkinson-linked mutations on the rate of its formation and its stability, new methods must be developed and applied (97).

Could alpha-synuclein fibrils and Lewy bodies be harmless epiphenomena of a pathogenic oligomerization pathway? The possibility that protofibrils and/or protofibril rings are actually the pathogenic species and that fibrils are innocuous (or less toxic) is consistent with pathological studies that have suggested that Lewy bodies may be neuroprotective. This study has several important consequences. First, the most pathogenic mutations, which presumably would be selected against by evolution, would promote protofibril formation and prevent fibrillization. This information could be useful in constructing animal models in which pathogenesis is accelerated. Second, animal models should be evaluated based on neuronal loss in the substantia nigra, rather than on the

presence of Lewy bodies, because the two are correlated but not necessarily linked. Finally, compounds that inhibit alpha-synuclein fibrillization, but allow protofibril formation, may promote disease. This is an important consideration for high-throughput screening efforts in which compounds are selected for their ability to inhibit fibrillization (98).

2.3 Modern therapy for Parkinsonism

2.3.1 Modern gene therapy

Gene delivery is a development of technologies for delivery of tyrosine hydroxylase gene to the nigrostiatal pathway, to promising treatments for Parkinson disease. Gene therapy is the development of treatment for many neurodegenerative diseases. Striatal delivery of a transgene encoding tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of dopamine, to supply dopa locally is an alternative to the pharmacological treatment of Parkinson disease.

Before such therapy can be applied to humans, adequate gene delivery systems need to be developed. In the past few years, recombinant adenovirus has emerged as a promising tool for direct gene transfer to the brain. An adenovirus vector encoding human tyrosine hydroxylase under the negative control of tet-off regulatory system AdPGK·tet·hTH1 was functional in infected human neural progenitor cells both in culture and after transplantation to the rat brain by direct injection in a rat model of Parkinsonism (99). The long-term ability of this vector to drive production of tyrosine hydroxylase in striatal cells and it's controlled by doxycycline (dox).

Gene product was controlled by exogenous control. Use the gene regulatory system of tetracycline-regulatable adenoviral system for on-off switching (100). Tyrosine hydroxylase contents can detect in the rat striatum by Western blotting within 2 weeks, and the use of lower doses of dox to inhibit transgene expression. The result in gene therapy, gene vector can be transfer tyrosine hydroxylase gene to striatal cells and produce tyrosine hydroxylase enzyme to maintain the levels of dopamine (101). Although the tyrosine hydroxylase expression can be controlled by dox but the result of gene therapy is still too short time.

2.3.2 Minocycline as the neuroprotective in the MPTP model of Parkinson disease

Parkinson disease is a chronic neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra, decreased striatal dopamine levels, and consequent extrapyramidopaminergicl motor dysfunction. Minocycline is a semisynthetic tetracycline, recently shown to have neuroprotective effects in animal models. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) mouse model of Parkinson's disease.

After parenteral administration, MPTP readily enters the brain and is metabolized by astroglia to 1-methyl-4-phenylpyridinium (MPP+). MPP+ is a substrate of the dopamine transporter and is concentrated in nigral dopaminergic neurons where it inhibits complex I of the mitochondrial electron transport chain, resulting in ATP depletion and subsequent cell death. This proposed mechanism of MPTP toxicity implies that dopaminergic neurons are the direct cellular targets of MPTP's neurotoxic action.

Minocycline treatment also blocked dopamine depletion in the striatum as well as in the nucleus accumbens after MPTP administration. After administration, minocycline and chemically related neuroprotective tetracyclines may be effective in preventing and/or treating Parkinson's disease. Eight-week-old male C57BL/6 mice (5–7 per group) were administered minocycline (60, 90, or 120 mg/kg per dopaminergicy in 5% sucrose; Sigma) by oral gavages before, during, and after MPTP administration. An untreated control group and MPTP-only group were included. The MPTP-treated groups received four injections of MPTP-HCl (20 mg/kg, i.p.) in saline at 2-h intervals in a single dopaminergicy (four injections total) as described and killed at 7 dopaminergicys after the last injection.

The brains were analyzed by immunohistochemistry to quantify TH-positive neurons in the substaintia nigra MPTP treatment reduced the number of TH-positive neurons by ≈63% compared with saline-treated controls. Mice that received dopaminergicily treatments of minocycline at either 90 or 120 mg/kg, and MPTP showed increased viable TH-positive neurons in the substaintia nigra , ranging from 37% of control (no minocycline treatment) to 56% (90 mg/kg) and 77% (120 mg/kg) of control after minocycline treatment (102). The neuroprotective effect of minocycline was dose-dependent as the dose of 60 mg/kg failed to protect dopaminergic neurons from MPTP toxicity.

2.4 Objectives of this thesis

In this study, the study of tyrosine hydroxylase enzyme and alpha-synuclein protein in normal and Parkinsonism conditions in common tree shrew brain (*Tupaia glis*) is emphasized in the aspects of the followings.

1. The study is interested in localization and characterization of tyrosine hydroxylase enzyme and alpha-synuclein protein in common tree shrew brain between normal and Parkinsonism condition.

2. The study of the localization and characterization of tyrosine hydroxylase enzyme and alpha-synuclein protein is detected by Western blot analysis using the antigen-antibody reaction. The previous histological study confirmed about the localization of dopaminergic neurons in substaintia nigra, caudate, and putamen.

3. Study in common tree shrew (*Tupaia glis*) is used to develop a new animal model to be representative of Parkinsonism condition.

4. This molecular detection is the basic science study for development of drug therapy and understanding in Parkinsonism pathway.

Chapter 3

Materials and Methods

3.1 Animal treatment

Ten-week-old male Common tree shrews (weight 80 to 100 g) were used in all experiments. Five animals were controlled group and five animals were MPTP-treated group. The controlled group was in normal condition and the MPTP-treated group was in Parkinsonism condition. The MPTP-treated groups were received two injections of MPTP-HCl (10 mg/kg, i.p.) in normal saline at 2 hours intervals in a single day (two injections total). The animals became to express Parkinsonism within 3 hours. Animals in MPTP-treated group were killed at 10 days after the last injection.

3.1.1 Personal protection

Protective equipment must be worn whenever working with MPTP or MPTP-treated animals and their waste within 72 hours of administration. Preparing MPTP solutions, the researcher must wear two pairs of chemical-resistant gloves (e.g., nitrile, latex). Administering MPTP to animals, ones again have to wear two pairs of chemical-resistant gloves (e.g., nitrile, latex), lab coat, wrist-guards, and mucous membrane protection (e.g., chemical goggles and surgical mask).

Transporting MPTP-treated animals within 72 hours post-injection, it is required to wear two pairs of chemical-resistant gloves (e.g., nitrile, latex), lab coat, wrist-guards, and mucous membrane protection (e.g., chemical goggles and surgical mask). Frequently changing the gloves were recommended. Change gloves immediately if their integrity has been compromised (e.g., punctured or torn). Washing hands with soap and water after removing gloves is recommended.

Protective clothing should be removed in the anteroom or immediately prior to exiting any room in which MPTP was in use, if no anteroom was available. Remove garments first, and then remove gloves, chemical goggles, and respirator, respectively. Wash chemical goggles with a mild detergent and water; thoroughly wash hands, face, and neck (61).

3.1.2 MPTP preparation

MPTP must be purchased in hydrochloride or titrate salt form rather than as free base. MPTP and MPTP-solutions must be stored in labeled, tightly capped containers. The container must be properly labeled with the identity of the hazardous contents (i.e., MPTP) and the appropriate hazard warning (i.e., neurotoxin). MPTP as received from the manufacturer/vendor in its original undiluted form must be stored in a certified chemical fume hood in a labeled container. The hood must be posted with an appropriate hazard label.

MPTP (M0896, Sigma) solutions must be prepared in a certified chemical fume hood. All work surfaces must be covered with absorbent, plastic-backed, disposable bench paper. If it was not possible to weigh MPTP in a certified chemical fume hood, then: tare an empty container with its cap. In a certified chemical fume hood, transfer 10 mg of MPTP-HCl into the container; cap and weigh the container; add 5 ml of normal saline to achieve the desired concentration.

Disposable lab ware should be used when preparing MPTP solutions. If non-disposable glassware was used, it must be single rinsed in 0.1 N HCl prior to washing. MPTP solutions must be collected and disposed of as chemical waste. When all work with MPTP was complete, carefully removed all bench paper. Wipe all surfaces with a 1% bleach solution (72).

3.1.3 Administration of MPTP

Administration of MPTP-HCl to common tree shrew must be conducted in a certified chemical fume hood by i.p injection. All work surfaces, except the down-draft table, must be covered with absorbent, plastic-backed, disposable bench paper. Use only needle-locking syringes or disposable syringe-needle units (i.e., needle was integral to the syringe). Used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated before disposal (70).

After the completion of each injection, immediately place the syringe-needle unit in a sharps disposal container. The MPTP-treated groups were received two injections of MPTP-HCl (10 mg/kg, i.p.) in normal saline at 2 hours intervals in a single day (two injections total). The animals became to express Parkinsonism in 3 hours. Animals in MPTP-treated group were killed at 10 days after the last injection.

3.1.4 Animal housing

For the first 72 hours after last injection, the door to each MPTP-animal room must be posted in such a manner that it was clear that the room contained MPTP-treated animals. The animal cages must also be properly labeled. Use clean and disposable cages; place cages in a certified chemical fume hood, plastic-backed, absorbent pads. If pan liners were not used, adequately wet the bedding with water to keep the dust down.

Changing the cage should not be performed for the first 72 hours post-injection. Seventy-two hours after the final MPTP injection, the animals may be transferred to clean, standard caging and the MPTP door signs and cage tags may be removed. This local exhaust ventilation should be wiped down with detergent and water after use. Dirty cages and racks should be covered with a full drape and moved to the cage wash for immediate cleaning (74).

3.2 Sample preparation

Common tree shrews (*Tupaia glis*) were separated to two groups. One group was a control that was in normal condition. The other was a MPTP-treated group that was in Parkinsonism condition.

3.2.1 Controlled group

Common tree shrew (*Tupaia glis)* in the controlled group was a representative of a normal condition of this experiment. These animals were used to determine the difference of tryrosine hydroxylase enzyme and alpha-synuclein protein, compared with Parkinsonism condition in MPTP-treated group.

Animals were weighed (80-100 g), anesthetized by 15 mg/kg of Nembutal (Nembutal, Sanofi), and decapitation suddenly was performed. After Isolation of animal's brain, the brains were weighed, and incubated into PBS buffer (see appendix) at 20°C for 30 minutes. Then the brain was moved into brain box (51384, Stoelting), dissected by using the cold blades (10 blades or more/brain). Collect the interested areas that were substaintia nigra, caudate, and putamen, and then weight the interested area in 1.5 ml tube.

Substaintia nigra, caudate, and putamen were homogenized with a little amount of PBS buffer in homogenizer, mixed 400 ul of the sample with 200 ul of 2x treatment buffer (see appendix) and stored the sample at 20°C until use.

3.2.2 MPTP-treated group

Common tree shrew (*Tupaia glis)* in the MPTP-treated group was a representative of a Parkinsonism condition of this experiment. The experiment used 5 animals to determine the different of tyrosine hydroxylase enzyme and alpha-synuclein protein, compared with normal condition in controlled group.

The MPTP-treated groups were received two injections 10 mg/kg, i.p of MPTP-HCl in normal saline at 2 hours intervals in a single day. The animals became to express Parkinsonism in 3 hours and killed at 10 days after the last injection. Animals were weighed (80-100 g), anesthetized by Nembutal (15 mg/kg), and decapitation suddenly. Isolation of animal's brain, weighed, and incubated the brain into PBS buffer at 20°C for 30 minutes. Then moved the brain into brain box, dissected it by using the cold blades (10 blades or more/brain). Collect the interested area that was substaintia nigra, caudate, and putamen, weighed a part of interested area in 1.5 ml tube.

Substaintia nigra, caudate, and putamen were homogenized with a little amount of PBS buffer in homogenizer, mixed 400 ul of the sample with 200 ul of 2x treatment buffer, and stored the sample at 20°C until use.

3.3 Molecular weight protein markers

Molecular weight of protein was characterized by using standard protein marker that was known the molecular weight of protein bands. In this study, three sets of series of known proteins were used as molecular weight marker for SDS-PAGE and Western blot analysis as the followings: MW-SDS 200 (SDS-6H, Sigma) identified the molecular weight at 29-205 kDa, rainbow marker (RPN756, Amersham biosciences) identified the molecular weight at 14-220 kDa, and prestained protein marker (P1677, Sigma) identified the molecular weight at 26-116 kDa. Moreover, some specific well known proteins were stilled used to be confirmed markers. These specific proteins were mentioned respectively as: bovine serum albumin marker (A7517, Sigma) identified the molecular weight at 66 kDa, ovalbumin marker (A7643, Sigma) identified the molecular weight at 45 kDa, carbonic anhydrase marker (C2273, Sigma) identified the molecular weight at 29 kDa, and lysozyme marker (US18645, Amersham biosciences) identified the molecular weight at 14 kDa. The experiment used the standard protein markers to characterize and identify the molecular weight of tyrosine hydroxylase enzyme and alpha-synuclein protein in SDS-PAGE and Western blot analysis.

3.4 SDS-PAGE analysis

3.4.1 Casting the separating gel

Assemble the gel caster (mini VE, Hoefer). Use the clean and transparent glass plate by clean it with soft detergent, alcohol, and allow to air dry.

Table 2 Casting the separating gel.

10 % Separating gel	
Acrylamide solution	5.00 ml
0.5 M Tris-Cl, pH 8.8	3.75 ml
10% SDS	0.15 ml
Deionized water	6.00 ml
Mixed	
10% (w/v) ammonium persulfate	90.00 ul
TEMED	10.00 ul
Total monomer	15.00 ml

10% Polyacrylamide gel was suitable for protein separation in tyrosine hydroxylase enzyme and alpha-synuclein protein. Prepare the separating gel solution for two gels by combine acrylamide solution 5.00 ml (see appendix), 1.5 M Tris-Cl (pH8.8) 3.75 ml (see appendix), 10% SDS 0.15 ml (20783, Serva), and deionized water 6.00 ml. Mix the combination in test tube by vortex and added 10% APS (freshly) 100 ul (see appendix) and TEMED 10 ul (35925, Serva) in mixture (Table 2). Pipette the separating gel solution to about 3 cm below the top of glass plat by use auto pipette (Glison). Overlay the separating gel with a thin layer of water-saturated n-butanol (see appendix) to prevent gel exposure to oxygen and made gel surface to smooth. Apply separating gel solution and water-saturated n-butanol near the spacer at the one side. Allow the separating gel to polymerize for 45 minutes.

3.4.2 **Casting the stacking gel** (4%polyacrylamide gel)

After the separating gel has polymerized, remove water-saturated n-butanol by rinsing the top of the gel with distilled water for three times.

Table 3 Casting the stacking gel.

4 % Stacking gel	
Acrylamide solution	1.00 ml
0.5 M Tris-Cl, pH 6.8	1.88 ml
10% SDS	0.08 ml
Deionized water	4.50 ml
Mixed	
10% (w/v) ammonium persulfate	30.00 ul
TEMED	10.00 ul
Total monomer	7.50 ml

Prepare the stacking gel solution by added acrylamide solution 1.40 ml, 1.5 M, Tris-Cl (pH6.8) 1.25 ml (see appendix), 10% SDS 0.05 ml, and deionized water 3.00 ml. Mixed in test tube and add 10% APS (freshly) 25 ul, TEMED 5 ul (Table 3). Pour the stacking gel solution onto the separating gel. Introduce a comb into the stacking gel, taking care not to trap air under the comb.

Allow a minimum of 20 minutes for the stacking gel to complete polymerize. Remove a comb by carefully pulling on comb. Rinse the wells with electrophoresis buffer to remove an unpolymerized acrylamide.

3.4.3 Electrophoresis

Assemble electrophoresis apparatus (mini VE, Hoefer); wash out wells with electrophoresis buffer (see appendix). Thaw the sample (substaintia nigra, caudate, and putamen) in the ice and boiled the samples (substaintia nigra, caudate, and putamen), protein marker in boiling water for 2-5 minutes. Add electrophoretic buffer in upper chamber. Load 40 ul of the sample (substaintia nigra, caudate, and putamen) and protein marker (10 ul of MW-SDS 200 marker or/and 7.5 ul of rainbow marker) in one gel. For the other one applied 40 ul of the sample (substaintia nigra, caudate, and putamen), protein marker (20 ul of prestained protein marker or/and 7.5 ul of rainbow marker). Add electrophoresis buffer in lower chamber to maximum level, final assembly the electrophoresis unit. Plug the color-code leads into the jacks of an approved power supply (red to red, black to black). Set electrophoretic condition to 300V, 150mA, and 100W.

3.4.4 Coomassie Blue staining

Place gel into the coomassie blue container that contained 500 ml of Coomassie Blue stain solution (see appendix) for 1 hour. Remove Coomassie Blue stain solution from container and destaine gel in destining solution I (see appendix) for 1 hour. Do not destain too longer because it remove too much stain from protein banding. If this happens, the gel can be restain in coomassie blue stain solution again. Finally, destaine gel in destainning solution II (see appendix) for 8 hour or until the background was clear.

Wrap gel in sealed a plastic bag and made triples seal, stored at 4°C or gel can be dried on gel drier.

3.5 Western blot analysis (Immunoblotting)

3.5.1 Transferation of protein onto nitrocellulose membrane

Protein samples (total proteins) were subjected to polyacrylamide gel electorphoresis (SDS-PAGE). Equilibrate the gel in transfer buffer (see appendix) for 10 minutes. When equilibration allowed the gel to swell or shrink before it contacted the transfer membrane. Pre-wet nitrocellulose membranes (Hybond, Amersham pharmacia) in deionized water, taking care not to trap air bubble by dip one end of nitrocellulose membrane into the transfer buffer and slowly submerge. After pre-wetting, soak nitrocellulose membrane in transfer buffer for 5 minutes, and wet the two pieces of blotting paper (RPN6101M, Amersham pharmacia) and the three of sponges in transfer buffer.

Assemble the Electro blotter (mini VE, Hoefer), center a packing sponge on the black cathode site, lay one piece of blotting paper on the sponge, put the equilibrated gel on the blotting paper try to place the gel correctly at the first time because proteins may begin to transfer immediately; once transfer has begun, moving the gel will distort results or cause "shadow band" on the blot.

Wet the gel surface with a few drops of transfer buffer. Lay the nitrocellulose membrane on the gel. Do not reposition the nitrocellulose membrane once it contacts the gel. Lay one piece of wet blotting paper on the nitrocellulose membrane, and lay two packing of sponges on the blotting paper. Close the Electro blotter; pour 300 ml of transfer buffer into the top of module. Put in the tank the black cathode site faces forward the center, the red anode side faces toward the outside tank wall. Add the cool deionized water to the tank. Place the safety lid on the tank, plug the color-code leads into the jacks of an approved power supply. Set electorphoretic transfer conditions to 25 V, 350 mA, and 25 W for 2 hours.

Turn off the power supply and disconnect the leads. Remove the safety lid and lift out the tank. Open the Electro blotter module, and remove the gel from the nitrocellulose membrane.

3.5.2 Detection of tyrosine hydroxylase enzyme

The protein in the gel was electrophoretically transferred to the nitrocellulose membrane. The nitrocellulose membrane was then soaked in blocking solution (see appendix). Place nitrocellulose membrane into the small container and add 20 ml of blocking solution to "block" it ability to non-specifically bind protein. Shake for 90 minutes at room temperature. *After protein transfer to membrane, gel may be stained in Coomassie Blue stain) to determine the efficiency of protein transfer.

Monoclonal anti-tyrosine Hydroxylase (T2928, Sigma) was a primary antibody for tyrosine hydroxylase enzyme detection. It recognizes an epitope in the N-terminal region of both rodent (~60 kDa) and human (62-68 kDa) and may be use for the localization of tyrosine hydroxylase enzyme. Dilute the anti-tyrosine hydroxylase (mouse anti rat) in blocking solution to 1:10,000. Prepare 10 ml of the anti-tyrosine hydroxylase by dilute 10 ul of anti-tyrosine hydroxylase with 9,990 ul of PBS buffer. The concentration became to 1:1,000. The stock solution (1:1,000) was aliquot to 20 tubes (500 ul per tube). Dilute 1,000 ul (2 tube of stock 1:1,000) of anti-tyrosine hydroxylase with 9,000 ul of blocking solution. Mix the combination, final dilution was 1:10,000 for 10 ml.

Remove blocking solution and wash the nitrocellulose membrane three times by shaking in 10 ml of PBS, 5 minute for each washing. Then add 10 ml of anti-tyrosine hydroxylase in the tyrosine hydroxylase container. Incubate nitrocellulose membrane with anti-tyrosine hydroxylase and shake for overnight at room temperature.

Wash three times for 5 minutes each with 10 ml of PBS-Tween 20 (see appendix). Incubate nitrocellulose membrane with 10 ml of biotinylated–conjugated polyclonal anti-mouse secondary antibody of solution B (85-8943, Zymed), shake for 45 minutes.

Wash 2 minute for three times with 10 ml of PBS without Tween 20. Incubates membrane with streptavidin-conjugated HRP (solution C) (85-8943, Zymed) for 45 minutes, wash 2 minutes for three times with 10 ml of PBS without Tween 20. Finally, incubate membrane with 10 ml of DAB chromogen (00-2014, Sigma) for 15 minutes or until desired intensity was reached.

3.5.3 Detection of alpha-synuclein protein

The protein in the gel was electrophoretically transferred to the nitrocellulose membrane. The nitrocellulose membrane was then soaked in blocking solution. Place nitrocellulose membrane into the small container and add 20 ml of blocking solution to "block" it ability to non-specifically bind protein. Shake for 90 minutes at room temperature. *After protein transfer to membrane, gel may be stained in Coomassie Blue stain to determine the efficiency of protein transfer.

Monoclonal anti-synuclein protein (S3062, Sigma) was used as a primary antibody for alpha-synuclein protein detection. It recognizes an epitope in the N-terminal region of mamal (~19kDa) and may be used for the localization of alpha-synuclein protein. Dilute the anti- alpha-synuclein (rabbit anti human) in blocking solution to 1:1,000. Prepare 10 ml of the anti- alpha-synuclein

Remove blocking solution and wash the nitrocellulose membrane three times by shaking in 10 ml of PBS, 5 minute for each washing. Then add 10 ml of anti-alpha-synuclein in the alpha-synuclein container. Incubate nitrocellulose membrane with anti-alpha-synuclein and shake for overnight at room temperature.

Wash three times for 5 minutes each with 10 ml of PBS-Tween 20. Incubate nitrocellulose membrane with 10 ml of anti-rabbit IgG secondary antibody conjugated peroxidase (A0545, Sigma) diluted to 1:10,000 in PBS, shake for 90 minutes.

Wash 5 minute for three times with 10 ml of PBS without Tween 20. Incubates membrane with 10 ml of DAB chromogen for 15 minutes or until desired intensity was reached.

Chapter 4

Results

In this study, common tree shrew was used as a new animal model to study of tyrosine hydroxylase enzyme and alpha-synuclein protein in normal and Parkinsonism conditions. The animals were divided to two groups, one group was controlled in normal condition and the other group was treated in Parkinsonism condition. In Parkinsonism condition, common tree shrew was administrated by 10 mg/kg of MPTP-HCl. The toxic effects of MPTP-HCl induced common tree shrew into Parkinsonism condition.

SDS-PAGE and Western blot analysis were used as molecular techniques for characterization of tyrosine hydroxylase enzyme and alpha-synuclein protein compared in normal and Parkinsonism conditions. In this experiment, three important areas of common tree shrew brain, substantia nigra, caudate and putamen, were included in the studies. These areas were interested because they represented the locations of dopaminergic neurons and their connections.

Tyrosine hydroxylase was characterized by SDS-PAGE and Western blot analysis. In SDS-PAGE analysis, the total proteins of extracted proteins from substantia nigra, caudate and putamen were separately electrophoresed on 10% SDS-PAGE and demonstrated on gel by using coomassie blue stainning. Western blot analysis established the specific protein on nitrocellulose membrane by using monoclonal anti-tyrosine hydroxylase antibody and biotinylated secondary antibody as specific antibodies. In negative control, monoclonal anti-tyrosine hydroxylase antibody was used as primary negative control and biotinylated secondary antibody was used as secondary negative control to confirm the results of this experiment, respectively.

Alpha-synuclein protein was characterized by SDS-PAGE and Western blot analysis. In SDS-PAGE analysis, the total proteins of extracted proteins from substantia nigra, caudate and putamen were separately electrophoresed on 10% SDS-PAGE and demonstrated on gel by using coomassie blue stainning. Western blot analysis was used to detect alpha-synuclein protein on nitrocellulose membrane by using monoclonal anti-alpha-synuclein antibody and anti-rabbit IgG secondary antibody as specific antibodies. In negative control, monoclonal anti-alpha-synuclein antibody was used as primary negative

control and anti-rabbit IgG secondary antibody was used as secondary negative control to confirm the results of this study, respectively.

4.1 Common tree shrew brain (*Tupaia glis*)

After decapitation, the common tree shrew brain was weighted and incubated into cold-PBS for 30 minutes in 20°C. The brain size had approximately 2.5 x 3.6 cm (Figure 11 A-B). Then, the brain was serially dissected in brain box by cold-blades. The coronal sections had thickness approximately to 2 mm. and were moved on filter paper for selection of the interested areas. The sections were separately isolated for the substantia nigra, caudate, and putamen. Substantia nigra was prominently observed as the dark-brow area in the lower part of the brain that contains dopaminergic neurons for dopamine synthesis. This area was located over the crus cerebri (Figure 11C). Caudate nucleus was the gray matter sitting on the lateral side of the lateral ventricles and appeared to blend into another nucleus, the putamen. Mostly, these two nuclei were divided by a band of axons called the internal capsule (Figure 11D). The small bridge which connects between caudate and putamen ventromedially was named the nucleus accumbens. All three nuclei were always collectively called the striatum. According to the photographs shown in the Figure 11A-D of the common tree shrew brain verified many areas in the midbrain as the followings: anterior commissure (AC), cerebral aqueduct (CA), caudate nucleus (Ca), cerebrum (CB), corpus callosum (CC), crus cerebri (Cc), cerebellum (CL), globus pallidus (GP), internal capsule (IC), longitudal fissure (LF), lateral ventricle (LV), olfactory bulb (OB), pons (Pons), putamen (Pu), and substantia nigra (Sn).

4.2 Characterization of tyrosine hydroxylase enzyme in normal condition

4.2.1 SDS-PAGE of tyrosine hydroxylase enzyme in normal condition

The proteins extracted from substaintia nigra, caudate, and putamen of the controlled group were separated on a 10% SDS-PAGE. Coomassie blue staining showed many bands of total protein samples at molecular weight approximately 20, 30-45, and 45-60 kDa. One band at molecular weight approximately 45-66 kDa (red arrow head) was expected to be tyrosine hydroxylase enzyme (Figure 12). Total proteins samples from common tree shrew brain in normal condition were separated on 10% SDS-PAGE as the followings: 10 ul of MW-SDS 200 marker (lane 1), 15 ul of MW-SDS 200 marker (lane 2), 2 ul of lysozyme marker (14 kDa) (lane 3 and 7), 40 ul of protein extracted from substantia

nigra (lane 4), 40 ul of protein extracted from caudate (lane 5), and 40 ul of protein extracted from putamen (lane 6).

4.2.2 Western blot analysis of tyrosine hydroxylase enzyme in normal condition

On Western blot analysis of tyrosine hydroxylase enzyme, interested protein band at molecular weight approximately 45-66 kDa was observed by using the monoclonal anti-tyrosine hydroxylase antibody. The antibody directed against the tyrosine hydroxylase subunit: a 60 kDa band corresponding to rodent tyrosine hydroxylase and 62-68 kDa band corresponding to human tyrosine hydroxylase. The monoclonal anti-tyrosine hydroxylase antibody was diluted in blocking solution (5% dry milk solution); the final dilution was 1:10,000. Western blot analysis established the specific protein band at molecular weight approximately 62 kDa on nitrocellulose membrane in three protein samples from substaintia nigra, caudate, and putamen in common tree shrew brain (Figure 13). It should represent tyrosine hydroxylase enzyme. The substaintia nigra revealed the higher amount of tyrosine hydroxylase by representing the higher intensity of protein band on nitrocellulose membrane.

Western blot analysis demonstrated the expected tyrosine hydroxylase enzyme from common tree shrew brain in normal condition by using anti-tyrosine hydroxylase antibody as following sequences: 20 ul of Prestained protein marker (lane 1), 7.5 ul of rainbow marker (lane 2), 40 ul of protein extracted from substantia nigra (lane 3), 40 ul of protein extracted from caudate (lane 4), and 40 ul of protein extracted from putamen (lane 5).

4.2.3 Western blot analysis of negative control of primary and secondary antibodies in normal condition

Monoclonal anti-tyrosine hydroxylase was used as primary negative control in this experiment. The monoclonal anti-tyrosine hydroxylase antibody (primary antibody) dilution to 1:10,000 was used as primary antibody and incubated with DAB chromogen. After the incubation, there was no any protein bands representing on nitrocellulose membrane (Figure 14). Western blot analysis verified the primary negative control of anti-tyrosine hydroxylase antibody as the followings: 20 ul of Prestained protein marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

Biotinylate–conjugated polyclonal anti-mouse secondary antibody and streptavidin-conjugated HRP (Histostain plus kit) was used as secondary negative control to confirm the result of Western blot analysis. After incubation of nitrocellulose membrane with secondary antibody and DAB chromogen, the membrane hasn't shown any protein bands (Figure 15). Western blot analysis revealed the secondary negative control of secondary antibody in normal condition as following sequences: 20 ul of Prestained protein marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

4.3 Characterization of alpha-synuclein protein in normal condition

4.3.1 SDS-PAGE of alpha-synuclein protein in normal condition

The proteins extracted from substaintia nigra, caudate, and putamen of the controlled group were separated on 10% SDS-PAGE. Coomassie blue staining showed many bands of total protein samples at molecular weight approximately 20, 30-45, and 45-60 kDa (Figure 16). The protein band at molecular weight approximately 20 kDa was expected to be alpha-synuclein protein (red arrow head).

Total proteins samples from common tree shrew brain in normal condition were separately electrophoresed on 10% SDS-PAGE as the followings: 10 ul of MW-SDS 200 marker (lane 1), 15 ul of MW-SDS 200 marker (lane 2), 2 ul of lysozyme marker (14 kDa) (lane 3 and 7), 40 ul of protein extracted from substantia nigra (lane 4), 40 ul of protein extracted from caudate (lane 5), and 40 ul of protein extracted from putamen (lane6).

4.3.2 Western blot analysis of alpha-synuclein protein in normal condition

On Western blot analysis of alpha-synuclein were observed by using the monoclonal anti-alpha-synuclein antibody. The antibody directed against the alpha-synuclein subunit: a 19-kDa band corresponding to human alpha-synuclein protein. The monoclonal anti-alpha-synuclein antibody was diluted in blocking solution (5% dry milk solution); the final dilution was 1:1,000. Western blot analysis demonstrated inconsiderable protein band on nitrocellulose membrane at molecular weight approximately 45 kDa in substaintia nigra but didn't revealed in caudate, and putamen samples (Figure 17).

In this experiment, Western blot analysis was used to detect alpha-synuclein protein from common tree shrew brain in normal condition by using anti-alpha-synuclein antibody as following sequences: 7.5 ul of rainbow marker (lane 1), 40 ul of protein

extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

4.3.3 Western blot analysis of negative control of primary and secondary antibodies in normal condition

Monoclonal anti-alpha-synuclein was used as primary negative control of this experiment. The only monoclonal anti-alpha-synuclein antibody (primary antibody) dilution to 1:1,000 was used as primary antibody and incubated with DAB chromogen. After the incubation, there was no any protein bands representing on nitrocellulose membrane (Figure 18). Western blot analysis showed primary negative control of anti-alpha-synuclein antibody as the followings: 7.5 ul of rainbow marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

Anti-rabbit IgG antibody was conjugated with peroxidase (secondary antibody) dilution to 1:10,000 was used as secondary negative control to confirm the result of Western blot analysis. After incubation of nitrocellulose membrane with secondary antibody and DAB chromogen, the membrane hasn't established any protein bands (Figure 19). Western blot analysis verified secondary negative control of secondary antibody in normal condition as following sequences: 7.5 ul of rainbow marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4)).

4.4 Characterization of tyrosine hydroxylase enzyme in Parkinsonism condition

4.4.1 SDS-PAGE of tyrosine hydroxylase enzyme in Parkinsonism condition

The proteins extracted from substaintia nigra, caudate, and putamen of the the MPTP-treated group were separated on a 10% SDS-PAGE. Coomassie blue staining demonstarted many bands of total protein samples at molecular weight approximately 20, 30-45, and 45-66 kDa. One band at molecular weight approximately 45-66 kDa was expected to be tyrosine hydroxylase enzyme (Figure 20). Total proteins samples from common tree shrew brain in Parkinsonism condition were separated on 10% SDS-PAGE as the followings: 7.5 ul of rainbow marker (lane 1), 10 ul of MW-SDS 200 marker (lane 2), 3 ul of bovine serum albumin marker (66 kDa) (lane3), 2 ul of cabonic anhydrase marker (29

kDa) (lane4), 40 ul of protein extracted from substantia nigra (lane 5), 40 ul of protein extracted from caudate (lane 6), and 40 ul of protein extracted from putamen (lane7).

4.4.2 Western blot analysis of tyrosine hydroxylase enzyme in Parkinsonism condition

On Western blot analysis of tyrosine hydroxylase enzyme revealed one band at molecular weight approximately 45-66 kDa was observed by using the monoclonal anti-tyrosine hydroxylase antibody. The antibody directed against the tyrosine hydroxylase subunit: a 60 kDa band corresponding to rodent tyrosine hydroxylase and 62-68 kDa band corresponding to human tyrosine hydroxylase. The monoclonal anti-tyrosine hydroxylase antibody was diluted in blocking solution (5% dry milk solution); the final dilution was 1:10,000. Western blot analysis was no any protein bands representing on nitrocellulose membrane (Figure 21).

Western blot analysis of tyrosine hydroxylase enzyme hasn't demonstrated any protein bands from common tree shrew brain in Parkinsonism condition by using anti-tyrosine hydroxylase antibody as following sequences: 20 ul of Prestained protein marker (lane 1), 7.5 ul of rainbow marker (lane 2), 40 ul of protein extracted from substantia nigra (lane 3), 40 ul of protein extracted from caudate (lane 4), and 40 ul of protein extracted from putamen (lane 5).

4.4.3 Western blot analysis of negative control of primary and secondary antibodies in Parkinsonism condition

Monoclonal anti-tyrosine hydroxylase was used as primary negative control in this experiment. The monoclonal anti-tyrosine hydroxylase antibody (primary antibody) dilution to 1:10,000 was used as primary antibody and incubated with DAB chromogen. After the incubation, there was no any protein bands representing on nitrocellulose membrane (Figure 22). Western blot analysis verified primary negative control of anti-tyrosine hydroxylase antibody in Parkinsonism condition as the followings: 20 ul of Prestained protein marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

Biotinylate–conjugated polyclonal anti-mouse secondary antibody and streptavidin-conjugated HRP (Histostain plus kit) was used as secondary negative control to confirm the result of Western blot analysis. After incubation of nitrocellulose membrane with secondary antibody and DAB chromogen, the membrane hasn't established any protein

bands (Figure 23). Western blot analysis showed secondary negative control of secondary antibody in Parkinsonism condition as following sequences: 20 ul of Prestained protein marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

4.5 Characterization of alpha-synuclein protein in Parkinsonism condition

4.5.1 SDS-PAGE of alpha-synuclein protein in Parkinsonism condition

The proteins extracted from substaintia nigra, caudate, and putamen of the MPTP-treated group were separated on a 10% SDS-PAGE. Coomassie blue staining demonstrated many bands of total protein samples at molecular weight approximately 20, 30-45, and 45-66 kDa (Figure 24). Total proteins samples from common tree shrew brain in Parkinsonism condition were separately electrophoresed on 10% SDS-PAGE as the followings: 7.5 ul of rainbow marker (lane 1), 10 ul of MW-SDS 200 marker (lane 2), 2 ul of lysozyme marker (14 kDa) (lane3), 2 ul of cabonic anhydrase marker (29 kDa) (lane4), 40 ul of protein extracted from substantia nigra (lane 5), 40 ul of protein extracted from caudate (lane 6), and 40 ul of protein extracted from putamen (lane7).

4.5.2 Western blot analysis of alpha-synuclein protein in Parkinsonism condition

On Western blot analysis, the specific protein bands were observed by using the monoclonal anti-alpha-synuclein antibody. The antibody directed against the alpha-synuclein subunit: a 19-kDa band corresponding to human alpha-synuclein protein. The monoclonal anti-alpha-synuclein antibody was diluted in blocking solution (5% dry milk solution), the final dilution was 1:1,000. Western blot analysis verified the specific band on nitrocellulose membrane at molecular weight approximately 19 kDa in all sample of substaintia nigra, caudate, and putamen (Figure 25). It should represent alpha-synuclein protein which was dominantly expressed and triggered after the animal had been induced into Parkinsonism condition. Morever, Western blot analysis revealed the other protein band at molecular weight approximately 45 kDa only in substaintia nigra but hasn't shown in caudate and putamen. This protein demonstrated in same manner as in normal condition.

Western blot analysis established the expected alpha-synuclein protein from common tree shrew brain in Parkinsonism condition by using anti-alpha-synuclein antibody as the followings: 20 ul of MW-SDS 200 marker (lane 1), 7.5 ul of rainbow marker (lane 2),

40 ul of protein extracted from substantia nigra (lane 3), 40 ul of protein extracted from caudate (lane 4), and 40 ul of protein extracted from putamen (lane 5). Western blot analysis of alpha-synuclein showed protein band at molecular weight approximately 19 kDa (red arrow head) in three samples (lane 2, 3, and 4) and one band at molecular weight approximately 45 kDa (black arrow head) in substantia nigra on nitrocellulose membrane).

4.5.3 Western blot analysis of negative control of primary and secondary antibodies in Parkinsonism condition

Monoclonal anti-alpha-synuclein was used as primary negative control of this experiment. The only monoclonal anti-alpha-synuclein antibody (primary antibody) dilution to 1:1,000 was used as primary antibody and incubated with DAB chromogen. After the incubation, there was no any protein bands representing on nitrocellulose membrane (Figure 26). Western blot analysis revealed primary negative control of anti-alpha-synuclein antibody in Parkinsonism condition as the followings: 7.5 ul of rainbow marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

Anti-rabbit IgG antibody was conjugated with peroxidase (secondary antibody) dilution to 1:10,000 was used as secondary negative control to confirm the result of Western blot analysis. After incubation of nitrocellulose membrane with secondary antibody and DAB chromogen, the membrane didn't establish any protein bands (Figure 27). Western blot analysis verified secondary negative control of secondary antibody in normal condition as following sequences: 7.5 ul of rainbow marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

Table 4 Western blot analysis of tyrosine hydroxylase enzyme and alpha-synuclein proteins by using anti-tyrosine hydroxylase and anti-alpha-synuclein antibodies stained

Condition	Anti-tyrosine hydroxylase Dilution 1:10,000			Anti-alpha-synuclein dilution 1:1,000		
	Control primary	Control secondary	Treatment	Control primary	Control secondary	Treatment
Normal	-	-	S C P +++ ++ ++ (at 62 kDa)	-	-	S C P - - - (at 19 kDa) +* - - (at 45 kDa)
Parkinsonism	-	-	S C P - - - (at 62 kDa)	-	-	S C P +++ ++ ++ (at 19 kDa) +* - - (at 45 kDa)

S: substantia nigra

C: caudate

P: putamen

-: no intensity of protein band

+: low intensity of protein band

++: high intensity of protein band

+++: higher intensity of protein band

+*: low intensity of protein band at 45 kDa

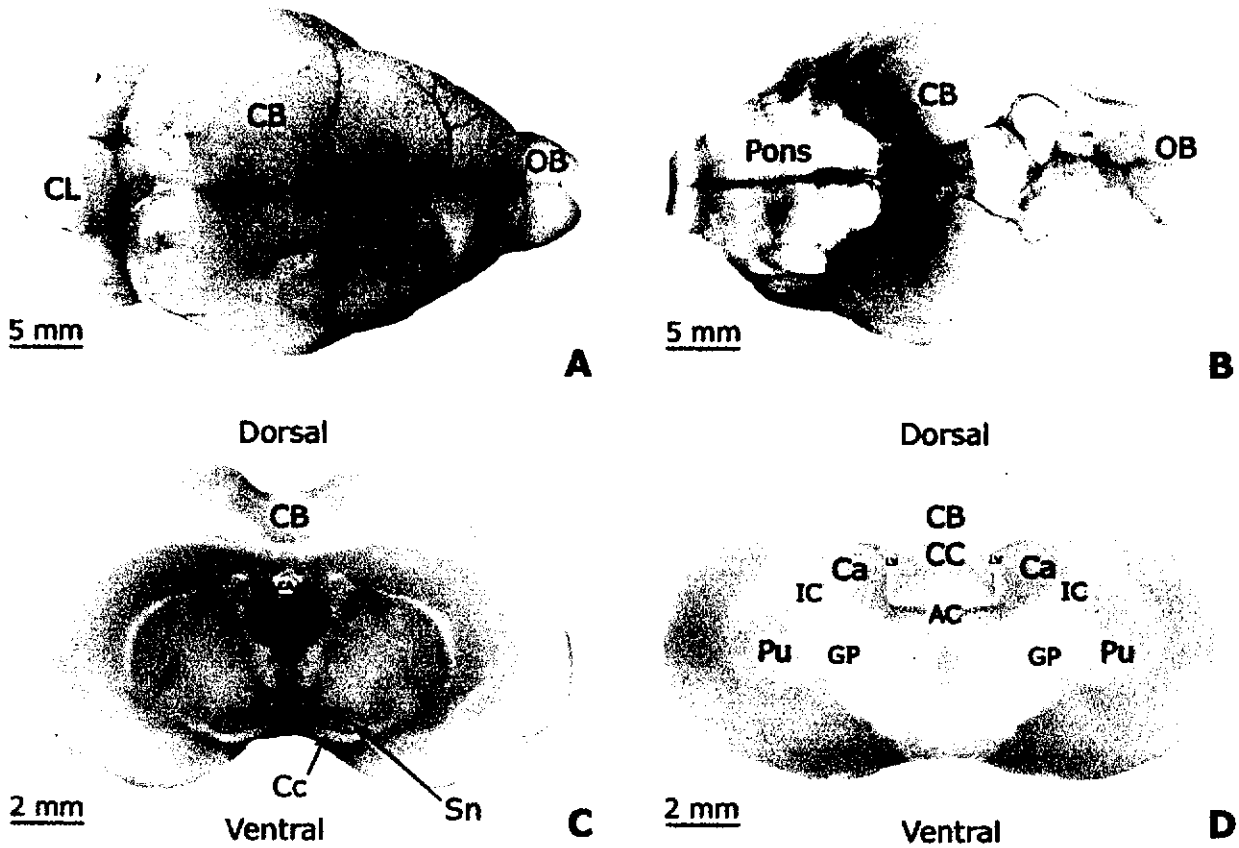

Figure 11 Photographs of the common tree shrew brain (*Tupaia glis*) A: dorsal aspect as the followings; cerebrum (CB), cerebellum (CL), longitudal fissure (LF), and olfactory bulb (OB). B: ventral aspect as the followings; cerebellum (CB), pons (Pons), and olfactory bulb (OB). C: Coronal section through the midbrain demonstrating as the followings; cerebrum (CB), cerebral aqueduct (CA), crus cerebri (Cc), and the substantia nigra (Sn). D: Coronal section through the midbrain showing as the followings; anterior commissure (AC), caudate nucleus (Ca), cerebrum (CB), corpus callosum (CC), globus pallidus (GP), internal capsule (IC), lateral ventricle (LV), and putamen (Pu).

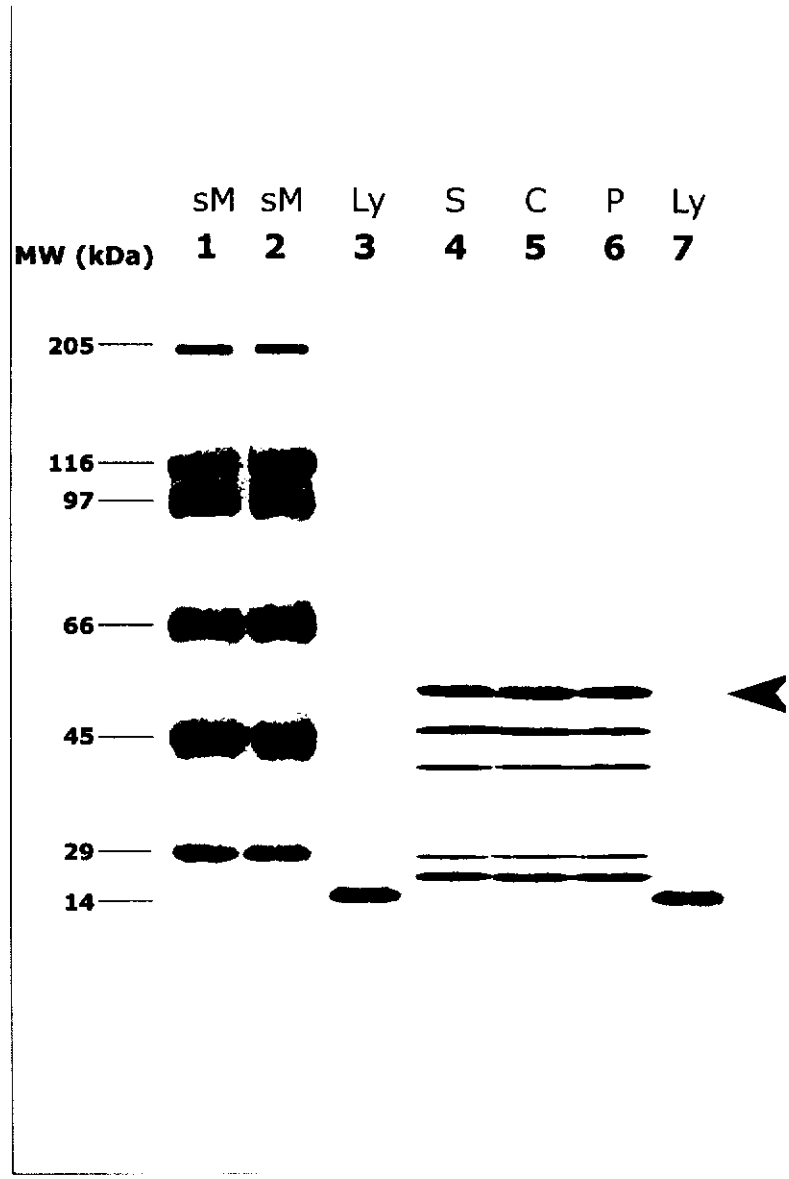

Figure 12 10% SDS-PAGE analysis demonstrated total proteins samples from common tree shrew brain in normal condition as the followings: 10 ul of MW-SDS 200 marker (lane 1), 15 ul of MW-SDS 200 marker (lane 2), 2 ul of lysozyme marker (14 kDa) (lane 3 and 7), 40 ul of protein extracted from substantia nigra (lane 4), 40 ul of protein extracted from caudate (lane 5), and 40 ul of protein extracted from putamen (lane 6). Protein band at molecular weight approximately 45-66 kDa (red arrow head) was expected to be tyrosine hydroxylase enzyme.

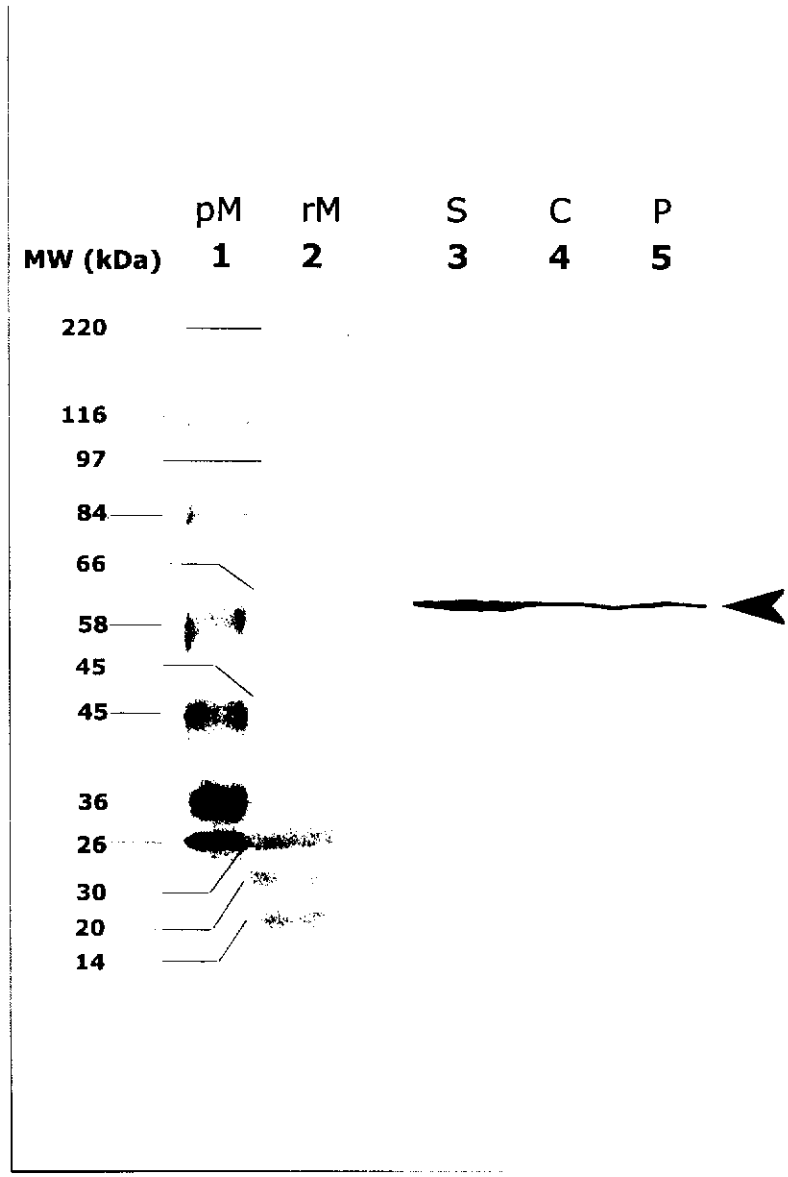

Figure 13 Western blot analysis revealed tyrosine hydroxylase enzyme from common tree shrew brain in normal condition as following sequences: 20 ul of Prestained protein marker (lane 1), 7.5 ul of rainbow marker (lane 2), 40 ul of protein extracted from substantia nigra (lane 3), 40 ul of protein extracted from caudate (lane 4), and 40 ul of protein extracted from putamen (lane 5). Specific protein bands at molecular weight approximately 62 kDa (red arrow head) should represent tyrosine hydroxylase enzyme (lane 3, 4, and 5).

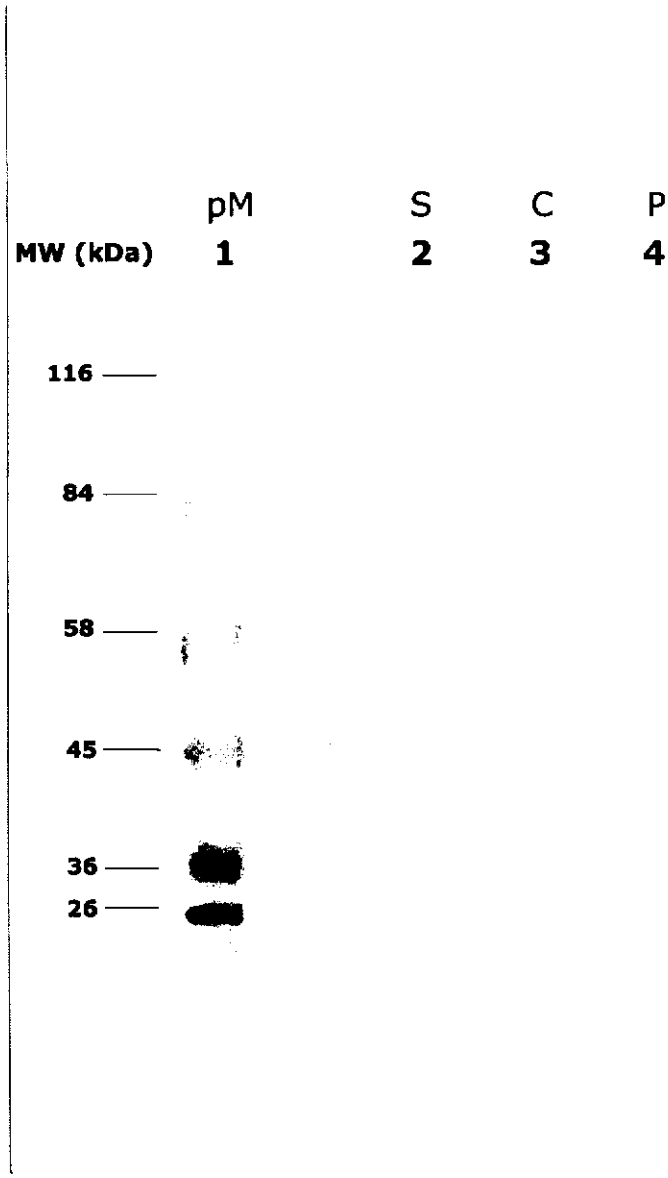

Figure 14 Western blot analysis of primary negative control of anti-tyrosine hydroxylase antibody, 20 ul of Prestained protein marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

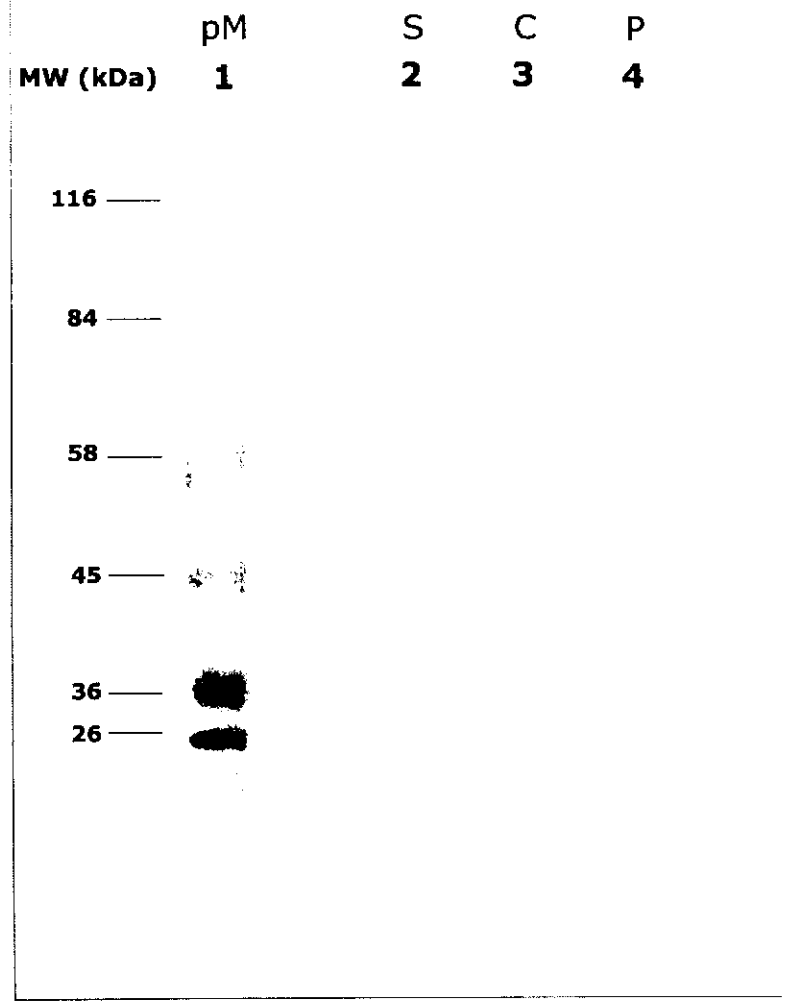

Figure 15 Western blot analysis of secondary negative control of Biotinylated secondary antibody in normal condition, 20 ul of Prestained protein marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

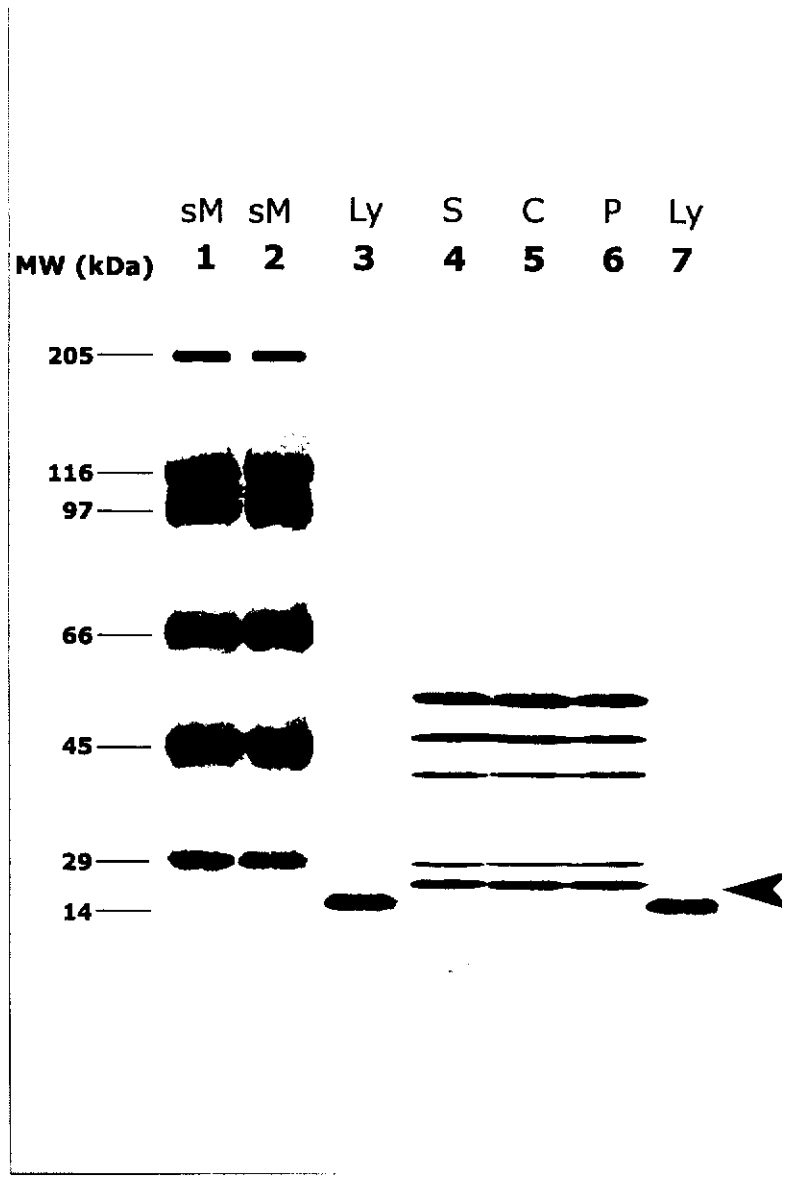

Figure 16 10% SDS-PAGE analysis demonstarted total proteins samples from common tree shrew brain in normal condition as the followings: 10 ul of MW-SDS 200 marker (lane 1), 15 ul of MW-SDS 200 marker (lane 2), 2 ul of lysozyme marker (14 kDa) (lane 3 and 7), 40 ul of protein extracted from substantia nigra (lane 4), 40 ul of protein extracted from caudate (lane 5), and 40 ul of protein extracted from putamen (lane6). Separated protein band at molecular weight approximately 20 kDa was expected to be alpha-synuclein protein (red arrow head).

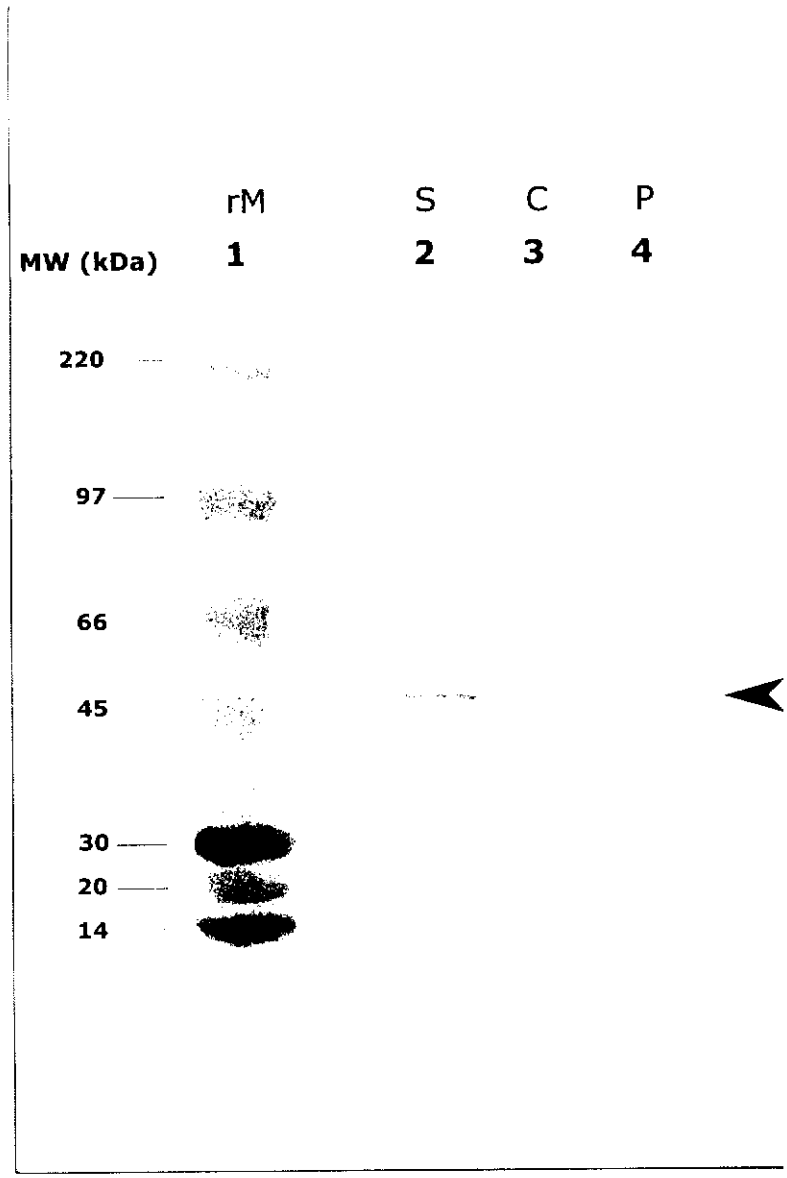

Figure 17 Western blot analysis verified alpha-synuclein protein from common tree shrew brain in normal condition as following sequences: 7.5 ul of rainbow marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4). Western blot analysis of alpha-synuclein showed inconsiderable protein band at molecular weight approximately 45 kDa (red arrow head) in substantia nigra (lane 2) on nitrocellulose membrane.

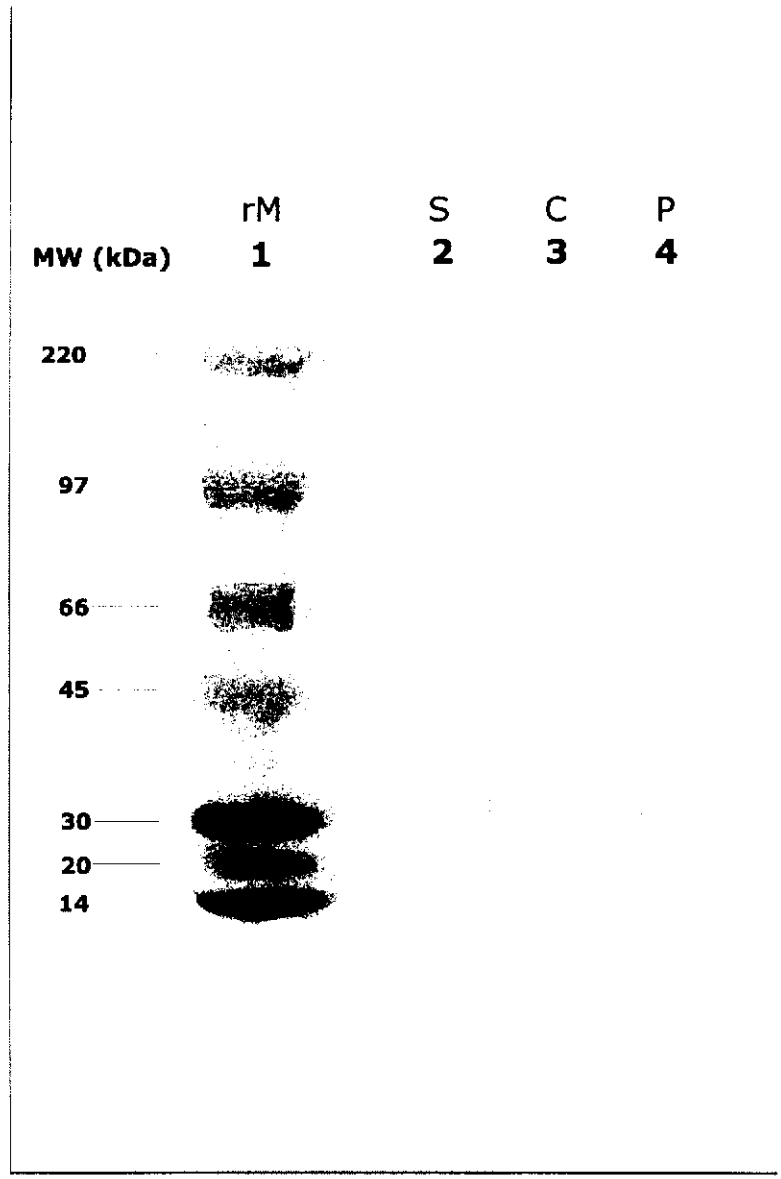

Figure 18 Western blot analysis of primary negative control of anti-alpha-synuclein antibody, 7.5 ul of rainbow marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

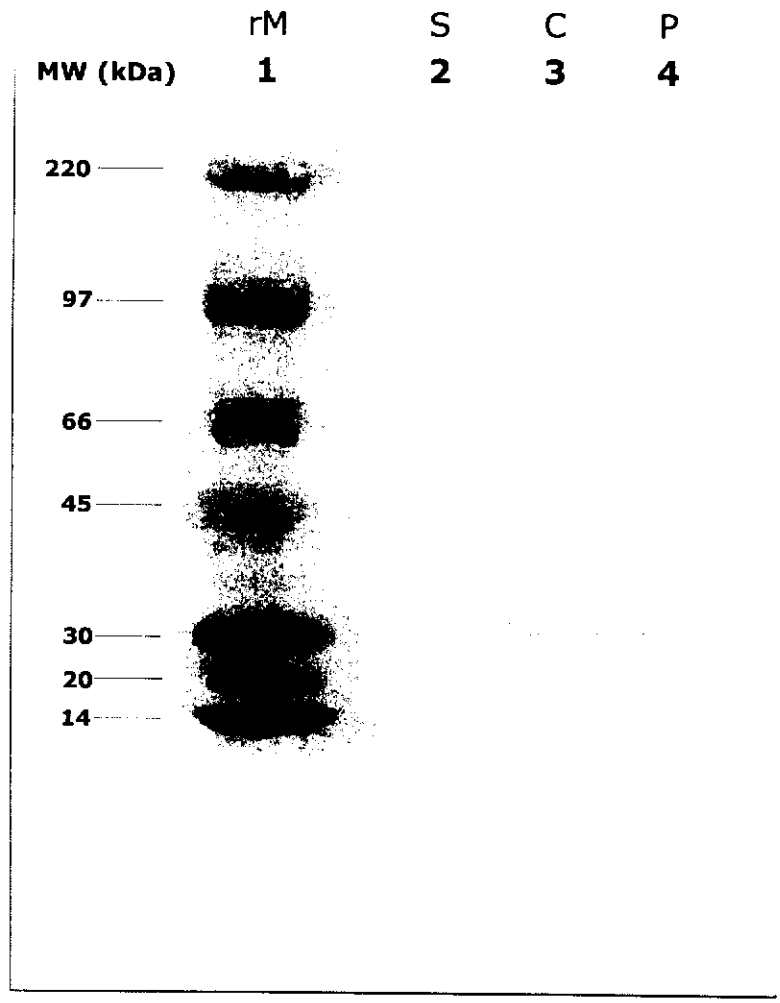

Figure 19 Western blot analysis of secondary negative control of Biotinylated secondary antibody in normal condition, 7.5 ul of rainbow marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

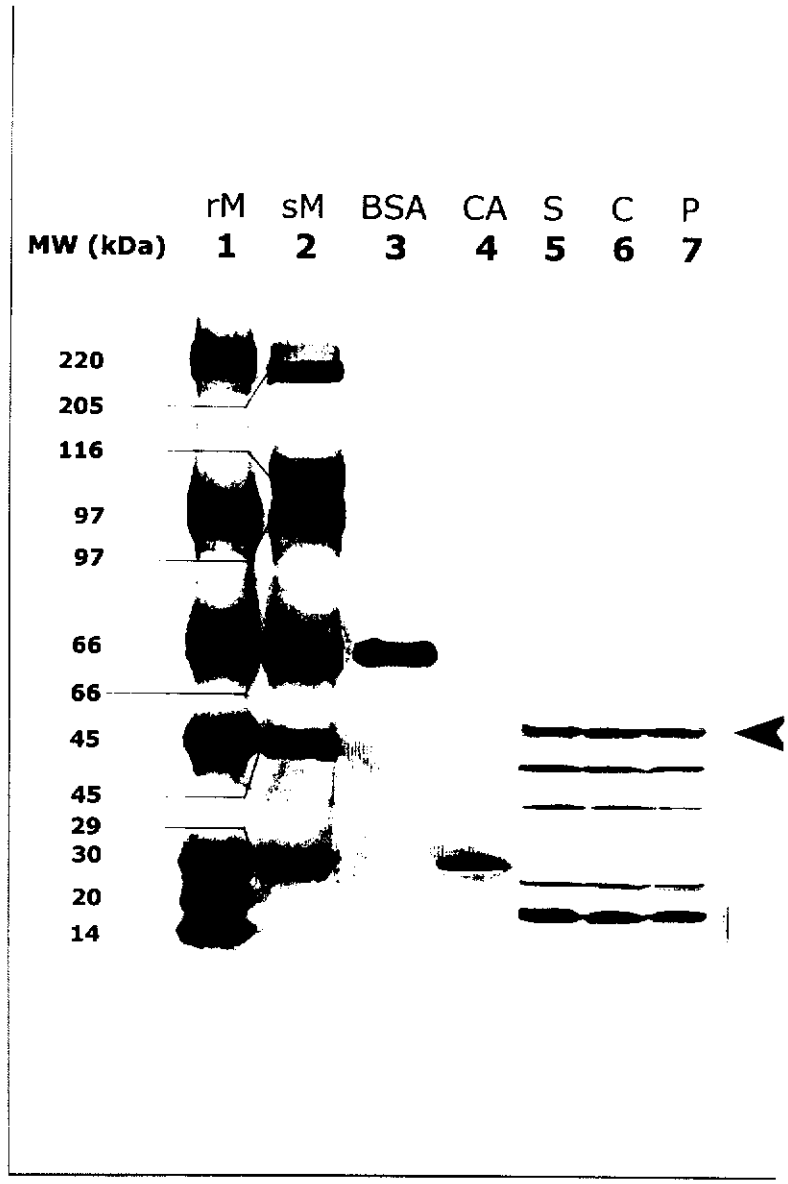

Figure 20 10% SDS-PAGE analysis established total proteins samples from common tree shrew brain in Parkinsonism condition as the followings: 7.5 ul of rainbow marker (lane 1), 10 ul of MW-SDS 200 marker (lane 2), 3 ul of bovine serum albumin marker (66 kDa) (lane3), 2 ul of cabonic anhydrase marker (29 kDa) (lane4), 40 ul of protein extracted from substantia nigra (lane 5), 40 ul of protein extracted from caudate (lane 6), and 40 ul of protein extracted from putamen (lane7). Protein band at molecular weight approximately 45-66 kDa (red arrow head) was expected to be tyrosine hydroxylase enzyme.

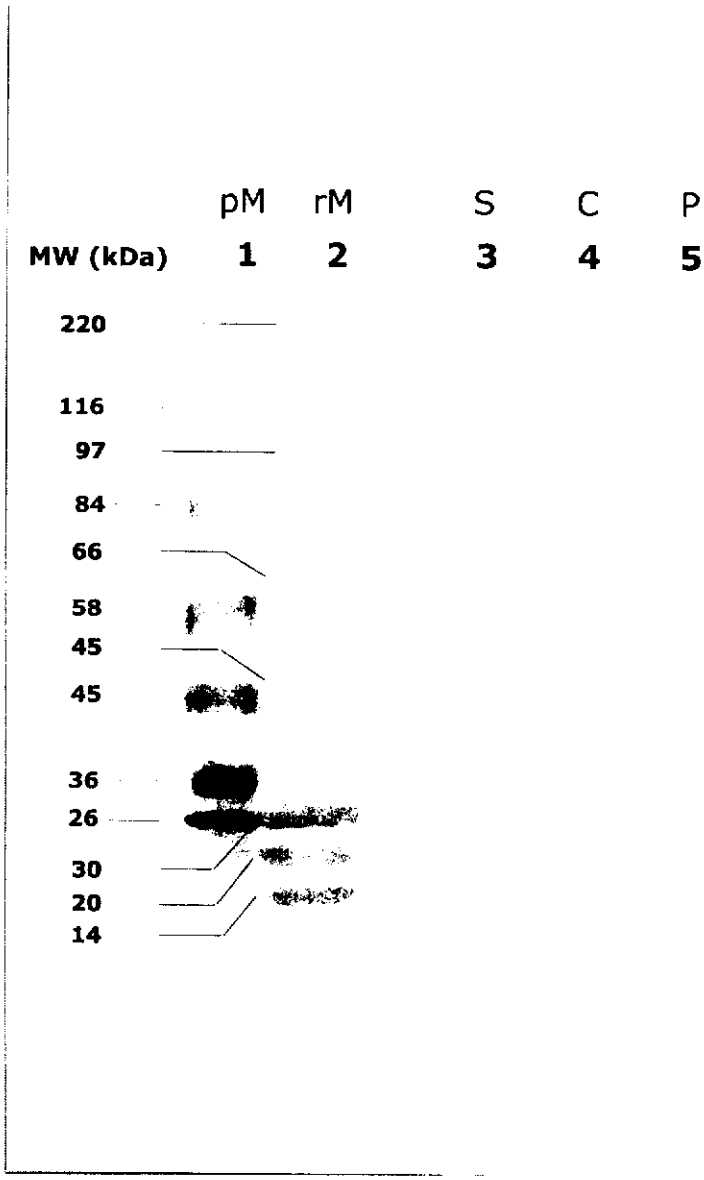

Figure 21 Western blot analysis hasn't revealed tyrosine hydroxylase enzyme from common tree shrew brain in Parkinsonism condition by using anti-tyrosine hydroxylase antibody as following sequences: 20 ul of Prestained protein marker (lane 1), 7.5 ul of rainbow marker (lane 2), 40 ul of protein extracted from substantia nigra (lane 3), 40 ul of protein extracted from caudate (lane 4), and 40 ul of protein extracted from putamen (lane 5). There was no any protein band representing on nitrocellulose membrane.

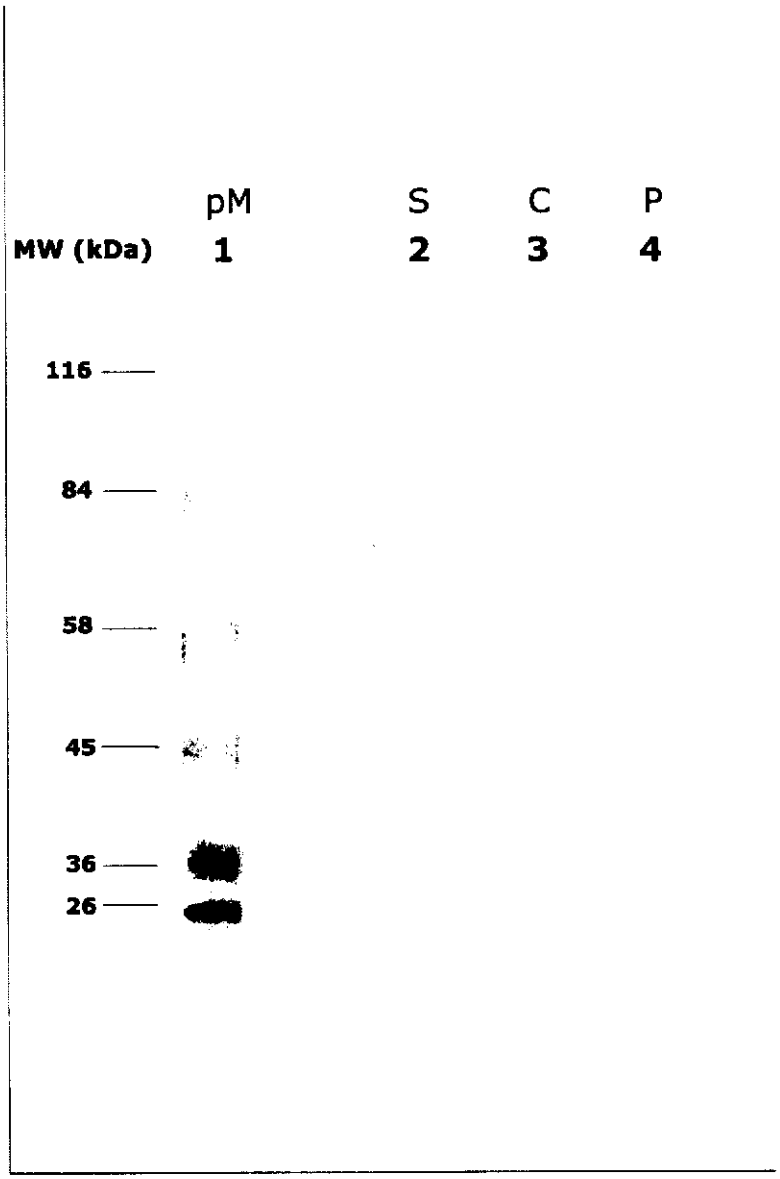

Figure 22 Western blot analysis of primary negative control of anti-tyrosine hydroxylase antibody in Parkinsonism condition, 20 ul of Prestained protein marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

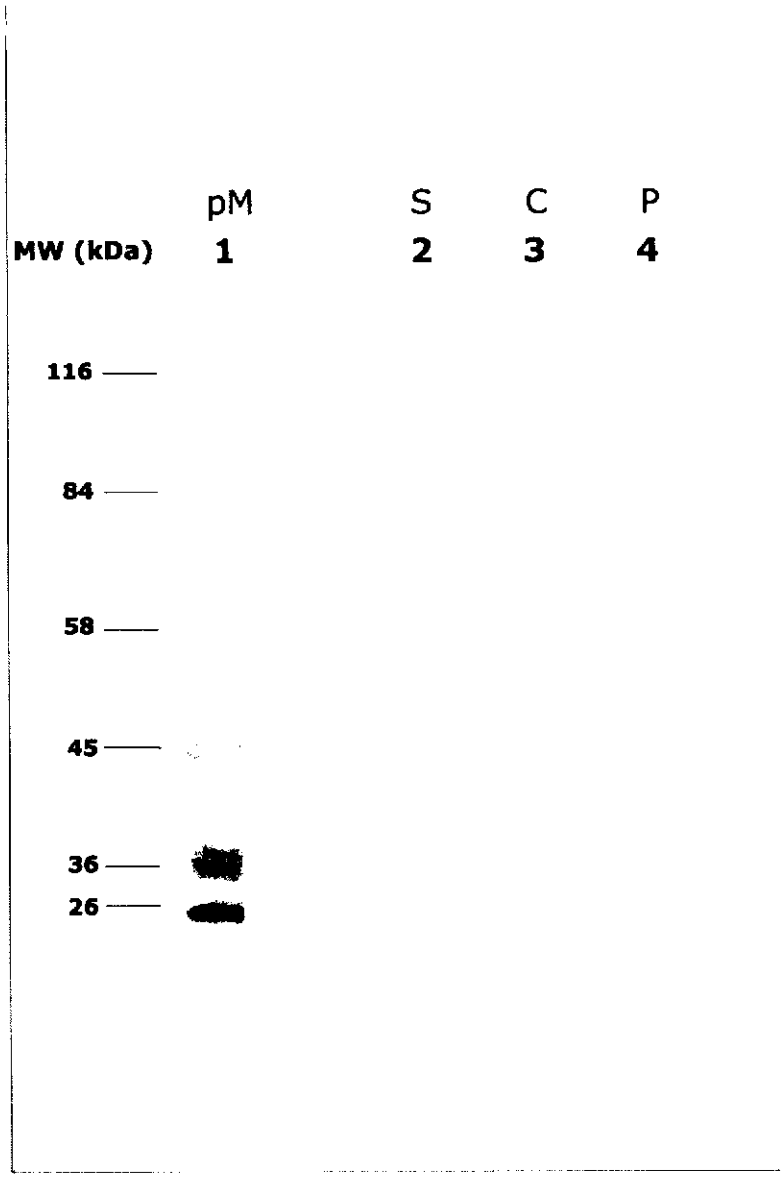

Figure 23 Western blot analysis of secondary negative control of Biotinylated secondary antibody in Parkinsonism condition, 20 ul of Prestained protein marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

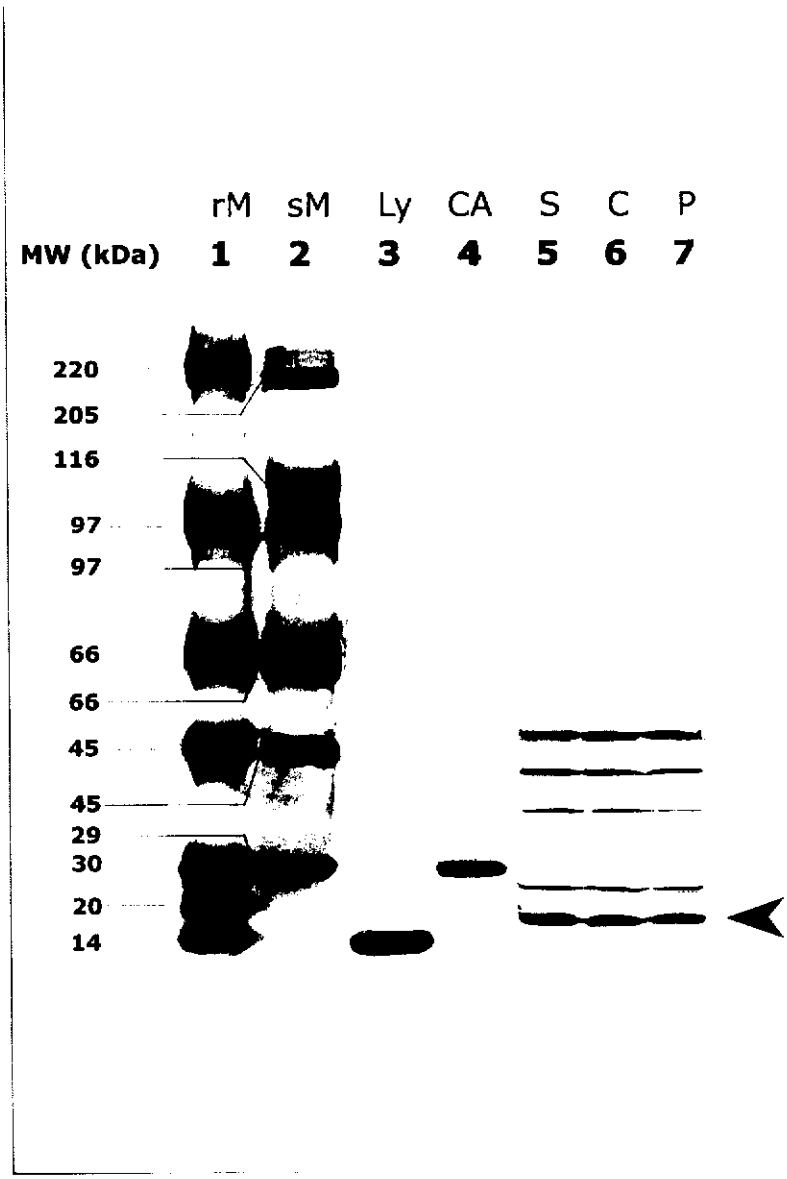

Figure 24 10% SDS-PAGE analysis demonstrated total proteins samples from common tree shrew brain in Parkinsonism condition as the followings: 7.5 ul of rainbow marker (lane 1), 10 ul of MW-SDS 200 marker (lane 2), 2 ul of lysozyme marker (14 kDa) (lane3), 2 ul of cabonic anhydrase marker (29 kDa) (lane4), 40 ul of protein extracted from substantia nigra (lane 5), 40 ul of protein extracted from caudate (lane 6), and 40 ul of protein extracted from putamen (lane7). Interested protein band at molecular weight approximately 14-20 kDa (red arrow head) was expected to be alpha-synuclein protein.

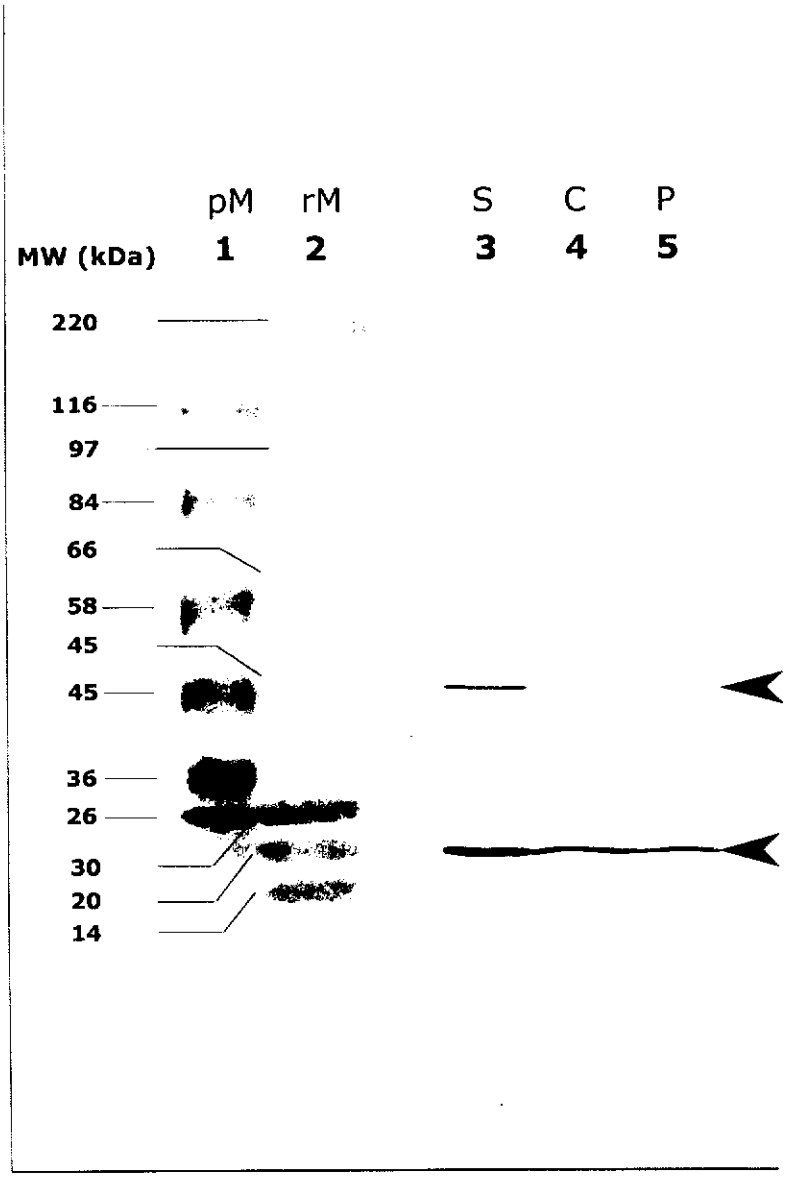

Figure 25 Western blot analysis revealed alpha-synuclein protein from common tree shrew brain in Parkinsonism condition by using anti-alpha-synuclein antibody as followind sequences: 20 ul of MW-SDS 200 marker (lane 1), 7.5 ul of rainbow marker (lane 2), 40 ul of protein extracted from substantia nigra (lane 3), 40 ul of protein extracted from caudate (lane 4), and 40 ul of protein extracted from putamen (lane 5). Western blot analysis of alpha-synuclein demonstrated specific protein band at molecular weight approximately 19 kDa (red arrow head) in three samples (lane 2, 3, and 4) and inconsiderable band at molecular weight approximately 45 kDa (black arrow head) in substantia nigra on nitrocellulose membrane, there should represent alpha-synuclein proein.

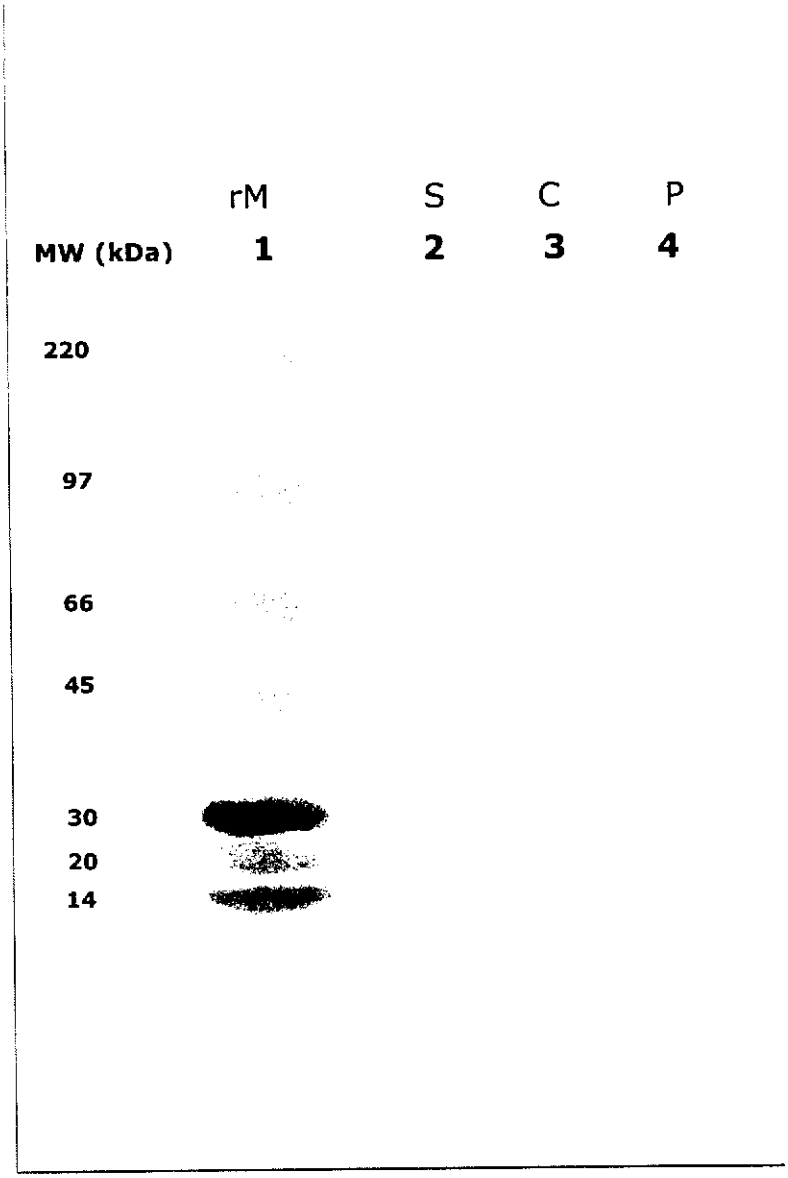

Figure 26 Western blot analysis of primary negative control of anti-alpha-synuclein antibody in Parkinsonism condition, 7.5 ul of rainbow marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

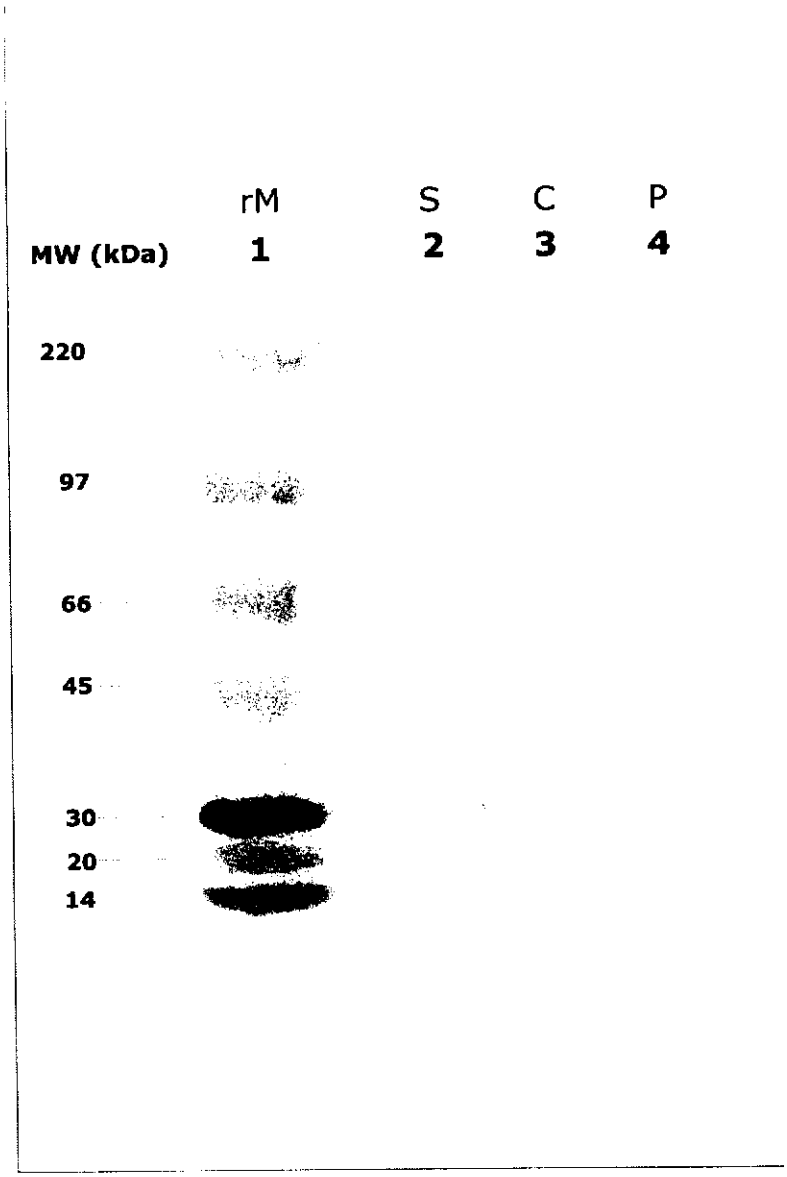

Figure 27 Western blot analysis of secondary negative control of Biotinylated secondary antibody in normal condition, 7.5 ul of rainbow marker (lane 1), 40 ul of protein extracted from substantia nigra (lane 2), 40 ul of protein extracted from caudate (lane 3), and 40 ul of protein extracted from putamen (lane 4).

Chapter 5

Discussion

5.1 The new animal model for Parkinsonism condition

Common tree shrews (*Tupaia glis*) are the animal in family: Tupaiidae, order: Scandentia (climbing mammals), subclass: Eutheria (true mammals), class: Mammalia (animals with milk glands) (51, 52). In this study, this kind of animal is interested and used as the Parkinsonism model.

In the last decade, male tree shrews have proven to be a powerful model to study the central nervous system. This animal has more relationship to human than rat and mice models (103). The previous studies, revealed position of dopamine receptors in the tree shrew brain bears great similarity to position described earlier in various primates, such as human and monkey (104). The evidence fits in with the already existing idea that tree shrews are phylogenetically related to primates. The other studies, demonstrated the phylogenetic trees of tree shrew, human, rat, and mice involving the 12S rRNA and 16S rRNA in mitochondrion (105). The phylogenetic analysis establishs that the tree shrew has closer evolutionary association to human than rodent animals which are rat and mice (106). In this study, common tree shrews are interested to be demonstrated as the new animal model for Parkinsonism condition. This animal should be the better model than rodent models to studies of Parkinsonism-like condition because it has more related evolution to human (107). In this experiment, common tree shrews were administrated by 10 mg/kg of MPTP-HCl. The animals became to have characteristic of movement disorder in two hours after injection and were killed at 10 days after the last injection. The proteins related to Parkinsonism condition in common tree shrew were characterized and confirmed by Western blot analysis. These interested proteins are tyrosine hydroxylase enzyme and alpha-synuclein protein. In normal condition, the recent studies showed the pattern of tyrosine hydroxylase at 62-68 kDa in human, 58-60 kDa in mice, and 60 kDa in rat (108). In common tree shrew, Western blot analysis verified tyrosine hydroxylase band at molecular weight approximately 62 kDa in substantia nigra, caudate, and putamen. This band is same the pattern of tyrosine hydroxylase in human (92). In Parkinsonism condition, Western blot analysis established the hallmark of Parkinsonism condition which is fibrillar

alpha-synuclein protein on nitrocellulose membrane at molecular weight approximately 19 kDa in common tree shrew brain as similar as human, rat, and mice brains (109, 110).

Moreover, the new models are more benefit, involving the mechanisms and pathways in Parkinsonism condition. This model might be used and be useful for understanding in the reduction of tyrosine hydroxylase activities, mutation of alpha-synuclein gene, and depletion of dopamine levels. The results of these molecular studies are used as the basic science studies and may be applied for development of prevention and treatments in Parkinson-like syndrome in the future.

5.2 The inactivation of tyrosine hydroxylase by MPTP

Parkinson's disease is a common neurodegenerative disorder characterized by disabling motor abnormalities attributed to a profound deficit in dopamine. The decline in dopamine level has been thought to arise solely from the severe loss of dopaminergic neurons in the nigrostriatal pathway (5). Animal models of Parkinsonism condition demonstrating that the reduction in dopamine metabolism-related markers such as tyrosine hydroxylase is far greater than the loss of neuronal cell bodies (73).

The previous studies demonstrated that MPTP impairs dopamine metabolism in mice (80). In the present study, the molecular mechanism for the MPTP-impairment of dopamine production and the potential role of this process in the pathogenesis of MPTP model of Parkinsonism condition was investigated. The rate-limiting enzyme in the dopamine synthesis, tyrosine hydroxylase, is a target for MPTP-induced tyrosine nitration, which is the adding of nitrogen into the aromatic ring of tyrosine hydroxylase (111). The nitration of a single tyrosine residue within this enzyme appears to be sufficient to impair its catalytic activity. Nitration of tyrosine residues *in vitro* results in inactivation of a vast number of mammalian proteins whose activity is dependent on tyrosine residues. The production of nitrotyrosine provides the biochemical explanation for the inhibition in the rate of phosphorylation by tyrosine kinases as well as for the inactivation of protein function (Figure 28). The rat tyrosine hydroxylase enzyme contains 17 tyrosine residues of 498 total amino acid residues, and 15 of these tyrosine residues are found in the catalytic domain. The human enzyme contains 15 tyrosine residues with 14 of them in the catalytic domain (112). The event of tyrosine nitration in catalytic domain has the effect of inactivation of tyrosine hydroxylase enzyme and finally disturbs the enzymatic function.

MPTP produced a rapid and profound loss in striatal dopamine content that was closely matched by the loss in tyrosine hydroxylase activity (113). Tyrosine hydroxylase inactivation and dopamine synthesis failure is an early event in MPTP neurotoxic process that precedes loss in tyrosine hydroxylase and dopaminergic neurons (114). The existence of nitrated tyrosine hydroxylase implies the formation of a nitrating agent after injection of MPTP. The results of MPTP effect may lead to conformation change in tyrosine hydroxylase molecule and decrease the molecular weight of separated protein bands in SDS-PAGE analysis in Parkinsonism condition.

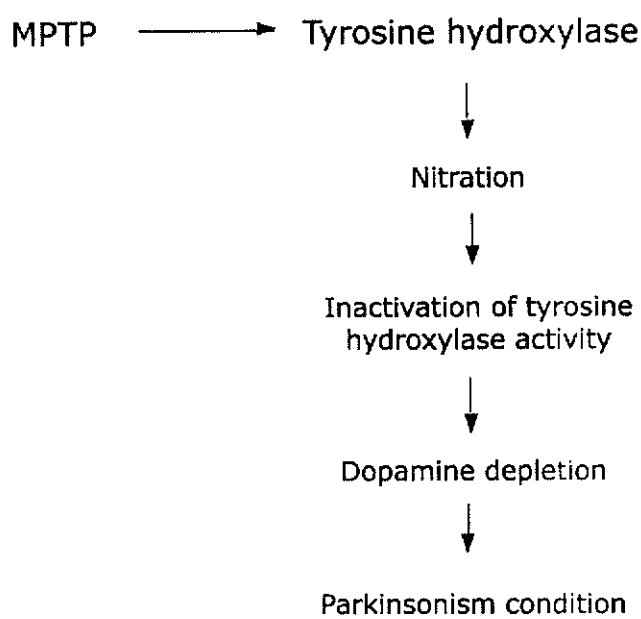

Figure 28 Proposed model for the inactivation of tyrosine hydroxylase by nitration following exposure to MPTP neurotoxin.

5.3 The effects of MPTP for reduction of tyrosine hydroxylase in Parkinsonism condition

Tyrosine hydroxylase is the importance enzyme in dopamine biosynthesis. It can change amino acid "tyrosine" to be final neurotransmitter "dopamine". The previous histological study showed the location of tyrosine hydroxylase enzyme in substiantia nigra, caudate, and putamen by using anti-tyrosine hydroxylase stained the brain sections of substiantia nigra, caudate, and putamen (data not shown) (22).

In the study, Western blot analysis was used to detect tyrosine hydroxylase enzyme in normal and Parkinsonism condition. The study revealed tyrosine hydroxylase band at molecular weight approximately 62 kDa on nitrocellulose membrane in substiantia nigra, caudate, and putamen. Especially in substiantia nigra, the band showed very high intensity because the substiantia nigra has a large number of dopaminergic neurons for dopamine production (4). The result of Western blot analysis is used to confirm the histological technique. There have shown the same patterns and molecular weights that were demonstrated in substiantia nigra, caudate, and putamen respectively.

Then Western blot analysis is used to detect tyrosine hydroxylase enzyme in Parkinsonism condition by using same condition that was studied in normal group. There is no any band representing tyrosine hydroxylase in all samples. A key question relates to the reduction of live dopaminergic neurons in Parkinsonism condition from the toxic effects of MPTP administration.

So the comparative studies in normal and Parkinsonism condition can be show the effects of MPTP act to the expression of tyrosine hydroxylase enzyme. In normal, the expression of tyrosine hydroxylase is normal, but in Parkinsonism, MPTP should have some effect on tyrosine hydroxylase expression (70). It means that MPTP destroys live dopaminergic neurons where is the location of tyrosine hydroxylase and acts decrease of tyrosine hydroxylase in these neurons.

5.4 Detection of tyrosine hydroxylase enzyme by different antibody dilutions

The study demonstrates that the MPTP can effectively destroy dopaminergic neurons in the nigro-striatal pathway (61). This pathway runs from the substantia nigra to produce dopamine to the basal ganglia, such as caudate and putamen. In normal, Western blot analysis shows tyrosine hydroxylase band at 62 kDa in substantia nigra, caudate, and putamen. The substaintia nigra reveals the higher amount of tyrosine hydroxylase by representing the higher intensity on nitrocellulose membrane. In the study of MPTP-treated group or Parkinsonism condition, Western blot analysis doesn't show any protein band when using anti-tyrosine hydroxylase dilution to 1:10,000.

The new experiment is decided to characterize amount of tyrosine hydroxylase from the toxic effects of MPTP in Parkinsonism. The study is expected to see the level expression of tyrosine hydroxylase in live dopamiergic neurons after MPTP administration. Anti-tyrosine hydroxylase antibody has many dilution are used in Western blot analysis.

Western blot analysis reveals tyrosine hydroxylase band at 62 kDa by using anti-tyrosine hydroxylase dilution to 1:5,000. This band is low intensity in caudate and putamen and lower intensity in substantia nigra when comparing with normal group that studied in same dilution (data not shown). The low amount of tyrosine hydroxylase is represented by low intensity in caudate and putamen and lower intensity in substantia nigra that is results from MPTP toxicity and alpha-synuclein effect.

In 1:15,000 dilutions, Western blot analysis shows tyrosine hydroxylase band at molecular weight approximately 62 kDa in substantia nigra, caudate, and putamen. The substantia nigra reveals the higher intensity in normal condition but doesn't show any protein band in three samples in Parkinsonism condition (data not shown). This result is similar to 1:10,000 dilutions but the intensity is less than 1:10,000 dilutions. Western blot analysis has shown the low intensity of tyrosine hydroxylase by using the high dilution of anti- tyrosine hydroxylase antibody when it is compared between normal and Parkinsonism conditions (Table 5). So, it should be possible that MPTP disturb the function of dopaminergic neurons in substantia nigra, caudate, and putamen and has the effect to decrease the amount of tyrosine hydroxylase production in Parkinsonism condition. As the result, the normal production of dopamine should be disturbed continuously. Then the function of dopaminergic neurons has been lost and could cause the condition of Parkinsonism.

Table 5 Western blot analysis of tyrosine hydroxylase enzyme in normal and Parkinsonism condition. Characterization of tyrosine hydrpxylase enzyme by using anti-tyrosine hydroxylase in varies dilution.

Condition	Anti-tyrosine hydroxylase dilution		
	1:5,000	1:10,000	1:15,000
	S C P	S C P	S C P
Normal	++++ +++ +++	+++ ++ ++	++ + +
	(data not shown)		(data not shown)
	S C P	S C P	S C P
Parkinsonism	+* + +	- - -	- - -
	(data not shown)		(data not shown)

S; substantia nigra

C: caudate

P: putamen

-: no intensity of protein band

+*: lower intensity of protein band

+: low intensity of protein band

++: high intensity of protein band

+++: higher intensity of protein band

++++: highest intensity of protein band

5.5 Mitochondrial dysfunction related to neuronal death in Parkinsonism condition

Mitochondria are the cellular organelles of a number of important cellular functions, including essential pathways of intermediate metabolism, amino acid biosynthesis, fatty acid oxidation, steroid metabolism, and apoptosis. The mitochondrion is the only organelle in the cell, aside from the nucleus, that contains its own genome and genetic machinery (59).

At present, it has been demonstrated that mitochondrial respiratory chain dysfunction has been seriously related in the Parkinsonism condition (115). The mitochondrial connection to Parkinsonism condition began almost 20 years ago, when it was discovered that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes Parkinsonism condition in humans and in laboratory animals. In other neurodegenerative disorders, the cellular patterns are related about the destruction of mitochondria, then proceeding up to the final cellular apoptosis. Interestingly, in Parkinsonism condition, not only a lot of cellular mitochondria have been destroyed, but also there is the event of genetic mutation of mitochondrial DNA. Moreover, the mutations of other genes in mitochondria, for example cytochome c oxidase, cytochome b, etc. finally lead the neurons to the condition of apoptosis (71).

MPTP indirectly inhibits complex I of the respiratory chain, disturbs ribosomal function, then causes the mitochondrial damage. Infact, *N*-methyl-4-phenylpyridinium ion (MPP^+), which is MPTP metabolite and produced by the mitochondrial outer membrane protein monoamine oxidase B (MAO-B) and/or astroglia cells, is the major cause of this damage. Moreover, complexes I and III are the principal sources of free radicals in the cell, which are related to process of program cell death. The altered complex I function in the substantia nigra pars compacta could be responsible for the increased DNA damage and lipid peroxidation found in Parkinsonism brains (65).

In relation to Parkinsonism condition, the effect of mitochondrial respiratory chain dysfunction continuously send the signal to cellular nucleus and then causes mutations of the genes encoding alpha-synuclein and parkin (116) which is one of the genes related to early-onset Parkinson's disease, including exonic deletions, insertions, and several missense mutations (Figure 29). The precise rolls of these polypeptides in neurodegeneration are still unknown. However, it has been established that the overexpression of alpha-synuclein gene produce the large amount of mutated fibrillar alpha-

synuclein protein (117). The signal from mitochondrial dysfunction then activates the aggregation of this fibrillar alpha-synuclein protein (118, 119). These aggregations represent and perform as cellular inclusion, named Lewy body, which is a very well-known hallmark of Parkinsonism condition for a long time.

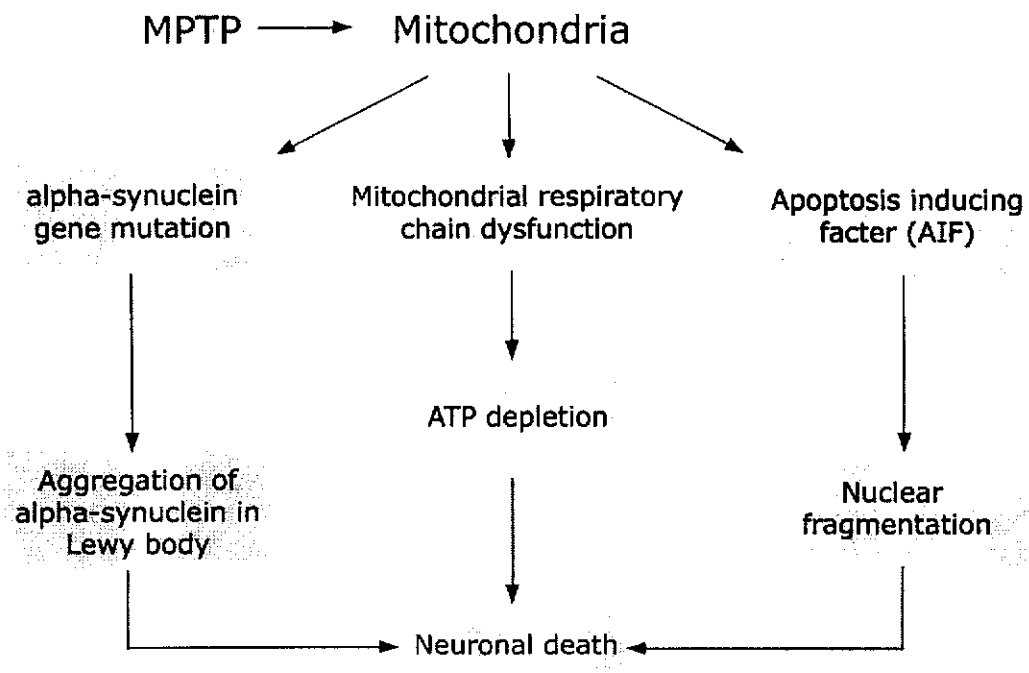

Figure 29 Proposed model for the effects of MPTP for mitochondrial respiratory chain dysfunction and alpha-synuclein mutation in dopaminergic neurons.

5.6 Role of alpha-synuclein protein in Parkinsonism condition

The proteins carry out many crucial functions in animal's body and brain. Recent studies indicated that toxic effect of MPTP can turn the normal protein function, alpha-synuclein protein, into a major contributor of the body movement disorder, Parkinson's disease (54, 75). A lot of studies about alpha-synuclein have been revealed as the following. Expression of alpha-synuclein shows has stimulation from alpha-synuclein gene mutation (27, 67, 95, 120, 121). In normal, the alpha-synuclein aids brain function, possibly by helping cells communication. In the other hand, the alpha-synuclein is implicated in the pathogenesis of Parkinson's disease. Although alpha-synuclein function is unclear, many

reports support and reveal that alpha-synuclein has effect and is concerned to be related in the Parkinsonism condition. In addition, the overexpresssion plays a role in the regulation of dopamine biosynthesis, acting to disturb and reduce the activity of tyrosine hydroxylase enzyme (32, 114, 122).

This experiment is decided to study the effect of MPTP acting to change the common tree shrew to be a Parkinsonism models. The study reveals alpha-synuclein protein is overexpression in Parkinsonism or MPTP-treated group (62, 72, 119). The alpha-synuclein protein is characterized by molecular technique, Western blot analysis. In normal condition, Western blot analysis shows one protein band at molecular weight approximately 45 in substaintia nigra but doesn't show in caudate and putamen samples. It could be a one larger or preprotein form which is at high molecular weight of alpha-synuclein protein that expressed in normal (33, 46). Because of alpha-synuclein has many forms of protein structure. The mutation can change the protein structure to different form (123-125).

In Parkinsonism, Western blot analysis shows one band of alpha-synuclein protein at molecular weight approximately 19 kDa in three samples from substantia nigra, caudate, and putamen and the other band at molecular weight approximately 45 only in substaintia nigra. The protein band at 19 kDa is representing to the fibrous form of alpha-synuclein protein that take place in Parkinson's disease and Parkinsonism condition (33). A lot of recent studies showed the fibrous alpha-synuclein protein is used as the Parkinson marker (26, 30, 37, 38, 126). This protein is the production of alpha-synuclein mutation that more expressed in Parkinsonism.

The protein band at 45 kDa can be found in substantia nigra in both conditions. It will be the normal form of alpha-synuclein protein (27, 30). The intensity of 45 kDa band in Parkinsonism is decreased when comparing with normal because the mutation in Parkinsonism changes the protein structure to fibrous form (47, 48).

In this report, MPTP may probably induce common tree shrew to have Parkinsonism condition by stimulation to cause mutation in alpha-synuclein gene. The possible mutation demonstrates the alpha-synuclein band at molecular weight approximately 19 kDa, this protein similar to human alpha-synuclein protein that is found in Lewy bodies which is the landmark of Parkinsonism condition.

5.7 The relation of tyrosine hydroxylase and alpha-synuclein in Parkinsonism model

From recent studies, tyrosine hydroxylase enzyme is the rate limiting enzyme in dopamine biosynthesis and can be found this enzyme in dopaminergic neurons (127). The dopamine is produced to control the body movement. The lacks of dopaminergic neurons from MPTP effects reduce the amount of tyrosine hydroxylase enzyme and dopamine synthesis (74, 128).

Alpha-synuclein protein is the normal protein in normal condition but the protein function is not clear (123, 129). However, the scientists know the mutation of alpha-synuclein gene is leaded to Parkinson's disease (130-132). The alpha-synuclein gene mutation induces the overexpression of alpha-synuclein protein and changes protein structure to fibrous form. The mutant protein can bind to tyrosine hydroxylase molecule and change the protein structure of tyrosine hydroxylase (133, 134). Moreover, the interaction between alpha-synuclein protein and tyrosine hydroxylase structure is still unclear, this effect can block and reduce the activity of tyrosine hydroxylase enzyme (112, 131) (Figure 30). So tyrosine hydroxylase can't change dopa to dopamine. The depletion of dopamine level leads to the loss of body movement and control, resulting in Parkinsonism.

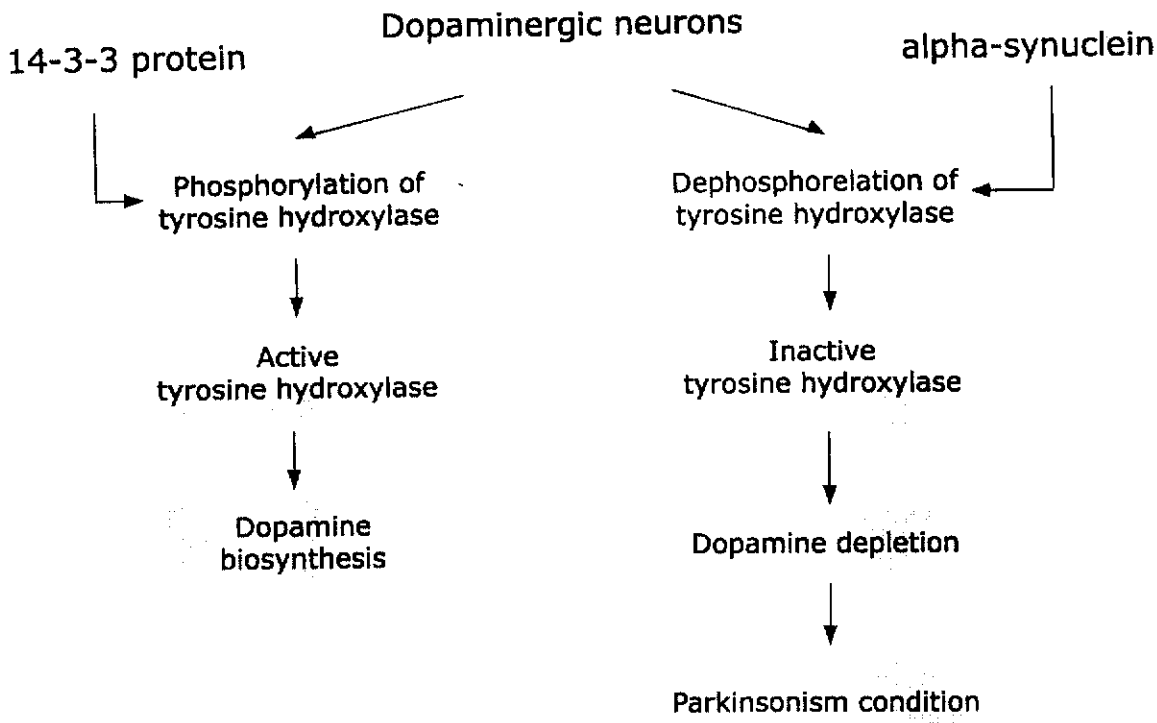

Figure 30 Proposed model for the interaction of tyrosine hydroxylase and alpha-synuclein in dopamine biosynthesis of Parkinsonism condition.

5.8 Summary

In summary, the common tree shrew (*Tupaia glis*) was used as the new animal model for Parkinsonism condition, it became to have characteristic of movement disorder and revealed the sign of Parkinson-like syndrome in two hours. This model was more benefit, involving the mechanisms and pathways in Parkinsonism condition. In normal condition, Western blot analysis demonstrated tyrosine hydroxylase at 62 kDa and alpha-synuclein at 45 kDa which should be preprotein or normal form of alpha-synuclein protein. In Parkinsonism condition, Western blot analysis didn't verified tyrosine hydroxylase in normal dilution (1:10,000) of anti-tyrosine hydroxylase. Moreover, in high dilution (1:5,000) of antibody, the band of tyrosine hydroxylase was recognized, but showed in very low intensity. Interestingly, Western blot analysis showed 19 kDa band of expected fibrillar alpha-synuclein protein which is a dominant hallmark of inclusion protein in Lewy body in Parkinsonism condition.

This thesis is the basic science study and may be applied for development of prevention and treatments in Parkinson-like syndrome in the future.

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Appendix

Appendix

1. Acrylamide stock solution:

Acrylamide powder		15	g
Bis-acrylamide		0.4	g
Deionized-water	adjust to	50	ml
*store at 4 °C away from light			

2. 1.5 M Tris-HCl buffer pH 8.8:

Tris powder		36.32	g
4 N HCl	adjust to	pH8.8	
Deionized-water	adjust to	200	ml

3. 0.5 M Tris-HCl buffer pH 6.8:

Tris powder		6.06	g
4 N HCl	adjust to	pH6.8	
Deionized-water	adjust to	100	ml

4. 10% SDS:

SDS powder		10	g
Deionized-water	adjust to	100	ml
*away from light			

5. 10% APS:

Ammonium persulphate		0.05	g
Deionized-water	adjust to	0.5	ml

6. PBS:

NaCl		8.0	g

KCl		2.0	g
Na_2HPO_4		1.44	g
KH_2PO_4		0.24	g
H_2O		800	ml
	adjust to	pH7.4	
H_2O	adjust to	1,000	ml

*store at 4 C

7. Blocking solution:

Dried milk		5.0	g
PBS	adjust to	100	ml

8. Electrophoresis: Tank buffer pH8.3

Tris powder		6.04	g
Glycine		28.8	g
SDS powder		2.0	g
Deionized-water	adjust to	2,000	ml

9. Transfer buffer: Towbin buffer pH8.3

Tris powder		3.0	g
Glycine		14.4	g
SDS powder		1.0	g
Methanol (100%)		200	ml
Deionized-water	adjust to	1,000	ml

10. Coomassie blue stain solution:

Coomassie blue	0.25	g
Methanol	400	ml
Glacial acetic acid	70	ml

Deionized-water	adjust to	1,000	ml

11. Destaining solution I:

Methanol		200	ml
Glacial acetic acid		35	ml
Deionized-water	adjust to	500	ml

12. Destaining solution II:

Methanol		25	ml
Glacial acetic acid		35	ml
Deionized-water	adjust to	500	ml

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STUDIES OF TYROSINE HYDROXYLASE ENZYME AND ALPHA-SYNUCLEIN PROTEIN IN NORMAL AND PARKINSONISM CONDITIONS IN COMMON TREE SHREW BRAIN (*Tupaia glis*)

AN ABSTRACT

BY

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Presented in partial fulfillment of the requirements for the
Master of Science degree in Molecular Biology
at Srinakharinwirot University
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Cheewan Sunthonwipat. (2003). *Studies of Tyrosine Hydroxylase Enzyme and Alpha-Synuclein Protein in Normal and Parkinsonism Conditions in Common Tree Shrew Brain (Tupaia glis).* Master thesis, M.Sc. (Molecular Biology). Bangkok: Graduate School, Srinakharinwirot University. Advisor Committee: Assist. Prof. Dr. Vipavee Anupunpisit, Dr. Suwadee Chanchaiyakul, Assist. Prof. Dr. Udomsri Showpittapornchai.

Parkinson's disease is a common neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra. The loss of dopaminergic afferents from the substantia nigra to caudate and putamen results in decreased dopamine levels lead to body movement and control disorder. Common tree shrew (Tupaia glis) is induced to Parkinsonism condition by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration. MPTP selectively destroys dopaminergic neurons in substantia nigra, resulting in a Parkinson-like syndrome. SDS-PAGE and Western blot analysis are used as molecular techniques to characterize tyrosine hydroxylase enzyme, the rate-limiting enzyme in dopamine synthesis and alpha-synuclein protein, the major component of Lewy body which is the hallmark of Parkinsonism condition. In normal condition, Western blot analysis demonstrated protein band at 62 kDa in substantia nigra, caudate, and putamen identified as tyrosine hydroxylase by using anti-tyrosine hydroxylase antibody. Moreover, the alpha-synuclein protein band at 45 kDa is verified only in substantia nigra sample which should represent the normal larger form. In Parkinsonism condition, amount of tyrosine hydroxylase is gradually decreased in all samples, especially in substantia nigra. In the other hand, the new 19 kDa alpha-synuclein protein, which should be fibrillar alpha-synuclein, is dominantly demonstrated in all samples, specifically in substantia nigra. Interestingly, there is only 45 kDa alpha-synuclein expression in normal condition whereas both 19 kDa and 45 kDa alpha-synuclein forms are revealed in Parkinsonism condition. In summary, tyrosine hydroxylase is decreased in Parkinsonism condition because of the effects of MPTP while and alpha-synuclein protein should act to disturb and inhibit the activity of tyrosine hydroxylase enzyme. These results are loss of dopamine levels in the brain and lead to Parkinson-like syndrome.

การศึกษา Tyrosine Hydroxylase Enzyme และ Alpha-Synuclein Protein ในสภาวะปกติและสภาวะพาร์กินสันในสมองของสัตว์ประเภทกระแต (*Tupaia glis*)

บทคัดย่อ

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มกราคม 2547

ชีวัน สุนทรวิภาต. (2546). *การศึกษา Tyrosine Hydroxylase Enzyme และ Alpha-Synuclein Protein ในสภาวะปกติและสภาวะพาร์กินสันในสมองของสัตว์ประเภทกระแต (Tupaia glis).* ปริญญานิพนธ์ วท.ม.(อณูชีววิทยา). กรุงเทพฯ: บัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ. คณะกรรมการควบคุม: ผู้ช่วยศาสตราจารย์. ดร. วิภาวี อนุพันธ์พิศิษฐ์, อาจารย์ ดร. สุวดี ชวนไชยะกูล, ผู้ช่วยศาสตราจารย์. ดร. อุดมศรี โชว์พิทธพรชัย.

โรคพาร์กินสัน เป็นโรคที่เกิดจากความผิดปกติของระบบประสาทที่เกี่ยวข้องกับเซลล์ประสาทที่สร้างสารสื่อประสาทโดปามีนในสมองส่วน substantia nigra และการสูญเสียของแนวประสาท nigrostrial pathway ซึ่งมีผลกระทบต่อการลดลงของปริมาณโดปามีนในสมองส่วน substantia nigra caudate และ putamen เป็นสาเหตุให้ร่างกายสูญเสียความสามารถในการควบคุมการเคลื่อนไหวและการทรงตัว นำไปสู่พยาธิสภาพของโรคพาร์กินสัน การศึกษาในครั้งนี้ได้ทำการชักนำให้สัตว์ประเภทกระแตมีลักษณะคล้ายกับโรคพาร์กินสันโดยการฉีดสาร MPTP ซึ่งเป็นสารพิษต่อระบบประสาท สาร MPTP มีผลในการทำลายเซลล์ประสาท dopaminergic ในสมองส่วน substantia nigra อย่างจำเพาะเจาะจงทำให้สัตว์ทดลองเข้าสู่สภาวะพาร์กินสัน การทดลองได้อาศัยวิธีการ SDS-PAGE และ Western blot analysis เป็นเทคนิคสำคัญในการวิเคราะห์การแสดงออกและขนาดของเอนไซม์ tyrosine hydroxylase ซึ่งมีบทบาทสำคัญในกระบวนการสังเคราะห์สารสื่อประสาทโดปามีน และของโปรตีน alpha-synuclein ซึ่งเป็นองค์ประกอบหลักของ Lewy body ภายในเซลล์ที่ใช้เป็นลักษณะสำคัญในการบ่งชี้การเกิดสภาวะพาร์กินสัน จากการศึกษาพบว่าในสภาวะปกติพบเอนไซม์ tyrosine hydroxylase ในสมองส่วน substantia nigra caudate และ putamen โดยมีขนาดประมาณ 62 กิโลดัลตัล ซึ่งทำการตรวจสอบโดยการใช้แอนติบอดีต่อเอนไซม์ tyrosine hydroxylase เป็นตัวบ่งชี้ แต่เมื่อใช้แอนติบอดีต่อโปรตีน alpha-synuclein เป็นตัวบ่งชี้ พบโปรตีนที่ 45 กิโลดัลตัล ซึ่งคาดว่าน่าจะเป็นกลุ่มของโปรตีน alpha-synuclein ที่พบทั่วไปในสภาวะปกติ โดยจะพบเฉพาะในสมองส่วน substantia nigra เท่านั้น สำหรับการศึกษาในสภาวะพาร์กินสัน พบว่าปริมาณของเอนไซม์ tyrosine hydroxylase มีปริมาณลดลงในสมองทั้งสามส่วนและลดลงมากในสมองส่วน substantia nigra แต่ในขณะเดียวกันมีการแสดงออกของโปรตีน alpha-synuclein ในสมองส่วน substantia nigra caudate และ putamen โดยมีขนาดประมาณ 19 กิโลดัลตัล ซึ่งน่าจะอยู่ในโครงสร้างประเภทเส้นใย เป็นที่น่าสนใจว่าในสภาวะปกติ สมองส่วน substantia nigra มีการแสดงออกของโปรตีน alpha-synuclein ที่มีขนาด 45 กิโลดัลตัลเท่านั้น แต่ในสภาวะพาร์กินสันกลับมีการแสดงออกของโปรตีน alpha-synuclein ที่มีขนาด 19 กิโลดัลตัล และ 45 กิโลดัลตัล ผลจากการศึกษาคาดว่าปริมาณของเอนไซม์ tyrosine hydroxylase ที่ลดลงในสภาวะพาร์กินสันน่าจะมีผลกระทบมาจากสาร MPTP ในขณะที่โปรตีน alpha-synuclein มีผลในการรบกวนและยับยั้งการทำงานของเอนไซม์ tyrosine hydroxylase ซึ่งส่งผลกระทบต่อการผลิตสารโดปามีนทำให้ปริมาณโดปามีนมีปริมาณลดลงและนำไปสู่สภาวะพาร์กินสันในที่สุด