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Pathological changes in mesostriatal neurons in a PKC-gamma mutant rat

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PUBLICATIONS

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LIST OF ABBREVIATIONS

α–Syn	α–synuclein
5-HT	Serotonin
5-HIAA	5-hydroxyindole acetic acid
6-OHDA	6-Hydroxydopamine
Aa	Area fraction
AD	Alzheimer's disease
APP	Amyloid precursor protein
AR-JP	Autosomal recessive juvenile parkinsonism
ATP	Adenosine triphosphate
AS	Albino Swiss
AS/AGU	Albino Swiss/Anatomy Glasgow University
BH4	Tetrahydrobiopterin (pteridine)
CAG	polyglutamine repeat within exon 1 of the gene in chromosome 4 that codes for the Huntingtin protein
CBD	Corticobasal Degeneration
CM/PF	Centromedian-Parafasicular
CNS	Central Nervous System
COMT	Catechol-O-Methyl Transferase
CSF	Cerebrospinal fluid
D1	Dopamine receptor 1
D2	Dopamine receptor 2
DA	Dopamine
DAB	Diaminobenzidine
DAT	Dopamine Transporter
DBS	Deep Brain Stimulation
DCPU	Dorsal caudate-putamen
DLB	Dementia with Lewy Bodies
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenyalanine
DOPAC	3,4-Dihydroxyphenylacetic Acid
E1	ubiquitin–activating enzyme
E2	ubiquitin–conjugating enzyme
E3	ubiquitin–protein ligase
EM	Electron Microscopy
Enk	Enkephalin
EPN	Entropenduncular Nucleus
ER	Endoplasmic Reticulum
Fe^{+2}	Ferrous iron
FTDP-17	Familial front temporal dementia and Parkinsonism linked to chromosome 17
FPD	Familial Parkinson 'disease
GABA	Gamma-Aminobutyric Acid
GP	Globus Pallidus
GPe	External Globus Pallidus
GPi	Internal Globus Pallidus
GPv	Ventral pallidum
H_2O_2	Hydrogen Peroxide
HD	Huntington's Disease
HPLC-ECD	High Performance Liquid Chromatography with Electrochemical Detection
HVA	Homovanillic Acid
ICC	Immunocytochemistry
kPa	Kilopascal
L-DOPA	L-3,4 Dihydroxyphenyalanine
LB	Lewy Body
LCPU	Lateral caudate-putamen
LM	Light Microscopy
MOA	Monoamine Oxidase
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
MSA	Multiple System Atrophy
NA	Noradrenaline
NE	Norepinephrine
NFT	Neurofibrillary Tangle
PB	Phosphate Buffer

PBS Phosphate Buffered Saline
PD Parkinson's Disease
PKCγ Protein Kinase C gamma
PNS Peripheral Nervous System
PPN Pendunculopontine Tegmental Nucleus
PSI Pound per square inch
PSP Progressive Supranuclear Palsy
RER Rough Endoplasmic Reticulum
SN Substantia Nigra
SNC Substantia Nigra Pars Compacta
SNL Substantia Nigra Pars Lateralis
SNR Substantia Nigra Pars Reticulata
SP Substance P
STN Subthalamic Nucleus
TB Toluidine Blue
TH Tyrosine Hydroxylase
TPH Tryptophan hydroxylase
TUNEL Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling
Ub Ubiquitin
UCHL1 Ubiquitin-C-Hydrolase-1
UPS Ubiquitin-Proteasome System
VCPU Ventral caudate-putamen
VM-VL Ventromedial/Ventrolateral Complex
VTA Ventral Tegmental Area
Vv Volume fraction

ABSTRACT

The AS/AGU rat originated as a recessive mutation (*agu*) in a closed colony of Albino Swiss (AS) rats. The mutation is in the gene coding for the gamma isoform of protein kinase C. It is characterized by movement impairments and progressive dysfunction of the nigrostriatal dopaminergic (DA) and raphe striatal serotonergic (5-HT) systems. The movement impairments including rigidity of the hind limbs, a staggering gait, a tendency to fall over every few steps, a slight whole body tremor and difficulty in initiating movements. The dysfunction in both systems is characterised by a failure to release DA or 5-HT within the striatum and cell loss within the substantia nigra pars compacta (dopaminergic cells) and the dorsal raphe nuclei (5-HT+ve cells).

In this study, three experiments were carried out to examine the possible pathological responses of midbrain cell groups to the *agu* mutation in the gene coding for protein kinase C-gamma (PKC-γ).

Experiment 1 was carried out to examine levels of two groups of molecules in the midbrain cell groups using quantitative immunofluorescence microscopy of cell bodies or their surrounding neuropil (a) those molecules giving information about the capacity of midbrain aminergic cell bodies to synthesis transmitters; tyrosine hydroxylase (TH) in the dopaminergic neurons and serotonin (5-HT) in the serotonergic neurons (b) those which have been found to occur in human neurodegenerative conditions such as Parkinson's disease: ubiquitin, parkin and α-synuclein (Lewy body proteins). Immunofluorescence levels of tyrosine hydroxylase (in dopaminergic cells of the SNC) and serotonin (in 5HT+ve cells of the dorsal raphe nuclei) were both significantly increased in AS/AGU (mutant) compared to the AS (control) rats aged 6 months and older. TH and 5-HT immunofluorescence levels were both significantly decreased in the striatum in the AS/AGU (mutant) compared to the AS (control) rat aged 12 months. Ubiquitin immunofluorescence show a gradual increase with age in AS and AS/AGU rats and the increase was much greater in the mutant in every region except the oculomotor and pontine nuclei. Parkin immunofluorescence show increases in the mutant within the SNC and the dorsal raphe nucleus and this increase was significant at older ages. Alpha-synuclein does not occur in the cell bodies of the substantia nigra or VTA but outside in the neuropil. Alpha-synuclein immunofluorescence levels progressively increased with age in both strains in the SN and VTA and were higher in the mutant. The levels of those molecules (ubiquitin, parkin and alpha-synuclein) do not differ in the striatum of mutants compared to controls.

Experiment 2 examined SNC cell bodies to look for possible strain differences in cell size or ultrastructure or any sign of cell death using light and transmission electron microscopy. The diameter (maximum and minimum) of the SNC cells and nuclei were measured in toluidine blue paraffin wax and immunoperoxidase DAB staining for TH sections. Cell diameter was reduced in the AS/AGU mutant compared to the AS control. No obvious ultrastructural differences were seen in nigrostriatal neurons

of both strains. The volume fractions of mitochondria and rough endoplasmic reticulum were significantly higher in the mutant. No Lewy bodies were present.

Experiment 3 examined TH+ve nigrostriatal dopaminergic terminals in the dorsal caudate-putamen to determine whether there are (a) differences in the percentages and numbers of TH+ve terminals and (b) differences in synaptic vesicles numbers. In 12-month AS/AGU mutant, there are reduction in TH+ve terminals (40%) together with a reduction in vesicle numbers (40%) in such terminals where in 3-month AS/AGU mutant, the reduction in TH+ve terminals was more (50%) and a reduction in vesicles numbers by three quarters. TH-ve terminals are also reduced in numbers in 12 months aged AS/AGU mutant rats. In 12-month AS/AGU rats, there were significantly reduced numbers of synaptic terminals in the striatum compared to AS controls. This applied to both dopaminergic terminals (which make up 15% of the total) and to non-dopaminergic terminals. In 3-month AS/AGU rats, there is a reduction in terminal numbers, but this is restricted to the dopaminergic terminals only: non-dopaminergic ones are unaffected.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview

This study was designed to take advantage of a unique mutation in a population of Albino Swiss (AS) rats. The mutant AS/AGU rat has a stop codon in the gene for expression of protein kinase C-gamma **(Craig *et al*., 2001)** which lead to disordered movement, a major loss in dopamine and serotonin release in the striatum and, with time, a loss of midbrain aminergic neurons **(Campbell *et al*., 1997, 1998; Al-Fayez *et al*., 2005)**.

Many aspect of human conditions such as Parkinson's disease (PD) can not be properly assessed until after the death of patient, and many Laboratory models of PD have therefore been introduced to mimic the disorder. However, since the neurological events leading up to symptomatic PD are unknown, these models rely on hypotheses which remain unproven. By contrast, the AS/AGU rat is a naturally-occurring model **(Payne *et al*., 2000)** which is under examination.

This study is designed to explore three aspects of the AS/AGU rat which have not been examined before:

I. Do midbrain neurons show changes in molecules which are elevated in human PD?
II. What are the morphological changes in the midbrain aminergic neurons in the mutant. Can these changes point to a particular pathology?
III. What changes occur in the synaptic terminals of these cells within the caudate-putamen?

1.2 The Basal ganglia

The term basal ganglia has no precise definition but, in general, the basal ganglia are a group of subcortical nuclei located bilaterally in the inferior cerebrum, diencephalon, and midbrain. They appear in a fifth week of development in the floor of the telencephalic vesicle **(FitzGerald and FitzGerald, 1994; Carlson, 2004)**. The basal ganglia play a prominent role in the planning, initiation and execution of movement **(Albin *et al*., 1989)**.

The term basal ganglia is currently used to describe the large nuclear masses in the deep forebrain and midbrain that (a) share a similar motor function and (b) which are connected to the cerebral cortex and thalamus by various loops. These include the caudate, putamen, globus pallidus, subthalamic nucleus, and substantia nigra. It is convenient to describe these components initially before discussing their functional circuitry.

1.2.1 Compartmental organization of the basal ganglia

Two levels of compartmental organization have been deduced by using techniques such as retrograde tracing, histochemistry, immunohistochemistry, and histopharmacology.

Striatal patch-matrix systems have been demonstrated by specific neurochemical markers. The patch compartment is defined by areas of dense μ-opiate receptor binding **(Herkenham and Pert, 1981)**, and by low expression of acetylcholinesterase **(Butcher and Hodge, 1976)**. **Graybiel and Ragsdale (1978)** was used the term striosome to describe these areas of low cholinesterase activity. Five-nucleotidase was

also found to be an excellent marker for striosome in the rat **(Schoen and Graybiel, 1992)**.

The striatal matrix compartment can be detected by positive staining for the calcium binding protein calbindin, and by the somatostatin immunoreactive positive fibers **(Gerfen and Baimbridge, 1985)**. Patch-matrix organization is mainly found in the caudate-putamen, extending into the dorsolateral and ventromedial areas.

In the rat, the compartmental organization of cortico-striatal afferent was related to their lamina of origin rather than to their cortical areas of origin **(Gerfen, 1989)**.

The striatal medium spiny neurons can be arranged into separate populations to form patch and matrix compartments that have their connections related to the laminae **(Gerfen, 1989)** and regional **(Donoghue and Herkenham, 1986)** organization of the cortex, and they can be also categorized by their projections to the globus pallidus, entopeduncular nucleus (EPN) and substantia nigra, with two type of neurons: the striatopallidal neurons within the globus pallidus, and the striatonigral neurons extending to EPN and substantia nigra **(Parent *et al*., 1984)**.

Striatopallidal projections to many cholinergic and substance P-expressing areas of the ventral pallidum arise mainly from patch areas, whereas matrix neurons tend to project mainly to the GABAergic enkephalin areas of the dorsal pallidum (globus pallidus) **(Gerfen, 1992)**.

In the rat both types of neurons project to the substantia nigra, patch neurons project to the dopaminergic cells in the SNC, and cell islands in SNR, whereas matrix neurons project to areas containing GABAergic neurons in the SNR **(Gerfen, 1984; 1985)**.

1.2.2 Striatum

The striatum or neostriatum is the largest component of the basal ganglia and the major receiving area **(Parent, 1990)**. It receives a glutamatergic projections from the whole cerebral cortex **(McGeorge and Faull, 1989)**, from the intralaminar nuclei of the thalamus **(Berendse and Groenewegen, 1990)**, and from dopaminergic neuron from substantia nigra pars compacta (SNC). It receives smaller projections from the globus pallidus (GP), the subthalamic nucleus (STN), the serotonergic dorsal raphe nucleus and the pendunculopontine tegmental nucleus (PPN) **(Parent, 1990)**.

The striatum sends projections to the globus pallidus (GP) and substantia nigra pars reticulata (SNR) **(Parent and Hazrati, 1995)**.

The striatum generally refers to both the putamen and caudate nucleus that are separated by the internal capsule in primates but united in rodents.

In man, the caudate nucleus is a large C-shaped mass of gray matter that form the floor of the lateral ventricle, lies lateral to the thalamus, and lies medial to the internal capsule. It is divided into a head that is large, rounded and forms the lateral wall of the anterior horn of the lateral ventricle, and a body and tail that terminates in the amygdaloid nucleus.

The putamen is a large, dark mass of gray matter and both putamen and globus pallidus called the lentiform nucleus.

The striatum is composed of a large number of medium spiny projection neurons, which use γ-amino-butyric acid (GABA) as a neurotransmitter **(Kita and Kitai, 1988; Parent and Hazrati, 1995a)** and a small number of large and medium sized interneurons **(Wilson and Groves, 1980).**

1.2.3 Globus pallidus

The globus pallidus represents the principal efferent side of the basal ganglia.

The globus pallidus (GP) can be subdivided into internal (medial) and external (lateral) segments, separated by an internal lamina of myelinated fibers. The GP also contains a ventral subcommissural portion termed the ventral pallidum (GPv) **(Heimer *et al*., 1982)**.

In rodents, the entopeduncular nucleus (EPN) represents the internal segment of globus pallidus **(Morgan, 1927)**. The GP receives afferent projections from the striatum and subthalamic nucleus and it sends efferent projections to the thalamus, subthalamic nucleus, SN and pedunculopontine nucleus **(Parent and Hazrati, 1995b)**. The principal neurons of GP are large cells with long, thick and generally smooth dendrites **(Fox *et al*., 1974; DiFiglia *et al*., 1982)** and they use γ-aminobutyric acid (GABA) as a neurotransmitter **(Oertel and Mugnaini, 1984)**.

1.2.4 Substantia nigra

The substantia nigra (SN) is a layer of grey matter containing numerous, deeply pigmented, multipolar nerve cells and extending throughout the whole length of the midbrain, (i.e from the rostral end of the pons to the subthalamic region) and it is considered part of the basal ganglia due to its close ties with the striatum. In sections of the midbrain in human, it is easily recognized by the black pigment from which its name derives. It is semilunar on transverse section; its concave surface being directed toward the tegmentum and its convex surface direct toward the crus cerebri; it is thicker medially than laterally.

Classically, the SN has been divided into (a) a dorsal pars compacta (SNC) composed of numerous medium-sized dopaminergic neurons (A9), containing large amounts of melanin pigament–with a few non-dopaminergic neurons; and (b) a ventral pars reticulata (SNR) composed of fewer neurons, some of which are also dopaminergic and contain small amount of melanin pigament **(Fallon and Loughlin, 1995)**, though the majority are GABAergic neurons **(Oertel and Mugnaini, 1984; Parent, 1990)**.

A lateral extension of the SNC is called the pars lateralis (SNL). It is insignificant in man **(Huber and Crosby, 1933)** and has a variety of neurons, some of which are dopaminergic **(Fallon and Loughlin, 1995)**.

The SN has extensive connections with the cortex, spinal cord, hypothalamus, and basal ganglia **(Table 1.1)**. Medial to the substantia nigra is the ventral tegmental area (VTA) composed of dopaminergic neurons (A10) and non- dopaminergic neurons **(Fallon and Loughlin, 1985;1995)**: their terminations are shown in **Table 1.1**.

	INPUT	OUTPUT
SNC	● Striatum (GABAergic) (human, primate, rat) ● Globus pallidus (GABAergic) (human, primate, rat) ● SNR (GABAergic) (rat) ● Cerebral cortex (glutamatergic) (human, primate, rat) ● Subthalamic nucleus (glutamatergic) (human, primate, rat) ● Pedunculopontine nucleus (glutamatergic and Cholinergic) (human, primate, rat) ● Dorsal raphe (serotonergic) (human, primate, rat)	● Striatum (A9 dopaminergic) (mainly) (human, primate, rat) ● Subthalamic nucleus (A9) (sparse) ((human, primate, rat) ● Globus pallidus (A9) (sparse) (human, primate, rat) ● Amygdala (dopaminergic) (a few) (human, primate, rat) ● Cerebral cortex (dopaminergic) (a few) (rat) ● Striatum (GABAergic) (a few) (rat)
SNR	● Striatum (GABAergic) (human, primate, rat) ● Globus pallidus (GABAergic) (rat) ● Accumbens nucleus (GABAergic) (rat) ● Ventral pallidum (GABAergic) (rat) ● Subthalamic nucleus (glutamatergic) (rat) ● Cerebral cortex (glutamatergic) (rat) ● Amygdala (rat)	● Striatum (A9 dopaminergic) (a few) (rat) ● VA-VL thalamus (GABAergic) (human, primate, rat) ● Superior colliculus (GABAergic) (human, primate, rat) ● Pedunculopontine nucleus (GABAergic) (human, primate, rat)
SNL	● Amygdala (rat)	● Striatum (A9 dopaminergic) (a few) (rat) ● Amygdala (dopaminergic) (rat) ● Inferior colliculus (non-dopaminergic (rat)
VTA	● Accumbens nucleus (GABAergic) (rat) ● Locus coeruleus (noradrenergic) (rat) ● Dorsal raphe (serotonergic) ((human, primate, rat)	● Ventral striatum (A10 dopaminergic) ((human, primate, rat) ● Amygdala (A10) (human, primate, rat) ● Cerebral cortex (A10) (rat) ● Visual cortex (non-dopaminergic) (rat) ● Pedunculopontine nucleus (non-dopaminergic) (rat)

Table 1.1 : Inputs and outputs connections of the substantia nigra (SN) and the ventral tegmental area (VTA). (from Fallon and Loughlin, 1985;1995; Flaherty and Graybiel, 1994; Blandini *et al*., 2000).

1.2.5 The subthalamic nucleus

The subthalamic nucleus (STN) is a small, densely populated and highly vascularized structure located dorsomedial to the junction between the crus cerebri and the internal capsule, ventral to the thalamus. The STN receives projections from both the frontal cortex and GPe and sends projections back to GPe and GPi.

The STN consists of a multitude of medium-sized and densely packed neurons with long, sparsely spiny dendrites radiating from the cell body **(Chang *et al*., 1983; Kita *et al*., 1983).** The subthalamic nucleus is the only glutamatergic nucleus of the basal ganglia circuit **(Smith and Parent, 1988)** and most of their neurons are projection neurons **(Van der Kooy and Hattori, 1980a)**.

1.3 Basal ganglia circuitry (See figure 1.1)

Early studies using autoradiography suggested that cortical afferents arising from the somatomotor cortex preferentially innervate the putamen **(Kunzle, 1977)**, while association cortex afferents innervate the caudate nucleus **(Goldman and Nauta, 1977)**. Different areas of cerebral cortex reach the striatum by excitatory glutamatergic projection **(Young *et a*l., 1981)**. The subthalamic nucleus is the only other structure in the basal ganglia receiving direct afferents from the premotor and motor cortices **(Afsharpour, 1985; Stanton *et al*., 1988)**. The basal ganglia are not isolated structures but form part of neural circuits organized in parallel called cortico-striato-thalamo-cortical loops. Five such loops have been described **(Alexander and Crutcher., 1990)**.

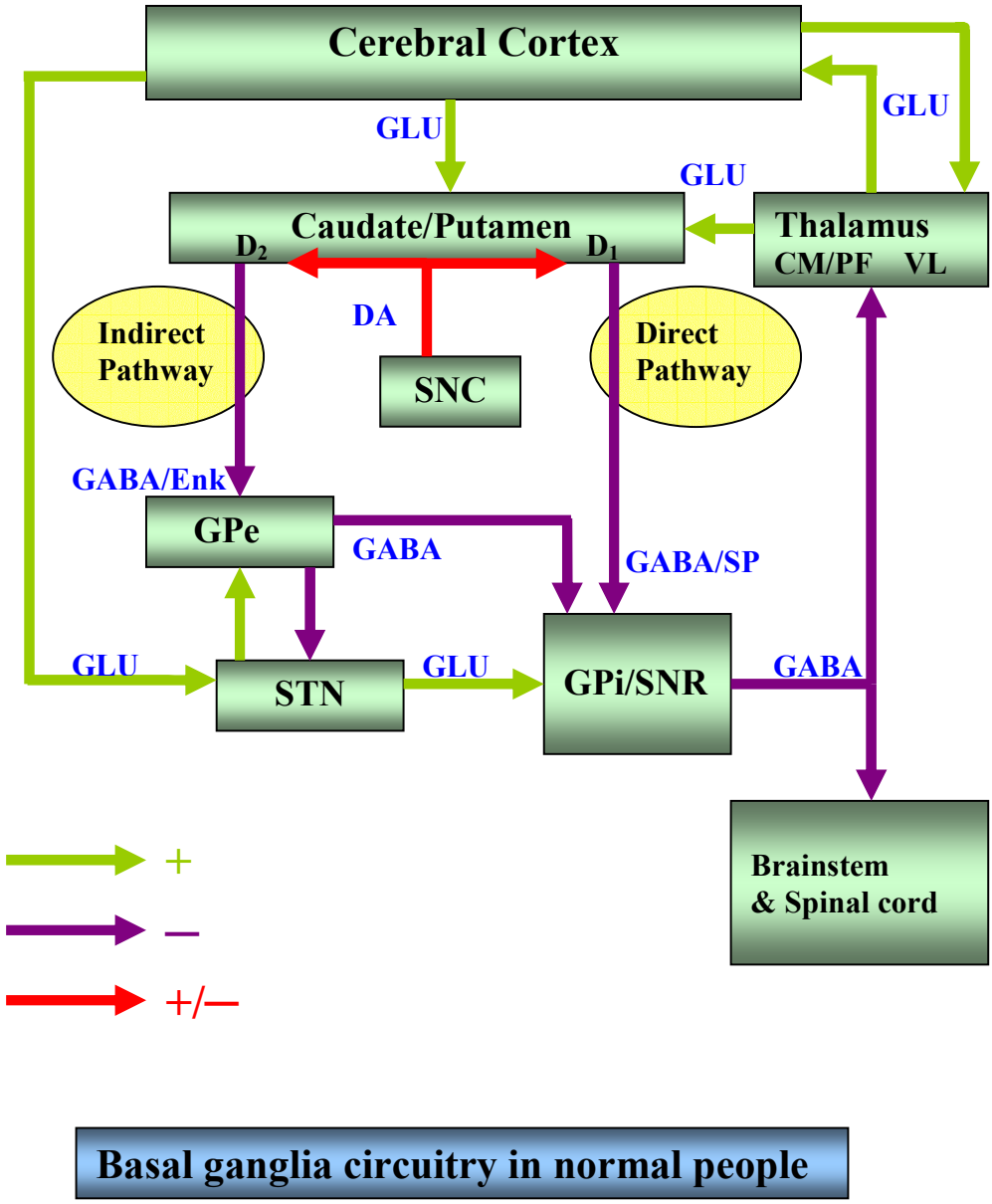


Figure 1.1 **Basal ganglia circuitry in normal people**, **showing the direct and indirect pathways of basal ganglia. Excitatory projections are shown in green; inhibitory projections are shown blue; dopaminergic nigrostriatal projections are shown red. (CM/PF: centromedian/farafasicular nucleus of thalamus; D1: dopamine receptor 1; D2: dopamine receptor 2; DA: dopamine; Enk: enkephalin; GABA: gamma-aminobutyric acid; GLU: glutamate; Gpe: external segment of globus pallidus; Gpi: internal segment of globus pallidus; SNC: substantia nigra pars compacta; SNR: substantia nigra pars reticulata; STN: subthalamic nucleus; SP: substance P; VL: ventrolateral thalamus) (Partially adapted from Alexander and Crutcher, 1990; Blandini *et al.*, 2000).**

1.3.1 Motor circuit or loop

The motor circuit is the loop most related to general movement. In the motor circuit information is somatotopically organized into distinct zones representing the leg, face and arm **(Kunzle, 1975; Crutcher and DeLong, 1984; Alexander and DeLong, 1985)**. The premotor cortex, the supplementary motor area and the somatosensory cortex all send excitatory information to the putamen **(Kunzle, 1977; Selemon and Goldman-Rakic, 1985)**, which projects topographically to specific areas of the internal and external segment of globus pallidus and the substantia nigra pars reticulata **(Johnson and Rosvold, 1971; Parent *et al*., 1984)**. Topographic projections reach thalamic nuclei including nucleus ventralis lateralis pars oralis, lateral nucleus ventralis anterior pars parvocellularis, lateral nucleus ventralis anterior pars magnocellularis and the centromedian nucleus **(DeVito and Anderson 1982; Kinsky *et al*., 1985)**. The motor loop ends with thalamo-cortical projections to the premotor and the supplementary motor cortical area **(Wiesendanger and Wiesendanger, 1985; Matelli *et al*., 1989)**. There are two main pathways through the basal ganglia.

a) **The direct pathway** is an inhibitory efferent pathway which projects from the striatum to the output nuclei (the internal segment of globus pallidus and the substantia nigra pars reticulata). The direct pathway contains GABA, substance P **(Albin *et al*., 1989)**, and dynorphin **(Vincent *et al*., 1982)** and expressed D_1 dopamine receptors.

b) **The indirect pathway** projects from the striatum to the external segment of the globus pallidus; this in turn projects to the subthalamic nucleus through purely GABAergic neurons **(Albin *et al*., 1989; Hamani *et al*., 2004)** and

from the subthalamic nucleus to the output nuclei through an excitatory glutamatergic projection **(Nakanishi *et al*., 1987; Smith and Parent, 1988)**.

The direct and indirect pathways have opposite effects on the output nuclei (Their connections to the thalamus) via an inhibitory GABAergic pathway **(Penney and Young, 1981; Chevalier *et al*., 1985) (Figure 1.1)**.

1.4 Extrinsic monoaminergic system

Monoaminergic pathways which originate in the midbrain project rostrally and make contact with key components of basal ganglia loops or circuits. Even though they are not part of the loop, they may exert a considerable effect on its activity and disturbances in their input underlie common motor disorders.

1.4.1 Dopamine

Dopamine (DA) is a member of the catecholamine family and was found in the brain in 1959. It has many functions around the body but has an especially important role as a neurotransmitter in the brain.

The existence of dopaminergic innervation within the brain was suggested by biochemical studies **(Thierry *et al*., 1973 a,b)** and confirmed by anatomical work utilizing glyoxylic acid-induced histofluorescence **(Lindvall and Bjorklund, 1974 a,b)**, histofluorescence in combination with tract-tracing **(Tork and Turner, 1981)**, autoradiography **(Descarries *et al*., 1987)**, immunohistochemistry against the synthesizing enzyme tyrosine hydroxylase (TH) **(Berger *et al*., 1985)** and

immunohistochemistry with antibodies against dopamine (DA); the problem of distinguishing between DA and NA in these studies is acknowledged **(Papadopoulos *et al*., 1989)**.

The amino acid tyrosine is the starting point for dopamine synthesis. It is converted into dihydroxyphenyalanine (dopa) by the rate-limiting enzyme tyrosine hydroxylase, after which dopa can be converted to dopamine by the enzyme dopa decarboxylase.

Dopamine synthesis is modulated by the end product inhibition of tyrosine hydroxylase (TH) through negative feedback; in addition, depolarization of dopaminergic cells results in TH activation, thus maintaing the balance between DA synthesis and release **(Joh *et al*., 1978) (Figure 1.2)**.

Dopamine can be taken back up into presynaptic terminals via the dopamine transporter **(Hitri *et al* 1994)**, or catabolized by monoamine oxidase (MAO) to 3,4,-dihydroxyphenylacetic acid or to 3-methoxytyramine by Catechol-O-methyltransferase (COMT).

Dopamine receptors can be divided into five subtypes (D1 to D5) all of which belong to the G-protein coupled type **(for review, see Wolfarth and Ossowska, (1995)**.

The vast majority of all the brain dopamine (some 80%) is found in the corpus striatum **(Coyle and Snyder, 1981)**.

The midbrain dopaminergic neurons form three groups of cells A8, A9, and A10 and three major dopaminergic pathways arise from them. This classification groups the cells according to the transmitter (class "A" cells contain dopamine or norepinephrine while class "B" contain serotonin) while numbers (e.g. 8,9,10) represent regions: thus, A9 is a group of cells containing dopamine in the SNC **(Dahlstrom and Fuxe, 1964)**.

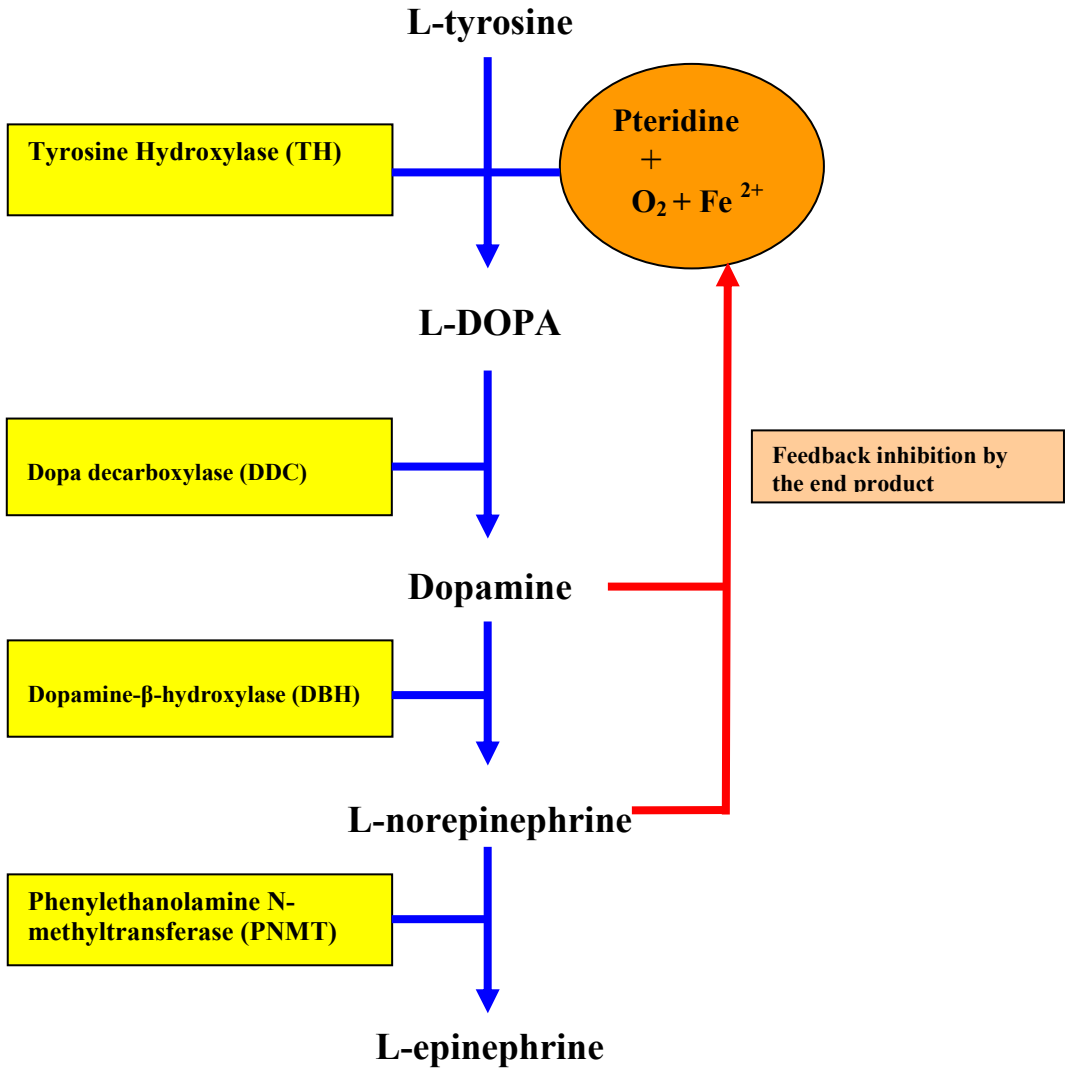


Figure 1.2 Biosynthetic pathway of catecholamine synthesis. The enzymes needed for this pathway are shown in yellow rectangles. The feedback regulation by the end product on TH is shown in red. (TH requires cofactors pteridine or tetrahydrobiopterin (BH4) and molecular oxygen and Fe^{+2}) partially adapted from Kumer and Vrana (1996).

The A9 cell group in the SNC, the A8 cell group in the retrorubral region, and the A10 cell group in the medial region of the ventral tegmental area are the main sources of dopamine for the ventral striatum, cerebral cortex and limbic system although these regions also receive some connections from the medial SNC **(Fallon and Loughlin, 1995)**.

The most important dopaminergic pathways are the nigrostriatal, mesolimbic, and mesocortical pathways. These all originate from the dopaminergic cells (A9, A8, and A10) so, neurons of the A9 and A8 groups supply the nigrostriatal system whereas A10 supplies the mesolimbic and mesocortical systems **(Prensa and Parent, 2001)** **(see Table 1.2)**.

Cell groups	Regions	Pathways	Targets
A8	Retrorubral field (RRF)	Mesostriatal Mesolimbic	Caudate-putamen (human, primate, rat) Amygdala (human, primate, rat)
A9	SNC ventral tier (lateral)	Nigrostriatal	Caudal putamen (striosome) (human)
A9	SNC ventral tier (medial)	Nigrostriatal	Dorsal caudate nucleus (human)
A9	SNC dorsal tier	Nigrostriatal	Rostral putamen (human) Caudate nucleus (matrix) (human)
A9	SNR	Nigrostriatal	Caudate-putamen (human, primate, rat)
A9	SNL	Nigrostriatal Mesolimbic	Caudate-putamen (rat) Amygdala (rat)
A10	VTA	Mesolimbic Mesocortical	Ventral striatum (human, primate, rat) Amygdala (human, primate, rat) Prefrontal/Anterior cingulate cortices (rat)

Table 1.2: Dopaminergic cell groups in the midbrain and their targets. (from Dahlstrom and Fuxe, 1964; Fallon and Loughlin, 1985;1995; Gibb and Lees, 1994; Prensa and Parent, 2001).

1.4.2 Dopamine interactions

Dopamine interacts with many other neurotransmitters in the basal ganglia circuitry. In particular, the interaction of dopamine with glutamate has an important excitatory effect on the neostriatum **(Shimizu *et al*., 1990; Garcia-Munoz *et al*., 1991)** from the converging of glutamatergic and dopaminergic afferents onto the same striatal GABA neuron **(Bouyer *et al*., 1984)**. Changes in dopamine and other transmitters during cell stress may be due to a global response, or may signal a true interaction. Thus, ischemia and hypoxia have been found to produce an increase in the concentration of dopamine, glutamate and aspartate in the neostriatum of rat **(Globus *et al*., 1988; Damsma *et al*., 1990; Akiyama *et al*., 1991)**, while dopamine depletion and glutamatergic receptor blockade have both been shown to attenuate neuronal death following hypoxic and ischemic injury **(Weinberger *et al*., 1985; Gill *et al*., 1987; Clemens and Phebus, 1988)**. Conversely, increasing dopamine concentration may lead to the release of glutamate and aspartate as a toxic cascade **(Barbeito *et al*., 1989; Carlsson and Carlsson, 1990)**, while the inhibitory amino acid GABA and taurine are thought to exert a protective role during a hypoxic and ischemic insult **(Sternau *et al*., 1989)**.

There are also important interactions between acetylcholine and dopamine. The striatum has very high expression of acetylcholine receptors and enzymes needed for the synthesis or metabolism of acetylcholine such as Choline acetyltransferase and acetylcholinesterase within pedunculopontine afferents and cholinergic interneurons in the striatum. In normal conditions there is a balance between the inhibition of acetylcholine release by dopamine receptor D2 and excitation of it by dopamine receptor D1 **(Jabbari and Pazdan, 2005)**.

A further important interaction is between DA and 5-HT. There are projections from the serotonergic dorsal raphe nucleus to dopaminergic (DA) cell bodies and dendrites in the substantia nigra, ventral tegmental area and striatum **(Van der Kooy and Hattori, 1980b; Steinbusch *et al*., 1981; Herve *et al*., 1987; Nedergaard *et al*., 1988; Corvaja *et al*., 1993)**. Moreover, dopamine from the SN and VTA may increase 5-HT release in the DRN **(Ferre and Arigas, 1993)**. Other studies show that dopamine release is facilitated by serotonin **(Benloucif and Galloway, 1991)** and the regulation of serotonin in the DRN is mediated by dopamine D2 receptor **(Ferre *et al*., 1994)**.

1.4.3 Serotonin

The midbrain raphe nuclei came to attention in the time of Cajal who described the cells in that area as large multipolar neurons with uncertain projections. 5-HT was considered as a CNS neurotransmitter when it found in significant but varying quantities in different regions of brain. 5-HT cell bodies and their axon terminals have been visualized by different methods including autoradiography using light microscopy **(Conrad *et al*., 1974; Bobillier *et a*l., 1976; 1979; Azmitia and Segal, 1978; Moore *et al*., 1978)**, electron microscopy **(Aghajanian and Bloom, 1967; Descarries *et al*., 1990)**, histochemistry **(Ungerstedt, 1971)** or immunohistochemistry with antibodies against 5-HT **(Steinbusch *et al*., 1978; Lidov *et al*., 1980; Lidov and Molliver, 1982a,b)**.

The 5-HT cell bodies in the brain are located in the raphe nuclei groups in the brain stem **(Morgan *et al*., 1987; Jacobs and Azmitia, 1992; Chojnacka-Wojcik, 1995)**. The areas are classified into nine regions (B1-B9) **(Dahlstrom and Fuxe, 1964;**

Steinbusch, 1981; Tork, 1990). Serotonin receptors are currently classified into 7 types (5HT1-5HT7) **(Boess and Martin, 1994; Wesolowska, 2002)**. Some of these project to the striatum e.g. dorsal raphe nucleus **(see Table 1.3) (Steinbusch *et al*., 1981; Vertes, 1991)**.

Midbrain raphe nuclei	Cell groups	Targets
Dorsal raphe nucleus	**B6 and B7**	● Striatum ● Amygdala ● Locus coeruleus
Median raphe nucleus	**B5 and B8**	● Hippocampus ● Anterior hypothalamus ● Mammillary bodies

Table 1.3: Serotonergic cell groups in the midbrain raphe nuclei and their targets. (from Dahlstrom and Fuxe, 1964; Azmitia and Segal, 1978; Imai *et al*., 1986; Jacobs and Azmitia, 1992; McQuade and Sharp, 1997).

1.5 Neuropathology of neurodegenerative diseases

Neurodegenerative diseases are characterized by the slowly progressive loss of neurological function without obvious causes such as infection, neoplasms, localized vascular disease or toxicity **(Maimone *et al*., 2001; Bossy-Wetzel *et al*., 2004)**. Neurodegenerative diseases usually affect older age groups, although the young may be affected. Many neurodegenerative diseases are sporadic and a few are inherited. The specific clinical characteristics of any particular neurodegenerative disease can stem from the anatomical location of the affected region and the pathological changes occurring. Two major classes of neurodegenerative disorder based on biochemical and structural abnormalities in certain molecules such as tau or α-synuclein and are known as **tauopathies** and **synucleinopathies**. Tau is a microtubule-associated protein which plays an important role in microtubule assembly and stabilization **(Weingarten *et al*., 1975; Cleveland *et al*., 1977)** and it has six different isoforms

(Buee *et al*., 2000; Shahani and Brandt, 2002). Deposition of hyperphosphorylated tau is a characteristic feature of neurofibrillary tangles found in Alzheimer's disease **(Alonso *et al*., 2001; Geschwind, 2003; Klucken *et al*., 2003)**.

Filamentous tau pathology is also characteristic of other disorders such as Pick's disease, Progressive Supranuclear palsy, Corticobasal degeneration, familial front temporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) **(Spilliantini and Goedert, 1998)**. Synucleinopathies are a group of disorders characterised by α-synuclein lesions including Parkinson's disease, Alzheimer's disease, dementia with Lewy bodies, MSA, Down syndrome, Hallervorden-Spatz disease and Prion disease **(Trojanowski and Lee, 1999)**.

1.5.1 Abnormal protein aggregation and neurodegenerative diseases

Normal cellular functions may lead to the production of significant levels of abnormal (e.g. misfolded) proteins **(Sherman and Goldberg, 2001; Goldberg, 2003; McNaught and Olanow, 2006)**. Furthermore, within neurons, auto-oxidation of neurotransmitters such as dopamine can produce free radicals that can damage proteins **(McNaught and Olanow, 2006)**. Abnormal protein aggregations interfere with intracellular processes and are frequently associated with neurodegenerative diseases such as Alzheimer's and Parkinson's diseases **(Agorogiannis *et al*., 2004; McNaught and Olanow, 2006)**. A wide variety proteins implicated in the pathology of neurodegenerative diseases and aggregated in inclusions are shown in **table 1.4**. The aggregations known as Lewy bodies contain may proteins, including α-synuclein **(Zhou *et al*., 2004) (See table 1.4)**.

Protein	Function	Disease genes	Protein deposits	Disease
14-3-3	Chaperone-like function	Associate with α–Syn (10)	α–Synuclein inclusion	α - Synucleinopathies
Aβ (2)	Unknown	APP, Presenilin1.2 (8) Associate with AS and LB (10)	Extracellular plaques	AD
Ataxin1	Unknown	CAG repeat expansion (8)	Inclusion	Spinocerebellar ataxia type1
Androgen receptor	Allow body respond to androgens	CAG repeat expansion (8)	Inclusion	Spinal and bulbar muscular atrophy
Atrophin 1	Unknown	CAG repeat expansion (8)	Inclusion	Dentatorubral pallidoluysian
Bc12-antagonist of cell death (BAD)	Regulate cell death	Associate with α–Syn (10)	α–Synuclein inclusion	α -Synucleinopathies
Calmodulin (CaM)	Calcium binding protein	Associate with α–Syn (10)	α–Synuclein inclusion	α -Synucleinopathies
Calcium/Calmodulin-dependent protein kinase	Modulated the cellular response to calcium	Associate with LB (10)	LB	PD
Clusterin/apolipo-protein J (6)	Cell-aggregating factor	Associate with LB (10)	LB	AD
Copper/ zinc-SOD	Scavenging free-radicals	Associate with LB (10)	LB	PD
Cytochrome oxidase	Respiratory chains enzyme	Associate with α–Syn (10)	α–Synuclein inclusion	α -Synucleinopathies
Cdk5 (4)	Phosphorylation a molecular component of LB	Associate with LB (10)	LB	AD
DJ-1 (3)	Involved in oxidative stress response	PARK7	LB	FPD (autosomal recessive)
Plasma membrane dopamine transporter (DAT)	Terminating dopamine by reuptake it into presynaptic neurons	Associate with α–Syn (10)	α–Synuclein inclusion	α -Synucleinopathies
Extracellular signal-regulated protein kinases (ERKs)	Involve in many cellular function	Associate with α–Syn (10)	α–Synuclein inclusion	α -Synucleinopathies
Non-selenium glutathione peroxidase	Antioxidant enzyme	Associate with LB (10)	LB	PD
Heme oxygenise	Cellular stress protein	Associate with LB (10)	LB	PD
Heat shock proteins	Chaperone-like function	Both (α–Syn +LB) (10)	LB+ α–Syn inclusion	α -Synucleinopathies
Huntingtin	Unknown	CAG repeat expansion (8)	Huntington inclusion	Huntington disease
LRRK2	Unknown	PARK8	LB	FPD (autosomal dominant)
Lysosomes associate proteins	Markers of lysosomes	Associate with LB (10)	LB	PD
Microtubule-associated protein 1 (MAP1A, B)	α-SN binding proteins	Associate with α–Syn (10)	α–Synuclein inclusion	α -Synucleinopathies
Microtubule-associated protein 2(MAP2)	Stabilize microtubule assembly	Associate with LB (10)	LB	PD
Mitogen-activated protein kinases (MARKs)	Stress response, Receptor signalling activity	Both (α–Syn +LB) (10)	LB+ α–Syn inclusion	α -Synucleinopathies
Myotonin kinase	Unknown	CAG repeat expansion (8)	Inclusion	Myotonic Dystrophy
Neurofilaments	Cytoskeleton of neuron	Associate with LB (10)	LB	PD
P62	Ubiquitin-binding protein	Both (α–Syn +LB) (10)	LB+ α–Syn inclusion	α -Synucleinopathies
Parkin	Enzyme E3 ligase in UPS	PARK2	LB+ α–Syn inclusion No LB	PD FPD (autosomal recessive)

Protein kinase C isoforms	**Receptor signalling activity**	**Associate with α–Syn (10)**	**α–Synuclein inclusion**	**α -Synucleinopathies**
Prion protein (PrP)	**Unknown**	**Mutation in PrP (8)**	**Prion plaque**	**Prion disease**
Phospholipase D2	**Enzyme involve in phosphatidic acid**	**Associate with α–Syn (10)**	**α–Synuclein inclusion**	**α -Synucleinopathies**
Proteasome subunits	**Proteasome possess the proteolytic site and stabilized the proteasome complex**	**Associate with LB (10)**	**LB**	**PD**
Rab5A	**Neuronal endocytosis**	**Associate with α–Syn (10)**	**α–Synuclein inclusion**	**α -Synucleinopathies**
Septin	**Involve in cytokinesis and exocytosis**	**Associate with LB (10)**	**LB**	**PD**
Superoxide dismutase 1(SOD1)	**Enzyme calayzes the conversion of toxic superoxide radicals to hydrogen peroxide and O2**	**Mutation in SOD1 (8)**	**Bunina bodies**	**Familial amyotrophic lateral sclerosis**
Synphilin-1 (9)	**α-SN binding proteins**	**Both(α–Syn +LB) (10)**	**LB+ α–Syn inclusion**	**α -Synucleinopathies**
TAT-binding protein1 (5)	**Proteosome activator**	**Associate with α–Syn (10)**	**α–Synuclein inclusion**	**α -Synucleinopathies**
Tau	**Microtubule-associated protein that stabilize neuronal microtubules**	**Tau gene mutation Associate with α–Syn (10)**	**Cytoplasmic tangles**	**AD+Tauopathies**
Torsin A (7)	**Chaperone-like function**	**Associate with LB (10)**	**LB**	**PD**
Tyrosine hydroxylase	**Catalyse the first step in biosynthesis of catecholamine**	**Associate with α–Syn (10)**	**α–Synuclein inclusion**	**α -Synucleinopathies**
Tubulin (1)	**Accelerate AS aggregation**	**Both (α–Syn +LB) (10)**	**LB+ α–Syn inclusion**	**α -Synucleinopathies**
Ubiquitin carboxyl-terminal hydrolase LI (UCH-L1)	**De-ubiquitinating Enzyme**	**PARK5**	**LB**	**FPD (autosomal dominant)**

Table 1.4: Proteins that aggregates in inclusions in neurodegenerative diseases, in Lewy bodies in Parkinson disease and proteins that associated with α-synuclein (AD: Alzheimer's disease; α-Syn: alpha-synuclein; FPD; familial Parkinson 'disease; LB: Lewy body; PD: Parkinson's disease; UPS: Ubiquitin-proteasome system). (1) Abdul Alim *et al.* (2002); (2) Arai *et al.* (1992); (3) Bonifati *et al.* (2003); (4) Brion *et al.* (1995); (5) Ghee *et al.* (2000); (6) Sasaki *et al.* (2002); (7) Shashidharan *et al.* (2000); (8) Taylor *et al.* (2002); (9) Wakabayashi *et al.* (2000); (10) Zhou *et al.* (2004).

1.6 Movement disorders and their neuropathology

In this introduction I am chiefly concerned with neurodegenerative conditions of aminergic neurons and the basal ganglia which lead to movement disorders. These include:

1.6.1 Multiple System Atrophy

The term multiple system atrophy (MSA) was introduced by **Graham and Oppenheimer (1969)** to describe a neurodegenerative disorder occurring sporadically and characterized by Parkinsonism, cerebellar dysfunction, and autonomic insufficiency **(Wenning *et al*., 1995; Kaufmann, 1998)**.

Neuronal loss and gliosis affect the substantia nigra, putamen, locus coeruleus, pontine nuclei, cerebellar Purkinje cells, inferior olive, intermediolateral columns of spinal cord and the dorsal motor nucleus of the vagus. There is evidence of loss of dopaminergic neurons in SNC (confirmed by the loss of pigment, but without Lewy bodies) as well as loss of dopamine and it's synthetic enzyme TH; in addition, there is loss of noradrenergic neurons in the locus ceruleus and adrenergic neurons in the rostral ventrolateral medulla **(Burn and Jaros, 2001; Rehman, 2001)**. The characteristic neuropathology of MSA is the presence of many cytoplasmic inclusions in glia (glial cytoplasmic inclusion) and, later, in neurons in the absence of Lewy bodies **(Lantos and Papp, 1994)**. Immunocytochemistry shows that glial cytoplasmic inclusions are ubiquitin, tau and alpha-synuclein positive **(Gai *et al*., 1998; Lantos, 1998; Terni *et al*., 2007)**.

Data from PD brain banks showed that up to 10% of patients diagnosed with PD turn out to have MSA **(Colosimo *et al*., 1995; Kaufmann., 1998)**.

1.6.2 Progressive Supranuclear Palsy

Progressive Supranucelear palsy (PSP) is commonly known as Steele-Richardson syndrome and was described by **Steele *et al* (1964)**.

Postural instability, gaze palsy, Parkinsonism and subcortical dementia clinically characterize PSP. The degeneration is principally in the brain stem, midbrain and basal ganglia. The main lesions are in the substantia nigra, globus pallidus, subthalamic nucleus and pons **(Litvan, 1996)**. The major neurons affected are the dopaminergic nigrostriatal neurons in the SNC (particularly the ventromedial portion) and decreases in dopamine and homovanillic acid levels in the striatum; the mesolimbic and mesocortical systems are not affected. There is loss of the postsynaptic dopamine D2 receptors in basal ganglia, GABAergic neurons in the striatum and the cholinergic neurons in brainstem and other areas of the brain **(Ruberg *et al*., 1985; Lowe *et al*., 1997; Rehman, 2000)**. Pathological ultrastructural changes centre around neurofibrillary degeneration, particularly the deposition of hyperphosphorylated tau protein as neurofibrillary tangles **(Schmidt *et al*., 1996)**. The treatment of PSP with L-dopa has little effect due to loss of the postsynaptic dopamine D2 receptors in basal ganglia **(Collins *et al*., 1995; Lowe et al., 1997)**.

1.6.3 Corticobasal degeneration

Corticobasal degeneration (CBD) is a rare and slowly progressive neurological disease, first described by **Rebeiz (1968)**. CBD is characterized clinically by an asymmetrical akinetic-rigid syndrome associated with cognitive problems (apraxia and aphasia), extrapyramidal motor dysfunction (rigidity and dystonia) and moderate dementia late in the course of the disease **(Rinne *et al*., 1994)**. The neuropathological

changes in CBD include gliosis, nerve cell loss and atrophy of the posterior frontal or parietal lobes of the cerebral cortex with the presence of swollen, achromatic tau positive Pick-like cells **(Dickson *et al*., 2000)** and loss of pigmented nigral neurons in the lateral portion of the SN; Lewy bodies are absent **(Rebeiz *et al*., 1968; Gibb *et al*., 1989; Riley *et al*., 1990; Lowe *et al*., 1997)**.

1.6.4 Huntington's disease

Huntington's disease (HD) is a hyperkinetic, autosomal dominant inherited disorder characterized by progressive chorea, rigidity, dystonia, dementia, cognitive deficits and psychological disturbance. Huntington's disease takes its name from the American physician George Huntington who described it in 1872. The age of onset is normally 30 to 45, but the extreme range is 2-80 years. The mutation which is responsible for HD is an expanded polyglutamine repeat (CAG) within exon 1 of the gene in chromosome 4 that codes for the Huntingtin protein **(Hedreen and Folstein, 1995; Reddy *et al*., 1999; Myers, 2004)**.

Neuropathological changes in HD include degeneration of the caudate and putamen **(Vonsattel *et al*., 1985)** and selective loss of GABA-and enkephalin-positive medium spiny neurons that project from the striatum to the external segment of the globus pallidus **(Perry *et al*., 1973; Sapp *et al*., 1995; Mitchell *et al*., 1999) (Figure 1.3)**. Some studies also report a loss in both nigrostriatal and in nonpigmented cells in the substantia nigra **(Oyanagi *et al*., 1989; Bohnen *et al*., 2000)**. Dopamine levels have been found to be normal or increased, whereas homovanillic acid was found to be low **(Bird and Iversen, 1974; Spokes, 1980; Kish et al., 1987)**.

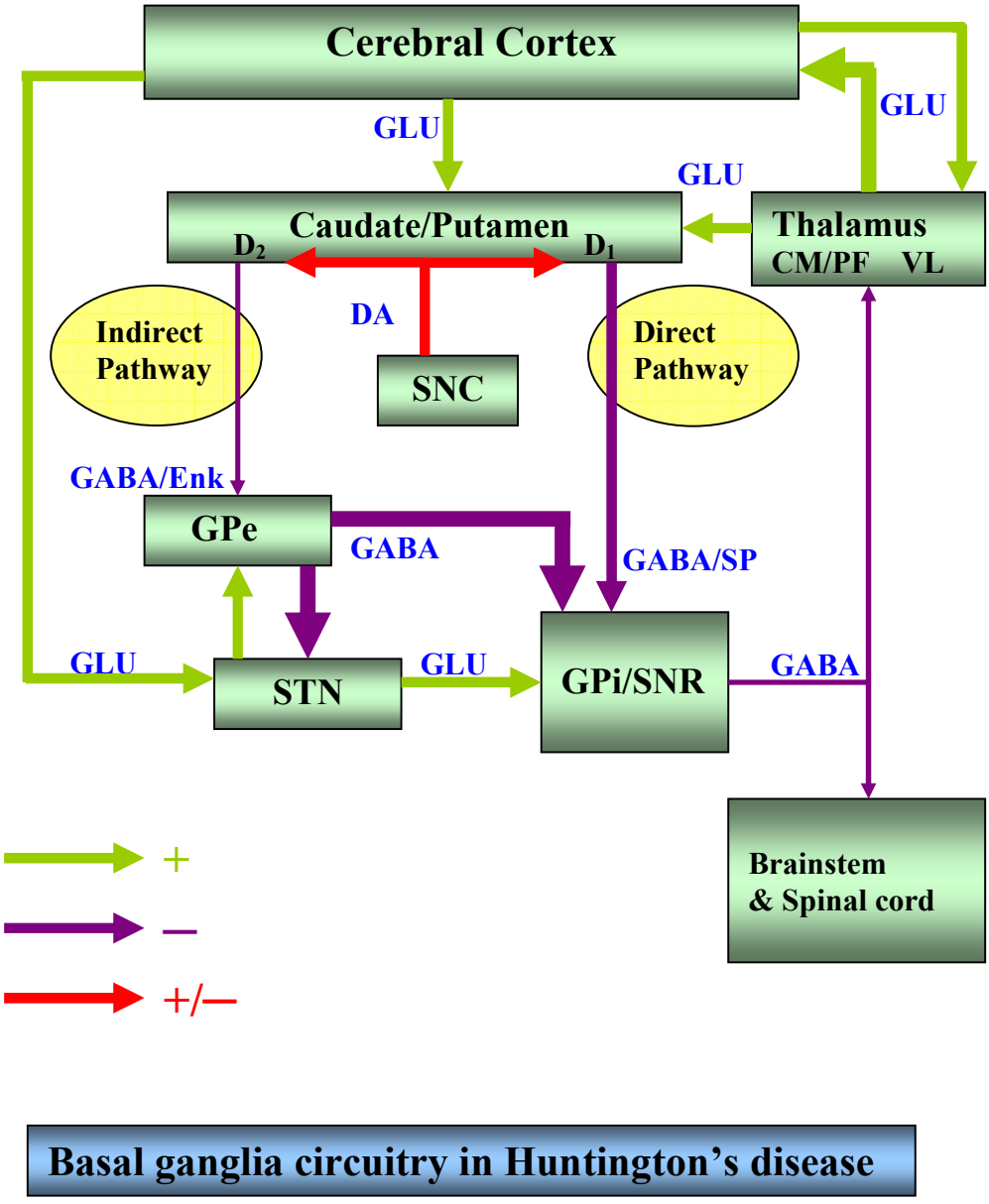


Figure 1.3: Basal ganglia circuitry in Huntington's disease, showing the direct and indirect pathways of the basal ganglia. Excitatory projections are shown in green; inhibitory projections are shown in blue; dopaminergic nigrostriatal projections are in shown red. Differences in the thickness of arrows indicates the relative degree of activation. (CM/PF: centromedian/farafasicular nucleus of thalamus; D1: dopamine receptor 1; D2: dopamine receptor 2; DA: dopamine; Enk: enkephalin; GABA: gamma-aminobutyric acid; GLU: glutamate; Gpe: external segment of globus pallidus; Gpi: internal segment of globus pallidus; SNC: substantia nigra pars compacta; SNR: substantia nigra pars reticulata; STN: subthalamic nucleus; SP: substance P; VL: ventrolateral thalamus) (Partially adapted from DeLong, 1990).

A major ultrastructural pathological change is the aggregation of inclusions in neurons of the striatum and cortex **(Roizin *et al*., 1974; DiFiglia *et al*., 1997)**. These inclusions contain aggregations of Huntingtin protein and ubiquitin **(Davies *et al*., 1997; DiFiglia *et al*., 1997)**. The mechanisms of neuronal degeneration resulting from the mutation in Huntingtin protein are poorly understand and many hypotheses have been advanced including glutamatergic excitotoxicity **(Young *et al*., 1988; Beal *et al*., 1991; Ferrante, 1993)**, mitochondrial dysfunction **(Horton *et al*., 1995; Gu *et al*., 1996; Koroshetz *et al*., 1997)**, endoplasmic reticulum stress **(Nishitoh *et al*., 2002)** apoptosis **(Saudou *et al*., 1998; Ona *et al*., 1999)** and dysfunction of the ubiquitin-proteasome system **(Seo et al., 2004, 2007)**.

1.6.5 Parkinson's disease

Parkinson's' disease (PD) is a progressive neurodegenerative movement disorder, first described by James Parkinson in 1817; it is the second most common neurodegenerative disease affecting 1-2% of people over 65 years of age **(de Rijk *et al*., 1997)**. There are two major classes of PD (i) the late onset sporadic form which occurs over the age of 55 and (ii) the early onset familial form **(Gwinn-Hardy, 2002)**. The majority of cases of PD (90%) are sporadic **(McNaught *et al*., 2006; Olanow and McNaught, 2006)**.

PD is characterized clinically by tremor that occurs at rest but decreases with voluntary movement ("resting tremor"), rigidity (increased limb resistance to passive movement), bradykinesia (slowness of movement), gait dysfunction, postural instability **(Dauer and Przedborski, 2003)**, depression and dementia.

The clinical symptoms appear after massive reduction of striatal dopamine levels (80%) **(Bernheimer *et al*., 1973; Hornykiewicz, 1998)** associated with severe loss of

dopaminergic neurons in the substantia nigra pars compacta (A9 dopaminergic neurons) **(Hirsch *et al*., 1988; German *et al*., 1989; Pakkenberg *et al*., 1991)**. The ventrolateral tier of the SNC which projects to the putamen is more affected **(Gibb and Lees, 1991)**. The dopaminergic neurons (A10) in the VTA (that project to cortical and limbic areas) show less severe cell loss **(Jellinger, 2005)**. Levels of dopamine are decreased in the substantia nigra, and ventral tegmental area and there is decreased TH activity in the SN and in the striatum **(Fahn *et al*., 1971; Rinne *et al*., 1974; McGeer and McGeer, 1976; Ploska *et al*., 1982; Javoy-Agid *et al*., 1990)**. The level of dopamine metabolites (such as DOPAC and HVA) are also decreased in SN and the striatum **(Sian *et al*., 1999)**.

Loss of nigrostriatal dopaminergic neurons promotes an activation of the indirect pathway through the basal ganglia circuit **(Filion *et al*., 1988)**. The initial part of this pathway is inhibitory with GABA/Enkephalin striatal neurons that project to the external segment of the globus pallidus. Inhibition of GABAergic neurons of GPe will release the subthalamic nucleus from it's inhibition (= disinhibition) by GPe. Increased activity in the subthalamic nucleus will, in turn, cause excitation of the basal ganglia output nuclei (internal segment of globus pallidus and the substantia nigra pars reticulata) via the excitatory glutamatergic pathway that connects both of them. Reduced activity of GABA/substance P neurons of the direct pathway will also result in disinhibition of the output nuclei projections leading to inhibition of thalamocortical neurons via the GABAergic projection which reduces activity of the glutamatergic neurons projecting to the motor areas of the cerebral cortex. This will result in many of the hypokinetic symptoms of Parkinson's disease **(Filion *et al*., 1988) (Figure 1.4)**. Many observations support this mechanism, such as reduced substance P in the output nuclei of the basal ganglia in Parkinson's disease patients

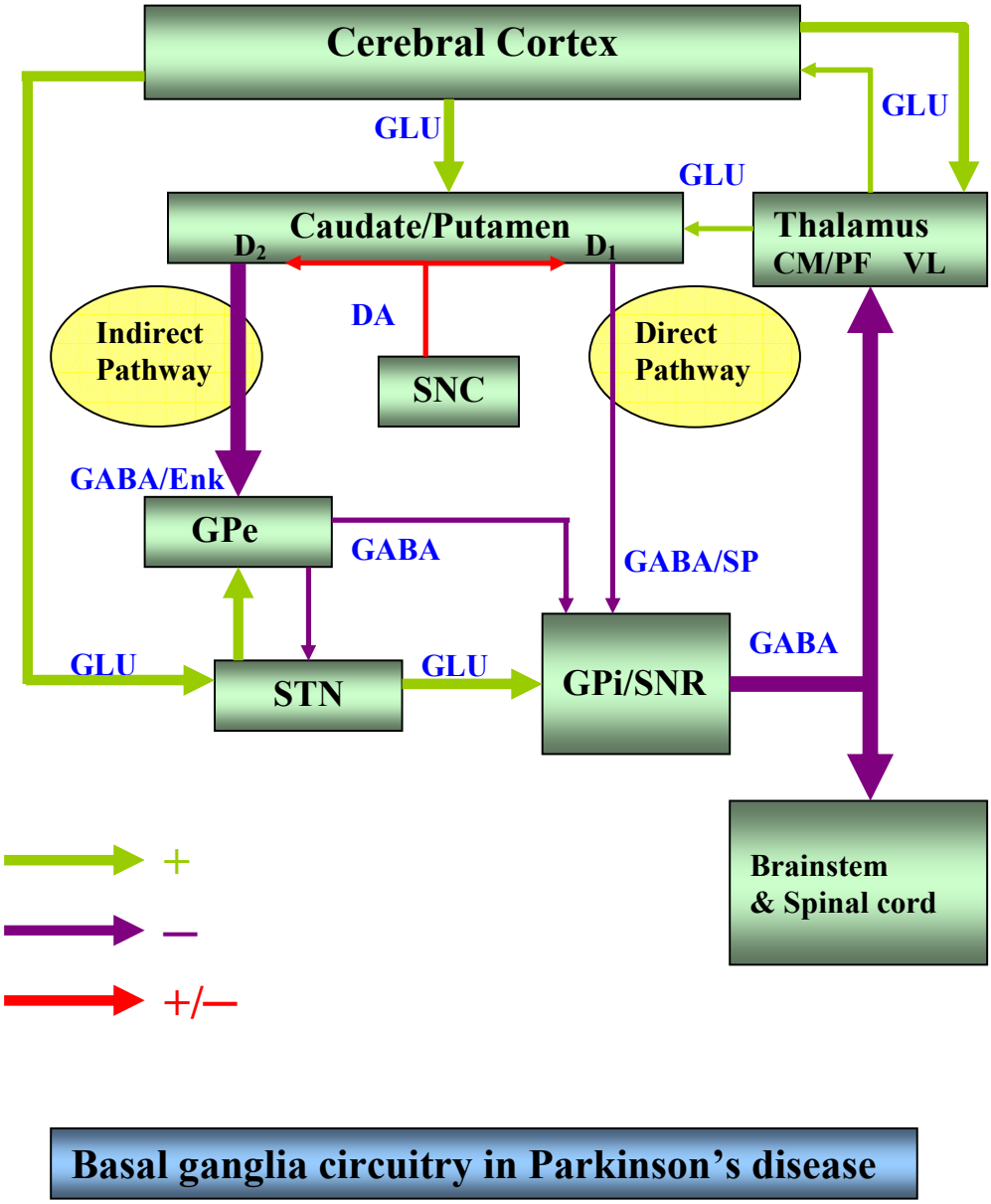


Figure 1.4: Basal ganglia circuitry in Parkinson's disease, showing the direct and indirect pathways of the basal ganglia. Excitatory projections are shown in green; inhibitory projections are shown in blue; dopaminergic nigrostriatal projections are shown in red. Differences in the thickness of arrows indicates the relative degree of activation. (CM/PF: centromedian/farafasicular nucleus of thalamus; D1: dopamine receptor 1; D2: dopamine receptor 2; DA: dopamine; Enk: enkephalin; GABA: gamma-aminobutyric acid; GLU: glutamate; Gpe: external segment of globus pallidus; Gpi: internal segment of globus pallidus; SNC: substantia nigra pars compacta; SNR: substantia nigra pars reticulata; STN: subthalamic nucleus; SP: substance P; VL: ventrolateral thalamus) (Partially adapted from DeLong, 1990; Blandini *et al.*, 2000).

(Agid *et al*., 1987; Waters *et al*., 1988) and unchanged enkephalin levels in the external segment of the globus pallidus **(Agid *et al*., 1987)**. Moreover, ablation surgery performed on the subthalamic nucleus reduces the symptoms of Parkinson's disease suggesting that excessive subthalamic nucleus activity is a key feature of the disorder **(Bergman *et al*., 1990; Aziz *et al*., 1991; Limousin *et al*., 1995)**. **Chesselet and Delfts (1996)** suggest that the model may need correcting following evidence that activity in the external segment of globus pallidus is increased after nigrostriatal dopamine depletion by MPTP, probably due to the increased neuronal discharge rate of excitatory glutamatergic projections from the subthalamic nucleus **(Pan and Walters, 1988; Tremblay and Filion, 1989)**. A similar finding was noted in 6-hydroxydopamine-lesioned animals **(Porter *et al*., 1994)**.

1.6.5.1 Etiology of Parkinson's disease

The etiology of PD is not fully understood, but many cases may involve an interaction between genetic and environmental factors **(Duvoisin, 1999; Mizuno *et al*., 1999; Le Couteur *et al*., 2002; Sherer *et al*., 2002; Schapira, 2006)**. Epidemiological studies suggest that exposure to environmental agents, such as pesticides, may increase the risk of PD **(Gorell *et al*., 1998; Menegon *et al*., 1998)**. In addition to the involvement of environmental factors in PD, genetic factors are also involved, since about 5-10 % cases are believed to have a familial Parkinsonism **(Olanow and Tatton, 1999; McNaught *et al*., 2006)**. Familial cases of PD have been associated with mutations in proteins such as α-synuclein on chromosome 4 **(Polymeropoulos *et al*.,1997)** and parkin on chromosome 6 **(Kitada *et al*., 1998)**. Mutation also in UCH-L1 **(Leroy *et al*., 1998)**, PINK1 **(Valente *et al*., 2004)**, DJ-1 **(Bonifati *et al*., 2003; Abou-Sleiman *et al*., 2004)** and LRRK2 **(Funayama *et al*., 2002)**. For reviews see, **Cookson, (2005); Jain *et al*., (2005); Schapira, (2006)**. Many different genes involved in PD

may relate to protein mis-folding **(McNaught *et al*., 2002; Ryu *et al*., 2002)**. For reviews, see **Soto, (2003); Agorogiannis *et al*., (2004); McNaught and Olanow, (2006)**. This topic will be discussed later.

Many factors lead to neurodegeneration in PD **(Olanow and Tatton, 1999)**. These include:

1) **Oxidative stress**, where damaging levels of hydrogen peroxide and then reactive oxygen species are increased as a result of:

Indirect

a- Increased dopamine turnover,

b- A deficiency in glutathione,

c- A build up of reactive iron which can lead to formation of hydroxyl radicals.

2) **Mitochondrial dysfunction**.
3) **Excitotoxicity**, which results from increased glutamate formation.
4) **Apoptosis**.

Alongside these factors, protein aggregation or mis-folding is implicated in the cell death mechanisms in PD and other degenerative conditions **(Moore *et al*., 2005; McNaught *et al*., 2006)**.

1.6.5.2 Neuropathology of Parkinson's disease

There are no changes in the gross morphology of the brains of PD patients. However, gross slices or histological sections reveal loss of neuromelanin pigmention in the substantia nigra (DA) and locus ceruleus (NE). Other regions such as the striatum and the globus pallidus appear normal.

On histopatholgical examination there is loss of the dopaminergic neurons of the substantia nigra pars compacta. In typical PD (as well as other diseases where

parkinsonism is a component) the neuronal loss is usually most marked in the ventrolateral tier of neurons which mainly project to the striatum (putamen) **(Bernheimur *et al*., 1973)**. The substantia nigra contains about 550,000 dopaminergic neurons (and approximately 450,000 dopaminergic one are in pars compacta) and are reduced by at least two thirds in PD patients and nondopaminergic neurons are 260,000 and reduced by about a quarter in PD patients **(Pakkenberg *et al*., 1991; Lang and Lozano, 1998a)**. The neuronal loss in the substantia nigra is accompanied by astrocytosis and microglial activation **(Teismann *et al*., 2003)**.

Other systems are affected in PD, and there is loss of (a) noradrenergic neurons and depletion of noradrenaline concentration in the locus ceruleus **(Mann *et al*., 1983; Cash *et al*., 1987; Jellinger, 2005)**, (b) cholinergic neurons in the nucleus basalis of Meynert, pedunculopontine nucleus, Edinger-Westphal nucleus and the dorsal motor nucleus of the vagus **(Nakano and Hirano, 1983; Zweig *et al*., 1989; Gai *et al*., 1992)** together with depletion of the cholinergic enzymes (such as Choline Acetyltransferase) in the putamen, globus pallidus and the SNC **(Nishino *et al*., 1988)**, (c) serotonergic neurons of the dorsal raphe nucleus **(Jellinger, 2005)** and a reduction in serotonin concentration in areas such as the striatum, substantia nigra, and hippocampus **(Rinne *et al*., 1974; Scatton *et al*., 1983; Agid *et al*., 1987; Mizuno, 2005)**. There is also a reduction in tryptophan hydroxylase activity in Parkinson's disease patients **(Sawada *et al*., 1985)**, as well as depleted 5-HT and its metabolites in the cerebrospinal fluid (CSF) **(Tohgi *et al*., 1993)**.

Other histopatholgical characteristics are the presence of neuronal intracytoplasmic inclusions called Lewy bodies, Lewy neurites (ubiquitin-positive degenerating neuronal processes), pale bodies which are considered as precursors of Lewy bodies **(Dale *et al*., 1992)** and a variable amount of extracellular neuromelanin and gliosis

(McGeer *et al*., 1988; Fearnley and Lee, 1991; Cornford *et al*., 1995; Forno, 1996).

1.6.5.3 Lewy body

The Lewy body is a neuronal intracytoplasmic inclusion body and it is widely considered as the histopatholgical hallmark of PD **(Pollanen *et al*., 1993; Cornford *et al*., 1995; Forno, 1996; Galvin *et al*., 1997; Shults, 2006)**. Lewy bodies were first described in the neurons of the substantia inominata and the dorsal vagal nucleus in PD by **Friederich Lewy in 1912** and bear his name **(Gibb and Poewe, 1986)**.

Lewy bodies can be seen in the surviving dopaminergic neurons of the substantia nigra pars compacta in all most every case of PD **(Jellinger, 1987; Hughes *et al*., 1993; Pollanen *et al*., 1993; Cornford *et al*., 1995; Forno, 1996)**, dead and they are also seen in other groups of neurons including dopaminergic mesolimbic neurons, the cholinergic neurons of the nucleus basalis of Meynert, the noradrenergic neurons of the locus coeruleus, the serotonergic neurons of the raphe nuclei, the motor vagal nuclei, pedunculopontine nucleus, the Edinger-Westphal nucleus, the intermediolateral cell column of the spinal cord, the hypothalamus and autonomic ganglia **(Jellinger, 1991)**. Neurons with Lewy bodies which do not stain for TH may be either non-dopaminergic cells or dopaminergic cells that may be defective or non functional **(Iravani *et al*., 2006)**.

Morphologically, Lewy bodies can be divided into two types which are found in different locations in the brain:

1) **classical brainstem LB** is a spherical intraneuronal eosinphilic inclusion that has a diameter of 8-30 μm with a central dense core and a pale peripheral halo **(Lowe *et al*., 1997; Jellinger, 2005)**.

2) **Cortical Lewy bodies** are a round intraneuronal eosinphilic inclusions without a halo but they have also angular and reniform shapes **(Gibb *et al*., 1987; Lowe *et al*., 1997; Jellinger, 2005)** and were first described by **Okazaki (1961)**.

Most affected neurons have a single LB, though multiples do occur LB **(Dickson, 2005)**. Ultrastructurally, both classical and cortical Lewy bodies are composed of radially arranged intermediate filaments (7-20 nm) associated with granular electron dense material **(Rajput and Rozdilsky, 1976; Pirozzolo *et al*., 1982; Crystal *et al*., 1990; Xuereb *et al*., 1990; Forno, 1996; McKeith *et al*., 1999; Jellinger *et al*., 2001) (Figure 1.5)**. Both classical and cortical Lewy bodies are immunopositive for ubiquitin, α-synuclein **(Lennox *et al*.,1989; Love and Nicoll, 1992; Irizarry *et al*., 1998; Spillantini *et al*., 1997;1998a) (Figure 1.5)** and neurofilaments **(Goldman *et al*., 1983; Hill *et al*., 1991; Schmidt *et al*., 1991)** in addition to other components **(see table 1.4).** Lewy neurites are ubiquitin-positive degenerating neuronal processes first described in the hippocampus **(Dickson *et al*., 1991)** and also found in other brain regions such as amygdala, cingulate gyrus and temporal cortex **(Dickson, 2005)**. Pale bodies are rounded granular pale eosinophilic areas seen in neurons of the substantia nigra and the locus coeruleus **(Lowe *et al*., 1997)**. Lewy bodies are widely accepted pathological hallmark of both sporadic and familial PD, as well as dementia with Lewy bodies **(Gibb *et al*., 1987; Gomez-Tortosa *et al*., 2000)**. Lewy bodies can be also seen in a number of other disorders, such as Alzheimer's disease, Down syndrome and Hallervorden-Spatz disease **(Arawaka *et al*., 1998; Lippa *et al*., 1999; Wakabayashi *et al*., 1999; Yokota *et al*., 2007)**.The mechanism by which Lewy bodies are formed and their relationship to neurodegenerative disease or age process

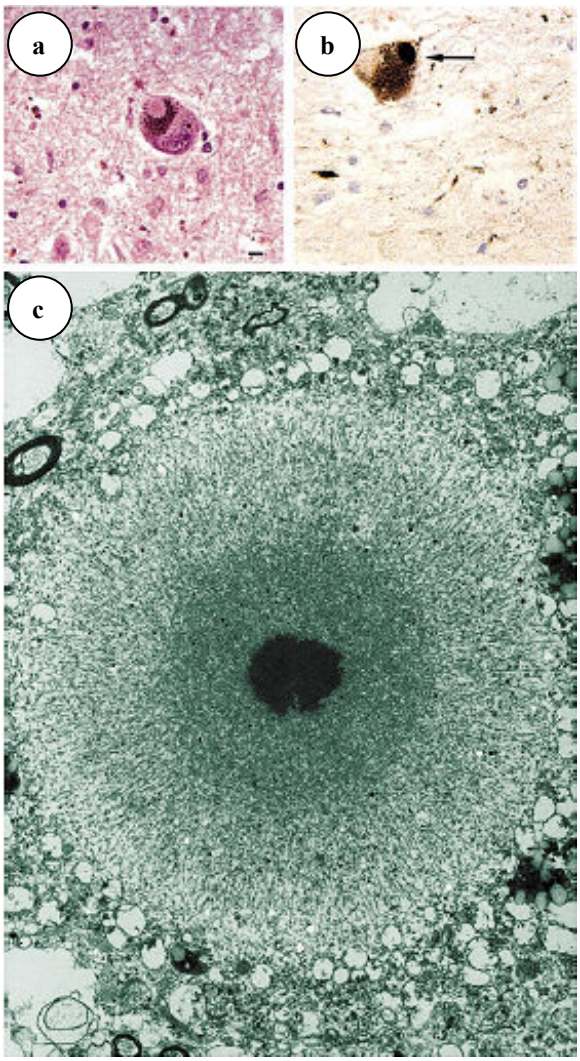


Figure 1.5: Lewy bodies are a characteristic feature of Parkinson's disease. Lewy bodies are small spherical inclusions that (a) can be stained with haematoxylin/eosin, and (b) contain the protein α-synuclein, which in this specimen was detected with a specific antibody. (c) Lewy bodies consist of radiating filaments that can be seen in this electron micrograph. (Panels a and b © Macmillan Magazines Ltd; panel c is adapted with permission from (Forno, 1996) American Association of Neuropathologists). The set of three photos is from Beal, (2001).

remained unknown. One possibility is that Lewy bodies reflect damage since proteins such as α-synuclein or ubiquitin will accumulate and aggregate if the ability of cells to degradate these proteins are exceeded or proteasomal function impaired **(Sherman and Goldberg, 2001)**; those aggregated proteins will subsequently provide a nucleation center for the formation of inclusion bodies such as Lewy bodies **(Chung *et al*., 2001a)** and the accumulation of these inclusion bodies might induce further neuronal dysfunction and/or cell death leading to neurodegeneration **(Alves-Rodrigues *et al*., 1998; Bence *et al*., 2001; Chung *et al*., 2001a)**. Anther possibility is that Lewy bodies are a protective device as they form and function in a way similar to an aggresome **(McNaught *et al*., 2002; Olanow *et al*., 2004)**. Aggresomes are cytoplasmic inclusion bodies that form at the centrosome (a perinuclear structure linked to the microtubular system) as a cytoprotective response to high levels of misfolded proteins **(Johnston *et al*., 1998; Kopito, 2000)**. There is evidence that Lewy bodies in PD resemble aggresomes (cytoprotective) since they stain positively for specific markers of aggresome such as γ-tubulin and pericentrin as well as UPS components **(McNaught *et al*., 2002)**. In PD, the aggresome might be an intermediate stage in the formation of Lewy bodies which form if there is continued failure to clear abnormal proteins **(McNaught *et al*., 2002; Olanow *et al*., 2004)**. Mutations in parkin (ubiquitin-ligase) cause dysfunction in UPS components required for protein ubiquitination leading to accumulation of poorly degraded cytotoxic proteins as well as impaired transport of ubiquitinated proteins to the aggresome; this may explain the lack of Lewy bodies in autosomal recessive juvenile parkinsonism and the lack of a cytoprotective response may further explain the early age of onset, and the rapidity and severity of neurodegeneration in such patients **(McNaught and Olanow, 2003)**. Inclusion bodies are not always found in neurodegenerative

conditions. For example, they do not occur in most laboratory models of Parkinson's disease such as those produced through 6-OH-dopamine or MPTP toxicity **(Forno *et al*., 1993; Dauer and Przedborski, 2003)**, although they are present in rotenone-induced degeneration in rat **(Betarbet *et al*., 2000)**. Moreover, some human Parkinsonian conditions occur without Lewy body formation. As mentioned above, this is especially true of patients with mutations in parkin (E3 ligase of UPS) and suggests that the E3 ligase may be critical for Lewy body formation.

1.6.5.4 Treatments of Parkinson's disease

(A) Drugs treatments used in PD include:

i. **Levo-dopa** has been commonest drug in the treatment of Parkinson's disease since 1967 **(Cotzias *et al*., 1967)**. Levodopa or L-dopa (3,4 dihydroxyphenyalanine) is the precursor of the neurotransmitter dopamine and can easily cross the blood-brain barrier where it is converted into dopamine by aromatic L-amino acid decarboxylase. Unfortunately, efficacy reduces with prolonged treatment and motor complications such as dyskinesias and fluctuations **(Lang and Lozano, 1998b)**.

ii. Bromocriptine, Pramipexole and Apomorphine are **dopamine agonists** which directly bind to and activate dopamine receptors in the brain and therefore have the same effect as dopamine, for review, see **Lang and Lozano, (1998b); Kuniyoshi and Jankovic, (2005)**.

iii. **Monoamine oxidase-B (MAO-B) inhibitors** such as Selegiline, prevent the metabolism of dopamine, and so will increase the availability of dopamine in the brain, for review, see **Lang and Lozano, (1998b); Bertoni and Elmer, (2005)**.

iv. **Catechol-O-methyl transferase (COMT)** inhibitors such as Entacapone, prevent the peripheral metabolism of dopamine thus allowing additional L-DOPA to gain access to the brain, for review, see **Wahba *et al*., (2005)**.

v. **Anticholinergic drugs** such as Benztropine and Trihexyphenidyl have been found effective in the treatment of Parkinson's disease and may reduce the tremor and rigidity due to the imbalance in cholinergic and dopaminergic systems interaction, for review, see **Jabbari and Pazdan, (2005)**.

(B) Surgical treatment has also been used to alleviate Parkinson's disease and four procedures have been used :

a. lesions of the basal ganglia such as **pallidotomy** which leads to improved parkinsonism symptoms and suppresses L-dopa induced dyskinesias **(Laitinen *et al*., 1992; Dogali *et al*., 1995)**,

b. **thalamotomy** to relieve tremor and L-dopa induced dyskinesias **(Ohye *et al*., 1982; Narabayashi *et al*., 1984)**.

c. **Subthalamotomy** to relieve contra-lateral tremor, rigidity, and bradykinesia **(Alvarez *et al*., 2001; Patel *et al*., 2003; Su *et al*., 2003)** and

d. **Deep Brain Stimulation (DBS)**- a surgical technique used to improve motor symptoms and L-dopa induced dyskinesias by placing a small electrode tip in target areas such as subthalamic nucleus, thalamus and globus pallidus to block abnormal nerve signals that cause motor symptoms in PD. The electrode is connected to a battery-operated neurostimulator placed in near the clavicle **(for review see Kumar, 2002)**.

1.7 Lewy body proteins

Lewy bodies contain a wide variety of proteins, but those that have received most attention (and which have been linked to human disorders such as PD) include ubiquitin, parkin and α-synuclein. I shall review each.

1.7.1 Ubiquitin and the ubiquitin–proteasome system

1.7.1.1 Ubiquitin:

Ubiquitin (Ub) is a small protein (8.5 KDa) made up of 76 amino acids; due to its ubiquitous nature, it was named ubiquitin. It is expressed in human by three genes **(Mayer *et al*., 1991)**. It was originally extracted from bovine thymus in the early 1970 S where it was thought to have properties relating to the differentiation of T and B lymphocytes **(Goldstein *et al*., 1975)** but it has now been found in all eukaryotic cells. It has a central role in the degradation of cytosolic, nuclear and endoplasmic reticulum proteins **(Hochstrasser, 1996)**, so it acts as a covalent tag to mark damaged or short-lived proteins for degradation by the ubiquitin–proteasome system. Studies indicate that It is implicated in cell functions such as the mediation of stress responses, regulation of differential gene expression, repair of damaged DNA **(Goldstein *et al*., 1975)** and control of the cell cycle.

1.7.1.2 The degradation of proteins by the ubiquitin–proteasome system:

The ubiquitin–proteasome system (UPS) is an ubiquitous, multienzymatic proteolytic pathway that removes misfolded, ubiquitinated proteins **(Ciechanover *et al*., 2000; Betarbet *et al*., 2005; Olanow and McNaught, 2006)**. The UPS plays an important

role in rapid degradation of 30 % or more of newly made proteins within the cell **(Schubert *et al*., 2000)** and it also plays a crucial role in a number of cellular events such as signal transduction, cell cycling, metabolism and the immune response **(Pagano, 1997; Ben-Neriah, 2002; Mukhopadhyay and Riezman, 2007)**.

Ubiquitin is first activated by the ubiquitin–activating enzyme (E1) in its C- terminal glycine residue to the thiol group of a cysteine residue on the activating enzyme (E1) via an ester bond. After that it is transferred to a cysteine thiol group on a ubiquitin carrier protein or ubiquitin–conjugating enzyme (E2) and, finally, it is ligated to a protein substrate by a ubiquitin–protein ligase (E3). At this step, two options exist; either ubiquitin is transferred to a protein substrate via the ubiquitin - protein ligase or the ubiquitin – protein ligase accepts both protein substrate and ubiquitin–conjugating enzyme for direct transfer of ubiquitin from the ubiquitin-conjugating enzyme to the protein substrate **(Hershko and Ciechanover, 1998)**.

The degradation of proteins is enhanced when more than one ubiquitin binds covalently to the target protein to form polyubiquitin chains **(Cook *et al*., 1994)**. The proteins attached by polyubiquitin chains are usually degraded by ubiquitin / ATP – dependent proteinase known as 26S proteasome. The 26S proteasome is a large multiprotein complex (2.5 MDa) **(Voges *et al*., 1999)**, which is mainly cytosolic but can also be found in nuclei **(Palmer *et al*., 1996)**. Ultrastructurally, the 26S proteasome is composed of a central catalytic 20S core complex, comprising 28 subunits in a cylindrical arrangement and containing the protease active sites, with 19S regulator complexes at each end **(Coux *et al*., 1996)**. The 19S particle contains at least 17 subunits, including ATPases, de-ubiquitinating enzyme and polyubiquitin-binding subunits **(Pickart, 1997)**. The ubiquitin molecule can be removed from the

ubiquitin-protein by de-ubiquitinating enzymes **(Wilkinson, 1997; Betarbet *et al*., 2005; Olanow and McNaught, 2006) (Figure 1.6)**.

1.7.1.3 The role of the ubiquitin–proteasome system in neurodegenerative disorders:

Many neurodegenerative disorders are characterized by the presence of intraneural cytoplasmic inclusions containing ubiquitinated filamentous protein aggregates.

These inclusions are found in the cytoplasm (e.g. endosomes, lysosomes) in Alzheimer's disease and Prion encephalophathies **(Mayer *et al*., 1996)** and in nuclei in Huntington's disease and spinocerebellar ataxias **(Davies *et al*., 1997)**.

Aggregations of ubiquitin protein were first detected in the neurofibrillary tangle (NFT) of Alzheimer's disease **(Mori *et al*., 1987)** and have since been shown to be a feature of Lewy bodies (LB) in Parkinson's disease and dementia with Lewy bodies (DLB), as well as inclusions in Pick's disease and amyotrophic lateral sclerosis **(Lowe *et al*., 1988)** and polyglutamine expansion diseases such as Huntington's disease **(DiFiglia *et al*., 1997)**.

The ubiquitinated filamentous protein aggregates within these inclusions result from a dysfunction or overload of the ubiquitin–proteasome system, structural changes in the protein substrates **(Alves-Rodrigues *et al*., 1998)**, mutations that impair the normal ubiquitin pathway e.g. in ubiquitin-ligase enzymes (parkin), or de-ubiquitinating enzymes (UCHL1) or protein substrates of UPS (α-synuclein) or in transcripts for ubiquitin itself **(Layfield *et al*., 2001)**, mitochondrial dysfunction and oxidative stress **(Reinheckel *et al*., 2000; Shamoto-Nagai *et al*., 2003)**.

For example, mutant genes encoding for proteins of the ubiquitin–proteasome system were found to be responsible for inherited forms of familial Parkinson's disease (PD)

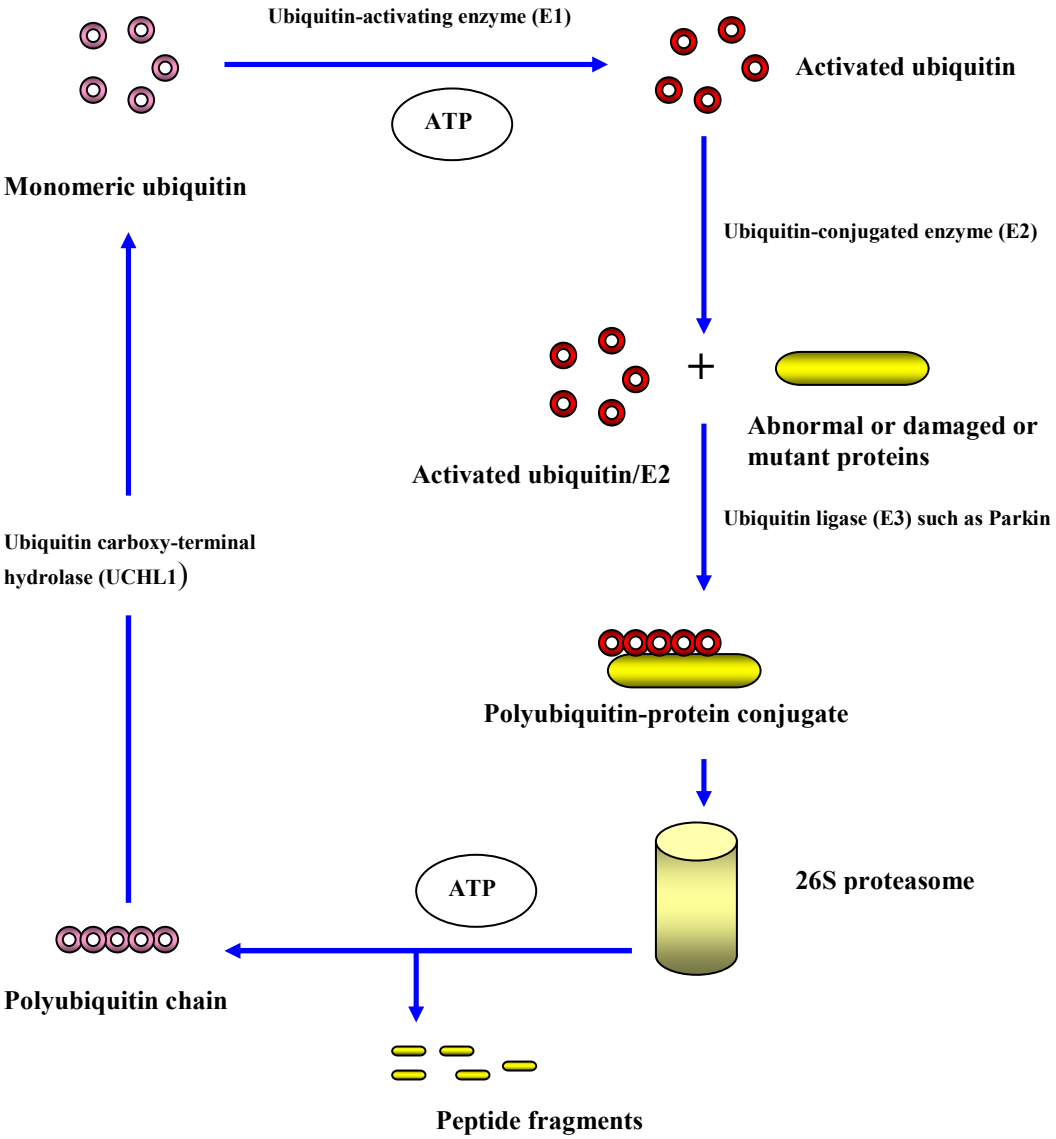


Figure 1.6: Degradation of abnormal proteins by the ubiquitin-proteasome system. Proteins are ubiquitinated by a series of enzymatic reactions: E1 enzymes activate ubiquitin monomers, E2 enzymes conjugate ubiquitin to proteins or to E3, which are in turn a series of ubiquitin ligases and attach chains of ubiquitin to specific protein substrate. Labelling of proteins with multiple ubiquitin molecules are recognized by proteasome and degraded in an ATP manner. In the final step, ubiquitin monomers are removed from ubiquitin-protein adducts by deubiquitinating enzymes (such as UCHL1) so they can recycle to degradate additional abnormal proteins. Partially adapted from McNaught *et al.* (2001).

(Polymeropoulos *et al*., 1997; Kitada *et al*., 1998; Leroy *et al*., 1998; Shimura *et al*., 2000). These genes include PARK1 of α-synuclein, PARK 2 of parkin and PARK 5 of ubiquitin-C-hydrolase-1 (UCHL1), all of which decrease the activity of the ubiquitin-proteasome system **(Kitada *et al*., 1998; Leroy *et al*., 1998; Chung *et al*., 2001a; Steece-Collier *et al*., 2002; Betarbet *et al*., 2005; Olanow and McNaught, 2006)**.

A mutant form of ubiquitin called ubiquitin^{+1} has been detected in the brain of Alzheimer's patients **(Van Leeuwen *et al*., 1998)**. In this case, polyubiquitin chains made by ubiquitin^{+1} are completely resistant to disassembly by deubiquinitinating enzymes. This causes an accumulation and aggregation of ubiquitinated proteins, leading to neurodegeneration **(Lam *et al*., 2000)**. In sporadic Parkinson's disease there is a reduction in mitochondrial complex 1 activity in the substantia nigra pars compacta **(Schapira *et al*., 1990b)**; inhibition of complex 1 activity might lead to impairment of the UPS which is an ATP dependent, leading to accumulation of ubiquitin proteins **(DeMartino and Slaughter, 1999)**.

Impairment of the ubiquitin-proteasome system leading to accumulation and aggregation of ubiquitinylated proteins does not appear to be a primary event in inclusion formation, but rather a secondary protective cellular response, so oxidative damage **(Giasson *et al*., 2000)** or glycosylation **(Shimura *et al*., 2001)** may be the initiating events in aggregation and inclusion formation, with ubiquitination forming a later stage in inclusion biogenesis as part of the normal cellular response to disease states. Immunohistochemical studies give insights in the relationship between UPS and inclusion formation. These shows that, while ubiquitin is often found in inclusions, it is not obligatory. For example, the tau protein is the main component of NFTs but ubiquitin appears to become associated later with the more mature lesions

(Bancher *et al*., 1989) although it should be noted that tau protein can form fibrils in the absence of ubiquitin **(Goedert *et al*., 1996)**. In Parkinson's disease, not all Lewy bodies are ubiquitin-positive **(Spillantini *et al*., 1998b)** and α-synuclein can also form fibrils in the absence of ubiquitin **(Hashimoto *et al*., 1998)**. The degradation of non-ubiquitinylated forms of α-synuclein and tau proteins by proteasome in the absence of ubiquitin **(Tofaris *et al*., 2001; David *et al*., 2002)** is probably possible because both belong to natively unfolded proteins **(Schweers *et al*., 1994)**.

1.7.1.4 The ubiquitinylated inclusions and neurodegenerative disorders:

One study **(Kakizuka, 1998)** has suggested that the formation of these inclusions might induce neural dysfunction and/or cell death because ubiquitin protein aggregations cause neural toxicity and apoptosis. If so, the aggregation of proteins directly impairs the function of the ubiquitin-proteasome system (UPS) which is likely to be involved in the positive feedback mechanism induced by the protein accumulation that altered its function, which in turn increases the production of aggregated proteins and ultimately results in neuronal cell death **(Bence *et al*., 2001)**. Conversely, other concept have suggested that the formation of these inclusions is one of the protective strategies of the cell to process damaged, misfolded, mutant proteins. For example, the Lewy bodies in Parkinson's disease may represent a protective cellular response to protein aggregates as they behave structurally and functionally as aggrosome **(Forno, 1996; Goldberg and Lansbury, 2000; McNaught *et al*., 2002; Olanow *et al*., 2004)**. Similarly, in a mouse model of Huntington's disease, nuclear inclusions are present in surviving neurons, suggesting that (at worse) the inclusion bodies are sub-lethal or (at best) they might be protective **(Reddy *et al*., 1998)**.

Inclusion bodies are not always found in neurodegenerative conditions. For example, they do not occurs in most laboratory models of Parkinson's disease such as those produced through 6-OH-dopamine or MPTP toxicity **(Forno *et al*., 1993; Dauer and Przedborski, 2003)**, through they are present in rotenone-induced degeneration in rat **(Betarbet *et al*., 2000)**. Moreover, some human Parkinsonian conditions occur without Lewy body formation. This is especially true of patients with parkin (E3 ligase of UPS) mutation, who lack Lewy bodies: it also suggest that the E3 ligase may be critical for Lewy body formation.

The AS/AGU rat does not appear to produce Lewy bodies yet has dopamine release deficits and cell loss.

1.7.2 Parkin

Parkin is a 465 amino acid protein that has a molecular weight of 52 KDa **(Kitada *et al*., 1998; Shimura *et al*., 1999)**, contains a ubiquitin-like homology domain at its N-terminus and may be involved in the recognition of the substrates (such as Pael-R, synphilin-1, Cdc-Rel 1 and O-glycosylated from of α-synuclein, see below) **(Shimura *et al*., 2000)**, it has a central domain with unknown function and two ring fingers at the C-terminus involved in E2 and substrate recognition and binding. Parkin has E3 ubiquitin-ligase activity **(Imai *et al*., 2000; Shimura *et al*., 2000; Zhang *et al*., 2000)**, and is a component of ubiquitin-proteasome system that identifies and degradates misfolded proteins **(reviewed by Sherman and Goldberg, 2001; Cookson, 2005)**.

Mutation in the gene encoding parkin has been implicated in autosomal-recessive parkinsonism **(Kitada *et al*., 1998)** and the gene responsible was mapped on chromosome 6 **(Matsumine *et al*., 1997)**. Parkin mutations are found in patients with early-onset Parkinson's disease which has the similar clinical signs to Parkinsonism

but where disordered sleep and abnormal dystonic movement can be alleviated by Levodopa **(Dauer and Przedborski, 2003)**. Pathologically it is characterized by loss of SNC dopaminergic neurons but without Lewy bodies **(Mizuno *et al*., 2001)**.

Parkin has been implicated in the ubiquitination of four proteins, the putative G-protein-coupled transmembrane receptors Pael-R **(Imai *et al*., 2001)**, the interacting protein synphilin-1 (a protein with unknown function present in Lewy body) which interacted to α-synuclein **(Chung *et al*., 2001b)**, the synaptic vesicle protein Cdc-Rel 1(it have roles in synaptic vesicle transport, fusion and recycling) **(Field and Kellogg, 1999; Zhang *et al*., 2000; Kartmann and Roth, 2001)** and an O-glycosylated from of α-synuclein **(Shimura *et al*., 2001)**.

The loss of parkin function leads to the toxic accumulation of substrate protein **(Dev *et al*., 2003a)**; overexpression of mutated forms of parkin causes oxidative stress and lead to cell death via proteosomal inhibition **(Hyun *et al*., 2002)**.

1.7.3 Alpha-synucleins

Synucleins are a family of 15-20 KDa proteins that have been described in vertebrates, are present in neurons, and are especially abundant at presynaptic terminals. Originally, synuclein proteins were identified in the electric organ of the electric ray (Torpedo california) and the name synuclein is a contraction of "synapse and nucleus" **(Maroteaux *et al*., 1988; Jakes *et al*., 1994)**.

Three genes produce synucleins whose client forms include α-synuclein, β-synuclein and γ-synuclein **(Lavedan, 1998)**.

Alpha-synuclein is a presynaptic protein made up of 140-amino acids and is encoded by a gene on chromosome 4 **(Ueda *et al*., 1993; Chen *et al*., 1995)**. Normally, α-synuclein is abundant in presynaptic terminals **(Lee and Trojanowski, 2006)**. Originally, α-synuclein was isolated from amyloid plaques of Alzheimer's disease

(Ueda *et al*., 1993) and it has been identified as the major component of inclusions in many neurodegenerative disorders. Such as the Lewy body and Lewy neurites of Parkinson's disease **(Spillantini *et al*., 1997)**, the Lewy body variant of Alzheimer's disease, dementia with Lewy body **(Spillantini *et al*., 1997; Wakabayashi *et al*., 1997; Arawaka *et al*., 1998; Baba *et al*., 1998; Irizarry *et al*., 1998; Takeda *et al*., 1998)** and glial neuronal cytoplasmic inclusions as in multiple system atrophy **(Gai *et al*., 1998; Tu *et al*., 1998)**.

The normal cellular functions of α-synuclein are unknown, but several observations suggest synaptic functions and neural plasticity **(Clayton and George, 1998)**. It is associated with synaptic vesicles in cultured rat hippocampal neurons **(Withers *et al*., 1997; Murphy *et al*., 2000)** and it linked to dopaminergic transmission (e.g. inhibition of tyrosine hydroxylase activity, binding to the presynaptic dopamine transporter (DAT) and being translocated to the cell surface where it accelerates dopamine uptake) **(Lee *et al*., 2001; Perez *et al*., 2002)**.

1.7.3.1 Alpha-synuclein aggregation

Alpha-synuclein is naturally unfolded **(Weinreb *et al*., 1996)** and assumes an α-helical structure in association with membranes **(Davidson *et al*., 1998)**. By contrast, α-synuclein aggregations exist in β-sheet structure **(El Agnaf *et al*., 1998; Narhi *et al*., 1999; Conway *et al*., 2000a)**.

Alpha-synuclein tends to form fibrils and unfolded α-synuclein can adopt a partially folded intermediate and protofibril during fibril formation. Changes in cellular conditions such as elevated temperature and decreased pH accelerate α-synuclein fibril formation and tend to result in a partially folded intermediate structure **(Uversky *et al*., 2001)**.

Abnormal aggregation of α-synuclein is a feature of many neurodegeneration disorders. Several mechanisms lead to aggregation: impairment of breakdown due to failure in the ubiquitin-proteasome system **(McNaught *et al*., 2003)** such as proteosomal inhibition leads to α-synuclein accumulation **(Bennett *et al*., 1999)** and aggregation **(Rideout *et al*., 2001)** and also an accumulation and aggregation α-synuclein interact with ubiquitin-proteasome system lead to impair it is function **(Bence *et al*., 2001; Tanaka *et al*., 2001; Snyder *et al*., 2003; Lee *et al*., 2004)**.

1.7.3.2 Alpha-synuclein and dopamine

Aggregation of α-synuclein also mediated by the oxidation environment in dopaminergic neurons such as oxidative stress and mitochondrial impairment. Overexpression of α-synuclein may increase generation of intracellular ROS that induced dopaminergic cell death **(Junn and Mouradian, 2002)**.

Alpha-synuclein plays a role in the regulation of dopamine biosynthesis by interacting directly with tyrosine hydroxylase **(Perez *et al*., 2002)** or deceasing it is gene expression and a wild-type α-synuclein inhibit enzyme that producing BH4 which is a cofactor required for tyrosine hydroxylase activity **(Baptista *et al*., 2003)**.

Anther hand, a loss of α-synuclein due to decreasing on the expression **(Neystat *et al*., 1999)** or aggregation **(El Agnaf and Irvine, 2000)** lead to more dopamine production.

1.7.3.3 Alpha-synuclein aggregation and Parkinson's disease

Two α-synuclein mutants the A53T **(Polymeropoulos *et al*., 1997)** and A30P **(Kruger *et al*., 1998)** cause rare inherited forms of familial Parkinson's disease and stimulate α-synuclein fibril formation **(Conway *et al*., 1998; Narhi *et al*., 1999)** as does another mutation E46K discovered by **Zarranz *et al* (2004)** in a Spanish family.

1.8 Animal models in Parkinson's disease

To understand the pathophysiology of Parkinson's disease (PD), as well as to test potential therapeutics, a number of animal models of PD have been developed. Many of these are based on the simple premise that the disease state is due to the death of dopaminergic neurons, so the most widespread models have employed neurotoxins such as 6-OHDA, MPTP and Rotenone.

1.8.1 Neurotoxin based models

1.8.1.1 6-hydroxydopamine (6-OHDA)

6-hydroxydopamine was the first chemical agent used to produce an animal model of PD that had specific neurotoxic effects on catecholaminergic neurons **(Ungerstedt, 1968)**. Usually the 6-OHDA is injected unilaterally or bilaterally into the substantia nigra or striatum, so that it accumulates in dopaminergic neurons and induces degeneration probably through the generation of hydrogen peroxide and hydroxyl radicals in the presence of iron **(Sachs and Jonsson, 1975; Glinka *et al*., 1997; Bove *et al*., 2005)**. The greatest loss was in the dopaminergic neurons of the SNC (A9), followed by the retrorubral field (A8) and the VTA (A10) **(Rodriguez *et al*., 2001)**. There was a reduction in striatal dopamine and animals showed characteristic symptoms such as rotation (provided the 6-OHDA injection was unilateral) and akinesia (with a bilateral 6-OHDA injection) **(Betarbet *et al*., 2002; Schober, 2004)**. 6-OHDA lesions do not result in Lewy body formation in the substantia nigra **(Betarbet *et al*., 2002; Shimohama *et al*., 2003; Melrose *et al*., 2006)**.

1.8.1.2 1-methyl-4-phenyl-1, 2,3,6-tetrahydro-pyridine (MPTP)

1-methyl-4-phenyl-1, 2,3,6-tetrahydro-pyridine (MPTP) was discovered accidentally in 1982 when a group of drug addicts developed sub acute severe parkinsonism **(Langston *et al*., 1983)**. MPTP crosses the blood-brain barrier and is metabolized to 1-methyl-4-phenylpyridin (MPP^+) by monoamine oxidase B. MPP^+ is taken up via the dopamine transporter and accumulates in dopamine neurons, where it inhibits complex I of the electron transport chain leading to oxidative stress **(Nicklas *et al*., 1985; Tipton and Singer, 1993; Betarbet *et al*., 2002) (Figure 1.7)**. In primates, MPTP causes severe Parkinsonism symptoms including degeneration of dopaminergic neurons; there is also the appearance of micro-inclusions which are unlike the Lewy bodies characteristic of idiopathic Parkinson's disease pathology **(Forno *et al*., 1993; Betarbet *et al*., 2002; Shimohama *et al*., 2003)** but which nevertheless contain α-synuclein aggregations **(Kowall *et al*., 2000)**. MPTP administration to mice and nonhuman primates causes a greater loss in dopaminergic neurons in SNC (A9) and mainly in the ventrolateral segment **(Sirinathsinghji *et al*., 1992; Varastet *et al*., 1994; Jackson-Lewis *et al*., 1995)**. As in PD, the neurons of the VTA (A10) and locus coeruleus are less affected **(Mitchell *et al*., 1985; Forno *et al*., 1986; 1993;1996)** but there is massive loss of the dopamine (90-99%) in the striatum **(Hantraye *et al*., 1993; Jackson-Lewis *et al*., 1995)**; degeneration of dopaminergic neurons terminals was greater in the putamen than in the caudate **(Moratalla *et al*., 1992; Snow *et al*., 2000)**. Mice have some locomotor defect comparable to PD such as hypokinesia, bradykinesia and akinesia **(Sedelis *et al*., 2001)** and primates develop motor dysfunctions such as tremor, bradykinesia, rigidity and postural impairment **(Langston *et al*., 1984; Schultz *et al*., 1985; Stern, 1990; Jenner, 2003)**. Rodents such as mice and rats are less sensitive to MPTP neurotoxicity **(Betarbet *et al*., 2002;**

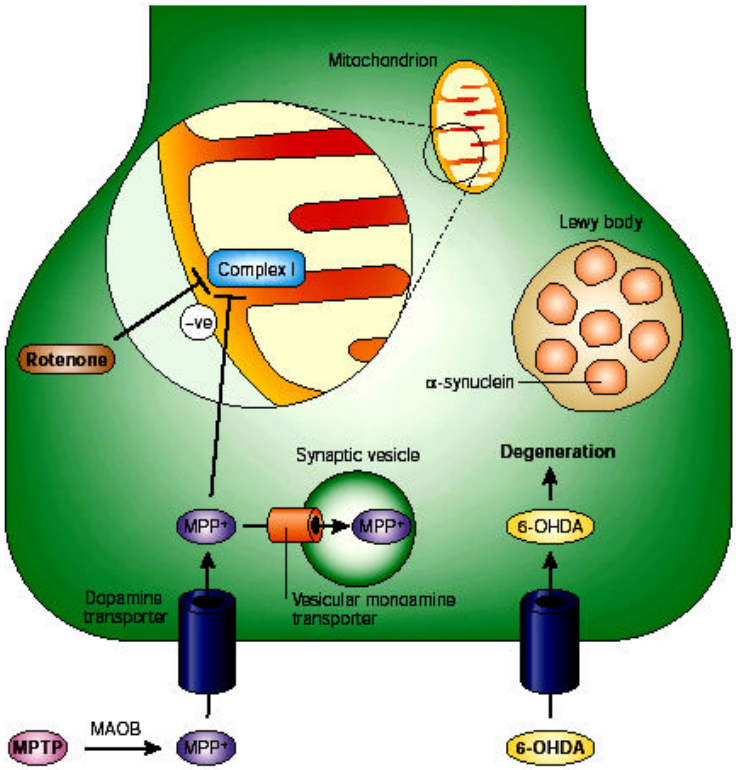


Figure 1.7: Pathogenesis of neuronal dysfunction produced by neurotoxins that affect dopamine neurons. The mechanisms by which neurotoxins kill dopamine neurons involve mitochondrial dysfunction and oxidative damage. 6-hydroxydopamine (6-OHDA) is taken up by the dopamine transporter and it then generates free radicals. 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) is converted by monoamine oxidase B (MAOB) to 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ is taken up by the dopamine transporter and can then be accumulated by mitochondria, leading to complex 1 inhibition and the generation of free radicals, or by the vesicular monoamine transporter, thus reducing toxicity. Rotenone is a direct inhibitor of complex 1, which also leads to free-radical generation. MPTP and rotenone treatment increase the expression of α-synuclein and, in later case, this leads to the formation of Lewy bodies. From Beal, (2001).

Shimohama *et al*., 2003; Bove *et al*., 2005) and it's use has generally been in primate studies. The main difficulty with MPTP as a model of PD is that it is an acute process, whereas PD is a slowly progressive disease **(Shimohama *et al*., 2003)**.

1.8.1.3 Rotenone

Another model of PD is based on systemic exposure of rats to the pesticide rotenone which is an inhibitor of mitochondrial complex 1. In rodents, rotenone caused selective death of nigrostriatal dopaminergic neurons and the formation of ubiquitin and α-synuclein-positive inclusions and treated animals showed rigidity, bradykinesia, postural instability, unsteady gait and tremor **(Betarbet *et al*., 2000; Sherer *et al*., 2003)**. The major disadvantages of this model are its variability, with some animals developing lesions and other not. For this reason bilateral lesions are not as predictably effective as bilateral treatment with 6-OHDA or MPTP **(Betarbet *et al*., 2002)**.

1.8.2 Genetic based models

A second groups of animal models has been developed from the knowledge that genetic defects can cause some forms of PD.

1.8.2.1 Parkin genetic model

Mutations in the gene encoding parkin are linked to the familial PD known as autosomal recessive juvenile parkinsonism (AR-JP) **(Kitada *et al*., 1998)**.

Drosophila which has parkin mutation has been developed to investigate the role of parkin loss in degeneration of dopaminergic neurons. The results suggest that the

mutation does not cause degeneration of dopaminergic neurons and the locomotor problems (climbing and flying defects) are caused by mitochondrial defect in muscles. The flies exhibit reduced life span, muscle degeneration and males have spermatid defects **(Greene *et al*., 2003)**. Several studies have described mutations and knockout of the parkin gene in mice and, again, there was no evidence for a reduction of nigrostriatal dopaminergic neurons. Brain morphology appeared normal and extracellular dopamine levels were increased in the striatum while synaptic excitability was reduced in medium-sized striatal spiny neurons **(Goldberg *et al*., 2003; Itier *et al*., 2003; Von Coelln *et al*., 2004; Perez and Palmiter, 2005)**. These models give some insight into parkin function but do not explain the role of parkin in PD pathogenesis since Parkinsonian symptoms are largely lacking **(Betarbet *et al*., 2005)**.

1.8.2.2 Alpha-synuclein genetic model

Mice which lack the α-synuclein gene show normal brain structure and only modest alterations in dopaminergic pathways such as (a) increased dopamine release following strong electrical stimuli, (b) a small reduction in striatal dopamine content and (c) attenuation of the dopamine-dependent locomotor response to amphetamine **(Abeliovich *et al*., 2000)**. However, the over-expression of both mutant and wild-type α-synuclein in mice led to the formation of cytoplasmic and nuclear micro-inclusions containing α-synuclein and ubiquitin in the neocortex, hippocampus and substantia nigra (albeit lacking fibrillar aggregates that are a characteristic of Lewy bodies). It led also to loss of nigrostriatal dopaminergic terminals in the striatum (but no loss of the nigrostriatal dopaminergic cells in the SNC) and reduced motor activity as examined by the rotorod test **(Beal, 2001; Masliah *et al*., 2000; van der Putten *et***

***al*., 2000; Giasson *et al*., 2002; Lee *et al*., 2002; Fleming *et al*., 2004)**. The over-expression of wild type or mutant α-synuclein in Drosophila led to the formation of Lewy body-like inclusions containing synuclein, loss of dopaminergic neurons and locomotor dysfunction in the form of a progressive decline in climbing ability **(Feany and Bender, 2000)**. There was retinal degeneration (which is not a feature of PD) **(Feany and Bender, 2000)**. It remains unclear whether the motor defect can be attributed to the dysfunction of dopaminergic neurons **(Beal, 2001)**. Lentiviral-mediated overexpression of wild type α-synuclein in rats led to intraneuronal cytoplasmic aggregates but no cell loss **(Lo Bianco *et al*., 2002)**.

1.8.3 Spontaneous rodent mutant models

In addition to neurotoxic and genetically modified models, there have been a number of spontaneous rodent mutant models including the weaver, lurcher, reeler, Tshrhyt, tottering, and coloboma mice and circling (ci) rat. These models display altered dopaminergic function or neurodegeneration that occur in PD and have deficits in motor behavior **(Heintz and Zoghbi, 2000)**. The AS/AGU rat belong to this category of model.

1.9 The AS/AGU rat

The AS/AGU rat arose as a spontaneous mutation within a closed inbred colony of Albino Swiss (AS) rats in the Laboratory of Human Anatomy at Glasgow University. The mutation is recessive **(Campbell *et al*., 1996)**, and AS/AGU rats have been isolated as a true breeding substrain. The phenotypic differences result from a single point mutation in the gene coding for the gamma isoform of protein kinase C (PKC-γ) **(Craig *et al*., 2001)**. The AS/AGU mutant exhibits serious movement impairments

including hindlimb rigidity, a staggering gait, a tendency to fall over every few steps, a slight whole body tremor and difficulty in initiating movement **(Clark and Payne, 1994; Payne *et al*., 1998)**. Previous studies including histological, neurochemical, immunocytochemical, and pharmacological and locomotor analysis revealed that in the mutant AS/AGU rats there are:

A. No obvious gross morphological differences between the brain of the normal AS and the mutant AS/AGU rats. The neocortical and cerebellar histology looks normal.

B. Neurochemical and immunocytochemical studies showed a loss in tyrosine hydroxylase immunoreactive cell bodies in the SNC of the mutant strain at aged beyond one year **(Clark and Payne, 1994)**.

C. There is 20-30% reduction in whole tissue dopamine levels of 6 months AS/AGU mutants compared to the normal AS rats at same age measured in striatal micropunches using High Performance Liquid Chromatography with electrochemical detection (HPLC-ECD) **(Campbell *et al*., 1996)**.

D. Microdialytic studies found a great reduction (80-90%) in extracellular dopamine in the dorsal caudate putamen of 3 months AS/AGU mutants and older compared to the normal AS strain when measured with HPLC-ECD **(Campbell *et al*., 1998)**.

E. Behavioural studies found that AS/AGU rats have a marked difficulty in initiating movement, and generally the mutant rats perform badly in simple locomotor tests such as rotating in mid-air and walking down a variety of inclined ramps of various widths compared to AS controls. The deficits are significant at 6 and 12 months. Locomotor deficiencies in AS/AGU rats can be ameliorated by L-dopa administration **(Campbell *et al*., 1998)**.

F. There is a marked depletion in utilization of 2-deoxy-glucose in the substantia nigra pars compacta, subthalamic nucleus and ventrolateral thalamus **(Lam *et al*., 1998)**.

G. These characteristics have been reviewed by **Payne *et al*. (2000)**. They have been work out primarly on male AS/AGU rat because movement deficent are more obvious.

H. The serotonergic (5-HT) raphe-striatal system has a reduction in the extracellular levels of 5-HT (70%) in the dorsal caudate-putamen of the mutant compared to the controls and an increase in the levels of the 5-HT metabolite (5-HIAA) at 3 months and older. At later ages, there are reductions in whole tissue 5-HT and increases in 5- HIAA in the striatum and the dorsal raphe nucleus region, companied by a decrease in the number of 5-HT-immunoreactive cells in the dorsal raphe nucleus. The median raphe nucleus was not affected **(Al-Fayez *et al*., 2005)**.

1.9.1 The AS/AGU mutation

Genetic analysis involving multiple backcrossing, has shown a very tight linkage between the *agu* mutation and a marker in the 3' untranslated region of protein kinase C- γ isoform encoding gene. The protein kinase C-γ gene is a single point mutation resulting from a stop codon between the regulatory and catalytic domains **(Craig *et al*., 2001)**.

Protein kinase C is a family of calcium-activated, phospholipid-dependent enzymes that are found in a wide variety of tissues **(Saito and Shirai, 2002)**. In the nervous system, PKC is involved in widely diverse functions including activation of PKC in nerve cells which it linked to modulation of ion channels **(Baraban *et al*., 1985;**

Madison *et al*., 1986; Shearman *et al*., 1989), the desensitization of receptors **(Huganir and Greengard, 1990)**, modification of neuronal plasticity **(Routtenberg, 1985; Akers *et al*., 1986)**, the enhancement of neurotransmitter release **(Malenka *et al*., 1986; 1987; Tanaka and Nishizuka, 1994; Stevens and Sullivan, 1998)**, implicated in general cellular processes such as apoptosis **(Zirpel *et al*., 1998; Lee, 2001; Ghoumari *et al*., 2002)** and cell surface signal transduction **(for review see Nishizuka, 1984 a,b; 1986)**. PKC has at least 10 isoforms that have been identified in mammalian tissue one of them is PKCγ **(Ohno and Nishizuka, 2002; Popp *et al*., 2006)**.

PKCγ is a member of the PKC family which was originally thought to be restricted to brain and spinal cord **(Huang *et al*., 1988; Nishizuka, 1988; Tanaka and Saito, 1992; Saito and Shirai, 2002)**. It is highly expressed in purkinje cells and in the medium-sized neurons of the striatum and globus pallidus that project to the substantia nigra **(Chen *et al*., 1995)**, in perikarya and neuropil in the striatum, and neuropil in substantia nigra **(Yoshihara *et al*., 1991)**. Within the nervous system, it has been associated with a variety of functions including neuron-glial plasticity **(Narita *et al*., 2004)**, memory **(Abeliovich *et al*., 1993)**, alcohol intake **(Bowers and Wehner, 2001)**, sensory processing **(Martin *et al*., 2001; Narita *et al*., 2001)**, anxiety **(Bowers *et al*., 2000)** and cerebellar afferent regulation **(Chen *et al*., 1995)**. Now known to be found in the heart **(Rouet-Benzineb *et al*., 1996)**, pulmonary epithelial cells **(Lin *et al*., 2000)**, pulmonary fibroblast **(Ludwicka-Bradley *et al*., 2000)** and immortalized mammary epithelial cells **(Mazzoni *et al*., 2003)** so it is clearly not restricted to the nervous system.

There is a PKCγ-ko mutant mouse which was developed to give insight into the effect of the mutation on cerebellum and this mouse is characterized by motor impairment and defects of Long-term potentation (LTP), due to the persistence in adulthood of multiple climbing fibres from the inferior olivary nucleus to Purkinje cells **(Abeliovich *et al*., 1993; Chen *et al*., 1995)**. There is no evidence of a similar problem in the AS/AGU rat: equally, there is no evidence of basal ganglia involvement in the PKCγ-ko mouse.

However, it is of interest that the protein kinase C-gamma gene is mutated in spinocerebellar ataxia type 14 which is an autosomal dominant neurodegenerative disorder characterized by slowly progressive cerebellar dysfunction including gait ataxia, dysarthria and abnormal eye movements **(Verbeek *et al*., 2005)**.

1.10 Aims of the study

In this study, three complimentary experiments were carried out to examine the possible pathological responses of midbrain cell groups to the *agu* mutation in the gene coding for protein kinase C-gamma (PKC-γ).

The three experiments were:

1) **Experiment 1** to examine levels of certain molecules in the midbrain cell groups using quantitative immunofluorescence microscopy of cell bodies or their surrounding neuropil. These molecules consisted of two groups:
    a) Those giving information about the capacity of midbrain aminergic cell bodies to synthesise transmitters: tyrosine hydroxylase for dopaminergic neurons and serotonin for serotonergic ones.

b) Those which have been found to increase in human neurodegenerative conditions such as Parkinson's disease: ubiquitin, parkin and α-synuclein.

2) **Experiment 2** to look at cell bodies in the SNC of the AS (control) and AS/AGU (mutant) rats using light and transmission electron microscopy (TEM) to determine whether there are any strain differences in cell size or ultrastructure or any signs of cell morbidity.

3) **Experiment 3** to look at TH+ve nigrostriatal dopaminergic terminals in the dorsal caudate-putamen of the AS (control) and AS/AGU (mutant) rats using TEM to determine whether there are:

a) Differences in the percentages and numbers of such terminals.

b) Differences in synaptic vesicles numbers.

CHAPTER 2

EXPERIMENT 1:

Quantification of transmitter synthesis and degeneration-associated molecules in midbrain and striatum: a comparison of AS and AS/AGU rats

2.1 Introduction

The AS/AGU rat has been characterised as a mutation in the gene coding for protein kinase C-γ **(Craig *et al*., 2001)** which results in an inability to release biogenic amines such as dopamine and serotonin in the striatum **(Campbell *et al*., 1997; Al-Fayez *et al*., 2005)**.

The physiological failure, measured by in vivo microdialysis and HPLC-ECD, occurs from an early age. Only in later life is there a measurable decrease in whole tissue dopamine or serotonin levels **(Campbell *et al*., 1998, 2000; Payne *et al*., 2000; Al-Fayez *et al*., 2005)** or a decrease in aminergic cell numbers **(Clarke and Payne, 1994; Scott *et al*., 1994; Stewart *et al*., 1994; Al-Fayez *et al*., 2005)**. This suggests that synthesis of amines is not compromised in early stages of the disorder, even though release is. However, overall levels of amines (as analysed by micropunch and HPLC-ECD) could mask considerable variation between non-functional senescent neurons and hyperactive compensatory ones. The first aim, therefore, was to examine the synthetic capabilities of cells in the substantia nigra (dopamine) and midbrain raphe nuclei (serotonin) at different ages. A second aim was to examine levels of molecules associated with neurodegeneration in Parkinson's disease, especially those associated with Lewy bodies such as ubiquitin, parkin and α-synuclein **(Love and Nicoll, 1992; Irizary *et al*., 1998)**. This is because most laboratory models of nigrostriatal dysfunction do not exhibit the cellular inclusions which are traditionally thought of as a defining characteristic of the human condition **(Forno *et al*., 1993; Dauer and Przedborski, 2003)**. Even if no such pathological features occur, it is important to know if there are elevations in intracellular levels of these molecules

since this would imply kinship between the laboratory model and the human disorder and, perhaps, similar mechanisms of dysfunction.

2.1.1 Tyrosine hydroxylase (TH)

Tyrosine hydroxylase (TH) has frequently been used as a marker of dopaminergic neurons **(Pearson *et al*., 1983)**. TH catalyses the first and rate-limiting step in biosynthesis of catecholamines **(Nagatsu *et al.*, 1964; Kuhar *et al*., 1999; Lehmann *et al*., 2006)** responsible for the conversion of L-tyrosine to L-DOPA. In the rat it is a homotetrameteric protein composed of 498 identical amino acid residue subunits **(Grima *et al,* 1985)**. It appears to be encoded by a single gene in all species **(Kumer and Vrana, 1996; Fitzpatrick, 1999)**. The conversion of L-tyrosine to noradrenaline and adrenaline was first shown in the adrenal medulla **(Blaschko, 1939)** and later confirmed in the CNS and PNS **(Flatmark, 2000)**. TH is present in a soluble and a membrane-bound form **(Nagatsu *et al.,* 1964; Kuczenski and Mandell, 1972; Kuhn *et al.,* 1990)**. TH activity is regulated by various mechanisms including induction of TH gene transcriptional regulation, phosphorylation by different kinase systems (PKA, PKC and PKG) and feedback inhibition by the end product; it also requires tetrahydrobiopterin or pteridine (BH4) as a co-factor, molecular oxygen and ferrous iron (Fe^{+2}) for it is reaction **(for review see, Kumer and Vrana, 1996)**.

TH levels and distribution have been studied enzymatically **(McGeer et al., 1971; McGeer and McGeer, 1976; Sawada *et al*., 1987)**, by using immunohistochemical techniques **(Gaspar *et al*., 1985; Martin *et al*., 1991 ; Holt et al., 1997; Hedreen, 1999)** and by fluorescence microphotometry in rat **(Sutoo *et al*., 1991)** and human brain **(Sutoo *et al*., 2001)**.

TH immunofluorescence levels have been determined in normal and pathological brains by TH messenger RNA (detected by in situ hybridization) and TH protein content in the cells **(Kastner *et al*., 1993; Sutoo *et al*., 1994)**.

Many points must be considered in analysing TH immunostaining in normal dopaminergic neurons of the mesencephalon as described by **Kastner *et al*. (1993)**. TH immunostaining was variable from one neuron to another in the same region and from region to region (e.g. dopaminergic neurons of A8 have higher TH content). TH immunolabelling of the midbrain are related to their projection organization within the striatum as in the striosome that has lower TH immunoreactivity. TH concentrations are not related to the neuromelanin content and are not age dependent. TH immunostaining levels are reduced in PD patients as is TH mRNA in surviving dopaminergic nigral neurons and in TH-ve dopaminergic neurons.

2.1.2 Serotonin (5-HT)

Serotonin (5-HT) is an aminergic neurotransmitter synthesized in several cell groupings located in the midbrain and brain stem **(Morgan *et al*., 1987; Jacobs and Azmitia, 1992; Chojnacka-Wojcik, 1995)**. Like dopamine, midbrain serotonergic neurons project mainly to the striatum and other forebrain regions **(Azmiti and Segal, 1978; Imai *et al*., 1986; Jacobs and Azmitia, 1992)** and are depleted in human PD **(Halliday et al., 1990; Paulus and Jellinger, 1991)**.

Serotonin is formed from the amino acid tryptophan which is converted to 5-hydroxy-tryptophan by the rate-limiting enzyme tryptophan hydroxylase **(Fitzpatrick, 1999)**, then converted to 5-HT by aromatic-L-amino acid decarboxylase. Serotonin can be metabolised initially into 5-hydroxy-indole-acetaldehyde by monoamine oxidase

(MAO) and is further oxidised by aldehyde dehydrogenase to 5-hydroxy-indole acetic acid **(see Osborne, 1982)**.

A reduction in serotonin concentration has been detected in the striatum, substantia nigra, hippocampus, and other regions of the Parkinsonian brain **(Rinne *et al*., 1974; Scatton *et al*., 1983; Agid *et al*., 1987; Mizuno, 2005)**. Also, a reduction in tryptophan hydroxylase activity occurs in Parkinson's disease patients **(Sawada *et al*., 1985)**, together with depleted 5-HT and its metabolites in the cerebrospinal fluid (CSF) **(Tohgi *et al*., 1993)**. The situation in laboratory models of PD can only be said to be highly variable and confusing. In animals treated with MPTP, the serotonin concentration in the striatum and the raphe nuclei has been reported as decreased **(Pifl *et al*., 1991; Frechilla *et al*., 2001)**, unchanged **(Rose *et al*., 1989)** or increased **(Gaspar *et al*., 1993)**. Rats treated neonatally with 6-OHDA exhibit serotonergic fibre hyperinnervation in the striatum accompanied by increased 5-HT and its metabolite (5-HIAA) in the striatum **(Kostrzewa *et al*., 2006)**. Conversely, others have found that the tissue content of 5-HT and 5-HT innervation were significantly decreased in the striatum of 6-OHDA-treated rat **(Takeuchi *et al*., 1991)**.

2.1.3 Lewy body proteins

Many age-related neurodegenerative disorders including PD involve abnormal aggregation and deposition of mis-folded proteins within affected neurons.

In PD, the aggregation and deposition of mis-folded proteins in the nigrostriatal dopaminergic neurons of the substantia nigra is a noted characteristic. Mis-folded proteins such as ubiquitin, parkin and α-synuclein are accumulated in the Lewy bodies that are a pathological hallmark feature of PD **(Pollanen *et al*., 1993; Cornford *et al*., 1995; Forno, 1996; Galvin *et al*., 1997; Shults, 2006)**.

(i) Ubiquitin is a 76 amino acid protein that has a major role in regulated protein degradation by the ubiquitin-proteasome pathway **(Hershko and Ciechanover, 1998)**. This pathway plays a major role in the degradation of abnormal proteins that result from oxidative stress, neurotoxicity and mutations **(Alves-Rodrigues *et al*., 1998)**.

Ubiquitin appears to be incorporated into inclusion bodies which occur in the major neurodegenerative diseases including Alzheimer's disease and Parkinson's disease **(Lowe *et al*., 2001)**.

(ii) Parkin is a protein that has ubiquitin-ligase a activity **(Imai *et al*., 2000; Shimura *et al*., 2000; Zhang *et al*., 2000)** and therefore also plays an important role in the ubiquitin-proteasome pathways. A mutation in the parkin gene is implicated in a familial type of PD called autosomal recessive juvenile parkinsonism **(Kitada *et al*., 1998)**.

(iii) Alpha-synuclein is a presynaptic protein **(Clayton and George, 1998; Spillantini *et al*., 1998a)**. Like parkin, a mutation of α-synuclein has been identified in one form of autosomal dominant familial PD **(Polymeropoulos *et al*., 1997; Kruger *et al*., 1998)**.

Lewy body proteins have been discussed in depth in **chapter 1** (the general introduction).

2.2 Rationale for measurement technique

Levels of compounds can be measured in brain regions by traditional techniques such as competitive protein binding or high pressure liquid chromatography **(Thorpe and Thorpe, 2000; Wilson, 2000)**. Similarly, enzymes can be measured by incubation of tissue slices with radiolabelled precusors **(Thorpe and Thorpe, 2000)**.

However, in each case, measurements are being made on a heterogeneous tissue sample, whether it be a core or a slice. This has the disadvantage of including neurons and glial cells of many types- as well as the inability to distinguish between cell bodies and neuropil. To get round these obvious disadvantages, the decision was made to sample large numbers of individual cells from known regions of CNS. One way to do this is to employ immunocytochemistry where the primary antibody is directed toward a molecule of interest (e.g. ubiquitin) and the secondary antibody is a fluorescent one. The amount of fluorescence coming from each cell gives a quantitative measure of the abundance of the molecule. Provided measurements are made on tissues which have been prepared, processed, treated and measured in exactly the same way, this allows good comparisons to be made between individuals or groups **(Kastner *et al*., 1993)**. This is especially true where a pair of animals (control and mutant) are analysed together at every stage. This was the case here. All inter-group comparisons are then made by dependent (i.e. paired) statistics.

The aim of **Experiment 1** is therefore to examine levels of molecules that give information about

i. synthesis of the aminergic neurotransmitters TH and 5-HT and
ii. levels of molecules which have been found in intracellular inclusions in Parkinson's disease using quantitative immunofluorescence microscopy.

2.3 Materials and Methods

2.3.1 Animals

18 AS control and 18 AS/AGU mutant male rats were used in this experiment.

The numbers in each age group were:

(1) 6 AS and 6 AS/AGU aged 6 months old.

(2) 6 AS and 6 AS/AGU aged 12 months old.

(3) 6 AS and 6 AS/AGU aged 18 months old.

2.3.2 Tissue preparation

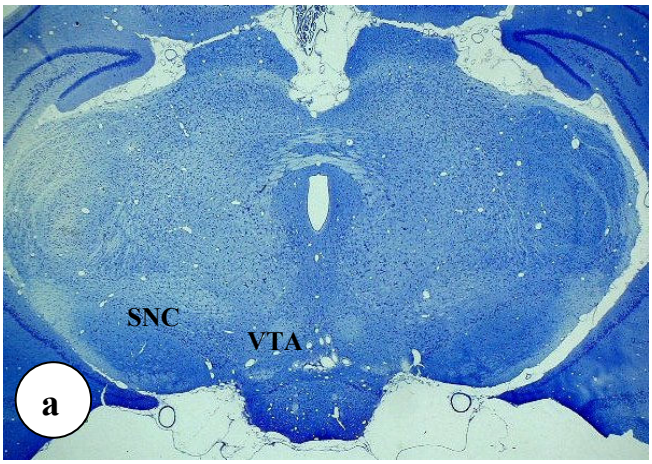
All rats were deeply anaesthetized with an overdose of sodium pentobarbitone B.P (Vet.), (2 ml of 60 mg/ml, Rhone-Merieux, Spire Greencentre, Harlow, Essex) injected intraperitoneally, and then the thoracic cavity was opened. One hundred ml of mammalian Ringer solution containing the vasodilator Lignocaine was injected into the left ventricle followed by 500ml 4% paraformaldehyde (Sigma- Aldrich Inc, P6148) in 0.1M phosphate buffer, excess fluid being drained via an incision through the right atrium **(Appendix 1)**. The brains were dissected out and immersion-fixed in 4% paraformaldehyde in 0.1M of phosphate buffer overnight. Pieces of brain containing areas of interest were dehydrated through an ascending ethanol series using a Histokinette 2000 automatic tissue processor (Reichert-Jung, Germany) then embedded in paraffin wax at 57ºC and serially sectioned at 7µm using a Microtome (Spencer, 820, USA) **(Appendix 2)**. The ribbons of sections were laid out in parallel rows on a tray.

2.3.3 Toluidine blue Staining

Three sections from the middle and ends of each row were collected and stained with 1% aqueous toluidine blue (R.ALAMB, UK) **(Appendix 3A)**. They were then examined under a light microscope to locate the rostral, central and caudal ends of the substantia nigra, dorsal and median raphe nuclei and the striatum in order to provide reference sections for matching sections of the AS and the AS/AGU rats using a standard atlas of the rat brain **(Paxinos and Watson, 1982)**.**(Figure 2.1)**.

2.3.4 Immunocytochemistry (ICC) on paraffin sections

It is an essential feature of this experiment that AS and AS/AGU sections were processed and assessed together as pairs. Adjacent sections were chosen, collected and stretched in a paraffin section-mounting bath for 1-3 mins at 40ºC. They were then mounted on APES (3-aminopropltriethoxysilane)-coated slides and dried in a 37ºC oven overnight and then at 56ºC for 2 hours. The sections were deparaffinized and rehydrated before undergoing a heat-mediated antigen retrieval technique **(Shi *et al*., 1991)**. Slides were immersed in boiling 0.01M sodium citrate buffer (pH 6.0) in a Prestige stainless steel pressure cooker **(Norton *et al*., 1994)**. The temperature was then raised to 120ºC for 1 minute. Sections were rinsed in distilled water followed by 0.01M phosphate buffered saline (PBS, 1x5 mins), and then incubated in 1.5% hydrogen peroxide (H_2O_2) for 10 minutes to inactivate endogenous peroxidase. After rinsing in distilled water (2x5 mins) and in PBS (2x5 mins), sections were treated with 1% normal goat serum (NGS, Sigma-Aldrich, G9023, UK) in PBS with 3% Triton X-100 (Sigma Chemical. CO) for 60 min at room temperature to reduce nonspecific background staining **(Appendix 3B)**.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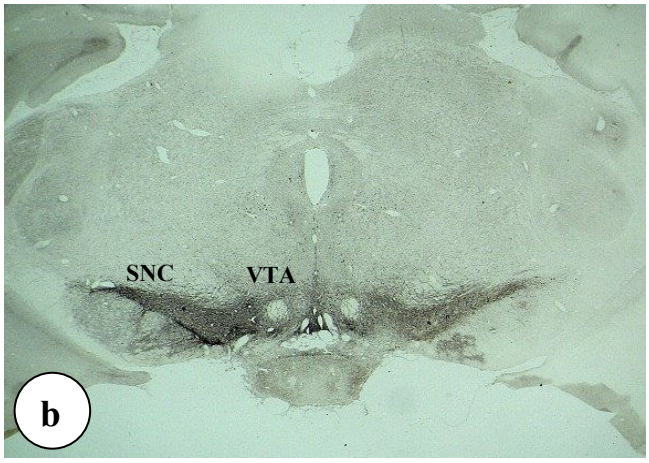
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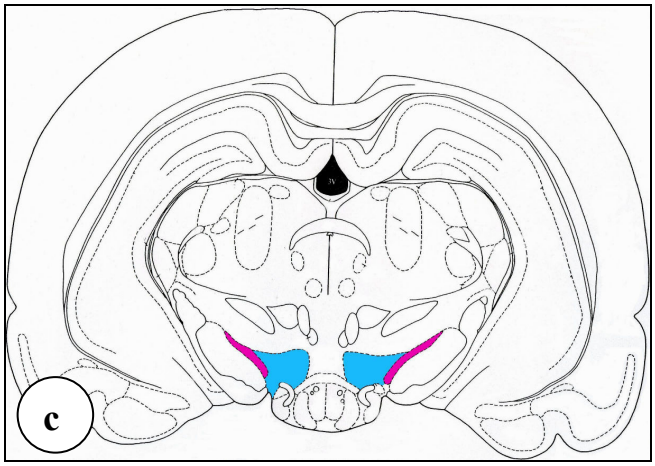
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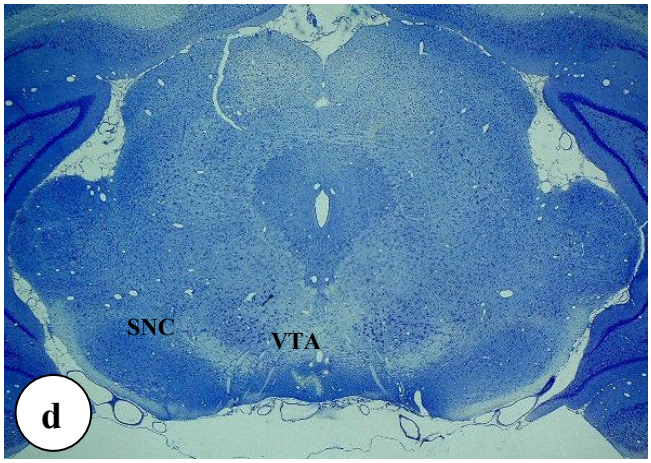
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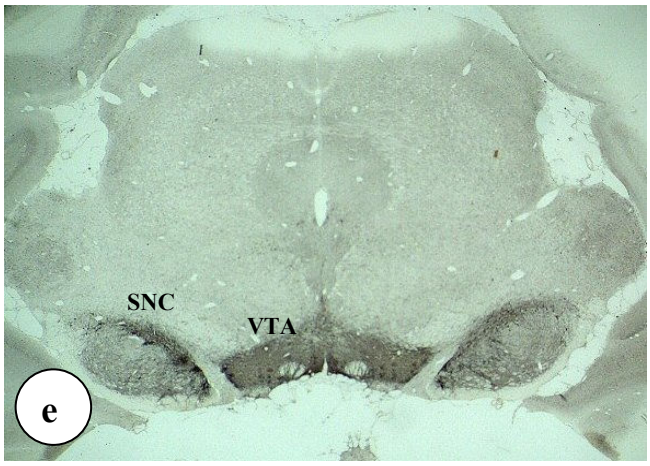
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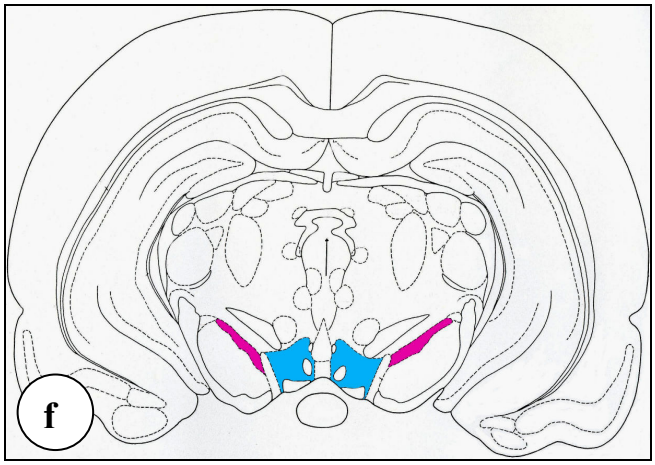
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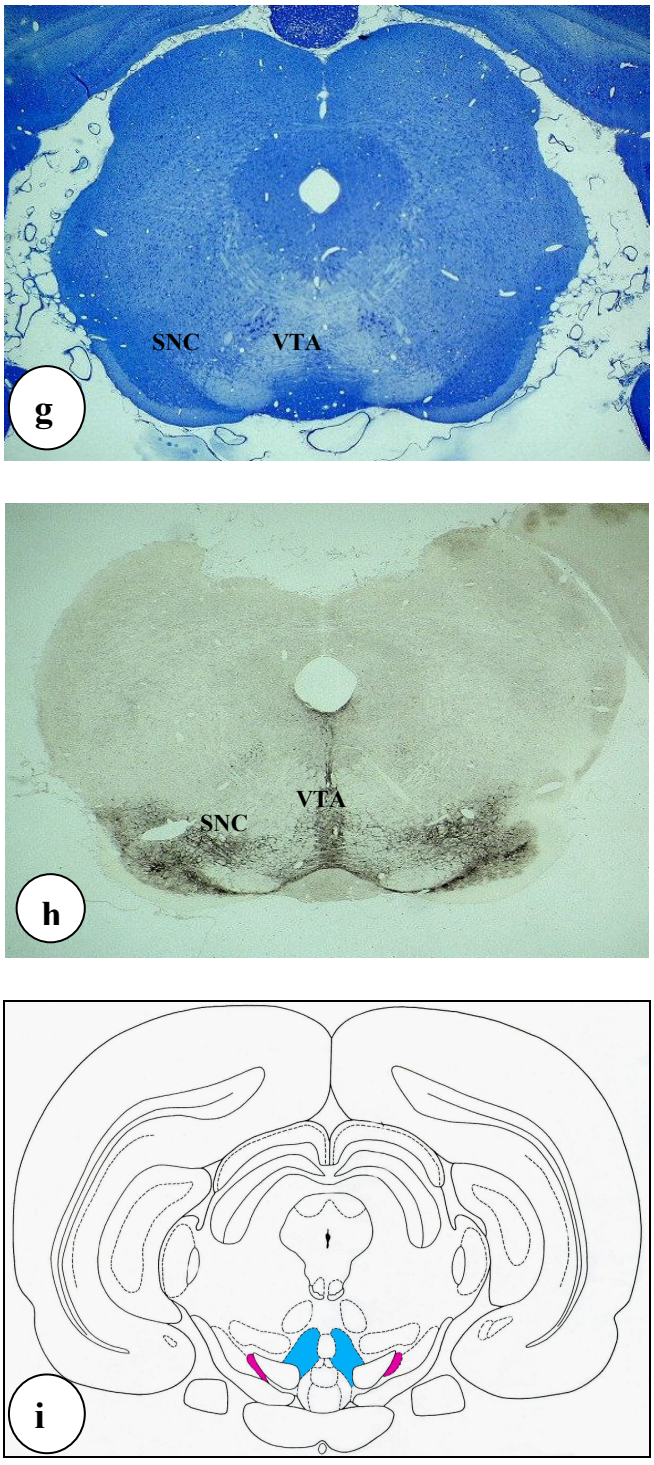


Figure 2.1: Coronal sections of midbrain to show substantia nigra:

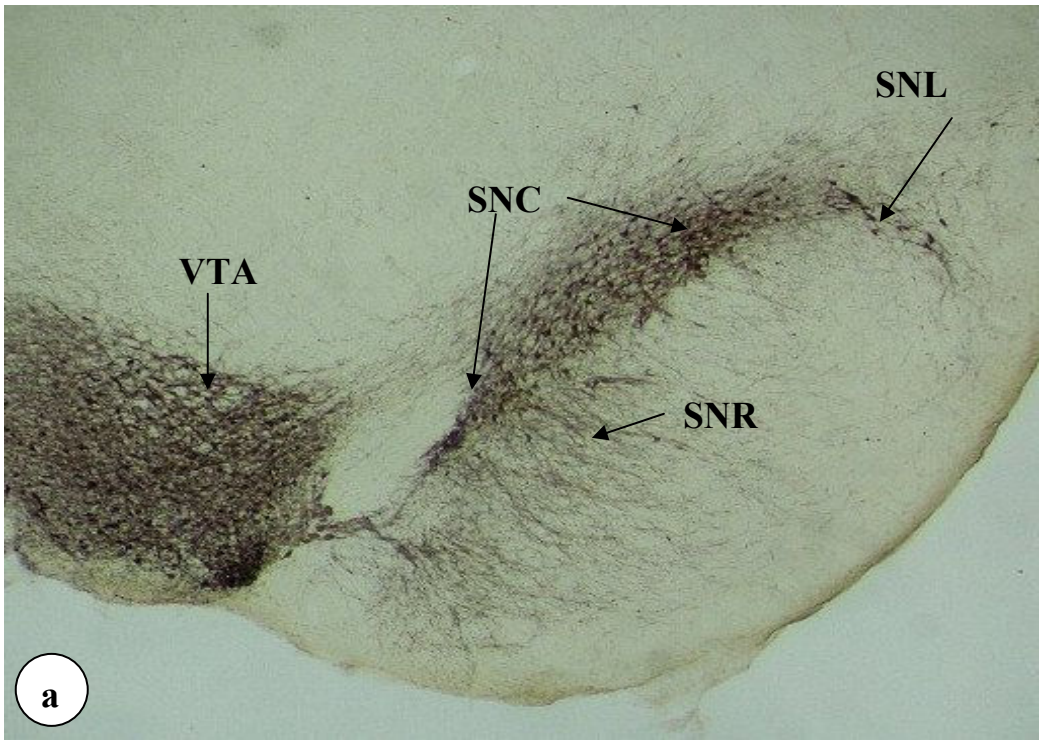
Sections (a, d, and g) were stained with toluidine blue. Sections (b, e and h) were stained with immunoperoxidase DAB for TH.
The sections show the rostral (a and b) central (d and e) and caudal (g and h) regions of the substantia nigra (x2.5). (c, f and i from Paxinos and Watson, 1982).

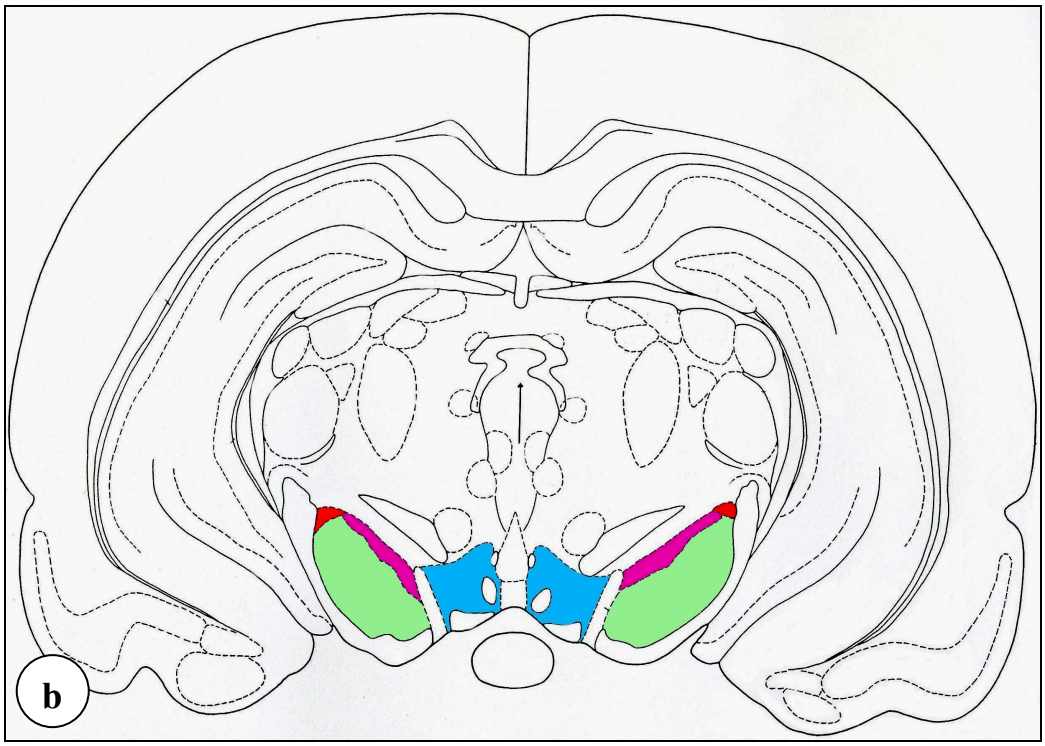
SNC **VTA**

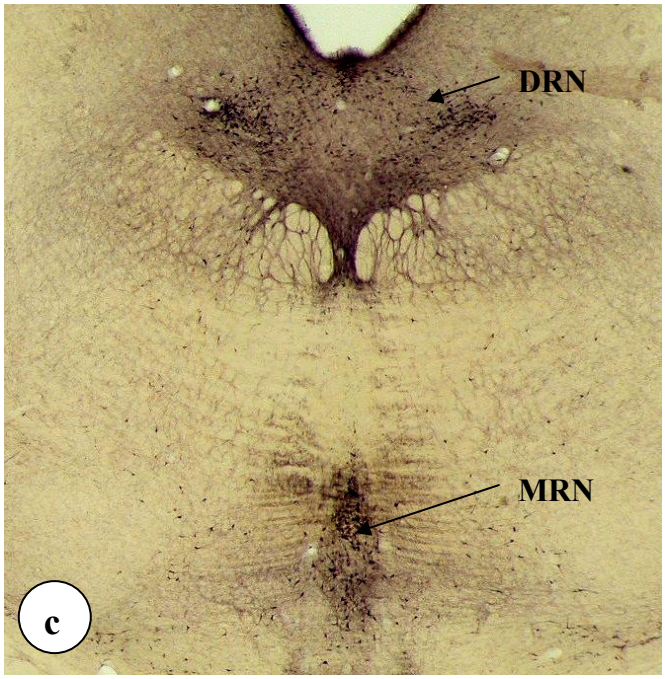
2.3.5 Immunoperoxidase staining

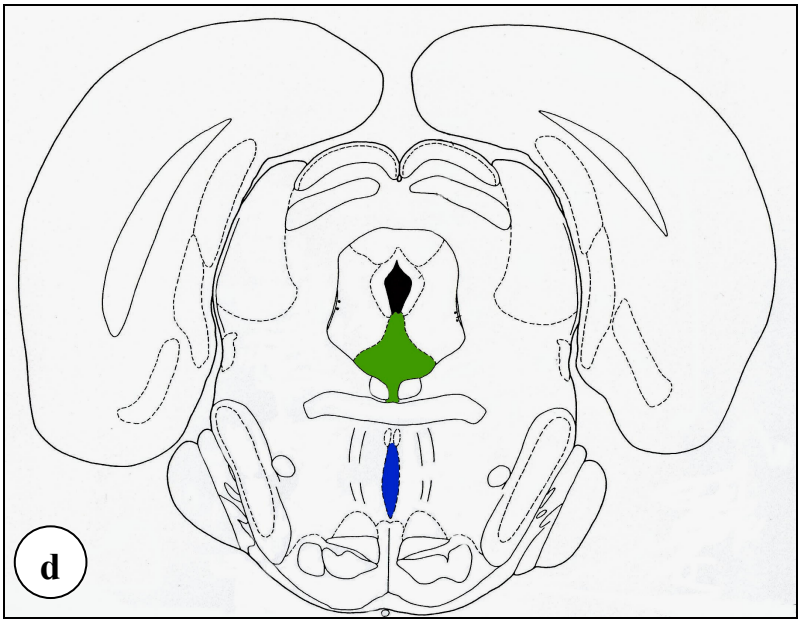
This method of ICC staining was carried out according to a protocol that has been in use for 25 years **(Hsu *et al*., 1981)** and was used to confirm the identify of the substantia nigra, midbrain raphe nucleus and the striatum in matching sections of the AS and the AS/AGU rats by staining for the rate-limiting enzyme tyrosine hydroxylase (TH) (within the SN and striatum) and serotonin (5-HT) (within the midbrain raphe nucleus),**(Figure 2.1 and 2.2)**.

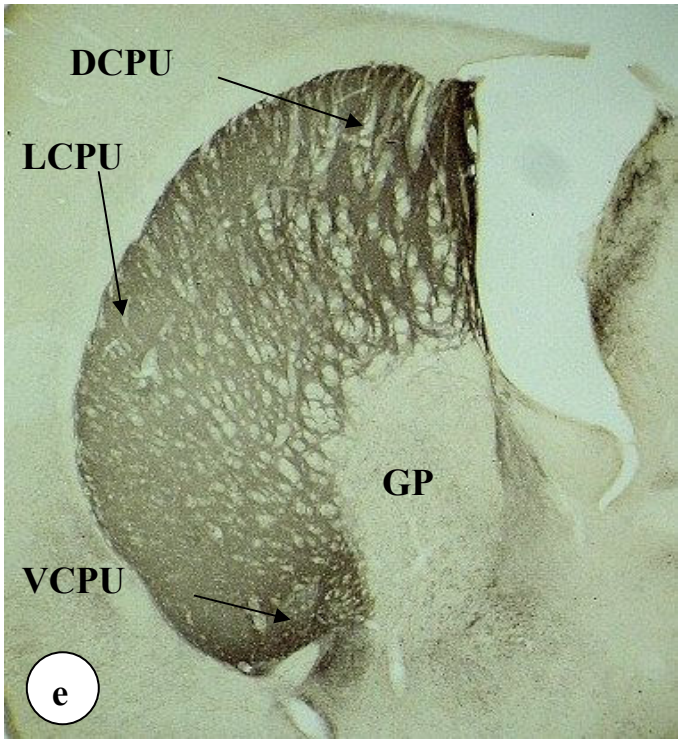
Sections were incubated for 24 h in a humidity chamber at 4ºC with the primary antibody to TH or 5-HT diluted in blocking serum (1% NGS in PBS with 0.3% Triton X- 100). The rest of the procedure was carried out at room temperature. After rinsing in PBS (3x5 mins), sections were incubated for 60 min in a biotinylated secondary antibody. Following incubation for 60 min in Avidin-Biotin-Complex (ABC) reagent (1:50; 20µl of solution A and 20µl of solution B in 1ml of PBS; Vectastain ABC kit, Vector Laboratories, INC, PK-6100, Peterborough UK), sections were then rinsed in PBS (3x5 mins) and 0.1 M phosphate buffer (PB, 1x5 mins). The location of the antigen-antibody complex was visualized by incubating sections in a medium containing 0.05% 3,3-diaminobenzidine (DAB substrate KIT for peroxidase, Vector Laboratories, INC, SK-4100, Peterborough UK) for 2-5 min. This step was carried out with care in fume cupboard and all equipment which came in contact with DAB was soaked with bleach in order to denature it, as DAB is potentially carcinogenic (International Agency for Research on Cancer, 1972). Finally, the sections were rinsed in distilled water, dehydrated and mounted with glass coverslips using histomount (RALAMB, HS-103, UK). Slides were then examined under a light microscope using a standard atlas of the rat brain **(Paxinos and Watson, 1982)**.


SNL
SNC
VTA
SNR
a


b


DRN
MRN
c


d



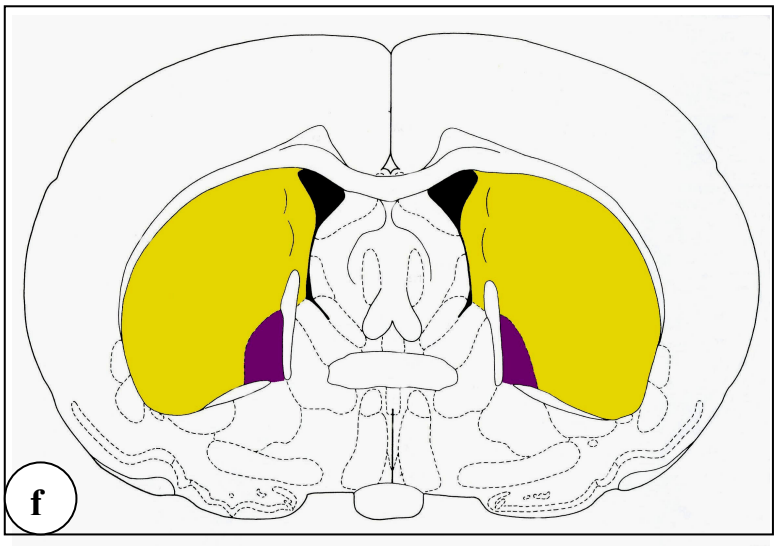


Figure 2.2 : Coronal sections of (a) substantia nigra (x40), (c) dorsal and median raphe nuclei (DRN and MRN),(x20) and (e) striatum,(x20) from a control (AS) male rat aged 12 months. Sections have been immunostained for TH. VTA, ventral tegmental area; SNC, substantia nigra pars compacta; SNR, pars reticularis; SNL, pars lateralis; DCPU, dorsal caudate-putamen; LCPU, lateral caudate-putamen; VCPU, ventral caudate-putamen; GP, globus pallidus. (b, d and f from Paxinos and Watson, 1982).

SNC VTA SNR SNL DRN

MRN Caudate-putamen Globus pallidus

Control sections were obtained by removing the primary antibody to test for any non–specific background staining.

Areas of interest were recorded using a digital camera, NIKON; Coolpix 995 (3.34 Mega pixel).

2.3.6 Immunofluorescence staining

Whilst toluidine blue and immunoperoxidase DAB staining for TH were used to confirm the position of sections, strain differences were assessed by quantitative immunofluorescence. The same procedures used in ICC were followed to prepare sections for the primary antibody. Sections were incubated in a humidity chamber for 24 h at 4ºC with primary antibody diluted in the blocking serum. After rinsing in PBS (3x5 mins), the sections were incubated in a humidity chamber for 24 h at 4ºC with a fluorescent secondary antibody at a dilution of 1:100 in PBS. Slides were covered with a caterwrap foil to give protection from bleaching in this step and during the rest of the procedure. The sections were then mounted with glass coverslips using Vectashield (H-1400, UK) after rinsing in distilled water (3x5 mins) and were ready for fluorescence quantification that was carried out on the same day. The area of interest was recorded using a Leica Wild MP52 photo-automat camera fitted to the microscope.

2.3.7 Double labeling Immunofluorescence staining

This method was used to confirm the present of Lewy body proteins such as ubiquitin, parkin and α-synuclein in the (TH) +ve cells or neuropil in the substantia nigra in the adjacent sections of the AS and the AS/AGU rats.

Sections were incubated in a humidity chamber for 24 h at 4ºC with a mixture of two primary antibodies (e.g. anti-tyrosine hydroxylase and anti-ubiquitin) diluted at 1:500

in the blocking serum. After rinsing in PBS (3x5 mins), the sections were incubated in a humidity chamber for 24 h at 4ºC with a fluorescent secondary antibody (Fluorescein and Rhodamine), at dilution of 1:100 in PBS for both. Slides were covered to give protection from bleaching in this step and the rest of the procedure. The sections were then mounted with glass coverslips using Vectashield (H-1400, UK) after rinsing in distilled water (3x5 mins).

Areas of interest were recorded using a Leica Wild MP52 photo-automat camera fitted to the microscope.

2.3.8 Fluorescence and the quantitative fluorescence microscope:

The physiological or pathological changes that occur in cells can be studied by sensitive techniques involving the use of fluorescent compounds coupled with a photometric fluorescence microscope.

Fluorescence is a type of luminescence where the absorption of light energy raises a molecule to a higher energy state, with subsequent releasing of energy in the form of light. In practical terms the wavelength of the emitted light is always longer than the excitatory light, so excitation in the ultraviolet leads to fluorescence in the visible range (usually in the blue or green) and excitation in the blue or green will usually lead to emission in the red. The advantage of using fluorescence is that it is possible to quantify the uptake of probe into a cell surface membrane or into cell organelles without damaging the cell.

The sensitivity and selectivity of fluorescence detection has excited the interest of microscopists since the1950s **(for review see Taylor and Salmon, 1989; Wampler and Kutz, 1989)**.

Immunofluorescence has been the most common application of fluorescence microscopy in cell biology. It combines the specificity and sensitivity of fluorescence microscopy with the selective binding of antibodies to restricted regions of antigen molecules.

Immunofluorescence quantification in these studies was achieved by using a Leitz Laborlux S microscope and photometric system **(Figure 2.3)**, which consisted of a photometer attachment with photomultiplier (ERNST LETZ WETZLAR GMBH, 301-289,133, Germany), which converts the light flux into electric signals. A control panel **(Figure 2.4)** allows the starting of the automatic measuring process, and coarse and fine amplification of the measuring signal, digital display unit and the power units for stabilizing the light sources. I followed immunofluorescence quantification techniques used over several years by researchers in the Division of Biochemistry and Molecular Biology in Glasgow University **(Modha *et al*., 1997; Ribeiro *et al*., 1998 ; Al-Adhami *et al.* , 2001 ; Akhkha *et al*, 2002 ; Al-Adhami *et al.* , 2003)**.

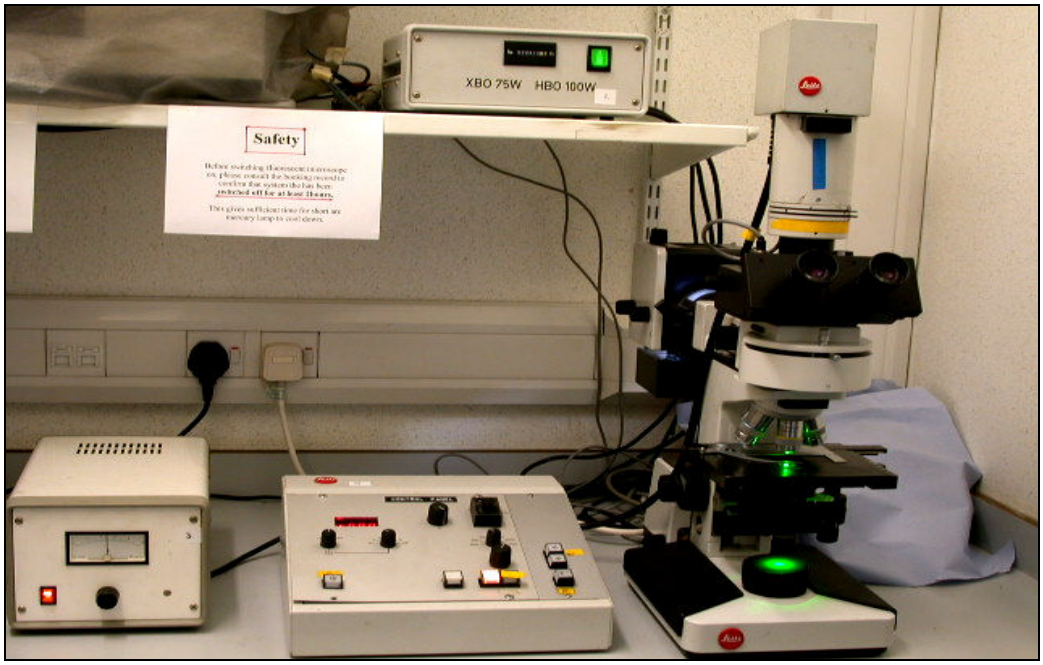


Figure 2.3: Leitz Laborlux S microscope photometer showing the major components (photometer, control panel and the power units).

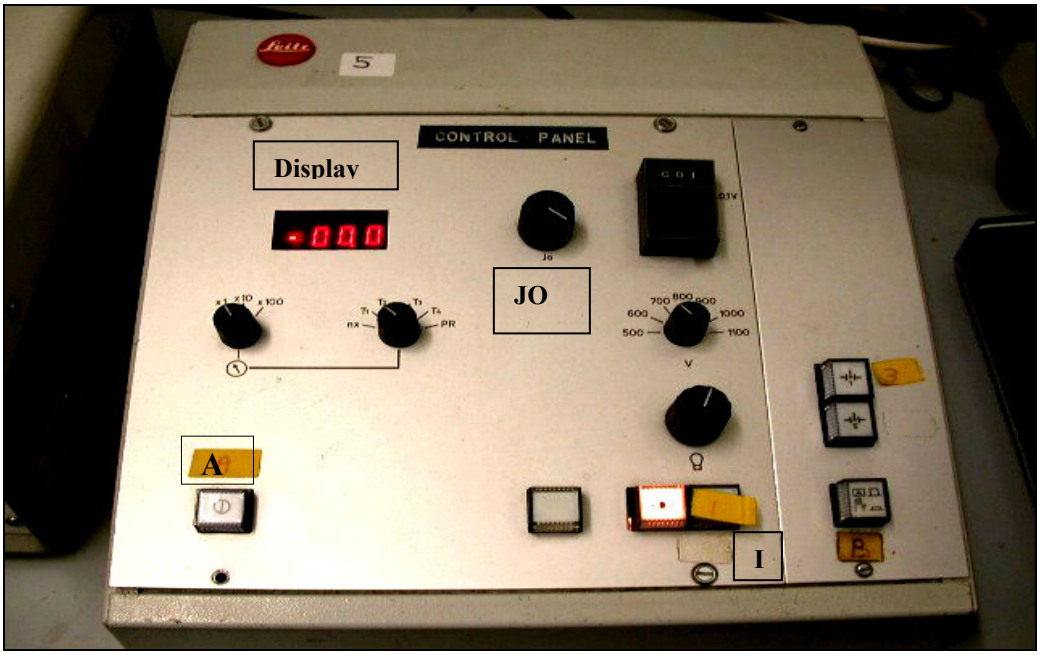


Figure 2.4: Leitz Laborlux S microscope photometer control panel showing the Display, A button for the starting the measuring process and I button to take the measurement and JO knob (adjust display reading to zero).

2.3.9 Fluorescence quantification

Fluorescence immunoreactivity of certain molecules in the midbrain cell groups or the striatum was quantified using a Leitz Laborlux S microscope photometer equipped with the filter set for Rhodamine Red-X (excitation, 570 nm and emission, 590 nm) an objective lens x50 (Fluoreszenz) and with a measurement field set at 150 μm^2 (The measurement was confirmed using a graduated slide from Graticules LTD Tonbridge Kent, England). Immunofluorescence was quantified in cells of the rostral substantia nigra pars compacta (SNC; *Approximately -4.8mm relative to bregma*), central SNC (*Approximately -5.3mm relative to bregma*), caudal SNC (*Approximately -6.3mm relative to bregma*), pars lateralis (SNL), pars reticularis (SNR), the ventral tegmental area (VTA), the dorsal raphe nucleus (DR; *Approximately -7.8 mm relative to bregma*) and the median raphe nucleus and dorsal, lateral and the ventral parts of the caudate-putamen (*Approximately -0.3 mm relative to bregma*) **(Paxinos and Watson, 1982)** of both AS (control) and AS/AGU (mutant) rats. The central SNC (the largest part) was also subdivided into medial and lateral halves and measurement started from the medial half and progressed toward the lateral half.

After the sections were put on the Leitz Laborlux S microscope stage, a bright sampling frame, viewed down the microscope eyepiece, was adjusted to a constant size of 15x10 µm throughout the experiment as this size was found to enclose the cell body satisfactorily. The only exception was α-synuclein since this is a pre-synaptic protein and therefore not found in cell bodies; here, the sampling frame was adjusted to enclose an area of neuropil of constant size (120x120 µm) throughout the experiment **(Figure 2.5 and 2.6)**.

As well as immunofluorescence-positive cells or neuropil areas the background fluorescence was also measured and subtracted from the recorded measurements.

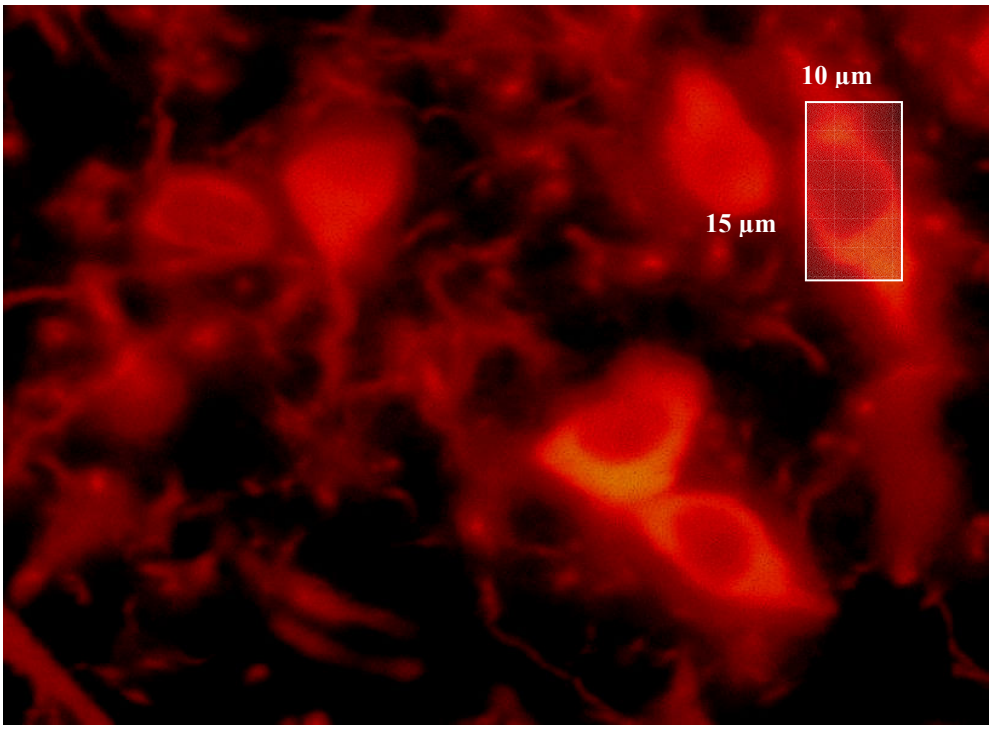


Figure 2.5: **A coronal section of substantia nigra shows a bright sampling frame and its size (10x15 µm) that is adjusted to enclose a TH-immunostained cell (x1000).**

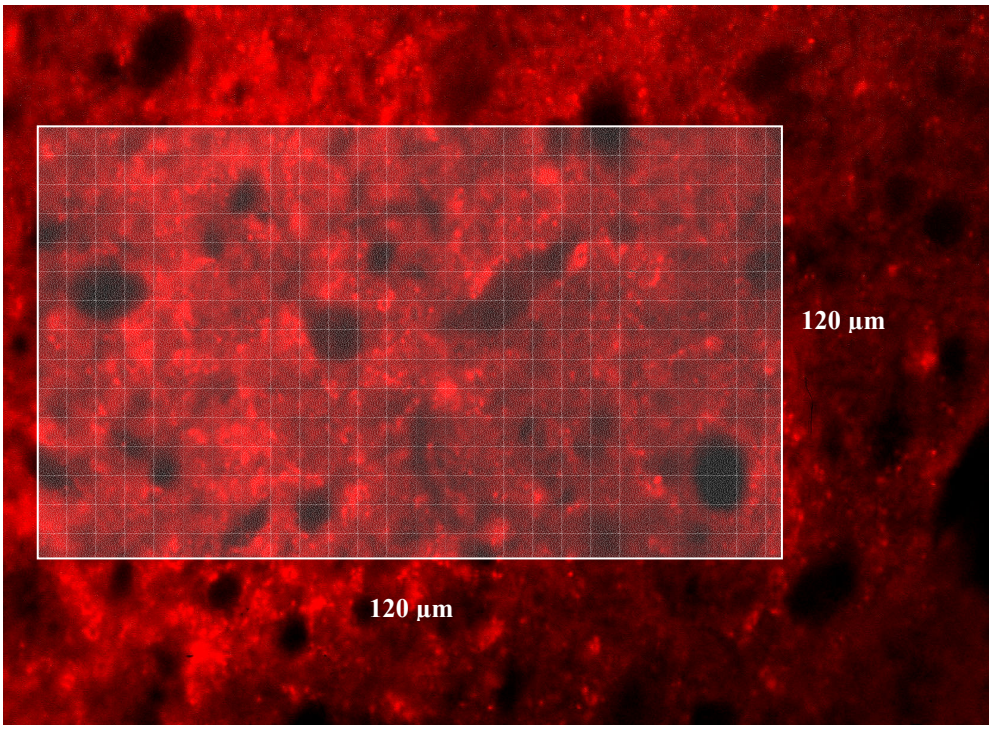


Figure 2.6: **A coronal section of substantia nigra shows a bright sampling frame and its size (120x120 µm) that is adjusted to enclose α-Synuclein-immunostained neuropil (x500).**

(Appendix 5).

The number of measured cells per animal was at least 50 in each area. This being the approximate numbers on a single section which showed a nucleus and nucleolus.

The fluorescence measurements are shown in arbitrary units, the range of values obtained being from 0.01-30. Tyrosine hydroxylase immunofluorescence per volume of cell was measured (the volume of SNC cells measured in **chapter 3 page 154**).

2.3.10 Statistical analysis

The results were analysed by paired t-test using the Minitab statistics package (MINITAB Release 13.30).

2.3.11 Calibration of fluorescent measurements

It was not possible to calibrate the arbitrary units against objects of known fluorescent emission. However, it was possible to test the relationship between fluorescence observed and primary antibody for TH on adjacent midbrain sections. Concentrations ranged between 1:50 and 1:2000 and show a strong liner relationship **(Table 2.1 and Figure 2.7)**.

Primary antibody concentrations (TH)	Fluorescence (arbitrary units)
1:50	20.48
1:100	19.44
1: 250	16.86
1: 500	13.83
1: 1000	11.85
1: 2000	8.16

Table 2.1: Calibration of fluorescent measurements: first column a series of primary antibody concentrations against TH and their fluorescent measurements (arbitrary units) in a second column.

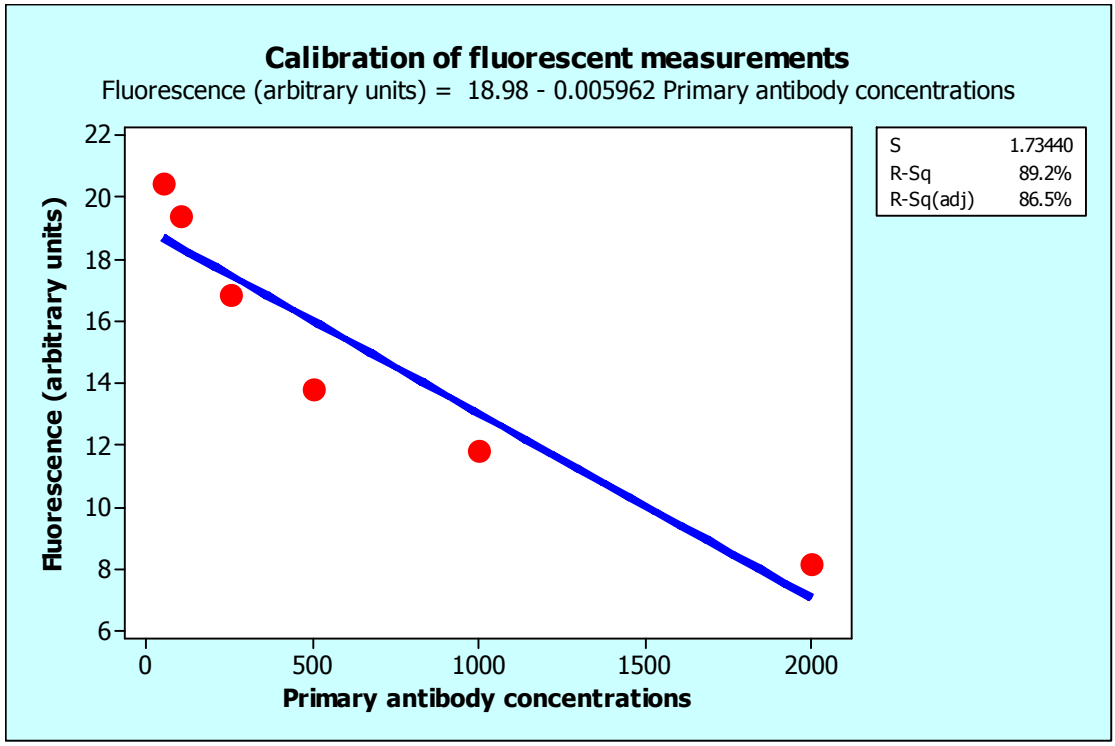


Figure 2.7: Calibration of fluorescent measurements: a series of primary antibody concentrations (TH) and their fluorescent measurements (arbitrary units).

Correlations: Primary antibody concentrations, Fluorescence (arbitrary units)

```
Pearson correlation of Primary antibody concentrations
and Fluorescence
(arbitrary units) = -0.944
```

P-Value = 0.005

2.4 Results for tyrosine hydroxylase

2.4.1 Quantified tyrosine hydroxylase immunofluorescence in the midbrain of AS and AS/AGU rats aged six months.

The results for tyrosine hydroxylase (TH) immunofluorescence show a statistically significant difference between the two groups in the rostral and caudal ends of the SNC, and the lateral half of the central SNC. ($P<0.05$ or higher). There are no statistical differences between the two groups in the levels of TH in the medial half of the central SNC, SNL, VTA or the SNR. In each case where there is a significant difference, the mutant rats have higher levels than the control strain. Tyrosine hydroxylase immunofluorescence per volume of cell show a statistically significant difference between the two groups in the rostral, cental and caudal ends of the SNC. The results are summarized **in table 2.2**.

	AS	AS/AGU	P
Rostral SNC	8.53 ± 1.40	12.44 ± 2.21	< **0.05**
Flu/cell volume	0.015 ± .002	0.061 ± .009	< **0.01**
Central SNC (a) overall	13.98 ± 2.19	18.45 ± 3.37	**< 0.05**
Flu/cell volume	0.021 ± 0.003	0.077 ± 0.014	< **0.05**
(b) medial	16.81 ± 2.50	18.88 ± 3.40	**NS**
Flu/cell volume	0.017 ± 0.003	0.075 ± 0.014	< **0.01**
(c) lateral	11.17 ± 2.02	18.02 ± 3.43	**< 0.05**
Flu/cell volume	0.026 ± 0.004	0.079 ± 0.014	< **0.05**
Caudal SNC	10.20± 1.38	14.54 ± 2.05	**< 0.01**
Flu/cell volume	0.013 ± 0.002	0.052 ± 0.009	< **0.01**
SNL	16.73 ± 2.46	17.18 ± 1.94	**NS**
SNR	17.83 ± 2.20	18.06 ± 1.79	**NS**
VTA	14.10 ± 1.67	17.81 ± 3.57	**NS**

Table 2.2: Tyrosine hydroxylase (TH) quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged six months (n=6 per group),(Flu/cell volume: fluorescence per cell volume). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.4.2 Quantified tyrosine hydroxylase immunofluorescence in the midbrain of AS and AS/AGU rats aged 12 months.

The results for tyrosine hydroxylase (TH) immunofluorescence show a statistically significant difference between the two groups in all TH +ve cells in the SNC. ($P<0.05$ or greater). In the SNL, SNR and the VTA there are no statistical differences between the two groups. Tyrosine hydroxylase immunofluorescence per volume of cell show a statistically significant difference between the two groups in the rostral, cental and caudal ends of the SNC. The results are summarized in **table 2.3**. In each case where there is a significant difference, the mutant rats have higher levels than the control strain.

	AS	AS/AGU	P
Rostral SNC	11.09 ± 0.47	16.36 ± 0.57	**< 0.001**
Flu/cell volume	0.017 ± 0.001	0.068 ± 0.003	**< 0.001**
Central SNC (a) overall	11.93 ± 0.47	15.02 ± 0.67	**< 0.01**
Flu/cell volume	0.018 ± 0.001	0.062 ± 0.003	**< 0.001**
(b) medial	11.32 ± 0.53	14.89 ± 1	**< 0.05**
Flu/cell volume	0.019 ± 0.001	0.062 ± 0.002	**< 0.001**
(c) lateral	12.53 ± 0.43	15.14 ± 0.42	**< 0.01**
Flu/cell volume	0.017 ± 0.001	0.061 ± 0.004	**< 0.001**
Caudal SNC	10.59 ± 0.55	15.39 ± 0.64	**< 0.001**
Flu/cell volume	0.016 ± 0.001	0.064 ± 0.003	**< 0.001**
SNL	10.13 ± 0.26	10.18 ± 0.26	**NS**
SNR	12.09 ± 0.65	12.82 ± 0.84	**NS**
VTA	11.92 ± 0.30	12.11 ± 0.31	**NS**

Table 2.3: Tyrosine hydroxylase (TH) quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 12 months (n=6 per group),(Flu/cell volume: fluorescence per cell volume). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.4.3 Quantified tyrosine hydroxylase immunofluorescence in the midbrain of AS and AS/AGU rats aged 18 months.

The results for tyrosine hydroxylase (TH) immunofluorescence show a statistically significant difference between the two groups in all regions except the SNL ($P<0.05$ or greater). Tyrosine hydroxylase immunofluorescence per volume of cell show a statistically significant difference between the two groups in the rostral, cental and caudal ends of the SNC. The results are summarized in **table 2.4**. In all regions, the mutant rats have higher levels than the control strain.

	AS	AS/AGU	P
Rostral SNC	9.29 ± 0.54	18.56 ± 1.63	**< 0.01**
Flu/cell volume	0.014 ± 0.001	0.063 ± 0.012	**< 0.01**
Central SNC (a) overall	10.28 ± 1.33	19.26 ± 2.75	**< 0.01**
Flu/cell volume	0.016 ± 0.002	0.08 ± 0.011	**< 0.01**
(b) medial	10.34 ± 1.56	18.64 ± 3.15	**< 0.01**
Flu/cell volume	0.016 ± 0.002	0.075 ± 0.014	**< 0.01**
(c) lateral	10.25 ± 1.25	19.93 ± 2.38	**< 0.001**
Flu/cell volume	0.015 ± 0.001	0.083 ± 0.01	**< 0.001**
Caudal SNC	11.30 ± 2.18	19.14 ± 2.58	**< 0.01**
Flu/cell volume	0.017 ± 0.003	0.08 ± 0.012	**< 0.01**
SNL	9.85 ± 1.17	15.46 ± 3.03	**NS**
SNR	9.68 ± 0.84	15.58 ± 2.11	**< 0.05**
VTA	9.88 ± 0.93	16.01 ± 2.38	**< 0.05**

Table 2.4. Tyrosine hydroxylase (TH) quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 18 months (n=6 per group),(Flu/cell volume: fluorescence per cell volume). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.4.4 A comparison of tyrosine hydroxylase(TH) levels in the midbrain of AS and AS/AGU rats at different ages.

Tyrosine hydroxylase levels decrease with age in AS (control) rats in the central SNC, SNL, SNR and VTA. In general, the decreases are progressive but modest. In other regions such as the rostral and caudal SNC, there are no effects of age and TH levels are constant over the range examined in this experiment.

By contrast, TH levels in AS/AGU (mutant) rats may rise with age (rostral SNC, caudal SNC) or fall initially but then rise again, so that TH levels at 18 months resemble those at 6 months (all other regions).

2.5 Results for serotonin

2.5.1 Quantified serotonin (5-HT) immunofluorescence in the midbrain of AS and AS/AGU rats aged six months

The results for serotonin (5-HT) immunofluorescence show a statistically significant difference between the two groups for the dorsal raphe nucleus ($P<0.05$). AS/AGU (mutant) rats had higher levels than AS (control). There was no statistical difference between the two groups for the median raphe nucleus. The results are summarized **in table 2.5**.

	AS	**AS/AGU**	**P**
Dorsal raphe nucleus	4.81 ± 0.23	6.07 ± 0.23	**< 0.05**
Median raphe nucleus	4.36 ± 0.13	4.74 ± 0.19	**NS**

Table 2.5. Serotonin quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged six months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant) .

2.5.2 Quantified serotonin (5-HT) immunofluorescence in the midbrain of AS and AS/AGU rats aged 12 months

The results for serotonin (5-HT) immunofluorescence show a statistically significant difference between the two groups in the dorsal raphe nucleus ($P<0.01$) with mutants having higher levels than controls. There is no statistical difference between the two groups in the levels of serotonin in the median raphe nucleus. The results are summarized in **table 2.6**.

	AS	**AS/AGU**	**P**
Dorsal raphe nucleus	3.93 ± 0.33	5.45 ± 0.47	**< 0.01**
Median raphe nucleus	3.51 ± 0.58	5.58 ± 1.07	**NS**

Table 2.6: Serotonin quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 12 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are by paired t-tests (NS, not significant).

2.5.3 Quantified serotonin (5-HT) immunofluorescence in the midbrain of AS and AS/AGU rats aged 18 months

The results for serotonin (5-HT) immunofluorescence show a statistically significant difference between the two groups in the dorsal raphe nucleus ($P<0.001$) with mutants having higher levels than controls. There is no statistical difference between the two groups in the levels of serotonin in the median raphe nucleus. The results are summarized in **table 2.7**.

	AS	**AS/AGU**	**P**
Dorsal raphe nucleus	3.23 ± 0.4	7.98 ± 0.24	**< 0.001**
Median raphe nucleus	3.46 ± 0.36	4.46 ± 0.59	**NS**

Table 2.7: Serotonin quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 18 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are by paired t-tests (NS, not significant).

2.5.4 A comparison of serotonin (5-TH) levels in the midbrain of 6, 12 and 18 months AS and AS/AGU rats

5-HT levels progressively decrease with age in the AS (control) rats in the dorsal raphe nucleus, whereas they increase at the oldest age in AS/AGU rats. 5HT levels do not change with age in the median raphe nucleus in either group.

2.6 Results for ubiquitin

2.6.1 TH and ubiquitin (Ub) labelling of adjacent sections

In the case of senescence-associated molecules, it was essential to confirm that measurements were being made on aminergic cells. This was achieved by a labelling carried out in adjacent sections. For both strains, adjacent sections were stained respectively for TH and ubiquitin using fluorescent secondary antibodies.

A double labelling with TH and ubiquitin showed that about 90% of the cells in the SNC stained with both TH and ubiquitin and only 10% stained with only one of them **(table 2.8 and figure 2.8)**. Only those cells positively confirmed for TH were measured for ubiquitin fluorescence.

Cell stained		Percentage %	
		AS SEM	AS/AGU SEM
TH and Ub	6 M	91.5 % (±1.4)	90.9 % (± 0.43)
	12 M	88.9 % (± 0.05)	88.3 % (± 0.04)
	18 M	89.7 % (± 0.08)	88.1% (± 0.12)
Ub not TH	6 M	7.5 % (± 0.06)	8.1% (± 0.04)
	12 M	9.6 % (± 0.01)	10.2 % (± 0.03)
	18 M	9.1 % (± 0.01)	9.9 % (± 0.01)
TH not Ub	6 M	1 % (± 0.13)	1 % (± 0.05)
	12 M	1.5 % (± 0.005)	1.5 % (± 0.007)
	18 M	1 % (±0.025)	2 % (± 0.026)

Table 2.8: Percentage of cells in the SNC that immunofluorescence stained with tyrosine hydroxylase (TH) or ubiquitin (Ub) or both of them.

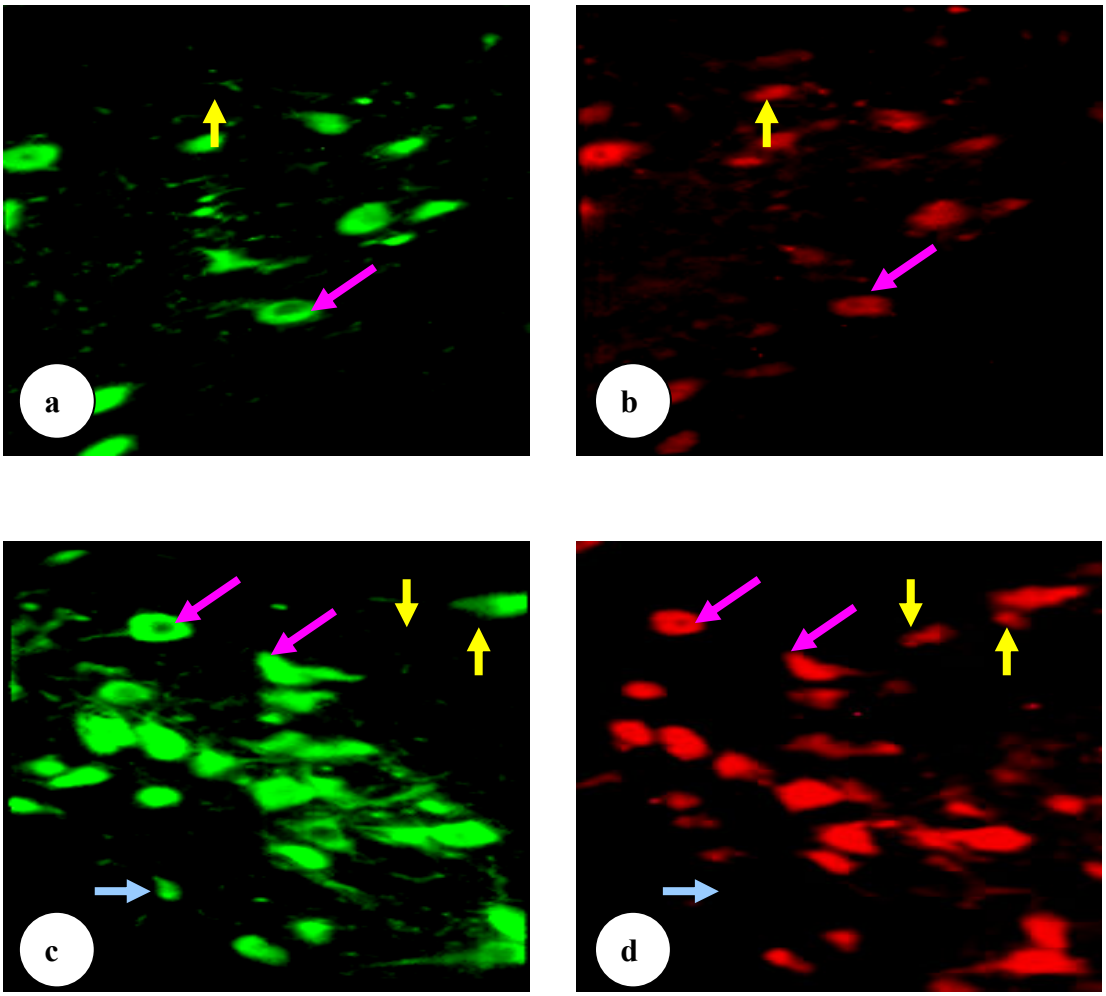


Figure 2.8: **TH and ubiquitin (Ub) labelling of adjacent sections. (a) AS TH-immunostained cells of substantia nigra (green color). (b) AS Ub-immunostained cells of substantia nigra (red color). (c) AS/AGU TH-immunostained cells of substantia nigra (green color). (d) AS/AGU Ub-immunostained cells of substantia nigra (red color). (x500).**

Examples

Cell bodies immunostained with both TH (a and c) and ubiquitin (b and d).

Cell bodies immunostained with TH only (c) which appear black in (d).

Cell bodies immunostained with ubiquitin only (b and d) which appear black in (a and c).

2.6.2 Quantified ubiquitin immunofluorescence in the midbrain of AS and AS/AGU rats aged 6 months

The results for ubiquitin (Ub) immunofluorescence show a statistically significant difference between the two groups in the rostral SNC, central SNC (overall, medial and lateral regions), caudal SNC, and the dorsal raphe nucleus (DRN) ($P<0.05$ or greater). Ubiquitin levels are significantly higher in the AS/AGU mutant. There are no statistical differences between the two groups in the levels of Ub ($P>0.05$) in the SNL, SNR, VTA, median raphe nucleus (MRN), oculomotor nucleus or pontine nucleus. The results are summarized in **table 2.9**.

	AS	AS/AGU	P
Rostral SNC	4.16 ± 0.25	5.74 ± 0.15	**< 0.01**
Central SNC (a) overall	5.79 ± 0.35	7.79 ± 0.35	**< 0.001**
(b) medial	5.75 ± 0.46	7.21 ± 0.39	**< 0.05**
(c) lateral	5.82 ± 0.28	8.35 ± 0.50	**< 0.001**
Caudal SNC	4.55 ± 0.25	6.14 ± 0.19	**< 0.001**
SNL	5.54 ± 0.66	5.90 ± 0.29	**NS**
SNR	5.23 ± 0.45	5.56 ± 0.44	**NS**
VTA	5.79 ± 0.44	6.57 ± 0.59	**NS**
Oculomotor nucleus	4.56 ± 0.60	4.88 ± 0.24	**NS**
DRN	4.26 ± 0.32	6.05 ± 0.24	**< 0.01**
MRN	4.32 ± 0.23	4.45 ± 0.29	**NS**
Pontine nucleus	3.77 ± 0.25	4.11 ± 0.41	**NS**

Table 2.9: Ubiquitin quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 6 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.6.3 Quantified ubiquitin immunofluorescence in the midbrain of AS and AS/AGU rats aged 12 month

The results for ubiquitin (Ub) immunofluorescence show a statistically significant difference between the two groups in the rostral SNC, central SNC (overall and lateral region), caudal SNC and the dorsal raphe nucleus (DRN) ($P<0.05$ or greater). In each case, AS/AGU mutants had higher levels than controls. There are no statistical differences between the two groups in the levels of Ub in the central SNC (medial region), SNL, VTA, median raphe nucleus (MRN), oculomotor nucleus or pontine nucleus. The results are summarized in **table 2.10**.

	AS	AS/AGU	P
Rostral SNC	4.21 ± 0.35	6.97 ± 0.79	**< 0.05**
Central SNC (a) overall	4.25 ± 0.50	6.00 ± 0.58	**< 0.001**
(b) medial	5.06 ± 0.61	5.85 ± 0.55	**NS**
(c) lateral	3.69 ± 0.63	6.34 ± 0.79	**< 0.01**
Caudal SNC	4.77 ± 0.47	6.97 ± 0.71	**< 0.01**
SNL	4.94 ± 0.05	4.96 ± 0.04	**NS**
SNR	4.87 ± 0.13	4.92 ± 0.12	**NS**
VTA	4.79 ± 0.26	5.19 ± 0.37	**NS**
Oculomotor nucleus	4.99 ± 0.69	5.01 ± 0.58	**NS**
DRN	3.68 ± 0.46	7.05 ± 0.95	**< 0.05**
MRN	4.43 ± 0.27	4.47 ± 0.26	**NS**
Pontine nucleus	4.59 ± 0.31	4.74 ± 0.17	**NS**

Table 2.10: Ubiquitin quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 12 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.6.4 Quantified ubiquitin immunofluorescence in the midbrain of AS and AS/AGU rats aged 18 month

The results for ubiquitin (Ub) immunofluorescence show a statistically significant difference between the two groups in the rostral SNC, central SNC (overall, medial and lateral regions), caudal SNC, SNL, SNR, VTA, DRN and the MRN. ($P<0.05$ or higher). In each case, AS/AGU mutants had higher levels than AS controls. There are no statistical differences between the two groups in the levels of Ub ($P>0.05$) in the oculomotor nucleus or pontine nucleus.. The results are summarized in **table 2.11**.

	AS	AS/AGU	P
Rostral SNC	7.94 ± 0.20	16.26 ± 0.46	**< 0.001**
Central SNC (a) overall	7.28 ± 1.02	15.75 ± 0.93	**< 0.001**
(b) medial	7.85 ± 1.14	17.18 ± 0.77	**< 0.001**
(c) lateral	6.60 ± 0.89	14.30 ± 1.18	**< 0.001**
Caudal SNC	6.59 ± 0.47	14.26 ± 0.84	**< 0.001**
SNL	8.37 ± 0.59	11.00 ± 0.66	**< 0.01**
SNR	8.61 ± 0.57	11.09 ± 0.52	**< 0.001**
VTA	7.06 ± 0.40	11.53 ± 0.49	**< 0.001**
Oculomotor nucleus	4.59 ± 0.24	4.83 ± 0.19	**NS**
DRN	6.15 ± 0.38	13.47 ± 1.03	**< 0.001**
MRN	7.46 ± 0.42	9.60 ± 0.57	**< 0.05**
Pontine nucleus	6.41 ± 0.35	7.08 ± 0.67	**NS**

Table 2.11: Ubiquitin quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 18 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.6.5 A comparison of ubiquitin(Ub) levels in the midbrain of 6, 12 and 18 months AS and AS/AGU rats

In every region except the oculomotor nucleus, cells show a gradual increase in ubiquitin immunofluorescence with age in AS (control) rats. However, the increase with age is much greater in AS/AGU (mutant) rats, leading to significant differences between the two strains at 18 months in all regions except the pontine and oculomotor nuclei.

2.7 Results for parkin

2.7.1 TH and parkin labelling of adjacent sections

Both TH and parkin labelling were carried out in which adjacent sections from AS and AS/AGU brains were stained for TH and parkin using fluorescent secondary antibodies.

Labelling with TH and parkin showed that about 90% of the cells in the SNC stained with both TH and parkin and the remainder with only one of them **(Table 2.12)**. **(Figure 2.9)**. In dopaminergic areas, such as the SNC and VTA, only cells which were TH positive were counted for parkin.

Cell stained		Percentage %			
		AS	SEM	AS/AGU	SEM
TH and parkin	6 M	89.6 %	(± 0.01)	89.1 %	(± 0.24)
	12 M	88.9 %	(± 0.02)	88.2 %	(± 0.03)
	18 M	87.8 %	(± 0.04)	87.7 %	(± 0.05)
Parkin not TH	6 M	8.9 %	(± 0.05)	9.9 %	(± 0.04)
	12 M	9.6 %	(± 0.02)	10.3 %	(± 0.03)
	18 M	10.7 %	(± 0.03)	11.3 %	(± 0.05)
TH not parkin	6 M	1.5 %	(± 0.02)	1 %	(± 0.03)
	12 M	1.5 %	(± 0.01)	1.5 %	(± 0.03)
	18 M	1.5 %	(± 0.01)	1 %	(± 0.05)

Table 2.12.: Percentage of cells in the SNC that immunofluorescence stained with tyrosine hydroxylase (TH) or parkin or both of them.

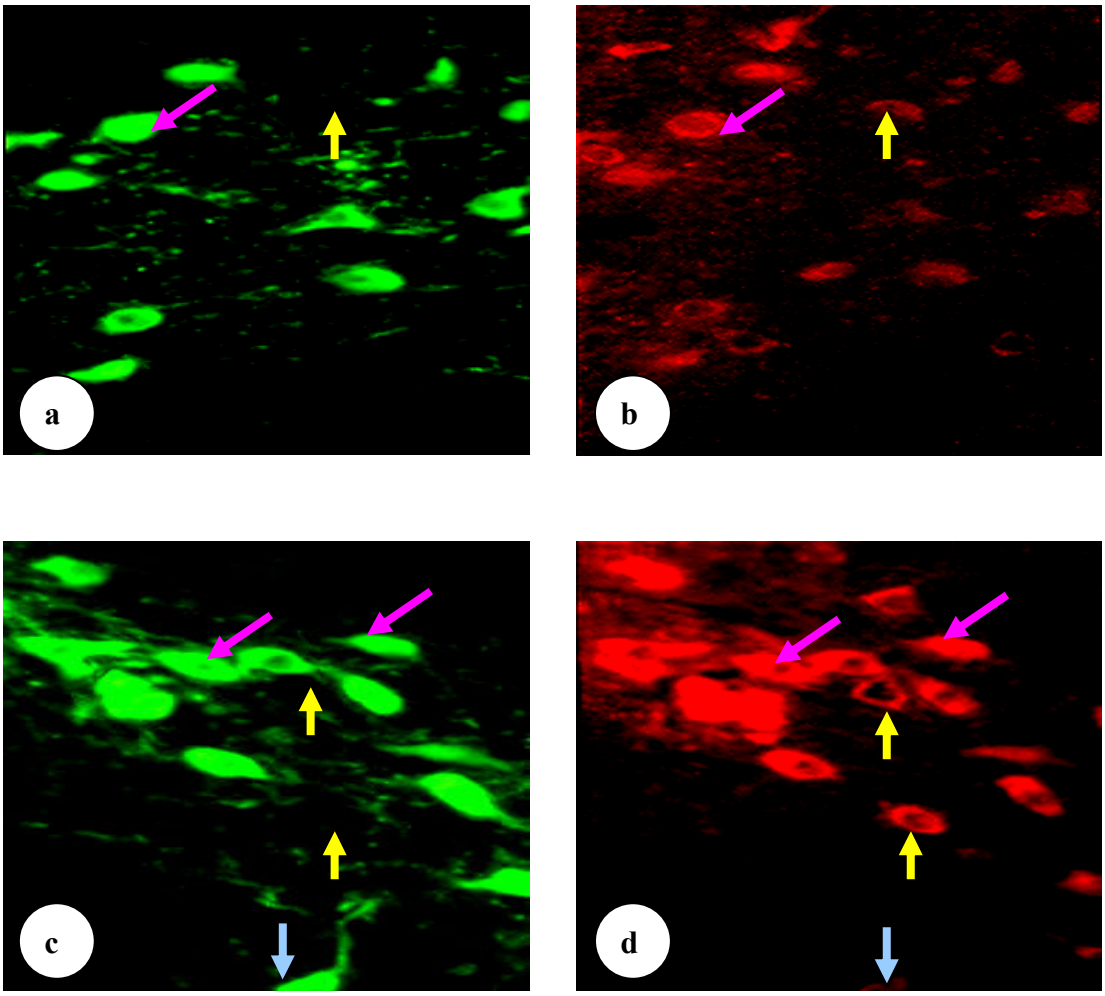


Figure 2.9: **TH and parkin labelling of adjacent sections. (a) AS TH-immunostained cells of substantia nigra (green color). (b) AS parkin immunostained cells of substantia nigra (red color). (c) AS/AGU TH-immunostained cells of substantia nigra (green color). (d) AS/AGU parkin immunostained cells of substantia nigra (red color). (x500).**

Examples

Cell bodies immunostained with both TH (a and c) and parkin.(b and d).

Cell bodies immunostained with TH only (c) which appear black in (d).

Cell bodies immunostained with parkin only (b and d) which appear black in (a) and (c).

2.7.2 Quantified parkin immunofluorescence in the midbrain of AS and AS/AGU rats aged 6 months

The results for parkin immunofluorescence show a statistically significant difference between the two groups for the central SNC, caudal SNC and the DRN. ($P<0.05$ or greater). AS/AGU (mutant) rats have higher levels than AS (control). There are no statistical differences between the two groups in the rostral SNC, SNL, SNR, VTA, median raphe nucleus (MRN), oculomotor nucleus or pontine nucleus. The results are summarized in **table (2.13)**.

	AS	AS/AGU	P
Rostral SNC	4.88 ± 0.27	6.29 ± 0.70	**NS**
Central SNC (a) overall	4.19 ± 0.17	5.32 ± 0.26	**< 0.05**
(b) medial	4.19 ± 0.11	5.44 ± 0.62	**NS**
(c) lateral	4.21 ± 0.33	5.96 ± 0.89	**NS**
Caudal SNC	5.35 ± 0.74	6.44 ± 0.67	**< 0.01**
SNL	4.38 ± 0.39	4.66 ± 0.25	**NS**
SNR	3.85 ± 0.27	4.64 ± 0.24	**NS**
VTA	3.76 ± 0.25	4.55 ± 0.40	**NS**
Oculomotor nucleus	4.11 ± 0.17	4.19 ± 0.10	**NS**
DRN	3.18 ± 0.19	4.31 ± 0.10	**< 0.01**
MRN	3.15 ± 0.08	3.33 ± 0.24	**NS**
Pontine nucleus	3.41 ± 0.07	3.43 ± 0.09	**NS**

Table 2.13: Parkin quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 6 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.7.3 Quantified parkin immunofluorescence in the midbrain of AS and AS/AGU rats aged 12 months

The results for parkin immunofluorescence show a statistically significant difference between the two groups for the rostral SNC, central SNC (overall, medial and lateral regions), caudal SNC and the DRN ($P<0.05$ or greater). In each case, AS/AGU mutants had higher levels than AS controls. There are no statistical differences between the two groups in the levels of parkin in the SNL, SNR, VTA, MRN, oculomotor nucleus or pontine nucleus. The results are summarized in **table 2.14**.

	AS	AS/AGU	P
Rostral SNC	3.17 ± 0.13	5.02 ± 0.15	**< 0.001**
Central SNC (a) overall	3.55 ± 0.66	5.18 ± 0.39	**< 0.01**
(b) medial	3.70 ± 0.75	5.14 ± 0.40	**< 0.05**
(c) lateral	3.41 ± 0.59	5.23 ± 0.41	**< 0.001**
Caudal SNC	2.79 ± 0.18	4.78 ± 0.14	**< 0.001**
SNL	3.64 ± 0.58	3.64 ± 0.57	**NS**
SNR	3.67 ± 0.59	3.74 ± 0.59	**NS**
VTA	3.53 ± 0.58	3.54 ± 0.43	**NS**
Oculomotor nucleus	3.71 ± 0.30	3.79 ± 0.26	**NS**
DR	2.65 ± 0.09	4.69 ± 0.45	**< 0.01**
MR	2.99 ± 0.12	3.16 ± 0.09	**NS**
Pontine nucleus	3.01 ± 0.04	3.07 ± 0.06	**NS**

Table 2.14: Parkin immunofluorescence (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 12 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.7.4 Quantified parkin immunofluorescence in the midbrain of AS and AS/AGU rats aged 18 months

The results for parkin immunofluorescence show a statistically significant difference between the two groups in the rostral SNC, central SNC (overall, medial and lateral regions), caudal SNC and the DRN ($P<0.05$ or greater). In each case, AS/AGU mutants had higher levels than AS controls. There are no statistical differences between the two groups in the levels of parkin in the SNL, SNR, VTA, MRN, oculomotor nucleus or pontine nucleus. The results are summarized in **table 2.15**.

	AS	AS/AGU	P
Rostral SNC	3.02 ± 0.16	5.73 ± 0.63	**< 0.01**
Central SNC (a) overall	3.48 ± 0.39	6.40 ± 1.06	**< 0.01**
(b) medial	3.37 ± 0.32	6.29 ± 1.11	**< 0.05**
(c) lateral	3.58 ± 0.46	6.51 ± 1.02	**< 0.01**
Caudal SNC	3.33 ± 0.19	6.29 ± 0.17	**< 0.001**
SNL	3.11 ± 0.26	4.58 ± 0.63	**NS**
SNR	3.53 ± 0.37	4.74 ± 0.67	**NS**
VTA	4.08 ± 0.37	4.12 ± 0.56	**NS**
Oculomotor nucleus	3.33 ± 0.22	3.42 ± 0.34	**NS**
DRN	2.56 ± 0.31	5.64 ± 0.33	**< 0.001**
MRN	2.86 ± 0.23	3.43 ± 0.26	**NS**
Pontine nucleus	3.26 ± 0.25	3.37 ± 0.23	**NS**

Table 2.15: Parkin quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 18 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.7.5 A comparison of parkin levels in the midbrain of 6, 12 and 18 months AS and AS/AGU rats

In AS (control) rats, there was a gradual, slight decrease in parkin immunofluorescence with age in virtually all brain regions. By contrast, there was a gradual, slight rise in parkin immunofluorescence with age in the AS/AGU mutant rats for regions such as the central SNC and dorsal raphe nucleus. In some cases this led to a statistically significant difference between the two groups at older ages, but not at earlier ones.

2.8 Results for α-synuclein

2.8.1 Double labelling

The staining with alpha-synuclein was in the neuropil of the SNC. A double labelling was carried out on a same sections from AS and AS/AGU rats using fluorescent secondary antibodies for TH and alpha-synuclein.

A double labelling with TH and alpha-synuclein showed that most of the cells in the SNC of both AS(control) and AS/AGU(mutant) rats were stained with TH. In the adjacent sections, stained for alpha-synuclein, the SNC cells are non-fluorescing and appear brown or black. **(Figure 2.10)**.

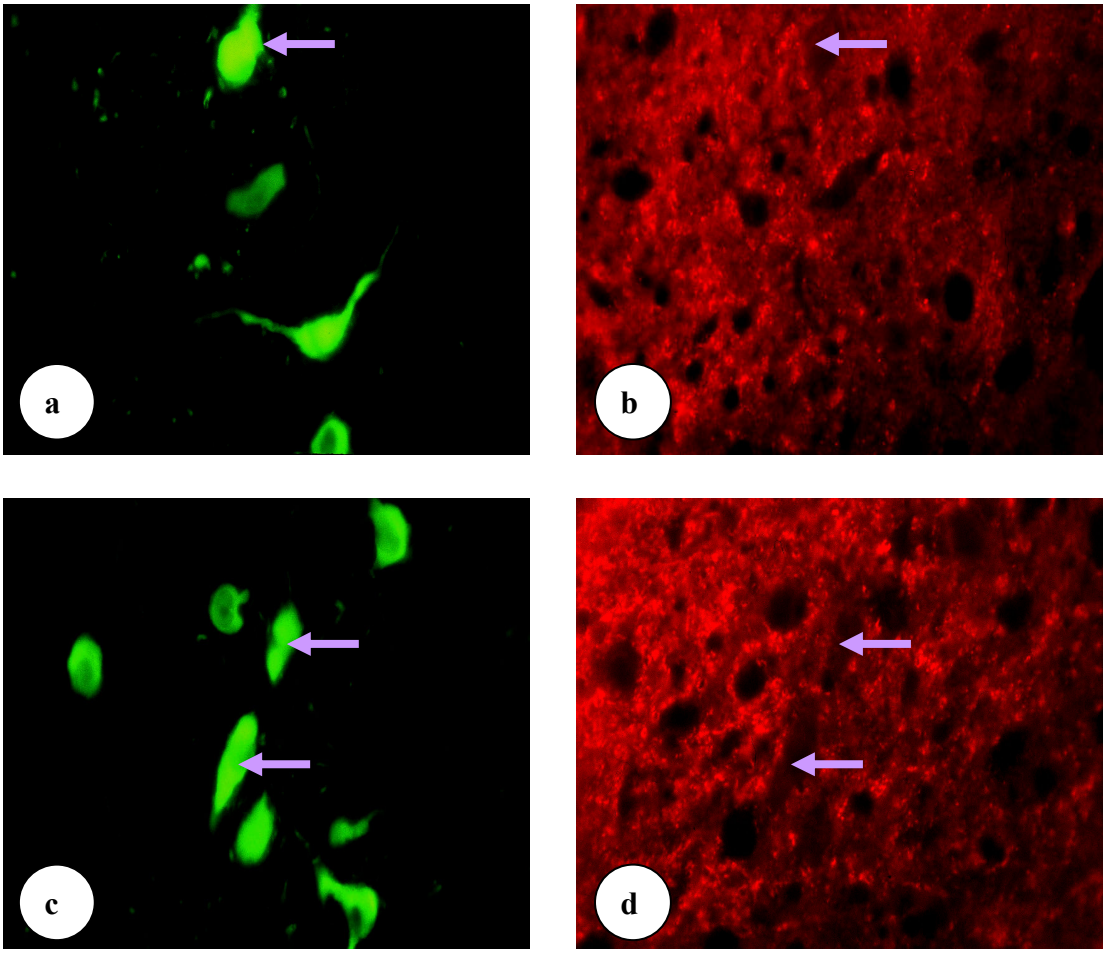


Figure 2.10: **TH and Alpha-synuclein double labelling. (a) AS TH-immunostained cells of substantia nigra (green color). (b) AS alpha-synuclein immunostained in the substantia nigra (red color). (c) AS/AGU TH-immunostained cells of substantia nigra (green color). (d) AS/AGU alpha-synuclein immunostained in the substantia nigra (red color). (x500).**

Examples

→ **Cell bodies immunostained with TH (a and c) not stained with alpha-synuclein and appear black or brown (b and d). Alpha-synuclein immunostained in the neuropil.**

2.8.2 Quantified α-Synuclein immunofluorescence in the midbrain of AS and AS/AGU rats aged 6 months

Alpha-Synuclein immunofluorescence of the neuropil showed a statistically significant difference between the two groups in the rostral SNC, central SNC and the caudal SNC. ($P<0.05$) with levels being highest in AS/AGU mutants. There are no statistical differences between the two groups in the levels of α-Synuclein in the SNR and the VTA. The results are summarized in **table 2.16**.

	AS	**AS/AGU**	**P**
Rostral SNC	1.33 ± 0.4	1.67 ± 0.3	**< 0.05**
Central SNC	1.15 ± 0.11	1.98 ± 0.41	**< 0.05**
Caudal SNC	0.64 ± 0.08	0.97 ± 0.08	**< 0.05**
SNR	1.43 ± 0.19	1.98 ± 0.32	**NS**
VTA	0.48 ± 0.06	0.52 ± 0.08	**NS**

Table 2.16: Alpha-Synuclein quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 6 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.8.3 Quantified α-Synuclein immunofluorescence in the midbrain of AS and AS/AGU rats aged 12 months

Alpha-Synuclein immunofluorescence of the neuropil showed a statistically significant difference between the two groups in the rostral SNC, central SNC and the caudal SNC ($P<0.01$ or greater) with levels being highest in AS/AGU mutant. There are no statistical differences between the two groups in the levels of α-Synuclein in the SNR and the VTA. The results are summarized in **table 2.17**.

	AS	**AS/AGU**	**P**
Rostral SNC	1.07 ± 0.14	2.46 ± 0.35	**< 0.01**
Central SNC	1.88 ± 0.51	4.56 ± 0.61	**< 0.01**
Caudal SNC	1.51 ± 0.16	3.26 ± 0.12	**< 0.001**
SNR	4.25 ± 0.72	5.42 ± 0.45	**NS**
VTA	1.13 ± 0.11	1.16 ± 0.10	**NS**

Table 2.17: Alpha-Synuclein quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 12 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.8.4 Quantified α-Synuclein immunofluorescence in the midbrain of AS and AS/AGU rats aged 18 months

Alpha-Synuclein immunofluorescence of the neuropil showed a statistically significant difference between the two groups in the central part of the SNC. ($P<0.05$). There are no statistical differences between the two groups in the levels of α-Synuclein in the rostral SNC, the caudal SNC, SNR or the VTA. In some cases, this may be due to the large standard errors within the group. The results are summarized in **table 2.18**.

	AS	AS/AGU	P
Rostral SNC	1.58 ± 0.28	3.74 ± 0.69	**NS**
Central SNC	2.78 ± 0.75	6.12 ± 0.8	**< 0.05**
Caudal SNC	1.79 ± 0.45	4.66 ± 1.06	**NS**
SNR	3.91 ± 1.04	6.43 ± 1.41	**NS**
VTA	1.55 ± 0.36	1.55 ± 0.39	**NS**

Table 2.18: Alpha-Synuclein quantification (arbitrary units of fluorescence) in the midbrain of AS control and AS/AGU mutant rats aged 18 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.8.5 A comparison of α-synuclein levels in the midbrain of AS and AS/AGU rats aged 6, 12 and 18 months

Alpha-synuclein levels progressively increase with age in the AS (control) and the AS/AGU (mutant) in the central SNC, caudal SNC and the VTA.

2.9 Results in the striatum

2.9.1 Quantified Tyrosine hydroxylase (TH) immunofluorescence in the striatum of AS and AS/AGU rats aged 12 months

The results for TH immunofluorescence show a statistically significant difference between the two groups in the dorsal caudate-putamen (DCPU) ($P<0.01$) with TH levels significantly reduced in the AS/AGU rats. There are no statistical differences between the two groups in the levels of TH in the lateral or ventral caudate-putamen (LCPU,VCPU). The results are summarized in **table 2.19**.

	AS	**AS/AGU**	**P**
DCPU	24.75 ± 2.87	13.5 ± 4.02	**< 0.01**
LCPU	17.45 ± 3.55	14.58 ± 4.55	**NS**
VCPU	10.6 ± 3.33	9.57 ± 3.88	**NS**

Table 2.19: Tyrosine hydroxylase quantification (arbitrary units of fluorescence) in the striatum of AS control and AS/AGU mutant rats aged 12 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS not significant).

2.9.2 Quantified serotonin (5-HT) immunofluorescence in the striatum of AS and AS/AGU rats aged 12 months

The results for 5-HT immunofluorescence show a statistically significant difference between the two groups in the dorsal caudate-putamen ($P<0.05$) with 5-HT levels significantly reduced in the AS/AGU rats. There are no statistical differences between the two groups in the levels of 5-HT in the lateral striatum or ventral striatum. The results are summarized in **table 2.20**.

	AS	**AS/AGU**	**P**
DCPU	6.7 ± 0.99	4.72 ± 0.94	**< 0.05**
LCPU	4.25 ± 1.50	3.68 ± 0.98	**NS**
VCPU	4.22 ± 1.00	4.00 ± 1.00	**NS**

Table 2.20: Serotonin quantification (arbitrary units of fluorescence) in the striatum of AS control and AS/AGU mutant rats aged 12 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.9.3 Quantified ubiquitin (Ub) immunofluorescence in the striatum of AS and AS/AGU rats aged 12 months

There are no statistical differences between the two groups in the levels of Ub in the dorsal, lateral or the ventral caudate-putamen or in the striatum cells. The results are summarized in **table 2.21**.

	AS	**AS/AGU**	**P**
DCPU	6.8 ± 0.76	9.17 ± 2.29	**NS**
LCPU	6.6 ± 0.69	7.43 ± 2.24	**NS**
VCPU	6.37 ± 0.73	7.05 ± 2.27	**NS**
Striatum cells	1.13 ± 0.12	1.29 ± 0.25	**NS**

Table 2.21: Ubiquitin quantification (arbitrary units of fluorescence) in the striatum of AS control and AS/AGU mutant rats aged 12 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.9.4 Quantified parkin immunofluorescence in the striatum of AS and AS/AGU rats aged 12 months

There are no statistical differences between the two groups in the levels of Parkin in the dorsal, lateral or the ventral caudate-putamen or in the striatum cells. The results are summarized in **table 2.22**.

	AS	AS/AGU	P
DCPU	5.98 ± 0.29	9.80 ± 1.60	**NS**
LCPU	4.90 ± 0.41	7.15 ± 1.34	**NS**
VCPU	5.28 ± 0.61	6.42 ± 1.02	**NS**
Striatum cells	1.10 ± 0.04	1.19 ± 0.05	**NS**

Table 2.22: Parkin quantification (arbitrary units of fluorescence) in the striatum of AS control and AS/AGU mutant rats aged 12 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.9.5 Quantified α-Synuclein immunofluorescence in the striatum of AS and AS/AGU rats aged 12 months

There are no statistical differences between the two groups in the levels of α-Synuclein in the dorsal, lateral or the ventral caudate-putamen. The results are summarized in **table 2.23**.

	AS	**AS/AGU**	**P**
DCPU	5.33 ± 0.99	9.00 ± 1.02	**NS**
LCPU	3.53 ± 1.45	5.15 ± 1.55	**NS**
VCPU	4.30 ± 1.42	5.88 ± 1.65	**NS**

Table 2.23: Alpha-Synuclein quantification (arbitrary units of fluorescence) in the striatum of AS control and AS/AGU mutant rats aged 12 months (n=6 per group). All values are mean arbitrary units ± SEM. All comparisons are paired t-tests (NS, not significant).

2.10 Discussion

2.10.1 Tyrosine hydroxylase

The synthesis and the release of dopamine are modulated by known mechanisms including presynaptic and dendritic autoreceptors **(Nowycky and Roth, 1978; Santiago and Westerink, 1991)**, changes in the firing rate of dopamine neurons **(Farnebo and Hamberger, 1971)** and the feedback inhibition of TH by the end product **(Nagatsu *et al*., 1964 ; Lovenberg and Victor, 1974; Kumer and Vrana, 1996)**.

The above should be borne in mind when considering the results found in the present experiment for TH immunofluorescence in individual cells of the substantia nigra. These neurons are known to release very little dopamine in the striatum – with extracellular levels only 10-20 % of normal **(Campbell *et al*., 1996)**. However, there has never been any evidence that they lack the ability to synthesise dopamine - in fact whole tissue micropunches of the midbrain and striatum have shown that dopamine levels remain normal until six months or more **(Campbell *et al*., 1997)**. Here, I can now demonstrate that TH levels in the cell bodies of the SNC are actually elevated. This is what might be expected of a relatively normal neuron where the primary deficit was in synaptic release.

In the central part of the SNC, TH levels were higher in the lateral region than in the medial. Although it is unclear why this is so, it is of interest that **Goto *et al* (1989)** and **Suttoo *et al* (2001)** found the greatest loss of dopaminergic neurons in the lateral SNC region of Parkinson's patients that projects to the dorsal putamen **(Gibb and Lees, 1991; 1994)**. TH levels per volume of cell were higher in the rostral, central and

caudal. By contrast to the SNC, the SNL and VTA **(see chapter1 page 8)** show little change in TH levels with age in either groups. In the first description of the AS/AGU rat the SNL and VTA were not as severely affected as the neighbouring SNC **(Clarke and Payne, 1994)** and the VTA is also less affected in human PD **(Uhl *et al*., 1985)**.

Unlike the SNC, TH levels are considerably reduced in the dorsal caudate-putamen of the AS/AGU mutant rats compared to the controls. This where the dopaminergic terminals (rather than the cell bodies) are located. Whole tissue dopamine levels in the dorsal and lateral caudate-putamen are known to be reduced in the AS/AGU mutant rat compared to the AS control between 6 and 12 months of age using high performance liquid chromatography with electrochemical detection of micropunch samples **(Campbell *et al*., 1996; 1998)**. Similar effects reductions of dopamine levels in the dorsal striatum have been seen in the weaver mouse **(Roffler-Tarlov and Graybiel, 1984)**, in post-mortem Parkinson's disease patients **(Hornykiewicz, 1995)** and in living patients with the disorder **(Leenders *et al*., 1986,1990)**. MPTP exposure **(Moratalla *et al*., 1992; Snow *et al*., 2000)** can also greatly reduce striatal dopamine **(Campbell *et al*., 1998, 2000)**.

One possible conclusion from these results is that, whilst the terminals are clearly dysfunctional, the cell bodies of DA neurons are not only normal at these ages, but capable of normal physiological responses to depleted DA release.

2.10.2 Serotonin

Serotonin appears to mimic dopamine. Thus, 5-HT levels in individual cells of the dorsal raphe nucleus are elevated in the AS/AGU mutant compared to the AS control - even though 5-HT release in the striatum is known to be greatly reduced **(Al-Fayez *et al*., 2005)**. Again, this suggests that the neuron remains relatively normal in terms of its synthesising capacity and, perhaps, its feedback responses.

It is interesting that the dorsal raphe nucleus (which projects to the striatum) is affected in the AS/AGU mutant, but the median raphe nucleus (which does not) is not. The effects include not only the reduced ability to release 5-HT in the striatum under normal physiological conditions, but also the loss of serotonergic cell bodies in the dorsal raphe with age **(Al-Fayez *et al*., 2005)**.

Many studies have described a reduction in the levels of serotonin in several brain regions of Parkinson's disease patients **(Rinne *et al*., 1974; Scatton *et al*., 1983; Agid *et al*., 1987)** and lesions of the DRN lead to a decrease in serotonin levels in the striatum whereas lesions of MRN do not **(Van der Kar and Lorens, 1979)**. The AS (control) rats have reduced 5-HT levels with age in the dorsal raphe nucleus and, again, it is known that old rats show lower levels of serotonin and dopamine **(Goicoechea *et al*., 1997)**.

2.10.3 Lewy body proteins

Dysfunction of the ubiquitin-proteasome system (UPS) has been implicated in Parkinson's disease and other neurodegenerative disorders and ubiquitin (Ub) is a major component of inclusions such as Lewy bodies in PD **(Gai et al., 2000; McNaught *et al*., 2002)**. In the present study Ub, parkin and α-synuclein levels were increased in SNC cell bodies or striatal neuropil with age in both controls and mutants, but more in the mutant. In particular, the lateral half of the central SNC showed increasing levels of Ub and parkin with age compared to the medial half in the mutants. It is of interest that the lateral half of the SNC is also more affected in rats following treatment with proteasome inhibitors **(McNaught *et al*., 2004)**. The levels of Ub, parkin and α-synuclein do not change in the SNR, SNL and the VTA in the mutant rats. Again, rats treated with proteasome inhibitors demonstrate a lack of neurodegeneration in the SNR **(McNaught *et al*., 2004)**, and the VTA less is affected

in human PD **(Uhl *et al*., 1985)**. The levels of Ub and parkin also increase with age in AS/AGU mutant rats compared to AS controls in the dorsal raphe nucleus; however, there is no change in the median raphe nucleus, suggesting that the raphe-striatal serotonergic system is affected differentially. Despite this, levels of Ub, parkin and α-synuclein in the striatum were not changed with age, nor did they differ between the two strains. However, it must be remembered that the technique samples a mixture of cells and terminals so that a change in one population might be masked. The dorsal raphe nucleus and the caudal end of the SNC showed higher ubiquitin and parkin levels compared to other areas in the midbrain of AS/AGU mutant rats, perhaps indicating that these areas are especially affected. Neuronal death is more severe in lower regions of the midbrain and the rate increases with age **(Braak *et al*., 2003)**.

Elevations in molecules such as ubiquitin, parkin and alpha-synuclein in mutants (and with age) are difficult to interpret since the normal function of some of these molecules is poorly understood **(see chapter 1 page 38, 44, 45) (Clayton and George, 1998)**. The comparison here must be with neurodegenerative states, such as Parkinson's disease, where levels are elevated and the proteins incorporated into cell inclusions **(Lennox *et al*.,1989; Love and Nicoll, 1992; Spillantini *et al*., 1997;1998a; Irizary *et al*., 1998)**. Such inclusions have not been found in rodents - except under special treatments such as rotenone **(Betarbet *et al*., 2000)** - so it is uncertain whether the elevations found in present study suggest a pre-inclusion state. This will be one of the features looked for in the second experiment.

The first experiment shows that biogenic amine activity (DA and 5-HT) is lowered in the striatum, but that the midbrain cell bodies remain synthetically active. In addition, molecules associated with human Lewy bodies (ubiquitin, parkin and α-synuclein) are elevated in the mutant - even though inclusion bodies as such do not occur.

CHAPTER 3

EXPERIMENT 2:

Stereology of dopaminergic neurons in the SNC of the AS and AS/AGU rats using light and transmission electron microscopy

3.1 Introduction

The substantia nigra is implicated in Parkinson's disease through degeneration of its dopaminergic neurons **(Hornykiewicz and Kish, 1987; Lang and Lozano, 1998a; Hague *et al*., 2005; Jenner and Olanow, 2006)**. The cell bodies in the substantia nigra pars compacta can be classified chemically (dopaminergic or nondopaminergic) or morphologically (medium or small) **(Fallon and Loughlin, 1985)**. The dopaminergic neurons project to the striatum **(Chiodo, 1988; Parent and Hazrati, 1994; 1995a; Blandini *et al*., 2000; Crossman, 2000; DeLong and Wichmann, 2007)**.

The first description of the substantia nigra was made by **Vicqd'Azyr in 1786** and **Soemmering in 1791 (Hajdu et al., 1973)**. Many studies have described the population of cells in the substantia nigra according to their appearance using the light microscope **(Cajal, 1904; Rioch, 1929; Taber, 1961; Hanaway *et al*., 1970; Fallon and Loughlin, 1985; 1995)**. The first description of the ultrastructure of the substantia nigra using electron microscopy was carried out in the mouse by **Bak (1967)**, who examined different forms of pharmacological treatment on the substantia nigra and caudate nucleus. He described a single type of neuron (15 μm diameter) and characterized it as electron lucent. Shortly afterward **Hirosawa (1968)** and **Schwyn and Fox (1969)** also described a single cell type in the substantia nigra of monkeys with a diameter of 30-40 μm and a granular reticulum distributed in the periphery of the cytoplasm; in the cat, a single neuron type was described in both the substantia nigra pars compacta and reticulata, 15-50 μm in diameter and with a prominent rough endoplasmic reticulum **(Rinvik and Grofova, 1970)**. By contrast, three distinct

neurons were described in the rat substantia nigra, large neurons (25-40μm) with well developed RER in the substantia nigra pars reticularis, medium neurons (15-20μm) with an eccentric nucleus in the pars compacta and small neurons (10-12μm) in both nigral regions **(Gulley and Wood, 1971)**. In another rat study **Hajdu *et al*. (1973)** described two different types of neuron in the substantia nigra, including a large neuron up to 40μm in a diameter with well developed RER and a small neuron with pale cytoplasm. **D'Agostino and Luse, (1964)** described the ultrastructure of pigmented cells in human substantia nigra.

The death of dopaminergic neurons in the substantia nigra of PD patients can potentially occur in three main ways:

i. **Necrosis** is characterized by excessive ionic flux through the plasma membrane leading to swelling of cellular organelles and rupture of the outer membrane **(Jenner and Olanow., 1998; Murphy *et al*., 1999; Sapolsky, 2001)**.

ii. **Apoptosis**, or programmed cell death, is characterised by chromatin condensation, nuclear fragments and cytoplasmic shrinkage **(Kerr *et al*., 1972; Mochizuki *et al*., 1996; Anglade *et al*., 1997; Tompkins *et al*., 1997; Burke and Kholodilov, 1998; Tatton, 2000; Andersen, 2001; Jellinger, 2001; Tatton *et al*., 2003)**.

iii. **Autophagy** is characterised by nuclear chromatin condensation, numerous vacuoles in the cytoplasm, moderate vacuolation of endoplasmic reticulum; mitochondria remain intact **(Anglade *et al*., 1997)**.

Changes in ultrastructure in the substantia nigra have been studied in animal models of Parkinson's disease such as MPTP. A study by **Cochiolo *et al*. (2000)** showed

marked mitochondrial swelling in SNC neurons with deformation, disruption and disintegration of cristae, but no changes in other organelles in the SNC. By contrast, **Mizukawa *et al*. (1990)** found dilated RER and Golgi apparatus, and a decrease in the number of ribosomes in their RER as well as disarranged mitochondrial cristae. After 6-OHDA administration, the SNC showed round, homogeneous, electron-dense chromatin clumps in the nucleus and nuclear membrane invagination, but other organelles remained intact **(Marti *et al*., 2002)**.

It is difficult to establish whether such changes in cell structure are due to necrosis or apoptosis. One possible is that some insults can induce both necrosis and apoptosis (and could be responsible for neuronal loss in either PD or animal models) such as exposure to high glutamate concentration and ATP depletion or lipid and protein peroxidation induced by reactive oxygen species and depletion of glutathione **(Dawson and Dawson, 1996; Jenner, 2001; Sapolsky, 2001; Higuchi, 2004)**. A second possible is that improve methods of differentiating of apoptosis and necrosis suggest that both modes of cell death could be found in the same cell **(Proskuryakov *et al*., 2003; Wei *et al*., 2004)**.

Cell death involving apoptosis and necrosis can be detected by different techniques such as ISEL (e.g. TUNEL) or fluorescent DNA binding dyes, and electron microscopy **(Olanow and Tatton, 1999)** as well as by conventional gel electrophoresis **(Smyth and Berman, 2002)**. A few studies using TUNEL techniques have failed to detect apoptosis in Parkinson's disease patients **(e.g. Dragunow *et al*., 1995; Kosel *et al*., 1997; Wullner *et al*., 1999)**; nevertheless electron microscopy has demonstrated many features of this form of cell death **(Baba *et al*., 1994; Oztas and Topal, 2003)**, and a combination of TUNEL method and morphological identification

(e.g. electron microscopy) has been considered quite an effective way to detect apoptosis **(He *et al*., 2000)**.

Death by apoptosis has also been reported in other neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD) and Multiple system atrophy (MSA) **(Jellinger, 2000)** by necrosis in AD **(Akiyama *et al*., 2000)** and Creutzfeldt-Jakob disease **(Ferrer, 1999)** and by autophagy in AD **(Cataldo *et al*., 1994)** and Huntington's disease **(Roizin *et al*., 1974)**.

The death of dopaminergic neurons in animal models of PD can occur by (i) necrosis (such as MPP^+-induced necrosis in a mesencephalon-derived dopaminergic neuronal cell line) **(Choi *et al*., 1999)** (ii) apoptosis from agents such as MPTP **(Tatton and Kish, 1997; Spooren *et al*., 1998; Serra *et al*., 2002)**, 6-OHDA **(He *et al*., 2000; Marti *et al*., 2002)** and Rotenone and MPP^+ (in cultures of rat mesencephalic neurons) **(Lim *et al*., 2007)** and (iii) autophagy from agents such as MPTP **(Oztas and Topal, 2003)**.

The aim of **experiment 2** is therefore to look at cell bodies in the SNC to see if there are difference between the AS (control) and AS/AGU (mutant) rats and look for any signs of cell death using TEM.

3.2 Materials and Methods

3.2.1 Animals

Three AS control and 3 AS/AGU mutant male rats aged 12 months were used for TEM.

Five AS control and 5 AS/AGU mutant male rats aged 12 months were used for light microscopy.

3.2.2 Initial tissue preparation

3.2.2.1 for TEM

All rats were deeply anaesthetized with an overdose of sodium pentobarbitone B.P (Vet.), (2 ml of 60 mg/ml, Rhone-Merieux, Spire Greencentre, Harlow, Essex) injected intraperitoneally, and then the thoracic cavity was opened. A mammalian Ringer solution (200 ml) containing the vasodilator Lignocaine was injected into the left ventricle for one minute followed by 500 ml 3% glutaraldehyde (Agar- Aldrich Inc, P6148) in 0.1M phosphate buffer, blood and excess fluid being drained via an incision through the right atrium **(Appendix 1)**. The brain was dissected out and immersion-fixed in 3% glutaraldehyde in 0.1M of phosphate buffer overnight. Pieces of brain containing areas of interest were serially sectioned at 70µm using a Vibrotome (Agar Scientific, BNBA 010664, Agar Scientific LTD, UK).

3.2.2.2 for light microscopy

All rats were deeply anaesthetized with an overdose of sodium pentobarbitone B.P (Vet.), (2 ml of 60 mg/ml, Rhone-Merieux, Spire Greencentre, Harlow, Essex)

injected intraperitoneally, and then the thoracic cavity was opened. One hundred ml of mammalian Ringer solution containing the vasodilator Lignocaine was injected into the left ventricle followed by 500ml 4% paraformaldehyde (Sigma- Aldrich Inc, P6148) in 0.1M phosphate buffer, excess fluid being drained via an incision through the right atrium **(Appendix 1)**. The brains were dissected out and immersion-fixed in 4% paraformaldehyde in 0.1M of phosphate buffer overnight. Pieces of brain containing areas of interest were dehydrated through an ascending ethanol series using a Histokinette 2000 automatic tissue processor (Reichert-Jung, Germany) then embedded in paraffin wax at 57ºC and serially sectioned at 7µm using a Microtome (Spencer, 820, USA) **(Appendix 2)**. The ribbons of sections were laid out in parallel rows on a tray.

3.2.3 Tissue verification

In order to confirm the identity of the area from which semi-thin and ultra-thin sections would eventually be analysed, toluidine blue and anti-TH immunocytochemistry with DAB staining were carried out.

3.2.3.1 Toluidine blue staining

Sections from the central SNC (*Approximately -5.3mm relative to bregma*) **(Paxinos and Watson, 1982)** from AS and AS/AGU rats were stained with 1% aqueous toluidine blue (R.ALAMB, UK) **(Appendix 3A)**. Slides were then examined under a light microscope.

3.2.3.2 Immunoperoxidase staining

The sections containing the central SNC (*Approximately -5.3mm relative to bregma*) **(Paxinos and Watson, 1982)** from the AS (control) and the AS/AGU (mutant) rats

were incubated for 24 h in a humidity chamber at 4ºC with the primary antibody (Monoclonal mouse anti-tyrosine hydroxylase, MAB 5280, Chemicon Europe Ltd (1: 1000) diluted in blocking serum (1% NGS in PBS with 0.3% Triton X- 100). The rest of the procedure was carried out at room temperature. After rinsing in PBS (3x5 mins), sections were then incubated for 60 min in a biotinylated anti-mouse secondary antibody (1:200 dilution), (Vector Laboratories, INC, BA-2001, Peterborough UK). Following 60 min incubation in Avidin-Biotin-Complex (ABC) reagent (1:50; 20µl of solution A and 20µl of solution B in 1ml of PBS; Vectastain ABC kit, Vector Laboratories, INC, PK-6100, Peterborough UK), sections were then rinsed in PBS (3x5 mins) and 0.1 M phosphate buffer (PB, 1x5 mins). The location of the antigen-antibody complex was visualized by incubating sections in a medium containing 0.05% 3,3-diaminobenzidine (DAB substrate KIT for peroxidase, Vector Laboratories, INC, SK-4100, Peterborough UK) for 2-5 min; this step was carried out with care in a fume cupboard, and all equipment which came in contact with DAB was soaked with bleach in order to denature it, as DAB is potentially carcinogenic (International Agency for Research on Cancer, 1972). Finally, the sections were rinsed in distilled water, dehydrated and mounted with glass coverslips using histomount (RALAMB, HS-103, UK). Slides were then examined under a light microscope.

3.2.4 Electron microscopy

Once the area of the SNC had been confirmed with toluidine blue and anti-TH staining (see above). Sections containing the SNC from AS (control) and the AS/AGU (mutant) rats were rinsed with PB and placed in a solution of 1% Osmium tetroxide in PB for 20 minutes in an agitator. The sections were rinsed with distilled water (3x30 mins) and dehydrated through a series of graded concentrations of

acetone from 70 to 100%, followed by a descending ratio of acetone to durcupan resin (3:1, 1:1, 1:3) and two changes of durcupan resin. The sections were flat-embedded in durcupan resin between two small sheets of acetate, sandwiched between two glass slides, weighted down with metal weights and heated at 60ºC overnight in an oven **(Appendix 6)**. The top acetate sheet were peeled off, stock embedded sections attached onto the end of a blank embedding block using RS adhesive and left for at least 30 min in an oven. The block containing the area of interest was trimmed.

3.2.4.1 Semi-thin sections processing and staining

Semi-thin sections (1µm) containing the SNC from the AS (control) and AS/AGU (mutant) rats were cut from the block using diamond knives (Drukker International, Netherlands) on a Reichert-Jung Ultracut E ultramicrotome. The semi-thin sections were stained with 1% Toluidine Blue buffered to pH 8.5 with sodium borate and examined under the light microscope to determine the area to be thin sectioned and to be used in stereology.

3.2.4.2 Ultrathin sections processing and staining

Ultrathin sections (80-90nm thickness) from the SNC of AS (control) and AS/AGU (mutant) rats were cut from selected blocks using diamond knives (Drukker International, Netherlands) on a Reichert-Jung Ultracut E ultramicrotome. Ultrathin sections were collected on 300 mesh-coated copper grids (300 Mesh Thin Bar Copper 3.05mm, G2720C, Agar Scientific, UK) and double stained with uranyl acetate and lead citrate **(Reynolds, 1963)**. The grids containing sections were stained in lead citrate for 5 mins, placed in sodium hydroxide 7 times, washed in distilled water 7 times, stained in 12.5% methanolic uranyl acetate for 5 mins, washed in distilled

water 7 times, stained in lead citrate for 5 mins, placed in sodium hydroxide 7 times , washed in distilled water 7 times and left to dry for 20 mins.

Ultrathin sections were examined at a magnification of 5900X using a transmission electron microscope (JEOL JEM-100S, No. IEM 100S-4, JEOL LTD, Tokyo, Japan). All the SNC cells containing a nucleolus were photographed. This organell was used to confirm that the section is centrally placed through the cell body **(Oorschot, 1996)**.

3.2.4.3 Pre-embedding immunocytochemistry

See **chapter 4 page 163**.

Cells are often considered positive (and immunogold labelling specific) when two or more particles are located within the cytoplasm **(Mengual and Pickel, 2002)**. In the present study, I have used five or more as a criterion.

3.2.5 Image analysis

Images were captured on plate film. The films were put in a working dilution of 500ml D19 Kodak developer (Kodak-path, Paris) in 500ml of water for 3 mins, and then were rinsed in water for one minute before placing in Amfix (Amfix, Champion, UK) for 5 mins, rinsed in water for 20 mins and dried. The negatives of electron micrographs were scanned using an Epson scanner (Epson perfection 4990), and the contrast and exposure were adjusted using Paint Shop before printing.

3.2.6 Stereological techniques

Stereological methods are used to obtain quantitative information about three-dimensional structures from simple counts made on two-dimensional slice images to facilitate correlation between structure and function.

3.2.6.1 Volume fraction (Vv)

The stereological method was developed for use on geological specimens by **Delesse** in **1848**, who indicated that the volume fraction of mineral in the whole rock was equal to the area fraction of mineral on the cut surface of a sample of the rock. This confirmed that the area of the phase of interest per unit of area is equivalent to the volume of that phase per unit of volume.

Aa (area fraction) = Vv (volume fraction)

$$\mathbf{A_{phase} / A_{ref} = V_{phase} / V_{ref}}$$

Thomson in **1930** and **Glagolev** in **1933** showed that we could estimate volume fraction through a random point counting method. The volume fraction **Vv** and area fraction **Aa** can be estimated in an unbiased manner by using a randomly positioned point grid and counting the number of points hitting the phase of interest **P (phase)** and the number of points hitting the reference space **P (ref)**, the volume fraction of the phase of interest being estimated from

Vv = Aa = P (phase) / P (ref)

The volume of a particular phase V(phase) can be estimated from

V(phase) = V(ref) x Vv (phase, ref)

The volume reference **V(ref)** can be calcaulated by the Cavalieri principle.

3.2.6.2 Surface density (S_V)

Surface density **(S_V)** represents the amount of surface contained in a reference volume and it can be estimated by dividing the number of intersections **(I)** by the total length of test line **(L_T)**

$$\mathbf{S_V = 2 \times I / L_T}$$

3.2.6.3 Volume estimates

The volume (V) of any arbitrary object can be estimated using the Cavalieri principle **(Mayhew, 1991; 1992)**. The object has to be serially sectioned at known distance (d) where the sectiones are parallel and the first slice must be randomly placed. The areas of only one face of each section are estimated by randomly superimposing a systematic array of test points on each face.

The volume can be estimated using:

$$V = P \times a \times d$$

Where **(P)** is the sum of points falling on all section faces, **(a)** the area associated with each test point and **(d)** the distance between the sections **(Mayhew, 1991; 1992)**.

Other stereological methods can estimate volume-weight mean volume which provides an unbiased estimate of particle volume **(Mayhew, 1992)**.

The volume-weight mean volume can be estimated from

$$\text{Volume-weight mean volume} = (I_0)^3 \times \pi/3$$

Where I_0 is the point-sampled intercept length average.

3.2.6.4 Stereology using the light microscope

This method was used to estimate the diameter (maximum and minimum) of the dopaminergic neurons in the SNC of 3 AS (control) and 3 AS/AGU (mutant) rats.

One section (7 μm thick) per animal from the central SNC (*Approximately -5.3mm relative to bregma*) **(Paxinos and Watson, 1982)**.

Sections were stained with:

(a) 1% aqueous toluidine blue to analyse at least 50 cells per section **(Figure 3.1)**.

(b) Immunoperoxidase for tyrosine hydroxylase to analyse at least 50 cells per section **(Figure 3.2)**.

The sections were analysed using a NIKON light microscope with a drawing tube (NIKON, OPTIPHOT-2, Japan). Average sizes of dopaminergic neurons containing a nucleolus were calculated.

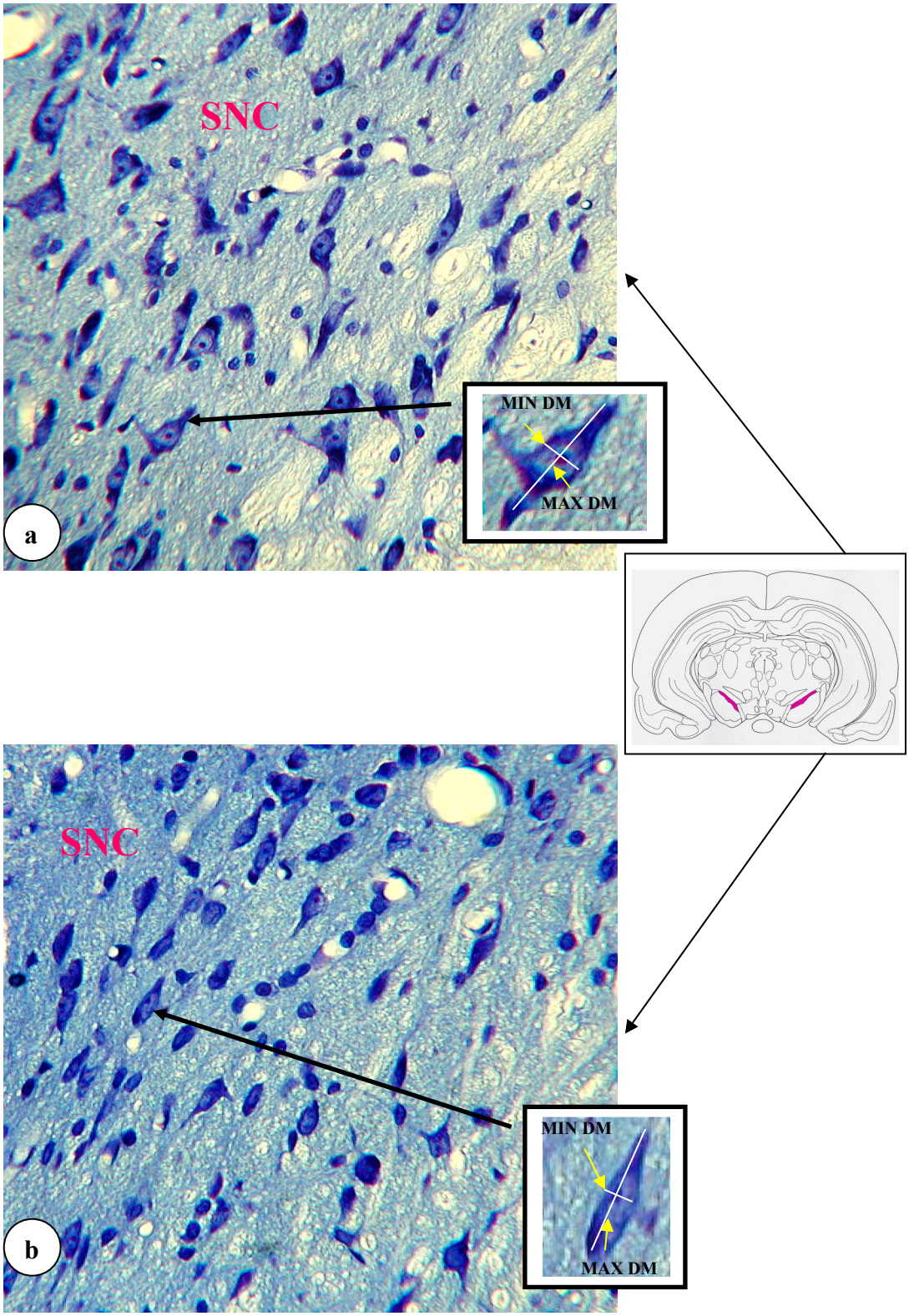


Figure 3.1: **Coronal sections of substantia nigra pars compacta (SNC) from (a) control (AS) and (b) mutant (AS/AGU) male rats aged 12 months. Sections have been stained with toluidine blue. In the inset box, lines show a maximum diameter (MAX DM) and a minimum diameter (MIN DM) (x400).**

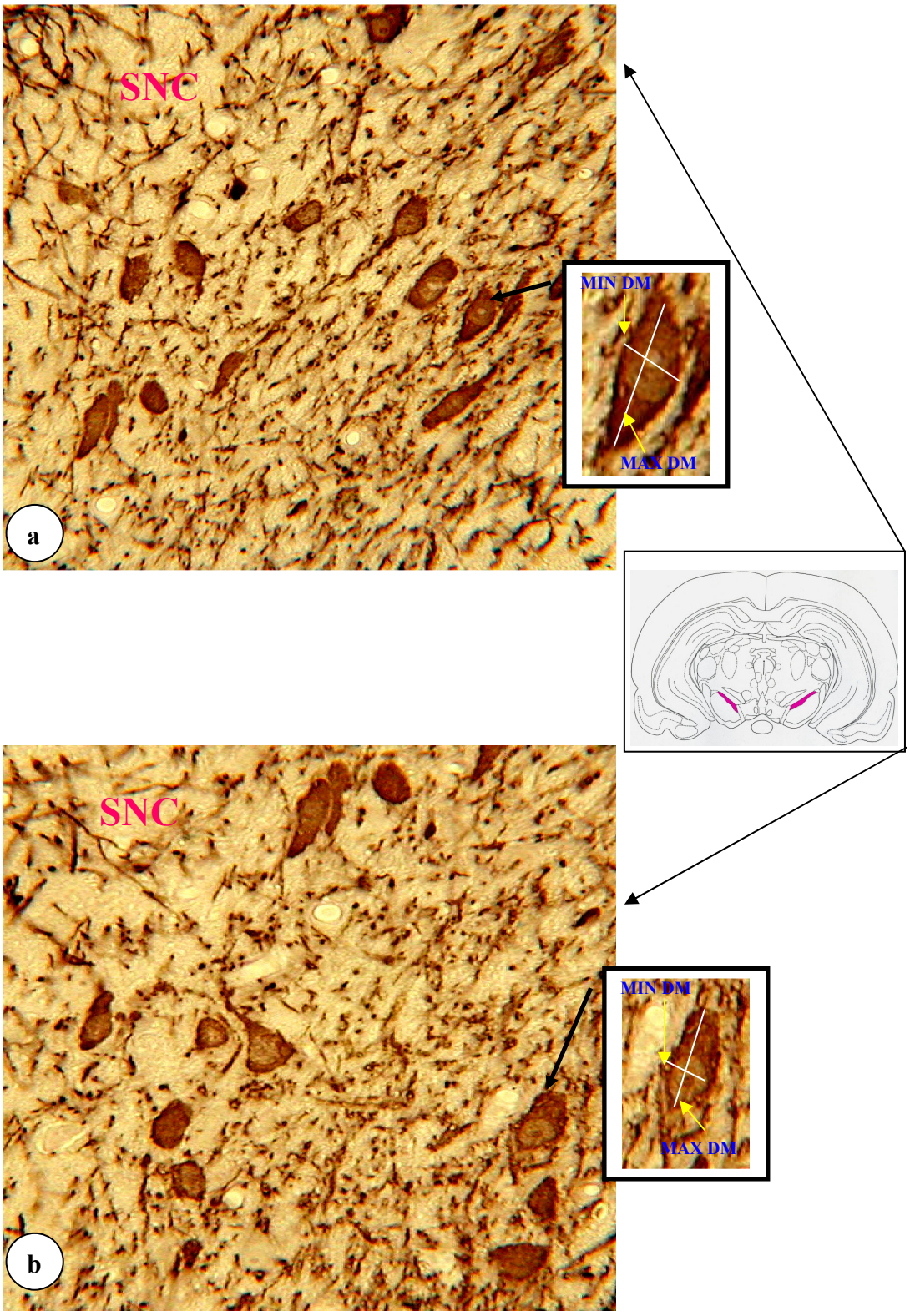


Figure 3.2: **Coronal sections of substantia nigra pars compacta (SNC) from (a) control (AS) and (b) mutant (AS/AGU) male rats aged 12 months. Sections have been DAB immunostained for TH. In the inset box, lines show a maximum diameter (MAX DM) and a minimum diameter (MIN DM) (x400).**

3.2.6.5 Stereology using the transmission electron microscope

In this stereological method, a simple point counting technique was used to estimate the **Vv** of mitochondria, rough endoplasmic reticulum and lipofuscin granules. The measurement was made on 30 SNC dopaminergic cells per animal from 3 AS (control) and 3 AS/AGU (mutant) rats.

The **Vv** was determined using a 1cm square grid. The square grid was superimposed on each micrograph randomly three times. The number of grid points that fell on the cell organelle (mitochondria, RER, lipofuscin granules) and the number of grid points that fell on the reference space (cell and cytoplasm) were counted **(Weibel, 1979; Mayhew, 1991)** and the **Vv** were calculated.

3.2.7 Statistical analysis

The results were analysed by two-sample t-test using the Minitab statistics package (MINITAB Release 13.30).

3.3 Results

3.3.1 The diameter of SNC cells and nuclei in paraffin wax sections stained with toluidine blue in the midbrain of AS and AS/AGU rats aged 12 months

The results show statistically significant differences between the two groups in the diameter (maximum and minimum) of the SNC cells and their nuclei with AS/AGU (mutant) rats having smaller neurons than the AS (control) animals. The results are summarized in **table 3.1**.

SNC cells	**AS**	**AS/AGU**	**P**
Maximum dimension of cell body (µm)	16.76 ± 0.64	11.76 ± 1.1	**< 0.01**
Minimum dimension of cell body (µm)	6.99 ± 0.24	5.06 ± 0.44	**< 0.01**
Maximum dimension of nucleus (µm)	5.74 ± 0.31	4.30 ± 0.05	**< 0.05**
Minimum dimension of nucleus (µm)	5.56 ± .0032	4.15 ± 0.04	**< 0.001**

Table 3.1: The diameter (maximum and minimum) of the SNC cells and their nuclei stained with toluidine blue in the midbrain of AS control and AS/AGU mutant rats aged 12 months (n=5 per group). All values are mean µm ± SEM. All comparisons are two-sample t-tests.

`

3.3.2 The diameter of SNC cells and nuclei in paraffin wax sections stained with immunoperoxidase DAB staining for TH in the midbrain of AS and AS/AGU rats aged 12 months

The results show statistically significant differences between the two groups in minimum cell body diameter and maximum nucleus diameter of the SNC cells with AS/AGU (mutant) rats being smaller than the AS (control). Differences in maximum cell diameter and minimum nucleus diameter did not reach significant. The results are summarized in **table 3.2**.

SNC cells	**AS**	**AS/AGU**	**P**
Maximum dimension of cell body (µm)	16.88 ± 0.99	14.27 ± 0.26	**NS**
Minimum dimension of cell body (µm)	7.26 ± 0.42	5.59 ± 0.13	**< 0.05**
Maximum dimension of nucleus (µm)	6.77 ± 0.46	5.32 ± 0.21	**< 0.05**
Minimum dimension of nucleus (µm)	5.98 ± 0.27	5.23 ± 0.19	**NS**

Table 3.2: The diameter (maximum and minimum) of the SNC cells and nuclei stained with immunoperoxidase DAB staining for TH in the midbrain of AS control and AS/AGU mutant rats aged 12 months (n=5 per group). All values are mean µm ± SEM. All comparisons are two-sample t-tests (NS, not significant).

3.3.3 A Comparison of ultrastructure of the SNC cells of AS (control) and AS/AGU (mutant) rats aged 12 months

No obvious morphological differences were seen in the nigrostriatal neurons of AS (control) compared to AS/AGU (mutant) rats. Neurons were of medium size with rounded nuclei exhibiting one or more indented envelopes, many mitochondria and considerable rough endoplasmic reticulum, Golgi apparatus and lipofuscin granules **(see figure 3.3-3.6)**.

There were no obvious Lewy body inclusions. Many microglial cells were seen near SNC cells in AS/AGU rats **(see figure 3.6)**. In **figure 3.7** a cell in the SNC has cytoplasm shrinkage, cell membrane budding and chromatin condensation at the nuclear membrane.

Most SNC cells stained with immunogold have more than 5 gold particles (at least two gold particles in cytoplasm are considered positive) and most of these gold particles are near the RER.

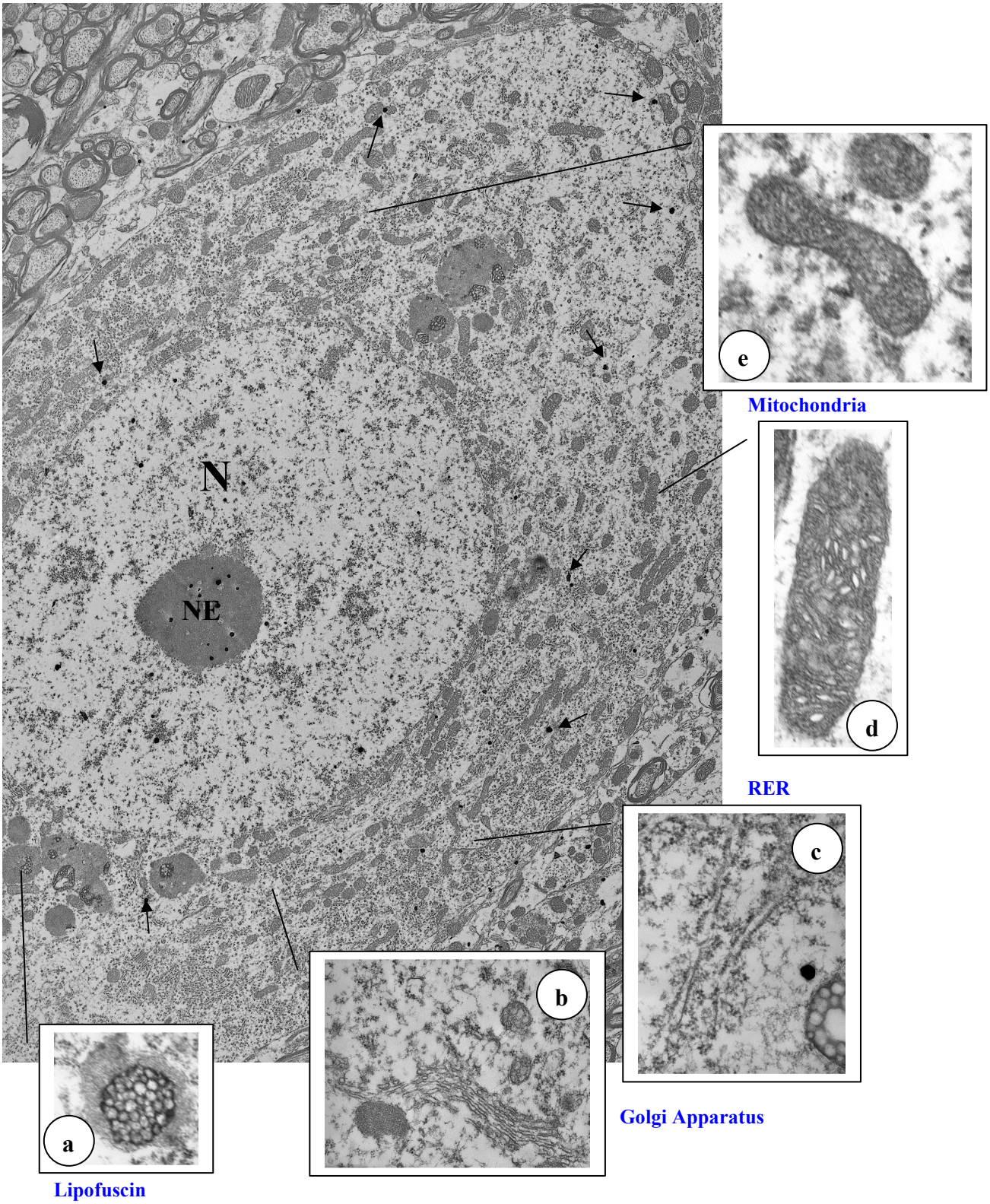


Figure 3.3: Electron micrograph showing SNC cell (immunogold stained with TH) in an AS (control) rat aged 12 months (x6000). In inset boxes (a) lipofuscin granule (b) Golgi Apparatus, (c) rough endoplasmic reticulum (RER) and (d and e) mitochondria. (N, Nucleus; NE, Nucleolus; small arrows show gold particles).

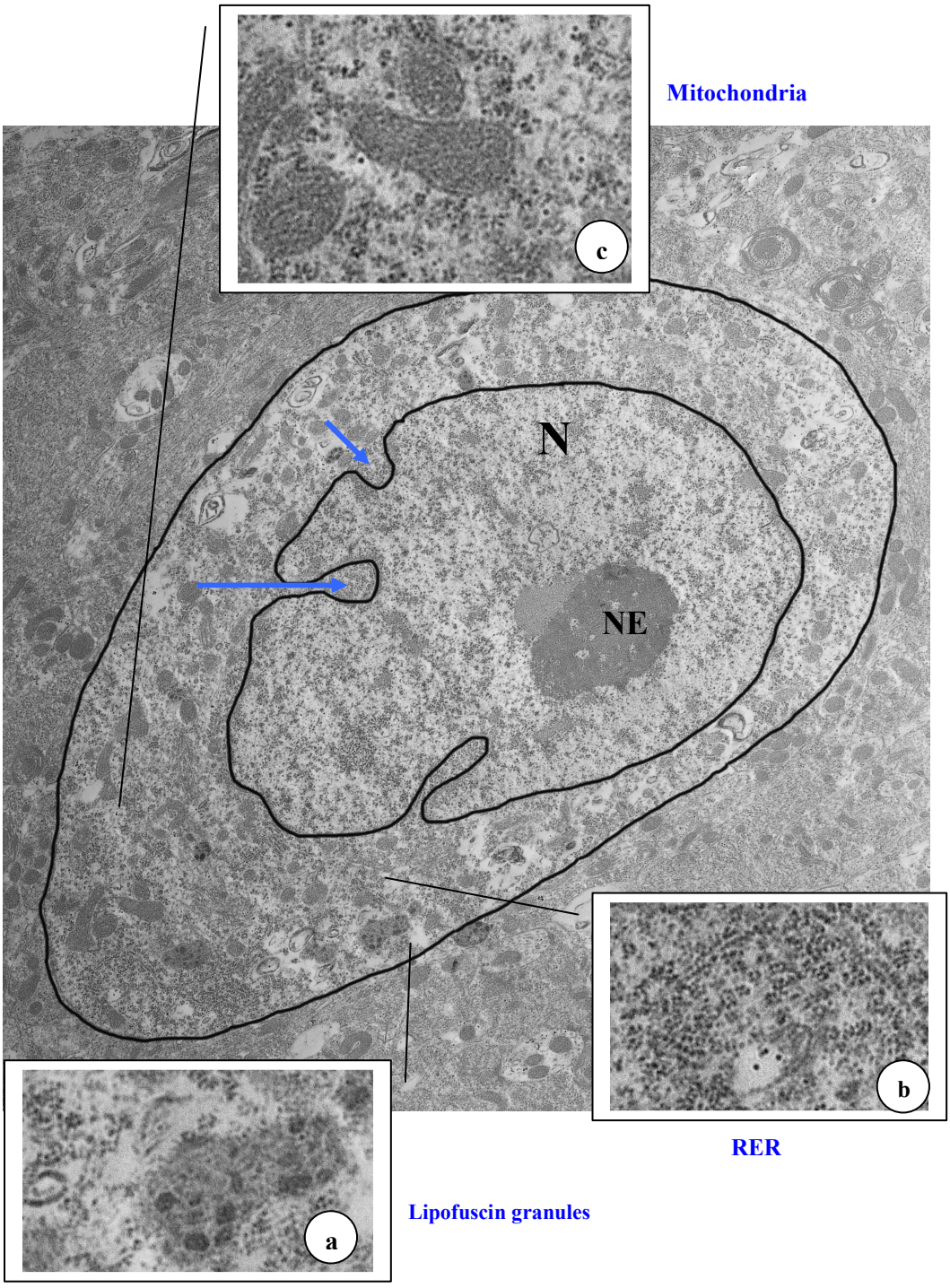


Figure 3.4: Electron micrograph showing SNC cell in an AS (control) rat aged 12 months (x5900). In inset boxes (a) Lipofuscin granules (b) RER and (c) mitochondria. (N, Nucleus; NE, Nucleolus; blue arrows show indented envelopes).

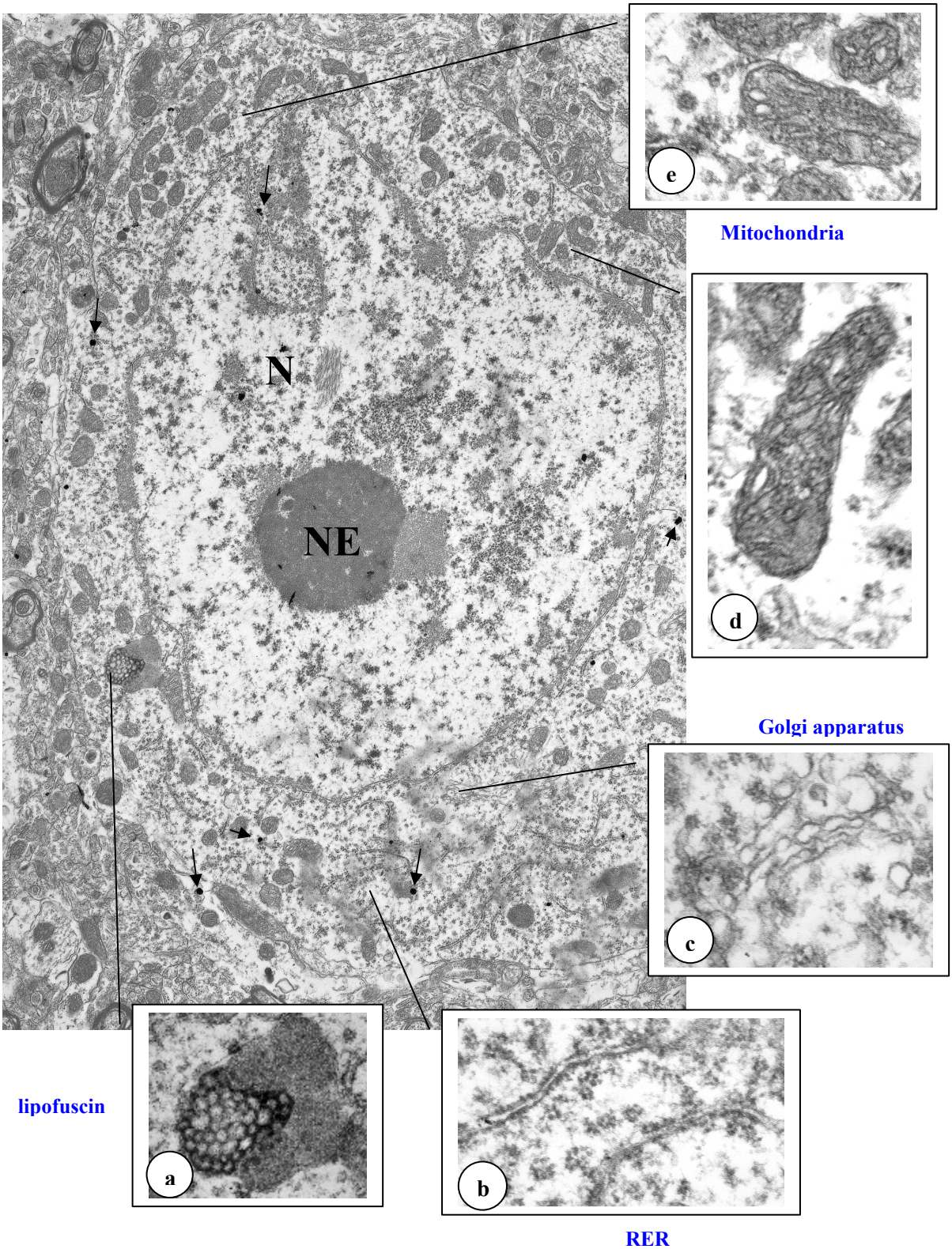


Figure 3.5: Electron micrograph showing SNC cell (immunogold stained with TH) in an AS/AGU (mutant) rat aged 12 months (x6000). In inset boxes (a) lipofuscin granule and (b) rough endoplasmic reticulum (RER) (c) Golgi apparatus and (d and e) mitochondria (x50,000). (N, Nucleus; NE, Nucleolus; small arrows show gold particles).

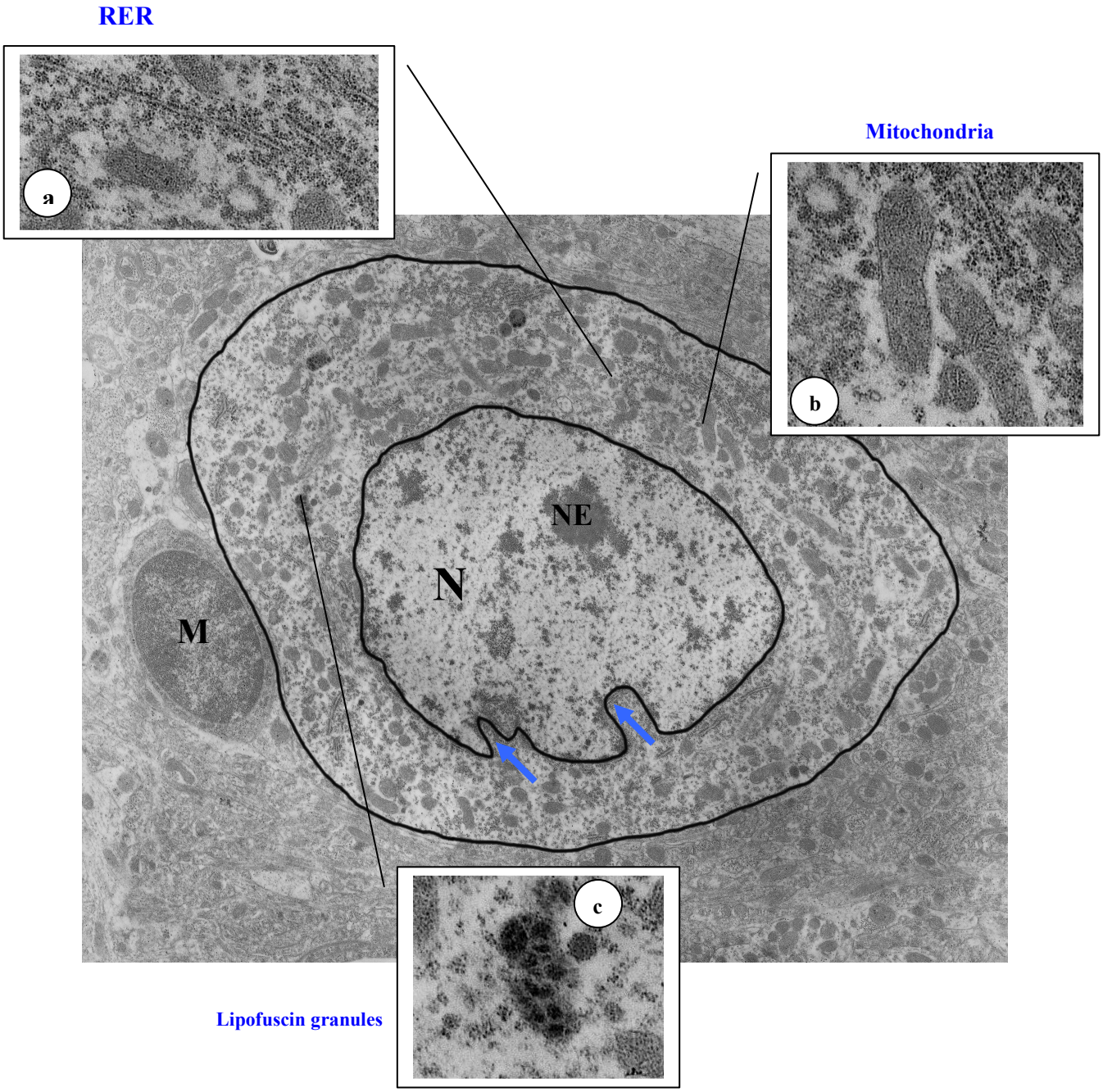


Figure 3.6: Electron micrograph showing SNC cell in an AS/AGU (mutant) rat aged 12 months (x5900). In inset boxes (a) RER (b) mitochondria and (c) Lipofuscin granules. (N, Nucleus; NE, Nucleolus; blue arrows show indented envelopes; M, microglial cell).

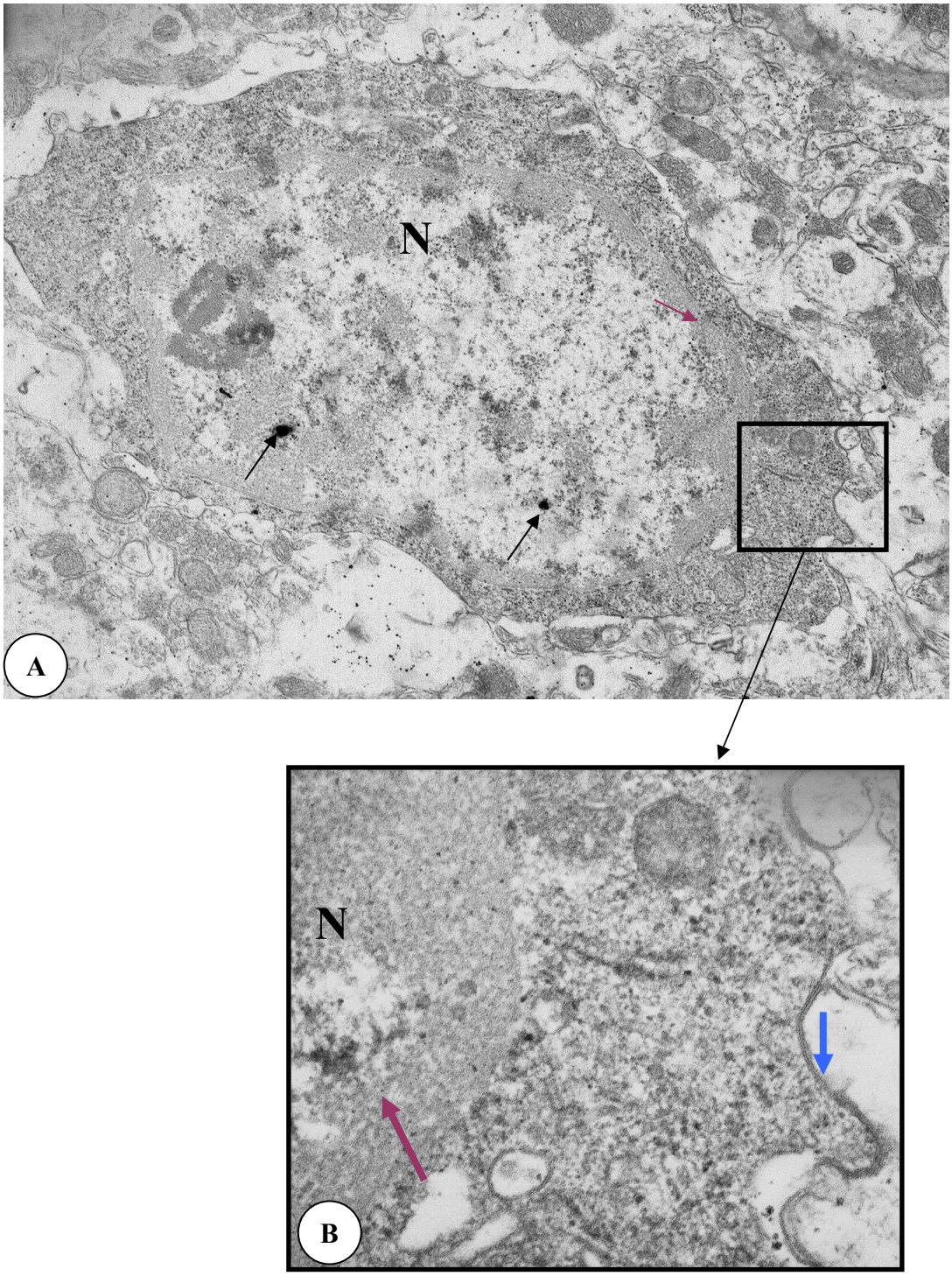


Figure 3.7: Electron micrograph (A) showing a shrunken SNC cell (immunogold stained with TH) in an AS/AGU (mutant) rat aged 12 months (x6000). (B) shows a detail of the same cell (x20,000). (N, Nucleus; small black arrows show gold particles).

→ Chromatin condensation at nuclear membrane.

→ Cell membrane budding.

3.3.4 The volume fraction (Vv), surface density (S_V) and volume (v) of SNC neuron organelles in the midbrain of AS and AS/AGU rats aged 12 months

The results show statistically significant differences between the two groups in the volume fraction of the mitochondria (in cell and cytoplasm) and the rough endoplasmic reticulum (in cell and cytoplasm), cell volume and the nuclear volume of SNC cells. The AS/AGU (mutant) rats had higher volume fractions, cell volume and nuclear volume than the AS (control). There were no statistical differences in the volume fraction of lipofuscin granules or the numbers of nuclear indentations and nuclear surface density. The results are summarized in **table 3.3**.

SNC cell organelles	AS	AS/AGU	P
Mitochondrial Vv: Cell	0.042 ± 0.002	0.051 ± 0.002	**< 0.05**
Mitochondrial Vv: Cytoplasm	0.0817 ± 0.001	0.089 ± 0.006	**< 0.05**
RER Vv: Cell	0.0247 ± 0.0003	0.0313 ± 0.0009	**< 0.05**
RER Vv: Cytoplasm	0.0463 ± 0.0023	0.0563 ± 0.011	**< 0.05**
Lipofuscin granules Vv: Cell	0.0067 ± 0.0003	0.017 ± 0.003	**NS**
Lipofuscin granules Vv: Cytoplasm	0.012 ± 0.001	0.031 ± 0.007	**NS**
Nuclear membrane S_V	0.09 ± 0.006	0.11 ± 0.01	**NS**
SNC cell volume (V)	664.1 ± 25	240.3 ± 22	**< 0.001**
SNC nuclear volume (V)	133 ± 7.3	53.13 ± 0.60	**< 0.001**

Table 3.3: Volume fraction (Vv), surface density (S_V) and volume (V) of the SNC cell and its organelles in the midbrain of AS control and AS/AGU mutant rats aged 12 months (n=3 per group). All values are mean ± SEM. All comparisons are two-sample t-tests (NS, not significant).

3.4 Discussion

The AS/AGU rat has a dysfunction of the midbrain monoaminergic systems projecting to the caudate-putamen, including the nigrostriatal dopaminergic system **(Clarke and Payne, 1994)** and the raphe striatal serotonergic system **(Al-Fayez *et al*., 2005)**. That dysfunction takes the form of a marked reduction in dopamine and serotonin release in the striatum **(Campbell *et al*., 1997; Al-Fayez *et al*., 2005)** as well as reduced whole tissue levels of dopamine and serotonin as revealed by micropunch **(Campbell *et al*., 1998; 2000; Al-Fayez *et al*., 2005)**. The dysfunction is known to occur before any gross morphological difference or cell loss can be identified **(Payne *et al*., 1998)**.

The animals examined in **Experiment 2** were aged 12 months. That is, they would be expected to display both physiological and morphological evidence of the disorder as this is in the beginning of the period of cell loss **(Payne *et al*., 2000)**. The movement disorder is, of course, also well established **(Payne *et al*., 2000)**.

The first point to make is that the ultrastructure of SNC neurons in AS animals resembled existing reports in other rat strains **(Gulley and Wood, 1971**; **Hajdu *et al*., 1973)** that have medium size neurons containing a round eccentric nucleus with slight nuclear indentations, and an abundance of cellular organelles including endoplasmic reticulum and Golgi apparatus.

Regarding possible pathological changes, no inclusion bodies can be seen in the AS/AGU (mutant) neurons, though these are a hallmark of Parkinson's disease **(Pollanen *et al*., 1993; Forno, 1996; Takahashi and Wakabayashi, 2001; Yokota *et al*., 2007)**. Inclusion bodies are not always found in neurodegenerative laboratory

models. For example, they do not occur in most laboratory models of Parkinson's disease such as those induced by 6-OH-dopamine or MPTP toxicity **(Forno *et al*., 1993; Dauer and Przedborski, 2003)**, though they are present in rotenone-induced degeneration in rat **(Betarbet *et al*., 2000)**.

Lewy bodies in human Parkinson's disease contain a complex mixture of mis-folded proteins-including α-synuclein, parkin and ubiquitin **(Lennox *et al*., 1989; Love and Nicoll, 1992; Spillantini *et al*., 1997; 1998a; Irizary *et al*., 1998; McNaught *et al*., 2006)**. It is of interest that, even though inclusion bodies were not seen in the AS/AGU rat, **Experiment 1** showed that levels of these molecules were elevated in cell bodies within the midbrain (or in the surrounding neuropil). There has been controversy over the role of some of these molecules in the pathological process **(McNaught and Olanow, 2006)**. Recently, **Periquet *et al*. (2007)** have shown that some truncated forms of α-synuclein lead reliably to aggregate formation whereas other do not. In the case of the AS/AGU rat, fluorescence readings show that α-synuclein is not present in the cell bodies in detectable amounts, so no inclusions would be expected. It is also possible that there could be changes in α-synuclein in the AS/AGU rat (and in other laboratory models) but they do not affect key regions of the molecule.

A robust finding is that the size and volume of nigrostriatal dopaminergic neuron cell bodies are reduced in AS/AGU mutant rats suggesting that they are shrinking. Similar observations were found in dopaminergic neurons in the SNC treated with MPTP **(Langston *et al*., 1984)** or 6-OHDA **(Chio *et al*., 1999)** as well as in the SNC of PD patients **(Anglade *et al*., 1997)**. The nucleus is also reduced in size and volume, suggesting that the whole cell is affected.

Regarding cell organelles, the volume fractions of mitochondria and rough endoplasmic reticulum of the SNC cells are higher in the AS/AGU mutants rats. There are many possible reasons for this observation:

i. The numbers of mitochondria and RER increase in AS/AGU (or decrease in AS) rats.

ii. The numbers of mitochondria and RER stay the same, but the organelles change their size. This would increase the chance of "hitting" a mitochondrion with a grid point. One possibility is that this is due to an increase in mitochondrial size as part of the swelling which occurs in the SNC neurons of other PD animal models as a characteristic feature of cell death **(Langston *et al*., 1984; Tanaka *et al*., 1988; Arai *et al*., 1990; Mizukawa *et al*., 1990; Rapisardi *et al*., 1990; Cochiolo *et al*., 2000)**.

iii. The number (and size) of mitochondria and RER stay the same, but the cell or its cytoplasm shrinks in AS/AGU rats or swells in AS ones. Again, this would increase the chance of "hitting" a mitochondrion and/or RER as they become packed together.

As mentioned above, the size of the SNC nuclei are greater in AS (control) than in AS/AGU (mutant) rats. However, nuclear indentation (a characteristic of pathological change) **(Anglade *et al*., 1997; Marti *et al*., 2002)** and the nuclear surface density (S_V) were not increased in the mutants nor was chromatin clumping seen, although it occurs in MPTP treatment **(Tanaka *et al*., 1988)**.

The nucleolus of both AS and AS/AGU TH+ve cells in the SNC frequently showed a few gold particles. We know that the nucleolus has a role in ribosomal RNA synthesis, processing and ribosome maturation **(Gerbi et al., 2003)**. It may also have

a role in TH regulation of gene expression **(Kumer and Vrana, 1996)**. In addition to that there is positive staining of the nucleolus in 6-OHDA-treated rats used silver impregnation methods **(Jeon *et al*., 1995)**.

Many microglial cells were seen near SNC cells in AS/AGU rats and the microglial activation accompanies the neuronal loss in the substantia nigra in the brains of PD patients **(Banati *et al*., 1998; Teismann *et al*., 2003)**, as well as MPTP-treated mice **(Czlonkowska *et al*., 1996; Kohutnicka *et al*., 1998)** and 6-OHDA-treated rats **(Akiyama and McGeer, 1989; Rodrigues *et al*., 2001)**. Ultrastructurally, microglial cells can be seen near the SNC cells in the brain of PD patients **(Anglade *et al*., 1997)**.

Taken collectively, these characteristic features may indicate that SNC cells are starting to die by apoptosis which occurs in many other animal model of PD **(Tatton and Kish, 1997; Spooren *et al*., 1998; He *et al*., 2000; Marti *et al*., 2002; Serra *et al*., 2002; Novikova *et al*., 2006)** and in Parkinson's disease patients **(Mochizuki *et al*., 1996; Anglade *et al*., 1997; Tompkins *et al*., 1997; Burke and Kholodilov, 1998; Tatton, 2000; Andersen, 2001; Tatton *et al*., 2003)**. It is possible that cells with more altered ultrastructure have died already and therefore can not be sampled.

CHAPTER 4

EXPERIMENT 3:

Nigrostriatal dopaminergic terminals in the AS and AS/AGU rat

4.1 Introduction

Nigrostriatal dopaminergic neurons mainly project to the striatum (for other connections, see **table 1.1, chapter 1 page 8**) **(Chiodo, 1988; Pickel *et al*., 1992a; Parent and Hazrati, 1994; 1995a; Blandini *et al*., 2000; Crossman, 2000; DeLong and Wichmann, 2007)** where they form synapses on medium spiny neurons (12-20μm) **(Smith and Bolam, 1990)**, which are the main neurons in the caudate-putamen **(DiFiglia *et al*., 1976; Bishop *et al*., 1982; Gerfen and Wilson, 1996)**. They form about 95% of striatal neurons **(Kemp and Powell, 1971)** and they use GABA as a neurotransmitter **(Parent and Hazrati, 1995a)**. The remainder of striatal neurons are interneurons **(DiFiglia *et al*., 1976; Bishop *et al*., 1982)** which are classified into large cholinergic neurons (25-40μm in diameter) **(Bolam *et al*., 1984)**, medium size (5-15 μm) neurons which use GABA as a neurotransmitter **(DiFiglia *et al*., 1976; Bishop *et al*., 1982; Bolam *et al*., 1984; Oertel and Mugnaini, 1984; Smith *et al*., 1987; Pasik *et al*., 1988; Kita, 1993)** and medium neurons containing neropeptide Y, Somatostatin or nitric oxide synthase **(Vincent *et al*., 1983 a,b; Smith and Parent, 1986; Dawson *et al*., 1991)**.

Another group of striatal cells are TH-positive and have been described in non-human primates by **Dubach *et al*. (1987)** and in the rat **(Tashiro *et al*., 1989)**. They have also been described in MPTP- and 6-OHDA- treated animals **(Betarbet *et al*., 1997)**, so they may be less susceptible to these toxins.

The degeneration of nigrostriatal dopaminergic neurons occurs in animal models treated with MPTP (e.g. monkey and baboons) **(Shimohama *et al*., 2003)** and in rats treated with 6-OHDA **(Ichitani *et al*., 1991; Betarbet *et al*., 2002)**. Regarding

ultrastructural changes in axons and terminals, the striatal degeneration that occur in animals treated with MPTP appears quite variable. For example, axonal swelling and myelin sheath disruption has been reported in the dog **(Rapisardi *et al*., 1990)** and terminals lacking synaptic vesicles occur in MPTP-treated mice **(Cochiolo *et al*., 2000)**. In 6-OHDA-treated rats there are increases in the terminal size and the number of vesicles **(Pickel *et al*., 1992b; Stanic *et al*., 2003)**.

The AS/AGU rat is characterised by its apparent inability to release DA in the striatum **(Campbell *et al*., 1997)**. This evidence comes from microdialysis experiments with indwelling cannulae. The fact that greatly reduced extracellular DA (10% of normal) is matched by greatly increased DA metabolites such as DOPAC and HVA, led **Payne *et al*. (2000)** to propose a dysfunction of synaptic packaging of DA, leaving it free within the cytoplasm to be acted upon by mitochondrial enzymes. However, there is no information on (a) the abundance of dopaminergic terminals in the striatum of the AS/AGU mutant, (b) the abundance of synaptic vesicles in dopaminergic terminals or (c) their zonation within the terminal relative to the synaptic cleft and the "readily-releasable pool".

The aim of **experiment 3** is therefore to look to TH+ve dopaminergic terminals in the dorsal caudate-putamen to see if there are difference between the AS (control) and AS/AGU (mutant) rats using TEM.

4.2 Materials and Methods

4.2.1 Animals

Three AS control and three AS/AGU mutant male rats aged 12 months.

Three AS control and three AS/AGU mutant male rats aged 3 months.

It is essential to be able to distinguish dopaminergic terminals within the striatum from other terminals. This involves immunocytochemical labelling of terminals with an antibody to tyrosine hydroxylase followed by secondary labelling by 3,3-diaminobenzidine (DAB) or immuno-gold particles for visualization. To ensure labelling, fixation must be with the lowest possible percentage of glutaraldehyde that is consistent with the retention of ultrastructural detail. After considerable trial-and-error a mixture of 1% glutaraldehyde: 4% paraformaldehyde was chosen as permitting visualization with both

(a) DAB, and

(b) Immunogold particles (1 nm) with silver enhancement.

4.2.2 Tissue preparation for transmission electron microscopy

All rats were deeply anaesthetized with an overdose of sodium pentobarbitone B.P (Vet.), (2 ml of 60 mg/ml, Rhone-Merieux, Spire Greencentre, Harlow, Essex) injected intraperitoneally, after which the thoracic cavity was opened. A mammalian Ringer solution (200 ml) containing the vasodilator Lignocaine was perfused via the left ventricle for one minute followed by 500 ml mixture of 4% paraformaldehyde and 1% glutaraldehyde (Agar- Aldrich Inc, P6148) in 0.1M phosphate buffer, blood and excess fluid being drained via an incision through the right atrium **(Appendix 1)**. The

brain was dissected out and immersion-fixed in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1M of phosphate buffer overnight. Pieces of brain containing areas of interest were serially sectioned at 70µm using a Vibrotome (Agar Scientific, BNBA 010664, Agar Scientific LTD, UK).

4.2.3 Tyrosine hydroxylase immunoperoxidase staining

The sections containing the dorsal region of the caudate-putamen *(Approximately -0.3 mm relative to bregma* **Paxinos and Watson, 1982**), from the AS (control) and the AS/AGU (mutant) rats were incubated for 24 h in a humidity chamber at 4ºC with the primary antibody (Monoclonal mouse anti-tyrosine hydroxylase, MAB 5280, Chemicon Europe Ltd (1: 1000) diluted in blocking serum (1% NGS in PBS with 0.3% Triton X- 100). The rest of the procedure was carried out at room temperature. After rinsing in PBS (3x5 mins), sections were then incubated for 60 min in a biotinylated anti-mouse secondary antibody (1:200 dilution), (Vector Laboratories, INC, BA-2001, Peterborough UK). Following 60 min incubation in Avidin-Biotin-Complex (ABC) reagent (1:50; 20µl of solution A and 20µl of solution B in 1ml of PBS; Vectastain ABC kit, Vector Laboratories, INC, PK-6100, Peterborough UK), sections were then rinsed in PBS (3x5 mins) and 0.1 M phosphate buffer (PB, 1x5 mins). The location of the antigen-antibody complex was visualized by incubating sections in a medium containing 0.05% 3,3-diaminobenzidine (DAB substrate KIT for peroxidase, Vector Laboratories, INC, SK-4100, Peterborough UK) for 2-5 min; this step was carried out with care in a fume cupboard, and all equipment which came in contact with DAB was soaked with bleach in order to denature it, as DAB is potentially carcinogenic (International Agency for Research on Cancer, 1972). Finally, the sections were rinsed in distilled water, dehydrated and mounted with glass

coverslips using histomount (RALAMB, HS-103, UK). Slides were then examined under a light microscope.

4.2.4 Pre-embedding immunocytochemistry

The sections containing the dorsal region of the caudate-putamen (*Approximately -0.3 mm relative to bregma* **Paxinos and Watson, 1982**), from the AS (control) and the AS/AGU (mutant) rats were treated with 1% sodium borohydride for 30 min **(Kosaka *et al*., 1986)**, rinsed in PBS many times (9x10 min), incubated in blocking solution **(Appendix 7)** for 30 minutes and incubated overnight in mouse antiserum to tyrosine hydroxylase (Monoclonal mouse anti-tyrosine hydroxylase, MAB 5280, Chemicon Europe Ltd) diluted to 1:300 in incubation buffer **(Appendix 7)**. Sections were then processed using a silver-enhanced immunogold method **(Chan *et al*., 1990)**. After the sections were rinsed in washing buffer (3x10 min), they were incubated for 4 hours in 1nm goat anti-mouse IgG immunogold (Amersham UK) diluted 1:50 in incubation buffer. Sections were then rinsed in washing buffer (3x15 min) and in PBS (3x5 min), postfixed in 2% glutaraldehyde in PBS for 10 minutes, rinsed in distilled water, and treated with a silver enhancement solution (IntenSE M kit, Amersham UK) according to the manufacturer's instructions. The optimal time for the silver enhancement step was found to be between 12 and 15 minutes at approximately 20^{o}C. The sections were then rinsed in distilled water (3x5 min), osmicated, dehydrated and embedded in Durcupan.

4.2.5 Electron microscopy

Sections containing the dorsal region of the caudate-putamen from AS (control) and the AS/AGU (mutant) rats were rinsed with PB and placed in a solution of 1% Osmium tetroxide in PB for 20 minutes in an agitator. The sections were rinsed with

distilled water (3 x 30 mins) and dehydrated through a series of graded concentrations of acetone from 70 to 100%, followed by a descending ratio of acetone to durcupan resin (3:1, 1:1, 1:3) and two changes of 100% durcupan resin. The sections were flat-embedded in durcupan resin between two small sheets of acetate, sandwiched between two glass slides, weighted down with metal weights and heated at 60ºC overnight in an oven **(Appendix 6)**. The top acetate sheet was peeled off, and stock embedded sections were attached onto the end of a blank embedding block using RS adhesive and left for at least 30 min in an oven. The block containing the area of interest was trimmed.

4.2.5.1 Ultrathin sections processing and staining

Ultrathin sections (50-70nm thickness) from the dorsal region of the caudate-putamen of AS (control) and AS/AGU (mutant) rats were cut from selected blocks using diamond knives (Drukker International, Netherlands) on a Reichert-Jung Ultracut E ultramicrotome. Ultrathin sections were collected on 300 mesh-coated copper grids (300 Mesh Thin Bar Copper 3.05 mm, G2720C, Agar Scientific, UK) and double stained with uranyl acetate and lead citrate **(Reynolds, 1963)**. The grids containing sections were stained in lead citrate for 5 mins, placed in sodium hydroxide 7 times, washed in distilled water 7 times, stained in 12.5% methanolic uranyl acetate for 5 mins, washed in distilled water 7 times, stained in lead citrate for 5 mins, placed in sodium hydroxide 7 times, washed in distilled water 7 times and left to dry for 20 mins.

Ultrathin sections were examined at a magnification of 10,000X and 50,000X using a transmission electron microscope (JEOL JEM-100S, No. IEM 100S-4, JEOL LTD, Tokyo, Japan). All areas of interest were photographed.

4.2.6 Data analysis

4.2.6.1 The classification of neuronal elements

Tyrosine hydroxylase +ve neuronal elements can be positively identified with immunoperoxidase DAB reaction when they showed a higher electron density in comparison to neighbouring elements. Similarly, they were considered positive with immunogold when one or more gold particles were located in the labeling area. The classification of neuronal elements was made according to the descriptions of **Peters *et al.* (1976)**. Axon terminals were 0.25 µm or larger in diameter and contained many small synaptic vesicles. A synapse can normally be defined as symmetric when it has a thin postsynaptic density and asymmetric when it has a thick postsynaptic density. Dendrites were identified by their large diameter, the presence of postsynaptic densities and/or an abundance of microtubules and endoplasmic reticulum.

4.2.6.2 Sampling of TH+ve and unlabelled terminals (Figure 4.1)

To determine the proportions of TH+ve terminals compared to unlabelled terminals in the dorsal caudate-putamen, at least 180 electron micrographs from 12 animals (at least 10 electron micrographs per animal) were photographed randomly at magnifications of x10,000 in areas (10.5 x 8.5 µm) in which immunogold or immunoperoxidase DAB for TH were present. For each animal (and stain) 10 squares from a 300 – square grid (G) were sampled in a manner which was uniform for all animals **(see figure 4.1)**.

The uppermost complete horizontal row was identified and

i. the first square (i.e. the one at the left hand end) was photographed in the top left corner.

ii. Five grid squares were omitted and the sixth grid photographed in the top right corner.

iii. The third and fourth grids were photographed in the bottom left and bottom right corners respectively. In each case, 5 grid squares were omitted between sample grids.

iv. The sequence began again.

v. Once a horizontal row had been completed, the next row was omitted and counts began again on the following row.

The aim was to cover as large an area on the grid as possible, but to sample in a standardised manner.

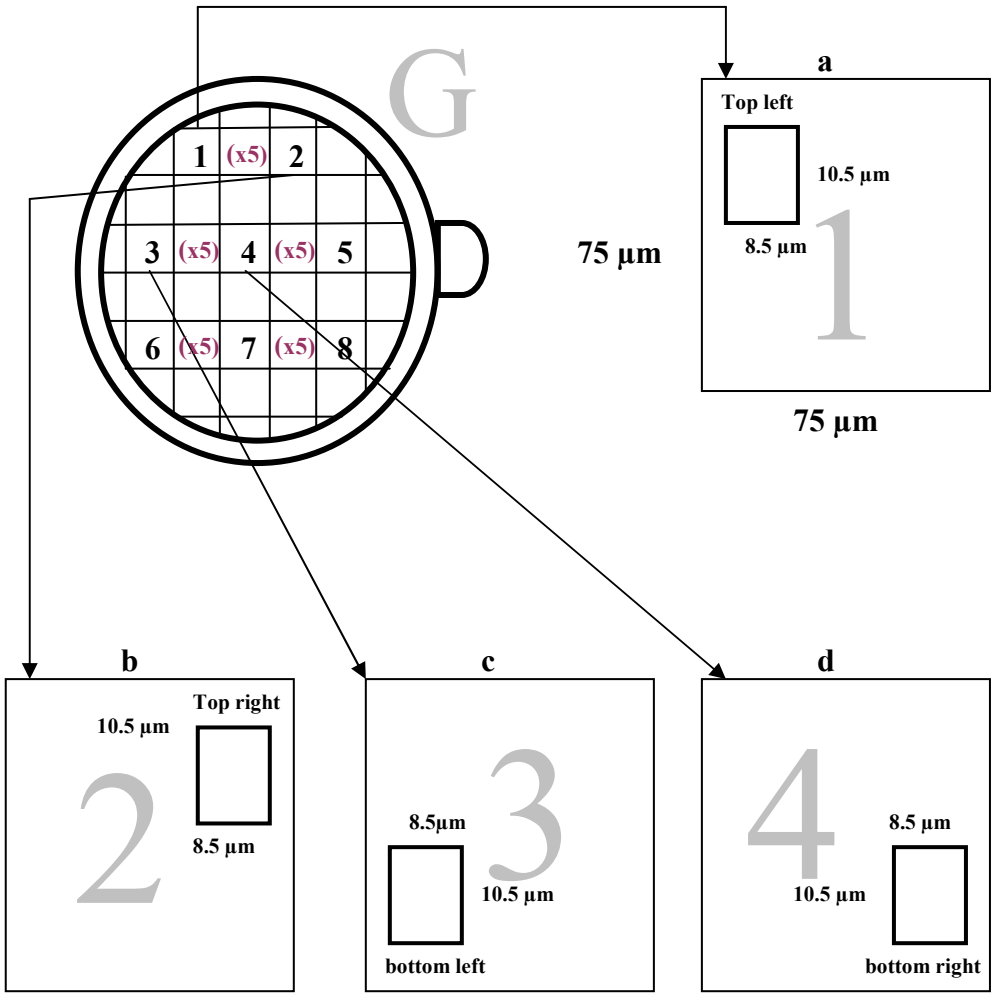


Figure 4.1 : A schematic diagram to show standard sampling methods for TEM photography and analysis (refer also to text).

4.2.6.3 Measurements of synaptic vesicle numbers

Figure 4.2 shows schematically the method for counting the number of vesicles and is partially modified from a recent study by **Tao-Cheng (2006)**. This anther studied the distributions of seven pre-synaptic proteins in the active zone using immunogold labelling. The active zone is a well-defined area in the presynaptic terminal directly apposed to the post-synaptic density (PSD) and a site of synaptic vesicle exocytosis and neurotransmitter release. He measured the numbers of immunogold particles and vesicles in three zones:

(i) Zones I and II (each 33 nm wide) contain the two rows of synaptic vesicles immediately adjacent to the presynaptic membrane known as the proximal or "readily releasable" pool. Active zone cytomatrix materials (pyramidal in shape and 50 nm wide) extend through these two zones (I and II).

(ii) Zone III is twice as wide as zone I and II together, and contains almost all the remaining synapses known as the distal or reserve pool.

In my study, vesicles were counted in four zones if they lay between the two parallel lines perpendicular to the synaptic cleft. The visible synapse length (L) of the synapse (determined by the post-synaptic density, PSD) was measured:

a) Zone I (0-41.7 nm).

b) Zone II (41.7-83.3 nm).

c) Zone III (83.3- 166.7 nm).

d) Zone IV (166.7- 300 nm).

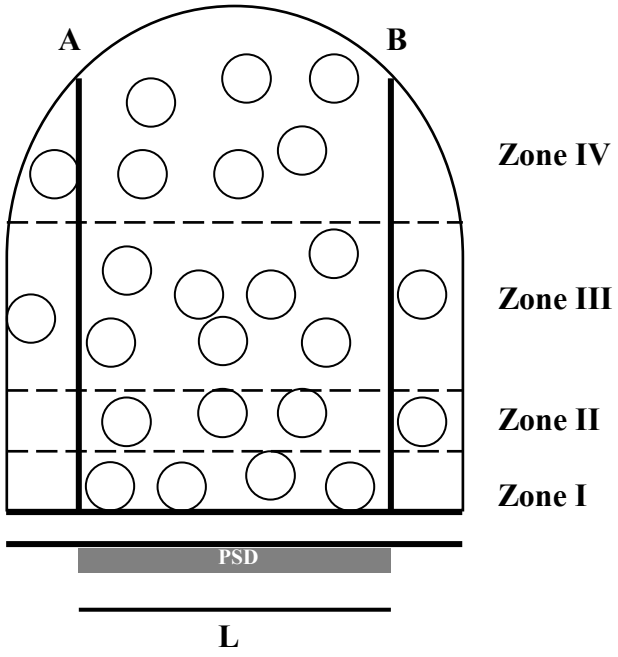


Figure 4.2: Schematic diagram of synaptic measurement zones. Two parallel lines (A + B) perpendicular to the segment of the presynaptic membrane define the two sides of the area of measurement; the distance between them is the index length (L). Three parallel bands with increasing distance from the presynaptic membrane were marked in dotted lines: Zone I, 0-41.7 nm; Zone II, 41.7-83.3 nm; Zone III, 83.3-166.7 nm and Zone IV, 166.7- 300 nm. The postsynaptic density (PSD) is shown as a dark gray rectangle. Partially modified from Tao-Cheng (2006).

Zone I (immediately adjacent to the presynaptic terminals) and Zone II are both 41.7 nm in wide. I choose this width to reflect the average diameter of vesicles (35-45 nm) in each zone. Zone III is four times as wide as Zone I. In my study a few vesicles were present further than 200 nm from the presynaptic membrane, so I added Zone IV (up to 300 nm from the presynaptic membrane) to cover the remaining vesicles.

The number of vesicles per synaptic length was calculated by dividing the number of vesicles in a zone by the length of the synapse (L). The number of synapses are at least 10 in each animal.

4.2.7 Image analysis

Images were captured on plate film which was then developed. The films were put in a working dilution of 500ml D19 Kodak developer (Kodak-path, Paris) in 500ml of water for 3 mins, and then were rinsed in water for one minute before placing in Amfix (Amfix, Champion, UK) for 5mins rinsed in water for 20 mins and dried. The negatives of electron micrographs were scanned using an Epson scanner (Epson perfection 4990), and the contrast and exposure were adjusted using Paint Shop before printing.

4.2.8 Statistical analysis

The results were analysed by two-sample t-test using the Minitab statistics package (MINITAB Release 13.30).

4.3 Results

4.3.1 Comparison of numbers and percentages of TH+ve nigrostriatal dopaminergic terminals in the dorsal caudate-putamen (DCPU) with immunoperoxidase DAB staining for TH in AS and AS/AGU rats aged 12 months

The results show statistically significant differences between the two groups in the total number of TH+ve terminals, unlabeled terminals and total terminals in the dorsal caudate-putamen (< 0.05). The AS/AGU (mutant) rats had fewer terminals than the AS (control). The results are summarized in **table 4.1**.

Despite the marked difference in absolute numbers the percentage of TH+ve nigrostriatal dopaminergic terminals as a proportion of the total number of terminals was approximately the same in both strains at this age **(Table 4.4)**.

The sampling methods used here led to the inclusion of 1-200 synaptic terminals per animal for analysis. Several feature emerged:

(i) There were simply fewer synaptic terminals per unit area in AS/AGU rats compared to the AS parent strain.

(ii) In both strain, about 15% of terminals were dopaminergic, as revealed by DAB staining.

(iii) The reduction in numbers of synapses in the AS/AGU rats included both the dopaminergic and non-dopaminergic ones.

Number	AS	AS/AGU	P
TH+ve terminals	35.7 ± 2.3	19.3 ± 0.9	**< 0.05**
Unlabeled terminals (TH-ve terminals)	182.3 ± 0.9	118 ± 5.3	**< 0.01**
Total terminals	218 ± 2.6	137.3 ± 6.1	**< 0.01**

Table 4.1: Number of terminals within the DCPU with immunoperoxidase DAB staining for TH in AS control and AS/AGU mutant rats aged 12 months (n=3 per group). All values are mean number of terminals ± SEM. All comparisons are two-sample t-tests.

4.3.2 Comparison of numbers and percentages of TH+ve nigrostriatal dopaminergic terminals in the DCPU with immunogold staining for TH in AS and AS/AGU rats aged 12 months

As with DAB staining, immunogold staining results show statistically significant differences between the two groups in the number of TH+ve terminals, unlabeled terminals and total terminals in the dorsal caudate-putamen (< 0.05). The AS/AGU (mutant) rats had fewer terminals than the AS (control). The results are summarized in **table 4.2**.

Despite the marked difference in absolute numbers the percentage of TH+ve nigrostriatal dopaminergic terminals as a proportion of the total number of terminals was only slightly less in AS/AGU (mutant) rats at this age (13%) than in AS controls (17%) **(Table 4.4)**.

It appears that DAB and immunogold staining give similar numbers of TH+ve and – ve terminals. For 3 – month old animals, only immunogold staining was used.

Number	**AS**	**AS/AGU**	**P**
TH+ve terminals	43.7 ± 2	23 ± 2.1	**< 0.01**
Unlabeled terminals (TH-ve terminals)	207.7 ± 9.3	148.3 ± 10	**< 0.05**
Total terminals	251.3 ± 11	171.3 ± 12	**< 0.05**

Table 4.2: Number of terminals within the DCPU with immunogold staining for TH in AS control and AS/AGU mutant rats aged 12 months (n=3 per group). All values are mean number of terminals ± SEM. All comparisons are two-sample t-tests.

4.3.3 Comparison of numbers and percentages of TH+ve nigrostriatal dopaminergic terminals in the DCPU with immunogold staining for TH in AS and AS/AGU rats aged 3 months

The results show statistically significant differences between the two groups in the number of TH+ve terminals in the dorsal caudate-putamen (< 0.05). The AS/AGU (mutant) rats had significantly fewer dopaminergic terminals than the AS (control) strain. The results are summarized in **table 4.3**. However, unlike the 12 month animals, the number of unlabelled terminals (and the total number of terminals) was similar in the two strains i.e. only the TH +ve terminals were reduced.

The percentage of TH+ve nigrostriatal dopaminergic terminals as a proportion of the total number of terminals was reduced in AS/AGU (mutant) **(Table 4.4)**. A chi-square (X^2) analysis of the percentage of TH+ve terminals (using absolute numbers) shows that the two strains are significantly different **(X^2 =7.19, df = 1, P<0.01)**.

	AS	AS/AGU	P
TH+ve terminals	54.67 ± 2.8	25.67 ± 0.33	**< 0.01**
Unlabeled terminals (TH-ve terminals)	254.3 ± 12	245 ± 9.7	**NS**
Total terminals	309 ± 15	270.7 ± 9.5	**NS**

Table 4.3: Number of terminals within the DCPU with immunogold staining for TH in AS control and AS/AGU mutant rats aged 3 months (n=3 per group). All values are mean number of terminals ± SEM. All comparisons are two-sample t-tests (NS: not significant).

Strains	AGE	Techniques	percentage
AS	12 month	TH immunoperoxidase DAB	16.4%
AS/AGU	12 month	TH immunoperoxidase DAB	14.1%
AS	12 month	TH immunogold	17.4%
AS/AGU	12 month	TH immunogold	13.4%
AS	3 month	TH immunogold	17.7%
AS/AGU	3 month	TH immunogold	9.5%

Table 4.4: percentages of TH+ve terminals within the DCPU with immunoperoxidase DAB and immunogold staining for TH in AS control and AS/AGU mutant rats aged 3 and 12 months (n=3 per group).

4.3.4 Comparisons of synaptic vesicles numbers of TH+ve nigrostriatal dopaminergic and unlabelled terminals in the DCPU (identified by immunogold staining for TH) of AS and AS/AGU rats aged 12 months

The results show statistically significant differences between the two groups in the number of synaptic vesicles in each zone and in the total numbers of vesicles and also in the number of vesicles per synaptic length in both TH+ve and unlabelled terminals of the DCPU at this age (< 0.05). The AS/AGU (mutant) rats had fewer vesicles than the AS (control) rats. The results are summarized in **table 4.5**.

	Terminals	AS	AS/AGU	P
Zone I (0-41.7 nm)	**TH +ve NV**	9.98 ± 0.94	5.08 ± 0.17	**< 0.05**
	NV/L	36.15 ± 2.7	14.2 ± 0.96	**< 0.05**
	Unlabeled (TH-ve) NV	7.28 ± 0.39	5 ± 0.1	**< 0.05**
	NV/L	29.59 ± 1.7	18.31 ± 0.79	**< 0.05**
Zone II (41.7-83.3 nm)	**TH +ve NV**	10.11 ± 0.78	5.57 ± 0.6	**< 0.05**
	NV/L	36.61 ± 1.7	15.64 ± 2.2	**< 0.01**
	Unlabeled (TH-ve) NV	7.72 ± 0.81	5.49 ± 0.003	**NS**
	NV/L	31.46 ± 3.7	20.16 ± 0.002	**NS**
Zone III (83.3- 166.7 nm)	**TH +ve NV**	26.48 ± 3.3	12.64 ± 2.6	**< 0.05**
	NV/L	95.7 ± 9.3	35.7 ± 8.2	**< 0.05**
	Unlabeled (TH-ve) NV	26.44 ± 1.5	14.39 ± 0.92	**< 0.01**
	NV/L	107.12 ± 1.5	52.65 ± 3.7	**< 0.01**
Zone IV (166.7- 300 nm)	**TH +ve NV**	16.63 ± 2.9	2.72 ± 0.87	**< 0.05**
	NV/L	60.9 ± 12	7.76 ± 2.6	**< 0.05**
	Unlabeled (TH-ve) NV	20.67 ± 0.35	6.28 ± 0.71	**< 0.01**
	NV/L	84.21 ± 5.1	23.16 ± 3.5	**< 0.01**
Total	**TH +ve NV**	63.20 ± 4.4	26.01 ± 4.1	**< 0.01**
	NV/L	229.4 ± 13	73.3 ± 14	**< 0.01**
	Unlabeled (TH-ve) NV	62.11 ± 1.3	31.17 ± 0.19	**< 0.01**
	NV/L	252.4 ± 7.3	114.3 ± 6.1	**< 0.01**

Table 4.5: Numbers of synaptic vesicles in TH+ve nigrostriatal dopaminergic and unlabelled terminals within the DCPU (identified by immunogold staining for TH) in AS control and AS/AGU mutant rats aged 12 months (n=3 per group). All values are mean number of synaptic vesicles (NV) or numbers per synaptic length (NV/L) ± SEM. All comparisons are two-sample t-tests (NS: not significant).

4.3.5 Comparison of synaptic vesicles numbers in TH+ve nigrostriatal dopaminergic and unlabelled terminals in the DCPU (identified by immunogold staining for TH) of AS and AS/AGU rats aged 3 months

The results show statistically significant differences between the two groups in the number of TH +ve synaptic vesicles in each zone and in the total number of vesicles and also in the number of vesicles per synaptic length in TH+ve nigrostriatal dopaminergic terminals of the DCPU. The AS/AGU (mutant) rats had fewer vesicles than the AS (control). By contrast, there were no significant differences between the two strains in the number of vesicles in unlabelled (i.e. non-dopaminergic) terminals. The results are summarized in **table 4.6**.

	Terminals	AS	AS/AGU	P
Zone I (0-41.7 nm)	**TH +ve NV**	7.89 ± 0.46	3.44 ± 0.29	**< 0.01**
	NV/L	29.97 ± 1.5	11.49 ± 0.51	**< 0.01**
	Unlabeled (TH-ve) NV	7.39 ± 0.63	6.72 ± 0.29	**NS**
	NV/L	30.23 ± 2.1	29.4 ± 3	**NS**
Zone II (41.7-83.3 nm)	**TH +ve NV**	8.83 ± 0.82	3.67 ± 0.35	**< 0.05**
	NV/L	33.43 ± 2.1	12.23 ± 0.62	**< 0.01**
	Unlabeled (TH-ve) NV	8.33 ± 1.1	7.72 ± 0.2	**NS**
	NV/L	34.64 ± 2.8	33.55 ± 1.7	**NS**
Zone III (83.3- 166.7 nm)	**TH +ve NV**	32.17 ± 2.7	7.89 ± 1.9	**< 0.01**
	NV/L	122.5 ± 12	26.2 ± 5.8	**< 0.05**
	Unlabeled (TH-ve) NV	32.56 ± 2.6	29.94 ± 2.1	**NS**
	NV/L	133.6 ± 11	129.33 ± 3.2	**NS**
Zone IV (166.7- 300 nm)	**TH +ve NV**	18.83 ± 3.3	2.95 ± 1.5	**< 0.05**
	NV/L	70.6 ± 8.1	9.51 ± 4.5	**< 0.01**
	Unlabeled (TH-ve) NV	13.89 ± 0.56	10.94 ± 2.7	**NS**
	NV/L	57.21 ± 4.4	46.1 ± 9.6	**NS**
Total	**TH +ve NV**	67.72 ± 5	17.95 ± 3.6	**< 0.01**
	NV/L	256.5 ± 8.8	59.4 ± 9.8	**< 0.001**
	Unlabeled (TH-ve) NV	62.5 ± 2.1	55.33 ± 4.5	**NS**
	NV/L	256.3 ± 9.5	238.42 ± 3.3	**NS**

Table 4.6: Numbers of synaptic vesicles in TH+ve nigrostriatal dopaminergic and unlabelled terminals within the DCPU (identified by immunogold staining for TH) in AS control and AS/AGU mutant rats aged 3 months (n=3 per group). All values are mean number of synaptic vesicles (NV) or numbers per synaptic length (NV/L) ± SEM. All comparisons are two-sample t-tests (NS: not significant).

4.3.6 Distributions of vesicles and average length of synaptic terminals in AS and AS/AGU rats aged 3 and 12 months.

The percentage zonal distribution of vesicles in TH+ve and unlabelled terminals are approximately the same in both strains at both ages **(Figure 4.3-4.6)**. Zone III often thought of as the "reserve pool" has the highest percentage of vesicles. The actual synapse length is significantly greater in AS/AGU mutants than AS controls at 12 months, otherwise there are no strain differences **(Figure 4.7)**.

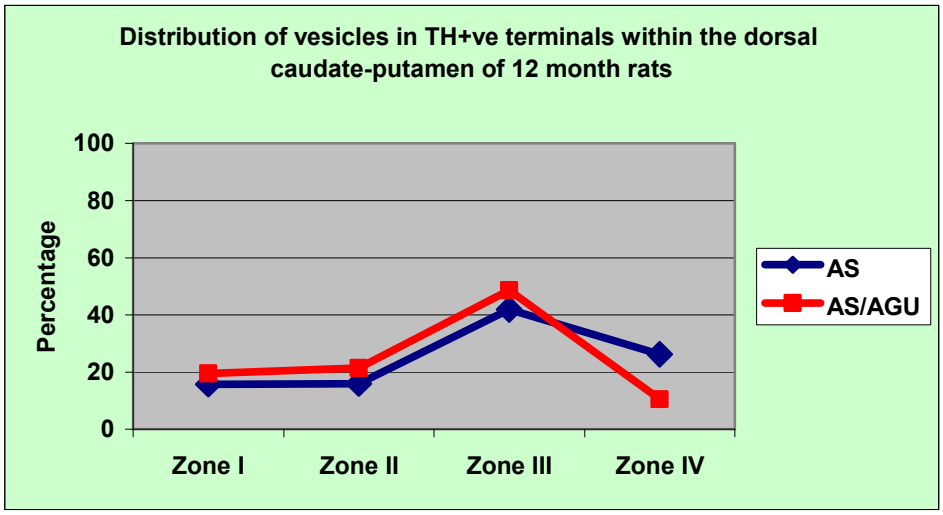


Figure 4.3: Distribution of vesicles of TH+ve terminals within the DCPU (identified by immunogold staining for TH) in control (AS) and mutant (AS/AGU) male rats aged 12 months. The figures are expressed as percentages (n=3 per group).

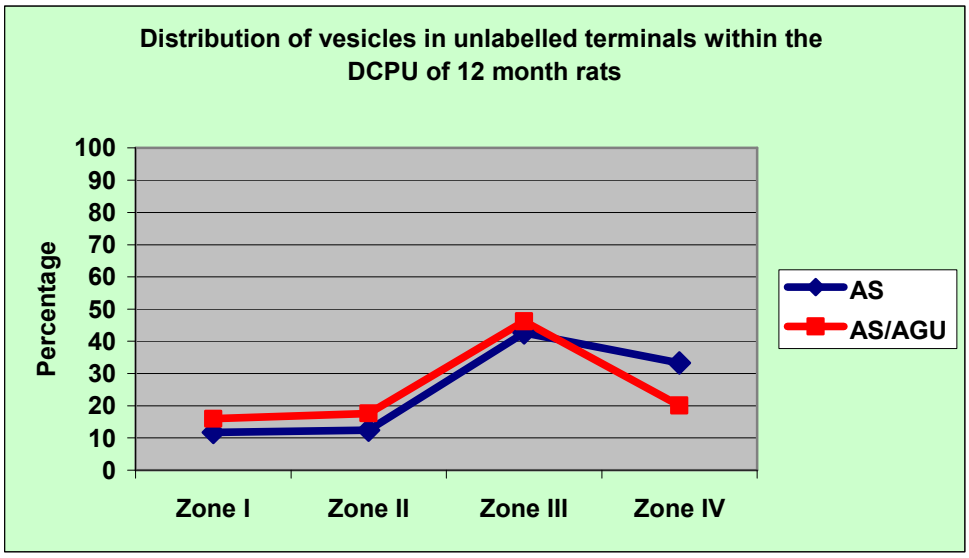


Figure 4.4: Distribution of vesicles of unlabelled terminals within the DCPU (identified by immunogold staining for TH) in control (AS) and mutant (AS/AGU) male rats aged 12 months. The figures are expressed as percentages (n=3 per group).

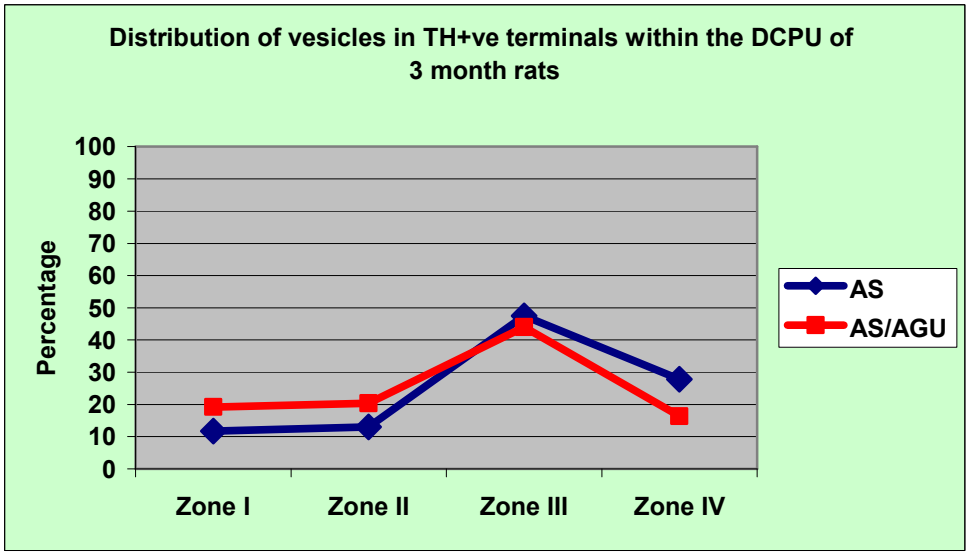


Figure 4.5: Distribution of vesicles of TH+ve terminals within the DCPU (identified by immunogold staining for TH) in control (AS) and mutant (AS/AGU) male rats aged 3 months. The figures are expressed as percentages (n=3 per group).

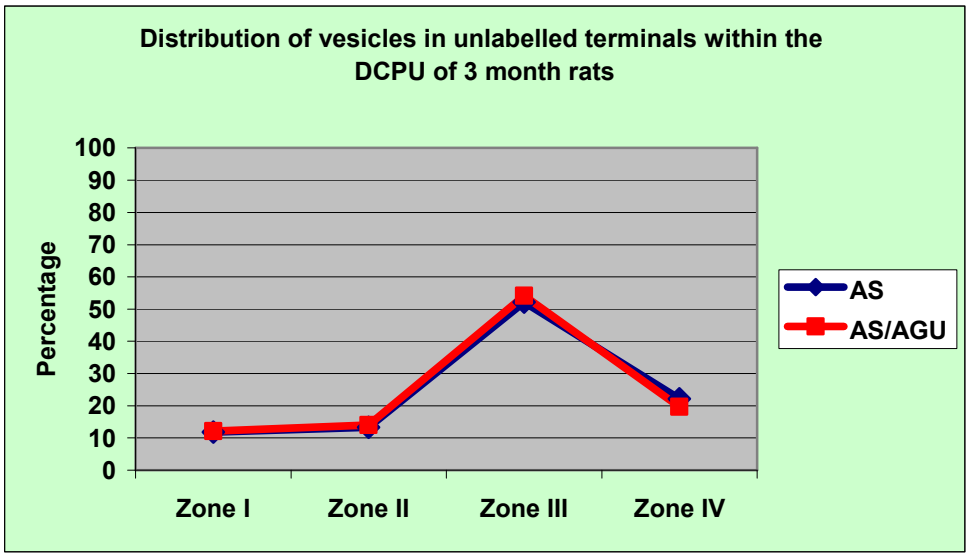


Figure 4.6: Distribution of vesicles of unlabelled terminals within the DCPU (identified by immunogold staining for TH) in control (AS) and mutant (AS/AGU) male rats aged 3 months. The figures are expressed as percentages (n=3 per group).

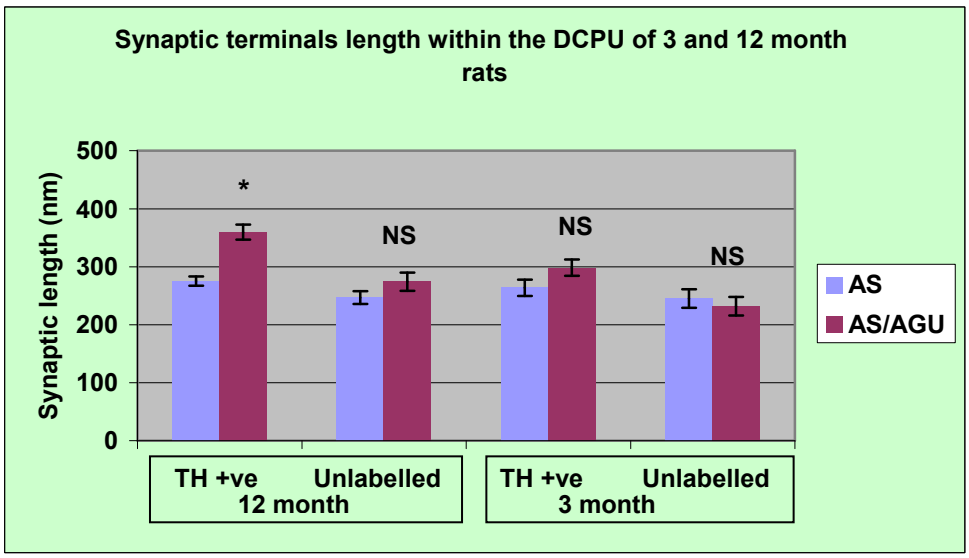
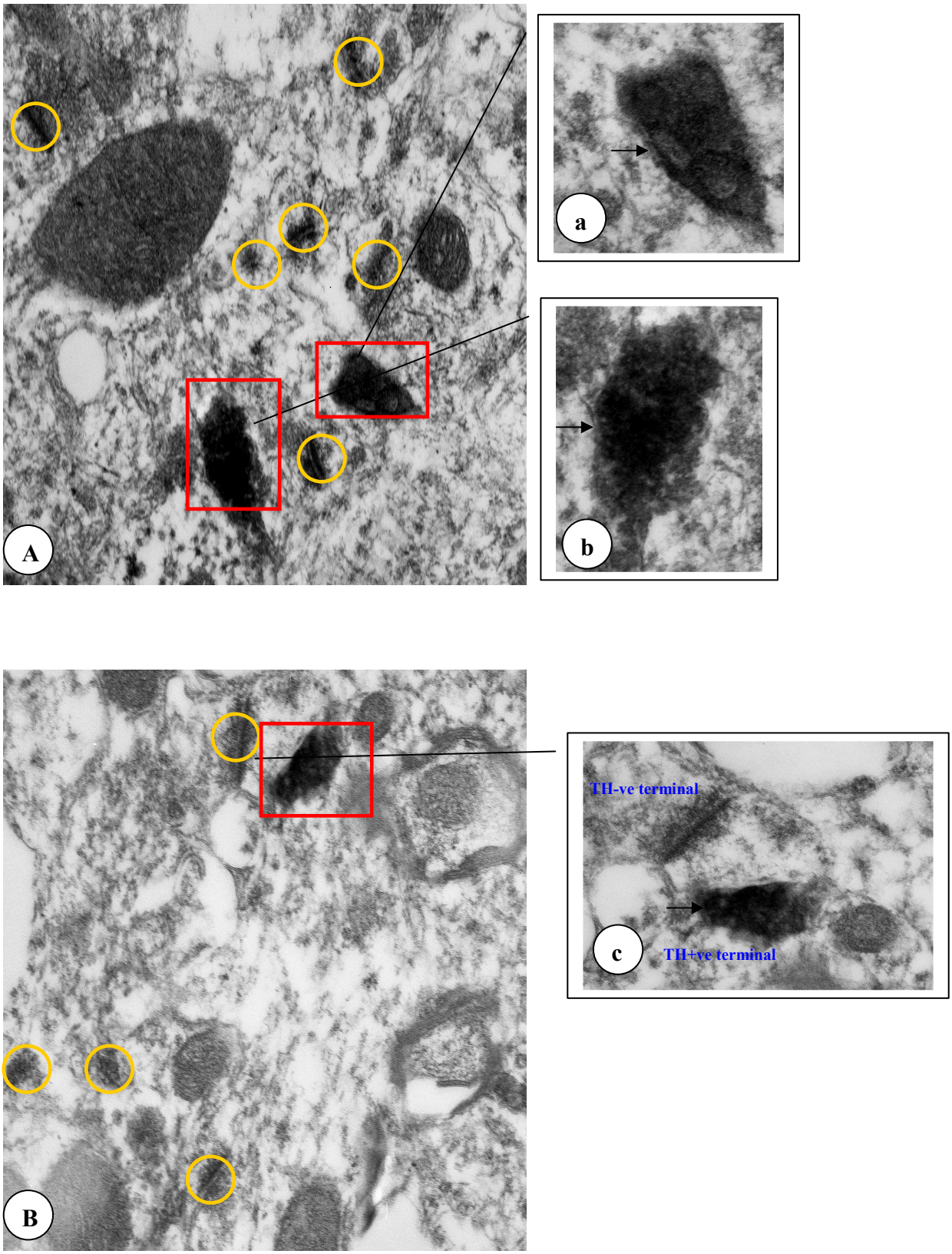


Figure 4.7: Synaptic terminals length within the DCPU (identified by immunogold staining for TH) in control (AS) and mutant (AS/AGU) male rats aged 3 and 12 months. Means ± SEM (n=3 per group). (* P < 0.05; NS: not significant).

4.3.7 Electron micrographs of nigrostriatal dopaminergic terminals in the AS and AS/AGU rat

Figure 4.8 shows TH+ve and TH-ve terminals identified by DAB staining for TH. The quality of this figure reflects the small amount of glutaraldehyde used in the fixative (1% : 4% paraformaldehyde).

Figures 4.9-4.13 show TH+ve and TH-ve terminals identified by immunogold staining for TH in both AS and AS/AGU rats aged 3 and 12 months.


A
a
b
B
TH-ve terminal
c
TH+ve terminal

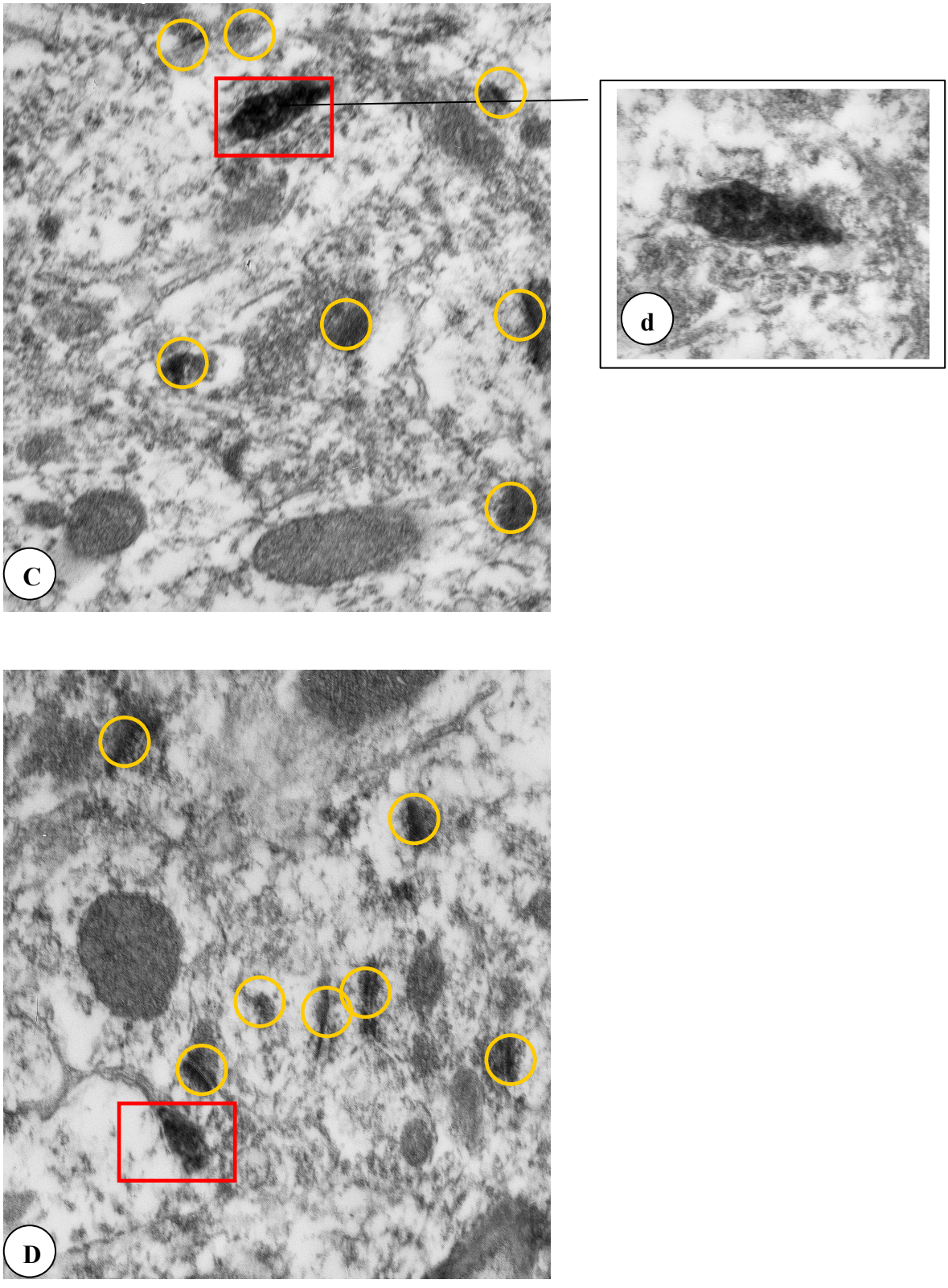


Figure 4.8: Electron micrograph (A,B,C and D) showing TH+ve terminals (red rectangular) stained with DAB for TH and TH-ve terminals (yellow circle) of AS rats aged 12 months, (x20,000). In inset boxes (a,b,c and d) a high magnification of TH+ve terminals and there synapses (arrows) (x50,000).

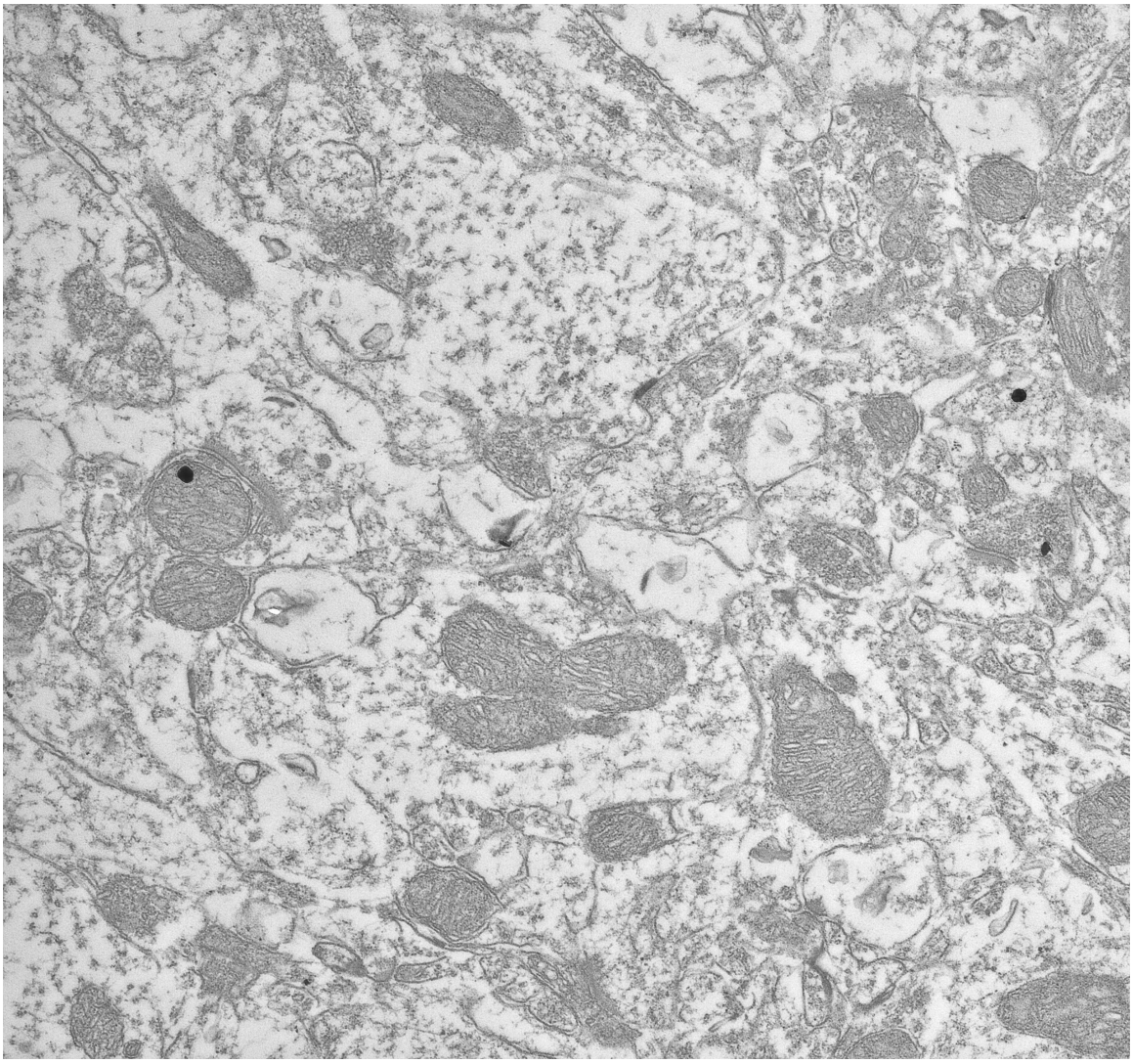
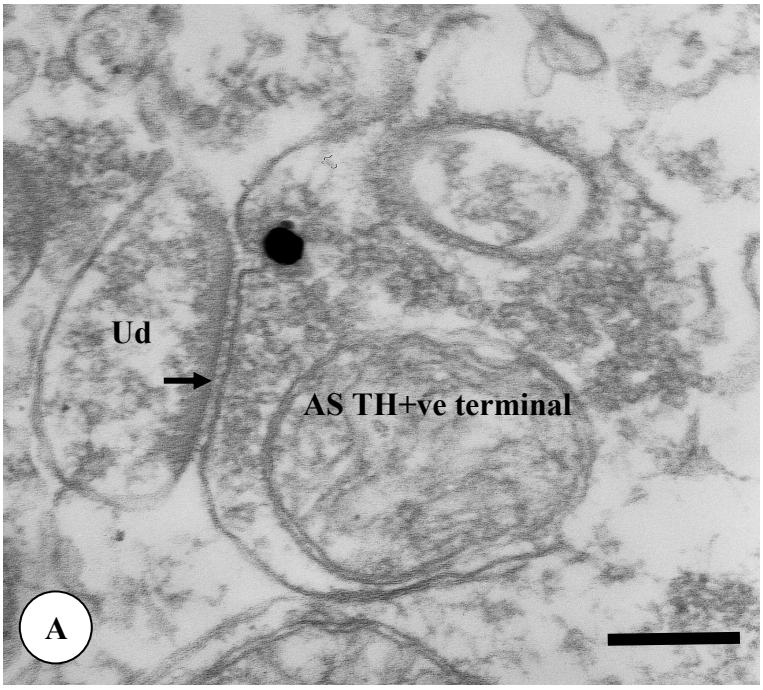
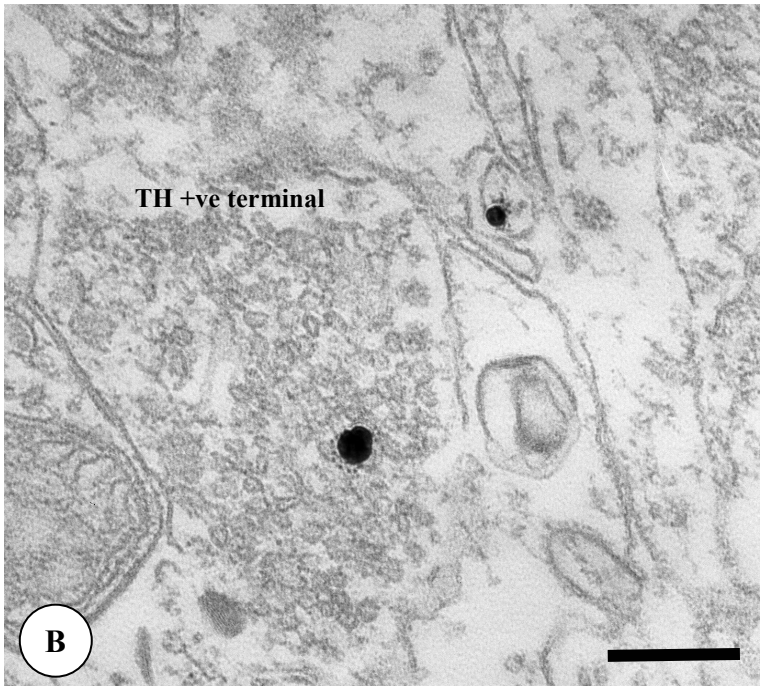


Figure 4.9: Low magnification electron micrograph showing TH+ve terminals stained with immunogold and TH–ve terminals of an AS rat aged 12 months, (x10,000).


Ud
AS TH+ve terminal
A


TH +ve terminal
B

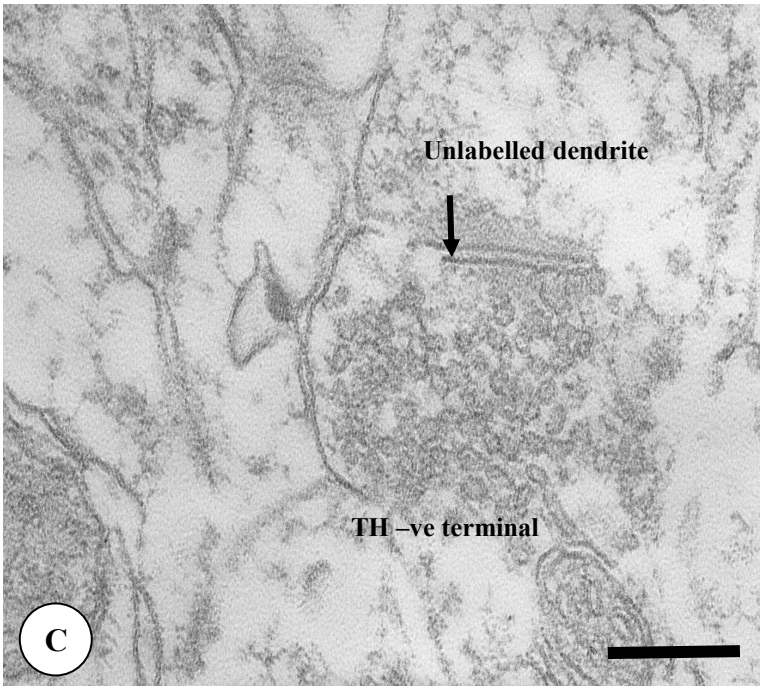
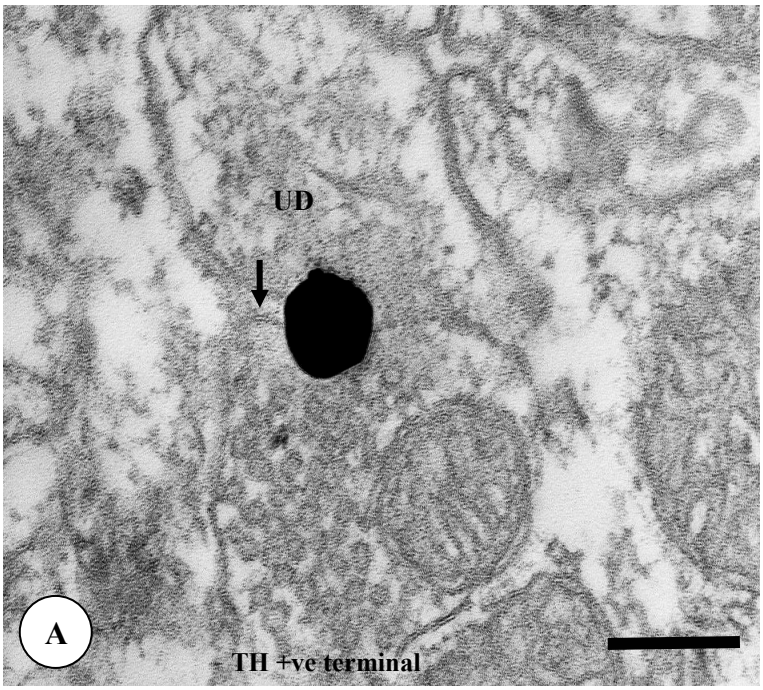
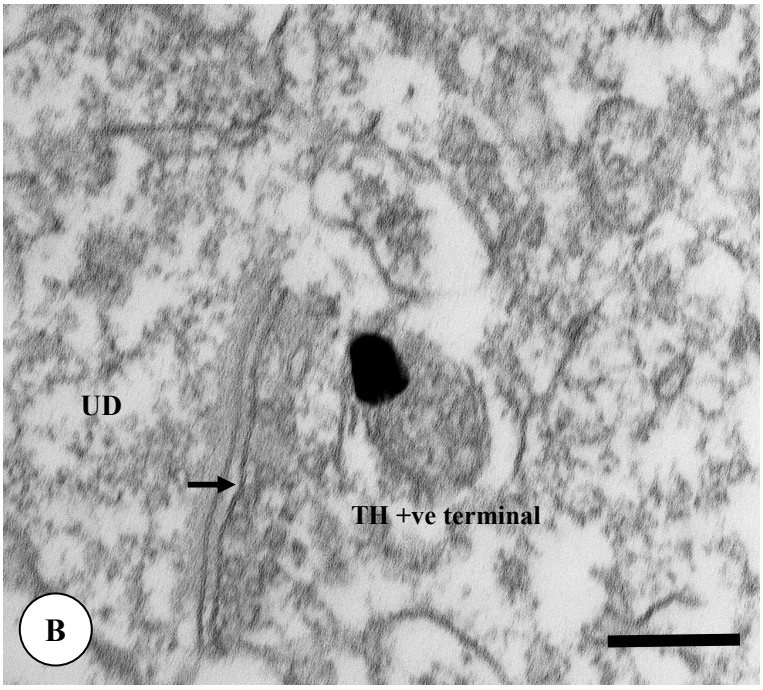


Figure 4.10: Electron micrograph showing TH+ve terminals (A and B) stained with immunogold for TH and TH –ve terminal (C), unlabelled dendrite (Ud) and synapses (arrows) of AS rats aged 12 months, (x50,000). Scale bars = 0.2 µm.


UD
A
TH +ve terminal


UD
TH +ve terminal
B

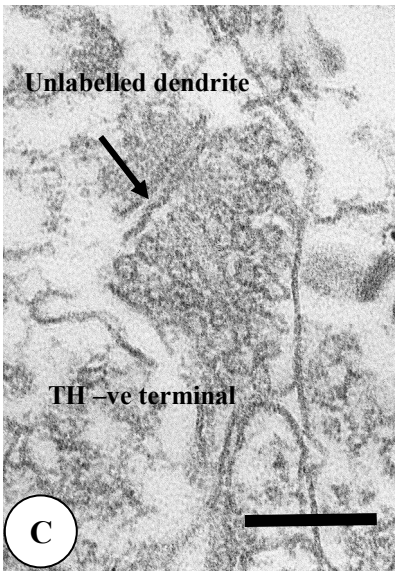


Figure 4.11: Electron micrograph showing TH+ve terminals (A and B) stained with immunogold for TH and TH –ve terminal (C), unlabelled dendrite (Ud) and synapses (arrows) of AS/AGU (mutant) rats aged 12 months, (x50,000). Scale bars = 0.2 µm.

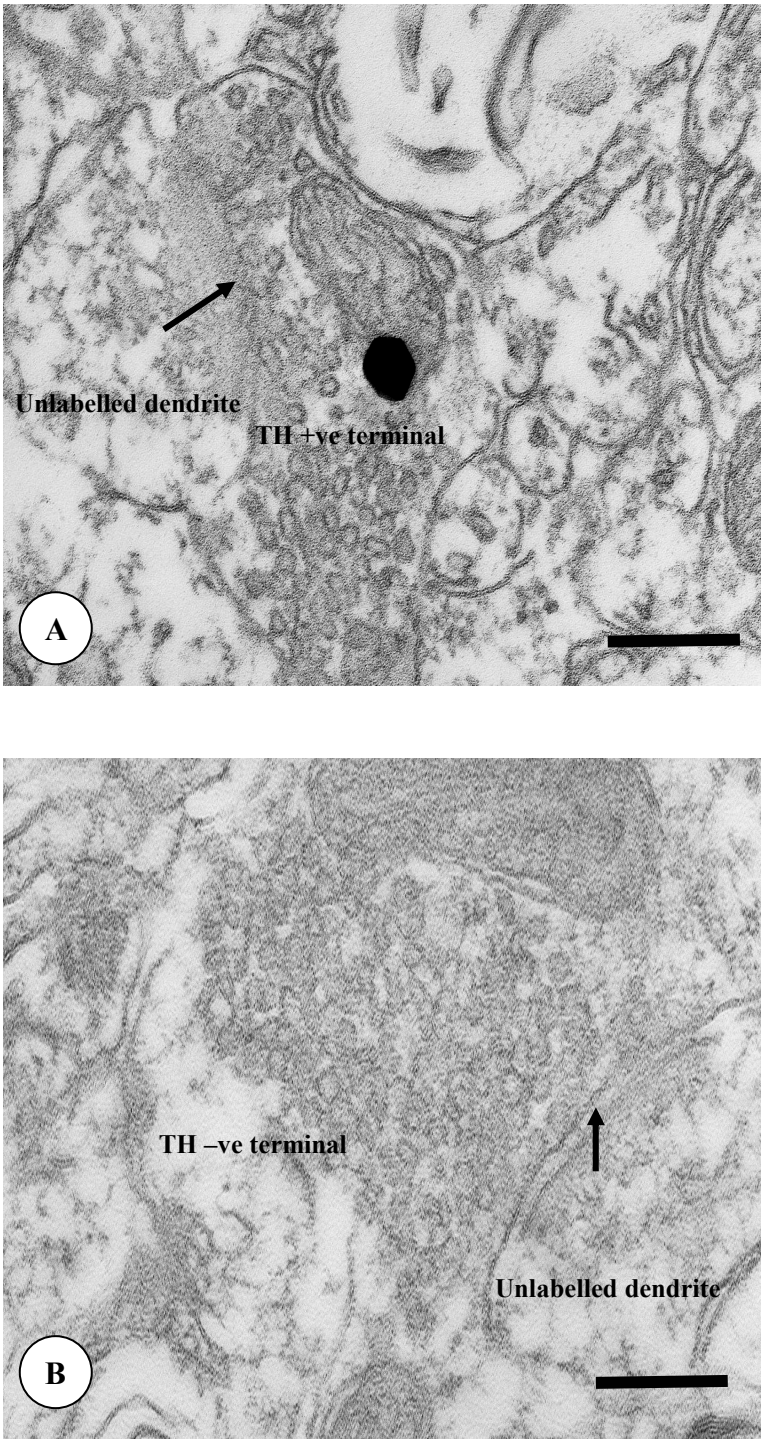


Figure 4.12: Electron micrograph showing TH+ve terminals (A) stained with immunogold for TH and TH –ve terminal (B), unlabelled dendrite (Ud) and synapses (arrows) of AS (control) rats aged 3 months, (x50,000). Scale bars = 0.2 µm.

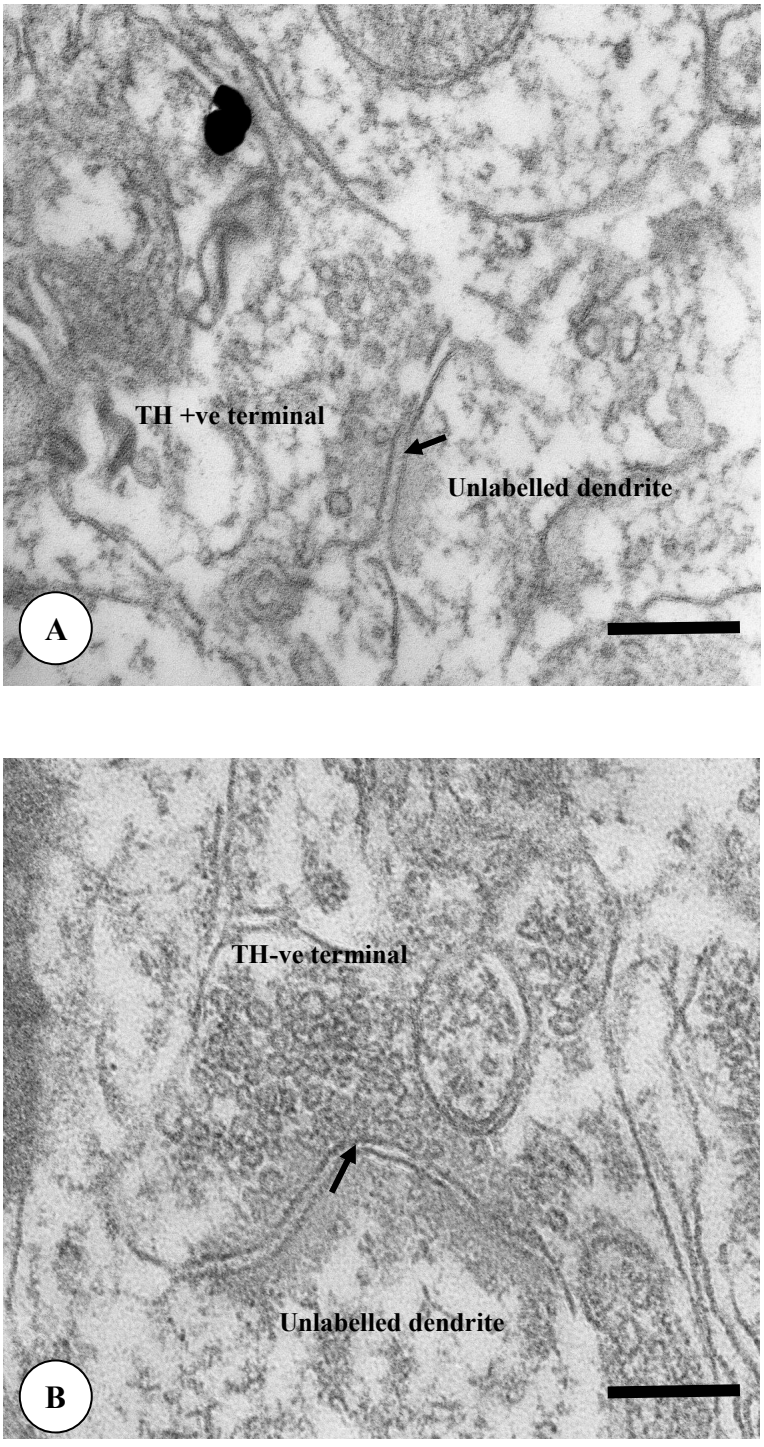


Figure 4.13: Electron micrograph showing TH+ve terminals (A) stained with immunogold for TH and TH –ve terminal (B), unlabelled dendrite (Ud) and synapses (arrows) of AS/AGU (mutant) rats aged 3 months, (x50,000). Scale bars = 0.2 µm.

4.4 Discussion

It is well established that the midbrain dopaminergic system is affected in AS/AGU rats **(Payne *et al*., 2000)**. The levels of dopamine in the dorsal and lateral caudate-putamen are reduced in both post-mortem micropunches which measures intra - and extra-cellular dopamine and by in vivo microdialysis which measures extra-cellular, released, dopamine **(Campbell *et al*., 1997, 1998)**.

Terminals were considered to be labeled (a) with immunoperoxidase when they showed a higher electron density in comparison to neighboring elements **(Mengual and Pickel, 2002)** and (b) with immunogold-silver when they contained one or more silver particles which appear as small black aggregates in pre-synaptic terminals **(Mengual and Pickel, 2002)**. Terminals were only included in the analysis if they showed pre-synaptic, post-synaptic membranes and ten or more vesicles.

The first control used in this experiment were sections from the same region (dorsal striatum) which underwent the same procedure for preembedding immunocytochemistry except for the omission of TH primary antibody. In this type of control, no gold particles were seen. The second control used in this experiment was the examination of the lumen of blood vessels in same sections. Sections had to reach the criterion that no gold particles were seen in the blood vessels lumen.

This study can be improved by (a) decreasing the concentration of glutaraldehyde fixative to less than 1% but this may effect the good morphology of the tissue or (b) the use cryosections treated with immunogold with silver enhancement **(Monaghan and Atherton, 1992)**.

There are some limitations of using immunocytochemistry : (a) TH protein is altered by fixation, dehydration and heating which lead to decreased recognition by

antibodies **(Chan *et al*., 1990)**, (b) using glutaraldehyde in high concentration leads to increased non-specific staining and decreased antigenicity of some protein **(Kosaka *et al*., 1986)**, (c) Avidin-biotin-peroxidase labelling is diffuse and may lead to artifactual labelling **(Beier, 1992)** and (d) immunogold-silver labelling is more localized but less deeply penetrating into the tissue **(Chan *et al*., 1990)**.

My results show firstly that the number of TH+ve terminals in the striatum of AS (control) rats aged 3 and 12 month (16-17%) are consistent with the figures obtained previously in human (approximately 16%) **(Kung *et al*., 1998)** and rat studies (9-21%) **(Hokfelt, 1968; Tennyson *et al*., 1974; Pickel *et al*., 1981; Descarries *et al*., 1996)**. Secondly, AS/AGU (mutant) rats aged 12 months have a reduction in numbers of TH+ve terminals and in unlabelled terminals and also in the number of synaptic vesicles compared to AS (control) rats. However, in younger, 3-month animals, the reduction is in TH+ve terminals only; TH-ve terminals are unaffected. Several issues are raised by this:

(i) Extracellular dopamine levels are reduced in AS/AGU rats by 80-90% compared to the control (AS) strain **(Campbell *et al*., 1998)**. In 12-month animals studied in the present experiment, the arithmetic sum of a 40% reduction in TH+ve terminals, together with a 40% reduction in vesicle numbers in these TH+ve terminals which remain, could account entirely for this reduction in released DA within the striatum. The figures are equally compelling for AS/AGU animals aged 3 months where there is a 50% reduction in TH+ve terminals and a reduction in vesicles by three quarters.

(ii) The susceptibility of TH+ve terminals in both young and old AS/AGU rats (while TH-ve terminals are only affected in old animals) suggests (a) that dopaminergic neurons are unusually vulnerable to the effect of the PKC-γ mutation but that (b) the eventual reduction in all terminals suggests a very fundamental mechanism underlies terminal loss.

(iii) The zonal figures for synaptic vesicle numbers suggest that the loss is general, is not linked particularly to the readily-releasable in the storage pool, and is probably not linked to a molecularly characteriseable population of vesicles. In particular, there is no evidence that the region closest to the synaptic cleft is devoid of vesicles.

The loss of nigrostriatal dopaminergic neurons that results in a marked reduction of dopaminergic nerve terminals in the striatum is a characteristic post-mortem feature of PD **(Frost *et al*., 1993)** and may be confirmable in living Parkinson's patients **(Leenders *et al*., 1990)**. The loss of dopaminergic terminals in the striatum occurred also in animal models such as MPTP-treated monkeys and baboons **(Herkenham *et al*., 1991; Hantray *et al*., 1993)** and in rats treated with 6-OHDA **(Pickel *et al*., 1992b; Ichitani *et al*., 1994)**.

Reduced vesicle counts have been seen in mice treated with MPTP. Low doses cause axon terminals to swell and show reduced vesicles; with high doses, terminals disappear **(Cochiolo *et al*., 2000)**. Paradoxically, in rats treated with 6-OHDA terminal size and vesicle numbers may increase **(Pickel *et al*., 1992b; Stanic *et al*., 2003)** a finding very much at variance with the present study.

CHAPTER 5

GENERAL DISCUSSION

General Discussion

Each of the three experiments reported in this thesis has its own Discussion section. This General Discussion seeks to place the findings in context by posing a series of questions.

(1) Do the aminergic cells in the substantia nigra and midbrain raphe nuclei have a normal capacity to synthesise their neurotransmitters in the AS/AGU mutant ?

This question arises because of an apparent paradox. It has been previously shown (using in vivo microdialysis of caudate-putamen and HPLC/ECD) that, at all ages, the AS/ AGU rat only possesses 10-20% of the extracellular dopamine levels found in the AS parent strain under normal physiological conditions **(Campbell *et al*., 1998; 2000)**. Conversely, whole tissue micropunches of the caudate-putamen (which will include a wide and heterogeneous variety of cells and cell parts) show no strain differences in dopamine until six months of age, and relatively modest differences thereafter **(Campbell *et al*., 1996; 1997)**. Since the strain difference in whole tissue dopamine levels is of order of 20-30%, whilst the strain differences in released (extracellular) dopamine is of the order of 80-90%, this could suggest either

(a) that there is a fundamental dysfunction of release (as has been proposed **Payne *et al*. 2000**) or

(b) that there is a fundamental deficiency of synthesis such that the relatively normal levels are built up over a lengthy period.

It should be pointed out that these data were obtained from rats of an age where a major decrease in SNC cell numbers would not be expected **(Clarke and Payne, 1994)** - and I used rats of this age range in my own experiments also.

(a) Tyrosine hydroxylase

My results **(Experiment 1)** suggest that there is no deficit in levels of tyrosine hydroxylase within the cell bodies of the substantia nigra pars compacta and, since TH is the rate-limiting enzyme for catecholamine synthesis **(Blanchard *et al*., 1995)**, it is difficult to conclude that there is any deficiency in dopamine synthesis in the mutant strain at the ages tested.

Is the use of a quantitative immunofluorescent technique a suitable one to answer this question? A widely used method of measuring TH activity would involve incubations of homogenized post mortem brain with radiolabelled precursors followed by measurement of labeled catecholamines **(Nagatsu *et al*., 1964; McGeer *et al*., 1971)**. However, this type of approach has drawbacks such as (a) a lack of knowledge of how many catecholaminergic neurons are actually present in the micropunches / culture (and , therefore, the "dilution factors" of other tissue components) (b) ignorance of the possible contribution made by other neurons and glial cells to dopamine synthesis and (c) ignorance of whether these are marked interneuronal differences in synthetic capacity e.g. between healthy and senescent neurons. The method I have used enabled me to sample individual dopaminergic neurons only. The main conclusions are:

i. that, despite being unable to release dopamine at striatal terminals **(Campbell *et al*., 1998)** dopaminergic cell bodies in the SNC of the AS/AGU mutant did not show reduced TH activity. Rather,
ii. they exhibited enhanced TH activity compared to AS controls, suggesting that they were operating a feedback control mechanism and
iii. that, whilst there was variation in TH activity from cell to cell, this was no greater in AS/AGU mutants than in AS controls.

The fact that TH levels are actually increased in the SNC neurons of mutant rats also requires some discussion. The simplest explanation is that it is due to feedback inhibition by the enzymatic end products **(Nagastu *et al*., 1964; Spector *et al*., 1967; Kumer and Vrana,1996)**. In the light of this, it is interesting to note that a reduction in dopamine levels in the striatum increases the ability of pteridine (which is a cofactor for TH) to bind to TH thus decreasing its ability to form a complex with ferric iron and resulting in an increase in TH activity **(Okuno and Fujisawa, 1985,1991; Andersson *et al*., 1988,1992; Haavik *et al*., 1991; Almas *et al*., 1992; Daubner *et al*., 1992; Ribeiro *et al*., 1992)**.

The possible loss of transmission of dopaminergic neurons with age in rats or mice could be due to one or more of the following: a reduction in dopamine release, a change in dopamine synthesis or a loss of dopaminergic cells in the SNC and VTA; it could even be due to a loss of port synaptic receptor density **(McNeill and Koek, 1990; Tatton *et al*., 1991; Della *et al*., 1992; Hamdi *et al*., 1992)**.

Unlike the cell bodies of the SNC, TH levels in the striatum (where nigrostriatal terminals are located) are diminished in AS/AGU rats. This may reflect a decreased activity in dopaminergic terminals themselves, or a decreased activity in cells of the caudate-putamen, some of which are TH +ve **(Tashiro *et al*., 1989)**, or a decreased number of dopaminergic terminals per unit area within the striatum: the results obtained in my **third experiment** (see below) suggest that the latter explanation is the correct one.

In the caudate-putamen, the sampling area is a large rectangle which will be heterogeneous in its content. In such circumstances, an immuno-fluorescent approach is less beneficial than in the case of the mid-brain where individual neurons could be sampled. However, I believe that the benefits of sampling cell bodies and striatum

with the same methodology outweighs this disadvantage (which is, in any case, no greater than that occurring in other techniques such as tissue slice incubation).

Moreover, the fact that a statistically significant difference could be found is indicative of a very real effect, given the "dilution" provided by the vast mass of surrounding cells and neuropil in which the dopaminergic terminals are embedded. Previous studies have shown that reduced dopamine release in the striatum of the AS/AGU rat is accompanied by elevated dopamine metabolites such as DOPAC and HVA **(Campbell *et al*., 1996; 1997; 1998; 2000)**. It has been suggested that this is due to an underlying dysfunction of vesicle formation and packaging, leaving dopamine free in the cytoplasm to be acted upon by mitochondrial monoamine oxidase **(Payne *et al*., 2000)**. An alternative explanation, that there is massive over-production of dopamine at the terminal, which the vesicular packaging system cannot cope with, appears unlikely from my data.

(b) Serotonin

Like dopaminergic neurons, serotonergic neurons in the dorsal raphe are reduced in number (and striatal release greatly impaired) in the AS/AGU mutant **(Al-Fayez *et al*., 2005)**. A loss of serotonergic neurons in the dorsal raphe nucleus **(Jellinger, 2005)** and a reduction in serotonin concentration in the striatum, substantia nigra and hippocampus have been documented in the brains of the Parkinson's patient **(Rinne *et al*., 1974; Scatton *et al*., 1983; Agid *et al*., 1987; Mizuno, 2005)** as has a depletion of 5-HT and its metabolites in the cerebrospinal fluid (CSF) **(Tohgi *et al*., 1993)**. Nevertheless, the present experiment shows that (like the data for TH) the 5-HT content of the cell bodies in the midbrain is higher in AS/AGU than in AS rats. This implies continual activity and (perhaps) a regulatory feedback mechanism. Whilst

end-product inhibition can apply to TH in the synthesis of dopamine, it is not so certain that it applies to tryptophan hydroxylase (TPH) which is the rate-limit enzyme of serotonin synthesis **(Martinez *et al*., 2001; Wang *et al*., 2002)**. In assessing the activity of serotonergic neurons, it is more common to measure 5-HT directly, rather than TPH **(Tappaz and Pujol, 1980; Moret and Briley, 1992; Martinez *et al*., 2001; Wang *et al*., 2002)**.

(2) Are typical Lewy body proteins elevated in the dopaminergic and serotonergic systems of PKC-γ mutant rats?

This question is important for two reasons:

i. in general, whilst Lewy bodies are a major feature of Parkinson's disease **(Pollanen *et al*., 1993; Cornford *et al*., 1995; Forno, 1996; Galvin *et al*., 1997; Shults, 2006)**, rodent models such as 6-OHDA do not show them. Nevertheless, it may be possible to measure levels of the molecules most commonly associated with Lewy bodies to see it if there are elevated.

ii. Because it remains a controversial issue whether the Lewy body and its contents are a harmful, pathological feature of Parkinson's disease, or whether they represent an attempt by the neuron to counteract the degenerative process **(Alves-Rodrigues *et al*., 1998; Bence *et al*., 2001; Chung *et al*., 2001a; McNaught *et al*., 2002; Olanow *et al*., 2004)**. The matter is made more difficult by a lack of information on the normal physiological role of some molecules characteristic of Lewy bodies.

(A) Ubiquitin

The immunofluorescence levels of ubiquitin are higher in the SNC (especially the lateral region) and the dorsal raphe nuclei of AS/AGU mutants compared to AS controls. These are cell groups which are affected in AS/AGU mutant rats (loss of cell numbers in the midbrain and depletion of striatal transmitters) **(Clarke and Payne, 1994; Campbell *et al*., 1997; 1998; Al-Fayez *et al*., 2005)**. Other regions such as SNR, SNL, VTA and MRN are less affected this suggest that, despite the lack of Lewy bodies in rats, elevated ubiquitin is associated with loss of function in very specific cell groups.

Ubiquitin is present in the Lewy bodies in Parkinson's disease **(Lennox *et al*.,1989; Love and Nicoll, 1992)**.

Lewy bodies (neuronal intracytoplasmic inclusion bodies) can be seen in the surviving dopaminergic neurons of the substantia nigra pars compacta in all most every case of PD **(Jellinger, 1987; Hughes *et al*., 1993; Pollanen *et al*., 1993; Cornford *et al*., 1995; Forno, 1996)** and isolated Lewy bodies (presumably the remains of dead neurons) can be found in the neuropil **(Jellinger, 1991; Mackenzie, 2001)**. Lewy bodies can also be seen in other non-dopaminergic neurons such as cholinergic neurons in the nucleus basalis of Meynert, noradrenergic neurons in the locus coeruleus, serotonergic neurons in the raphe nuclei, the motor vagal nuclei, the pedunculopontine nucleus, the Edinger-Westphal nucleus, in the intermediolateral cell column of the spinal cord, the hypothalamus and the autonomic ganglia **(Jellinger, 1991)**. They due therefore not restricted to a transmitter-specific neuron population.

The mechanism by which Lewy bodies are formed and their relationship to neurodegenerative disease, or the ageing process, remains unknown. One hypothesis is that Lewy bodies are harmful. Proteins such as α-synuclein or ubiquitin will

accumulate and aggregate if the ability of cells to degradate these protein is exceeded or if proteasomal function is impaired **(Sherman and Goldberg, 2001)**. Further, aggregated proteins will provide a nucleation center for the formation of inclusion bodies such as Lewy bodies **(Chung *et al*., 2001a)** and the accumulation of these inclusion bodies might in turn induce neuronal dysfunction and/or cell death **(Alves-Rodrigues *et al*., 1998; Bence *et al*., 2001; Chung *et al*., 2001a)**. A quite different hypothesis is that Lewy bodies are beneficial and protective for neurons because they behave structurally and functionally as aggresomes **(McNaught *et al*., 2002; Olanow *et al*., 2004)**. Aggresomes are cytoplasmic inclusion bodies formed at the centrosome (a perinuclear structure linked to the microtubular system) as a cytoprotective response to high levels of misfolded proteins **(Johnston *et al*., 1998; Kopito, 2000)**. Ubiquitin accumulation may suggest a failure of the ubiquitin-proteasome system (UPS) leading to accumulated abnormal proteins as ubiquitin aggregates or inclusion bodies associated with cell degeneration **(Alves-Rodrigues *et al*., 1998)**. Many investigators have assumed that the immunopositive ubiquitin staining in Lewy bodies must represent elevated levels of intracellular ubiquitin-protein conjugates **(e.g. Andersen, 2000)** in both sporadic and hereditary neurodegenerative diseases **(Ciechanover *et al*., 2000)**. However, it must be remembered that most forms of immunocytochemistry do not lend themselves to easy quantitative interpretation. If ubiquitin levels are indeed elevated (and if that elevation is injurious) then a cascade could follow in which a primary failure of UPS leads to aggregated proteins, which could further inhibit the UPS by direct interaction with the proteasome **(Akopian *et al*., 1997; Bence *et al*., 2001)** or by saturating the capacity of one or more molecular chaperones which are required for UPS function **(Bercovich *et al*., 1997)**.

Many factors in the SNC in the midbrain of PD patients could potentially contribute to failure of the UPS these include:

a) high levels of protein oxidation **(Alam *et al*., 1997)**,

b) high levels of 3-nitrotyrosine in the SNC which may indicate excitoxicity **(Good *et al*., 1998)**,

c) reduction in the levels of α-subunits of 20S proteasome and levels of 26S proteasome enzyme activities **(McNaught *et al*., 2001)**,

d) inhibition of complex 1 activity **(Schapira *et al*., 1990a,b; DeMartino and Slaughter, 1999)** since UPS is an ATP-dependent system **(Alves-Rodrigues *et al*., 1998)**,

e) dopamine itself can produce free radicals by auto-oxidation or through metabolism by monoamine oxidase **(Olanow, 1990; Jenner and Olanow, 1998; Jenner, 1998)** and

f) 4-hydroxy-2-nonenal (HNE) is a product of lipid peroxidation present in surviving dopaminergic neurons in PD patients **(Yoritaka *et al*., 1996)**; it leads to enhanced cross-linking of proteins and mediated inhibition of proteasomal function **(Okada *et al*., 1999)**.

In the present study, elevated levels of ubiquitin can be found inside the cell bodies of the SNC and dorsal raphe – cell bodies which appear to remain viable and responsive in terms of their transmitter synthesising capacity (see above), but whose terminals are incapable of releasing physiological normal amounts of DA or 5-HT in the striatum **(Campbell *et al*., 1997; 1998; Al-Fayez *et al*., 2005)**.

(B) Parkin

The immunofluorescence levels of parkin are higher in the cell bodies of both SNC and dorsal raphe neurons in the mutants compared to the controls. Other areas such as SNR, SNL, VTA and MRN are less affected. The affected regions are also those which exhibit loss of neurons and depletion of their neurotransmitters (dopamine and serotonin) in the striatum in AS/AGU mutant rats **(Clarke and Payne, 1994; Campbell *et al*., 1997; 1998; Al-Fayez *et al*., 2005)** and in PD **(Bernheimer *et al*., 1973; Hornykiewicz, 1998; Jellinger, 2005)**.

Parkin staining is characteristically found in the cytoplasm of neuronal cell bodies **(Shimura *et al*., 1999; Gu *et al*., 2000; Horowitz *et al*., 2001)**, in rough endoplasmic reticulum **(Imai *et al*., 2002)**, Golgi apparatus **(Shimura *et al*., 1999)** and also in the nucleus **(Stichel *et al*., 2000; Horowitz *et al*., 2001)**.

In addition, parkin is present in Lewy bodies in the brain of PD patients **(Schlossmacher *et al*., 2002)** except in an autosomal recessive juvenile parkinsonism patients that, interestingly, have a mutation in the parkin gene and no Lewy bodies **(Takahashi *et al*., 1994; Mori *et al*., 1998; Shimura *et al*., 1999; Hayashi *et al*., 2000)**. The ubiquitin-homology domain at the N-terminal of parkin is involved in substrate recognition **(Shimura *et al*., 2000)** and it has importance in multi-ubiquitin formation **(Finley and Chau, 1991)**.

This correlation between ubiquitin and parkin is strengthened by examples of the lack of parkin (e.g. by mutation) leading to accumulation and overexpression of substrate proteins such as Pael-R and CDcrel-1 **(Zhang *et al*., 2000; Imai *et al*., 2001)** and to dysfunction of UPS and the death of dopaminergic neurons **(Dong *et al*., 2003; Yang *et al*., 2003)**.

Moreover, the overexpression of mutated forms of parkin causes oxidative stress and cell death via proteasomal inhibition **(Hyun *et al*., 2002)**.

Like ubiquitin (see above) there are diverse views on the significance of supra-normal levels of parkin within neurons. Thus, overexpression of parkin has been associated with reduced ubiquitinated proteins levels **(Hyun *et al*., 2002)**, parkin may be playing a protective role against apoptotic cell death by delaying mitochondrial swelling and reducing Cytochrome C release **(Darios *et al*., 2003; Feany and Pallanck, 2003; Greene *et al*., 2003)**, parkin may mitigate α-synuclein-induced neuronal cell death **(Yamada *et al*., 2005)** and may have aggresome-like properties **(Ardley *et al*., 2003)**. These proposal would suggest that over-expression of parkin (such as was found in my study) may have a protective role.

(C) Alpha-synuclein

In my study, staining with alpha-synuclein was found in the neuropil of the SNC and striatum. This is to be expected, give that α-synuclein normally has a pre-synaptic distribution **(Lee and Trojanowski, 2006)** except in pathological conditions when it is found in the Lewy bodies **(Spillantini *et al*., 1997)** which neither occur in conventional rat models of PD (e.g. 6-OHDA) **(Betarbet *et al*., 2002; Shimohama *et al*., 2003; Melrose *et al*., 2006)** nor in the AS/AGU mutant (see chapter 2 and above). Alpha-synuclein immunofluorescence levels were higher in the SNC neuropil of the AS/AGU mutants compared to the AS controls rats and levels progressively increase with age in the SNC of both strains. The neuropil of the SNC contains projections from several different regions such as GABAergic neurons from the striatum, globus pallidus and the adjacent SNR **(Ribak *et al*., 1980; Smith and Bolam, 1989; Tepper *et al*., 1995)**, glutamatergic neurons from the prefrontal cortex, subthalamic nucleus

and the pedunculopontine tegmental nucleus (which also provides cholinergic projections) **(Flaherty and Graybiel, 1994; Naito and Kita, 1994; Reese *et al*., 1995; Smith *et al*., 1996; Bezard and Gross, 1998; Blandini *et al*., 2000)** and serotonergic neurons from the dorsal raphe nucleus **(Flaherty and Graybiel, 1994; Blandini *et al*., 2000)**.

There is little doubt that the SNC and its projections are particularly affected in Parkinson's disease **(Bernheimer *et al*., 1973; Spillantini *et al*., 1997; Hornykiewicz, 1998; Sian *et al*., 1999)** and in the AS/AGU mutant rat **(Clarke and Payne, 1994; Campbell *et al*., 1997; 1998)**.

However, the link between α-synuclein, Lewy bodies and toxicity in rodents remains unclear. Transgenic mice that over-express α-synuclein show remarkably little neuropathology **(Matsuoka *et al*., 2001)**. Although, Lewy bodies occur in rotenone-treated mice, they do not seen to occur in rats, nor do they occur with other treatments designed to mimic PD (such as 6-OHDA) **(Betarbet *et al*., 2002; Shimohama *et al*., 2003; Melrose *et al*., 2006)**. The most parsimonious explanation is that α-synuclein levels are not high enough to lead to Lewy body formation, but another factors could be increasing levels of parkin which may prevent the accumulation of α-synuclein in dopaminergic cells in the SNC **(Lo Bianco *et al*., 2004)**. Recently, **Periquet *et al*. (2007)** have shown that some truncated forms of α-synuclein lead reliably to aggregate formation, whereas others do not. In the case of the AS/AGU rat, fluorescence readings show that α-synuclein is not present in the cell bodies in detectable amounts, so no inclusions would be expected.

As alpha-synuclein is not present in cell bodies but in the neuropil of the SNC (with AS/AGU rats having higher levels), there are many possible explanations:

a) alpha-synuclein is present in glial cells and is seen in the form of glial cytoplasmic inclusions in PD **(Arai *et al*., 1999; Piao *et al*., 2000; 2001; Hishikawa *et al*., 2001; Takahashi and Wakabayashi, 2001; Mori *et al*., 2002)** and MSA **(Braak and Braak, 1999; Burn and Jaros, 2001; Wenning and Jellinger, 2005)**.

b) Alpha-synuclein is present in dendrites or axons or blood vessels.

c) Alpha-synuclein is present in the terminals as it is a pre-synaptic protein **(Withers et al., 1997; Lee and Trojanowski, 2006)**. High levels of alpha-synuclein in the terminals leads to:

   i. toxicity **(Conway *et al*., 2000 a,b; Hegde and Jagannatha Rao, 2003; Yamada *et al*., 2004)**.

   ii. Loss of the physiological function of alpha-synuclein **(Rajagopalan and Andersen, 2001)**. Both factors may affect the ability of terminals to carry out their functions (such as the packaging and release of transmitters).

(3). What are the possible mechanisms of death of SNC cells in PKC-γ mutant rats?

This question is discussed because, although my experiments did not examine cell death, they did examine:

a) Cell structure which may give insight into degenerative morphology.

b) Molecules associated with pathological change.

The progressive loss of dopaminergic nigrostriatal neurons in the SNC is a major pathological feature of Parkinson's disease. One of the degenerative mechanisms that

occurs in human SNC cells is apoptosis **(Mochizuki *et al*., 1996; Anglade *et al*., 1997; Tompkins *et al*., 1997; Tatton, 2000)**. A similar mechanism also occurs in laboratory animal models such as treatment with MPTP **(Tatton and Kish, 1997; Spooren *et al*., 1998; Serra *et al*., 2002)**, 6-OHDA (**He *et al*., 2000; Marti *et al*., 2002)** or rotenone and MPP^+ **(Lim *et al*., 2007)**.

The dopaminergic cells of the SNC number approximately 450,000 **(Lang and Lozano, 1998a)**. The process of apoptosis in general takes a few hours to a day **(Barret and Preston, 1994)** and post-mortem studies on the SNC of PD patients suggest a timing of 8 hours **(Anglade *et al*., 1997)**. Whilst it is highly likely to find numerous apoptotic cells in the immediate aftermath of a neurotoxic treatment (MPTP, 6-OHDA), it is highly unlikely that significant numbers of such cells will be found in a naturally-occurring model of cell loss. However, it is possible to use stereological techniques to identify differences in ultrastructure between the dopaminergic cell bodies of mutant AS/AGU rats and the parent AS strain which may suggest decreased viability. Regarding cell organelles, the volume fraction of mitochondria and rough endoplasmic reticulum of the SNC cells is higher in the AS/AGU mutants rats. There are many possible reasons for this observation:

a) The number of mitochondria and RER increase in AS/AGU or decrease in AS rats.
b) The number and size of mitochondria and RER stay the same, but the cell or its cytoplasm shrinks in AS/AGU rats or swells in AS rats.
c) The number of mitochondria and RER stay the same, but the organelles change their size.

Several studies indicate that the mitochondria and rough endoplasmic reticulum are affected in the SNC in neurodegenerative situations:

i. endoplasmic reticulum stress occurs due to the accumulation of mis-folded proteins that interferes with protein degradation **(Friedlander *et al*., 2000)**, and occurs after treatment with MPTP, 6-OHDA and rotenone **(Ryu *et al*., 2002)**. It is known that endoplasmic reticulum stress may, in turn, affect different components of mitochondrial Cytochrome C oxidase leading to mitochondrial dysfunction **(Hori *et al*., 2002)**,

ii. mitochondrial dysfunction is an indication of apoptosis and leads to cell death **(Deckwerth and Johnson, 1993; Vayssiere *et al*., 1994; Petit *et al*., 1995; Zamzami *et al*., 1995 a,b; Liu *et al*., 1996; Schinder *et al*., 1996; Susin *et al*., 1996; White and Reynold, 1996; Ellerby *et al*., 1997)**, and future studies could profitably examine mitochondrial activity, for example, how oxidative stress due to dopamine, plays an important role in sporadic PD **(Mouradian, 2002)** by causing a degenerative cascade in which increasing ROS production and an increase in mis-folded proteins leads to impairment of the ubiquitin-proteasome system, endoplasmic reticulum stress, mitochondrial dysfunction and cell death **(Parker *et al*., 1989; Schapira *et al*., 1990 a,b; Shoffiner *et al*., 1991; Mann *et al*., 1992; Martin *et al*., 1996; Sheehan *et al*., 1997; Bence *et al*., 2001; Nishtoh *et al*., 2002)**.

iii. Furthermore, loss of parkin (which is an ubiquitin ligase) **(Shimura *et al*., 2000))** leads to accumulation of parkin substrate in the endoplasmic reticulum, endoplasmic reticulum stress and cell death **(Imai *et al*., 2001)**.

iv. Other effects of dopamine and its metabolites lead to the loss of oxidative phosphorylation function and mitochondrial swelling that allow the opening of the mitochondrial transition pore (MTP) **(Cohen *et al*., 1997; Kim *et al*., 1999; Berman and Hastings, 1999)**.

AS/AGU rats have smaller neurons and less volume than AS (control) rats within the SNC **(see chapter 3)** which may indicate cell shrinkage. One example of cytoplasmic shrinkage, together with cell membrane budding and chromatin condensation at the nuclear membrane is shown in **figure 3.7 (page 151)**. All these features may be morphological characteristics of apoptosis **(Anglade *et al*., 1997; Jellinger, 2001)**. No signs of necrosis were found in AS/AGU rats.

(4)- How can a mutation in PKC-γ lead to a pathological dysfunction of dopaminergic and serotonergic systems?

The PKC family is thought to be involved in widely diverse functions including modulation of ion channels **(Baraban *et al*., 1985; Madison *et al*., 1986; Shearman *et al*., 1989)**, the desensitization of receptors **(Huganir and Greenyard, 1990)**, modification of neuronal plasticity **(Routtenberg, 1985; Akers *et al*., 1986)**, the enhancement of neurotransmitter release **(Malenka *et al*., 1986; 1987),** vesicle packaging and release **(Tanaka and Nishizuka, 1994; Stevens and Sullivan, 1998)** and cell surface signal transduction **(for review see Nishizuka, 1984 a,b; 1986)**.

The mutation in PKC-γ in the AS/AGU rat must underlie dysfunction of both dopaminergic and serotonergic systems, although it is unclear whether the mutation directly underlies dysfunction of both systems or whether one system is primarily affected and the other secondarily.

Dopamine levels in the striatum are reduced in AS/AGU (mutant) compared to AS (control) rats as measured by micropunches and by microdialysis **(Campbell *et al*.,**

1996; 1997; 1998). The reduction of extracellular dopamine (as measured using microdialysis) is greater than the reduction in whole tissue dopamine levels (as measured by micropunch) suggesting that the main impairment is in the release rather than in synthesis and/or storage, a suggestion which is supported by pharmacological interventions **(Campbell *et al*., 2000)**. In addition, dopamine metabolites are greatly increased in the AS/AGU (mutant) rats **(Campbell *et al*., 1998; 2000)**. One possibility is that dopamine may be free within the cytoplasm of the nerve terminal where it can be metabolized by mitochondrial enzymes such as monoamine oxidase. This is a strong possibility given (a) normal-or increased-TH activity for synthesising DA (b) greatly reduced numbers of vesicles in identified DA terminals in the striatum. Free dopamine can cause inhibition of the dopamine transporter system **(Berman *et al*., 1996)** as well as being cytotoxic through the formation of dopa-quinone **(Graham, 1978; Hastings *et al*., 1996; Stokes *et al*., 1999)** or reactive oxygen species **(Cohen *et al*., 1997)**. Like dopamine, serotonin levels are reduced in several areas of the striatum as well as in the dorsal raphe nuclei. Extracellular levels in the striatum (as measured by microdialysis) are especially reduced, and there is also a reduction in serotonin neuron numbers in the DRN by age 12 months while the MRN is unaffected **(Al-Fayez *et al*., 2005)**. The level of 5-HT metabolites are also elevated in the striatum and midbrain of the AS/AGU (mutant) rats **(Al-Fayez *et al*., 2005)**. If this means that, like DA, 5-HT is free in the cytoplasm, it may also be toxic since it can be metabolized to toxic dimmers and quinone-imines **(Perez-Reyes and Mason, 1981)**. Moreover, the aberrant oxidation of 5-HT is known to occur in conditions such as Alzheimer's disease **(Wong *et al*., 1993)**.

The reduction in the number of TH+ve terminals and synaptic vesicles in the caudate-putamen of AS/AGU mutant rats as seen in **Experiment 3** may explain the reduction

in the levels of extracellular dopamine. This reduction is greater than the loss of dopaminergic cell bodies in the SNC **(Payne *et al*., 2000)** and suggests that the reduction in extracellular dopamine in the caudate-putamen of AS/AGU mutant rats may be due to the failure in dopamine packaging and release. We hypothesize that the mutation of PKC-γ interferes with this process, because the PKC family are involved in vesicles packaging and release **(Tanaka and Nishizuka, 1994; Stevens and Sullivan, 1998)**.

Does the AS/AGU rat continue to be a useful model that give insight into conditions such as Parkinson's disease?

The AS/AGU rat has been suggested as model that gives insight into conditions such as Parkinson's disease **(Petzinger and Jankowec, 2003; 2005; Dev *et al*., 2003b; Huang *et al*., 2004)**. It is a spontaneous animal model with progressive movement disorder and dysfunction of aminergic transmitters release which, together with the parent AS control strain, provides a useful model to investigate the factors that lead to (or prevent) reductions in both dopaminergic and serotonergic function. Previous studies have shown that the *agu* mutation in the gene coding for PKCγ affects both the dopaminergic and serotonergic nigro-striatal systems. The major effects (reduction in striatal transmitter release, elevation in metabolite levels and loss of cell numbers in the midbrain) are the same for both systems and mimic characteristics of PD **(Bernheimer *et al*., 1973; Jellinger, 1990; 2005; Hornykiewicz, 1998; Mizuno, 2005)**. In the present study, I show that, whilst the terminals are clearly dysfunctional, the cell bodies of DA and 5-HT neurons remain relatively normal at these ages, and may well be responsive to depleted DA and 5-HT release. This suggest that the cell bodies may be capable of being rescued by an appropriate therapeutic treatment –

unlike the situation with toxic treatments such as 6-OHDA or MPTP – which gives the AS/AGU rat a positive advantage. Nevertheless, molecules associated with human Lewy bodies (ubiquitin, parkin and α-synuclein) are elevated in the mutant - even though inclusion bodies do not occur. This confirms the similarity of the model to human PD, but may also give insight into the significance of raised levels. The size of nigrostriatal dopaminergic neuron cell bodies are reduced in AS/AGU mutant rats. The number of TH+ve terminals and vesicles are reduced in AS/AGU mutant rats. This demonstrates that dysfunction can stem from a reduction in terminal numbers and/or terminal function – and not necessarily from a reduction in midbrain cell numbers. This is important because cell counts of nigral dopaminergic neurons can only be mode post mortem. It thus remains unclear what stage in the course of the Parkinson's disease cell loss has occurred.

CHAPTER 6

CONCLUSIONS

CONCLUSIONS

Despite being unable to release dopamine and serotonin at striatal terminals **(Campbell *et al*., 1998)** dopaminergic cell bodies in the SNC and serotonergic cell bodies in the DRN of the AS/AGU mutant did not show reduced TH or 5-HT activity. Rather, they exhibited enhanced TH and 5-HT activity compared to AS controls, suggesting that they were operating a feedback control mechanism. Whilst there was variation in TH and serotonin activity from cell to cell, this was no greater in AS/AGU mutants than in AS controls.

Lewy body proteins, ubiquitin and parkin, are elevated in the cell bodies of the SNC and raphe nuclei of AS/AGU rats and increase with age within the SNC area of both AS and AS/AGU rats. Control areas (such as oculomotor and pontine nuclei) are unaffected. Alpha-synuclein does not occur in the cell bodies of the midbrain nuclei, but in the surrounding extracellular tissue. It is elevated in the neuropil of the SNC of AS/AGU rats but other areas are unaffected.

AS/AGU (mutant) rats have smaller SNC neurons than AS (control) rats and their volume was decreased. However, within that overall decreased size, no obvious ultrastructural morphological differences were seen between the two strains.

Both the number of TH+ve terminals and the number of vesicles within them were decreased within the dorsal caudate-putamen of AS/AGU mutant rats aged 3 and 12 months compared to AS controls. In 12 month AS/AGU mutant rats, there are also reductions in the number of non-dopaminergic terminals but there is no evidence of a reduction at 3 months. There is no evidence that the region closest to the synaptic cleft is devoid of vesicles.

The AS/AGU rat provides a useful spontaneous experimental model for studying factors leading to the reduction in dopaminergic and serotonergic function, and for

testing the capability of cell bodies of being rescued by appropriate therapeutic treatments. The elevation in levels of Lewy body-associated molecules (ubiquitin, parkin and alpha-synuclein), even without Lewy bodies being present, confirm the similarity of the model to human PD.

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APPENDICES

Appendix1

Preparing paraformaldehyde fixative:

To prepare 1000 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB):

1) Heat 500 ml of distilled water in a conical flask until water temperature reaches 70º C.
2) Weigh 40 mg of paraformaldehyde then add to the flask.
3) Add concentrated 1% sodium hydroxide solution drop by drop until all is dissolved.
4) Add 500 ml of 0.2 M PB to make 1000 ml.
5) Filter the solution.

Preparing glutaraldehyde fixative:

To prepare 1000 ml of 3% glutaraldehyde in 0.1 M phosphate buffer (PB):

1) Add 1000 ml distilled water to a conical flask.
2) Measure and add 120 ml of 25% glutaraldehyde to the flask.
3) Filter the solution.

Preparing a mixture of 1%glutaraldehyde and 4% paraformaldehyde fixative:

To prepare 1000 ml of mixture of 1% glutaraldehyde and 4% paraformaldehyde

1) Prepare 4% paraformaldehyde using the same procedures as above.
2) Add to it 40 ml of 25% glutaraldehyde.

Preparing mammalian ringer :

Add the following substances to 1000 ml distilled water:

1)	Sodium Chloride	9g.
2)	Potassium Chloride	0.4 g
3)	Calcium Chloride	0.25g
4)	Magnesium Chloride	0.005g
5)	Sodium Hydrogen Carbonate	0.5g
6)	Sodium Dihydrogen Phosphate	0.05g
7)	D-glucose	1g

Appendix 2

Histokinette automatic tissue processor and Wax embedding

1) Remove the specimens from the fixative.
2) Rinse the specimens with 0.1 M PB.
3) Label and place the specimens into a Histokinette basket.
4) The sequence for the Histokinette is as follows:

I.	70% ethanol	(1x2 hours)
II.	90% ethanol	(1x2 hours)
III.	Absolute alcohol	(3x2 hours)
IV.	Amyl acetate	(3x2 hours)
V.	Wax bath	(2x4 hours)

5) Place the specimens into a vacuum embedder for 20 minutes to get rid of air that may be present and allow further penetration of wax within the specimens.
6) Leave to cool for 30 min before sectioning at 7 µm with microtome.

Appendix 3

(A) Staining

Toluidine blue Staining

1) Place slides in histoclear for 10 minutes.

2) Hydration

First Absolute Alcohol	1 min
Second Absolute Alcohol	1 min
90% Alcohol	1 min
70% Alcohol	2 min

3) Wash in water.

4) Place slides in 1% aqueous toluidine blue for 30 seconds.

5) Wash in water.

6) Dehydration

70% Alcohol	1 min
90% Alcohol	1 min
First Absolute Alcohol	1 min
Second Absolute Alcohol	2 min

7) Place in Histoclear for 10 minutes.

8) Mount in Histomount.

Appendix 3

(B) Immunocytochemistry on wax sections

1) Cut and mount the sections on slides coated with APES.
2) Place the slides into a 37º C oven overnight.
3) Place the slides into a 56º C oven for 2 hours.
4) Boil 1600 ml of 0.01 M sodium citrate buffer (pH 6.0) in a stainless steel pressure cooker.
5) Position the slides onto metal staining racks and lower into the pressure cooker, ensuring the slides are totally immersed in citrate buffer solution, and close the lid.
6) When the pressure cooker reaches its operating temperature (120º C) and pressure (103 kPa/ 15 PSI) (after about 4 minutes) start a timer for 1 minute.
7) Remove the pressure cooker from the heat source and run cold water until the pressure decreases.
8) Place the slides into distilled water.
9) Place the slides into PBS (1x5 min).
10) Place the sections into 1.5% H_2O_2 for 10 minutes.
11) Rinse the sections in distilled water (2x5 min), followed by PBS (2x5 min).
12) Circle the individual sections with a wax pen, cover sections with blocking serum for 60 minutes and incubate overnight in primary antibody diluted in blocking serum.
13) Rinse the sections in PBS (3x5 min).

14) Incubate the sections in biotinylated secondary antibody for 60 mins. (For a fluorescent secondary antibody the sections were incubated for 24 hours, then rinsed in distilled water (3x5 mins). Proceed to step 21).
15) Rinse the sections in PBS (3x5 min).
16) Incubate the sections in ABC complex for 60 mins
17) Rinse the sections in PBS (3x5 mins).
18) Rinse the sections in 0.1M PB (1x5 mins).
19) Incubate in DAB solution for 2-5 mins.
20) Rinse the sections in 0.01M PB (2x5 mins).
21) Dehydrate, clear and mount.

Appendix 4

Solutions

1) 0.01M Sodium Citrate buffer (pH 6.0)

Add 3.84g of Citric acid (anhydrous) to 1.8 L of distilled water. Adjust to pH 6.0 using concentrated NaOH. Add 200 ml to make up 2 L.

2) 0.01 M Phosphate Buffered Saline (PBS)

Add 36g NaCl to 200 ml Phosphate buffer and add 1800 ml distilled water.

3) Phosphate buffer (PB)

(A) To make 0.2 M PB, Add 17.47g Sodium Dihydrogen Phosphate and 40.75g di-Sodium hydrogen Phosphate to two litres of distilled water.

(B) To make 0.1 M PB, add one litre of distilled water to one litre of 0.2 phosphate buffer.

4) Blocking serum

Add 10µl of 1% normal goat serum (NGS) to 990 µl of 0.3% Triton in PBS.

5) 0.3%Triton X-100 in PBS

Add 30µl Triton to 10 ml of PBS.

6) Primary antibody in antiserum diluent

1:500 Add 1µl of primary antibody to 0.5 ml blocking serum.

1:1000 Add 1µl of primary antibody to 1ml blocking serum.

7) Biotinylated secondary antibody

Add 5µl secondary antibody to 1ml blocking serum.

8) Fluorescent secondary antibody

Add 1µl secondary antibody to 0.1 ml PBS.

9) ABC Reagent

Add 20µl reagent A and 20µl reagent B to 1ml PBS. Mix well immediately and allow to stand for at least 30 minutes.

10) DAB Solution

1) Add 2 drops of Buffer Stock Solution to 0.5 ml of distilled water. Mix well.

2) Add 4 drops of DAB Stock Solution. Mix well.

3) Add 2 drops of Hydrogen Peroxide Solution. Mix well.

4) Add 2 drops of the Nickel Solution (to achieve a gray-black staining). Mix well.

Appendix 5

Fluorescence quantification using Leitz Laborlux S Fluorescence microscope (Figure 2)

1) Focus the square on specimen with UV light on.

2) Press **A,** adjust display reading to zero with knob **JO**.

3) Press **I** and record value from display.(Note, all values read negative).

4) Press **A** once. This resets display.

5) Press **A** again, zero should appear in the display.

6) Focus the square again (Remember to take background readings).

7) Press **I** and record new value.

8) Repeat procedure.

Points to note

1) If more sensitivity is required turn from

   x1 to x10
   or
   x10 to x100

2) If increased sensitivity is used either (x10 or x100 which is very sensitive) than remember that the background reading will also be higher. Take new background reading.
3) If values are higher than 100 a blank reading shows in the display. It is then necessary to decrease sensitivity.

Appendix 6

Processing tissue for Transmission electron microscopy

1) Rinse in 0.1 phosphate buffer (3x5 mins).
2) Osmicate with 1% osmium tetroxide in agitator 20 mins .
3) Rinse in a distilled water (3x30 mins).
4) Dehydrate:

   70% Acetone 15 mins

   90% Acetone 15 mins

   100% Acetone (4x15 mins)
5) Mixture of 75% Acetone and 25% Durcupan 2 hours.
6) Mixture of 50% Acetone and 50% Durcupan 2 hours.
7) Mixture of 25% Acetone and 75% Durcupan 2 hours.
8) 100% Durcupan overnight.
9) 100% Durcupan 3 hours.
10) Embed in durcupan and leave overnight in oven at 70^{o}C.
11) Stick embedded tissue onto the end of blank embedding block and leave in oven at 70^{o}C for at least 30 mins.

Durcupan resin

1) Add 10g of Durcupan (Durcupan, ACM Fluka, 44611, Netherlands).
2) Add 10g of DDSA (dodecenyl succinic anhydride, R1052, Agar Scientific LTD, UK).
3) Add 0.3g of DMP-30 (2,4,6-Tri-dimethylaminomethyl phenol, R1065, Agar Scientific LTD, UK).
4) Add 0.3g of Bibutyl Pthalate (R1071, Agar Scientific LTD, UK).
5) Mixed and leave for at least 30 min in oven.

Appendix 7

Pre-embedding immunocytochemistry

1) Incubate the sections in blocking solution for 30 minutes.

2) Rinse the sections in washing buffer for 5 min.

3) Incubate the sections overnight in primary antibody in incubation buffer.

4) Rinse the sections in washing buffer (3x10 min).

5) Incubate the sections in 1nm goat anti-mouse immunogold (1/50) in incubation buffer for 4 hours.

6) Rinse the sections in washing buffer (3x15 min) and in PBS (3x5 min).

7) Postfix the sections with 2% glutaraldehyde in PBS for 10 min.

8) Rinse the sections in distilled water (2x5 min).

9) Prepare the silver enhancement solution by mixing equal parts of Enhancer and Initiator of the IntenSE M kit just before use. Apply at least 4 drops of silver enhancement mixture on the sections for 10-15 min.

10) Rinse the sections in distilled water (3x5 min).

11) Dehydrate in acetone and embed in Durcupan.

Solutions

Blocking solution

0.8 % Bovine Serum Albumin (BSA), 0.1% gelatin IGSS, 5% normal goat serum (NGS) and 2mM NaN_3 in PBS (pH 7.4).

Washing buffer

0.8 % BSA , 0.1% gelatin IGSS and 2mM NaN_3 in PBS (pH 7.4).

Incubation buffer

0.8 % BSA , 0.1% gelatin IGSS, 1% NGS and 2mM NaN_3 in PBS (pH 7.4).

Appendix 8

The primary antibodies used in immunocytochemistry

Molecules	Primary antibody	Types	Concentration	Manufacturer
α-synuclein (ASN)	Rabbit anti-ASN	Polyclonal	1: 100	* Kind gift from Dr.Poul Henning
Parkin	Rabbit anti-parkin	Polyclonal	1: 100	* Kind gift from Dr.Poul Henning
Serotonin (5HT)	Rabbit anti-5HT	Polyclonal	1: 500	AFFINITI, UK
Tyrosine hydroxylase (TH)	Mouse anti-TH	Monoclonal	1:500	Chemicon Europe Ltd
Ubiquitin (Ub)	Rabbit anti-Ub	Polyclonal	1:500	DAKO Ltd

* Dr.Poul Henning. Institute of Medical Biochemistry, University of Aarhus, Denmark.

The secondary antibodies used in immunocytochemistry

(A) Fluorescence

Secondary antibody	Concentration	Fluorescence	Manufacturer
Anti-mouse	1: 100	Fluorescein (green)	Jackson ImmunoResearch
Anti-mouse	1: 100	Rhodamine (Red)	Jackson ImmunoResearch
Anti-rabbit	1: 100	Fluorescein (green)	Jackson ImmunoResearch
Anti-rabbit	1: 100	Rhodamine (Red)	Jackson ImmunoResearch

(b) Biotinylated

Secondary antibody	Concentration	Manufacturer
Anti-mouse	1: 200	Vector Laboratories
Anti-rabbit	1: 200	Jackson ImmunoResearch