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# Raphe-Striatal Serotonergic System in the AS/AGU Mutant Rat


**Musaed Al-Fayez**

**MSc UCL (LOND.)**


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Laboratory of Human Anatomy

Faculty of Medicine

University of Glasgow


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# DECLARATION

I declare that all of the work in this thesis is original and carried out by my self.

Musaed Alfayez

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# Publications

Deficiencies in the raphe-striatal serotonergic system of the AS/AGU rat. Al-Fayez M.A.S. , Russell D., Campbell J.M., Favor-Ankersen G., Baker P.J. , Gilmore D.P., Stone T.W., Davies R.Wayne, Sutcliffe R.G.and Payne A.P.. International Parkinson's Disease Symposium, Heklsinki, July 2001. (Parkinsonism & Related Disease, volume 7, S34)

The raphe-striatal serotonergic system is depleted in the AS/AGU mutant rat. M.A.S.Al-Fayez, D.Russell, J.M.Campbell, G. Favor-Ankersen, P. J. Baker , T.W.Stone, R.Wayne Davies, R.G.Sutcliffe, D.P.Gilmore and A.P.Payne. IUPS Meeting, Christchurch September 2001

An ideal animal model of age-progressive degeneration of dopaminergic neurones: the AS/AGU rat. Russell D., Al-Fayez M.A.S. , Campbell J.M., Gilmore D.P., Craig, N.J., Duran-Alonso, M.B., Shiels, P. Hawker, K.L., Glencorse, T.A., Stone T.W., Sutcliffe R.G. Davies R.Wayne and Payne A.P.. American Parkinson's Disease Society Meeting, San Diego, November 2001.

Aminergic deficits in a protein kinase C-gamma rat mutant. Al-Fayez M.A.S. , Russell D., Campbell J.M., Favor-Ankersen G., Baker P.J. #, Gilmore D.P., Stone T.W., Davies R.Wayne, Sutcliffe R.G.and Payne A.P.. Anatomische Gesellschaft Meeting, Dresden, March 2003. (Annals of Anatomy, 185, p 217-218)

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-HIAA | 5- Hydroxy-indole-acetaldehyde |
| 5-HT | 5-Hydroxytryptamine |
| 5-HTP | 5-Hydroxytryptophan |
| 6-OH-DA | 6-Hydroxydopamine |
| ACSF | Artificial Cerebrospinal Fluid |
| AMPA | Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate |
| AS | Albino Swiss |
| AS/AGU | Albino Swiss/Anatomy Glasgow University |
| CNS | Central Nervous System |
| DHBA | Dihydroxybenzylamine |
| DOPAC | 3,4-Dihydroxyphenylacetic Acid |
| EDTA | Diaminoethanetetra-acetic Acid |
| EPN | Entopenduncular Nucleus |
| GABA | Gamma-Aminobutyric Acid |
| GP | Globus Pallidus |
| Gpe | External Globus Pallidus |
| Gpi | Internal Globus Pallidus |
| HBN | Lateral Habenular Nucleus |
| HD | Huntington's Disease |
| HPLC-ECD | High Performance Liquid Chromatography with Electrochemical Detection |
| HVA | Homovanillic Acid |
| L-AAAD | L-Aromatic-amino Acid Decarboxylase |
| L-DOPA | L-3,4-Dihydroxyphenylalanine |
| MAO | Monoamine Oxidse |
| MDMA | Methlyene-dioxy-methamphetamine |
| MPTP | 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine |
| MSA | Multiple System Atrophy |
| NMDA | N-methyl-D-aspartate |

| | |
|---|---|
| PCA | π-Chloromamphetamine |
| PCPA | p-Chlorophenylalanine |
| PD | Parkinson's Disease |
| PKCγ | Protein Kinase C gamma |
| PPN | Pendunculopontine Tegmental Nucleus |
| PSP | Progressive Supranuclear Palsy |
| RF | Response Factor |
| SC | Superior Colliculus |
| SN | Substantia Nigra |
| SNpc | Substantia Nigra Pars Compacta |
| SNpr | Substantia Nigra Pars Reticulata |
| STN | Subthalamic Nucleus |
| Thal | Thalamus |
| VM-VL | Ventromedial/Ventrolateral Complex |
| VTA | Ventral Tegmental Area |

# ABSTRACT

The AS/AGU rat is a spontaneous recessive mutation which arose in a closed colony of Albino Swiss (AS) rats. It is characterised by disordered locomotion including a wide gait, whole body tremor and frequent falling over in continuous locomotion. It has been demonstrated that there is a substantial dysfunction of the nigro-striatal dopaminergic system which begins with a marked reduction in dopamine release in the striatum, followed by a reduction in whole tissue dopamine levels and, finally, a loss of dopaminergic cells.

The mutation has recently been shown to be a stop codon in the gene responsible for the activity of protein kinase C (PKC) -γ. Although few specific functions are known for this particular isoform (and those that are appear to cover widely disparate types of CNS activity), the PKC family in general has a role in transmitter packaging and release, as well as in ion channel modulation and receptor sensitivity. It is very likely, therefore, that the mutation will affect other transmitter systems in addition to the dopaminergic system already investigated. Furthermore, all human neurodegenerative conditions which combine disordered locomotion with basal ganglia and aminergic dysfunction have shown evidence of effects on several transmitter sysems.

This study was undertaken, therefore, to look at the integrity and functioning of the raphe-striatal serotonergic system in the mutant, using the parent strain as a control. This is an exciting model, since any differences found must stem directly or indirectly from a single point mutation.

Initial experiments were carried out to verify a) the nature of the mutation and b) the connectivity of the dorsal and median raphe nuclei to the striatum in these strains. Immunocytochemical investigations of the AS wild-type showed positive staining for PKC-γ in many parts of the brain, including cerebral and cerebellar cortices, striatum, substantia nigra and raphe regions; no staining could be found in the AS/AGU mutant. This confirms the identification of the gene mutation.

Injections of the tracer cholera toxin-B were made into the dorsal caudate-putamen (a region of the basal ganglia found to be particularly affected in previous work on the dopaminergic system). Cells of the dorsal raphe nucleus (but not the median raphe nucleus) were found to be retrogradely labelled. Labelling ocurred throughout the dorsal raphe nucleus, with peak cell numbers occurring in the centre of the rostral-caudal distribution of the nucleus. Approximately a third of the cell bodies were positively stained for CB-T. This confirms that the dorsal caudate-

putamen receives a strong afferent input from the dorsal raphe nucleus, but that the median raphe does not project to the striatum.

Three complimentary experiments were carried out to examine the integrity of the raphe nuclei and their serotonergic innervation of the striatum.
Firstly, the AS/AGU mutants were found (by conventional and stereological counting methods) to have reduced numbers of 5-HT-ir cells in the dorsal raphe nucleus compared to the wild-type. The reduction was some 20-25%. No such loss was found in the median raphe nucleus.
Secondly, experiments were carried out to assay serotonin, dopamine and their metabolites in post mortem micropunches of several brain regions using HPLC-ECD methods. Serotonin and dopamine were both significantly reduced in several parts of the caudate-putamen, as well as (respectively) the dorsal raphe nucleus and substantia nigra. By contrast, metabolite levels such as 5-HIAA and DOPAC were elevated. This suggests that both transmitter systems are affected as a result of the mutation, but that the deficit is in transmitter release rather than synthesis.
To test the effects of the mutation on release, microdialysis experiments were carried out with indwelling cannulae in the dorsal caudate-putamen which sampled the extracellular fluid. HPLC-ECD with a highly sensitive Intro detector showed that extracellular levels of both 5-HT and DA were extremely reduced in mutants compared to the parent strain but, again, metabolite levels were higher. Previous studies on dopamine have interpreted this as the result of dopamine being free within the terminal cytoplasm and a similar phenomenon could account for the present findings on serotonin.

Micropunch studies also revealed that administration of 5-HTP led to increased 5-HT and 5-HIAA levels in both striatum and raphe nuclei. Similarly, L-DOPA administration led to increased DA and DOPAC levels. A further finding, namely that administration of L-DOPA could reduce 5-HT levels, while administration of 5-HTP could elevate DA levels, points to important interactions between the two systems.

Since both AS and AS/AGU rats showed expected changes in 5-HT levels with 5-HTP administration, it was decided to use a simple behavioural test - total locomotion per unit time as recorded with an automated infra-red system - to see if 5-HT manipulation could affect locomotion in the two strains. In both strains, 5-HT depletion by pCPA reduced locomotion, while augmentation by 5-HTP increased it; saline administration was neutral. The percentage

changes were largely similar between the two strains, although mutants responded more positively to 5-HTP administration than controls.

The results demonstrate that the raphe-striatal serotonergic system of the AS/AGU rat is compromised in the same way as the nigro-striatal dopaminergic system. Because the two systems are inter-linked morphologically and physiologically, it is unclear whether the mutation exerts a direct affect on each system separately or whether one system (or each system) affects the other. The results continue to demonstrate that the symptoms of the AS/AGU rat and its neural deficiencies make it a useful animal to study human conditions involving cell loss, movement and the basal ganglia.

# 1 General Introduction

## 1.1 Introduction

The AS/AGU rat is a spontaneous recessive mutation with a stop codon in the gene for protein kinase C (PKC)–gamma and which exhibits locomotor dysfunction. The dopaminergic nigrostriatal system has already been shown to be affected by this mutation. Because the PKC family of enzymes may be involved in the packaging or release of neurotransmitters in vesicles, and because other aminergic neurons may also be involved in the basal ganglia and their disorders, I have investigated the raphe-striatal serotonergic system in these mutants and the parent AS strain.

So I intend to write in my introduction about the basal ganglia, movement disorders, aminergic input to the basal ganglia, models of neurodegenerative disorders such as Parkinson's disease and finally about the AS/AGU rat as a valuable model of neurodegenerative disease.

## 1.2 Basal ganglia

The basal ganglia are among the most complex and least understood structures in the mammalian forebrain (for major reviews see Alexander et al.,1986; Parent, 1990; Parent and Hazrati, 1995; Crossman, 2000; Pollack, 2001; Baev et al., 2002; Jose et al., 2002; Lehericy and Geradin, 2002; Yelnik, 2002; Wichmann and DeLong, 2003) The definition of 'basal ganglia' is subject to some variation, but key structures include the corpus striatum (caudate nucleus, putamen and globus pallidus), subthalamic nucleus and the substantia nigra. These subcortical nuclei interconnect and their afferent and efferent connections extend throughout the telencephalon, diencephalon and midbrain. Together with motor cortical areas and the cerebellum, the basal ganglia have an important role in motor integration whereby sensory information modulates the timing and trajectory of movement to produce both

accurately aimed and smoothly executed actions. These ideas came initially from observation in Parkinsonian patients where damage to the basal ganglia not only caused a slowing of voluntary movement but also frequently resulted in uncontrollable involuntary movements.

The neostriatum consists of the caudate nucleus and putamen; these are separate components in the primate brain, but united in rodents. They form the input nuclei of the basal ganglia, receiving almost all of the afferent information from the cerebral cortex. The globus pallidus has two segments, internal and external (sometimes called medial and lateral segments respectively). The subtantia nigra is located within the midbrain and has two cytologically distinct zones. The first, known as the pars reticulata, is located ventrally and is distinctly pale in nature and the second is a more dorsally-sited pigmented zone known as the pars compacta. It is this region which in humans gives the substantia nigra its characteristic dark appearance in post mortem specimens and is made up of neuromelanin-containing dopaminergic neurons. The internal segment of the globus pallidus and the substantia nigra pars reticulata are sometimes considered to be a single structure due to the similarities they share in cytology, connectivity and function. In fact these structures form the major output for the entire basal ganglia, directing information through the thalamus and on to the premotor, motor and prefrontal cortices. One of the major differences between the basal ganglia and other motor systems is that they don't make any direct connections with the spinal cord. In contrast, the basal ganglia contain several structurally and functionally distinct closed circuits (loops) connecting the cerebral cortex, basal ganglia, thalamus and cortex again. At least five circuits are thought to exist, all of which are organised in parallel and segregated from each other in that they engage different regions of the basal ganglia and thalamus and their output is centred on different areas of the frontal lobe.

### 1.2.1 The basal ganglia- circuity and parallel processing

The basic basal ganglia circuitry with inhibitory and excitatory connections is shown in figure 1. Different regions of cerebral cortex reach the striatum by excitatory glutamatergic projections (Young et al., 1981). Early studies using autoradiography showed 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). This helped form the theory that input from different cortical areas is segregated and remains so throughout the neostriatum. The premotor and motor cortices also send excitatory afferents to the subthalamic nucleus which is the only other structure within the basal ganglia receiving a direct cortical input (Afsharpour, 1985; Stanton et al., 1988). The striatum also receive afferents from the substantia nigra pars compacta which are mainly dopaminergic fibres (Moore et al., 1971) and there is a possibility that the centromedian-parafascicular nuclei of the thalamus give an indirect cortical input (Beckstead, 1984).

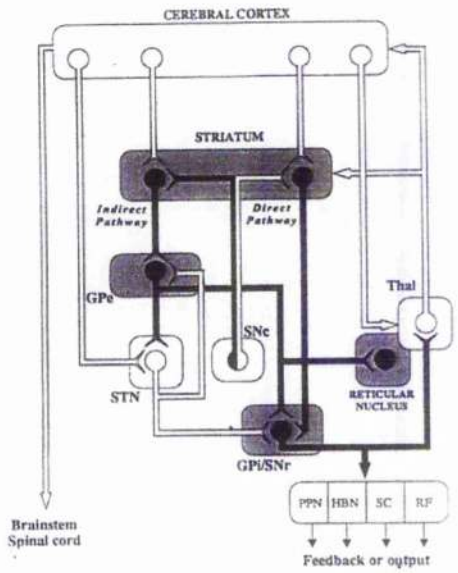



**Figure 1.1 An updated version of the basal ganglia circuitry originally represented by Alexander and Crutcher (1986), showing the connectivity of the direct and indirect pathways of the basal ganglia. Inhibitory projections are shown as filled arrows, excitatory projections as open arrows. Gpe = external segment of the globus pallidus; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; STN = subthalamic nucleus; Thal = thalamus; PPN = pedunculopontine tegmental nucleus; HBN = lateral habenular nucleus; SC = superior colliculus; and RF = reticular formation.**

The basic circuit contains a 'direct pathway' to the output nuclei (the internal segment of the globus pallidus and the substantia nigra pars reticulata) and an 'indirect pathway' (via the external segment of the globus pallidus and the subthalamic nucleus). The direct pathway from the striatum consists of GABAergic and substance P-containing neurons and is an inhibitory efferent pathway whereas the indirect pathway reaches the output nuclei via GABAergic and enkephalin-containing striatal projection neurons. These form the first part of the pathway innervating the external segment of the globus pallidus which in turn projects to the subthalamic nucleus via purely GABAergic neurons (Albin et al., 1989; Jose et al., 2002).

The final part of the indirect pathway which connects the subthalamic nucleus to the output nuclei is an excitatory glutamatergic projection (Nakanishi et al., 1987; Smith and Parent, 1988). These two striatal efferent systems have opposing effects on the

basal ganglia output nuclei and subsequently upon the thalamic nuclei each of the circuits innervate. The striatum has another efferent pathway this time innervating the substantia nigra pars compacta. This is thought to consist of GABA and substance P neurons as in the case of the direct pathway. The output nuclei project via an inhibitory GABAergic pathway to the thalamic nuclei (Penney and Young, 1981; Chevalier et al., 1985).

Both output nuclei have a high level of spontaneous basal activity which exerts an inhibitory effect on the thalamic nuclei and is modified by the opposing effects of the direct and indirect pathways. There are two possibilities as to how this parallel processing affects movement. Firstly, the direct and indirect pathways may both innervate the same set of output neurons. This could cause a 'smoothing' of the cortically-initiated motor pattern which is reinforced by the direct pathway. A second possibility is that the two pathways are directed to different output nuclei neurons, resulting in reinforcement of a selected motor pattern via the direct pathway while the indirect pathway filters out the conflicting patterns (Alexander and Crutcher. 1990c).

### 1.2.2 The compartmental organisation of the striatum

Regular Nissl staining of the striatum shows a largely homogeneous mass. With the benefit of techniques such as retrograde tracing, histochemistry, immunohistochemistry and histopharmacology, two levels of compartmental organisation emerged in the striatum: patch-matrix compartments, and the organisation of separate striatopallidal and striatonigral systems.

Striatal patch-matrix systems are demonstrated and determined by specific neurochemical markers and also by underlying neuronal connections. The patch compartment is defined by μ-opiate binding (Herkenham and Pert, 1981), and by low expression of acetylcholinesterase (Butcher and Hodge, 1976). The term striosome

("striatal body") was first used by Graybiel and Ragsdale (1978), to describe these areas of low cholinesterase activity. Five-nucleotidase (Schoen and Graybiel, 1992) was also found to be an excellent marker for striosomes in the rat.

The matrix compartment can be defined by staining for the calcium binding protein calbindin, and by the immunoreactivity of somatostatin positive fibres (Gerfen et al., 1985). Patch-matrix organisation is predominantly found in the caudate-putamen, extending into the dorsolateral and ventromedial areas.

In the rat, it was observed that compartmental organisation of cortico-striatal afferents was related to their laminar origin rather than to their cortical areas of origin (Gerfen, 1989), with different cortical laminae innervating patch and matrix compartments. Thus corticostriatal neurons in infragranular layers project principally to patches, with the matrix being the main recipient of axons from supragranular layers. A similar pattern exists in primates but is complicated by the fact that allocortical areas have a higher concentration of corticostriatal neurons arising from infragranular layers, whereas in neocortical areas, supragranular corticostriatal neurons are more numerous (Arikuni and Kubota, 1986). These corticostriatal projections terminate principally on the dendrites of medium spiny projection neurons where they have an excitatory effect due to glutamate. These findings are consistent with studies that show the developmental relationship between medium spiny neurons of the patches and matrix, and neurons of deep and superficial cortical laminae respectively (Fishell and Van der Kooy, 1987).

If striatal medium spiny neurons can be divided into two populations (patch and matrix) that have a relationship with the laminar (Gerfen, 1989) and regional (Donoghue and Herkenham, 1986) organisation of the cortex, they can also be characterised by their respective projections to globus pallidus, entopenduncular nucleus (EPN) and substantia nigra, with striatopallidal neurons arborizing within the

GP, and striatonigral neurons extending to the EPN and SN (Parent et al., 1984). Gerfen (1992) hypothesised that striatopallidal projections to many cholinergic and substance P-expressing areas of the ventral pallidum originate largely in the patch striatal area, whereas matrix neurons tend to project to the predominantly GABAergic enkephalin-containing neurons.

Tracing techniques *in* rats have shown that although both types of neurons project to the substantia nigra, patch neurons project to the dopaminergic cell groups in the SNpc, and cell islands in the SNpr, whereas matrix neurons project to areas in the SNpr containing GABAergic neurons (Gerfen, 1984; 1985).

### 1.2.3 The basal ganglia-motor circuit

The cortico-striato-thalamo-cortical loop described above is not unitary. Indeed, there appear to be several loops eg. motor, oculomotor, associative, limbic and orbitofrontal.

One of the most interesting circuits is the motor circuit which has received a lot of attention. In this circuit the putamen receives the majority of the information entering the basal ganglia from the cerebral cortex, particularly from the primary motor and sensory cortices, the arcuate premotor area and the supplementary motor area (Kunzle, 1977; Selemon and Goldman-Rakic, 1985). This information is somototopically organised into distinct zones i.e. there are separate zones for the arm and leg etc. (Kunzle, 1975; Crutcher and De Long, 1984; Alexander and De Long, 1985). Topographic projections from the putamen reach specific areas of the internal and external segment of the globus pallidus and the substantia nigra pars reticulata (Johnson and Rosvold, 1971; Parent et al., 1984). The degree of specificity amongst these projections is still maintained at the level of the thalamic nuclei where connections are made with the nucleus ventralis lateralis pars oralis, lateral nucleus

ventralis anterior pars parvocellularis, lateral nucleus ventralis anterior pars magnocellularis and centromedian nucleus (De Vito and Anderson, 1982; Kinsky et al., 1985). These nuclei in turn close the circuit with thalamocortical projections to the supplementary motor area, the premotor cortex and the motor cortex (Wiesendanger et al., 1985; Matelli et al., 1989).

The role of the basal ganglia in normal movement in primates remains unclear. A large proportion (30-50%) of neuronal activity within the supplementary motor area, motor cortex, putamen and both segments of the globus pallidus appears to code for the direction of limb movement (Mitchell et al., 1987; Crucher and De Long 1984; Alexander and Crutcher, 1990a) and there are individual cells which are direction-dependent. However, there are also cells which are range-dependent and ones which respond to complex variables such as movement linked to reward. Many studies have shown that the motor circuit is involved in movement planning with neuronal discharge when an instructional or anticipatory stimulus was presented to the animal (Alexander and Crutcher, 1990a; Wise et al., 1983; Tanji and Kurata, 1985; Georgopoulos et al., 1989). Movement preparation is thought to be mediated through a separate subchannel from movement execution and is believed to occur in each of the somatotopic motor circuit channels. Other experiments have demonstrated additional organisation within the somatotopic channels where further subchannels are thought to be specific for target location, limb trajectory/kinematics and muscle activity pattern. These processes are all thought to occur in parallel (Alexander and Crutcher, 1990a).

## 1.3 Movement disorders

Although there is uncertainty regarding the normal function of the basal ganglia, there is better understanding of dysfunction in the basal ganglia which can result in

many movement disorders. These disorders can be classified into two categories; hypokinetic and hyperkinetic. Many of them involve the amincrgic systems.

### 1.3.1 Multiple system atrophy

Multiple system atrophy (MSA) is a term introduced by Graham and Oppenheimer (1969). MSA is sporadic degenerative disease characterised by a combination of Parkinsonism, autonomic and cerebellar dysfunction and pyramidal signs (see major reviews by Wenning et al., 1995; Kaufmann, 1998).

Pathologically there are varying degrees of cell loss and gliosis principally affecting the substantia nigra and putamen (striatonigral degeneration), pontine nuclei, cerebellar Purkinje cells and inferior olives (olivopontocerebellar atrophy), intermediolateral cell columns of the thoracic spinal cord, and Onuf's nucleus in the sacral cord. Symptoms include ataxia, rigidity and general autonomic failure as well as problems with balance, coordination and speech.

In striatonigral degeneration, treatment with levodopa was found to be effective. Data from PD brain banks showed that up to 10% of those brains from patients diagnosed with PD turn out to have MSA (Kaufmann, 1998).

### 1.3.2 Progressive supranuclear palsy

Progressive supranuclear palsy (PSP) is a hypokinetic disease which predominantly occurs in men, with an onset of disease in middle to late life. The duration of the condition until death is relatively short (5.3 years).

The main symptoms of the disease are pronounced imbalance with frequent falls, bradykinesia, rigidity, 'pure akinesia' (Mastsuo et al., 1991; Imai et al., 1986),

vertical gaze palsy and dementia. Treatment with L-dopa has no or minimal effect (Collins et al., 1995).

Nigrostriatal dysfunction is the main cause of PSP with an 80-90% decrease in dopamine, tyrosine hydroxylase and D2 receptors in the striatum (Russell, 2001).

### 1.3.3 Corticobasal degeneration

There are similarities between corticobasal degeneration, idiopathic Parkinson's disease and PSP (Bergen et al., 1996).

Clinically, corticobasal degeneration is characterised by an asymmetrical akinetic-rigid syndrome, cognitive loss (apraxia and aphasia) and extrapyramidal motor dysfunction (rigidity and dystonia). This disease is very rare and dementia can be seen in late stages of the disease.

Neuropathologically, this disease is characterised by severe neuronal loss and gliosis in the cerebral cortex with the presence of swollen, achromatic, tau positive staining Pick cells, atrophy in frontoparietal cerebral cortex, loss of dopaminergic cells and gliosis in the substantia nigra. Like PSP, it is poorly responsive to L-dopa treatment (see review Watts et al, 1995).

### 1.3.4 Parkinson'disease

One of the most documented hypokinetic disorders is Parkinson's disease. This disease is more common with advancing age and affects approximately 1 per cent of the population over 65. The average duration from diagnosis of Parkinson's disease to death is thirteen years.

The main symptoms include akinesia, which is difficulty initiating movement, bradykinesia, which is a slowing of movement, muscular rigidity (commonly

referred to as cog-wheel rigidity), flexed posture and a postural tremor that is usually worse at rest. These features were found to be due to a selective striatal dopamine deficiency in the early 1960's though this has been more accurately described as degeneration of the nigrostriatal dopaminergic neurons (Hornykiewicz, 1966; Bernheimer et al., 1973). An important association occurs between Parkinson's disease and elderly dementia with the frequency being 6.6 times higher compared to non-Parkinsonian-patients (Mayeux et al., 1990). Many of the clinical, pathological and biochemical features of human Parkinson's disease have been demonstrated in animals treated with 1-Methyl-4-Phenyl-1,2,3,6 tetrahydropyridine, more commonly referred as MPTP (Bankiewicz et al., 1986; Burns et al., 1983). Therefore, the majority of the work done in this area is on MPTP-treated animals.

It has been demonstrated that a loss of striatal dopamine promotes increased transmission via the indirect pathway (Filion et al., 1988). The initial part of the indirect pathway is inhibitory, with GABA/enkephalin striatal neurons projecting to the external segment of globus pallidus. Therefore, decreased activity of the external segment of the globus pallidus will release the subthalamic nucleus from the inhibitory constraint produced by its GABA-mediated pathway. This in turn will result in increased firing by the subthalamic nucleus neurons causing excitation of the basal ganglia output nuclei (internal segment of the globus pallidus and the substantia nigra pars reticulata) via the excitatory gluatmatergic pathway that connects both of them. Reduced firing of the GABA/substance P neurons of the direct pathway will result in decreased inhibition of the basal ganglia output nuclei, again causing their excitation. A combination of the opposing effects on the direct and indirect striatal projections results in an overt increase in output nuclei firing causing inhibition of thalamocortical neurons via GABAergic projection. This in turn will reduce firing in the glutamatergic neurons projecting to the motor areas of the

cerebral cortex and may decrease the responsiveness of the precentral motor field engaged by the motor circuit thus producing the symptoms of Parkinson's disease (Filion et al., 1988). These features of increased activity in striatal neurons projecting to the external segment of the globus pallidus and reduced activity in the striatal neurons projecting to the internal segment of the globus pallidus were also reported in 6-hydroxydopamine lesioned animals frequently used as models of Parkinson's disease.

Further studies in support of this mechanism revealed that the concentration of substance P in the output nuclei of the basal ganglia was reduced in Parkinson's disease patents (Waters et al., 1988; Agid et al., 1987). However enkephalin levels in the external segment of the globus pallidus often remained unchanged (Agid et al., 1987). Surgery performed on the subthalamic nucleus was found to alleviate the symptoms of Parkinson's disease which supports the proposal that excessive subthalamic nucleus activity is a key step in this process (Bergman et al., 1990; Aziz et al., 1991; Limousin et al., 1995). More recently a paper by Chesslet and Delfs (1996) suggests that the model may need correcting following evidence that activity in the external segment of the globus pallidus is increased after nigrostriatal dopamine depletion by MPTP. This is thought to be due to the excitatory glutamatergic projection from the subthalamic nucleus which has an increased neuronal discharge rate (Tremblay et al., 1989; Pan and Walters, 1988). A similar finding was noted in 6-hydroxydopamine lesioned animals (Porter et al., 1994).

Olanow and Tatton (1999) have described the involvement of genetic factors in PD, since about 5-10% patients have a familial form of Parkinsonism with an autosomal dominant pattern of inheritance. The incidence of PD is greater in family members than in age-matched controls. There are large numbers of different genes involved in

PD and these may relate to protein misfolding (McNaught et al., 2002; Ryu et al., 2002) for reviews see (Soto, 2003)

Also, Olanow and Tatton (1999) have outlined many factors, which may cause neurodegeneration in PD:

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

a- Increased dopamine turnover,

b- A deficiency in glutathione,

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

2- Mitochondrial dysfunction, where an approximately 40% decrease in complex I activity of the mitochondrial respiratory chain has been found in the SNc of PD patients while other brain regions are unaffected.

3- Excitotoxicity, which results from increased glutamate formation.

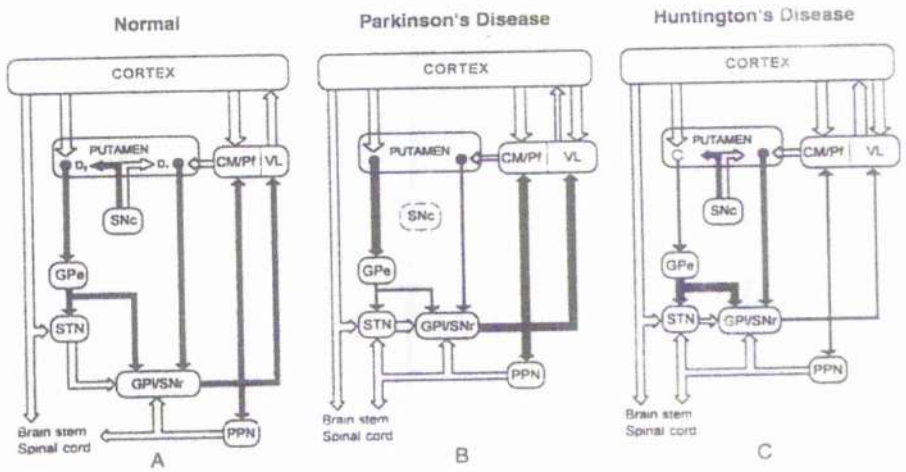



**Figure 1.2 Basal ganglia thalamocortical circuitry A) under normal conditions. B) with Parkinson's disease C) with Huntington's disease. Inhibitory projections are shown as filled arrows, excitatory projections as open arrows. Changes in arrow thickness reflect differential changes beteen disease states. Gpi = internal segment of the globus pallidus; Gpe = external segment of the globus pallidus; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; STN = subthalamic nucleus; VL = ventrolateral thalamus; PPN =pendunculopontine tegmental nucleus; CM/PF = centromedian/ farafasicular nucleus of thalamus. From Wichmann and DeLong (1998).**

### 1.3.5 Huntington's disease

This is named after George Huntington, who first described this disorder in 1872. Huuntington's disease (HD) typically begins in mid-life between the age of 30 and 45, though onset may occur as early as the age of 2. Children who develop the juvenile form of the disease rarely live to adulthood. HD affects males and females equally and can be seen in a variety of ethnic and racial groups.

HD is a hyperkinetic disorder characterised by progressive chorea, rigidity, dementia, dystonia, cognitive deficit and psychological disturbance. It is an autosomal dominant hereditary neurodegenerative disease. The mutation which is responsible for HD is an expanded polyglutamine repeat (CAG) within exon 1 of the gene that codes for the protein Huntingtin (Reddy et al., 1999; Hedreen and Folstein, 1995). The choreoathetosis of Huntington's disease is found to be the result of a selective loss of the striatal GABA/enkephalin neurons that project to the external segment of the globus pallidus (Albin et al., 1989). Reiner et al., (1988) also revealed that there was a selective loss of enkephalin-immunoreactive striatal terminals in the external segment of the globus pallidus in the early stages of Huntington's disease. This would cause excess GABAergic inhibition of the subthalamic nucleus which in turn produces a reduced excitatory input to the output nuclei of the basal ganglia. The output nuclei will thereafter project a reduced inhibitory outflow causing excessive disinhibition of the thalamus resulting in excessive positive feedback to the precentral motor fields in the supplementary motor area, premotor cortex and motor cortex. The end result of this mechanism is likely to be the choreiform movements that are seen in Huntington's disease. The substance P striatal terminals are well preserved until the later stages of the disease when they too are selectively lost.

## 1.4 Aminergic inputs to the basal ganglia

Although the core circuitry of the basal ganglia takes the form of cortico-striato-thalamo-cortical loops (see above) it is clear that there are aminergic inputs from midbrain cell groups, which exert a considerable effect on the activity of the loops. Most attention has focused on dopaminergic inputs.

### 1.4.1 Dopamine

Dopamine was found in the brain in 1959, since when a massive and varied body of work has been carried out.

The existence of a dopaminergic innervation of the brain was suggested by biochemical studies (Thierry et al., 1973a,b) and subsequently confirmed by anatomical work utilising glyoxylic acid-induced histofluorescence (Lindvall et al., 1974; Lindvall and Bjorklund, 1974), histofluorescence in combination with tract-tracing (Tork and Turner, 1981), autoradiography (Descarries et al., 1987), immunohistochemistry against the synthesising enzyme tyrosine hydroxylase (TH) in combination with destruction of the NA system (Berger et al., 1985) and, finally, immunohistochemistry with antibodies against dopamine (DA) itself, ending the problems of distinuishing between DA and NA that hampered earlier work (Papadopoulos et al., 1989b).

Dopamine is synthesised from the amino acid tyrosine, which is first converted into dihydroxyphenylalanine (dopa) by tyrosine hydroxylase, the rate-limiting enzyme in the biosynthetic pathway; dopa is, in turn, decarboxylated to dopamine. 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 maintaining the balance between DA synthesis and release

(Joh et al., 1978). Like the other monoamines, DA is catabolised by monoamine oxidase (MAO).

In studies using iontophoretic application, DA has been demonstrated to inhibit cortical neurons in the cat (Reader, 1978) and rat (Bunney and Aghajanian, 1976; Reader et al., 1979a) but, like 5-HT, it has also been shown to have excitatory action on some neurons.

The action of DA is terminated by reuptake into the presynaptic terminals by a DA-specific reuptake carrier (Coyle et al., 1969), which involves the dopamine transporter (Hitri et al., 1994; Zhou et al., 2003).

Dopamine receptors were originally divided into type D1 and D2 in the 1970's, since when more have emerged with the cloning of types D3, D4, D5 and their isoforms; they belong to G-protein coupled types (for review see Wolfarth and Ossowska, 1995).

Nearly 80% of all the brain's DA is located in the putamen of the corpus striatum, which is densely innervated by the pars compacta of the substantia nigra via the nigrostriatal pathway. The substantia nigra is not the only source of striatal dopamine. Other midbrain dopaminergic cell groups which project to the striatum include the retrorubral region (A8) and the medially situated ventral tegmental area (A10) which is the main source of dopamine for the ventral striatum, prefrontal cortex and limbic targets, although these regions also receive some connection from the medial SNc (Fallon and Loughlin, 1995).

Hornykiewicz (1966) and Bernheimer et al., (1973) revealed that the nigrostriatal dopaminergic projection is degenerated in Parkinson's disease and thereafter a great deal of research was devoted to devising pharmacological and surgical mechanisms to reverse the symptoms of this disease.

L-dihydroxyphenylalanine (L-dopa) is one of the earliest drugs used in the treatment of Parkinson's disease. It is converted by the enzyme aromatic L-amino acid decarboxylase to dopamine, the neurotransmitter deficient in the disease (Lloyd et al., 1975; Melamed et al., 1985). The dopamine that is formed is thought to substitute for the loss of dopaminergic input to the striatum that underlies the pathology of the condition. Not only dopamine agonists such as L-dopa and bromocriptine, but also muscarinic antagonists such as benzhexol and benztropine have been found to be beneficial in the treatment of the disease and this gave rise to the hypothesis that symptoms might be due to a striatal dopamine-acetylcholine imbalance. It was thought that degeneration of the nigrostriatal dopaminergic system lead to an overactivity of the striatal cholinergic system thus producing the symptoms of Parkinson's disease, which are reversed through the adminstration of L-dopa. Recent experiments with 6-hydroxydopamine lesioned rats using microdialysis where L-dopa was inserted into both the striatum and the substantia nigra demonstrated that the substantia nigra pars reticulata released a high concentration of dopamine. Experiments performed by Orosz and Bennett (1992) indicated that following L-dopa adminstration there was a far greater increase in the concentration of dopamine in the extracellular fluid of the substantia nigra pars reticulata than in the striatum. This indicates that the SN could be a major site of dopamine production in the hemiparkinsonian rat. Therefore, it may be assumed that the effects of L-dopa on basal ganglia function extend beyond the restoration of dopamine concentration within the striatum.

#### 1.4.1.1 Dopamine interactions

Many investigations have centred on the interaction of dopamine with other transmitters in the basal ganglia circuitry. Particular attention has been paid to the

interaction of dopamine with glutamate, an excitatory amino acid within the neostriatum. This is because glutamatergic and dopaminergic afferents have been found to converge upon the same striatal GABA neuron (Bouyer et al., 1984) and recent work indicates the possibility of direct interaction (Shimizu et al., 1990; Garcia-Munoz et al., 1991). Garcia-Munoz et al, (1991) propose that dopamine released from nigral afferents diffuses and stimulates the glutamatergic terminals via a process of non-synaptic volumetric transmission. On the other hand it is thought that there may be a striato-pallido-thalamo-cortico-striatal neural loop that releases glutamate and aspartate following dopamine release in the neostriatum (Barbito et al., 1989; Carlsson and Carlsson, 1990). Many of the experiments performed on the conscious rat involved acute injections of amphetamine and apomorphine which are dopamine receptor (both D1 and D2) agonists and thus release dopamine into the neostriatum on administration. These drugs were found to cause an increase in the extracellular concentration of both glutamate and aspartate as well as the inhibitory amino acid GABA, taurine and glycine. It has been proposed that these effects could be part of a protective alarm system for neurotoxic injury, since hypoxia and ischaemia have been found to produce an increase in both the concentration of dopamine and the excitatory amino acid glutamate and aspartate in the neostriatum (Golbus et al., 1988; Damsma et al., 1990; Akiyama et al., 1991). Moreover, dopamine depletion and glutamatergic receptor blockade have been shown to attenuate neuronal death following hypoxic/ischaemic injury (Weinberger et al., 1985; Gill et al., 1987; Clemens and Phebus, 1988). Therefore, increased dopamine concentration leading to the release of glutamate and aspartate may mediate the toxic effects that are produced. Both taurine and GABA are thought to exert a protective role during a hypoxic/ischaemic insult (Sternau et al., 1989).

Dopamine is known to interact with many other substances within the central nervous system, including opiods in the basal ganglia and forebrain, serotonin and GABA in the midbrain, norepinephrine and cholecystokinin elsewhere within the CNS.

### 1.4.2 Serotonin

The central or midline (raphe) location of the large neurons of the brain stem has attracted the attention of anatomists since the time of Ramon y Cajal, who described these cells as large multipolar neurons with uncertain projections. No one suspected that they contained the same chemical substance distributed throughout the body. For many years, investigators had known of a blood-borne chemical that produced vasconstriction (a 'Serum' factor that affected blood vessel 'tonus' hence the name Serotonin) and of a substance present in the gut that increased intestinal motility (enteramine). In the mid-twentieth century, serotonin or 5-hydroxy tryptamine (5-HT), the single compound producing both these effects, was isolated and synthesised, and its molecular structure was elucidated (Hamlin and Fisher, 1951). Shortly thereafter, 5-HT was found to be present in the mammalian CNS in significant quantities and to be concentrated in varying amount in different regions of the brain. This led to the proposal of 5-HT as a CNS neurotransmitter.
Localisation of the cell bodies and axon terminals was initially visualised in the 1960's with the Falck-Hillarp method of formaldehyde-induced histofluorescence (Dahlstorm and Fuxe, 1964). However, the instability of the fluorophore produced meant that this method could not provide information on the serotonergic innervation of the brain at the same level of sensitivity as it did for the catecholaminergic innervation. Alternative methods were subsequently used to localise 5-IIT in the brain: autoradiography either at the light microscope (Conral et al., 1974; Bobillier et

al., 1976, 1979; Azmitia and Segal, 1978; Moore et al., 1978) or at the electron microscope level (Aghajanian and Bloom, 1967; Descarries et al., 1975); lesion studies combined with biochemistry (Geyer et al., 1976) or histochemistry (Ungerstedt, 1971). More recently, the preferred method of immunohistochemistry with antibodies against 5-HT has demonstrated the full extent of serotonergic innervation (Steinbush et al., 1978; Lidov et al., 1980; Lidov and Moliver, 1982a,b) The serotonergic cell bodies are restricted to clusters in the brain stem, but their fibres, using most of the known longitudinal pathways, innervate nearly every area of the brain (Steinbusch, 1981; Steinbusch and Verhofstad, 1981; Tork, 1990). The neurons in the entire mammalian CNS number in billions, whereas serotonergic cells number in the thousands and they constitute ~1/1,000,000 of all CNS neurons. However, their influence on their target sites appears to go far beyond these numbers. In the rat brain it is estimated that there are ~$6x10^6$ serotonergic varicosities/mm cortical tissue. By extrapolation, this means that each serotonergic neurons projecting to the cortex may be responsible for $5x10^5$ serotonergic varicosities, that each of their cortical target neurons receives ~ 200 varicosities, and that serotonergic terminals may account for as many as 1/500 of all axon terminals in rat cortex (Audet et al., 1989).

The 5-HT cell bodies in the brain are located in the brain stem (Chojnacka-Wojcik, 1995; Jacobs and Azmitia, 1992; Morgan et al., 1987), in raphe nuclei groups which extend from the midbrain to the medulla oblongata. These areas are classified into nine regions (B1-B9) (Dahlstrom and Fuxe, 1964; Steinbusch, 1981; Tork, 1990; Weissmann et al., 1987) rostral 5-HT neurons have ascending projections that innervate virtually all areas of the brain, whereas the caudal cell groups project to the spinal cord (Goridis and Rohrer, 2002)

Serotonergic neurons synthesize 5-HT from dietary tryptophan, which is converted to 5-hydroxy-tryptophan by the rate limiting enzyme tryptophan 5-hydroxylase. 5-hydroxy-tryptophan is, in turn, converted to 5-HT by aromatic-L-amino acid decarboxylase. The production rate is believed to be adjusted by impulse-coupled regulation of tryptophan hydroxylase activity (for review see Cooper et al., 1986). Serotonin itself is metabolised initially into 5-hydroxy-indole-acetaldehyde by monoamine oxidase (MAO), the enzyme that oxidatively deaminates all monoamines to their corresponding aldehydes; further oxidation by aldehyde dehydrogenase leads to the formation of 5-hydroxy-indole acetic acid (see Osborne, 1982).

Serotonin is released by stimulation of the serotonergic cell bodies in the raphe nuclei. These cells possess 5-HT autoreceptors, suggesting that 5-HT regulates its own release. However, other neurotransmitters and modulators (dopamine, noradrenaline, acetylcholine) have also been implicated in this regulation. Drugs and experimental compounds like reserpine, π–chloroamphetamine (PCA), and methlyene-dioxy-methamphetamine (MDMA/ 'Ecstasy') can also cause release of 5-HT (for review, see Green et al., 1995). The action of 5-HT upon postsynaptic receptors, as studied by iontophoretic application, is chiefly inhibitory (Reader, 1978), although it can also have (or facilitate) excitatory activity (Nedegaardt et al., 1987). This action is terminated by reuptake into the presynaptic terminal; the uptake system has a high specificity and does not interact with other monoamines.

Serotonin receptors were originally classified as D and M types in the 1950's and later as 5-HT1, 5-HT2, 5-HT3 and more recently, 5-HT4. The advent of molecular cloning techniques brought about an explosive increase in the number of distinct 5-HT receptors subtypes that can be identified reaching the current number of 14. 5-HT3 is a ligand-gated ion channel, while the rest belong to G-protein coupled types (Teitler and Herrick-Davis, 1994, Leonard, 1996).

Serotonergic cell bodies appear very early (E12) in the embryonic development of the rat, when they form a cluster on either side of the floor plate of the rhombencephalon, accompanied by short, non-varicose fibers (Aitken and Tork, 1988). This cluster gives rise to the ascending fibres, while a second one, appearing caudally at E14, gives rise to the descending ones. The dorsal and the median raphe nuclei, which will be examined in this thesis, arise from the first cluster.

## 1.5 Animals models of Parkinson's disease

For the last 40 years, one research goal has been to find an animal model which can mimic Parkinson's disease. One of the first models was introduced by Ungersted (1968) who developed the 6-hydroxydopamine (6-OHDA) treated rat. The drug, injected into the substantia nigra or into the striatum, accumulates in dopaminergic neurons and kills them owing to toxicity that is thought to involve the generation of free radicals. 6-OHDA is frequently injected in one side of the striatum to produce a unilateral lesion, leaving the contralateral side as the control. Marmosets with a 6-OHDA lesion showed 98% depletion of DA in the neostriatum, 90% depletion in the prefrontal cortex, as well as a loss of noradrenaline and 5-HT compared to controls receiving saline (Annett et al., 1992). The model is complex because contralateral effects can complicate data interpretation (Jenner et al., 1992). Moreover, the motor deficits examined are usually apomorphine- or amphetamine-induced rotations which are not characteristic of PD patients.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment also induces Parkinsonism (Alexander et al., 1992; Jenner et al., 1992), particularly in primates where it causes a loss of DA neurons in the pars compacta of the SN, together with a loss of DA and DA uptake sites in the striatum (Alexander et al., 1992). It was first noticed when heroin addicts developed severe PD after using a 'designer drug'

contaminated with MPTP. MPTP is a lipophilic which can cross the blood brain barrier, where it is metabolised to 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B, an enzyme involved in catecholamine degradation (fig 1.3).

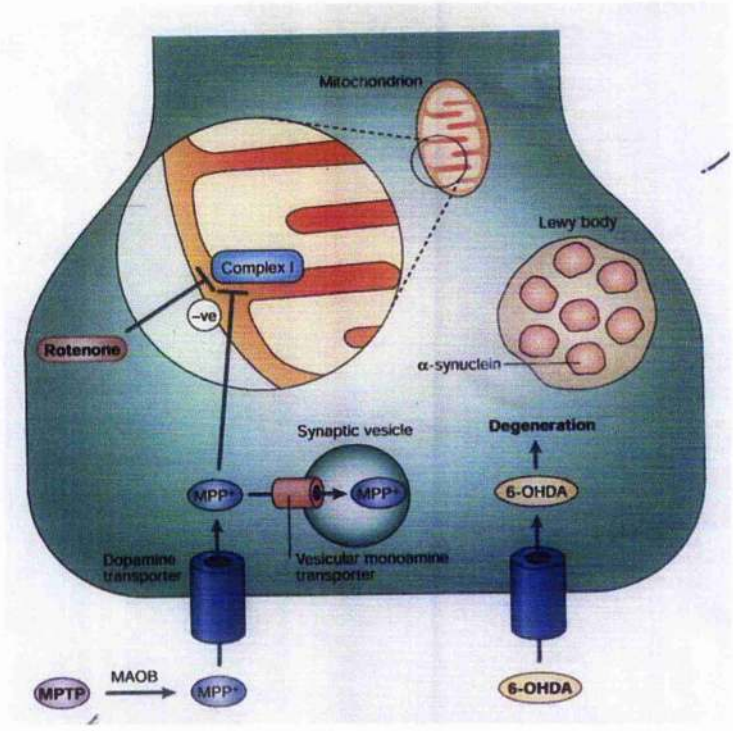



**Figure 1.3 Pathogenesis of neuronal dysfunction produced by neurotoxins that affect dopamine neurons. The mechanisms by which neurotoxins kill dopamine neurons involve mitochondrial dysfunction and oxidative damage. 6-hydroxydopamine (6-OHDA) is taken up by the dopamine transporter and it then generates 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 I inhibition and the generation of free radicals, or by the vesicular monoamine transporter, thus reducing toxicity. Rotenone is a direct inhibitor of complex I, which also leads to free-radical generation. MPTP and rotenone treatment increase the expression of α-synuclein and, in the latter case, this leads to the formation of Lewy bodies. From Flint Beal, (2001).**

In primates, MPTP-treated animals were found to have most of the signs of PD. In monkeys, the drug was able to produce the typical behavioural features, including the tremor which does not occur in most primate species following MPTP intervention (Elsworth et al., 1992). The main difficulty with MPTP toxicity as a model of PD is that it is an acute or subacute process, whereas PD is a slowly progressive illness.

Others experimental models of Parkinson's disease have been produced by infusing rats intravenously with rotenone - a lipophilic pesticide. This leads to dopaminergic nigrostriatal degeneration which produces hypokinesia and rigidity and, more interestingly, the animals accumulate fibrillar cytoplasmic inclusions that contain ubiquitin and α-synuclein as found in Lewy bodies (Betarbet et al., 2000). However, only half of the rats treated with rotenone developed lesions (Flint Beal, 2001). Alpha-synuclein models in Drosophila and mouse have been introducing as models of Parkinson's disease but they lack several of characteristic features of PD (for review see Flint Beal, 2001).

Mutant strains of laboratory rodents (especially mice) with locomotor disorders have arisen spontaneously. In general, they are poor breeders with low life expectancies, and are complicated in that they may have cerebellar deficiencies including a microcerebellum or loss of Purkinje or granule cells. These mice include weaver, staggerer and tottcrer strains (Gupta et al., 1986; Triahou et al., 1987; 1988).

Knockouts of DA receptor by types gene deletion have been used to produce other models. For example, Drago et al., (1996) reported $D_{1A}$ knockouts to show no gross neurological deficit. On assessing phenotype in terms of accumulation of visually observed rearing events and line-crossing in an open field over a 15 min period, $D_{1A}$ mutants were characterised by a reduced level of rearing but no significant reduction in locomotion. By contrast, Xu et al., (1994a,b) using a photo beam analysis reported that there were increases in activity with reduction only in visually observed grooming, but not in rearing or sniffing, over a 1h period. Such finding showed that $D_{1A}$ knockouts can not be characterised simplistically as either hypoactivity or hyperactivity but, rather, by complex, topographical shifts between individual elements of behaviour in the natural mouse repertoire that cannot be encapsulated by either term (for review see Waddington et al., 2001).

$D_{1B/5}$ knockouts showed increases in both horizontal and vertical activity in terms of photocell beam breaks; there was also enhanced performance on the rotarod (Sibley, 1999).

Baik et al., (1995) showed that $D_2$ knockout mice had an abnormal posture, with fore and hind paws flattened to the ground; impaired gait, with sprawled hind legs; impaired rotarod performance, with a shorter latency to fall.

$D_3$ knockout features include increased line-crossing, as an index of locomotor activity, and in rearing but not in grooming events (Steiner et al., 1997).

Knocking out $D_4$ led to reduced horizontal and vertical movements in terms of photocell beam interruptions over repeated testing (Rubinstein et al., 1997).

## 1.6 The AS/AGU rat

In the Laboratory of Human Anatomy at the University of Glasgow, the AS/AGU rat has arisen as a spontaneous mutation within a closed inbred colony of Albino Swiss (AS) rats. The mutation is recessive (Campbell et al., 1996), and AS/AGU rats have been isolated as a true breeding substrain. Phenotypic differences are therefore, the result of single point mutation. The mutant animals show serious movement impairments including rigidity, a staggering gait and a tendency to fall over every few steps, a slight whole body tremor and difficulty in initiating movement (Clarke and Payne, 1994; Payne et al., 1998).

Several studies including histological, neurochemical, immunocytochemical and locomotor analysis revealed that in the AS/AGU mutants there are:

A- No gross morphological difference between the mutant and the parent brains. Neocortical and cerebellar areas looks normal.

B- Immunocytochemical studies showed a reduction (c.60%) in TH-immunoreactive cell bodies in the SNc of the mutant strain at $1^+$ years of age (Clarke and Payne,

1994). Another preliminary study showed a reduction in noradrenergic cells in the locus coeruleus (Scott et al, 1994).

C- At 6 months, there is a reduction (20-30%) in whole tissue dopamine levels measured in striatal micropunches using High performance liquid chromatography with electrochemical detection (HPLC-ECD) (Campbell et al., 1996).

D- At 3 months, microdialytic studies showed a massive reduction (-80%) in extracellular dopamine in the dorsal caudate putamen, compared with AS controls when measured with HPLC-ECD.

E- Behavioural studies showed that AS/AGU rats have a marked difficulty in initiating movement, and are generally unable to perform simple non-invasive locomotor tests such as rotating in mid-air and walking down a variety of inclined ramps of various widths.The deficits are significant at 6 and 12 months. L-dopa was found to restore striatal dopamine levels and ameliorate many locomotor deficiencies in AS/AGU rats (Campbell et al., 1998).

F- There is a marked reduction in utilisation of 2-deoxy-glucose in several brain areas including substantia nigra pars compacta (SNc), subthalamic nucleus and ventrolateral thalamus (Lam et al., 1998).

### 1.6.1 The AS/AGU mutation

Genetic mapping, involving multiple backcross studies, has revealed the *agu* mutation to possess very tight linkage to a marker in the 3' untranslated region of the protein kinase c γ isoform encoding gene. Subsequently, sequencing of this gene revealed as far as we know, it is a single point mutation a substitution resulting in a stop codon between the regulatory and catalytic domains (Craig et al., 2001).

Truncation of the PKC γ coding region before the catalytic domain may lead to the expression of the regulatory domain only, or the inactivation of the messenger RNA. In any case PKC γ activity is complelety abolished in AS/AGU rats.

PKC γ has been labelled a CNS restricted isoform (Kano et al., 1995). But recent finding suggest expression ouside of the CNS.

Expression patterns of PKC γ within the CNS are largly unclear but it is known that levels are high in Purkinje cells, medium sized neurons of the striatum and globus pallidus, which project to the substantia nigra pars reticulata, and spinal cord (Chen et al., 1995). In addition, activition of PKC in nerve cells found to have affect in modulation of ion channels (Shearman et al., 1989), the desensitisation of receptors (Huganir and Greenyard, 1990), and the enhancement of neurotransmitter release.

The aim of this project is to examine a second aminergic neuronal system the raphe-striatal serotonergic system to establish whether it is degenerate or dysfuntional in the mutant AS/AGU rat compared to the parent AS strain. This will be carried out by a series of experiments.

Firstly, it is essential to determine if the genetic defect had been identified correctly, so different regions in the brain will be investigated by means of anti PKC γ immunocytochemistry in the mutant (AS/AGU) and parent AS rat.

Secondly it is important to confirm that rostal projections from the raphe do innervate the caudate-putamen as previous literature suggests and this will be done by a retrograde tracing study.

Experiments three onwards then examine the integrity of the serotonergic raphe striatal system in three ways

i) an evaluation of the numbers of 5-HT-ir cells in the dorsal and median raphe nuclei (Experiment 3)

ii) measurement of the levels of indole- and catecholamines in several striatal and medbrain regions by micropunch and HPLC-ECD (Experiment 4)

iii) measurement of extracellular levels of indole-and catecholamines within the caudate-putamen using in vivo microdialysis followed by HPLC-ECD (Experiment 5)

Experiment 4 will also include the effects of simple pharmacological manipulations on indole- and catecholamine levels in the mutant and parent strain. The final experiment (Experiment 6) will determine whether similar manipulations can affect behaivour in the two strains using a simple study of spontanous locomotion.

It is hoped by these means to establish whether similar dificits occur within the midbrain serotonergic system of the AS/AGU mutant as have already been demonstrated in the midbrain dopaminergic system? The expectation is that deficit are likely to occur since

a) naturally occurring syndromes in the humans which involve the basal ganglia and locomotion often lead to dysfunction of several transmitters systems, and

b) it is unlikely that a basic enzyme such as protein kinase C (whose - isoform is defective in the AS/AGU mutant) would be restricted in its action to one tranamitter system only.

Finally, it is hoped that the results obtained in these experiments will give insight into the potential use of the AS/AGU rat as a model for important human neurodegenerative conditions.

# 2 Experiment 1. PKCγ expression in AS and AS/AGU mutant rats

## 2.1 Introduction

Protein kinase C (PKC) is an enzyme which is located in a wide variety of tissues. Within the nervous system, PKC is involved in widely diverse functions. Thus, activation of PKC in nerve cells is found to be linked to modulation of ion channels (Shearman et al., 1989; Baraban et al., 1985; Madison et al., 1986), the desensitisation of receptors (Huganir and Greenyard, 1990), modification of neuronal plasticity (Routtenberg et al., 1985; Akers et al., 1986) and, most interestingly, the enhancement of neurotransmitter release (Malenka et al., 1986,1987). The role of PKC in cell surface signal transduction is now also widely accepted and well documented (for review, see Nishizuka, 1984 a,b; 1986).

There are at least 14 isoforms of PKC that have been identified in mammalian tissue, each of which has a specific expression pattern. One of them is PKC γ which is said to be restricted to the CNS, being expressed in Purkinje cells and medium sized neurons of the striatum and globus pallidus (Chen et al., 1995). Experiments on PKC γ knockout mice have shown a link with synaptic plasticity, control of long term potentiation and elimination of surplus climbing fibres in the developing cerebellum (Kano et al., 1995; Chen et al., 1995). PKC γ deficient mice exhibit ataxia (Kano et al., 1995) and impaired motor coordination, but are capable of discrete motor learning (Chen et al., 1995). In the basal ganglia there is dense localisation of PKC γ in the cytoplasm of medium sized neurons projecting to the substantia nigra; in perikarya and neuropil in the striatum; and neuropil in the substantia nigra (Yoshihara et al., 1991).

The aim of this chapter is to confirm the PKC γ mutation in the AS/AGU rat by immunocytochemistry (ICC).

Different neuronal regions were chosen because of the expected high expression of this enzyme in striatum, substantia nigra, dorsal raphe and cerebellum in normal animals. It

was expected that PKC γ would be absent in the mutant rat (AS/AGU) all in areas chosen if the genctic defect had been identified correctly (Craig et al., 2001). The same regions were analysed in the AS rat in order to assess the presence of PKC γ and to verify that the technique was appropriate to the study.

## 2.2 Material and Methods

### 2.2.1 Animals

One year old AS and AS/AGU male rats (3 in each group) were used in this experiment.

### 2.2.2 Immunocytochemistry (ICC)

An ICC technique was used in this experiment according to the protocol which was first employed by Hsu and colleagues (1981).

The rats were deeply anaesthetized with an overdose of Sodium pentobarbitone BP (Vet) (Rhone-Merieux, Spire Greencentre, Harlow, Essex, 60mg/ml). They were then perfused through the left ventricle with 100ml Ringer's solution containing the vasodilator Lignocaine followed by 500ml 4% paraformaldehyde (2944744 BDH) in 0.1M phosphate buffer. The brains were dissected out and post-fixed in formalin overnight. Pieces of brain containing areas of interest were dehydrated through an ascending ethanol series using a Histokinette automatic tissue processor, then processed for paraffin at 57 °C, serially sectioned at 6μm and mounted on APES (3-aminopropyltriethoxysilane) coated slides.

The sections were deparaffinized and rehydrated before undergoing a high temperature antigen unmasking technique. Slides were immersed in boiling 0.01M sodium citrate buffer (pH 6.0) in a Prestige stainless steel pressure cooker. The temperature was then raised to 121 °C for 1minute.

### 2.2.3 Immunocytochemistry on wax sections

Sections were incubated in 0.5% hydrogen peroxide ($H_2O_2$) for 10 minutes to eliminate endogenous peroxidase activity. After rinsing in 0.01M phosphate buffered saline (PBS, 3x5 mins), sections were treated with 1% normal goat serum (NGS) in PBS with 3% triton-x100 for 1 hour to clear background staining. They were then incubated in a humidity chamber overnight at 4°C with the primary antibody (anti-PKC-γ ; RDI) (1:1000) diluted in

PBS containing 3% triton-x100. The rest of procedure was carried out at room temperature. After rinsing in PBS (3x5 mins), a biotinylated anti-rabbit secondary antibody raised in goat (1:500 dilution) Jackson ImmunoReaearch Laboratories, INC, West Grove, PA, U.S.A) and then an Avidin-biotin complex (1:50; Vector Laboratories, INC, Burlingame, CA, U.S.A) were used to label the bound primary antibody. The location of the antigen-antibody complex was visualised by incubating sections in a medium containing 0.05% 3,3- diaminobenzidine, 0.01% $H_2O_2$ and 0.02% nickel chloride in 0.01M phosphate buffer (2-5 mins).

After dehydration sections were mounted in Histomount (Hughes & Hughes, LTD., Willington, Somerset, UK).

## 2.3 Results

### 2.3.1 PKC-γ immunostaining in cerebellum

PKC γ antibodies stained the cell bodies and dendrites of Purkinje cells in control (AS) rats. There was also obvious staining of the axon of these cells. Furthermore, there was no obvious staining of basket or stellate cells in the molecular layer or other cell types in the granule layer when PKC γ antibody was used.

In the mutant rat (AS/AGU) there was no specific staining in any layers of the cerebellum either in cell bodies or terminals.

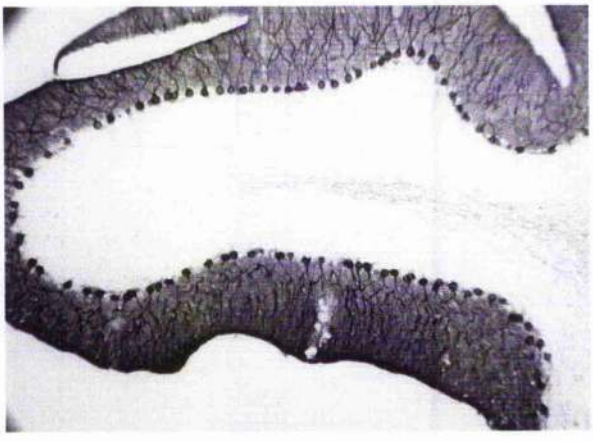


**Figure 2-1 A cross section of cerebellum from an Albino Swiss (AS) male rat aged 12 months. The section has been immunostained for protein kinase C (PKC)- γ and Purkinje cells are readily visualised. (x100).**

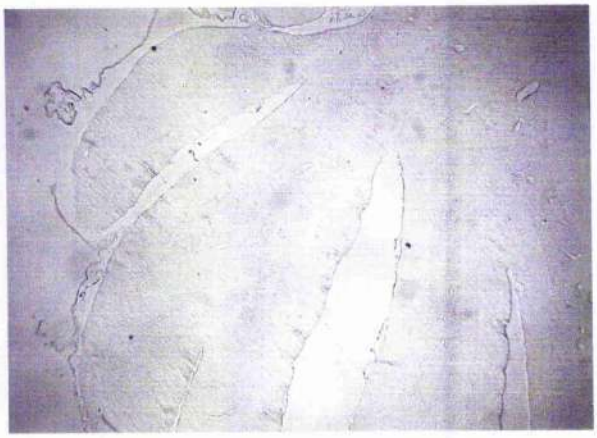


**Figure 2-2 A cross section of cerebellum from mutant (AS/AGU) male rat aged 12 months. The section has been immunostained for protein kinase C (PKC)-γ but no specific staining in any layers of the cerebellum was found. (x20)**

### 2.3.2 PKC-γ immunostaining in the Hippocampus

PKC γ immunoreactivity was distributed throughout the hippocampal formation. Large numbers of immunoreactive pyramidal cells were present in the CA1-CA3 layers. Intense immunoreactivity was found in the perikarya and there was moderate immunoreactivity in the nuclei of the pyramidal cells. The neuropil of the hippocampus was moderately stained and the most intense immunoreactivity was found in the proximal portion of the stratum radiatum of the CA3 region.

In the mutant rat (AS/AGU) there was no specific staining in any regions of the hippocampus.

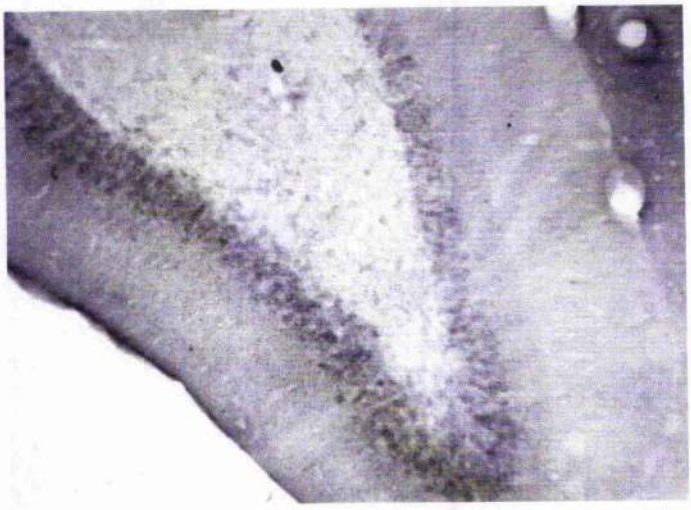


**Figure 2-3 A cross section of hippocampus from an AS male rat aged 12 months. The section has been immunostained for protein kinase C (PKC)-γ and dense immunoreactivity occurred in the fiber bundle-like structure in CA3. (x100)**

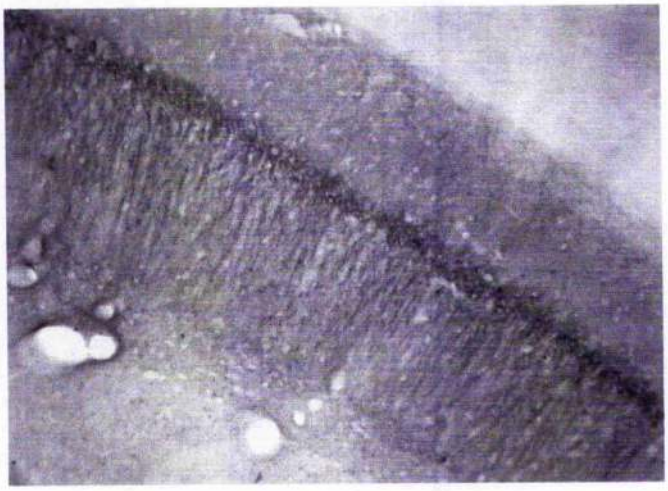


**Figure 2-4 AS hippocampus (x100)**

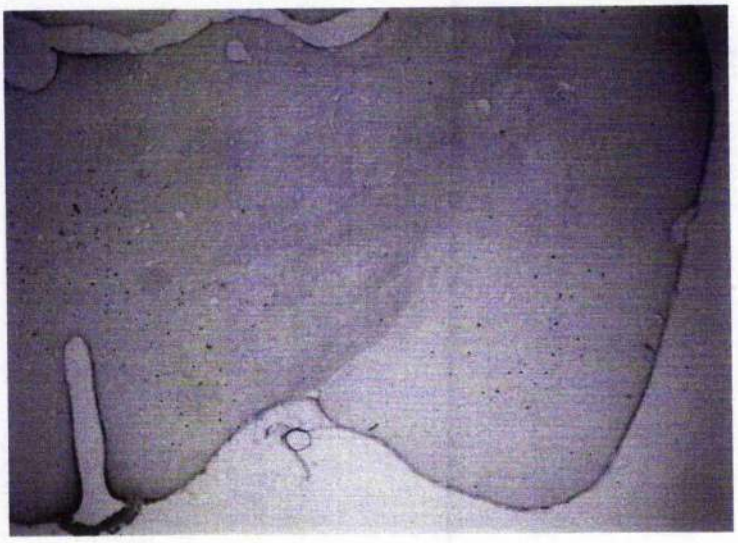


**Figure 2-5 A cross section of hippocampus from mutant (AS/AGU) male rat aged 12 monthes. The section has been immunostained for protein kinase C (PKC)-γ but no specific staining was found in any region of the hippocampus. (x20)**

### 2.3.3 PKC-γ immunostaining in the cerebral cortex

Staining occurred throughout the neocortex, although with varying intensities of immunoreactivity. PKC γ immunoreactivity showed positive staining in neurons resembling pyramidal cells and their apical dendrites in layers II to VI, while layer I was nearly devoid of staining. PKC γ immunopositive neuropil of AS (control) rats was seen in layers I, II, V, and VI.

In the mutant rat (AS/AGU) there was no staining in any layers of the cerebral cortex.

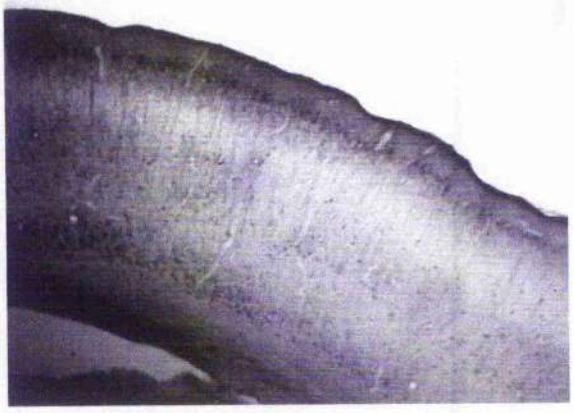


**Figure 2-6 A cross section of cerebral cortex from an AS male rat aged 12 months. The section has been immunostained for protein kinase C (PKC)-γ. (x40)**

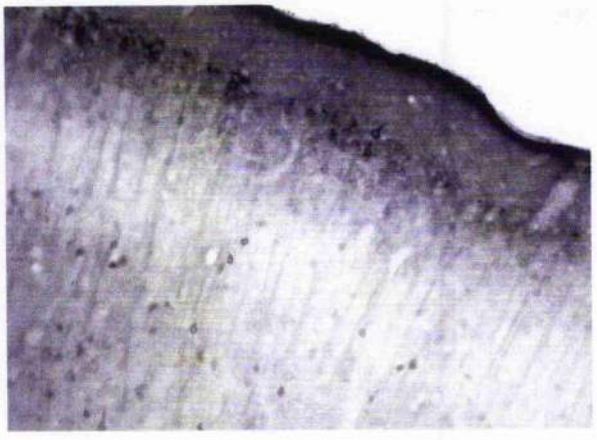


**Figure 2-7 Higher magnification of 2.6 showing PKC)-γ immunoreactivity. Immunopositive cells are seen in layer II. (x100)**

**Lack of staining for PKC-γ in cerebral cortex of mutant rat is shown in Fig 2.9**

## 2.3.4 PKC-γ immunostaining in the striatum

PKC γ immunoreactivity was abundant in the perikarya, neuropil and fibre bundles of the striatum of AS (control) rats. Staining was found in numerous medium sized neurons scattered throughout the CP but was less intense in the globus pallidus.

In the mutant rat (AS/AGU) there was no specific staining in any areas of the striatum.

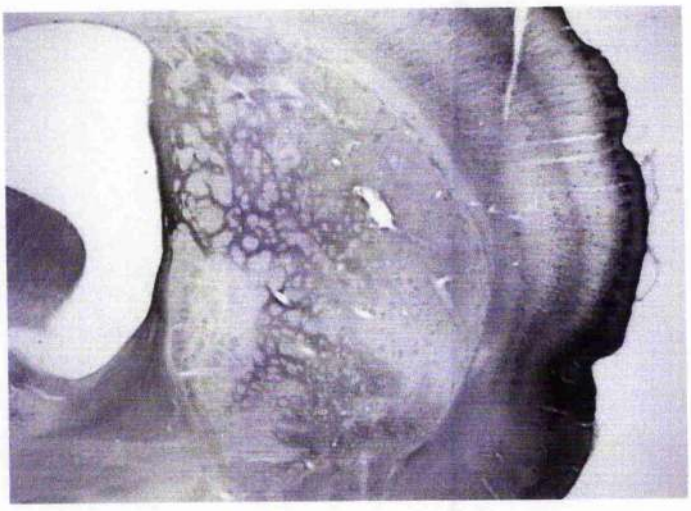


**Figure 2-8 A cross section of striatum from an Albino Swiss (AS) male rat aged 12 months. The section has been immunostained for protein kinase C (PKC)-γ. (x20)**

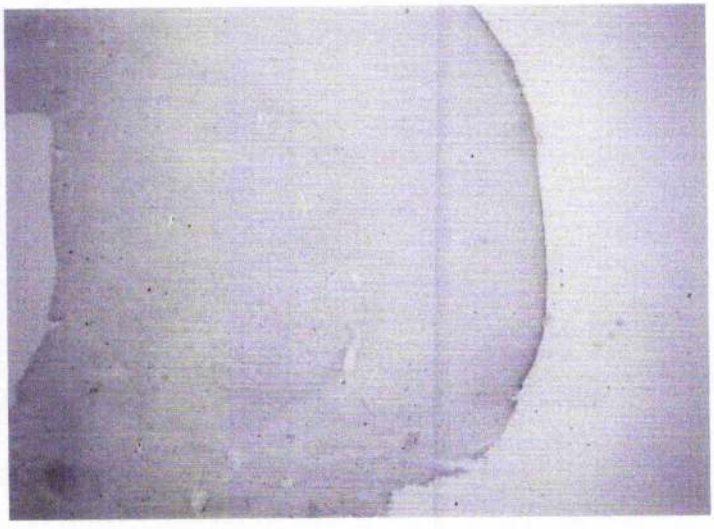


**Figure 2-9 A cross section of striatum from mutant (AS/AGU) male rat aged 12 months. The section has been immunostained for protein kinase C (PKC)-γ and no staining was detected in any areas of the mutant rat. (x20)**

### 2.3.5 PKC-γ immunostaining in the substantia nigra

PKC γ immunoreactivity was abundantly present in the neuropil of AS (control) rats which showed more intense immunoreactivity in the medial than in the lateral part of the pars reticulata, whereas the neuronal perikarya were not labelled.

In the mutant rat (AS/AGU) there was no specific staining in any layers of the substantia nigra.

**Figure 2-10 A cross section of substantia nigra from an Albino Swiss (AS) male rat aged 12 months. The section has been immunostained for protein kinase C (PKC)-γ and labelled neuropil throughout SN. (x30)**

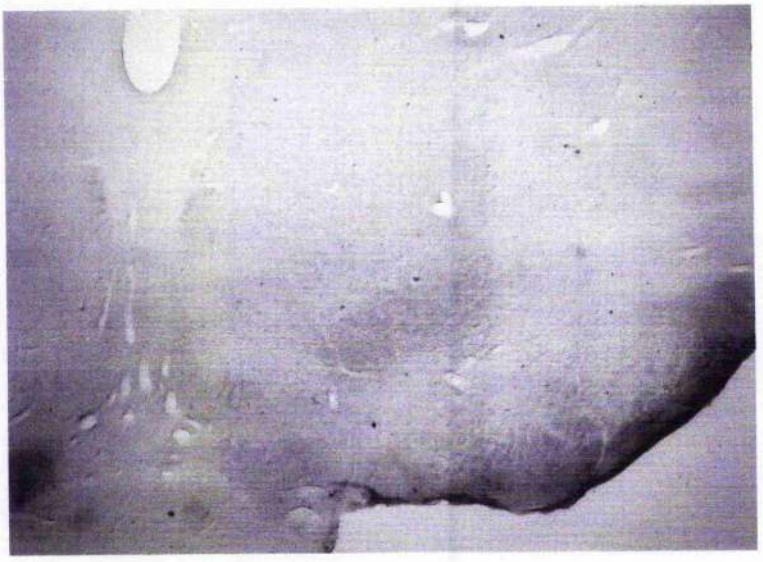


**Figure 2.11 A cross section of substantia nigra from mutant (AS/AGU) male rat aged 12 months. The section has been immunostained for protein kinase C (PKC)-γ and there no staining was found. (x30)**

## 2.3.6 PKC-γ immunostaining in DRN & MRN

PKCγ immunoreactivity was less abundant in the neuropil of the DRN where no labelled cells were seen in the control animals (AS). In contrast, there were a few labelled cells been in the MRN of control AS rats.

In the mutant rat (AS/AGU) there was no specific staining in any area of the DRN and MRN.

**Figure 2-12 A cross section of DRN & MRN from an Albino Swiss (AS) male rat aged 12 months. The section has been immunostained for protein kinase C-γ and labelled neuropil of DRN can be seen. (x40)**

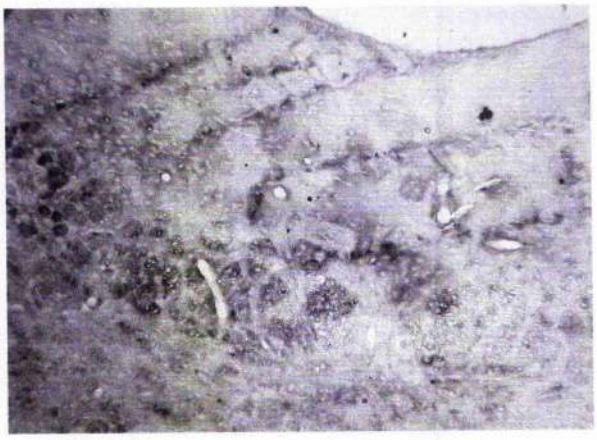


**Figure 2-13 Higher magnification of fig 2.14 showing the labelled neuropil of DRN from an Albino Swiss (AS) rat. (x100)**

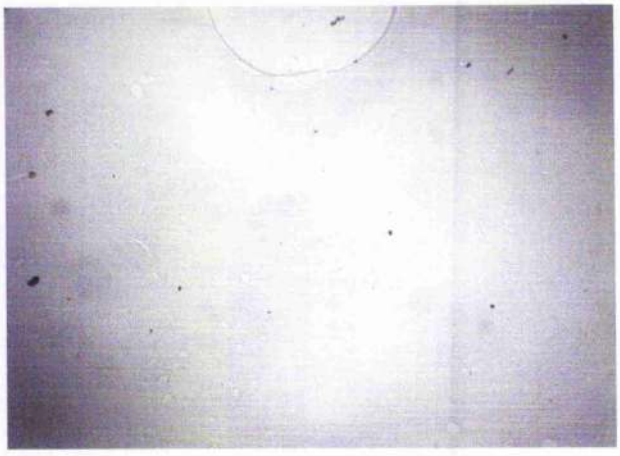


**Figure 2-14 A cross section of DRN and MRN from a mutant (AS/AGU) male rat aged 12 months. The section has immunostained for protein kinase C-γ and there no staining in both neuclei. (x25)**

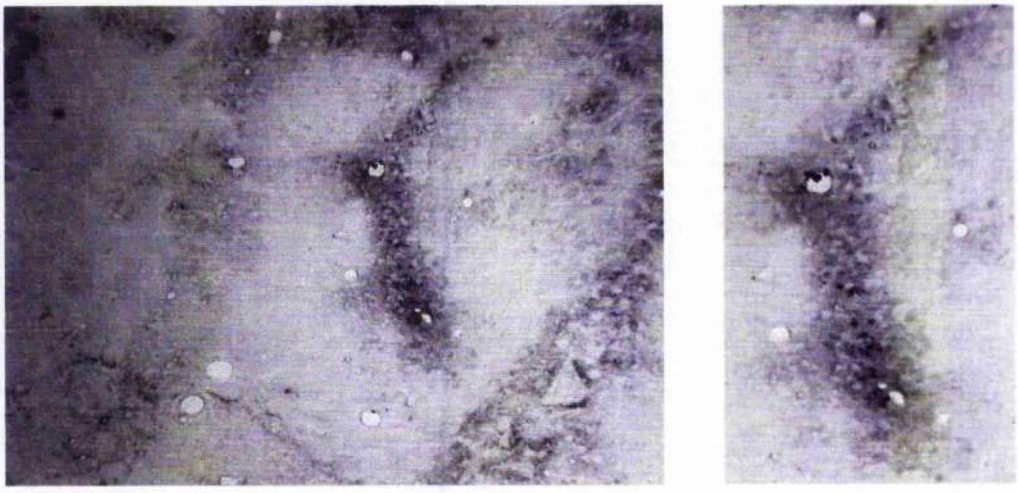


**Figure 2-15 Cross section of MRN of an Albino Swiss (AS) rat showing a few labelled cells. (x100)**

## 2.4 Discussion

In this discussion I will briefly review the following points:

A) Do my immunocytochemical studies support the identification of the defective gene as that for PKC-γ (Craig et al., 2001)?

B) In normal animals, does the pattern of PKC-γ reactivity reflect the previous literature?

C) What effects might a PKC-γ deficiency have?

ICC shows positive labelling of PKC γ in AS animals while AS/AGU rats show no labelling at all in any of the brain areas studied. This confirms the identification of the gene by Craig et al (2001).

In the case of the cerebellum, the labelling was within cerebellar Purkinje cell bodies and dendrites which agrees well with previously published data (Huang et al., 1988; Kano et al., 1995). Other immunoreactive areas in the present study also confirm previous reports of staining e.g. in the hippocampus (Huang et al., 1988; Saito et al., 1994), cerebral cortex (Huang et al., 1988; Tsujino et al., 1990), the caudate- putamen and substantia nigra (Yoshihara et al., 1991).

Electron microscopic studies on the hippocampus showed PKC-γ immunoreactivity in the presynaptic nerve terminals and also in postsynaptic dendrites. The PKC-γ was present homogeneously in presynaptic terminals of the shaft synapse and appeared to be associated with synaptic vesicles (Saito et al., 1994).

Studies comparing expression of the 3 types of isoforms, α,β,γ in cerebellum found that Purkinje cells were stained for all 3, but only γ was present in their dendritic trees which indicate that if both kinases are involved in release, γ may play a large role proximal to the cell body (Huang et al., 1988).

The specification of the antibody (anti-PKC-γ ; RDI) for the active site at the carboxyl terminus (the region which has been shown to be missing in the *agu* mutation Craig et al., 2001) leads to selective labelling of PKC-γ and shows no cross reactivity for other PKC isoforms. Mutant rats should not therefore show staining in any of the areas examined and this indeed was the case in this study,

Loss of activity of PKC-γ must be related to the neurochemical, neurodegenerative and locomotor disorders exhibited by these rats.

Hashimoto (1988) reported that PKC-γ was only expressed postnatally so there was no chance of a deficit producing substantial developmental errors. In contrast, other investigators have found the expression of PKC-γ in the embryonic chick brain (McIntyre et al, 1999). In addition more recent work on Xenopus has confirmed a role for PKC-γ in early brain development (Kramer et al., 2002).

Much available litertaure deals with the PKC family in general, rather than specific isoforms. However, both PKC and other subsets (alpha and beta) are known to be intrically involved in the packaging and release of neurotransmitters (Ben-Shlomo et al., 1991). The role of PKC-γ in synaptic mechanisms is unclear and made more complicated in that the absence of PKC-γ can lead to upregulated activity of other isoforms (e.g. alpha and beta) by cellular compensatory mechanisms (Chen. 1995).

Activation of PKC by phorbol esters causes phosphorylation and redistribution of dopamine and serotonin transporters in transfected LLC-PK1 and HEK293 cells respectively (Apparsundaram et al., 1998) and treatment with PKC activator induces movement of GABA transporter proteins between cytoplasmic membrane vesicles and the surface membrane in *Xenopus laevis* oocytes (Apparsundaram et al., 1998).

Even at low concentrations, glutamate release can be enhanced by PKC activators such as PMA and antagonised by PKC inhibitors (Nakamura et al., 2003). Also studies on σ 1 agonist-mediated inhibition of NMDA-stimulated [$^3$H] dopamine release is transduced by

a PKC signalling system. Regulation is abolished by pre-treatment with PMA inhibitors (Nuwayhid and Werling, 2003).

5-HT$_2$A receptor is enriched in many brain areas including the striatum (Pazos et al., 1985, 1987). Furthermore, experiments performed with PKC inhibitors confirmed that PKC was involved in the regulation of 5-HT$_2$A receptor mRNA by agonists and implicate the conventional subgroup of PKC isoforms.

Upon treatment of C6 glioma cells with 5-HT, levels of PKC-γ increased in the nuclear fraction suggesting that PKC-γ may be involved in the regulation of 5-HT$_2$A receptor expression in response to agonist treatment (Anji et al., 2001). Indeed, the post-synaptic regulation of GABA and 5-HT receptors by PKC-γ has been linked to anxiety (Bowers et al., 2000).

Downregulation of PKC by chronic exposure to PMA induced 5-HT secretion. PKC isoforms mediate secretion of 5-HT by Parafollicular cells (Pf cells) Liu et al., 2000). More recent work has confirmed the role of PKC-γ in 5-HT secretion (Liu et al., 2003). The findings on Pf cells are likely to be very relevant to other neuronal mechanisms induced by $Ca^{+2}$ entry, including synaptic transmission.

It is with the knowledge that PKC can influence 5-HT release (and the knowledge that this PKC-γ deficient mutant exhibits reduced DA release and subsequent loss of DA cells) that I have undertaken three experiments. These will respectively answer the questions

1- Are 5-HT-containg neurons reduced in the AS/AGU rat ?
2- Are 5-HT levels reduced in a variety of brain regions of the AS/AGU mutant rat and how does the mutant respond to simple manipulation of 5-HT by pharmacological means compared to the parent (AS) strain ?
3- Is 5-HT release in the striatum reduced in the AS/AGU mutant ?

As a prelude to experiments 2 & 3 it is necessary to confirm the projections from DRN to striatum and this is the next experiment I shall report.

# 3 Experiment 2. Investigation of the rostral projections from the raphe nuclei using the retrograde tracer cholera toxin

## 3.1 Introduction

The dorsal and median raphe nuclei in the midbrain are amongst the major nuclei composed of serotonergic cells (Azmitia and Segal, 1978; Chojnacka-Wojcik, 1995; Dray, 1981; Jacobs and Azmitia, 1992; Van der Kooy and Hattori, 1980).

The mapping of aminergic neurons started with the formaldehyde-induced fluorescence (FIF) technique, which allows microscopic visualization of monoamines in freeze-dried tissues (Falck et al., 1962). Subsequent modifications of the FIF techniques allowed a variety of authors (Dahlstrom and Fuxe, 1964; Fuxe and Jonsson, 1967; Bjorklund et al., 1971; Fuxe and Jonsson, 1974; Jonsson et al., 1975; Azmitia and Henriksen, 1976; Bjorklund et al., 1976; Loren et al., 1976; Smialowska, 1979) to demonstrate that the midbrain raphe nuclei contain numerous serotonin-synthesizing cells which give rise to both ascending and descending fibers.

In the midbrain, six ascending fibre tracts were identified, the dorsal raphe nucleus being the sole source of four tracts with one arising from the median raphe nucleus and one shared (Azmitia, 1978). The tracts can be classified as those lying within the medial forebrain bundle (MFB) a collection of ascending and descending connections between the forebrain and midbrain (for review Guillery, 1957). The dorsal raphe forebrain tracts lie in the ventrolateral aspect of the medial forebrain bundle and project mainly to lateral forebrain structures (e.g. basal ganglia, amygdala, and the pyriform cortex). The median raphe forebrain tracts lie in the ventromedial aspect of the MFB and project to medial forebrain areas (e.g., cingulate cortex, medial septum and hippocampus). The importance of the MFB as the ascending route for 5-HT projections in the rodent brain has been stressed by workers using electrolytic (Heller et al., 1962) and neurotoxic (Jacobs et al., 1977) lesioning techniques as well as by autoradiographic connection tracing (Azmitia and Segal, 1974; Conrad et al., 1974; Moore and Halaris, 1975). The latter technique has provided anatomical evidence for divergent projections from the midbrain nuclei (DRN and MRN) (Bobillier et al., 1975; Pierce et al., 1976). The descending fibers of the DRN

are distributed to the cerebellum, the lower brain stem and the spinal cord (Steinbusch et al., 1981).

A subsequent experimental tool involves the identification of neuronal cell bodies following injections into their target field. One possible mechanism is by the uptake of material by the axon terminal, and its transport in a centripetal or retrograde direction to the cell body. This technique started about thirty years ago when horseradish peroxidase was used to retrogradely label motoneurons supplying the gastrocnemius muscle and retinal ganglion cells innervating the optic tectum (Kristensson and Olsson, 1971; LaVail and LaVail, 1972).

Since then, retrograde tracing has become the main tool for studying neuronal connectivity in both the central and peripheral nervous system (Schwab et al., 1979; Stockel et al., 1977; Trojanowsky et al., 1982; Wan et al., 1982). Varieties of these tracers (often involving horseradish peroxidase conjugates with increased sensitivity) make it possible to suit almost any experimental design (Wan et al., 1982). They include retrogradely-transported molecules that fluoresce or can be detected immunocytochemically (Horikawa and Powell, 1986; Luppi et al., 1987), tracers linked to colloid gold particles (Basbaum and Menetrey, 1987; Llewellyn-Smith et al., 1990), fluorescent latex microspheres (Katz et al., 1984) and, most recently, viruses which reveal chains of neurons that innervate the target at which the virus was injected (Aston-Jones and Card, 2000) have been widely used.

A particularly efficient retrograde tracer is cholera toxin (CT), which is transported in both the peripheral and central nervous systems (Stockel et al., 1977; Luppi et al., 1987); it was introduced as a retrogradely transported marker in 1977 (Sawchenko and Gerfen, 1985). It is known that gangliosides represent a relatively large proportion of neuronal membrane lipids and cholera toxin is bound with high affinity to gangliosides (Stoeckel et al., 1977).

The aim of this study is to investigate the localization of possible serotonergic (5-HT) projections from the dorsal and median raphe nuclei within the neostriatum of AS and AS/AGU rats.

In order to determine the connectivity between the raphe and the caudate-putamen, the retrograde tracer cholera toxin b (CTb) was injected into 1- the anterior caudate-putamen (ACPU) and 2- the dorsal caudate-putamen (DCPU). ICC was carried out for CTb and 5-HT within the midbrain and cell counts were carried out.

These two regions were chosen as previous experimental work on dopamine depletion suggested that the DCPU was badly affected in the AS/AGU mutant whereas the ACPU was not (Campbell et al., 1996). Projections from the substantia nigra pars compacta (SNc) were also examined in CTb-treated animals, using tyrosine hydroxylase (TH) immunoreactivity to confirm that the cells were dopaminergic.

## 3.2 Material and Methods

Adult male rats aged 12 months were used, 4 AS and 5 AS/AGU in each case. Two µl of solution of choleragenoid (Cholera toxin B subunit) (Sigma) was injected stereotaxically by means of 10 µl Hamilton syringe into either the anterior (ACPU) or dorsal caudate-putamen (DCPU).

Rats were anaesthetized using Xylazine (Rompun; Bayer plc., Bury St. Edmund, UK., 37 mg/kg) and Ketamine (Vetalar; Park & Davies, Pontypool, UK., 73 mg/kg) in a ratio of 1:2. The injections were given at 1.1 ml/kg. In order to confirm deep anaesthesia, the "pad pinching" technique was used (where no reflexual withdrawing of the foot was observed when full anaesthesia was reached). It was very important to keep the eye moist until the end of the procedure.

A small area on the head was shaved and then wiped with alcohol. The rats were then placed into a Kopf stereotaxic frame (Munich, Germany) with ear bars, nose clips and teeth clips.

An incision was made using a scalpel and the skin retracted using curved Spencer Wells forceps. The skull was scraped using a scalpel blade to remove the periosteum and the area dried using a cotton bud. Bregma (the intersection of the coronal and sagittal sutures) was identified and marked using a pencil, and confirmed independently prior to taking the bregma co-ordinates.

Lateral, antero-posterior and dorso-ventral co-ordinates (+ 0.35mm, -0.1mm and –0.4mm respectively) were taken using the x,y, and z scales on the stereotaxic frame. From these co-ordinates, new co-ordinates were calculated depending on the area of interest using the Paxinos and Watson (1982) stereotaxic brain atlas.

A microsyringe was placed directly over the brain area corresponding to these new co-ordinates and again this was marked using a pencil and confirmed independently. A hole was drilled into the skull over the pencil mark and the area dried with a cotton bud. The microsyringe was then placed over the hole and lowered into the brain very slowly to reach

the appropriate ventral co-ordinate then allowed to rest for at least two minutes; 1 µl of cholera toxin b was then injected and after, another minute, another 1 µl of toxin was injected. The needle was then left in for a further two minutes to prevent leakage of the toxin and then withdrawn very slowly.

The hole was sealed with Redifast powder (Wright Health Group Ltd., Dundee, U.K.) and Redifast solution (Wright Health Group Ltd., Dundee, U.K.), the rat removed from the stereotaxic frame and the skin incision sutured. The rats were then given 0.1 ml atipamezole hydrochloride (antisedan 5mg/ml; Pfizer Ltd, Kent) in order to reverse the effects of the anaesthetic and allowed to recover before being replaced into clean cages.

One week following brain micro-injection of CTb the animals were sacrificed and their brains removed. The rats were deeply anaesthetised with phenobarbitone sodium BP (Rhone-Mericux, Spire Greencentre, Harlow, Essex, 60mg/1ml) and perfused with Ringer's solution via intracardiac cannulation, and fixed with 4% paraformaldehyde. The rats were decapitated using scissors and their brains removed carefully using ronguers (Merck Ltd., Leicester), before being post-fixed overnight in the same fixative.

Before any ICC procedures were carried out it was established whether or not the injection had gone into the correct area. This was checked via a light microscope. Signs of the injection site were a noticeable crack due to the shrinkage of the surrounding tissue and red blood cells along with a large deposit of cholera toxin itself due to leakage, if any. If the injection was not in the target area then that particular animal was not used.

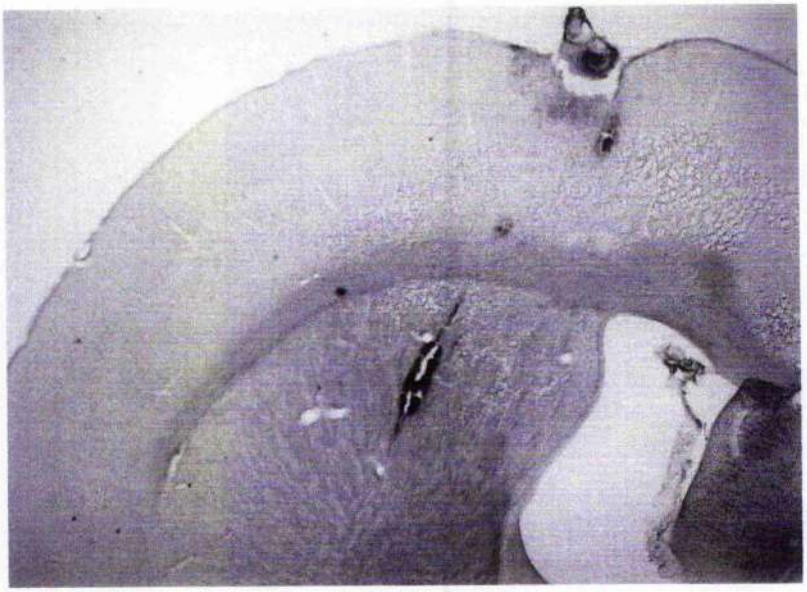


**Figure 3-1 Injection site in the DCPU. (x40 )**

## 3.2.1 Immunocytochemistry

### 3.2.1.1 5-HT staining

ICC for 5-HT (see experiment 3).

### 3.2.1.2 Cholera Toxin b staining

No blocking serum was required as the primary antibody was goat anti-cholera toxin B. The sections were incubated overnight in 1:40,000 primary antibody at 4 $^{o}$C then incubated for sixty minutes in biotinylated secondary antibody (1:500 biotinylated antigoat) before continuing as above.

### 3.2.1.3 Double labelling

After three PBS rinses, the sections were incubated overnight in mixture of primary antisera (Rabbit anti-serotonin and Goat anti cholera toxin b) the dilutions were (1/2,000 and 1/20,000 respectively). The sections were then incubated for two hours in fluorescent secondary antibodies (fluorescein (FITC)-conjugated Donkey anti-Rabbit 1gG and Texas red (TR)-conjugated Donkey anti-Goat 1gG), the dilution was 1/100 for both. After two rinses with PBS the sections were mounted with Vectashield.

# 3.3 Results

## 3.3.1 Retrograde staining using cholera-b-toxin

### 3.3.1.1 Staining of the dorsal raphe nucleus from the dorsal caudate-putamen

Serotonin- and cholera toxin- immunoreactive staining was easily seen within the dorsal raphe nucleus. When 5-HT-ir cell numbers in the DRN were plotted along a rostral-caudal axis (Fig. 3.2), the numbers followed a slightly skewed normal distribution with peak cell numbers marginally rostral of the mid-point of the range (approximately Bregma - 7.8mm). Although the numbers of CTb cells were less than those staining for 5-HT, the distributions mirrored one another (Fig. 3.2).

When total cell numbers were considered, it was found that CTb cells represented 40-50% of 5-HT-ir numbers in both AS and AS/AGU rats (Fig. 3.3). Allowing for the uncertainty of reaching all cells in the dorsal caudate-putamen by this method, this implies that at least half the serotonergic cells in the dorsal raphe nucleus have some connection with the DCPU in the area of the injection.

It is also possible to compare cell counts from AS and AS/AGU rats. This shows that AS/AGU rats have fewer 5-HT-ir cells than AS rats ($t = 12.89$, $df = 5$ $p < 0.0001$ T-TEST).

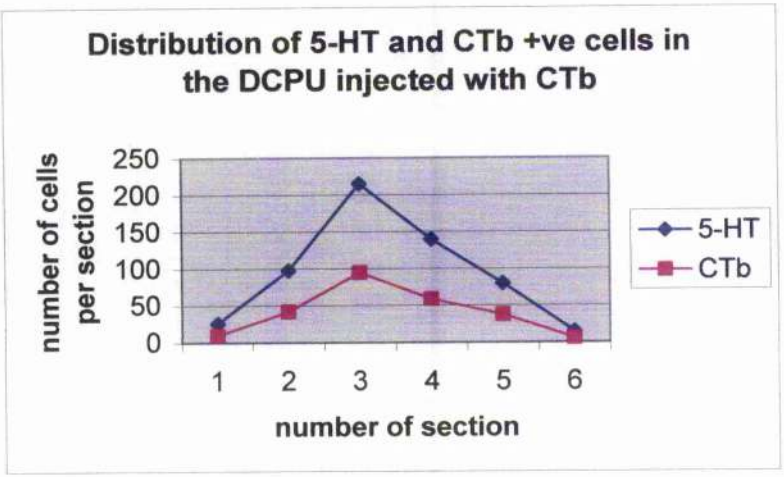



**Figure 3.2 Distribution of 5-HT and CTb +ve cells in the DCPU injected with CTb from rostral (1) to caudal (6).**

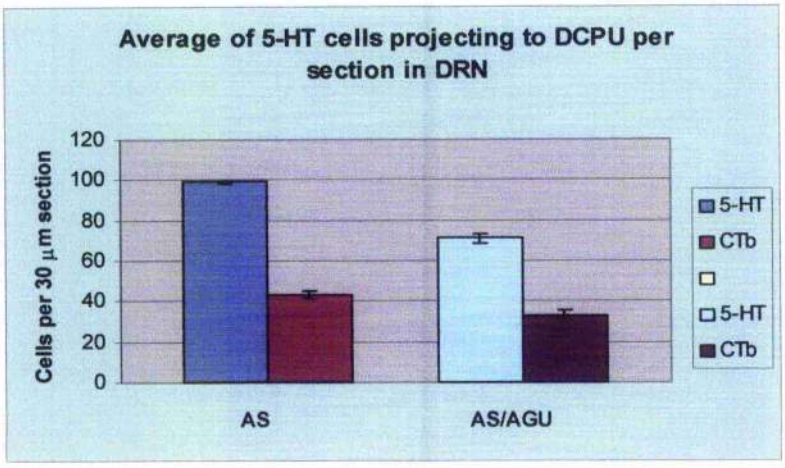



**Figure 3-3 The numbers of 5-HT-ir and CTb-ir cells per 30μm section in the dorsal raphe nucleus of AS and AS/AGU rats aged 12 months after CTb injection into the dorsal caudate-putamen (DCPU). No 6 per group. All figures are means**

### 3.3.1.2 staining of the median raphe nucleus from the dorsal caudate-putamen

No staining with cholera-b-toxin was found.

### 3.3.1.3 Staining of the dorsal raphe nucleus from the anterior caudate-putamen

Although not an objective of this experiment, I helped to supervise an Honours student who, as part of her work, examined a single AS rat with cholera-b-toxin injected into the anterior caudate-putamen. The data from this single animal is shown in Fig. 3.4 from which it can be seen that the number of cells staining positively for CTb were about a third of those staining for 5-HT.

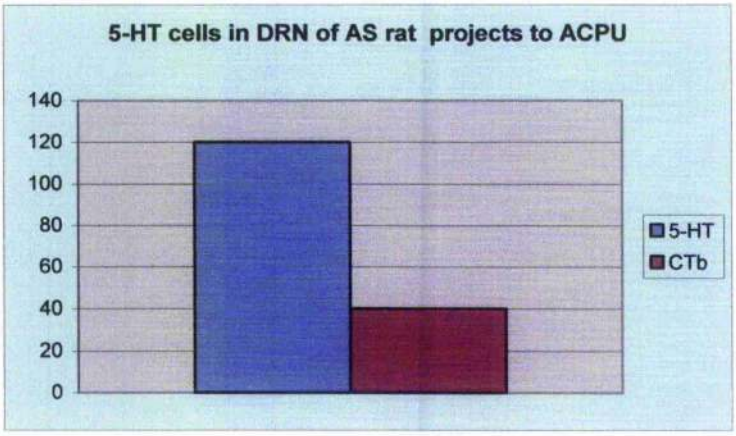



**Figure 3-4 5-HT-ir cells project to the ACPU (taken from L4 student whom I supervised).**

### 3.3.2 Double labelling

In the studies reported above, different sections were used for 5-HT and CTb immuno-staining. There remains, therefore, a remote theoretical possibility that these are different cell populations. To control for this, a double labelling experiment was carried out in which the same sections were stained for both 5-HT and CTb using fluorescent antibodies.

Although no counting were carried out, double labelling with 5-HT and CTb showed that most of the cells stained with CTb are 5-HT positive. Also it was clear that there are cells stained with 5-HT but not stained with CTb (Fig. 3.5) By contrast few cells stained with CTb that were not 5-HT positive cells.

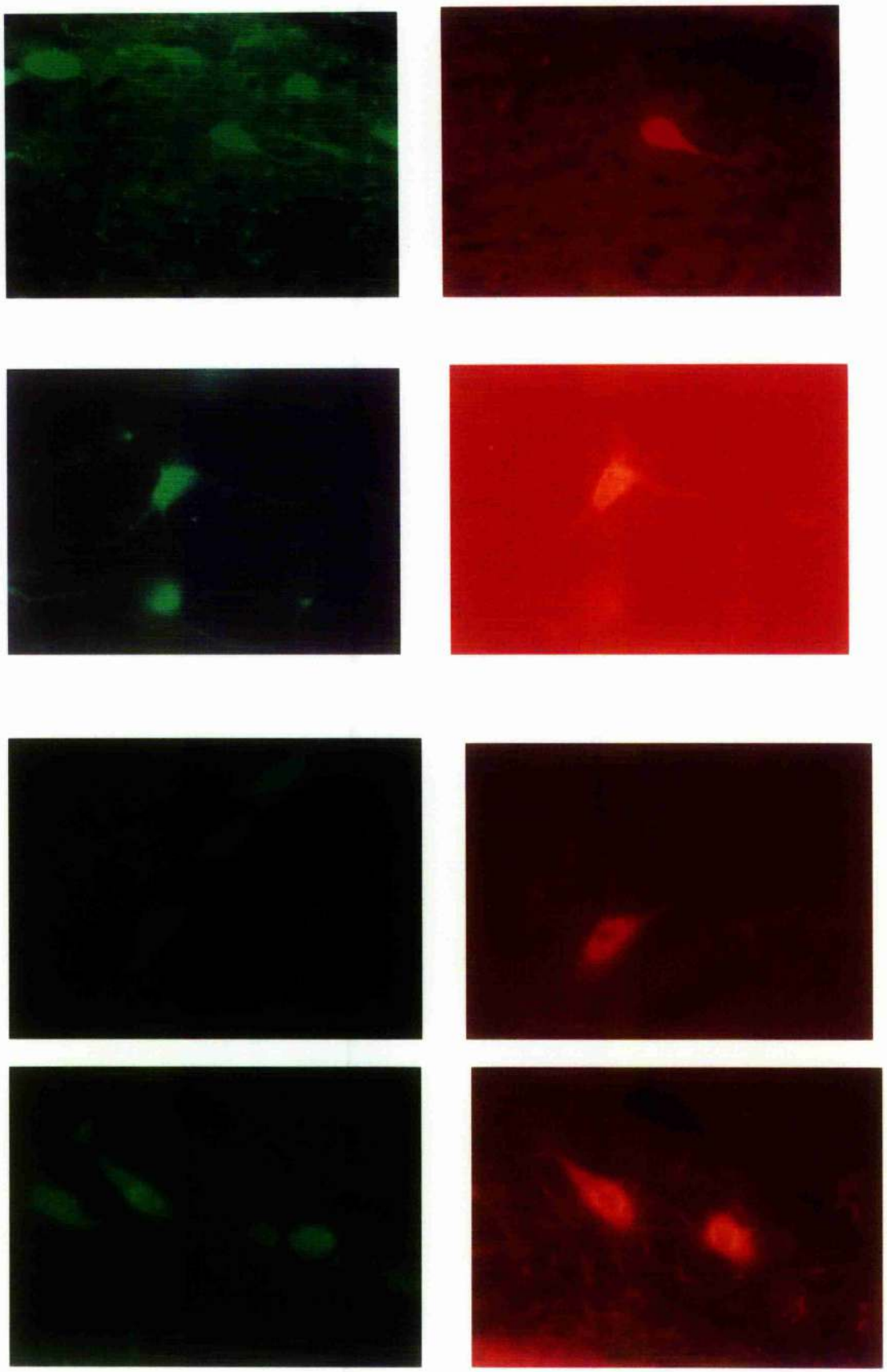


**Figure 3.5 Coronal section of DRN stained with both 5-HT (green) and CTb (red).**

## 3.4 Discussion

The main purpose of this study was to confirm that the dorsal raphe nucleus projected to the caudate-putamen and to see whether serotonergic cells projecting from this midbrain raphe nucleus to different parts of the caudate putamen are uniformly distributed within the raphe or occupy specific areas.

Mapping can be done with many techniques; one of them is lesioning but this has the disadvantage of potentially affecting fibre systems passing through or close to the area of the lesion (Conrad et al., 1974). Some other techniques like fluorescence appear less sensitive for serotonin than for catecholamines (Conrad et al., 1974).

The injection of a retrograde tracer like CTb will avoid such problems and has many additional advantages. The technique depends upon the non-toxic part of CTb binding specifically to monosialoganglioside receptors on the nerve terminal surface (Stockel et al., 1977). The binding capacity of CTb also results in a restricted injection site. Indeed, CT injection sites are at least twice as restricted as those of un-conjugated wheat germ agglutinin (WGA) with the same concentration and volume.

Many studies have shown greates sensitivity of CTb than other retrograde tracers such as (Peyron et al., 1998) who observed afferents to the DRN either not reported or underestimated in other studies used HRP or WGA-HRP (Aghajanian and Wang, 1977; Kalen et al., 1985). The technique can be carried out on paraformaldehyde or glutaraldehyde-fixed material or both.

CTb can be combined with immunohistochemistry of many neurotransmitter substances (Luppi et al., 1987). In contrast, some retrograde tracers like nerve growth factor are confined to sensory and adrenergic neurons (Stoeckel et al., 1977).

The more complete retrograde staining obtained with CTb cannot be attributed to an uptake of CTb by fibers of passage as suggested by Chen and Aston-Jones (1995).

Chen et al (1999) found that CTb did not appear to effect PRV transport or infectivity, also the dense deposit of CTb did not appear to change over time.

When considering rats as experimental subjects, one week after injection is long enough to provide a nice staining quality of the injected tracers (Lanciego et al., 2000). In the present study, ICC was carried out according to Peyron et al., (1998) with some minor modifications.

The cholera b toxin tracing experiments produced results that confirm the known inter-relationship between the DRN and the caudate putamen in terms of 5-HT neurotransmission in the rat. Both ACPU and DCPU were confirmed to receive inputs from the dorsal but not the median raphe nucleus. In both ACPU and DCPU, the sub-population of serotonergic cells reached by the cholera toxin injection was between 30% and 50% of the total number. In the case of the DCPU, this pattern is much the same in both AS controls and mutant animals, although total cell numbers are reduced in the mutant for both 5-HT (as will be shown in details in experiment 3) and cholera toxin. This finding suggests that although 5-HT cell numbers in the AS/AGU dorsal raphe nucleus are reduced by around 22%, the integrity of the projections to the DCPU by remaining cells seems to be relatively intact, and that a reduction in 5-HT in the striatum is probably more likely to be the result of a production/release problem than due to there being a deficit in the DRN-DCPU connections. It should be borne in mind, however, that the remaining cells may have survived due to their having more robust connections with the striatum, and that this does not rule out poor DRN-DCPU connectivity at an earlier stage in cells that were lost. It would be interesting to extend this study in future to an age range of animals in order to determine whether cell loss/striatal connection ratios were consistent with age.

The double fluorescent staining experiment demonstrates that all cholera-toxin positive neurons in the DRN are actually serotonergic, thus showing a proportional deficit between 5-HT-positive cells and 5-HT projections to the dorsal striatum in the AS/AGU rat.

# 4 Experiment 3. Cell numbers in the dorsal and median raphe nuclei of AS and AS/AGU rats

## 4.1 Introduction

The cell bodies of most serotonergic neurons are located within the brainstem (Chojnacka-Wojcik, 1995; Jacobs and Azmitia, 1992; Morgan et al., 1987). Those in the hindbrain project down to the spinal cord whilst another group in the midbrain projects rostrally. It is the group in the midbrain which was looked at in this experiment.

The area of the midbrain called the raphe nucleus can be divided into dorsal raphe and median raphe nuclei (DRN and MRN, respectively) and it is within these that the serotonergic neurons are concentrated (Azmitia and Segal, 1978; Chojnacka-Wojcik, 1995; Dray, 1981; Jacobs and Azmitia, 1992; Van der Kooy and Hattori, 1980), being most abundant in the dorsal raphe nucleus.

The cells of the dorsal and median raphe nuclei provide the vast majority of axonal processes containing serotonin (5-hydroxy tryptamine, 5-HT) that innervate the forebrain of the rat (Azmitia and Segal, 1978; Imai et al., 1986; Jacobs and Azmitia, 1992). However, the pattern of termination of 5-HT fibres to the forebrain differs between the DRN and MRN. Thus, whereas 5-HT cells within the DRN mainly innervate brain structures related to motor activity such as the basal ganglia, those in the MRN project preferentially, though not exclusively, to limbic regions such as the medial septum and hippocampus (Bobillier et al., 1975; Azmitia and Segal, 1978; Kohler et al., 1982; Imai et al., 1986; Jacobs and Azmitia, 1992; Mcquade and Sharp, 1997).

In addition, the axons of 5-HT cells of the MRN are thicker and have larger varicosities compared to serotonergic cells in the DRN (Kosofsky and Molliver, 1987; Mamounas et al., 1991). The two nuclei also show differences in their sensitivity to neurotoxins (Mamounas and Molliver, 1988; Blier et al., 1990), agonists at somatodendritic 5-HT1A autoreceptors (Sinton and Fallon, 1988; Hillegaart et al., 1990; Invernizzi et al., 1991; Casanovas et al., 1997) and in their activation as a response to aversive stimuli (Dilts and Boadle-Biber, 1995; Adell et al., 1997). However, conflicting studies exist which have

found no difference in the response to neurotoxins (Hensler et al., 1994; Gartside et al., 1996) or the sensitivity to 5-HT1A agonists or selective 5-HT reuptake inhibitors (Hajos et al., 1995).

As described in the Introduction, Parkinson's disease was initially defined as a loss of both dopaminergic and serotonergic neurons, with particular loss of large 5-HT cells in the dorsal raphe found post-mortem (Jellinger, 1992). Other related syndromes (e.g. MSA, PSNP) show cell loss in multiple aminergic systems (Hedera and Whitehouse, 1994). The purpose of this experiment, therefore, was to see if AS/AGU mutants had reduced 5-HT neurons as well as reduced DA neurons (as previously reported).

Both midbrain raphe nuclei, the dorsal raphe nucleus and median raphe nucleus were examined. The reasons for choosing these two nuclei is to see:

A/ whether the serotonergic system is affected at all in the AS/AGU rat and

B/ whether the damage is general or specific to neurons projecting to the basal ganglia.

So numbers of serotonergic cells have been counted in both nuclei in AS and AS/AGU rats.

The numbers of cells in particular brain regions or nuclei is a fundamental piece of information and cell counts have been carried out in many brain areas in many different species and using many different methods. Serotonergic neurons in the brain and the raphe nuclei in particular have been counted in rat (Dahlstrom and Fuxe, 1964; Beaudet and Descarries, 1976; Nygren and Olson, 1977; Iorez et al., 1978; Descarries et al., 1979; Jacobs and Azmitia, 1992), cat (Wiklund et al., 1981), monkey (Hatzidimitriou et al., 1999) and in human (Agid et al., 1987; Jellinger, 1992).

Quantitive methods for analyzing the morphology of the brain have undergone a revolution in the past fifteen years. The unique elements of this revolution include systematic random sampling, measuring three-dimensional quantities such as total neuronal number and volume, and using unbiased methods to estimate these three-dimensional quantities (Sterio, 1984; Gundersen, 1986; Swaab and Uylings, 1987; Gundersen et al., 1988; Bolender et al.,

1991; Oorschot et al., 1991; Mayhew, 1992; Oorschot, 1994). One of these unbiased methods is the Cavalieri principle, which enables the determination of the total volume of the brain structure of interest. Another is the optical disector method, which enables the determination of the number of neurons in a sub-volume of the brain structure. The product of the total volume and the number of neurons in a sub-volume (i.e., the neuronal density, or the Nv) by these methods yields an unbiased estimate of the total number of neurons. Such total number studies permit reliable comparisons to be made between species, between normal and diseased human conditions, and between control and experimentally injured animals (Pakkenberg et al., 1991; Bjugn, 1993; Oorschot, 1994; Raadsheer et al., 1994). Total neuronal numbers are also important for theoretical approaches such as the computer modeling of brain function.

Data on total neuronal numbers within the rat raphe nuclei (e.g. by Dahlstrom and Fuxe, 1964; BeauDet and Descarries, 1976; Nygren and olson, 1977; lorez et al., 1978; Descarries et al., 1979; Jacobs and Azmitia, 1992; Wiklund et al., 1981; Hatzidimitriou et al., 1999; Agid et al., 1987) as well as counts involving human control and PD subjects (e.g. Jellinger, 1992) have not been made using unbiased stereological methods. Therefore, I have analysed the DRN and MRN of AS and AS/AGU rats

A/ using conventional cell counts in an interrupted series of sections in order to compare findings with previous authors.

B/ using unbiased Cavalieri and optical disector methods.

## 4.2 Materials and Methods

### 4.2.1 Animals

Six male AS and six male AS/AGU rats were used, aged 12 months.

### 4.2.2 Immunocytochemistry (ICC)

In order to ensure that I was counting 5-HT cells, it was necessary to identify them by immunocytochemistry, a technique first employed by Hsu et al., (1981).

The rats were deeply anaesthetized with an overdose of Sodium pentobarbitone BP (Vet) (Rhone-Merieux, Spire Greencentre, Harlow, Essex, 60mg/1ml). They were then perfused through the left ventricle with 100ml Ringer's solution containing the vasodilator Lignocaine, followed by 500ml 4% Paraformaldehyde (2944744 BDH) in 0.1M phosphate buffer. The brains were removed and post-fixed in the perfusion fixative for 3 hours. They were then washed three times for 10 minutes in PBS and immersed in 7% Sucrose (10274, BDH) and Sodium azide (S2002, SIGMA) in PBS before storage overnight at 4 °C.

The brains were embedded in tissue-tek (4583, Miles) frozen in liquid-nitrogen and then cut at 30 μm thickness on a cryostat at –20 °C (Leica, Jung Frigocut 2800E). Coronal sections were taken in a rostral to caudal direction and collected serially in bottles containing 0.1M phosphate buffer (PB). The sections were processed for 5-HT immunostaining.

#### 4.2.2.1 Immunocytochemistry on cryostat sections

The sections were rinsed in PBS and incubated initially in Goat anti-serum in 0.3 Triton X-100 (30632, BDH) for one hour. This is used to bind charged proteins within the tissue and

allow enhanced penetration of primary antibody. This was followed by incubation with the Primary antibody, at an optimal dilution in PBS containing 0.3% Triton X-100.

The primary antibody was Rabbit anti-Serotonin (Z02597, AFFINITI) at a dilution of 1:10,000, which reliably labels serotonergic neurons in the midbrain. Initially, different dilutions of antiserum were tested under the same conditions in order to determine the optimum antibody concentrations.

PBS was used to dilute the antibodies and for all washes. Triton X-100 increased the penetration of antibodies (Grube, 1980). Incubation was performed in a humidified chamber at room temperature overnight.

The sites of polyclonal antibody-antigen reaction were visualized by the application of biotinylated donkey anti-rabbit immunoglobulin (IG) (RPN 1004, AMERSHAM) at a dilution of 1:250 for one hour at room temperature, followed by Avidin-Biotin-Peroxidase complex (20µl of solution A and 20 µl of solution B in 1ml of PBS Vector ABC elite kit) which binds to the secondary antibody as the Avidin portion of the complex contains 4 binding sites specific to biotin. Finally, 5-HT-immunoreactivity was visualised using 3,3-diaminobenzidine (DAB). This reacts with the peroxidase portion of the ABC complex to form a brown reaction product after 3-5 minutes. As DAB is a carcinogen, care was taken to ensure that this part of the experimental protocol was carried out in a fume cupboard, using disposable laboratory equipment.

## 4.2.3 Cell Counting techniques

### 4.2.3.1 Conventional counts

The rostral-caudal length of the DRN/MRN is approximately 3mm (Paxinos and Watson, 1982) thus yielding c. 100 x 30 µm thick coronal section. Every fifth section was collected, stained and counted using a microscope with a 10x eyepiece fitted with a

graticule. All stained cells were counted throughout the dorsal and median raphe nuclei, yielding a value of the average number of 5-HT-ir cells per 30μm section.

#### 4.2.3.2 Stereology

The optical disector technique (Wreford, 1995) was used to estimate 5-HT cell numbers in DRN & MRN. Total raphe nuclei volumes were estimated using the Cavalieri Principle (Mayhew, 1992). Slides used to estimate the number of cells were also used to estimate raphe nuclei volume to avoid any requirement for correction factors due to tissue shrinkage. A computer using AutoCADlt97 software (Autodesk Inc, San Rafael, CA) and a digitising tablet was used to estimate the surface area of selected sections (every fifteenth section). The total surface area of these selected sections is designated *SA*. The thickness of each section is known (h), as is the distance between the sections (d), and total Raphe nuclei volume (tv) can be calculated by $tv = SA \times h \times d$. The numerical density was estimated using an Olympus BX50 microscope fitted with a motorized stage (Prior Scientific Instruments, Cambridge) and *Stereologer* software (Systems Planning Analysis, Alexandria, VA).

The absolute number of cells in each nucleus was then calculated by multiplying its volume by the cells numerical density.

## 4.3 Results

To ensure optimal cell staining, it was first necessary to determine the appropriate concentration of primary antibody required. The concentration of primary antibody that resulted in clearly visible serotonergic cells was 1:10,000 and this was used throughout the successive ICC experiments. At this concentration, serotonergic cells within the dorsal and median raphe nuclei stained positively and could easily be identified (see fig 4.1). A clear distinction could readily be seen between the cells in the median raphe nucleus and those contained in the larger, dorsal raphe nucleus. Cells clusters were carefully examined at high magnification with repeated re-foucusing until the number of cells forming the cluster could be determined.

There was no difference in raphe nucleus size between two group of animals (AS and AS/AGU), nor any obvious difference in cell size or shape.

There appeared to be slight regional differences in cell morphology in the two raphe nuclei. Cells of the DRN were more densely stained, tended to have a triangular shape and their dendritic processes were highly visible (fig 4.2). In contrast, cell bodies contained in the MRN were rounder, smaller and paler and the neuronal projections were less visible. Nevertheless, all serotonergic cells could be easily counted in both regions.

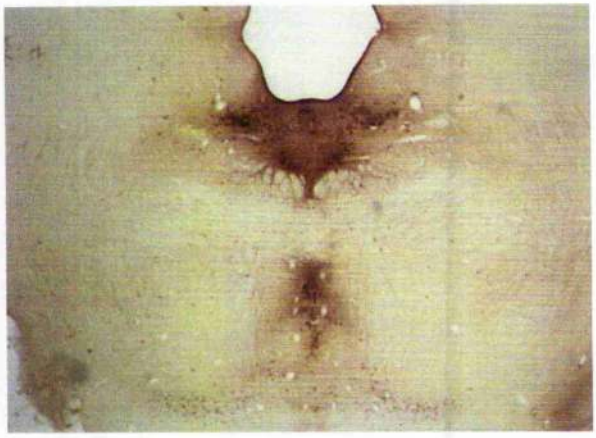


**Figure 4-1 Coronal section of the midbrain showing both dorsal (top) and median (bottom) raphe nuclei. This section is approximately –7.8mm relative to bregma. Magnification (x25)**

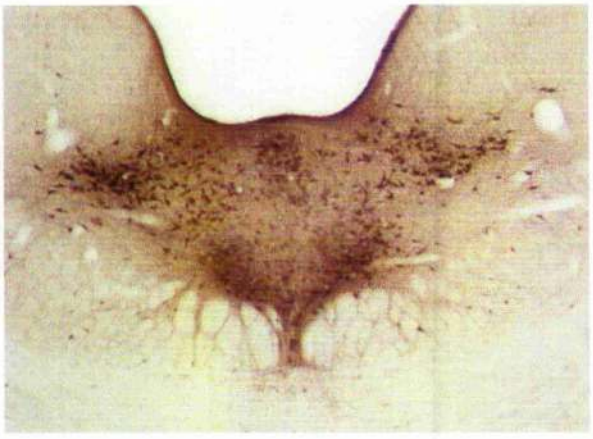


**Figure 4-2 Higher magnification of fig 4.1 shows the dorsal raphe nucleus after serotonin immunohistochemistry. (x65)**

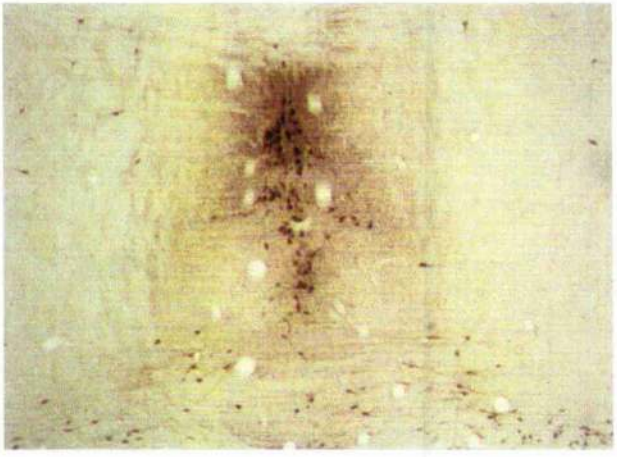


**Figure 4-3 Higher magnification of fig 4.1 shows the median raphe nucleus after serotonin immunohistochemistry. (x65)**

### 4.3.1 Conventional Cell counts

The number of serotonergic cell bodies within the DRN and MRN of AS and AS/AGU rats can be seen in fig 4.4 and fig 4.5 respectively. A student t-test for independent samples was performed, using the Minitab statistics package. The results are summarized in table 4.1 and shown pictorially in fig. 4.4. The data show a significant difference between the AS (control) and AS/AGU (mutant) rats, with mutants possessing c. 23% fewer 5-HT-ir cells in the dorsal raphe than controls ($p<0.001$).

By contrast, table 4.2 and fig. 4.5 show that there was no significant difference between AS and AS/AGU rats in the numbers of serotonergic cells in the median raphe.

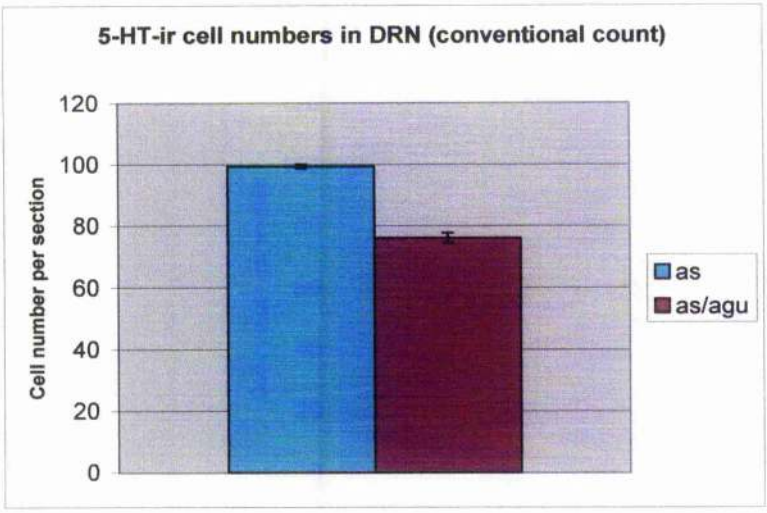



**Figure 4.4 The average number of 5-HT immunoreactive cells per 30μm section in the dorsal raphe nucleus of control (AS) and mutant (AS/AGU) male rats aged 12 months (n = 6 per group). Counting was made by conventional means.**

| Animal | AS | AS/AGU |
|---|---|---|
| 1 | 98 | 82 |
| 2 | 97.7 | 77.7 |
| 3 | 98.7 | 75.4 |
| 4 | 100 | 74 |
| 5 | 99.7 | 76 |
| 6 | 102 | 71 |
| Average | 99.35 | 76.02 |
| SEM | 0.65 | 1.50 |

t = 14.22 df = 10 P< 0.001

Table 4.1 The average number of 5-HT immunoreactive cells per 30μm section in the dorsal raphe nucleus of control (AS) and mutant (AS/AGU) rats.

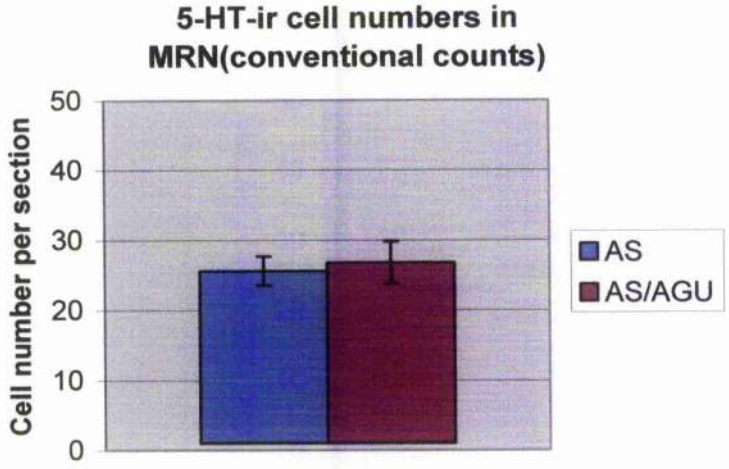



Fig 4.5 the average number of 5-HT-immunoreactive cells per 30μm section in the median raphe nucleus of control (AS) and mutant (AS/AGU) male rats aged 12 months (n= 6 per group). Counting was made by conventional means

| Animal | AS | AS/AGU |
|---|---|---|
| 1 | 30 | 25 |
| 2 | 22 | 19.82 |
| 3 | 23 | 28.78 |
| 4 | 25 | 32 |
| 5 | 26 | 25 |
| 6 | 25 | 24 |
| Average | 25 | 25.9 |
| SEM | 1.23 | 1.9 |

T = 0.33 P> 0.75 DF= 8

Table 4.2. The average number of 5-HT immunoreactive cells per 30μm section in the median raphe nucleus of control (AS) and mutant (AS/AGU) rats.

## 4.3.2 Cell count (using computerised stercology)

### 4.3.2.1 Estimation of the total volume of each raphe nucleus (Tv):

This was calculated according to the Cavalieri principle by multiplying three factors:

1- Surface area of selected sections (Sa)

2- The thickness of each section (H)

3- The distance between sections (D).

The surface area was measured by a computer with Auto CAD1t97 software and digitising tablet.

So the total volume (Tv) of each raphe nucleus is calculated as:

$$Tv = Sa \times H \times D$$

### 4.3.2.2 Estimation of the numerical density (Nv) of 5-HT-ir cells:

Pilot studies were performed to determine appropriate measurement factors, which will reduce a coefficient of error (CE) to less than 0.10 (Gundersen and Jensen, 1987). Then the full study of calculating the Nv was achieved automatically using an Olympus BX50 microscope fitted with a motorized stage and *stereologer* software.

### 4.3.2.3 Total neuronal number in dorsal and median raphe nuclei (Tn):

The absolute number of cells in each nucleus was calculated by multiplying the cell density by the volume $Tn = Nv \times Tv$

The total number of serotonergic cells in the dorsal raphe nucleus of the two groups is shown in Table 4.3.

| Animal | AS | AS/AGU |
|---|---|---|
| 1 | 8562 | 7196 |
| 2 | 8312 | 6850 |
| 3 | 8697 | 6611 |
| 4 | 8854 | 6484 |
| 5 | 9062 | 6991 |
| 6 | 8780 | 6749 |
| Average | 8711 | 6813 |
| SEM | 114.9 | 115.4 |

$t = 12.77$ $df = 10$ $P<0.001$

Table 4.3. The total number of 5-HT immunoreactive cells in the dorsal raphe nucleus of control (AS) and mutant (AS/AGU) rats, as measured by computerised stereological methods.

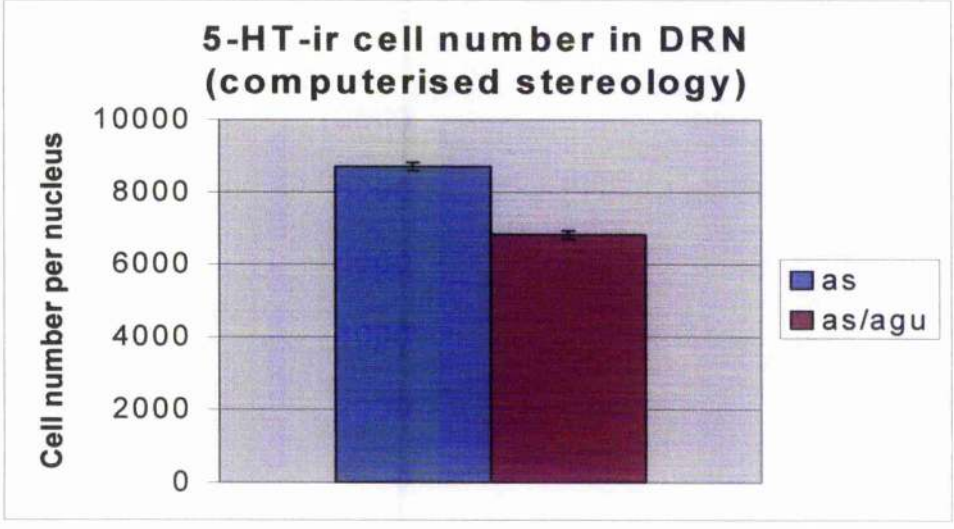



Fig. 4.6. The total numbers of 5-HT-immunoreactive cells per dorsal raphe nucleus in AS and AS/AGU male rats aged 12 months (n = 6 per group).

Counts were obtained by computerised stereological means.

From table 4.3 and fig. 4.6, it is apparent that there is a very significant difference in the number of 5-HT-ir cells in the dorsal raphe nucleus of AS (control) and AS/AGU (mutant) rats. Mutants possess 22% fewer serotonergic cells than controls.

By contrast, we can see from table 4.4 and fig. 4.7 that there are no differences between AS (control) and AS/AGU (mutant) rats with respect to the numbers of 5-HT-ir cells in the median raphe nucleus.

| Animal | AS | AS/AGU |
|---|---|---|
| 1 | 1022 | 1215 |
| 2 | 998 | 1017 |
| 3 | 1076 | 1002 |
| 4 | 1086 | 1042 |
| 5 | 1221 | 1053 |
| 6 | 985 | 1111 |
| Average | 1064 | 1073 |
| SEM | 38.8 | 35.3 |

$t = 0.18$ $df = 10$ $P<0.86$

**Table 4.4. The total number of 5-HT immunoreactive cells in the median raphe nucleus of AS (control) and AS/AGU (mutant) rats as measured by computerised stereological methods.**

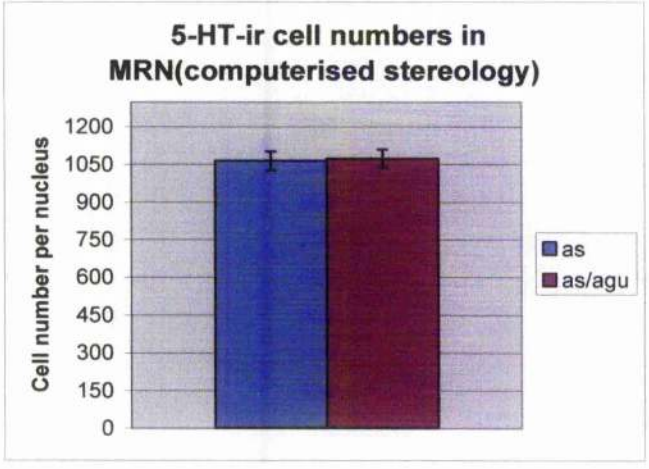



**Fig 4.7. The total number of 5-HT-immunoreactive cells per median raphe nucleus in control (AS) and mutant (AS/AGU) male rats aged 12 months (n = 6 per group).**

Counts were obtained by computerised stereological means.

## 4.4 Discussion

Two points require initial discussion. Firstly, are total cell numbers comparable to those found in previous studies and, secondly, are there differences in cell numbers between control and mutant animals?

Cell numbers in the midbrain have been counted using conventional methods in different species. Descarries et al. (1979) reported that the total population of serotonergic cells in the DRN of adult rat was 11,428. In cat DRN the number was estimated to be 24,257 (Wiklund et al., 1981). More recent work has indicated that there are 11,500 serotonergic neurons in the DRN of adult rat while there are 1100 serotonergic neurons in the MRN (Barry and Azmitia, 1992). Cell numbers in primates may be more equal in the two nuclei. Thus, 5-HT-ir cell counts in the DRN and MRN of squirrel monkeys showed that the numbers were 8,269 and 7,034 respectively (Hatzidimitriou et al., 1999).

In the present study the total numbers of serotonergic neurons were analysed using unbiased computerised sterological software and Cavalieri principle (Wreford, 1995; Mayhew, 1992 respectively). The data obtained by unbiased methods in the present study showed that serotonergic neuron numbers were 8,711 for the DRN and 1064 for the MRN of AS animals. These numbers are slightly lower than those reported with conventional methods (Descarries et al., 1979; Barry and Azmitia, 1992) but this may be accounted for by age and strain difference as well as the different methodology employed.

To answer the second question, this study shows that there are fewer 5-HT-ir cells in the DRN of the mutant rat compared to the AS control rats. The reduction is approximately 23% when the total numbers were obtained using unbiased methods and 22% when counted by more conventional means. By contrast with the DRN, counts of the MRN serotonergic cells showed no difference between strains when measured either by conventional counts or by unbiased methods.

The extent of 5-HT cell loss in the mutant rat is less than found previously for DAergic cells (C. 40%) in 1 year old animals (Payne and Clarke, 1994; Payne et al., 2000), but is consistent with a general depletion of aminergic cells.

The reduction of 5-HT cells in DRN along with 40% loss in DA ergic cells in the mutant rat strongly suggests that the AS/AGU rat is a valuable model for studying neurodegenerative processes. Cell reductions of this magnitude have been reported in many human conditions related to movement disorders.

The change in cell numbers is likely to affect the target regions to which these cells project. The DRN is the principal source of striatal and cortical serotonin (Srebro and Lorens, 1975) and axons from the DRN project mainly to the caudate-putamen, amygdala and substantia nigra pars compacta (Azmitia and Segal, 1978; Imai et al., 1986). There are also projections to the thalamus (Azmitia and Segal, 1978) and to the locus coeruleus (Imai et al., 1986). In contrast, the MRN was found to send projections mainly to the hippocampus and the anterior hypothalamus (Azmitia and Segal, 1978; Van der Kar and Lorens, 1979), and also the mammillary body (Bobiller et al., 1979). Additionally, there was no evidence of axonal projection from the MRN to the striatum or amygdala (Bobiller et al., 1979) or the substantia nigra (Imai et al., 1986). Moreover, lesions of the DRN were found to cause much greater decreases of serotonin in the striatum than lesions of the MRN (Jacobs et al., 1974; Van der Kar and Lorens, 1979).

The reduction in cell numbers in the mutant DRN leads to hypothesis that this will affect levels of 5-HT in the DRN and the striatum. To test this, I carried out the next experiment where levels of 5-HT were measured with HPLC-ECD using a micropunch extraction.

# 5 Experiment 4. Levels of serotonin, dopamine and their metabolites in post mortem micropunch samples of the striatum and raphe nuclei of AS and AS/AGU rats.

## 5.1 Introduction

Movement disorders associated with the basal ganglia often involve dysfunction of multiple transmitter systems due to neuroual loss together with a reduced synthetic capacity in the remaining neurons. For example, PD is classically associated with degeneration of the dopaminergic nigrostriatal system and it is widely accepted that dopamine deficiency in this pathway plays a key role in the genesis of motor dysfunction (Jellinger, 1987). However, it is also clear that several other systems are involved, especially in the later stages of the disease (Jellinger, 1987). In patients with PD, noradrenaline concentration has been found to be depleted in the locus coeruleus (Mann et al., 1983; Agid et al., 1987) and in the limbic system (Agid et al., 1987). There is also cholinergic (Ach) depletion in the nucleus basalis of Meynert, pedunculopontine nucleus and Edinger-Westphal nucleus (Nakano and Hirano, 1983 Agid et al., 1987). Reductions in serotonin concentration have been reported in the substantia nigra, hippocampus and other known serotonin-rich regions of Parkinsonian brains (Agid, 1987; Rinne et al., 1974; Scatton et al., 1983). In addition to the 5-HT reduction, decreased 5-HIAA levels have been reported in the cerebrospinal fluid (CSF) of PD patients (Tohgi et al., 1993). Other neurodegenerative disorders related to Parkinson's disease, such as supranuclear palsy and multiple system atrophy are also characterised by cell loss in a number of amine systems (Hedera and Whitehouse, 1994).

Previous studies on the AS/AGU rat using HPLC-ECD have shown a decrease of 30-40% in the concentration of dopamine in the dorsal and lateral corpus striatum (Campbell et al., 1995, 1997). The first part of this experiment was therefore undertaken to see if serotonin was depleted in the AS/AGU rat in the same way as dopamine, and to confirm the latter. The second part of this experiment was undertaken to determine how the two strains responded to the administration of precursors of serotonin and dopamine, either alone or in combination.

In principle there are at least three possible ways to increase DA:

1- administration of DA e.g. by transplanting embryonic dopaminergic cells,

2- inhibition of enzymes responsible for DA breakdown,

3- increase of local synthesis through administration of the immediate DA precursor L-dopa (see fig 5.1)

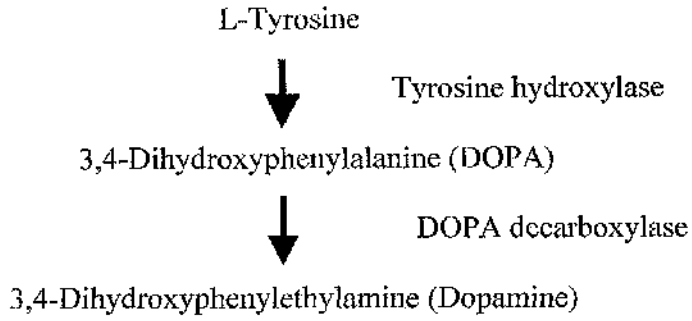



**Figure 5-1 Enzymatic pathway of dopamine synthesis.**

Even a single injection of L-dopa leads to a rapid increase of DA in the animal brain (Carlsson et al., 1958; Langelier et al., 1973; Pletcher and Gey, 1962; Poirier et al., 1967a,b) as well as in patients with Parkinson's disease (Davidson et al., 1971; Horyniewick, 1966; Rinne et al., 1972). In animals, the increase is most conspicuous within the neostriatum (Poirier et al., 1967b).

5-HT can be increased by systemic administration of the 5-HT precursor 5-hydroxytryptophan (5-HTP), which enters the brain and is decarboxylated by L-aromatic amino acid decarboxylase to form 5-HT (fig 5.2). In normal physiological conditions, this enzyme is not saturated with its substrate, so that administration of 5-HTP leads to increased synthesis of 5-HT (Moir and Eccleston, 1968) and an increase in levels of 5-HT (Moir and Eccleston, 1968; Okada et al, 1972) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in the brain (Moir and Eccleston, 1968; Okada et al, 1972; Cespuglio et al, 1981).

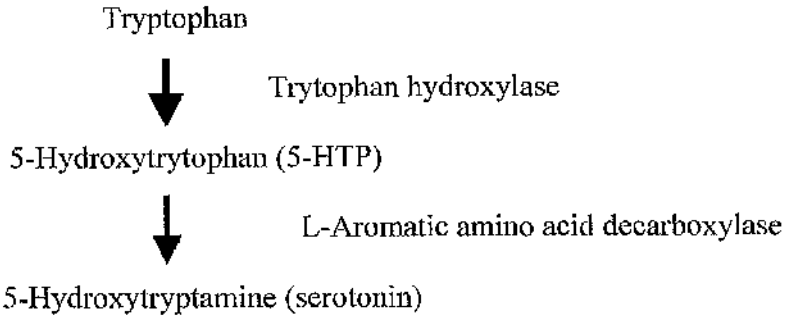



**Figure 5-2 Enzymatic pathway of 5-hydroxytryptamine (5-HT) synthesis.**

There are many procedures for the determination of serotonin (5-HT) and its major metabolite 5-HIAA in the brain, including fluorometric (Bogdanski et al., 1956; Snyder et al., 1965; Curzon and Green, 1970; Miller and Maickel, 1970) and radioenzymatic assays to measure 5-HT synthesis within discrete micropunched brain nuclei (Saavedra et al., 1973), as well as mass fragmentographic methods (Cattabeni et al., 1972). Usually the indoles are assayed separately and the preparation of the samples for analysis can be complicated and time consuming. Moreover, the reagents for radioenzymatic and mass fragmentographic methods are rather expensive. Furthermore, with radioenzymic assay, measurement of 5-HIAA- an indictor of 5-HT neuronal activity- cannot be achieved (Neff and Tozer, 1968). These methods have now been superceded by high performance liquid chromatography (HPLC) with electrochemical detection (ECD). Methods for the measurement of 5-HT and 5-HIAA were introduced by Lackovic et al., (1981), Lyness et al., (1980) and Mefford and Barchas (1980) in macrodissected brain regions. This method is very sensitive and can measure less than nanogram quantities of 5-HT and 5-HIAA in the same chromatogram.

## 5.2 Material and methods

### 5.2.1 Experiment 1. The basal levels of the serotonin and dopamine (and metabolites) in regions of the AS and AS/AGU rat brain.

Twelve AS and twelve AS/AGU male rats were used to measure the basal levels of serotonin and dopamine (and metabolites). All animals were killed by decapitation, the brains were removed and sectioned (see 5.2.2 below).

### 5.2.2 Experiment 2. Effect of serotonin and dopamine manipulation.

Ninety-six one year male rats were used:

12 AS and 12 AS/AGU received 5-HTP (50mg/kg) (i.p).

12 AS and 12 AS/AGU received L-DOPA (25mg/kg) (i.p).

12 AS and 12 AS/AGU received 5-HTP (50mg/kg) and L-DOPA (25mg/kg) in combination. (i.p).

12 AS and 12 AS/AGU received saline (control) (i.p).

All animals were killed either 30 or 60 minutes after injection. All received i.p injection of peripheral decarboxylase inhibitor (Carbidopa for 5-HTP 25mg/kg and Benserazide 2.5mg/kg) half an hour before the drug being administrated.

The rats were killed by decapitation, using a guillotine. This method was used to prevent any possible effect of anaesthetics on amines levels and the brains were rapidly removed from the skull and placed on a block of dry ice. The decapitation procedures were in accordance with the Home Office Guidelines and were specifically licensed under the Animal (Scientific Procedures) Act 1986; the Home Office project licence was 60/2167. These brains were then sectioned coronally (20μm) using a Reichert-Jung Frigocut 2800 cryostat. The Paxinos and Watson (1982) stereotaxic rat brain atlas was used as guide to determine specific landmarks close to the area of interest and sectioning continued until a cut surface was obtained corresponding to Bregma + 2.2 (fig. 5.3). A bilateral sample was

then taken from the anterior caudate putamen (ACPU) using a 1mm outside diameter corer, to a depth of 1mm.

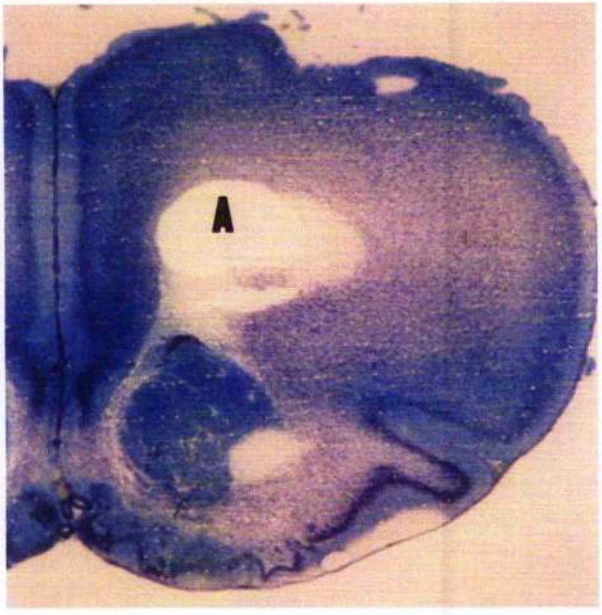



**Figure 5-3 A coronal section of rat brain at Bregma + 2.2 mm showing the area of anterior caudate-putamen (A) removed by micropunch for HPLC-ECD analysis. Staining by toluidine blue. (x 25)**

Sectioning continued until the fornix was seen to be above the decussation of the anterior commissures. This corresponded to bregma –0.8mm in the stereotaxic rat brain atlas. From here, bilateral samples were taken from the dorsal, lateral and ventral caudate putamen (DCPU, LCPU and VCPU) (fig.5.4). Finally bilateral samples were also taken from the dorsal and median raphe. This corresponded to bregma (-7.3mm).

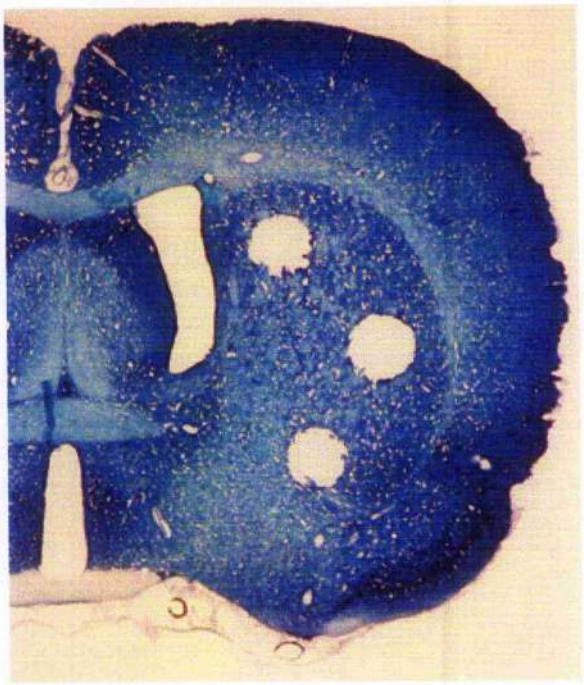


**Figure 5-4 A coronal section of rat brain at Bregma – 0.8 mm showing the areas of dorsal, lateral and ventral caudate-putamen removed by micropunch for HPLC-ECD analysis. Staining by toluidine blue. (x 25)**

All samples were placed into clean, clearly labelled Eppendorf tubes and then immediately frozen in liquid nitrogen.

Dihydroxy benzylamine hydrobromide (DHBA) was the internal standard used for this experiment as it is not present in the brain, and does not react with the neurotransmitters present in the samples. It is of known concentration and is stable, therefore it can be used as a reference point to measure unknown concentrations. HCL is an acid which releases neurotransmitters from membranes and once released it stabilises them.

DHBA was added at a known concentration (50µl: 1µg/20µl) to each sample along with hydrochloric acid (HCL) (0.1M; 100µl).

The contents of the tubes were mixed with a vortex mixer for 45 seconds each. Each sample was then centrifuged (3000 rpm/10 minutes at 4°C), and vortexed for 45 seconds to further homogenise the tissue; finally the samples were re-centrifuged for 10 min at 3500 rpm at 4°C.

The supernatants were transferred into clean Eppendorf tubes using plastic micropipettes and the remaining protein pellets were kept for protein estimation (Lowry, 1951).

All samples were kept at –80 °C until analysis by High performance liquid chromatography (HPLC) was carried out.

### 5.2.3 High performance liquid chromatography (HPLC):

HPLC is a form of chromatography with two distinct phases, which uses high pressure to increase the speed of separation and analysis of different compounds in the sample. It is a very valuable laboratory assay due to its speed, resolution and sensitivity of analysis. Many compounds can be measured using this method including proteins, neurotransmitters, blood products etc (Jussoffe et al., 1993). Therefore, this method of analysis was chosen for detection and evaluation of neurotransmitters found in the samples.

An HPLC system consists of 5 major components (see fig.5.5)

1- Pump (Gilson model 307)

2- Solvent reservoir

3- Analytical column

4- Data acquisition unit (Computer)

5- Detector (ECD) (Gilson model 141)

**Figure 5-5 An Image of HPLC-ECD system showing the major components (Pump, Solvent reservoir, Analytical column, Detector and Data acquisition unit (Computer).**

The mobile phase is generally an aqueous medium, which differs according to the chemistry of the compounds being separated and analysed. The pH also has an important influence on the separation procedure. The pump is responsible for delivering a reproducible and constant flow of mobile phase against pressure up to 3000 psi.

The analytical column is made from internally polished high-grade stainless steel and can vary from 10-25cm long and 3-5mm internal diameter. The packing gel can be 5 or 10 micron diameter. The packing gel that is most commonly used is consists of silica gel onto which organic chains (C18) are chemically bonded providing a non-polar surface. This is known as the stationary phase.

The selection of a suitable detector depends on the chemical properties of compounds to be analysed and likely sources of interference. For the analysis of neurotransmitters an Electrochemical Detection (ECD) system must be used due to the redox chemistry of these compounds (Knox, 1978). ECD detectors can be up to 1000 times more sensitive than UV detectors towards electro-active species. In ECD, an electrochemical reaction occurs at the surface of a working electrode when an electro-active species is present. The resulting current is amplified and presented to the computer. The current produced by the electrochemical reaction if passed through the reference electrode would alter it's potential. An auxiliary electrode (made of glassy carbon) is used whose potential is electronically controlled so as to maintain the desired potential difference between the working and reference electrodes. As no current flows through the reference electrode, it becomes a stable and reliable half-cell which can be used as a reference point for measurements (Rocklin, 1993).

The computer monitors the output of the detector and allows both a qualitative and a quantitative evaluation of the chromatographic separation. In terms of qualitative analysis, the computer produces a pictorial account of the separation procedure within the analytical column i.e. a chromatogram (see fig. 5.6). As can be seen, chromatograms give an indication of the retention times of compounds as a reference point to calculate response

factors, peak areas and peak concentrations. An internal standard (DHBA) at a known concentration is injected with each sample in order to provide a reference peak from which other "unknown" peaks can be calculated.

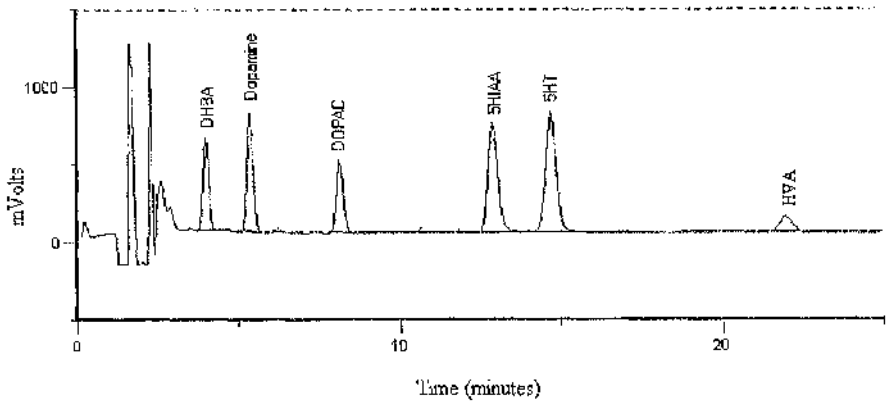
The solutions used with HPLC are shown in Appendix 2.

The sample was injected via the injector port and forced at high pressure by the pump (Gilson 306) and mobile phase through the column (Hichrom ODS) to the detector (Gilson 141). The neurotransmitters present in the sample were separated according to their polarity and size. The silica packing gel inside the column consists of non-polar C18 bonded phase. Compounds with a high degree of polarity within the sample will therefore pass through to the electrochemical detector (ECD) at a more rapid rate. The ECD consists of three electrodes: a reference electrode which was set to +0.7V, the threshold required to initiate oxidization of the compounds, a working electrode where the redox potential of the compounds was measured, and an auxillary electrode. The current produced at the surface of the working electrode was recorded by the data acquisition unit and displayed in the form of a peak. The computer stores information on peak concentration and retention time (time from injection to detection) in the form of a chromatogram.

Before analyzing the samples, 20 µl of composite standard was injected. This contains not only compounds which may be present in the sample tissue, but also the internal standard. From the composite standard chromatogram, the retention times and response factors of the compounds present were recorded and used for the calculation of neurotransmitter levels in the AS/AGU and control brain samples.

Typical composite standard and sample chromatograms are shown in Figures 5.6a and b).

a)




b)

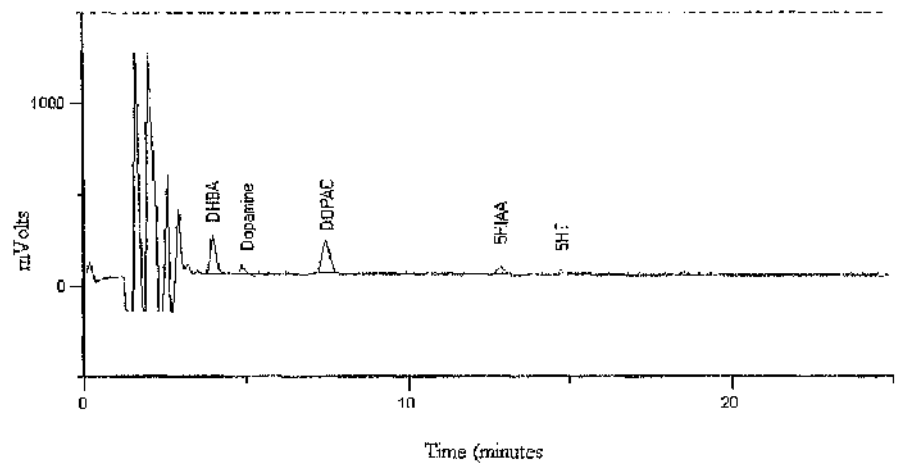



**Figure 5.6 Typical HPLC chromatograms from a) Composite standard, and b) sample. Peaks in order of elution are DHBA (internal standard), Dopamine, DOPAC, 5-HIAA, 5-HT.**

### 5.2.4 Protein Estimation

Because the micropunch samples are very small, weight is a relatively unreliable variable and it has become customary to quantify transmitters and their metabolites according to the protein content of the sample. The Lowry assay (Lowry, 1951) was used for protein estimation. It is used to estimate the amount of protein present in a sample when the sample is too small to be weighed. It is based on the absorbance of a dye, Coomassie Blue, in the ultraviolet region of the spectrum at 595nm.

A standard protein Bovine Serum Albumin (BSA) was made up in distilled water. The concentrations used ranged from 10 to 200 µg. Coomassie Blue (0.9ml) was added to each solution, mixed and the absorbance recorded at 595nm. A standard curve was obtained using these values.

To each protein pellet 210µl of 0.1M NaOH was added, the mixture vortexed for 45 seconds and left overnight. The samples were stored at 4 $^{o}$C.

Into a 2ml plastic cuvette, 1ml distilled water, 0.9ml of Coomassie Blue and 0.1ml of protein were added.

The contents were mixed up and the absorbance at 595nm was recorded using a spectrophotometer (LKB Biochrom Ultrospec 4050) to obtain the absorbance value. This was repeated twice to establish consistency between samples.

Using the equation of a straight line, $y = mx + c$, the unknown concentration of the protein was obtained from the standard curve by inserting the absorbance value of the protein (y) into the equation. The final concentration of each sample was in µg of protein.

All samples were injected freshly as 5-HT and 5-HIAA are said to be degraded quicker than other neurotransmitters.

Each sample was injected twice. A composite standard was injected after every six "unknown" samples. Results were analysed and quantified using the following equations:

$$\text{Peak Concentration} = \frac{\text{Area of unknown x Response factor}}{\text{Area of Standard}}$$

$$\text{Concentration of neurotransmitter (ng/wet weight)} = \frac{\text{Peak Concentration x 60 (dilution factor)}}{\text{Tissue Weight (g)}}$$

### 5.2.5 Statistical Analysis

In Chapters 5 and 7, it has been necessary to carry out two-way analyses of variance where the numerical data can be assorted by variables such as strain (AS v. AS/AGU) and also treatment (control v. treatment 1v. treatment 2 ...etc). It was decided that the same tests should be used throughout the thesis to give consistency. However, some experiments used equal-sized groups whereas other experiments used groups of different sizes. Written advice from Minitab Inc (State College, Pennsylvania, USA) suggested the use of the General Linear Model which allows both balanced and unbalanced designs. This procedure allows the initial identification of significant variance due to strain (but ignoring treatment) and treatment (but ignoring strain). It was therefore necessary to carry out a subsequent, second stage of analysis in which each individual group could be compared with each other using a one-way analysis of variance (ANOVA). Tukey's test (set at 95%) was used as a post hoc test to generate Confidence Intervals for individual inter-group comparisons.

## 5.3 Results

### 5.3.1 Comparison of serotonin and dopamine (and metabolites) levels in regions of the AS and AS/AGU rat brain

#### 5.3.1.1 Serotonin (5-HT) levels.

These are generally lower in all parts of the caudate putamen in mutant AS/AGU rats compared to the parent AS strain. The differences are statistically significant in the dorsal ($t = 6.69$, $df = 22$, $p < 0.001$) and ventral ($t = 2.18$, $df = 22$, $p < 0.05$) caudate-putamen, and approach significance in the lateral caudate-putamen ($t = 2.03$, $df = 22$, $p < 0.06$). Serotonin levels are much higher in the dorsal and median raphe nuclei than in the striatum. In the dorsal raphe, 5-HT levels are significantly higher in AS controls than in mutants ($t = 16.95$, $df = 22$, $p < 0.001$), but there are no strain differences in serotonin in the median raphe nucleus. **(see Table 5.1)**

#### 5.3.1.2 5-hydroxyindole acetic acid (5-HIAA) levels.

By contrast with serotonin, levels of its metabolite 5-HIAA are not reduced in mutant rats within the caudate-putamen. Indeed, in one region, the dorsal caudate-putamen, levels are significantly higher ($t = 8.25$, $df = 22$, $p < 0.001$). Levels of 5-HIAA are substantially higher in the dorsal and median raphe nuclei than in the striatum. In the dorsal raphe nucleus, 5-HIAA levels are significantly higher in the AS/AGU mutant than in the AS control ($t = 11.71$, $df = 22$, $p < 0.001$), but there are no strain differences in 5-HIAA levels in the median raphe nucleus. **(see Table 5.1)**

### 5.3.1.3 Dopamine (DA) levels.

Previous studies (Campbell et al 1997) have shown lower DA levels in the AS/AGU mutant compared to the parent AS control in several regions of the caudate-putamen. These differences are confirmed in the present study. Thus, AS controls had higher levels of DA than mutants in the dorsal caudate-putamen ($t = 17.88$, $df = 22$, $p < 0.001$) and the lateral caudate-putamen ($t = 11.22$, $df = 22$, $p < 0.001$). There were no strain differences in the anterior or ventral caudate-putamen. Dopamine levels were non-detectable in the region of the dorsal and median raphe nuclei. **(see Table 5.1)**

### 5.3.1.4 Dihydroxyphenylacetic acid (DOPAC) levels.

By contrast with dopamine, levels of its metabolite DOPAC are generally not lower in the AS/AGU mutant compared to the AS control. Indeed, levels of DOPAC were significantly higher in the mutant in the dorsal caudate-putamen ($t = 6.48$, $df = 22$, $p < 0.001$), lateral caudate-putamen ($t = 5.20$, $df = 22$, $p < 0.001$) and ventral caudate-putamen ($t = 5.03$, $df = 22$, $p < 0.001$). Like dopamine itself, DOPAC levels were undetectable in the regions of the dorsal and median raphe nuclei. **(see Table 5.1)**

| Brain Region | 5-HT | | 5-HIAA | | DA | | DOPAC | |
|---|---|---|---|---|---|---|---|---|
| | AS | AS/AGU | AS | AS/AGU | AS | AS/AGU | AS | AS/AGU |
| ACPU | 5.03 ± 0.45 | 4.86 ± 0.16 | 3.03 ± 0.17 | 3.01 ± 0.19 | 22.8 ±0.4 | 23 ±0.6 | 3.7 ±0.32 | 2.9 ±0.29 |
| t | 0.35 ns | | 0.06 ns | | -.25 ns | | 1.86 ns | |
| DCPU | 4.18 ± 0.11 | 2.67 ± 0.20 | 2.71 ± 0.13 | 4.18 ± 0.12 | 12.6 ±0.3 | 7.3 ±0.16 | 2.64 ±0.14 | 3.8 ±0.1 |
| t | 6.69, p < 0.001 | | -8.25 P<0.001 | | 17.9 P<0.01 | | -6.5 P<0.01 | |
| LCPU | 5.13 ± 0.37 | 3.77 ± 0.56 | 2.92 ± 0.23 | 3.13 ± 0.17 | 24.7 ± 0.4 | 19.1 ± 0.36 | 2.3 ± 0.1 | 2.93 ± 0.1 |
| t | 2.03 P<0.057 | | -0.73 ns | | 11.2 P<0.01 | | -5.2 P<0.01 | |
| VCPU | 4.75 ± 0.41 | 3.62 ± 0.32 | 3.56 ± 0.25 | 3.45 ± 0.26 | 22.1 ± 0.7 | 21 ± 0.74 | 2.2 ± 0.1 | 4 ± 0.4 |
| t | 2.18 P<0.041 | | 0.3 ns | | 1.2 ns | | -5.03 P<0.01 | |
| DRN | 31.25 ± 0.46 | 22.17 ± 0.27 | 23 ± 0.43 | 30 ± 0.47 | nd | nd | nd | nd |
| t | 16.95 P<0.001 | | -11.7 P<0.01 | | | | | |
| MRN | 12 ± 0.49 | 12.3 ± 0.57 | 18.75 ± 0.65 | 17.75 ± 0.45 | nd | nd | nd | nd |
| t | -0.33 ns | | 1.26 ns | | | | | |

Table 5.1. **Levels of serotonin (5-HT), 5-hydroxyindole acetic acid (5-HIAA), dopamine (DA) and dihydroxyphenylacetic acid (DOPAC) in the anterior (ACPU), dorsal (DCPU), lateral (LCPU) and ventral (VCPU) caudate-putamen, dorsal (DRN) and median (MRN) raphe nuclei of the Albino Swiss (AS) rat and the AS/AGU mutant. All values are mean ng/wet weight ± SEM. nd = non-detectable For definitions and co-ordinates of the brain regions, see Material and Methods. All comparisons are 2-tailed t-tests.**

### 5.3.2 Comparison of the affect of the drugs on serotonin and dopamine (and metabolites) levels in regions of the AS and AS/AGU rat brain

Between pages 97-145 There are a numbers of pages showing analysis of variance for transmitters or metabolites for seneral brain regions. Groups are identified in tables by numbers, as identified below.

Key

i) for group analysis, 1 = AS control 2 = AS/AGU mutant

ii) for treatment (treat A)

1 = saline 30 mins, 2 = saline 60 mins.

3 = 5-HTP 30 mins, 4 = 5-HTP 60 mins.

5 = L-dopa 30 mins 6 = L-dopa 60 mins.

7 = both 30 mins 8 = both 60 mins.

iv) for group x treatment (treatb)

11 = AS, saline 30 mins, 12 = AS/AGU, saline 30 mins.

21 = AS, saline 60 mins, 22 = AS/AGU, saline 60 mins

31 = AS, 5-HTP 30 mins, 32 = AS/AGU, 5-HTP 30 mins.

41 = AS, 5-HTP 60 mins, 42 = AS/AGU, 5-HTP 60 mins

51 = AS, L-dopa 30 mins 52 = AS/AGU, L-dopa 30 mins.

61 = AS, L-dopa 60 mins 62 = AS/AGU, L-dopa 60 mins.

71 = AS both 30 mins 72 = AS/AGU both 30 mins.

81 = AS both 60 mins 82 = AS/AGU both 60 mins.

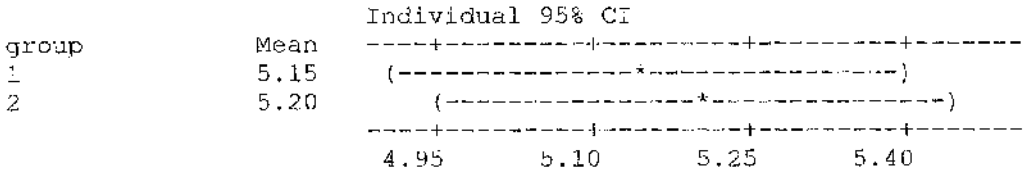
### 5.3.2.1 Serotonin (5-HT)

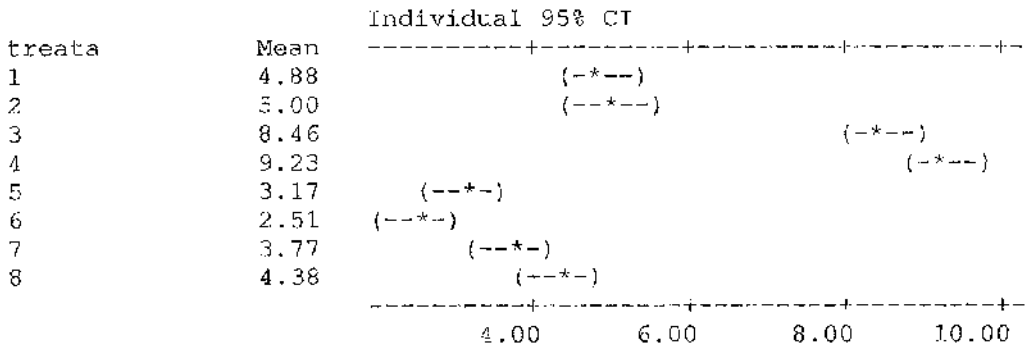
1) **Anterior caudate-putamen** A two-way analysis of variance (ANOVA) showed no significant effect of group (AS v AS/AGU) but a very significant effect of treatment ($F = 92.7$, $df = 7$, $p < 0.001$) (see p 99). Serotonin levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment. Unexpectedly, treating rats with L-DOPA significantly reduced 5-HT below saline control levels, both at 30 and 60 minutes ($p < 0.05$). The inhibitory effect of L-DOPA continued when both L-DOPA and 5-HTP were given together as a combined treatment. Thus, thirty minutes after treatment, 5-HT levels remained significantly below saline-treated controls ($p < 0.05$) although levels had become comparable to controls by one hour after injection. Even though the analysis had shown no effect of group on variance, the ANOVA was extended to compare the response of AS and AS/AGU rats to these treatments; this confirmed that there were no significant differences between the two groups (p 99).

## Two-way ANOVA: acp5-HT versus group, treata

Analysis of Variance for acp5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 0.060 | 0.060 | 0.08 | 0.779 |
| treata | 7 | 492.796 | 70.399 | 92.70 | 0.001 |
| Interaction | 7 | 9.977 | 1.425 | 1.88 | 0.084 |
| Error | 80 | 60.757 | 0.759 | | |
| Total | 95 | 563.590 | | | |



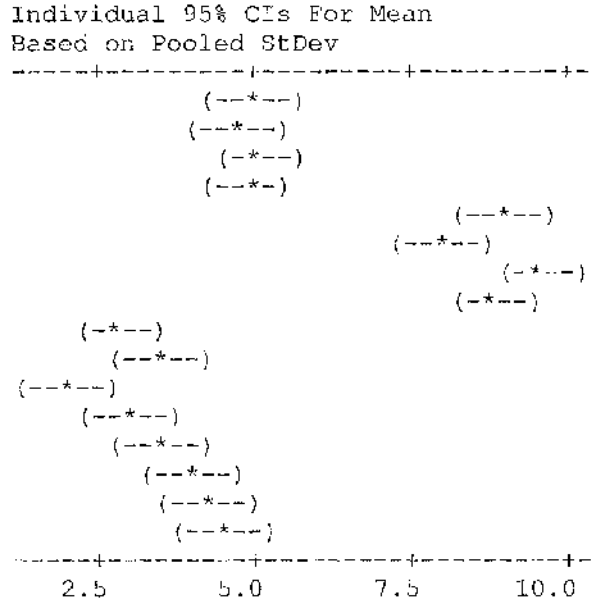

## One-way ANOVA: acp5-HT versus treatb

Analysis of Variance for acp5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 502.833 | 33.522 | 44.14 | < 0.001 |
| Error | 80 | 60.757 | 0.759 | | |
| Total | 95 | 563.590 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 4.950 | 1.793 |
| 12 | 6 | 4.817 | 0.449 |
| 21 | 6 | 5.100 | 1.435 |
| 22 | 6 | 4.900 | 0.681 |
| 31 | 6 | 8.950 | 1.309 |
| 32 | 6 | 7.967 | 0.635 |
| 41 | 6 | 9.600 | 1.115 |
| 42 | 6 | 8.850 | 0.288 |
| 51 | 6 | 2.867 | 0.656 |
| 52 | 6 | 3.467 | 0.656 |
| 61 | 6 | 2.000 | 0.303 |
| 62 | 6 | 3.017 | 0.674 |
| 71 | 6 | 3.533 | 0.857 |
| 72 | 6 | 4.000 | 0.400 |
| 81 | 6 | 4.183 | 0.662 |
| 82 | 6 | 4.567 | 0.850 |

Pooled StDev = 0.871

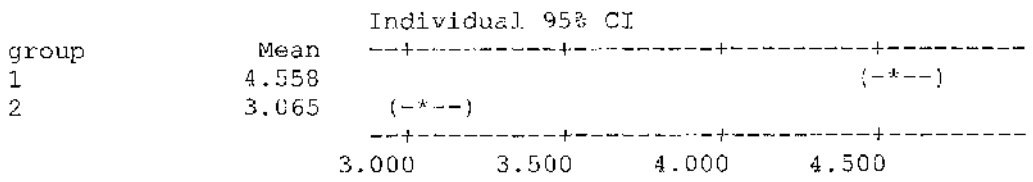


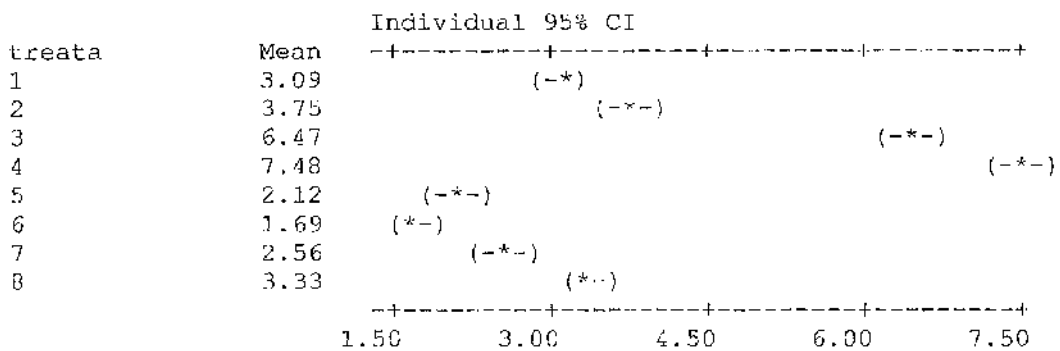
2) **Dorsal caudate-putamen** A two-way analysis of variance (ANOVA) showed a highly significant effect of group (AS v AS/AGU $F = 276$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 267$, $df = 7$, $p < 0.001$) (see p 101). Serotonin levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). As found in the anterior caudate-putamen, treating rats with L-DOPA significantly reduced 5-HT below saline control levels, both at 30 and 60 minutes ($p < 0.05$). The inhibitory effect of L-DOPA continued when both L-DOPA and 5-HTP were given together as a combined treatment, although rats given the combined treatment had levels of 5-HT which were just comparable to controls at both thirty minutes and one hour after injection. When the analysis was extended to compare AS and AS/AGU rats (p 101) it is clear that AS rats have higher levels of 5-HT than AS/AGU mutants a) when both groups received saline, b) when both groups received 5-HTP and c) when both groups received a combination of L-DOPA and 5-HTP. When both groups received L-DOPA alone, 5-HT levels were extremely low and no differences were found between mutant and control strains.

## Two-way ANOVA: dcp5-HT versus group, treata

Analysis of Variance for dcp5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 53.551 | 53.551 | 276.36 | 0.001 |
| treata | 7 | 362.633 | 51.805 | 267.35 | 0.001 |
| Interaction | 7 | 22.092 | 3.156 | 16.29 | 0.001 |
| Error | 80 | 15.502 | 0.194 | | |
| Total | 95 | 453.777 | | | |




## One-way ANOVA: dcp5-HT versus treatb

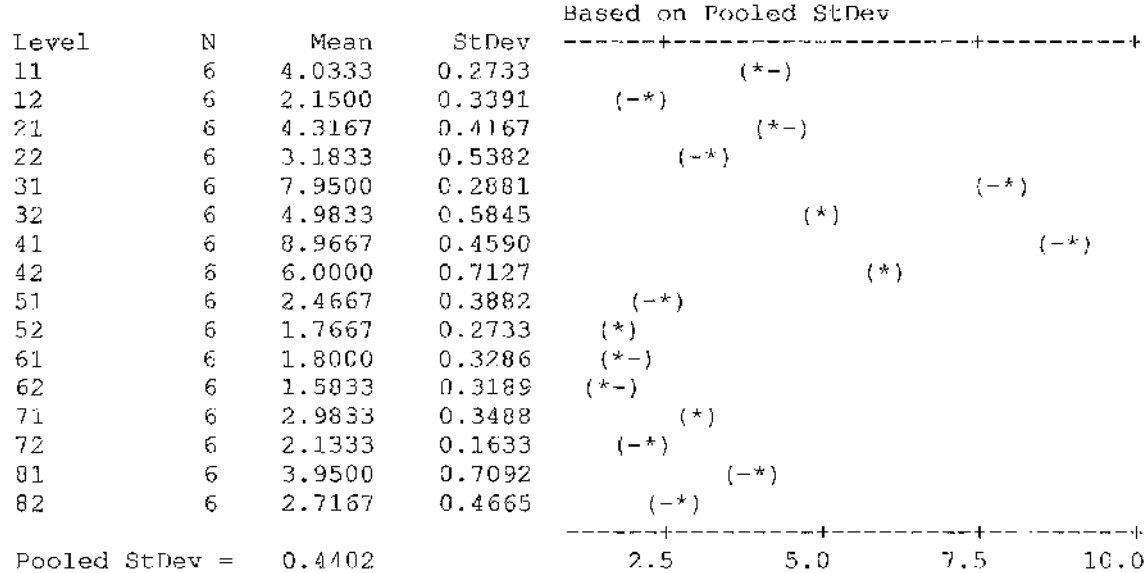
Analysis of Variance for dcp5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 438.276 | 29.218 | 150.79 | < 0.001 |
| Error | 80 | 15.502 | 0.194 | | |
| Total | 95 | 453.777 | | | |

Individual 95% CIs For Mean Based on Pooled StDev

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 4.0333 | 0.2733 |
| 12 | 6 | 2.1500 | 0.3391 |
| 21 | 6 | 4.3167 | 0.4167 |
| 22 | 6 | 3.1833 | 0.5382 |
| 31 | 6 | 7.9500 | 0.2881 |
| 32 | 6 | 4.9833 | 0.5845 |
| 41 | 6 | 8.9667 | 0.4590 |
| 42 | 6 | 6.0000 | 0.7127 |
| 51 | 6 | 2.4667 | 0.3882 |
| 52 | 6 | 1.7667 | 0.2733 |
| 61 | 6 | 1.8000 | 0.3286 |
| 62 | 6 | 1.5833 | 0.3189 |
| 71 | 6 | 2.9833 | 0.3488 |
| 72 | 6 | 2.1333 | 0.1633 |
| 81 | 6 | 3.9500 | 0.7092 |
| 82 | 6 | 2.7167 | 0.4665 |

Pooled StDev = 0.4402

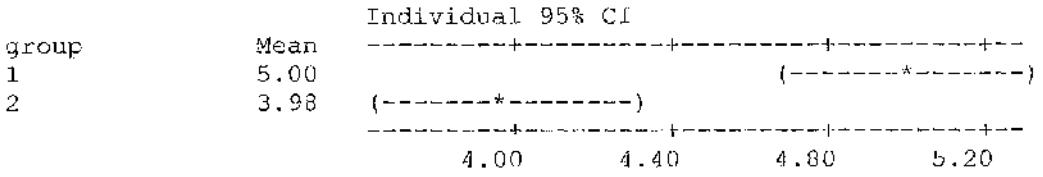


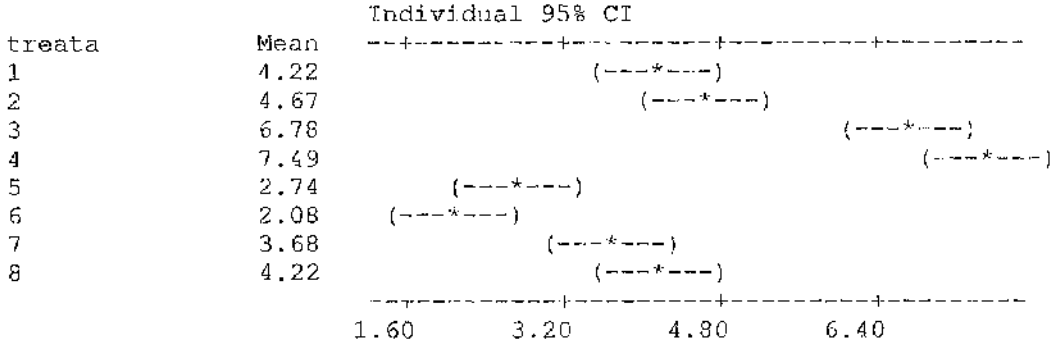
3) **Lateral caudate-putamen.** A two-way analysis of variance (ANOVA) showed a highly significant effect of group (AS v AS/AGU $F = 19.09$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 31.6$, $df = 7$, $p < 0.001$) (see p 103). Serotonin levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). As found in both the anterior and dorsal caudate-putamen (see above), treating rats with L-DOPA significantly reduced 5-HT below saline control levels, both at 30 and 60 minutes ($p < 0.05$). The inhibitory effect of L-DOPA was barely counteracted by 5-HTP when both were given together as a combined treatment; rats given the combined treatment had levels of 5-HT which were just comparable to controls at both thirty minutes and one hour after injection. When the analysis was extended to compare AS and AS/AGU rats (p 103) it is clear that AS rats have higher levels of 5-HT than AS/AGU mutants only when both groups received saline. When animals received 5-HTP, L-DOPA or a combination of the two, strain differences were not significant.

## Two-way ANOVA: lcp5-HT versus group, treata

Analysis of Variance for lcp5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 24.81 | 24.81 | 19.09 | 0.001 |
| treata | 7 | 287.37 | 41.05 | 31.60 | 0.001 |
| Interaction | 7 | 3.97 | 0.57 | 0.44 | 0.876 |
| Error | 80 | 103.93 | 1.30 | | |
| Total | 95 | 420.09 | | | |



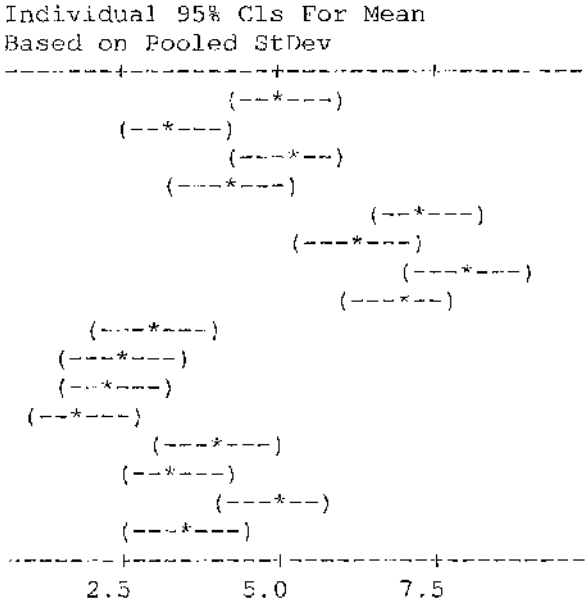

## One-way ANOVA: lcp5-HT versus treatb

Analysis of Variance for lcp5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 316.15 | 21.08 | 16.22 | < 0.001 |
| Error | 80 | 103.93 | 1.30 | | |
| Total | 95 | 420.09 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 5.100 | 1.565 |
| 12 | 6 | 3.333 | 1.846 |
| 21 | 6 | 5.150 | 1.089 |
| 22 | 6 | 4.200 | 2.081 |
| 31 | 6 | 7.367 | 1.164 |
| 32 | 6 | 6.200 | 0.660 |
| 41 | 6 | 8.050 | 0.742 |
| 42 | 6 | 6.933 | 0.864 |
| 51 | 6 | 3.033 | 0.423 |
| 52 | 6 | 2.450 | 0.782 |
| 61 | 6 | 2.317 | 0.454 |
| 62 | 6 | 1.850 | 0.675 |
| 71 | 6 | 4.033 | 0.855 |
| 72 | 6 | 3.333 | 1.846 |
| 81 | 6 | 4.917 | 0.736 |
| 82 | 6 | 3.533 | 0.441 |

Pooled StDev = 1.140



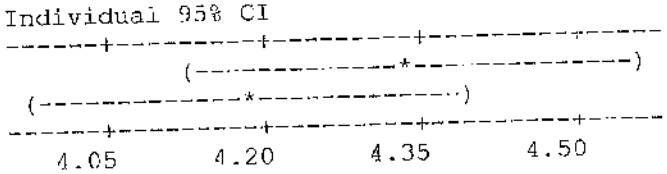
4) **Ventral caudate-putamen**. A two-way analysis of variance (ANOVA) showed no significant effect of group (AS v AS/AGU) but a very significant effect of treatment ($F = 56.98$, $df = 7$, $p < 0.001$) (see p 105). Serotonin levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment. As with all the other regions of the striatum examined (see above), treating rats with L-DOPA significantly reduced 5-HT below saline control levels, both at 30 and 60 minutes ($p < 0.05$). A combined treatment of L-DOPA plus 5-HTP resulted in 5-HT levels which were comparable to saline-treated controls. Even though the analysis had shown no effect of group on variance, the ANOVA was extended to compare the response of AS and AS/AGU rats to these treatments; this demonstrated only one significant difference, that mutants treated with saline for 60 minutes had lower levels of 5-HT than controls (p 105).

## Two-way ANOVA: vcp5-HT versus group, treata

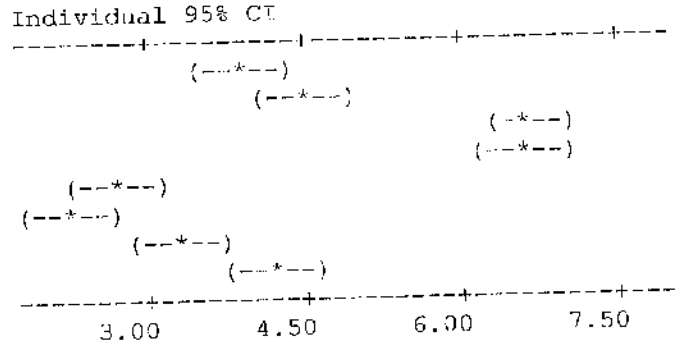
Analysis of Variance for vcp5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 0.570 | 0.570 | 1.02 | 0.316 |
| treata | 7 | 223.593 | 31.942 | 56.98 | 0.001 |
| Interaction | 7 | 29.135 | 4.162 | 7.42 | 0.001 |
| Error | 80 | 44.847 | 0.561 | | |
| Total | 95 | 298.145 | | | |

| group | Mean |
|---|---|
| 1 | 4.34 |
| 2 | 4.19 |




| treata | Mean |
|---|---|
| 1 | 3.89 |
| 2 | 4.47 |
| 3 | 6.66 |
| 4 | 6.58 |
| 5 | 2.72 |
| 6 | 2.27 |
| 7 | 3.27 |
| 8 | 4.22 |



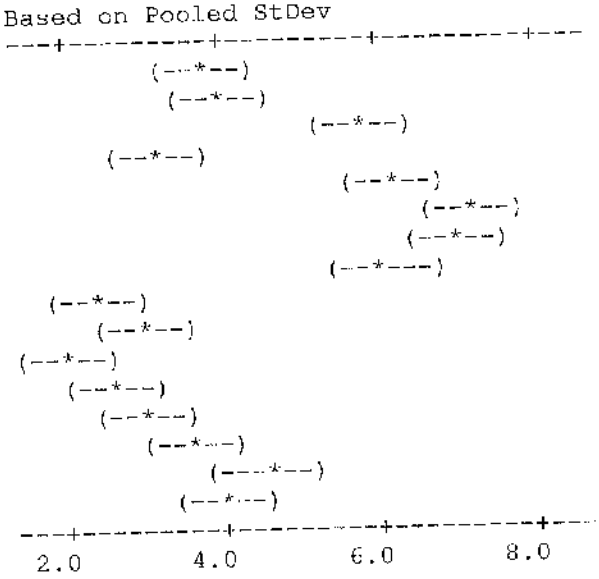

## One-way ANOVA: vcp5-HT versus treatb

Analysis of Variance for vcp5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 253.298 | 16.887 | 30.12 | < 0.001 |
| Error | 80 | 44.847 | 0.561 | | |
| Total | 95 | 298.145 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 3.7333 | 1.2660 |
| 12 | 6 | 4.0500 | 1.3442 |
| 21 | 6 | 5.7667 | 0.6501 |
| 22 | 6 | 3.1833 | 0.6047 |
| 31 | 6 | 6.1167 | 0.4956 |
| 32 | 6 | 7.2000 | 1.2050 |
| 41 | 6 | 7.0667 | 0.6055 |
| 42 | 6 | 6.1000 | 0.6229 |
| 51 | 6 | 2.4833 | 0.2229 |
| 52 | 6 | 2.9667 | 0.8017 |
| 61 | 6 | 2.0167 | 0.2483 |
| 62 | 6 | 2.5167 | 0.4916 |
| 71 | 6 | 3.0333 | 0.3933 |
| 72 | 6 | 3.5167 | 0.6735 |
| 81 | 6 | 4.5000 | 0.6066 |
| 82 | 6 | 3.9500 | 0.5891 |

Pooled StDev = 0.7487

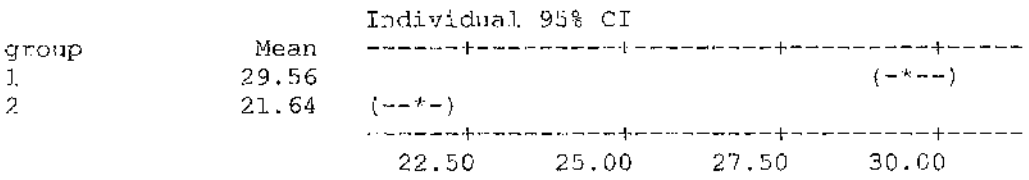


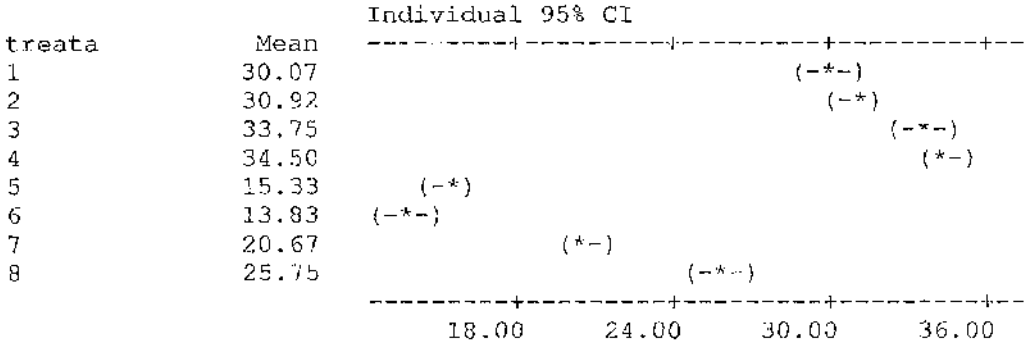
5) **Dorsal raphe nucleus**. A two-way analysis of variance (ANOVA) showed a highly significant effect of group (AS v AS/AGU $F = 384$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 202$, $df = 7$, $p < 0.001$) (see p 107). Serotonin levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). As found in the striatum (above), treating rats with L-DOPA significantly reduced 5-HT below saline control levels, both at 30 and 60 minutes ($p < 0.05$). The inhibitory effect of L-DOPA was only marginally counteracted by 5-HTP when both were given in combination, and rats given the combined treatment had levels of 5-HT which were still substantially and significantly lower than saline-treated controls at both thirty minutes and one hour after injection. When the analysis was extended to compare AS and AS/AGU rats (p 107) it is clear that AS rats have higher levels of 5-HT than AS/AGU mutants a) when both groups received saline, b) when both groups received 5-HTP, c) when both groups received L-DOPA and d) sixty minutes after both groups received a combination of L-DOPA and 5-HTP.

## Two-way ANOVA: Drn5-HT versus group, treata

Analysis of Variance for Drn5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 1504.96 | 1504.96 | 383.95 | 0.001 |
| treata | 7 | 5545.73 | 792.25 | 202.12 | 0.001 |
| Interaction | 7 | 793.23 | 113.32 | 28.91 | 0.001 |
| Error | 80 | 313.57 | 3.92 | | |
| Total | 95 | 8157.49 | | | |



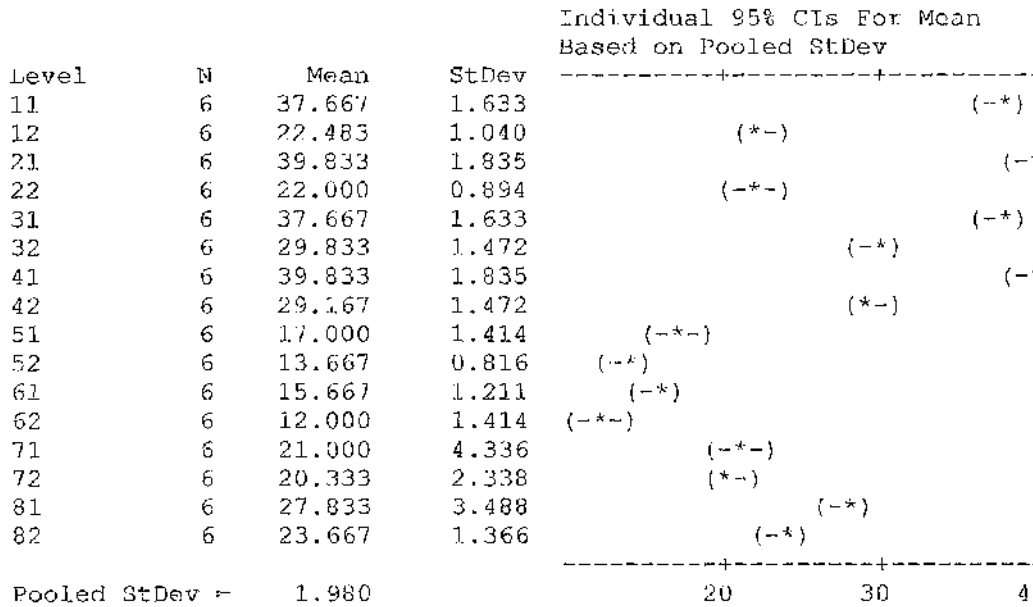

## One-way ANOVA: Drn5-HT versus treatb

Analysis of Variance for Drn5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 7843.91 | 522.93 | 133.41 | < 0.001 |
| Error | 80 | 313.57 | 3.92 | | |
| Total | 95 | 8157.49 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 37.667 | 1.633 |
| 12 | 6 | 22.483 | 1.040 |
| 21 | 6 | 39.833 | 1.835 |
| 22 | 6 | 22.000 | 0.894 |
| 31 | 6 | 37.667 | 1.633 |
| 32 | 6 | 29.833 | 1.472 |
| 41 | 6 | 39.833 | 1.835 |
| 42 | 6 | 29.167 | 1.472 |
| 51 | 6 | 17.000 | 1.414 |
| 52 | 6 | 13.667 | 0.816 |
| 61 | 6 | 15.667 | 1.211 |
| 62 | 6 | 12.000 | 1.414 |
| 71 | 6 | 21.000 | 4.336 |
| 72 | 6 | 20.333 | 2.338 |
| 81 | 6 | 27.833 | 3.488 |
| 82 | 6 | 23.667 | 1.366 |

Pooled StDev = 1.980

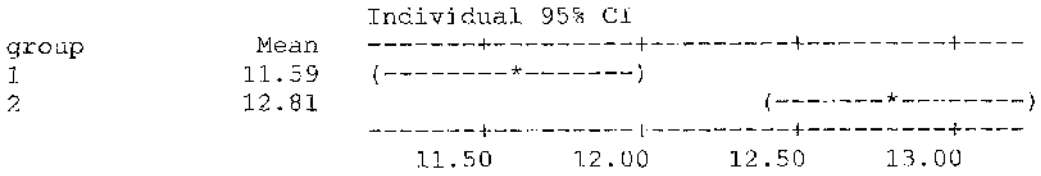


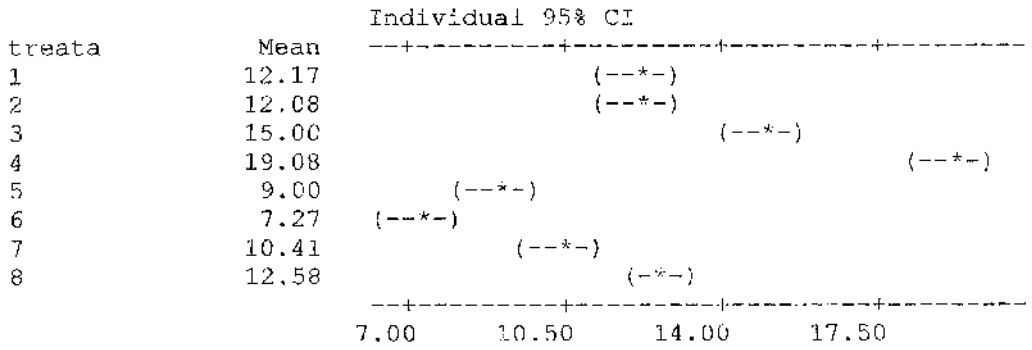
6) **Median raphe nucleus** A two-way analysis of variance (ANOVA) showed a highly significant effect of group (AS v AS/AGU $F = 36.3$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 159.6$, $df = 7$, $p < 0.001$) (see p 109). Serotonin levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). As found in the striatum and dorsal raphe (above), treating rats with L-DOPA significantly reduced 5-HT below saline control levels, both at 30 and 60 minutes ($p < 0.05$). Rats given a combined treatment of L-DOPA plus 5-HTP had levels of 5-HT which were comparable to saline-treated controls. When the analysis was extended to compare AS and AS/AGU rats (p 109) AS rats have lower levels of 5-HT than AS/AGU mutants thirty minutes after L-DOPA administration, and also after both groups received a combination of L-DOPA and 5-HTP.

## Two-way ANOVA: Mrn5-HT versus group, treata

Analysis of Variance for Mrn5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 36.26 | 36.26 | 16.47 | 0.001 |
| treata | 7 | 1117.05 | 159.58 | 72.47 | 0.001 |
| Interaction | 7 | 64.16 | 9.17 | 4.16 | 0.001 |
| Error | 80 | 176.17 | 2.20 | | |
| Total | 95 | 1393.64 | | | |



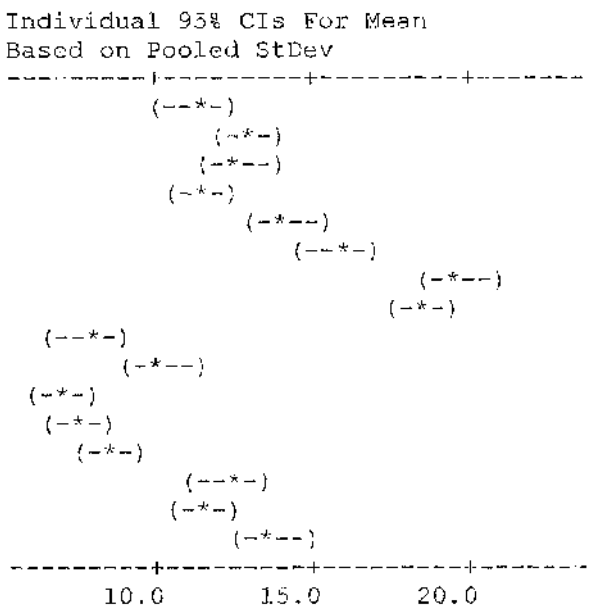

## One-way ANOVA: Mrn5-HT versus treatb

Analysis of Variance for Mrn5-HT

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 1217.47 | 81.16 | 36.86 | < 0.001 |
| Error | 80 | 176.17 | 2.20 | | |
| Total | 95 | 1393.64 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 11.333 | 1.633 |
| 12 | 6 | 13.000 | 2.098 |
| 21 | 6 | 12.667 | 1.633 |
| 22 | 6 | 11.500 | 1.643 |
| 31 | 6 | 14.167 | 1.169 |
| 32 | 6 | 15.833 | 1.602 |
| 41 | 6 | 19.667 | 1.633 |
| 42 | 6 | 18.500 | 1.871 |
| 51 | 6 | 7.833 | 0.753 |
| 52 | 6 | 10.167 | 1.229 |
| 61 | 6 | 7.033 | 0.816 |
| 62 | 6 | 7.517 | 0.508 |
| 71 | 6 | 8.483 | 1.125 |
| 72 | 6 | 12.333 | 1.862 |
| 81 | 6 | 11.500 | 1.517 |
| 82 | 6 | 13.667 | 1.633 |

Pooled StDev = 1.484



### 5.3.2.2 5-Hydoxyindole acetic acid (5-HIAA)

1) **Anterior caudate-putamen**. A two-way analysis of variance (ANOVA) showed a significant effect of group (AS v AS/AGU F= 23.24, df=1, p <0.001) and a very significant effect of treatment (F =33.25, df = 7, p < 0.001) (see p 111). 5-HIAA levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment (p < 0.05). Treating rats with L-DOPA resulted in 5-HIAA levels intermediate between saline and 5-HTP treatment (and significantly different from each for comparable times after administration, p < 0.05). When L-DOPA and 5-HTP were given together as a combined treatment, levels of 5-HIAA were comparable to giving 5-HTP alone. When the analysis was extended to compare AS and AS/AGU rats (p 111) AS rats had higher levels of 5-HIAA than AS/AGU mutants at both thirty and sixty minutes after both groups received a combination of L-DOPA and 5-HTP.

## Two-way ANOVA: AcpHIAA versus Treat, Group

Analysis of Variance for AcpHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treat | 7 | 133.712 | 19.102 | 33.25 | 0.001 |
| Group | 1 | 13.350 | 13.350 | 23.24 | 0.001 |
| Interaction | 7 | 30.463 | 4.352 | 7.58 | 0.001 |
| Error | 80 | 45.953 | 0.574 | | |
| Total | 95 | 223.478 | | | |

| Treat | Mean | Individual 95% CI |
|---|---|---|
| 1 | 3.02 | (---*---) |
| 2 | 3.02 | (---*---) |
| 3 | 5.44 | (---*---) |
| 4 | 6.15 | (---*---) |
| 5 | 4.14 | (---*---) |
| 6 | 4.74 | (---*---) |
| 7 | 5.39 | (---*---) |
| 8 | 6.13 | (---*---) |

3.00 4.00 5.00 6.00

| Group | Mean | Individual 95% CI |
|---|---|---|
| 1 | 5.13 | (------*------) |
| 2 | 4.38 | (------*------) |

4.20 4.50 4.80 5.10

## One-way ANOVA: AcpHIAA versus Treatb

Analysis of Variance for AcpHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treatb | 15 | 176.218 | 11.748 | 18.04 | < 0.001 |
| Error | 80 | 52.107 | 0.651 | | |
| Total | 95 | 228.325 | | | |

| Level | N | Mean | StDev | Individual 95% CIs For Mean Based on Pooled StDev |
|---|---|---|---|---|
| 11 | 6 | 3.1667 | 0.7062 | (---*---) |
| 12 | 6 | 2.8667 | 0.1966 | (---*---) |
| 21 | 6 | 2.8833 | 0.4446 | (---*---) |
| 22 | 6 | 3.1500 | 0.9566 | (---*---) |
| 31 | 6 | 5.9500 | 0.8019 | (---*---) |
| 32 | 6 | 4.9333 | 0.5574 | (---*---) |
| 41 | 6 | 6.6333 | 0.7607 | (---*---) |
| 42 | 6 | 5.6667 | 1.0328 | (---*---) |
| 51 | 6 | 4.0833 | 0.4708 | (---*---) |
| 52 | 6 | 5.1333 | 1.2028 | (---*---) |
| 61 | 6 | 4.3500 | 0.4231 | (---*---) |
| 62 | 6 | 5.1333 | 1.2028 | (---*---) |
| 71 | 6 | 6.5000 | 1.0488 | (---*---) |
| 72 | 6 | 4.2833 | 0.5947 | (---*---) |
| 81 | 6 | 7.4500 | 1.1309 | (---*---) |
| 82 | 6 | 4.8167 | 0.3817 | (---*---) |

Pooled StDev = 0.8071

3.2 4.8 6.4

2) **Dorsal caudate-putamen**. A two-way analysis of variance (ANOVA) showed a significant effect of group (AS v AS/AGU $F= 11.72$, $df=1$, $p <0.001$) and a very significant effect of treatment ($F =49.84$, $df = 7$, $p < 0.001$) (see p 113). 5-HIAA levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$) especially at the latter time. Treating rats with L-DOPA, or L-DOPA in combination with 5-HTP, resulted in 5-HIAA levels intermediate between saline and 5-HTP treatment ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 113) AS/AGU rats had higher levels of 5-HIAA than AS/AGU mutants when treated with a) saline b) 5-HTP c) L-DOPA and d) L-DOPA in combination with 5-HTP.

## Two-way ANOVA: DcpHIAA versus Treat, Group

Analysis of Variance for DcpHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treat | 7 | 129.008 | 18.430 | 49.84 | 0.001 |
| Group | 1 | 4.335 | 4.335 | 11.72 | 0.001 |
| Interaction | 7 | 34.893 | 4.985 | 13.48 | 0.001 |
| Error | 80 | 29.583 | 0.370 | | |
| Total | 95 | 197.820 | | | |

| Treat | Mean | Individual 95% CI |
|---|---|---|
| 1 | 3.41 | (--*--) |
| 2 | 3.48 | (--*--) |
| 3 | 6.14 | (--*--) |
| 4 | 7.05 | (--*--) |
| 5 | 4.98 | (-*--) |
| 6 | 5.34 | (--*-) |
| 7 | 4.49 | (-*--) |
| 8 | 5.10 | (--*-) |

3.60 4.80 6.00 7.20

| Group | Mean | Individual 95% CI |
|---|---|---|
| 1 | 4.785 | (--------*--------) |
| 2 | 5.210 | (--------*--------) |

4.800 5.000 5.200 5.400

## One-way ANOVA: DcpuHIAA versus Treatb

Analysis of Variance for DcpuHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treatb | 15 | 168.236 | 11.216 | 30.33 | < 0.001 |
| Error | 80 | 29.583 | 0.370 | | |
| Total | 95 | 197.820 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 2.6667 | 0.2338 |
| 12 | 6 | 4.1500 | 0.4593 |
| 21 | 6 | 2.7500 | 0.6473 |
| 22 | 6 | 4.2000 | 0.3795 |
| 31 | 6 | 6.2000 | 0.5550 |
| 32 | 6 | 6.0833 | 0.5636 |
| 41 | 6 | 7.2833 | 0.6014 |
| 42 | 6 | 6.8167 | 0.7333 |
| 51 | 6 | 4.1333 | 0.5820 |
| 52 | 6 | 5.8167 | 0.7333 |
| 61 | 6 | 4.4833 | 0.7679 |
| 62 | 6 | 6.2000 | 0.4427 |
| 71 | 6 | 5.0833 | 0.8976 |
| 72 | 6 | 3.9000 | 0.4472 |
| 81 | 6 | 5.6833 | 0.6969 |
| 82 | 6 | 4.5167 | 0.6401 |

Pooled StDev = 0.6081

Individual 95% CIs For Mean
Based on Pooled StDev

(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)
(--*--)

3.2 4.8 6.4

3) **Lateral caudate-putamen**. A two-way analysis of variance (ANOVA) showed a significant effect of group (AS v AS/AGU F= 8.83, df=1, p <0.01) and also a very significant effect of treatment (F = 21.57, df = 7, p < 0.001) (see p 115). 5-HIAA levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment (p < 0.05). Treating rats with L-DOPA resulted in 5-HIAA levels intermediate between saline and 5-HTP treatment - significantly different from saline at 60 minutes after administration (p < 0.05) and from 5-HTP at both 30 and 60 minutes (p < 0.05). When L-DOPA and 5-HTP were given together as a combined treatment, levels of 5-HIAA were comparable to giving 5-HTP alone. When the analysis was extended to compare AS and AS/AGU rats (p 115) AS rats had higher levels of 5-HIAA than AS/AGU mutants at sixty minutes after both groups received a combination of L-DOPA and 5-HTP (p < 0.05).

## Two-way ANOVA: LcpHIAA versus Treat, Group

Analysis of Variance for LcpHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treat | 7 | 109.515 | 15.645 | 21.57 | 0.001 |
| Group | 1 | 6.407 | 6.407 | 8.83 | 0.004 |
| Interaction | 7 | 23.345 | 3.335 | 4.60 | 0.001 |
| Error | 80 | 58.033 | 0.725 | | |
| Total | 95 | 197.300 | | | |

| Treat | Mean | Individual 95% CI |
|---|---|---|
| 1 | 2.97 | (----*----) |
| 2 | 3.07 | (----*----) |
| 3 | 5.33 | (----*----) |
| 4 | 5.68 | (----*----) |
| 5 | 3.81 | (----*----) |
| 6 | 4.15 | (----*----) |
| 7 | 4.63 | (----*----) |
| 8 | 5.93 | (----*----) |

3.00 4.00 5.00 6.00

| Group | Mean | Individual 95% CI |
|---|---|---|
| 1 | 4.71 | (----*----) |
| 2 | 4.19 | (----*----) |

4.00 4.25 4.50 4.75

## One-way ANOVA: LcpHIAA versus Treatb

Analysis of Variance for LcpHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treatb | 15 | 139.266 | 9.284 | 12.80 | < 0.001 |
| Error | 80 | 58.033 | 0.725 | | |
| Total | 95 | 197.300 | | | |

| Level | N | Mean | StDev | Individual 95% CIs For Mean Based on Pooled StDev |
|---|---|---|---|---|
| 11 | 6 | 2.8833 | 0.8035 | (---*---) |
| 12 | 6 | 3.0667 | 0.5574 | (---*---) |
| 21 | 6 | 2.9500 | 0.8894 | (---*---) |
| 22 | 6 | 3.1833 | 0.6463 | (---*---) |
| 31 | 6 | 5.6500 | 0.8432 | (---*---) |
| 32 | 6 | 5.0167 | 0.7468 | (---*---) |
| 41 | 6 | 6.0000 | 1.1472 | (---*---) |
| 42 | 6 | 5.3667 | 0.6861 | (---*---) |
| 51 | 6 | 3.6500 | 0.3886 | (---*---) |
| 52 | 6 | 3.9667 | 0.7339 | (---*---) |
| 61 | 6 | 4.0667 | 0.9092 | (---*---) |
| 62 | 6 | 4.2333 | 0.4131 | (---*---) |
| 71 | 6 | 5.1000 | 0.8532 | (---*---) |
| 72 | 6 | 4.1667 | 0.6408 | (---*---) |
| 81 | 6 | 7.3500 | 1.6047 | (---*---) |
| 82 | 6 | 4.5167 | 0.9928 | (---*---) |

Pooled StDev = 0.8517

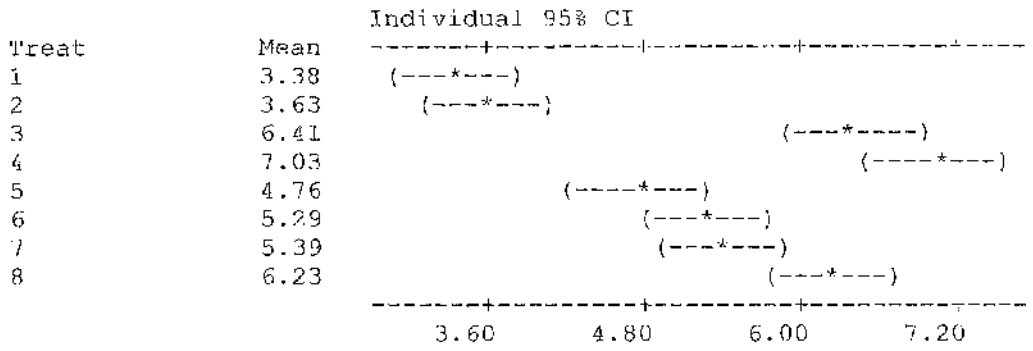
3.2 4.8 6.4

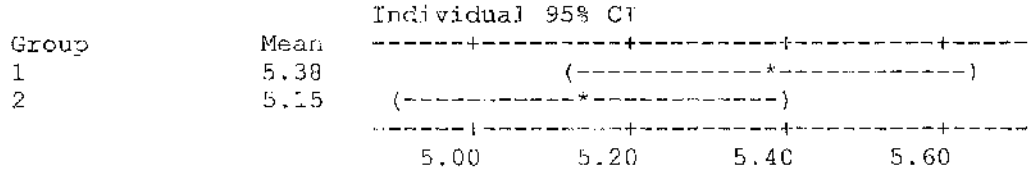
4) **Ventral caudate-putamen**. A two-way analysis of variance (ANOVA) showed no significant effect of group (AS v AS/AGU) but a very significant effect of treatment ($F = 25.90$, $df = 7$, $p < 0.001$) (see p 117). 5-HIAA levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). Treating rats with L-DOPA resulted in 5-HIAA levels intermediate between saline and 5-HTP treatment (and significantly different from each for comparable times after administration, $p < 0.05$). When L-DOPA and 5-HTP were given together as a combined treatment, levels of 5-HIAA were comparable to giving 5-HTP alone. When the analysis was extended to compare AS and AS/AGU rats (p 117) no differences between AS and AS/AGU rats were found.

## Two-way ANOVA: VcpHIAA versus Treat, Group

Analysis of Variance for VcpHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treat | 7 | 141.962 | 20.280 | 25.90 | 0.001 |
| Group | 1 | 1.283 | 1.283 | 1.64 | 0.204 |
| Interaction | 7 | 6.982 | 0.997 | 1.27 | 0.274 |
| Error | 80 | 62.635 | 0.783 | | |
| Total | 95 | 212.862 | | | |




## One-way ANOVA: VcpHIAA versus Treatb

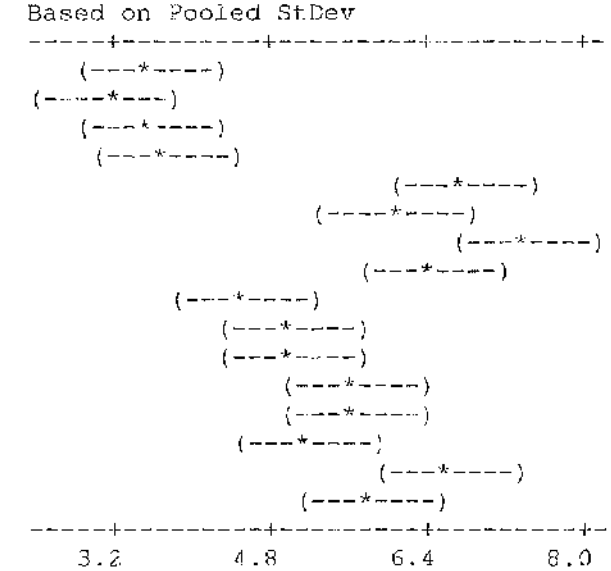
Analysis of Variance for VcpHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treatb | 15 | 147.644 | 9.843 | 12.39 | < 0.001 |
| Error | 80 | 63.535 | 0.794 | | |
| Total | 95 | 211.179 | | | |

Individual 95% CIs For Mean Based on Pooled StDev

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 3.5833 | 0.8796 |
| 12 | 6 | 3.1833 | 1.0610 |
| 21 | 6 | 3.5333 | 0.9070 |
| 22 | 6 | 3.7167 | 0.7360 |
| 31 | 6 | 6.7333 | 0.7691 |
| 32 | 6 | 6.0833 | 0.4708 |
| 41 | 6 | 7.4167 | 0.8134 |
| 42 | 6 | 6.4667 | 0.7394 |
| 51 | 6 | 4.5333 | 0.8262 |
| 52 | 6 | 4.9833 | 0.7782 |
| 61 | 6 | 4.9667 | 0.8116 |
| 62 | 6 | 5.6167 | 0.7223 |
| 71 | 6 | 5.6333 | 0.8959 |
| 72 | 6 | 5.1500 | 0.6189 |
| 81 | 6 | 6.6333 | 1.5227 |
| 82 | 6 | 5.8167 | 1.1907 |

Pooled StDev = 0.8912



5) **Dorsal Raphe Nucleus**. A two-way analysis of variance (ANOVA) showed an extremely significant effect of group (AS v AS/AGU, $F = 370.55$, $df = 1$, $p < 0.001$) and a very significant effect of treatment ($F = 71.35$, $df = 7$, $p < 0.001$) (see p 119). 5-HIAA levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). Treating rats with L-DOPA (or L-DOPA and 5-HTP together as a combined treatment) resulted in 5-HIAA levels intermediate between saline and 5-HTP treatment (and significantly different from each for comparable times after administration, $p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 119) AS/AGU mutants rats had higher levels of 5-HIAA than AS rats when treated with a) saline b) L-DOPA and c) L-DOPA + 5-HTP combined.

## Two-way ANOVA: DrnHIAA versus Treat, Group

Analysis of Variance for DrnHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treat | 7 | 1971.33 | 281.62 | 71.35 | 0.001 |
| Group | 1 | 1462.50 | 1462.50 | 370.55 | 0.001 |
| Interaction | 7 | 591.13 | 84.45 | 21.40 | 0.001 |
| Error | 80 | 315.75 | 3.95 | | |
| Total | 95 | 4340.70 | | | |

| Treat | Mean | Individual 95% CI |
|---|---|---|
| 1 | 26.42 | (--*--) |
| 2 | 27.00 | (--*--) |
| 3 | 38.00 | (--*--) |
| 4 | 39.75 | (--*--) |
| 5 | 29.83 | (--*--) |
| 6 | 31.12 | (--*--) |
| 7 | 33.17 | (--*--) |
| 8 | 34.67 | (--*--) |

28.00 32.00 36.00 40.00

| Group | Mean | Individual 95% CI |
|---|---|---|
| 1 | 28.59 | (-*--) |
| 2 | 36.40 | (--*-) |

30.00 32.50 35.00 37.50

## One-way ANOVA: DrnHIAA versus Treatb

Analysis of Variance for DrnHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treatb | 15 | 4024.96 | 268.33 | 67.99 | < 0.001 |
| Error | 80 | 315.75 | 3.95 | | |
| Total | 95 | 4340.70 | | | |

| Level | N | Mean | StDev | Individual 95% CIs For Mean Based on Pooled StDev |
|---|---|---|---|---|
| 11 | 6 | 22.333 | 1.211 | (-*-) |
| 12 | 6 | 30.500 | 1.871 | (--*-) |
| 21 | 6 | 23.667 | 1.506 | (-*-) |
| 22 | 6 | 30.333 | 1.506 | (-*--) |
| 31 | 6 | 37.667 | 1.633 | (-*-) |
| 32 | 6 | 38.333 | 1.862 | (--*-) |
| 41 | 6 | 39.833 | 1.835 | (-*-) |
| 42 | 6 | 39.667 | 1.633 | (--*-) |
| 51 | 6 | 25.317 | 1.372 | (-*-) |
| 52 | 6 | 34.333 | 3.204 | (-*-) |
| 61 | 6 | 25.900 | 1.367 | (-*-) |
| 62 | 6 | 36.333 | 1.751 | (-*-) |
| 71 | 6 | 26.167 | 2.137 | (-*--) |
| 72 | 6 | 40.167 | 2.041 | (-*--) |
| 81 | 6 | 27.833 | 3.488 | (--*-) |
| 82 | 6 | 41.500 | 1.871 | (-*--) |

Pooled StDev = 1.987 21.0 28.0 35.0 42.0

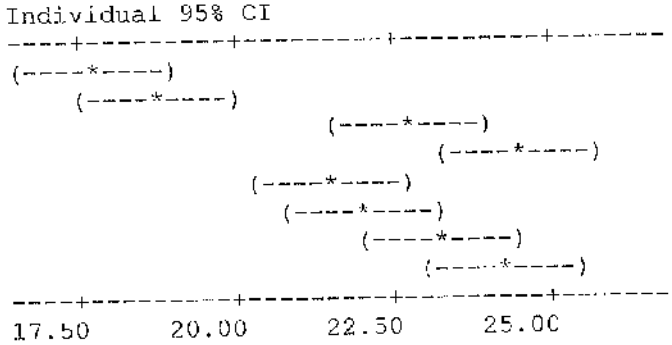
6) **Median Raphe Nucleus**. A two-way analysis of variance (ANOVA) showed a significant effect of group (AS v AS/AGU, F=10.32, df =1, p <0.01) and a very significant effect of treatment (F = 15.77, df = 7, p < 0.001) (see p 121). 5-HIAA levels were significantly elevated in 5-HTP-treated animals compared with saline-treated ones at both 30 and 60 minutes after treatment (p < 0.05). Treating rats with L-DOPA (or L-DOPA and 5-HTP together as a combined treatment) was not dissimilar to treatment with 5-HTP alone. When the analysis was extended to compare AS and AS/AGU rats (p 121) AS control rats had higher levels of 5-HIAA than AS/AGU rats when treated with L-DOPA + 5-HTP combined.

## Two-way ANOVA: MrnHIAA versus Treat, Group

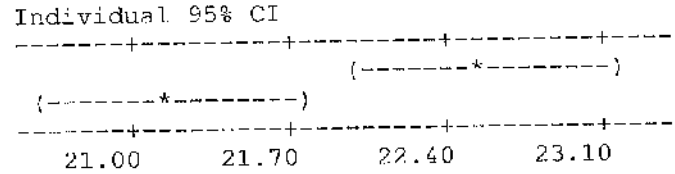
Analysis of Variance for MrnHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treat | 7 | 504.91 | 72.13 | 15.77 | 0.001 |
| Group | 1 | 47.18 | 47.18 | 10.32 | 0.002 |
| Interaction | 7 | 95.24 | 13.61 | 2.97 | 0.008 |
| Error | 80 | 365.91 | 4.57 | | |
| Total | 95 | 1013.25 | | | |

| Treat | Mean |
|---|---|
| 1 | 17.83 |
| 2 | 18.67 |
| 3 | 22.81 |
| 4 | 24.58 |
| 5 | 21.58 |
| 6 | 22.00 |
| 7 | 23.25 |
| 8 | 24.17 |




| Group | Mean |
|---|---|
| 1 | 22.56 |
| 2 | 21.16 |



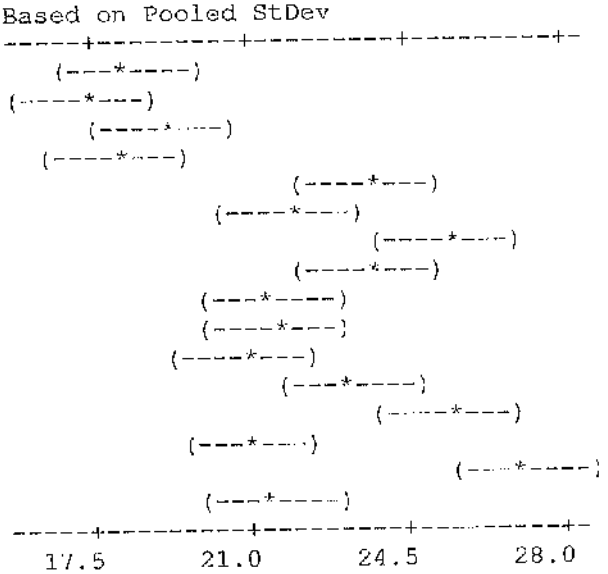

## One-way ANOVA: MrnHIAA versus Treatb

Analysis of Variance for MrnHIAA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Treatb | 15 | 702.44 | 46.83 | 12.57 | < 0.001 |
| Error | 80 | 298.08 | 3.73 | | |
| Total | 95 | 1000.51 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 18.333 | 1.966 |
| 12 | 6 | 17.333 | 1.633 |
| 21 | 6 | 19.167 | 2.639 |
| 22 | 6 | 18.167 | 1.472 |
| 31 | 6 | 23.667 | 1.366 |
| 32 | 6 | 21.950 | 1.420 |
| 41 | 6 | 25.500 | 1.871 |
| 42 | 6 | 23.667 | 1.033 |
| 51 | 6 | 21.500 | 2.258 |
| 52 | 6 | 21.667 | 1.751 |
| 61 | 6 | 20.833 | 2.317 |
| 62 | 6 | 23.167 | 1.835 |
| 71 | 6 | 25.500 | 1.049 |
| 72 | 6 | 21.000 | 2.449 |
| 81 | 6 | 27.000 | 1.414 |
| 82 | 6 | 23.500 | 3.082 |

Pooled StDev = 1.930



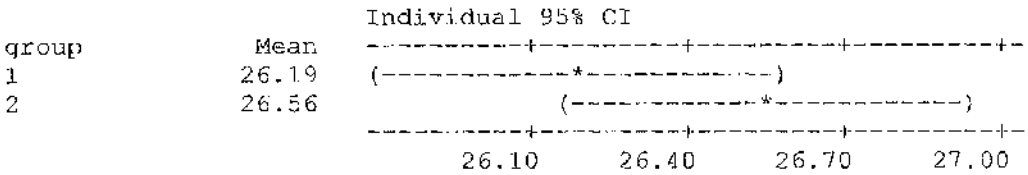
### 5.3.2.3 Dopamine

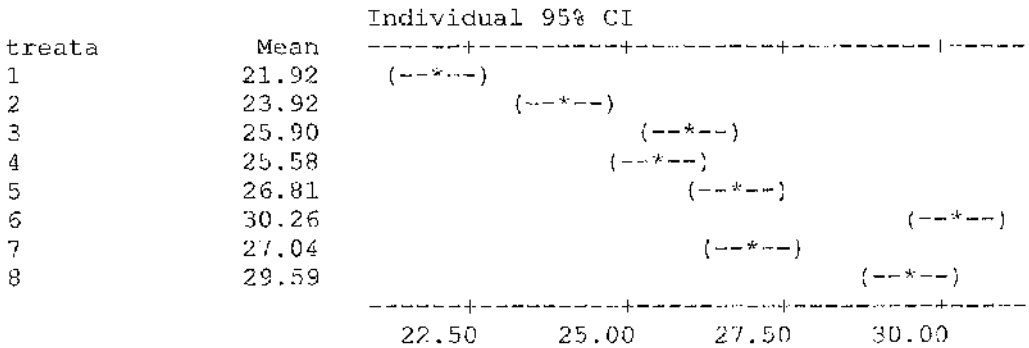
1) **Anterior caudate-putamen**. A two-way analysis of variance (ANOVA) showed no significant effect of group (AS v AS/AGU) but a very significant effect of treatment ($F = 50.29$, $df = 7$, $p < 0.001$) (see p 123). DA levels were significantly elevated in L-DOPA-treated animals (as well as in animals treated with both L-DOPA and 5-HTP simultaneously) compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). Unexpectedly, DA levels were also elevated in animals given 5-HTP alone ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 123) no differences were found between the two strains.

## Two-way ANOVA: AcpDA versus group, treata

Analysis of Variance for AcpDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 3.23 | 3.23 | 1.79 | 0.185 |
| treata | 7 | 633.98 | 90.57 | 50.29 | 0.001 |
| Interaction | 7 | 22.16 | 3.17 | 1.76 | 0.107 |
| Error | 80 | 144.08 | 1.80 | | |
| Total | 95 | 803.45 | | | |




## One-way ANOVA: AcpDA versus treatb

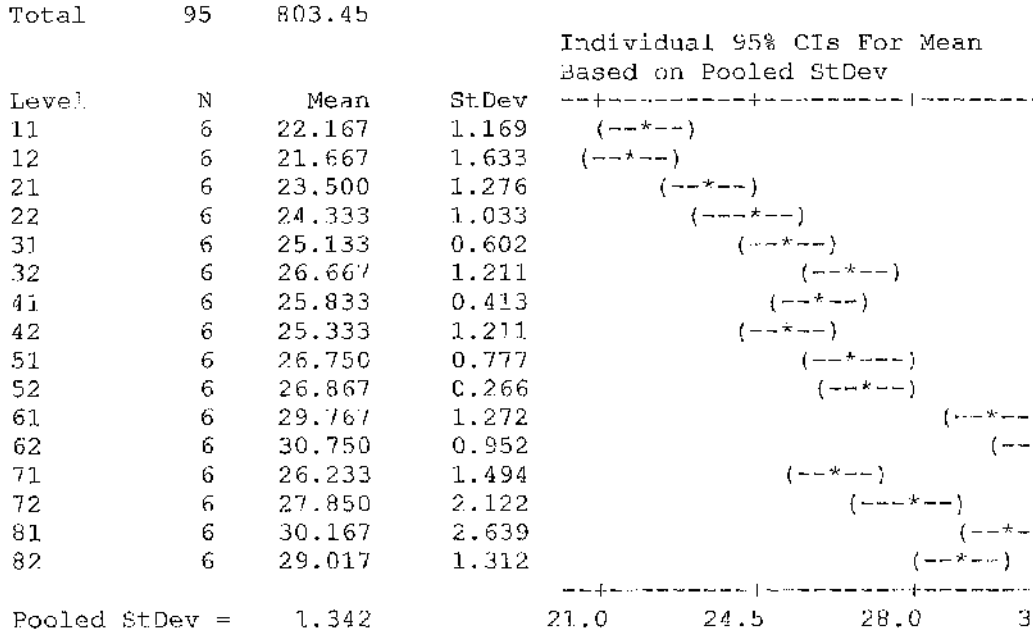
Analysis of Variance for AcpDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 659.37 | 43.96 | 24.41 | < 0.001 |
| Error | 80 | 144.08 | 1.80 | | |
| Total | 95 | 803.45 | | | |

Individual 95% CIs For Mean Based on Pooled StDev

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 22.167 | 1.169 |
| 12 | 6 | 21.667 | 1.633 |
| 21 | 6 | 23.500 | 1.276 |
| 22 | 6 | 24.333 | 1.033 |
| 31 | 6 | 25.133 | 0.602 |
| 32 | 6 | 26.667 | 1.211 |
| 41 | 6 | 25.833 | 0.413 |
| 42 | 6 | 25.333 | 1.211 |
| 51 | 6 | 26.750 | 0.777 |
| 52 | 6 | 26.867 | 0.266 |
| 61 | 6 | 29.767 | 1.272 |
| 62 | 6 | 30.750 | 0.952 |
| 71 | 6 | 26.233 | 1.494 |
| 72 | 6 | 27.850 | 2.122 |
| 81 | 6 | 30.167 | 2.639 |
| 82 | 6 | 29.017 | 1.312 |

Pooled StDev = 1.342



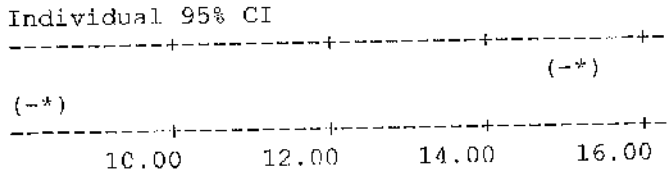
2) **Dorsal caudate-putamen**. A two-way analysis of variance (ANOVA) showed a highly significant effect of group (AS v AS/AGU $F = 946$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 48.79$, $df = 7$, $p < 0.001$) (see p 125). As with the anterior caudate-putamen (see above) DA levels were significantly elevated in L-DOPA-treated animals (as well as in animals treated with both L-DOPA and 5-HTP simultaneously) compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). Unlike the anterior caudate-putamen, no effect of 5-HTP alone could be found. When the analysis was extended to compare AS and AS/AGU rats (p 125) very large differences were found between the two strains under all treatments and times, with control AS rats having higher dopamine levels than AS/AGU mutants.

## Two-way ANOVA: DcpDA versus group, treata

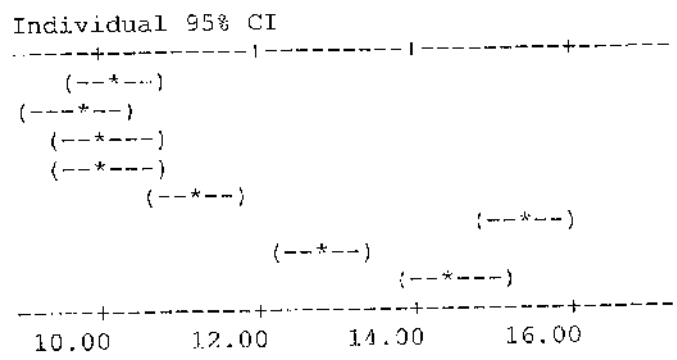
Analysis of Variance for DcpDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 1130.25 | 1130.25 | 946.41 | 0.001 |
| treata | 7 | 407.90 | 58.27 | 48.79 | 0.001 |
| Interaction | 7 | 130.13 | 18.59 | 15.57 | 0.001 |
| Error | 80 | 95.54 | 1.19 | | |
| Total | 95 | 1763.82 | | | |

| group | Mean |
|---|---|
| 1 | 15.18 |
| 2 | 8.31 |




| treata | Mean |
|---|---|
| 1 | 10.22 |
| 2 | 9.71 |
| 3 | 10.09 |
| 4 | 10.10 |
| 5 | 11.20 |
| 6 | 15.37 |
| 7 | 12.79 |
| 8 | 14.48 |



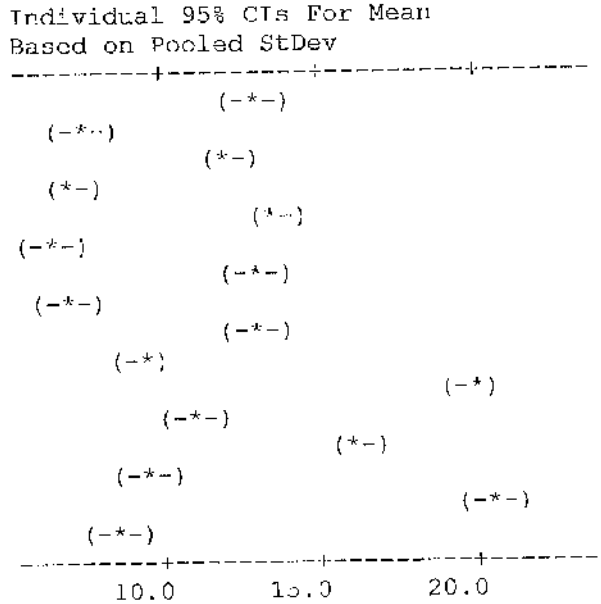

## One-way ANOVA: DcpDA versus treatb

Analysis of Variance for DcpDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 1668.28 | 111.22 | 93.13 | < 0.001 |
| Error | 80 | 95.54 | 1.19 | | |
| Total | 95 | 1763.82 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 12.967 | 0.859 |
| 12 | 6 | 7.467 | 0.557 |
| 21 | 6 | 12.217 | 0.749 |
| 22 | 6 | 7.200 | 0.540 |
| 31 | 6 | 13.683 | 0.646 |
| 32 | 6 | 6.500 | 0.645 |
| 41 | 6 | 13.117 | 1.650 |
| 42 | 6 | 7.083 | 0.671 |
| 51 | 6 | 13.100 | 1.010 |
| 52 | 6 | 9.300 | 1.305 |
| 61 | 6 | 19.750 | 1.507 |
| 62 | 6 | 11.000 | 1.271 |
| 71 | 6 | 16.200 | 1.518 |
| 72 | 6 | 9.383 | 0.496 |
| 81 | 6 | 20.383 | 1.933 |
| 82 | 6 | 8.583 | 0.549 |

Pooled StDev = 1.093



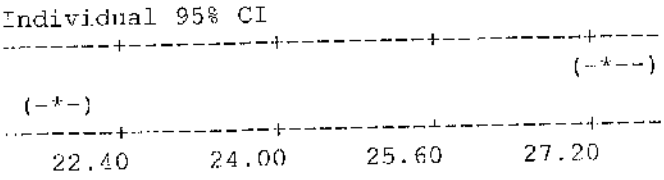
3) **Lateral caudate-putamen**. A two-way analysis of variance (ANOVA) showed a highly significant effect of group (AS v AS/AGU $F = 444$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 30.58$, $df = 7$, $p < 0.001$) (see p 127). As with other parts of the caudate-putamen (see above) DA levels were significantly elevated in L-DOPA-treated animals (as well as in animals treated with both L-DOPA and 5-HTP simultaneously) compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). Like the anterior caudate-putamen, 5-HTP administration alone also led to raised DA levels ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 127) very large differences were found between the two strains under all treatments and times, with control AS rats having higher dopamine levels than AS/AGU mutants.

## Two-way ANOVA: LcpDA versus group, treata

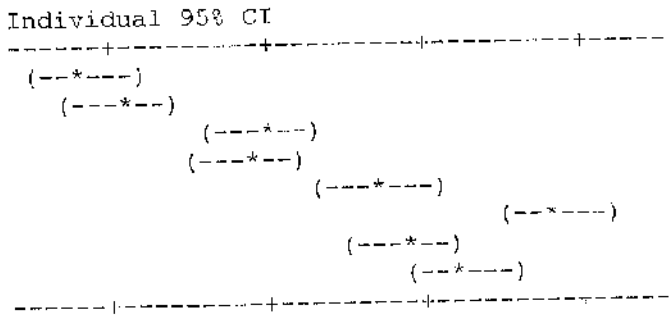
Analysis of Variance for LcpDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 761.63 | 761.63 | 444.08 | 0.001 |
| treata | 7 | 367.18 | 52.45 | 30.58 | 0.001 |
| Interaction | 7 | 129.37 | 18.48 | 10.78 | 0.001 |
| Error | 80 | 137.21 | 1.72 | | |
| Total | 95 | 1395.39 | | | |

| group | Mean |
|---|---|
| 1 | 27.39 |
| 2 | 21.76 |




| treata | Mean |
|---|---|
| 1 | 21.68 |
| 2 | 22.11 |
| 3 | 23.94 |
| 4 | 23.71 |
| 5 | 25.36 |
| 6 | 27.66 |
| 7 | 25.71 |
| 8 | 26.46 |



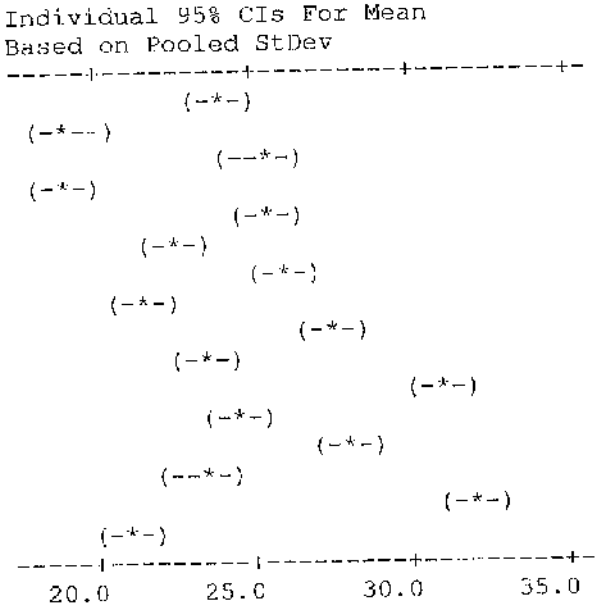

## One-way ANOVA: LcpDA versus treatb

Analysis of Variance for LcpDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 1258.18 | 83.88 | 48.91 | < 0.001 |
| Error | 80 | 137.21 | 1.72 | | |
| Total | 95 | 1395.39 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 24.133 | 1.172 |
| 12 | 6 | 19.217 | 1.463 |
| 21 | 6 | 25.283 | 1.011 |
| 22 | 6 | 18.933 | 1.129 |
| 31 | 6 | 25.383 | 1.150 |
| 32 | 6 | 22.500 | 2.011 |
| 41 | 6 | 26.017 | 0.671 |
| 42 | 6 | 21.400 | 1.020 |
| 51 | 6 | 27.350 | 0.912 |
| 52 | 6 | 23.367 | 0.950 |
| 61 | 6 | 30.833 | 2.401 |
| 62 | 6 | 24.483 | 1.221 |
| 71 | 6 | 28.150 | 1.531 |
| 72 | 6 | 23.267 | 0.848 |
| 81 | 6 | 32.000 | 1.414 |
| 82 | 6 | 20.917 | 0.850 |

Pooled StDev = 1.310



4) **Ventral caudate-putamen**. A two-way analysis of variance (ANOVA) showed a significant effect of group (AS v AS/AGU $F = 22.6$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 6.49$, $df = 7$, $p < 0.001$) (see p 129). DA levels were significantly elevated in L-DOPA-treated animals both 30 and 60 minutes after treatment as well as in animals treated with both L-DOPA and 5-HTP simultaneously 60 minutes after treatment (for all, $p < 0.05$). No effect of 5-HTP alone could be found. When the analysis was extended to compare AS and AS/AGU rats (p 129) control AS rats had higher dopamine levels than AS/AGU mutants 60 minutes after a combined L-DOPA + 5-HTP administration; all other comparisons were non-significant.

## Two-way ANOVA: VcpDA versus group, treata

Analysis of Variance for VcpDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 97.00 | 97.00 | 22.60 | 0.001 |
| treata | 7 | 194.96 | 27.85 | 6.49 | 0.001 |
| Interaction | 7 | 200.09 | 28.58 | 6.66 | 0.001 |
| Error | 80 | 343.38 | 4.29 | | |
| Total | 95 | 835.42 | | | |

| group | Mean | Individual 95% CI |
|---|---|---|
| 1 | 24.34 | (-----*-----) |
| 2 | 22.33 | (-----*-----) |

22.40 23.20 24.00 24.80

| treata | Mean | Individual 95% CI |
|---|---|---|
| 1 | 21.83 | (-----*-----) |
| 2 | 21.70 | (-----*-----) |
| 3 | 22.75 | (-----*-----) |
| 4 | 21.87 | (-----*-----) |
| 5 | 24.77 | (-----*-----) |
| 6 | 25.68 | (-----*-----) |
| 7 | 23.67 | (-----*-----) |
| 8 | 24.42 | (-----*-----) |

20.80 22.40 24.00 25.60

## One-way ANOVA: VcpDA versus treatb

Analysis of Variance for VcpDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 492.05 | 32.80 | 7.64 | < 0.001 |
| Error | 80 | 343.38 | 4.29 | | |
| Total | 95 | 835.42 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 21.500 | 2.665 |
| 12 | 6 | 22.167 | 1.941 |
| 21 | 6 | 22.733 | 1.796 |
| 22 | 6 | 20.667 | 2.733 |
| 31 | 6 | 23.333 | 2.066 |
| 32 | 6 | 22.167 | 1.941 |
| 41 | 6 | 21.667 | 1.506 |
| 42 | 6 | 22.083 | 1.625 |
| 51 | 6 | 25.167 | 2.317 |
| 52 | 6 | 24.367 | 1.169 |
| 61 | 6 | 26.500 | 2.074 |
| 62 | 6 | 24.867 | 1.451 |
| 71 | 6 | 24.833 | 1.472 |
| 72 | 6 | 22.500 | 1.378 |
| 81 | 6 | 29.000 | 3.950 |
| 82 | 6 | 19.833 | 1.169 |

Pooled StDev = 2.072

Individual 95% CIs For Mean
Based on Pooled StDev

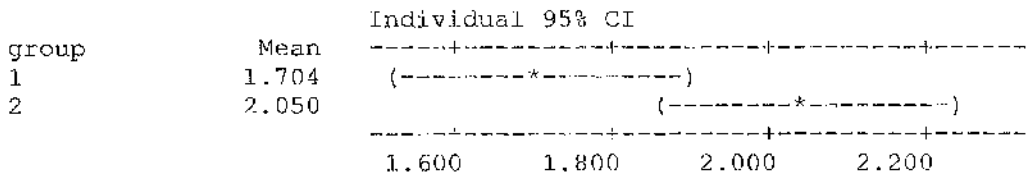
21.0 24.5 28.0

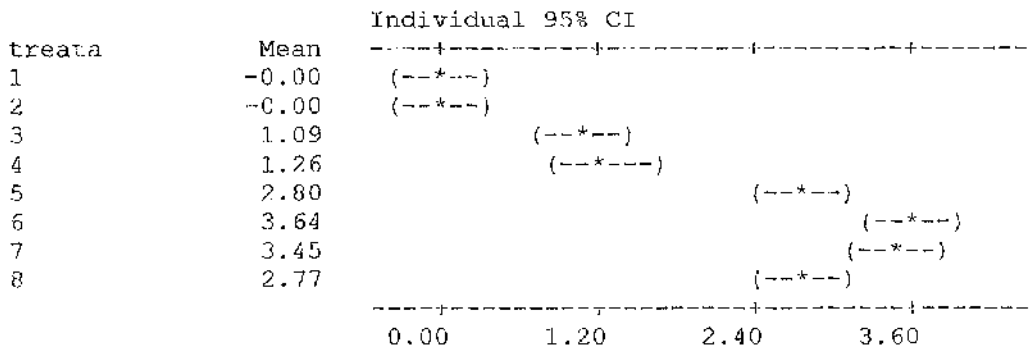
5) **Dorsal raphe nucleus**. A two-way analysis of variance (ANOVA) showed a significant effect of group (AS v AS/AGU $F = 6.66$, $df = 1$, $p < 0.05$) as well as a very significant effect of treatment ($F = 60.83$, $df = 7$, $p < 0.001$) (see p 131). DA levels were non-detectable in the region of the dorsal raphe in control saline-treated rats of either strain. However, DA levels became detectable (and thus significantly elevated) in L-DOPA-treated animals - as well as in animals treated with both L-DOPA and 5-HTP simultaneously - compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). Furthermore, administration of 5-HTP alone led to a rise in DA levels found ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (Fig. **) differences were found between the two strains in animals treated with L-DOPA and L-DOPA combined with 5-HTP. However, since these differences included higher levels of dopamine in AS rats on some occasions and in AS/AGU rats on others, it is unclear if they are of functional significance (see p 131). Levels of dopamine were always very low in the dorsal raphe.

## Two-way ANOVA: DrnDA versus group, treata

Analysis of Variance for DrnDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 2.870 | 2.870 | 6.66 | 0.012 |
| treata | 7 | 183.510 | 26.216 | 60.83 | 0.001 |
| Interaction | 7 | 36.073 | 5.153 | 11.96 | 0.001 |
| Error | 80 | 34.477 | 0.431 | | |
| Total | 95 | 256.930 | | | |



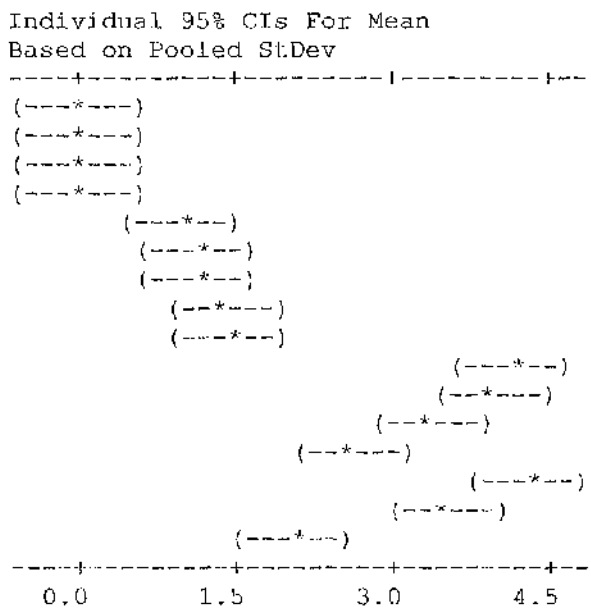

## One-way ANOVA: DrnDA versus treatb

Analysis of Variance for DrnDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 222.453 | 14.830 | 34.41 | < 0.001 |
| Error | 80 | 34.477 | 0.431 | | |
| Total | 95 | 256.930 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | -0.0000 | 0.0000 |
| 12 | 6 | -0.0000 | 0.0000 |
| 21 | 6 | -0.0000 | 0.0000 |
| 22 | 6 | -0.0000 | 0.0000 |
| 31 | 6 | 1.0333 | 0.4719 |
| 32 | 6 | 1.1500 | 0.5683 |
| 41 | 6 | 1.1333 | 0.7062 |
| 42 | 6 | 1.3833 | 0.4262 |
| 51 | 6 | 1.4333 | 0.3830 |
| 52 | 6 | 4.1667 | 1.4720 |
| 61 | 6 | 3.9667 | 0.6976 |
| 62 | 6 | 3.3167 | 0.6369 |
| 71 | 6 | 2.5833 | 0.6274 |
| 72 | 6 | 4.3167 | 0.9948 |
| 81 | 6 | 3.4833 | 0.8704 |
| 82 | 6 | 2.0667 | 0.5680 |

Pooled StDev = 0.6565



6) **Median raphe nucleus**. A two-way analysis of variance (ANOVA) showed no effect of group (AS v AS/AGU) but a very significant effect of treatment ($F = 47.74$, $df = 7$, $p < 0.001$) (see p 133). DA levels were non-detectable in the region of the median raphe in control saline-treated rats of either strain. However, DA levels became detectable (and thus significantly elevated) in L-DOPA-treated animals - as well as in animals treated with both L-DOPA and 5-HTP simultaneously - compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). Furthermore, administration of 5-HTP alone led to a rise in DA levels found ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 133) a single difference was found between the two strains in animals treated with 5-HTP where mutants had higher DA levels than controls.

## Two-way ANOVA: MrnDA versus group, treata

Analysis of Variance for MrnDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 0.220 | 0.220 | 1.41 | 0.239 |
| treata | 7 | 52.238 | 7.463 | 47.74 | 0.001 |
| Interaction | 7 | 4.475 | 0.639 | 4.09 | 0.001 |
| Error | 80 | 12.507 | 0.156 | | |
| Total | 95 | 69.440 | | | |

| group | Mean | Individual 95% CI |
|---|---|---|
| 1 | 1.002 | (-----------*-----------) |
| 2 | 1.098 | (-----------*-----------) |
| | | 0.960 1.040 1.120 1.200 |

| treata | Mean | Individual 95% CI |
|---|---|---|
| 1 | 0.00 | (--*--) |
| 2 | 0.00 | (--*--) |
| 3 | 0.85 | (--*--) |
| 4 | 0.94 | (--*---) |
| 5 | 1.23 | (---*--) |
| 6 | 2.25 | (--*--) |
| 7 | 1.34 | (--*--) |
| 8 | 1.78 | (--*---) |
| | | 0.00 0.70 1.40 2.10 |

## One-way ANOVA: MrnDA versus treatb

Analysis of Variance for MrnDA

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 56.933 | 3.796 | 24.28 | < 0.001 |
| Error | 80 | 12.507 | 0.156 | | |
| Total | 95 | 69.440 | | | |

| Level | N | Mean | StDev | Individual 95% CIs For Mean Based on Pooled StDev |
|---|---|---|---|---|
| 11 | 6 | 0.0000 | 0.0000 | (---*---) |
| 12 | 6 | 0.0000 | 0.0000 | (---*---) |
| 21 | 6 | 0.0000 | 0.0000 | (---*---) |
| 22 | 6 | 0.0000 | 0.0000 | (---*---) |
| 31 | 6 | 0.8500 | 0.2168 | (---*---) |
| 32 | 6 | 0.8500 | 0.2258 | (---*---) |
| 41 | 6 | 0.4333 | 0.1211 | (---*---) |
| 42 | 6 | 1.4500 | 0.4037 | (---*---) |
| 51 | 6 | 0.9833 | 0.5345 | (---*---) |
| 52 | 6 | 1.4833 | 0.3061 | (---*---) |
| 61 | 6 | 2.2667 | 0.6055 | (---*---) |
| 62 | 6 | 2.2333 | 0.8548 | (---*---) |
| 71 | 6 | 1.4667 | 0.4082 | (---*---) |
| 72 | 6 | 1.2167 | 0.4535 | (---*---) |
| 81 | 6 | 2.0167 | 0.2137 | (---*---) |
| 82 | 6 | 1.5500 | 0.5753 | (---*---) |
| | | | | 0.00 0.80 1.60 2.40 |

Pooled StDev = 0.3954

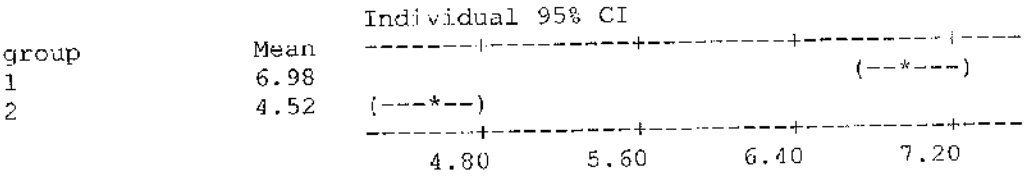
### 5.3.2.4 Dihydroxyphenylacetic acid (DOPAC)

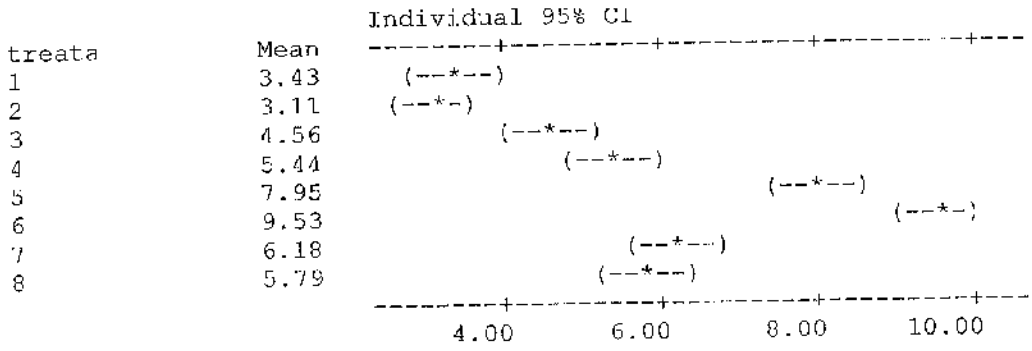
1) **Anterior caudate-putamen**. A two-way analysis of variance (ANOVA) showed a highly significant effect of group (AS v AS/AGU $F = 156$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 61.54$, $df = 7$, $p < 0.001$) (see p 135). DA levels were significantly elevated in L-DOPA-treated animals (as well as in animals treated with both L-DOPA and 5-HTP simultaneously) compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). The combined treatment was less effective than L-DOPA alone. 5-HTP alone also significantly raised DOPAC levels ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 135) very large differences were found between the two strains both 30 and 60 minutes after L-DOPA administration; in each case, control AS rats had higher levels of DOPAC than AS/AGU mutants. A similar response was found in rats given L-DOPA + 5-HTP 30 minutes after administration.

## Two-way ANOVA: acpDOP versus group, treata

Analysis of Variance for acpDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 144.305 | 144.305 | 156.04 | 0.001 |
| treata | 7 | 398.357 | 56.908 | 61.54 | 0.001 |
| Interaction | 7 | 210.136 | 30.019 | 32.46 | 0.001 |
| Error | 80 | 73.982 | 0.925 | | |
| Total | 95 | 826.780 | | | |



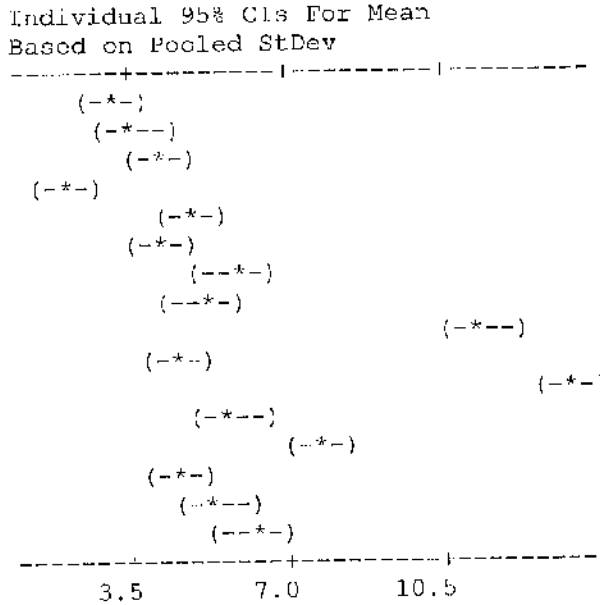

## One-way ANOVA: acpDOP versus treatb

Analysis of Variance for acpDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 752.798 | 50.187 | 54.27 | < 0.001 |
| Error | 80 | 73.982 | 0.925 | | |
| Total | 95 | 826.780 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 3.200 | 1.147 |
| 12 | 6 | 3.667 | 0.819 |
| 21 | 6 | 4.150 | 0.929 |
| 22 | 6 | 2.067 | 0.234 |
| 31 | 6 | 4.900 | 1.318 |
| 32 | 6 | 4.217 | 0.313 |
| 41 | 6 | 5.783 | 0.804 |
| 42 | 6 | 5.100 | 1.257 |
| 51 | 6 | 11.300 | 1.230 |
| 52 | 6 | 4.600 | 0.522 |
| 61 | 6 | 13.300 | 1.691 |
| 62 | 6 | 5.767 | 1.015 |
| 71 | 6 | 7.783 | 0.850 |
| 72 | 6 | 4.567 | 0.427 |
| 81 | 6 | 5.383 | 0.595 |
| 82 | 6 | 6.200 | 0.949 |

Pooled StDev = 0.962

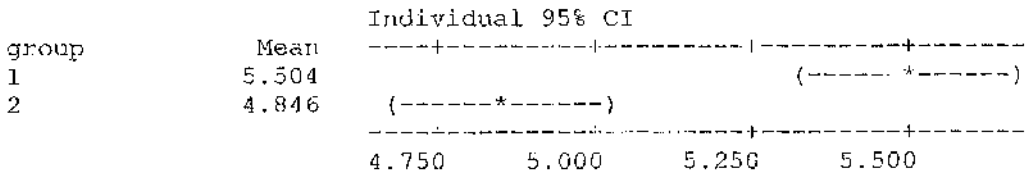


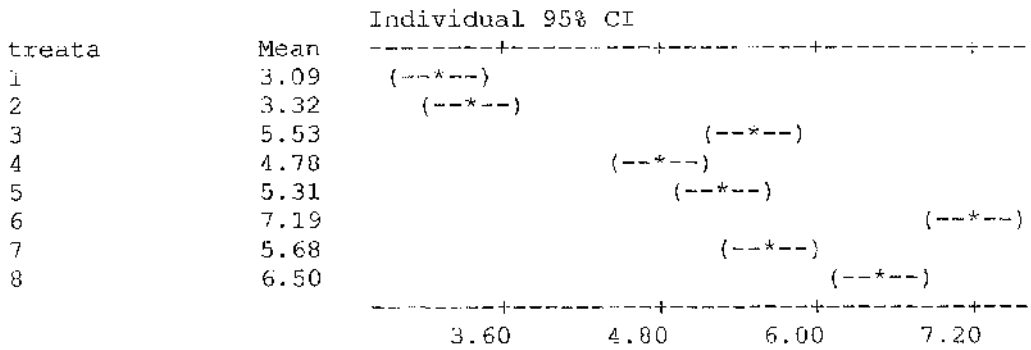
2) **Dorsal caudate-putamen**. A two-way analysis of variance (ANOVA) showed a highly significant effect of group (AS v AS/AGU $F = 26.8$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 62.57$, $df = 7$, $p < 0.001$) (see p 137). As with the anterior caudate-putamen (see above) DA levels were significantly elevated in L-DOPA-treated animals (as well as in animals treated with both L-DOPA and 5-HTP simultaneously) compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). 5-HTP alone also significantly raised DOPAC levels ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 137) many significant differences were found between the two strains. When the treatment was saline alone, DOPAC levels were higher in mutants than controls. However, when the treatment was a pharmacological intervention of any kind, the reverse was observed, with control AS rats having higher dopamine levels than AS/AGU mutants.

## Two-way ANOVA: dcpDOP versus group, treata

Analysis of Variance for dcpDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 10.402 | 10.402 | 26.80 | 0.001 |
| treata | 7 | 169.990 | 24.284 | 62.57 | 0.001 |
| Interaction | 7 | 37.742 | 5.392 | 13.89 | 0.001 |
| Error | 80 | 31.047 | 0.388 | | |
| Total | 95 | 249.180 | | | |



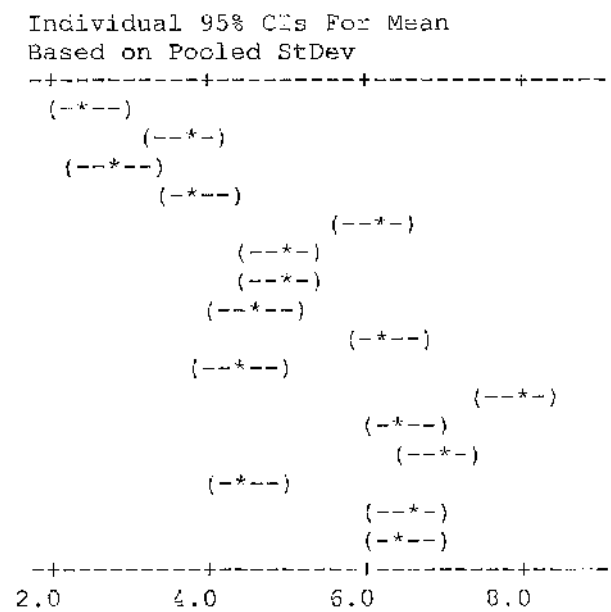

## One-way ANOVA: dcpDOP versus treatb

Analysis of Variance for dcpDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 218.133 | 14.542 | 37.47 | < 0.001 |
| Error | 80 | 31.047 | 0.388 | | |
| Total | 95 | 249.180 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 2.4833 | 0.4491 |
| 12 | 6 | 3.7000 | 0.3098 |
| 21 | 6 | 2.8000 | 0.5099 |
| 22 | 6 | 3.8333 | 0.4082 |
| 31 | 6 | 6.1500 | 0.6473 |
| 32 | 6 | 4.9167 | 0.3764 |
| 41 | 6 | 4.9667 | 0.6861 |
| 42 | 6 | 4.6000 | 0.4382 |
| 51 | 6 | 6.2167 | 0.6969 |
| 52 | 6 | 4.4000 | 0.4561 |
| 61 | 6 | 7.9333 | 0.9374 |
| 62 | 6 | 6.4500 | 0.8408 |
| 71 | 6 | 6.9333 | 0.6186 |
| 72 | 6 | 4.4167 | 0.4262 |
| 81 | 6 | 6.5500 | 0.6285 |
| 82 | 6 | 6.4500 | 1.0114 |

Pooled StDev = 0.6230



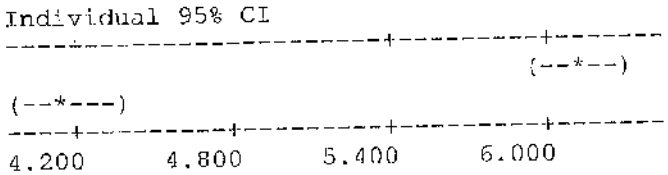
3) **Lateral caudate-putamen**. A two-way analysis of variance (ANOVA) showed a highly significant effect of group (AS v AS/AGU $F = 209$, $df = 1$, $p < 0.001$) as well as a very significant effect of treatment ($F = 87.25$, $df = 7$, $p < 0.001$) (see p 139). As with the anterior caudate-putamen (see above) DA levels were significantly elevated in L-DOPA-treated animals (as well as in animals treated with both L-DOPA and 5-HTP simultaneously) compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). 5-HTP alone also significantly raised DOPAC levels ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 139) many significant differences were found between the two strains. When the treatment was saline alone, DOPAC levels were higher in mutants than controls. However, when the treatment was a pharmacological intervention of any kind, the reverse was observed, with control AS rats having higher dopamine levels than AS/AGU mutants.

## Two-way ANOVA: lcpDOP versus group, treata

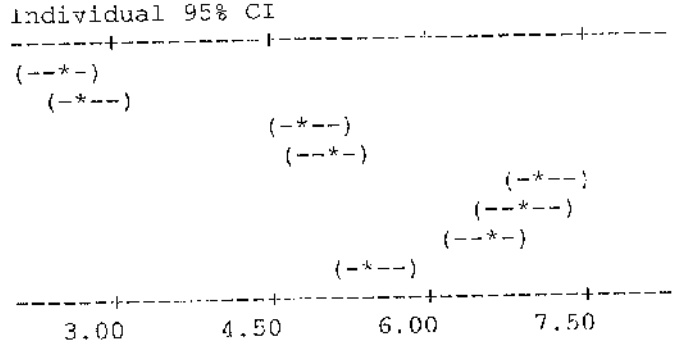
Analysis of Variance for lcpDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 90.229 | 90.229 | 209.47 | 0.001 |
| treata | 7 | 263.077 | 37.582 | 87.25 | 0.001 |
| Interaction | 7 | 109.390 | 15.627 | 36.28 | 0.001 |
| Error | 80 | 34.459 | 0.431 | | |
| Total | 95 | 497.156 | | | |

| group | Mean |
|---|---|
| 1 | 6.106 |
| 2 | 4.167 |




| treata | Mean |
|---|---|
| 1 | 2.50 |
| 2 | 2.71 |
| 3 | 4.83 |
| 4 | 5.08 |
| 5 | 7.08 |
| 6 | 6.90 |
| 7 | 6.55 |
| 8 | 5.45 |



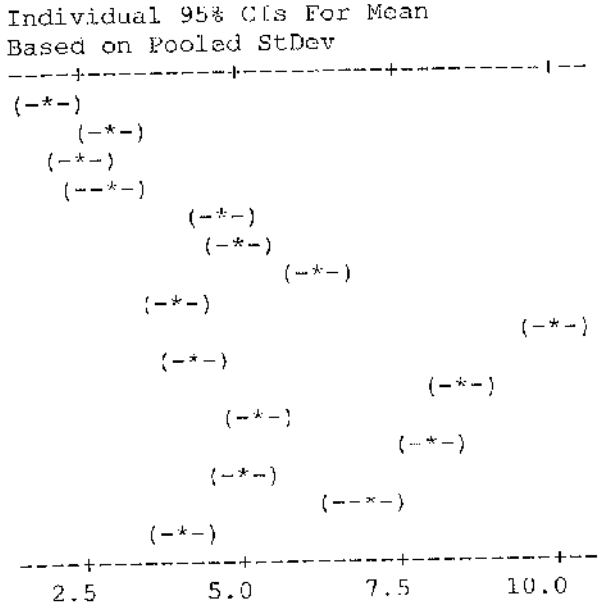

## One-way ANOVA: lcpDOP versus treatb

Analysis of Variance for lcpDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 462.697 | 30.846 | 71.61 | < 0.001 |
| Error | 80 | 34.459 | 0.431 | | |
| Total | 95 | 497.156 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 2.033 | 0.216 |
| 12 | 6 | 2.967 | 0.320 |
| 21 | 6 | 2.528 | 0.101 |
| 22 | 6 | 2.883 | 0.306 |
| 31 | 6 | 4.683 | 0.591 |
| 32 | 6 | 4.967 | 0.476 |
| 41 | 6 | 6.183 | 0.578 |
| 42 | 6 | 3.983 | 0.538 |
| 51 | 6 | 9.917 | 0.930 |
| 52 | 6 | 4.233 | 0.750 |
| 61 | 6 | 8.567 | 0.516 |
| 62 | 6 | 5.233 | 0.734 |
| 71 | 6 | 8.033 | 0.532 |
| 72 | 6 | 5.067 | 0.258 |
| 81 | 6 | 6.900 | 0.810 |
| 82 | 6 | 4.000 | 1.483 |

Pooled StDev = 0.656



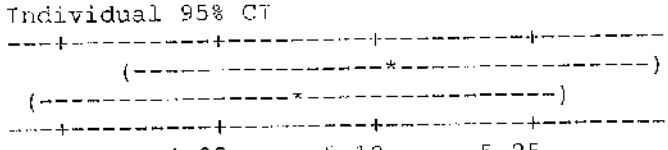
4) **Ventral caudate-putamen**. A two-way analysis of variance (ANOVA) showed no significant effect of group (AS v AS/AGU) but a very significant effect of treatment ($F = 26.29$, $df = 7$, $p < 0.001$) (see p 141). As with the anterior caudate-putamen (see above) DA levels were significantly elevated in L-DOPA-treated animals (as well as in animals treated with both L-DOPA and 5-HTP simultaneously) compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). 5-HTP alone also significantly raised DOPAC levels ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 141) many significant differences were found between the two strains. When the treatment was saline alone, DOPAC levels were higher in mutants than controls. However, when the treatment was a pharmacological intervention of any kind, the reverse was observed, with control AS rats having higher dopamine levels than AS/AGU mutants (significantly so with 5-HTP and L-DOPA treatments).

## Two-way ANOVA: vcpDOP versus group, treata

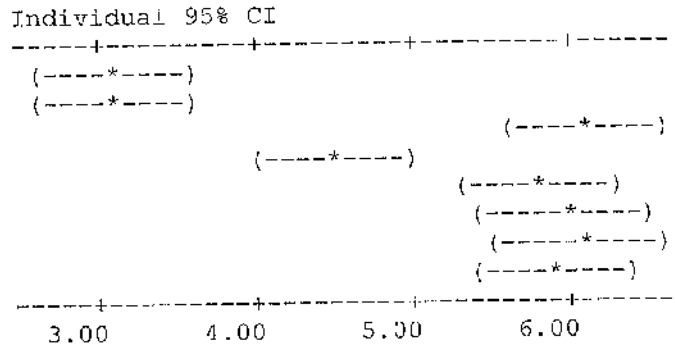
Analysis of Variance for vcpDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 0.215 | 0.215 | 0.27 | 0.602 |
| treata | 7 | 144.115 | 20.588 | 26.29 | 0.001 |
| Interaction | 7 | 42.584 | 6.083 | 7.77 | 0.001 |
| Error | 80 | 62.660 | 0.783 | | |
| Total | 95 | 249.574 | | | |

| group | Mean |
|---|---|
| 1 | 5.12 |
| 2 | 5.02 |




| treata | Mean |
|---|---|
| 1 | 3.08 |
| 2 | 3.13 |
| 3 | 6.08 |
| 4 | 4.53 |
| 5 | 5.83 |
| 6 | 5.96 |
| 7 | 6.05 |
| 8 | 5.89 |



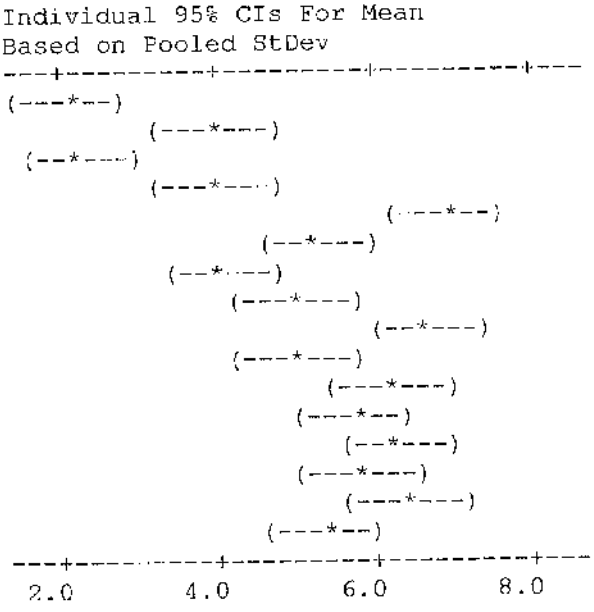

## One-way ANOVA: vcpDOP versus treatb

Analysis of Variance for vcpDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 186.914 | 12.461 | 15.91 | < 0.001 |
| Error | 80 | 62.660 | 0.783 | | |
| Total | 95 | 249.574 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | 2.1500 | 0.2665 |
| 12 | 6 | 4.0167 | 1.2875 |
| 21 | 6 | 2.2733 | 0.1796 |
| 22 | 6 | 3.9833 | 1.2497 |
| 31 | 6 | 6.9000 | 1.0640 |
| 32 | 6 | 5.2500 | 0.5683 |
| 41 | 6 | 4.0500 | 0.7314 |
| 42 | 6 | 5.0167 | 0.7705 |
| 51 | 6 | 6.6333 | 1.0857 |
| 52 | 6 | 5.0167 | 0.5565 |
| 61 | 6 | 6.2167 | 0.8424 |
| 62 | 6 | 5.7000 | 1.1349 |
| 71 | 6 | 6.2833 | 1.0304 |
| 72 | 6 | 5.8167 | 0.7859 |
| 81 | 6 | 6.4167 | 1.0944 |
| 82 | 6 | 5.3667 | 0.5125 |

Pooled StDev 0.8850



5) **Dorsal raphe nucleus**. A two-way analysis of variance (ANOVA) showed no significant effect of group (AS v AS/AGU) but a very significant effect of treatment ($F = 29.11$, $df = 7$, $p < 0.001$) (see p 143). DOPAC was not detectable within the raphe region of saline-treated control rats of either strain. However, levels became detectable (i.e. were significantly elevated) in L-DOPA-treated animals (as well as in animals treated with both L-DOPA and 5-HTP simultaneously) compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). 5-HTP alone also significantly raised DOPAC levels ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 143) no significant differences between the two strains could be found.

## Two-way ANOVA: DrnDOP versus group, treata

Analysis of Variance for DrnDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 0.023 | 0.023 | 0.03 | 0.864 |
| treata | 7 | 162.512 | 23.216 | 29.11 | 0.001 |
| Interaction | 7 | 9.937 | 1.420 | 1.78 | 0.103 |
| Error | 80 | 63.795 | 0.797 | | |
| Total | 95 | 236.267 | | | |

| group | Mean | Individual 95% CI |
|---|---|---|
| 1 | 2.05 | (---------*---------) |
| 2 | 2.08 | (---------*---------) |

1.80 1.95 2.10 2.25 2.40

| treata | Mean | Individual 95% CI |
|---|---|---|
| 1 | -0.00 | (---*---) |
| 2 | -0.00 | (---*---) |
| 3 | 3.82 | (---*---) |
| 4 | 2.57 | (---*---) |
| 5 | 1.83 | (---*---) |
| 6 | 2.42 | (---*---) |
| 7 | 2.88 | (---*---) |
| 8 | 2.98 | (---*---) |

0.00 1.20 2.40 3.60

## One-way ANOVA: DrnDOP versus treatb

Analysis of Variance for DrnDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 172.472 | 11.498 | 14.42 | < 0.001 |
| Error | 80 | 63.795 | 0.797 | | |
| Total | 95 | 236.267 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 6 | -0.0000 | 0.0000 |
| 12 | 6 | -0.0000 | 0.0000 |
| 21 | 6 | -0.0000 | 0.0000 |
| 22 | 6 | -0.0000 | 0.0000 |
| 31 | 6 | 3.4333 | 1.0783 |
| 32 | 6 | 4.2000 | 1.7855 |
| 41 | 6 | 2.6833 | 0.8998 |
| 42 | 6 | 2.4500 | 0.9482 |
| 51 | 6 | 1.4333 | 0.3830 |
| 52 | 6 | 2.2167 | 1.0666 |
| 61 | 6 | 2.5667 | 0.9352 |
| 62 | 6 | 2.2667 | 0.6743 |
| 71 | 6 | 2.6167 | 0.5879 |
| 72 | 6 | 3.1500 | 1.3795 |
| 81 | 6 | 3.6333 | 1.2972 |
| 82 | 6 | 2.3333 | 0.3933 |

Pooled StDev = 0.8930

Individual 95% CIs For Mean
Based on Pooled StDev

0.0 1.6 3.2 4.8

6) **Median raphe nucleus**. A two-way analysis of variance (ANOVA) showed a significant effect of group (AS v AS/AGU, $F = 13.11$, $df = 1$, $p < 0.001$) and a very significant effect of treatment ($F = 42.94$, $df = 7$, $p < 0.001$) (see p 145). DOPAC was not detectable in samples from the median raphe of saline-treated control rats. However, levels became detectable (i.e. were significantly elevated) in L-DOPA-treated animals (as well as in animals treated with both L-DOPA and 5-HTP simultaneously) compared with saline-treated ones at both 30 and 60 minutes after treatment ($p < 0.05$). 5-HTP alone also significantly raised DOPAC levels ($p < 0.05$). When the analysis was extended to compare AS and AS/AGU rats (p 145) some significant differences were found between the two strains with mutants having hifher levels of DOPAC than the parent strain.

## Two-way ANOVA: MrnDOP versus group, treata

Analysis of Variance for MrnDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| group | 1 | 7.370 | 7.370 | 13.11 | 0.001 |
| treata | 7 | 168.942 | 24.135 | 42.94 | 0.001 |
| Interaction | 7 | 33.143 | 4.735 | 8.42 | 0.001 |
| Error | 80 | 44.963 | 0.562 | | |
| Total | 95 | 254.418 | | | |

| group | Mean | Individual 95% CI |
|---|---|---|
| 1 | 1.88 | (--------*--------) |
| 2 | 2.43 | (--------*--------) |

1.75 2.00 2.25 2.50

| treata | Mean | Individual 95% CI |
|---|---|---|
| 1 | 0.00 | (---*---) |
| 2 | 0.00 | (---*---) |
| 3 | 3.00 | (---*---) |
| 4 | 3.86 | (---*---) |
| 5 | 2.47 | (---*---) |
| 6 | 2.39 | (---*---) |
| 7 | 2.38 | (---*---) |
| 8 | 3.14 | (---*---) |

0.00 1.20 2.40 3.60

## One-way ANOVA: MrnDOP versus treatb

Analysis of Variance for MrnDOP

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 15 | 209.455 | 13.964 | 24.84 | < 0.001 |
| Error | 80 | 44.963 | 0.562 | | |
| Total | 95 | 254.418 | | | |

| Level | N | Mean | StDev | Individual 95% CIs For Mean Based on Pooled StDev |
|---|---|---|---|---|
| 11 | 6 | 0.0000 | 0.0000 | (---*---) |
| 12 | 6 | 0.0000 | 0.0000 | (---*---) |
| 21 | 6 | 0.0000 | 0.0000 | (---*---) |
| 22 | 6 | 0.0000 | 0.0000 | (---*---) |
| 31 | 6 | 2.4000 | 0.6481 | (---*---) |
| 32 | 6 | 3.6000 | 1.1883 | (---*---) |
| 41 | 6 | 4.0500 | 1.0710 | (---*---) |
| 42 | 6 | 3.6667 | 0.7528 | (---*---) |
| 51 | 6 | 0.9833 | 0.5345 | (---*---) |
| 52 | 6 | 3.9500 | 0.8142 | (---*---) |
| 61 | 6 | 2.7000 | 0.7239 | (---*---) |
| 62 | 6 | 2.0833 | 0.6735 | (---*---) |
| 71 | 6 | 1.5667 | 0.6022 | (---*---) |
| 72 | 6 | 3.1833 | 1.4219 | (---*---) |
| 81 | 6 | 3.3167 | 0.8329 | (---*---) |
| 82 | 6 | 2.9667 | 0.6653 | (---*---) |

Pooled StDev = 0.7497

0.0 1.5 3.0 4.5

## 5.4 DISCUSSION

Over 100 animals were used in this part of the study, yielding some 400 individual pieces of data. In the analysis of such a large body of material (pp 93-144 ), it is recognised that complex and conflicting results may emerge and that some random differences will appear statistically significant by chance. For that reason, I wish to concentrate in the Discussion on the strongest themes which emerge from my results. These are :-

1. That mutants have reduced 5-HT in the dorsal raphe nucleus where the cell bodies of serotonergic neurons are concentrated. They also have reduced 5-HT in some parts of the striatum (where the terminals of these cells are located) but not in all. Interestingly, serotonin is not reduced in the median raphe which does not project to the striatum.
2. That, in general, mutants have elevated 5-HIAA in those parts of the brain where 5-HT is reduced. This is especially true of the dorsal raphe nucleus and the dorsal caudate-putamen.
3. That 5-HT and 5-HIAA levels are elevated by the administration of the precursor 5-HTP.
4. That 5-HT levels are markedly reduced by the administration of the dopamine precursor L-DOPA, a reduction which the simultaneous administration of 5-HTP is generally unable to compensate for.
5. That mutants have reduced DA levels compared with the parent AS strain in the dorsal and lateral caudate-putamen, but not in the anterior or ventral parts. This replicates almost precisely date reported some years ago (Campbell et al, 1997).
6. That DA levels are elevated by the administration of L-DOPA (alone or in combination with 5-HTP) in all areas of the caudate-putamen in both strains. Furthermore, L-DOPA administration led to detectable DA within the dorsal and median raphe nuclei; in saline-treated controls, DA was non-detectable. In general, increases in DA were more pronounced in control AS than in mutant AS/AGU rats.

7. That DA levels were also elevated by the administration of the 5-HT precursor 5-HTP, both within the striatum and within the raphe nuclei.
8. That DOPAC levels were higher in mutants than controls in several striatal regions, including the dorsal and ventral caudate-putamen.
9. That DOPAC levels are elevated by the administration of L-DOPA (alone or in combination with 5-HTP) in all areas of the caudate-putamen in both strains. Furthermore, L-DOPA administration led to detectable DOPAC within the dorsal and median raphe nuclei; in saline-treated controls, DOPAC was non-detectable. In general, increases in DOPAC were more pronounced in control AS than in mutant AS/AGU rats.

Different parts of the caudate-putamen 1) show intrinsic differences in levels of catechol- or indoleamines and their metabolites 2) show differences in response to simple pharmacological interventions. In particular, the dorsal and lateral caudate-putamen are more likely to show differences than the ventral and anterior regions.

Similarly, the dorsal raphe nucleus is much more likely to show differences in basal levels and resposes to intervention than the median raphe nucleus. This is not unexpected given that it is the dorsal raphe (rather than the median) which shows cell losses - see Chapter 3. Elevation of 5-HIAA in those brain regions where 5-HT is reduced supports the notion that there are changes in 5-HT release mechanisms rather than cell death. If so, this may be because vesicles are not forming propely (or are leaking) as has already been proposed for DA (Payne et al., 2000).

5-HTP administration increased both 5-HT and its major metabolite 5-HIAA. This indicates that the synthetic pathway is functioning propely. Administration of 5-HTP leads to increase synthesis of 5-HT (Moir and Eccleston, 1968) and an increase in levels of 5-HT (Moir and Eccleston, 1968; Okada et al., 1972) and its metabolite 5-HIAA in the brain (Moir and Eccleston, 1968; Okada et al., 1972; Cespuglio et al., 1981).

L-dopa administration decreased 5-HT levels. Although this may appear paradoxical, it agrees with previous findings (Bartholini et al., 1968; Everett and Borcherding, 1970). Also it has been reported that administration of L-dopa increases the release of 5-HT (Ng et al., 1970) and its metabolites from the striatum (Bjorklund and Dunnett, 1992), suggesting that a portion of administered L-dopa may enter serotonergic nerve terminals and undergo decarboxylation to DA with resultant displacement of endogenous 5-HT (Arai et al., 1994). At least one *in vitro* study has shown that L-dopa interferes with tryptophan hydroxylase activity and reduces 5-HT synthesis, while 5-HTP inhibits tyrosine hydroxylase activity and lowers DA synthesis (Manuyama et al., 1992).

It has been reported that tissue content of 5-HT and 5-HT innervation were significantly decreased in the striatum of 6-OHDA-lesioned rat (Dunnett et al., 1988; Takeuchi et al., 1991), and some animals with mesencephalic DA-rich grafts showed increases in 5-HT level and serotonergic hyperinnervation in the striatum (Dunnett et al., 1988; Takeuchi et al., 1991).

Reduction of DA levels in mutant animals compared with the parent AS strain confirms previous reports (Campbell et al., 1997). The reduction was noticed in two areas in mutant rats namely DCPU and LCPU while other areas were unaffected. This is similar to the regional pattern of striatal DA loss observed in patients with Parkinson's disease (Hornykiewicz, 1995).

Elevation of DA in the striatum after L-dopa administration agrees with preivous reports (Langelier et al., 1973; Watanabe, 1983). Moreover L-dopa was able to increase DA to detectable levels in DRN and MRN. It is possible that L-dopa may be using 5-HT cells to produce or facilitate DA production in the raphe nuclei. For example, when L-dopa and a peripheral AADC inhibitor were administrated, DA-ir cells were found in serotonergic nuclei (Barrett and Balch, 1971; Butcher et al., 1970; Lidbrink et al., 1974; Tison et al., 1991). More recently, Aria and colleagues (1994) examined rats which had received L-

dopa plus AADC; by using double-labeling they showed that serotonin-stained neurons of the dorsal raphe nucleus of rat were also immunoreactive to dopamine.

A final interesting result of the present study is that the mutant rats have more DOPAC than normal animals, and the levels of DOPAC can be increased with L-dopa in both strains. Again, this suggest that the dopaminergic system is capable of synthesis and metabolise and the increase was similar in both strains in all areas investigated. DOPAC levels were also elevated by administrating L-dopa and that has been reported before (Wesemann et al., 1993). Furthermore, DOPAC levels became detectable in DRN and MRN after L-dopa injection, as did DA levels when 5-HTP was administreted.

The present experiment clearly demonstrates that midbrain 5-HT and DA systems projecting to the basal ganglia are physically intact in mutants at this age (though they may show cell loss later-chapter 4). Moreover, they can respond to precursor administration with synthesis and elevate release. There does appear to be dysfunction of normal physiological release mechanisms (as repated previously for the nigrostriatal system and – now- for the raphestriatal system).

This does confirm my predictions

a) That a mutation in a basic enzyme such as PKC should affect multiple transmitter system.

b) That the AS/AGU rat continues to be a model, which can often insight into human conditions where several transmitters systems are affected.

The lack of deficit in 5-HT levels in the MRN leaves open the question of whether striatal afferent systems are especially vulnerable and, if so, why?

# 6 Experiment 5. Extracellular levels of 5-HT, 5-HIAA, DA and DOPAC in the dorsal striatum of AS and AS/AGU rats.

## 6.1 Introduction

Campbell and colleages recently documented reduced dopamine levels in the dorsal and lateral caudate-putamen of the AS/AGU rat compared to Albino Swiss (AS) controls between six and 12 months of age using high performance liquid chromatography with electrochemical detection of micropunch samples (Campbell et al., 1996; 1998). Similarly, Roffler-Tarlov and Graybiel, (1984) revealed that dopamine levels were severely depleted in the dorsal striatum of the weaver mouse. One difficulty with interpreting concentrations of substances in micropunch samples is that the technique cannot distinguish between intra- and extracellular levels of transmitters, so it was necessary to assess the extracellular levels of dopamine using microdialysis. In the AS/AGU rats, this technique demonstrated a massive (80-90%) reduction in extracellular DA (Campbell et al., 1998; 2000). The present experiment was undertaken to confirm these findings and to extend them to serotonin release.

The analysis of chemical events which occur between cells has been difficult. The chemical analysis of tissue samples represents a static reflection of synaptic events, mixing cells, organelles and extracellular fluid. To obtain a more dynamic picture of the chemical interplay between cells in living tissue, microdialysis has been introduced. Microdialysis involves perfusing a thin dialysis tube inserted in the tissue. The concentration of compounds such as neurotransmitters and metabolites in the emerging perfusate reflects their concentration in the extracellular fluid as a result of their diffusion across the dialysis membrane.

Microdialysis has advantages over older techniques such as the push-pull cannula and cortical cup techniques. The dialysis membrane over the tip of the probe is the main feature of difference between microdialysis and push-pull cannula. This difference means that in microdialysis there is no need to balance the push of the flow of liquid with the pull of the flow. In push-pull cannulae, liquid has to be infused through the tissue and be pulled out through the cannula. As a result, there may be damage to tissue which is minimised in

microdialysis. The dialysis membrane also acts as a barrier which improves the sterility of the fluid diffusing into the brain and excludes proteins and other macromolecules from coming out of the brain which would have to be removed before HPLC analysis. These are major advantages over earlier techniques and microdialysis can be performed on most organs of the body. An additional feature of microdialysis is the ability to stimulate the tissue locally by including substances such as pharmacological agents in the perfusion mixture. Neurotransmission and release processes may be examined at the same time as the stimulation of receptors by the local administration of a drug via the microdialysis probe (Westerink et al., 1987).

Ultrastructural studies of 5-HT have revealed both synaptic and nonsynaptic terminals. The paradigm of nonsynaptic 5-HT neurotransmission is the supraependymal axon located inside the cerebral ventricles (Chan-Palay, 1977). Similary, synaptic 5-HT terminals are rarely found in the median eminence and cerebral cortex (Calas et al., 1974; Descarries et al., 1975). On the other hand, electron microscopic studies reveal that >90% of 5-HT terminals in the substantia nigra pars reticulata (SNr) exhibit synaptic complexes (Moukhles et al., 1997). The ultrastructure of other serotonergic brain regions exhibits both synaptic and nonsynaptic 5-HT terminals (Beaudet and Descarries, 1981; Descarries et al., 1990; Maley et al., 1990).

A particularly complex situation occurs in the dorsal raphe nucleus (DRN), the primary site of 5-HT cell bodies in the CNS. In this region, 5-HT cell bodies and dendrites accumulate 5-HT and package it in vesicles, apparently in a releasable form (Hery and Ternaux, 1981; Iravani and Kruk, 1997; Bunin et al., 1998). Early studies suggested that 5-HT accumulation was restricted to cell bodies and dendrites (Fuxe, 1965; Loizou, 1972; Descarries et al., 1979, 1982; Baraban and Aghajanian, 1981), but 5-HT axon collaterals and terminals appear to exist as well (Mosko et al., 1977; Liposits et al., 1985; Chazal and Ralston, 1987). Ultrastructural studies suggest that release sites in the DRN are both synaptic and nonsynaptic.

5-HT is similar to dopamine in the nucleus accumbens in which ultrastructural studies have identified abundant synaptic specializations (Garris et al., 1994). These systems stand in dramatic contrast to the glutamate and GABA systems, in which the most evidence suggests classical synaptic transmission (Isaacson et al., 1993a; Clements et al., 1996). Much of our knowledge on the mechanisms by which central serotonergic neurons release 5-HT is derived from brain tissue preparations in vitro. From such studies there is considerable evidence that 5-HT, like other neurotransmitters in the brain, can be released from the nerve terminal via an excitation-secretion coupled event (for review see Sanders-Bush and Martin, 1982). For instance, electrical stimuli or depolarizing agents such as potassium ions, evoke the release of preloaded [$^3$H]5-HT or endogenous 5-HT from perfused or incubated rat brain slices and synaptosomes (Chase et al., 1969; Elks et al., 1979; Farnebo, 1971; Gothert and Weinheimer, 1979; Lane and Aprison, 1977; Mulder et al., 1975) .

The brain perfusion method, microdialysis, coupled to high performance liquid chromatography (HPLC) with electrochemical detection, offers a direct way to study release of 5-HT in the brain of the rat *in vivo* (Sharp et al., 1989a; Di Chiara, 1990; Sharp and Hijorth, 1990). Of great value is the fact that, using this approach, effects of drugs on release of 5-HT in the brain can be studied under conditions when exocytosis is limited by the omission of calcium from the perfusion medium (Carboni and Di Chiara, 1989; Sharp et al., 1990) and when serotonergic neuronal activity is selectively inhibited by administration of the 5-HT $_{1A}$ receptor agonist, 8- hydroxyl-2-(di-n-propylamine) tetralin (8-OHDPAT) (Sprouse and Aghajanian, 1986; Sharp et al., 1989b; Sharp and Hjorth, 1990; Hjorth and Sharp, 1991). Here this methodology has been used to measure the basal levels of 5-HT and its major metabolite 5-HIAA in dialysates of the dorsal striatum of the AS and AS/AGU rats.

## 6.2 Material and Methods

All animal procedures were in accordance with the Home Office Guidelines and were specifically licensed under the Animal (Scientific Procedures) Act 1986, The Home Office project licence was 60/2167.

Nine AS and nine AS/AGU animals aged three months were used to determine extracellular levels of serotonin and it's metabolite 5-HIAA in the striatum in freely moving rats, dopamine and DOPAC were also measured.

Microdialysis followed by HPLC was used according to the method adopted by Campbell et al. (1998). This technique allows the monitoring of normal basal neurotransmitter levels in conscious animals, and also has the benefit of being able to show the effects of drugs administered either before or during the microdialysis process. Samples can be collected at a chosen flow rate thus determining sample volume, and neurotransmitters can be detected at very low concentrations (picomoles) in protein-free samples.

There are three main stages to the extracellular sampling:

1- The surgical procedure.

2- Microdialysis procedure.

3- Transmitter and metabolite analysis.

### 6.2.1 The surgical procedure

AS and AS/AGU male rats were anaesthetised using Vetalar (Ketamine hydrochloride; 100mg/ml) and Rompun (Xylazine hydrochloride 2%) in a ratio of 2:1. I.P injection was given at 1.1ml/kg of body weight.

When the animal was deeply anaesthetised (usually after 5-10 minutes verfied by toe pinching) it was placed into ear bars on a Kopf stereotaxic frame (fig 6.1), and the head held in place using tooth and nose bars. This stabilised the head to allow a midline incision

to be made in a pre-shaved area of the scalp using a scalpel blade, the skin being held back from the skull using retractors.

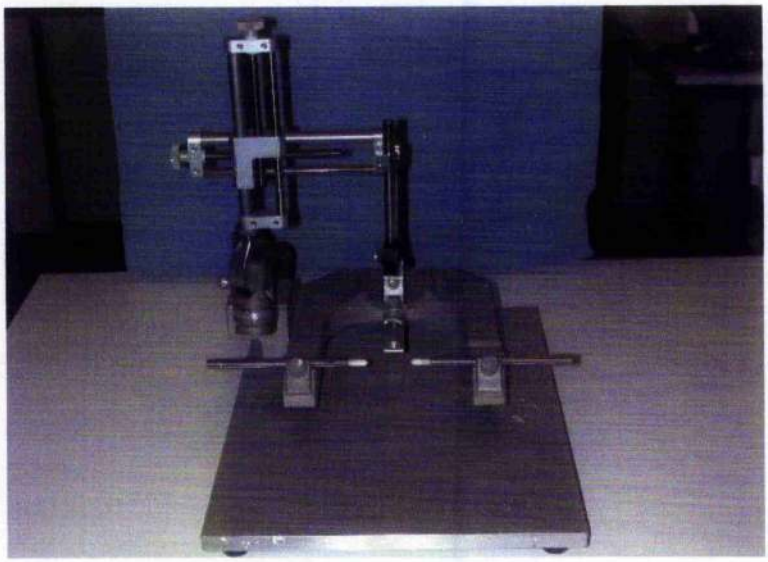


**Figure 6.1 A Kopf stereotaxic frame.**

The intersection point of coronal and sagittal cranial sutures (bregma) was identified by scraping of the skull using a scalpel blade, then marked with pencil.

An atlas of the rat brain (Paxinos and Watson, 1982) was used to locate the dorsal caudate-putamen. These coordinates were; 1mm (anterior/posterior), 3.5mm (lateral) and 4.5mm (ventral) (fig.6.2). All these coordinates were based on the bregma as starting point.

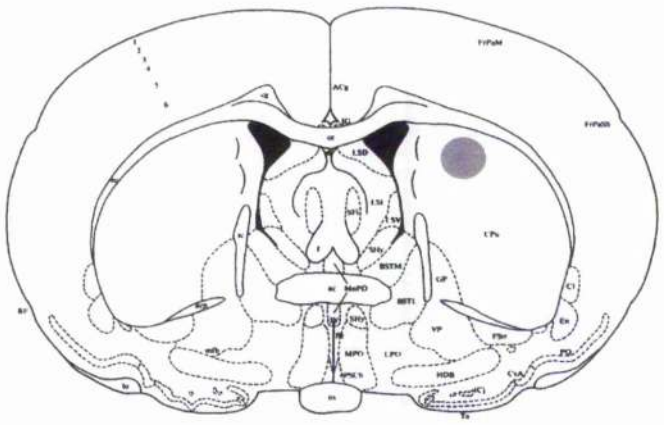


**Figure 6-2 Diagram of a coronal brain section at the level of the striatum where microdialysis samples were taken. The gray circle indicate the DCPU. From ' The rat brain in stereotaxic coordinates' (Paxinos and Watson, 1982).**

Anterior/posterior and lateral coordinates were used to locate the point on the skull where the hole should be drilled by a 1mm drill and marked again by a pencil. The hole was drilled very carefully avoiding damaging any tissue beneath the skull. Then the cannula was lowered very slowly into the hole until the appropriate ventral coordinate was reached. The cannula was secured in place until the end of the experiment by *Rediofast* dental cement. A further two holes were drilled into the skull in an area close to the probe, two screws were placed into these holes before the cement was poured over the whole area which was contained within a plastic "hat" made by cutting a 5ml plastic syringe to obtain a 1cm deep cylinder. The function of the screws was to secure the cannula in place. At the end of the operation the cannula was kept patent by inserting a guide cannula into the cannula.

Then the rat was removed from the stereotaxic frame and received subcutaneously 0.1ml of Antisedan (atipamezole hydrochloride, 5mg/ml) to reverse the effect of the anaesthetic. It was important to observe the animal in an incubator until recovery was complete, when it was moved back to its cage.

The whole procedure must not exceed 40 minutes to make sure that the animal did not recover before the operation finished.

### 6.2.2 Microdialysis procedure

Microdialysis was started two days after surgery, to allow the rat to fully recover. The rat is linked to the microdialysis system. This consisted of a CMA automatic microinjector, a series of lengths of narrow bore tubing, a microdialysis probe and a perspex box (42x23x41cm). In the box, the rat was free to walk normally on box bedding material with easy access to water and food. Also in the top of the Perspex box a metal lever (reflex arm) was attached which allowed the rat to move freely while ensuring the safety of the probe and tubes.

**Figure 6-3 An image of CMA automatic microinjector and Perspex box with reflex arm attached.**

The probe was inserted carefully into the cannula while the animal's head was held securely and immobilised. The mirosyringe was attached to the probe's inflow tubing via a connection at the collection port that was situated at the end of the reflex arm. The outflow probe tubing was connected to a plastic collecting vial at this port.

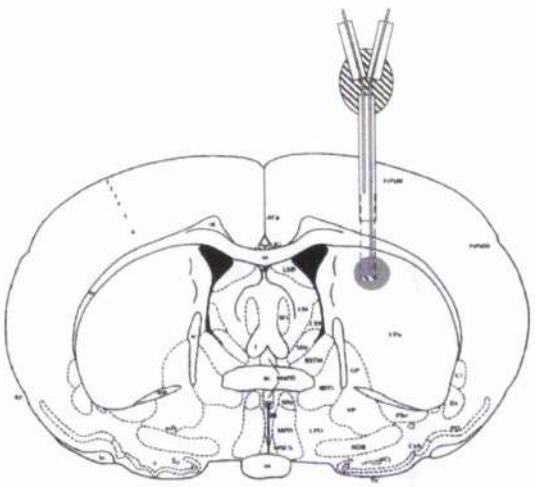


**Figure 6-4 Diagram of the same brain section as fig 6.2 with probe attached.**

The microdialysis probe is a single cannula type and is a variation on that used by Carswell and colleagues (1997). It consists of a series of tubing types of different diameters ranging

from a 1mm diameter plastic tube down to a silica glass tubing which is situated innermost in a concentric arrangement, and has an outer diameter of 25 μm.

The tip of the probe is the active area which consists of a 200 μm ID semi-permeable tubular dialysis membrane with a 40, 000 molecular weight cut-off. The pore size is 60Å. Microdialysis occurs over the exposed surface of this membrane, and its length can be adjusted to suit the size of the brain area under investigation. Artificial cerebrospinal fluid (ACSF) flows into the region where the microdialysis takes place and the dialysed liquid then flows back out of the probe via the silica glass tubing that is situated inside the microdialysis tubing, the tip of which is plugged with *RS* epoxy resin.

The dialysis membrane allows the two-way passage of fluid with exclusion of any high molecular weight protein matter that would contaminate the sample. Neurotransmitters are thus passed through a concentration gradient into the transmitter-free ACSF. This allows us to monitor basal extracellular concentrations under standardised conditions as well as following pharmacological interventions. ACSF is passed through the probe at a flow rate of 2μl per minute via a length of narrow bore perspex tubing (0.28mm internal diameter) and is collected after microdialysis in the plastic collecting vial. Samples are collected every 20 minutes giving a sample volume of 40μl. No pre-HPLC treatment is required for these pure samples, hence they can be either injected onto the HPLC system immediately or frozen in liquid nitrogen for injection at a later time.

The animals were allowed to move around freely in the perspex box for a period of 2 hours after implantation of the probe before samples were collected (Hjorth and Sharp, 1991; Gartside etal., 1992). After that basal samples were collected for period of 4 hours.

### 6.2.3 Transmitter and metabolite analysis

5-HT, 5-HIAA, DA and DOPAC analysis was achieved by using HPLC, which consisted of a Gilson 305 pump with 805 manometric module, a 7125I Rheodyne sample injector, a microbore column and an ANTEC *intro* electrochemical detector. The column (stationary phase) was a Hichrom microbore reverse phase column with a C18 ODS2 5μm packing gel. This allowed effective and consistent separation of indole- and catecholamines when used with a buffered mobile phase consisting of: Citric acid (83 mM), EDTA (1mM), Disodium Hydrogen Phosphate (43mM), Octane Sulphonic Acid (0.2mM) and Methanol (10%). PH was 3.5.

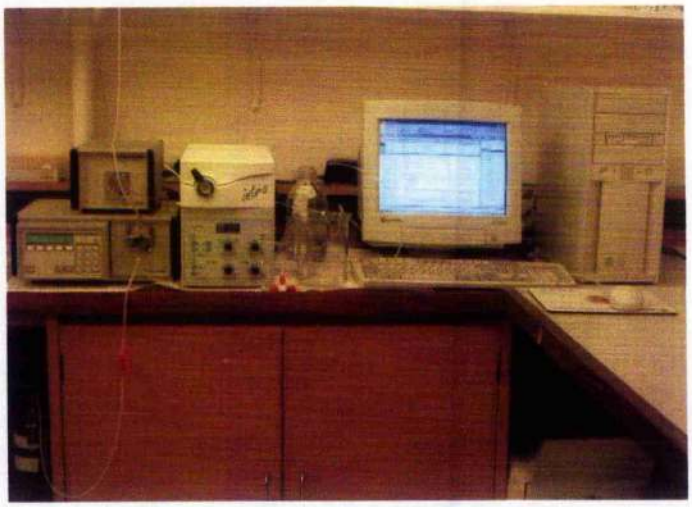


**Figure 6-5 An image of HPLC-ECD showing (from left to right) a Gilson pump, sample injector, *intro* electrochemical detector and data collection (computer).**

A 100μl sample loop was used on the injector to ensure that the whole sample (45μl) was injected onto the column. This maximised the amount of detectable neurotransmitter. Before injecting the samples, a composite standard which contained all of the detectable amines and metabolites (5-HT, 5-HIAA, DA and DOPAC) was injected at a concentration of 10 nanograms per ml for each. An internal standard (DHBA) was added to the composite mixture at the same concentration.

There are two main functions of using the composite mixture, 1- it was used to determine the time that it takes each substance to be eluted from the column, and detected by the

ECD (retention time). 2- the peak areas from the resulting composite chromatogram are used to calculate the response factor (RF) for each substance.

A typical 45µl microdialysis sample contains 40 µl of dialysate and 5 µl of DHBA at the same concentration as in the composite standard. With some experiments, a sample volume of slightly less than 45 µl was injected due to a smaller return per 20 minutes via the microdialysis probe. The 5 µl of DHBA (internal standard) was added in order to obtain a peak from each sample that represented a known concentration. This internal standard was chosen because it is stable, is not found in the brain, and it is not known to react with any of the indole- or catecholamines present in the sample. The DHBA peak area was used to calculate the unknown concentration of amines within the sample.

From the chromatograms, the order of the elution of the internal standard and neurochemicals under investigation can be seen. The peaks of 5-HT, 5-HIAA, DA and DOPAC are identifiable by their retention times, a line drawn underneath the peaks, and the areas calculated by computer software.

Results were analysed and quantified using the following equations:

.

$$\text{1- Response factor (RF)} = \frac{\text{Area of internal standard in composite standard}}{\text{Area of Amine in composite standard}}$$

2- RF used to calculate the final figures for each 5-HT and 5-HIAA as follow:

$$\text{Peak Concentration} = \frac{\text{Area of Amine}}{\text{Area of internal standard in sample}} \times \text{RF}$$

The final concentrations are expressed as picograms per 45µl.

## 6.3 Results

The dorsal caudate-putamen is an important projection area for serotonergic cells of the dorsal raphe nucleus (Azmitia and Segal, 1978; Bobillier et al., 1976; Dray et al., 1978; Parent et al., 1981; Steinbusch, 1981; Steinbusch and Nieuwenhuys, 1983) as well as for dopaminergic cells of the substantia nigra pars compacta. AS/AGU mutants have significantly reduced extracellular levels of serotonin and dopamine which are less than 30% and 20% respectively of levels found in the parent AS strain. By contrast, levels of their metabolites in the extracellular fluid of the dorsal caudate-putamen are substantially elevated in the AS/AGU mutant; thus, 5-HIAA levels are some three times higher in the mutant than in the parent strain, while DOPAC levels are approximately quadrupled.

| | AS | AS/AGU | t |
|---|---|---|---|
| 5-HT | 291 ± 34 | 82.9 ± 21 | 5.20 $p < 0.001$ |
| 5-HIAA | 81 ± 6.1 | 280 ± 36 | 5.42 $p < 0.001$ |
| DA | 126 ± 11 | 20.2 ± 1.9 | 9.82 $p < 0.001$ |
| DOPAC | 15.67 ± 1.1 | 57.9 ± 3.7 | 11.06 $p < 0.001$ |

**Table 6.1. Extracellular levels of serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), together with dopamine (DA) and its metabolite dihydroxyphenylacetic acid (DOPAC) in the dorsal caudate-putamen of Albino Swiss (control) and AS/AGU (mutant) rats. All figures are mean pg/20 mins ± SEM (n = 9 per group). Significance levels are based on a two-tailed distribution.**

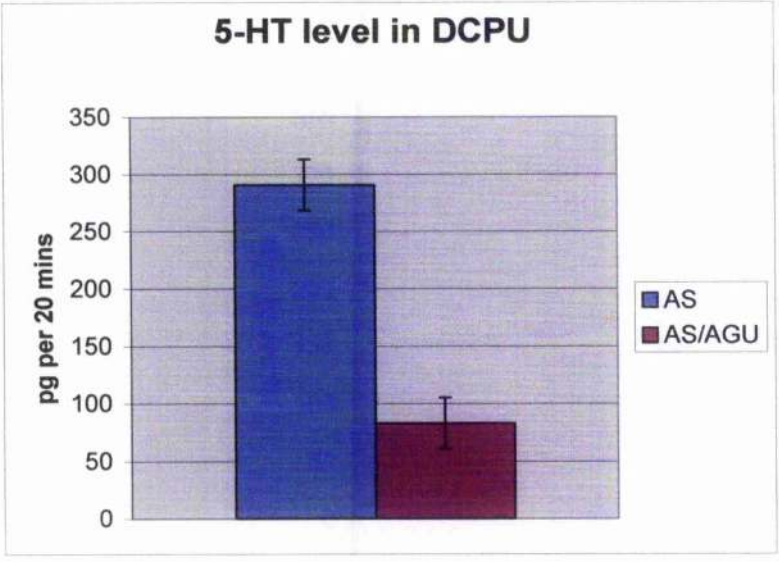



**Figure 6.6 Extracellular levels of 5-HT in the dorsal caudate-putamen measured with HPLC-ECD.**

## Two Sample T-Test and Confidence Interval

```
Two sample T for C13(AS) vs C14(AS/AGU)

            N      Mean     StDev   SE Mean
AS          9       291       102        34
AS/AGU      9      82.9      63.3        21

95% CI for mu C13 - mu C14: ( 121,  294)
T-Test mu C13 = mu C14 (vs not =): T = 5.20  P < 0.002  DF = 13
```

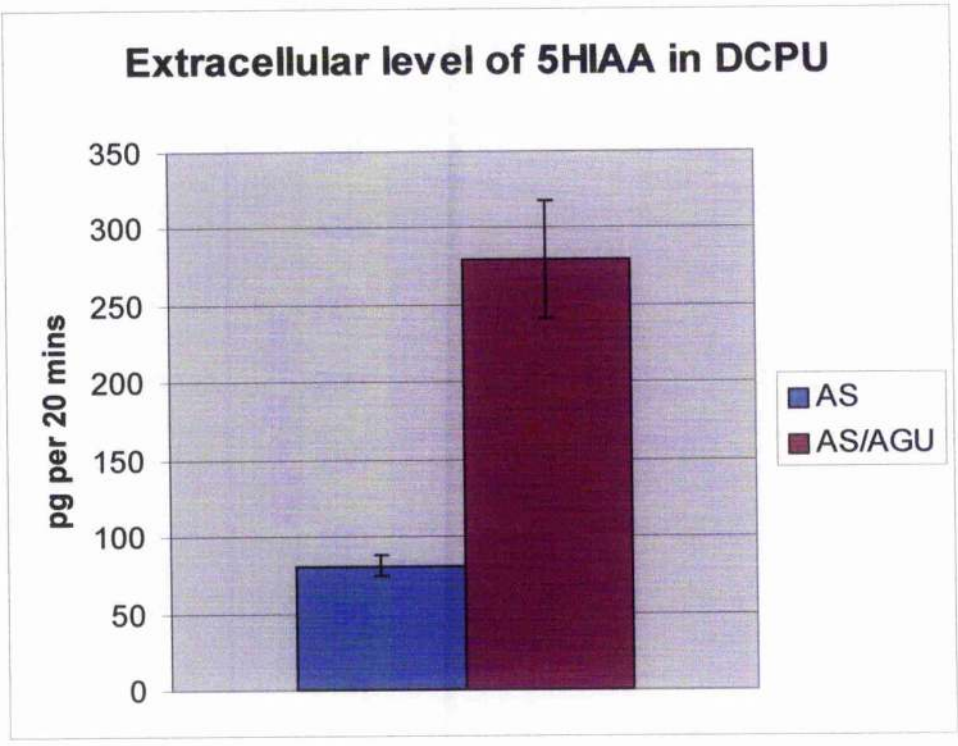



**Figure 6.7 Extracellular levels of 5-HIAA in the dorsal caudate-putamen measured with HPLC-ECD.**

## Two Sample T-Test and Confidence Interval

```
Two sample T for C16(AS) vs C17(AS/AGU)

          N      Mean     StDev   SE Mean
AS        9      81.0      18.4       6.1
AS/AGU    9       280       109        36

95% CI for mu C16 - mu C17: ( -283.5,  -114)
T-Test mu C16 = mu C17 (vs not =): T = -5.42   P < 0.006  DF = 8
```

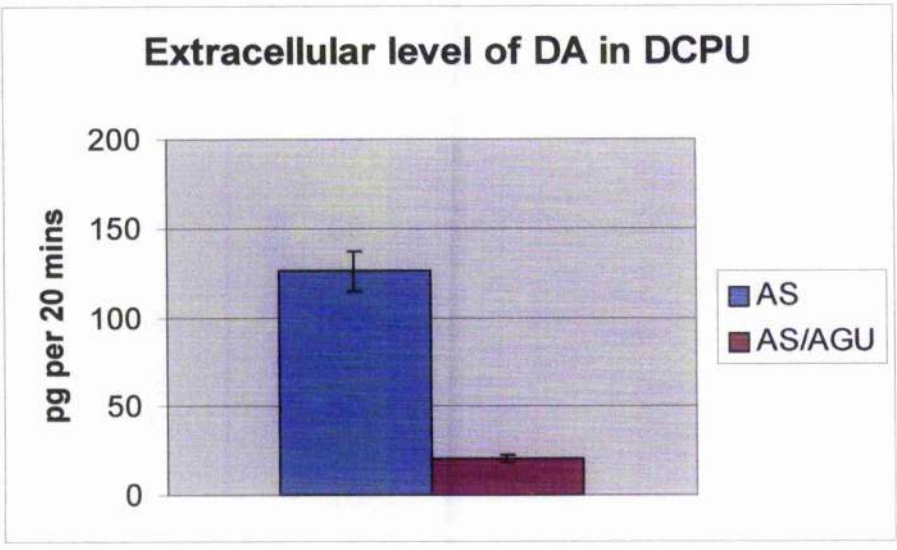



**Figure 6.8 Extracellular levels of DA in the dorsal caudate-putamen measured with HPLC-ECD.**

## Two Sample T-Test and Confidence Interval

```
Two sample T for C6(AS) vs C7(AS/AGU)

            N      Mean      StDev   SE Mean
AS          9     126.0       31.8        11
AS/AGU      9     20.17       5.67       1.9

95% CI for mu AS - mu AS/AGU: (81,  130.7)
T-Test mu AS = mu AS/AGU (vs not =): T = 9.82  P < 0.001  DF = 8
```

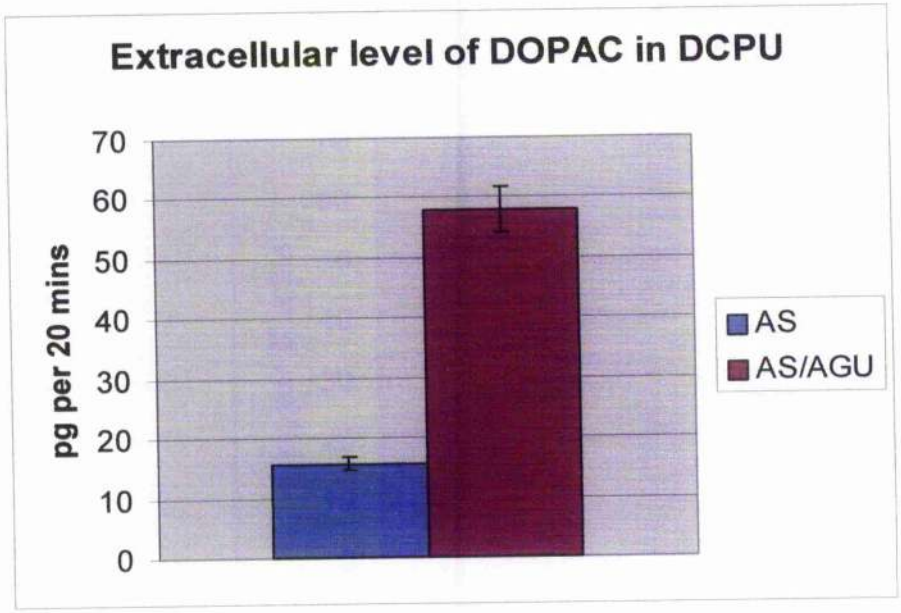



**Figure 6.9 Extracellular levels of DOPAC in the dorsal caudate-putamen measured with HPLC-ECD.**

## Two Sample T-Test and Confidence Interval

```
Two sample T for C10 (AS) vs C11 (AS/AGU)

            N      Mean     StDev   SE Mean
AS          9     15.67      3.32       1.1
AS/AGU      9      57.9      11.0       3.7

95% CI for mu C10 - mu C11: ( -50.9,  -33.6)
T-Test mu C10 = mu C11 (vs not =): T = -11.06  P < 0.001  DF = 9
```

## 6.4 Discussion

The dorsal raphe nucleus (DRN) is the origin of most ascending serotonergic neurons and is almost exclusively the source of the raphe-striatal pathway (Azmitia and Segal, 1978; Bobillier et al., 1976; Dray et al., 1978; Parent et al., 1981; Steinbusch, 1981; Steinbusch and Nieuwenhuys, 1983). Serotonin (5-HT) released in the caudate nucleus can be controlled by presynaptic mechanisms (Hamon et al., 1974) involving axoaxonic contacts. However, striatal amine release is also controlled by nerve activity generated at the cell body level, i.e. in the DRN (Hery et al., 1979). Thus, stimulation of DRN 5-HT cell bodies which modifies the activity of these neurons enhances 5-HT release at the nerve endings (Ashkenazi et al., 1972; Fujjiwara et al., 1981; Hery et al., 1979). Several neurotransmitters such as GABA (Aghajanian and Wang, 1978) or L-glutamic acid (Aghajanian, 1972; Baramwell and Gonye, 1976) control the activity of DRN 5-HT neurons. Among them, 5-HT alone plays an important role. Perfusion of 5-HT or 5-HT agonists into the DRN causes a rapid inhibition of the activity of serotonergic neurons (Aghajanian et al., 1968) and prevents 5-HT release at the nerve ending ( Sharp et al., 1989).

The fact that injection of 5-HT into the DRN is able to modify the electrical activity of the 5-HT ascending pathway is particularly interesting as has been demonstrated that 5-HT can be synthesised and released in the DRN (Hery et al., 1982).

The basal levels of 5-HT and its metabolite 5-HIAA in control AS rats were comparable with those reported by other groups (Price and Lucki, 2001; Balcioglu et al., 2003) and in the case of DA and its metabolite DOPAC in AS animals were also similar to those reported previously in the same lab some years ago (Campbell et al., 2000; Payne et al., 2000). AS/AGU mutants rats showed a reduction in both 5-HT and DA together with an increase in their metabolites 5-HIAA and DOPAC respectively.

One possible cause of the extracellular reduction in both amines in the mutant rats is the reduction in the cell numbers. However, in the case of 5-HT there were only a 23% reduction in serotonergic cells in DRN in the mutants while the drop in extracellular 5-HT

levels was about 70%. Moreover, the dopaminergic cells loss was about 40% in the mutant at one year old, while the drop in extracellular DA levels reached 90% in the mutant rats compare to the parent AS rats.

Furthermore, although transmitter levels declined, metabolite levels (5-HIAA and DOPAC) substantially increased. This again is not an obvious result of cell loss. It could, however, be the result of a failure of mechanisms associated with synaptic vesicle formation, in which case a) reduced amounts of transmitter would be available for release with b) transmitters located free within the cytoplasm of the synaptic terminal and available for metabolism via mitochondrial enzymes. Such a possibility has already been proposed for DA in the AS/AGU rat (Campbell et al., 2000; Payne et al., 2000). A similar disruption to normal vesicular sequestering could underlie the results obtained here for 5-HT and 5-HIAA.

What is clear from this present study is that 5-HT/5-HIAA levels in the striatum mirror those already found for DA/DOPAC and therefore, this suggests a shared mechanism of dysfunction.

# 7 Experiment 6. Spontaneous locomotion of the AS and AS/AGU rats: Strain differences and response to serotonin manipulation

## 7.1 Introduction

AS/AGU rats have serious movement impairments which particularly affect the hind limbs causing rigidity, a staggering gait and falling over every few steps (Clarke and Payne, 1994; Payne et al., 1998). A variety of simple tests have been useful tools to show the disruption of motor performance in adult AS/AGU rats compared to the parent AS strain (Payne et al, 1998). One such test is Mid-Air Righting where animals are held upside down 50 cm above a soft substrate and dropped. Rats are usually able to turn around within a vertical drop of 20 cm to land on all fours and thus the success rate of control AS animals is virtually 100% until one year old when success declines. In contrast, AS/AGU mutant rats have an initial success rate of only 60% and this shows a steep decline over the period of a year after birth. Another test is the inclined ramp test where rats attempt to walk down a series of inclined wooden ramps 85 cm long and of varying widths, into a large container filled with wood chips. This is best illustrated with a ramp of 70mm width angled downwards at 14° where AS rats show no decrease in performance over a year while AS/AGU rats were increasingly unable to negotiate the plank without falling off (Payne et al., 2000).

The role of 5-HT has been investigated in many behavioural activities. Thus, studies using raphe lesions showed that 5-HT is involved in sleep (Jouvet, 1972), general activity levels (Kostowski et al, 1968; Lorens et al, 1971; Neill et al, 1972; Vergnes et al, 1973; Vergnes et al, 1974), habituation (Davis and Sheard, 1974), aggression (Vergnes et al, 1973; Vergnes et al, 1974), pain sensitivity and morphine analgesia (Harvey et al, 1974; Hole and Lorens, 1975; Lorens and Yunger, 1974), avoidance behaviour (Hole and Lorens, 1975; Lorens, 1973; Lorens et al, 1971; Lorens and Yunger, 1974), self- stimulation (Lorens, 1971) and water consumption (Lorens et al, 1971; Lorens and Yunger, 1974).

Many methods have been used to reduce brain 5-HT levels, among them electrolytic lesioning of the 5-HT-containing cells of the midbrain raphe nuclei (Dahlstrom and Fuxe, 1964), systemic injection of p-chlorophenylalanine (PCPA), an inhibitor of tryptophan hydroxylase (Koe and Weissman, 1966) or the halogenated phenethylamine, p-chloroamphetamine (PCA), which is toxic to 5-HT neurons (Harvey et al, 1975; Kohler et al, 1978; Sander-Bush and Steranka, 1978; Massari et al, 1978), as well as intracerebral administration of the neurotoxic 5-HT analogs 5-6 and 5-7- dihydroxytryptamine (DHTs) which selectively destroy 5-HT nerve terminals in the CNS under specific conditions (Baumgarten et al, 1976; 1978; Rotman, 1977; Bjorklund et al, 1975).

On other hand, 5-HT can be increased by systemic administration of the 5-HT precursor 5-hydroxytryptophan (5-HTP), which enters the brain and is decarboxylated by L-aromatic amino acid decarboxylase to form 5-HT. In normal physiological conditions, this enzyme is not saturated with its substrate, so that administration of 5-HTP leads to increased synthesis of 5-HT (Moir and Eccleston, 1968) and an increase in levels of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in the brain (Moir and Eccleston, 1968; Okada et al, 1972;).

The purpose of this experiment is to observe the role of 5-HT on spontaneous locomotion within the cage environment. This was done by measuring the basal activity in a group of animals and then administering either a) the 5-HT precursor, 5-HTP or b) the 5-HT synthesis inhibitor p-chlorophenylalanine (PCPA) or c) Saline. In each case the activity level was measured by means of a computerised infrared sensor system in both AS (control) and the mutant rats AS/AGU.

## 7.2 Material and Methods

In this chapter two sets of experiments have been carried out. Initially, a comparison of the spontaneous locomotion of AS and AS/AGU rats was made using Infrared movement analysis. Experiment two was then undertaken to see the effect on locomotion of 5-HT depletion (using PCPA) and 5-HT increase by administration of 5-HTP.

### 7.2.1 Experiment 1. Comparison of spontaneous locomotion of AS and AS/AGU rats

Twenty-seven AS and twenty-seven AS/AGU male rats aged six months were used in this experiment where spontaneous locomotion activity within the cage environment was studied using the computerised infrared sensor system, which is illustrated in fig.7.1.

**Figure 7.1 An image of infra-red apparatus showing cages with infra-red sensors which are attached to the computer (data collection).**

With a Coulburn Instruments infrared motion activity system, the measurement of movements within the cages was detected in three dimensions, and recorded information included the number and duration of movements based on pre-programmed criteria. The infrared sensor was fixed firmly at the top of a cage whose dimensions were 50x32x18 cm. The sensor was facing into the cage at an angle of $45^{o}$ to make sure that activity in all areas of the cage was detectable. The sensors were connected to a computer with a software package where the data could be collected and then analysed. The activity was measured over a six hour time period, with data being reported every five minutes. The

data collected include small movements (1-3 seconds duration), and large movements (more than 3 seconds. One advantage of not recording movement lasting less than one second is to eliminate natural phenomena such as breathing, or tremor in the case of mutants.

### 7.2.2 Experiment 2. The effects of serotonergic manipulation on locomotion

Twenty seven AS control and twenty seven AS/AGU male rats aged six months were assessed. Activity was measured over a six hour time period, with data being reported every five minutes. The data collected include small movements (1-3 seconds duration), and large movements (more than 3 seconds) as described above.

When spontaneous movement had been quantified the animals were split into 3 groups which were tested on a subsequent occasion after receiving either

i) p-chlorophenylalanine

Ten AS and ten AS/AGU rats were given DL-p-chlorophenylalanine methyl ester hydrochloride (PCPA) (Sigma Aldrich Co Ltd., Poole, Dorset) intraperitoneally (i.p.) (100 mg/kg) dissolved in 0.9% NaCl or 0.9% NaCl vehicle alone. All doses were injected at 10:00 in the morning and the testing for six hours began at once.

ii) 5-hydroxytryptophan

Ten AS and ten AS/AGU rats were given 5-hydroxy-L-tryptophan, Hydrochloride (5-HTP) (Calbiochem., La Jolla, CA) 50 mg/kg dissolved in (i.p) 0.9% NaCl or 0.9% NaCl vehicle alone. All doses were injected at 10:00 in the morning and the testing for six hours started at once. Each injection was preceded by an injection of the peripheral decarboxylase inhibitor Carbidopa (Sigma Aldrich Co Ltd., Poole, Dorset) intraperitoneally (i.p.)

(Darwish and Furman, 1977; Koshikawa et al., 1990; Datla and Curzon, 1997)(25 mg/kg) dissolved in 0.9% NaCl, 30 minutes prior to 5-HTP treatment (Eble and Goodrich, 1987). This ensured that the administrated 5-HTP was being metabolised in the brain only.

iii) Saline

Fourteen AS and fourteen AS/AGU rats were given a saline (i.p) injection, All doses were injected at 10:00 in the morning and the testing for six hours started at once

## 7.3 Results

### 7.3.1 A comparison of spontaneous locomotion levels in AS and AS/AGU rats

Twenty-seven AS and 27 AS/AGU male rats were put in cages with infra-red monitors for 6 hours

AS/AGU mutants exhibit far more small movements ( < 3 seconds) and large movements (> 3 seconds) than AS controls. See Table 7.1. The differences are highly significant ($p < 0.001$).

| | **Small movements** | **Large Movements** |
|---|---|---|
| **AS** | 97.6 ± 12 | 15.9 ± 2.0 |
| **AS/AGU** | 193.5 ± 16 | 43.7 ± 6.6 |
| **t** | 4.81, df = 52, p< 0.001 | 4.03, df = 52, p < 0.001 |

**Table 7.1 The number of small (< 3 secs) and large (> 3 secs) movements made per six hours by AS and AS/AGU male rats aged six months.**

### 7.3.2 Second experiment. What are the effects of treating AS and AS/AGU rats with saline (control), pCPA or 5-HTP ?

Because of the differences in movement between the two groups found above, it was decided to analyse movement in response to pharmacological treatments in terms of percentage (%) change over basal levels. Animals were tested a) before treatment b) after treatment

a) small movements A general linear model analysis shows no significant variance for group (AS v AS/AGU) but a highly significant variance for treatment ($F = 166.23$, $df = 2$, $p < 0.001$). Saline treatment produced no significant change over basal levels, but pCPA treatment reduced movement (significantly so in the case of AS rats), while 5-HTP treatment significantly raised movement in both groups ($p < 0.05$).

b) large movements. A general linear model showed significant variance due to both group (AS v AS/AGU, $F = 16.57$, $df = 1$, $p < 0.001$) and treatment ($F = 134.14$, $df = 2$, $p < 0.001$). Saline treatment produced no significant change over basal levels, but pCPA treatment reduced movement significantly in both groups of rats, while 5-HTP treatment significantly raised movement in both both groups ($p < 0.05$). However, the percentage increase in response to 5-HTP was substantially greater in AS/AGU mutants than in controls, and this appears to be the contributing factor to the significant variance due to group.

| % change | AS | AS/AGU | |
|---|---|---|---|
| **i) Small movements** | | | |
| Saline | 2.5 ± 1.2 | - 5.86 ± 2 | ns |
| PCPA | - 55.0 ± 8 | - 32.7 ± 7 | ns |
| 5-HTP | 99.8 ± 4 | 101.5 ± 3 | ns |
| **ii) large movements** | | | |
| Saline | 3.63 ± 1 | 1.14 ± 0.8 | ns |
| PCPA | -51.7 ± 9 | - 43.8 ± 6 | ns |
| 5-HTP | 64.4 ± 5 | 150.0 ± 6.8 | $P < 0.05$ |
| | | | |

**Table 7.2 The percentage change in the number of small (< 3 secs) and large (> 3 secs) movements made over a six-hour period by AS and AS/AGU rats treated with saline, PCPA or 5-HTP.**

## General Linear Model: Small versus group, treat

Analysis of Variance for Small, using Adjusted SS for Tests

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| group | 1 | 722 | 574 | 574 | 0.87 | 0.354 |
| treat | 2 | 218342 | 218342 | 109171 | 166.23 | 0.001 |
| Error | 51 | 33495 | 33495 | 657 | | |
| Total | 54 | 252559 | | | | |

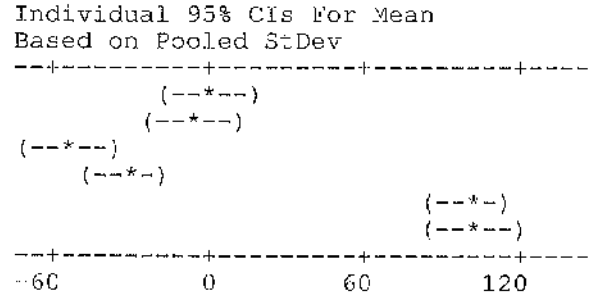
## One-way ANOVA: Small versus treatb

Analysis of Variance for Small

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 5 | 221252 | 44250 | 69.26 | 0.001 |
| Error | 49 | 31307 | 639 | | |
| Total | 54 | 252559 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 8 | 2.50 | 14.76 |
| 12 | 7 | -5.86 | 10.79 |
| 21 | 10 | -55.00 | 6.82 |
| 22 | 10 | -32.70 | 9.15 |
| 31 | 10 | 99.80 | 36.75 |
| 32 | 10 | 101.50 | 41.84 |

Pooled StDev = 25.28




Tukey's pairwise comparisons

## General Linear Model: Large versus group, treat

Analysis of Variance for Large, using Adjusted SS for Tests

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| group | 1 | 15944 | 15290 | 15290 | 16.57 | 0.001 |
| treat | 2 | 247546 | 247546 | 123773 | 134.14 | 0.001 |
| Error | 51 | 47057 | 47057 | 923 | | |
| Total | 54 | 310547 | | | | |

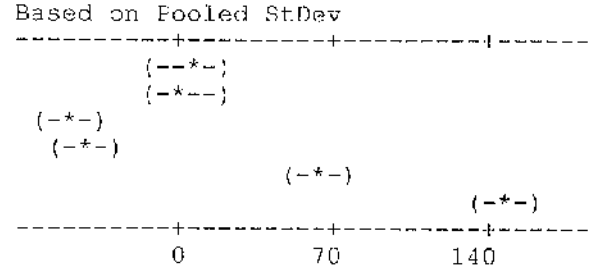
## One-way ANOVA: Large versus treatb

Analysis of Variance for Large

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| treatb | 5 | 285173 | 57035 | 110.14 | 0.000 |
| Error | 49 | 25375 | 518 | | |
| Total | 54 | 310547 | | | |

| Level | N | Mean | StDev |
|---|---|---|---|
| 11 | 8 | 3.63 | 18.88 |
| 12 | 7 | 1.14 | 17.92 |
| 21 | 10 | -51.70 | 8.50 |
| 22 | 10 | -43.80 | 11.44 |
| 31 | 10 | 64.40 | 21.77 |
| 32 | 10 | 150.00 | 40.64 |

Pooled StDev = 22.76




Tukey's pairwise comparisons

## 7.4 Discussion

As described previously (Payne et al., 2000) the AS/AGU mutant rat is characterized by many locomotor defects ranging from rigidity to falling every few steps. Simple tests like the inclined ramp test and mid-air righting show marked differences between the performance of AS/AGU and the parent AS rats. Administration of L-Dopa plus the peripheral Dopa decarboxylase inhibitor benserazide have improved the success in locomotor tests (Campbell et al, 1997; Russell et al, 1998).

A wide variety of locomotor tests have been used to examine animals which have received toxic substances (Jolicoeur et al., 1979; Ivens, 1990) or animals with mutations which lead to locomotor dysfunction such as the shaker rat (Wolf et al., 1996) or weaver mouse (Eisenman et al., 1998). Locomotor testing has been used intensively in models of Parkinson's disease such as the rat 6-OH-DA model (Sakia and Gash, 1994; Borlogan et al., 1996; Mukhida et al., 2001) and in non-human primates and mice with MPTP lesions (Herrero et al., 1993; Gnalalingham et al., 1995; Moratalla et al., 1992; Rosas et al., 1998; Fredriksson et al., 1990; Nishi et al., 1991). In most of these studies, spontaneous general locomotor activity has been observed and considered as a valuable tool for assessing treatment with drugs or toxins (Borbgan et al., 1996; Doan et al., 1999; Med et al., 1994). However, more specific tests have proved useful in some circumstances. For example, the handgrip test has been studied in monkeys (Rouiller et al., 1998; Lemon et al., 1996) and rodents (Ivens, 1990; Gad, 1982) to observe motor control of fingers. Gait analysis has been found to be a useful tool in measuring locomotors deficits, with parameters such as stride length, gait width, and gait symmetry used to observe the abnormal gait patterns (Wolf et al., 1996). Similar tests include placement of forelimb and hind limb (Wolf et al., 1996), the ability to walk along a rod, and to right in mid-air and on a surface (Wolf et al., 1996; Gad, 1982).

One interesting use of locomotor tests was to examine the improvement of spontaneous motor activity after introducing L-dopa treatment to MPTP-treated mice where the spontaneous motor activity had decreased (Fredriksson et al, 1990).

5-HT has been found to increase locomotor activity after administration of L- tryptophan or 5-HTP in combination with a monoamine oxidise inhibitor or peripheral decarboxylase inhibitor (Modigh, 1973; Magyar et al., 1978; Clarke et al., 1984). More recent work on lesioned cats found that serotonin, its precursor 5-hydroxytryptophan (5-HTP) and the agonist quipazine improved locomotion (Brustein and Rossignol, 1999). Furthermore, decreasing 5-HT levels by PCPA was found to reduce locomotion (Marsden and Curzon, 1977; Matte and Tornow, 1978) and exploration (Dringenberg et al., 1995).

Spontaneous locomotion measurement can be affected by external factors such as fear, frustration, sudden noise, light and dark and exploration to the novel environment. It is necessary to keep the environment and experimental conditions under strict control so that spontaneous movement is a reflection of treatment as much as possible.

The motivation for spontaneous locomotion may include feeding, exercise, or curiosity. In this study the mutant rat is far more active than the control; this could be because its clumsier movement causes more activition of the sensor but it could also be less able or willing to settle in the cages.

Adminstration of 5-HTP to both strains increased both small and large movements, which agrees with many previous studies (Grahame-Smith, 1971; Magyar et al, 1978; Modigh, 1972; Modigh and Svensson, 1972; Schlosberg and Harvey, 1979; Stewart et al., 1976). By contrast, administration of PCPA (5-HT synthesis inhibitor) has led to decreased locomotor activity in both strains in large and small movement to a smiliar degree with slightly greater effect in large movement in mutant rats.

Decreased movement with PCPA has been previously reported by Marsden and Curzon, (1976) Matte and Tornow, (1978) Steigrad et al., (1978). Moreover, lesioning midbrain

raphe nuclei leads to suppressed open-field activity (Bouhuys and Van den Hoofdakker, 1977; Dray et al., 1978; Jacobs et al., 1975; Kohler and Lorens, 1978; Lorens et al., 1976). The above drugs have opposing effects on 5-HT content of brain, with 5-HTP increasing the amine while PCPA decreasing it. In the case of 5-HTP the micropunch data (chapter 4) in this study confirm this.

It is wholly unclear what determines the amount of spontaneous locomotion carried out by an animal in a given time. When an animal is put into a new cage, as in my present experiment, the amount of locomtion could reflect any (or all) of :

2) Exploration of a novel environment
3) The establishment of territorial ownership
4) Assertiveness versus fear within a novel situation
5) Relative dexterity of (or ability to initiate) movement

Similiary, when any pharmacological treatment is administered, it is unclear whether the treatment affects locomotion directly or whether it is altering motivation and, hence, movement by indirect means. In this context, it is of interest to note that 5-HT has been reported to influence emotionality, territoriality and exploration.

A relationship between the 5-HT system and emotionality has been found in that hyper-emotionality is reportedly linked to the hyper-activity of serotonergic neurons (Li et al., 1995). Moreover, both 5-HT receptors and serotonin transporters also have influence on modulation of emotionality (Tsuji et al., 2000; Murphy et al., 2001)

The steady basal serotonergic activity recorded in the dorsal raphe neurons in cats has been shown to be decreased during sleep and increased with arousal (Jacobs and Fornal, 1999). Supporting data were obtained from microdialysis studies, which showed that various stressful and nonstressful conditions caused a similar increase in extracellular 5-HT in several forebrain sites (Rueter and Jacobs, 1996; Wilkinson et al., 1996). In addition, the relation between 5-HT and territoriality have been reported (Lasley and Thurmond, 1985).

Serotonin depletion causes long-term reduction of exploration in the rat (Lipska et al., 1992). Moreover, 5-$HT_{1A}$ agonists are found to enhance exploratory process in hamsters (Buhot et al., 1989) and the involvement of 5-HT in exploration has been reported by Meert et al., (1992; 1997) and reviewed by Insel and Winslow, (1998).

Furthermore, 5-HT has been reported to have an effect on anxiety, which may influnce movement (Graeff et al., 1996; Artaiz et al., 1998; Malleret et al., 1999).

Open field testing (roughly similar to our apparatus) is one of the most traditional and widely used methods for the assessment of emotionality (Tobach, 1969; for review; see Archer, 1973) exploratory behaviour (Cuomo et al., 1996) and anxiety (Henderson and Flint, 2001).

Although I canot say what the underlying motivation for the spontaneous locomotion seen here is,

a) Tests of spontaneous locomotion are amongst the most widely used simple neuropsychological studies.
b) Both strains respond similarly to 5-HT manipulation despite the massive differences in normal 5-HT content and release noted in experiments (4,5).

# 8 Chapter 8 General discussion

# 9 General Discussion

This study was undertaken primarily to see if the raphe-striatal serotonergic system was affected in the AS/AGU rat (which carries a mutation in the gene for protein kinase C-γ (Craig et al., 2001)) similar to the dysfunction already reported in the nigrostriatal dopaminergic system (Campbell et al., 1998; Payne et al., 2000). The programme of work consisted of several complimentary studies, each of which has been reported separately with its own Discussion. The purpose of this short overall Discussion is therefore to review these and to put the work in a broad context by posing a series of questions.

Has the mutation been correctly identified?

This was confirmed to be the case by finding positive immunostaining for PKC-γ in AS rats in several brain regions (cerebellum, hippocampus, cerebral cortex, striatum, substantia nigra and dorsal and median raphe nuclei) but negative staining in the AS/AGU mutant. The areas chosen for examination were those which were found positive for PKC-γ in previous studies (Huang et al., 1988; Saito et al., 1994; Tsujino et al., 1990; Yoshihara et al., 1991)

Is PKC-γ restricted to the nervous system and what other PKC isoforms occur there?

It used to be thought that PKC-γ was restricted to the nervous system (Saito et al., 1988) but Goodnight and colleagues (1995) have found it expressed in fibroblasts. More recent work has shown it to be expressed in kidney epithelial cells also (Nowicki et al., 2000). The function of PKC-γ is poorly understood in the nervous system (see Introduction) and wholly unknown in non-neuronal tissues.

Several studies have shown that other isoforms (α,β,γ,ε,θ,δ,η,λ) of PKC are also expressed in the brain (Saito et al., 1994; Tsujino et al., 1990; Yoshihara et al., 1991) and at least α and β have been shown to have role in transmitter on packaging and release (Ben-Shlomo et al., 1991). We have no information on whether other PKC isoforms are up-regulated as a result of the loss of PKC-γ.

Does the dorsal raphe nucleus provide afferents to the dorsal caudate-putamen?

The use of retrograde tracers (CTb) confirmed the rostral projection from the DRN to the striatum while the MRN projects elsewhere. This agrees with previous studies (Azmitia and Segal, 1978; Bobiller et al., 1979; Imai et al., 1986). Azmitia and Segal (1978), and Imai and colleagues (1986) found that axons from the DRN project mainly to the caudate-putamen, amygdala and substantia nigra pars compacta. By contrast, the MRN projects mainly to the hippocampus, hypothalamus and mammillary body (Azmitia and Segal, 1978; Van der Kar and Lorens, 1979; Bobiller et al., 1979).

The DRN cells which project to the DCPU are distributed throughout the length of the DRN. Even if the number of cells projecting to the DCPU seem to be small, their influence on their target sites can be substantial since it is estimated that there are up to 6x $10^6$ serotonergic varicosities/$mm^3$ in parts of the rat brain (Audet et al., 1989).

Does the mutation lead to decreased cell numbers in the midbrain raphe nuclei?

My first step to investigate the effect of the mutation on the serotonergic system was to compare the cell numbers of DRN and MRN in the mutant rat and the parent AS strain. Cell counts using both conventional and unbiased stereology methods showed that 5-HT cell numbers in the DRN of mutant rats were reduced by 23% compared to the parent AS strain. The change looks to be restricted to the DRN, since no change in cell numbers was found to occur in the MRN of the mutant rats.

As is well established, the DRN is the sole source of the serotonergic projections to the striatum. The reduction in cell numbers could affect the availability of 5-HT in the striatum and this will be discussed below.

It is not clear why one nucleus (DRN) is affected and the other (MRN) is not. However, recent paper by Kovacs and colleagues (2003) reported selective loss of cells in different 5-HT groups according to particular human disorders. It does appear, therefore, that we should not expect all 5-HT cell groups to react similarly to a single circumstance or condition.

It does not seem that serotonergic cell numbers have been counted in lab models designed to replicate PD (e.g. 6-OH-DA or MPTP models), but the loss of serotonergic cells in the DRN of human PD patients has been reported by Jellinger (1992) as 44% in post mortem brains. In this present study the loss of DRN cells was 23% in one-year-old animals which might subsequently become comparable with what Jellinger (1992) found with PD patients. We are restricted by HO licence in the age to which we can keep the mutant rats. On the other hand, we do not know when cell loss occurs. Animal may be born with reduced cell numbers or acquire loss subsequently. Nor do we know how cell numbers change in the AS rat with age. But serotonin neuron loss in normal human ageing has been linked to neuropsychiatric disorders (Meltzer et al., 1998). It is known that old rats show lower levels of serotonin and dopamine (Goicoechea et al., 1997).

Is there a decrease in available 5-HT in the striatum- a confirmed target of DRN cells?

The whole-tissue study conducted here found that 5-HT levels were reduced in the striatum and in the DRN, but no affect was noticed in the MRN. This is to be expected given the observations on differential cell loss.

The microdialysis study showed that the extracellular levels of 5-HT in the mutant rats are reduced by 70% compared with normal animals. Since 5-HIAA levels in the dialysate were greatly elevated, this does not suggest a simple relationship to cell loss or dysfunction.

Most of these results found with 5-HT systems in the AS/AGU rats are very similar to those found previously for the nigrostriatal dopaminergic system (Campbell et al., 2000; Payne et al., 2000). This indicates that the mutation of PKC γ may affect both dopaminergic and serotonergic systems in a similar fashion. It is not clear if the effect is a direct one on the two cell groups or if the effects on one cell group subsequently effects the other also.

To shed light on that we have to understand potential links between 5-HT and DA.

The interaction between DA and 5-HT systems are well known (Benloucif and Galloway, 1991; Parsons and Justice, 1993; Iyer and Bradberry, 1996) for review see Broderick and Phelin, (1997). These include: -

i) Anatomical connections between the two aminergic systems

There are projections from the DRN to DA cell bodies in the ventral tegmental area, substantia nigra and striatum (Steinbush et al., 1980; Van der Kooy and Hattori, 1980; Herve et al., 1987). More detailed studies have shown that serotonergic neurons make direct synaptic contact with DA cell bodies and dendrites in the SN and VTA (Corvaja et al., 1993; Herve et al., 1987; Needergaard et al., 1988). Other studies have demonstrated that 5-HT neurons projecting to the SN appear to be collaterals of 5-HT neurons projecting from the dorsal raphe to the striatum (Imai et al., 1986; Van der Kooy and Hattori, 1980). In addition, Ferre and Arigas (1993) have indicated that DA from projections from the substantia nigra or the ventral tegmental area may increase 5-HT release in the DRN

ii) Neurophysiological connections between the aminergic system

When the DRN is stimulated (or 5-HT agonists and antagonists are administered directly to the SN or the ventral tegmental area) modulation of the electrophysiological activity of dopaminergic neurons has been reported (Brodie and Bunney, 1996; Cameron et al., 1997; Kelland et al., 1990; Minabe et al., 1996; Pessia et al., 1994; Prisco et al., 1994; Trent and Tepper, 1991).

For example, experiments using microdialysis have shown that dopamine release is facilitated by serotonin agonists (Benloucif and Galloway, 1991) as is dopamine release in the cerebral cortex (Iyer and Bradberry, 1996). Similarly, Parsons and Justice (1993) have shown that serotonin perfusion leads to increased extracellular dopamine in the nucleus accumbens.

A reciprocal relationship has also been demonstrated. Thus dopaminergic regulation of 5-HT release has been reported *in vitro* within the SN (Benkirane et al., 1987; Hery et al., 1980; Iravani and Kruk1997; Kelly et al., 1985). *In vivo* studies (Chen et al., 1992) have also confirmed a link, showing that apomorphine can elevate tryptophan, serotonin and 5-

HIAA concentrations in the DRN and its corresponding projection site, the striatum, but not in the MRN and its terminal area, the hippocampus. These effects are mediated through DA in the SN. In addition, Ferre and Arigas (1993) have indicated that DA from projections from the substantia nigra or the ventral tegmental area may increase 5-HT release in the DRN. Ferre et al. (1994) have shown DA D2-like receptor-mediated regulation of serotonin in the DRN.

All these studies indicate that reciprocal interactions between serotonergic and dopaminergic systems occur in different brain areas including the midbrain nuclei and the striatum.

It is not possible, therefore, to decide between

a) The mutation having a direct affect on two cell groups independently or

b) The mutation affecting one cell group which, in turn, leads to a dysfunction of the other.

The fact that the mutation is in the gene for a protein kinase that may be involved in transmitter packaging and release might tend towards the first explanation, but this cannot be proposed with certainty at this stage.

Should we expect dysfunction of more than one transmitter system in conditions involving the basal ganglia and/or locomotor disorders?

Involvement of 5-HT in many neurodegenerative disorders has been reported. Jellinger (1992) has indicated that raphe cell numbers are reduced in Parkinson's disease. In addition, reductions in serotonin concentration have been reported in the substantia nigra, hippocampus and other known serotonin-rich regions of Parkinsonian brains (Agid, 1987; Rinne et al., 1974; Scatton et al., 1983). Moreover, reduced 5-HIAA levels have been reported in the cerebrospinal fluid of PD patients (Tohgi et al., 1993). Many other studies have reported the significant losses of serotonin and its major metabolite 5-HIAA in Parkinson's disease in several brain regions including the striatum, globus pallidus, thalamus and substantia nigra (Curzon, 1972; 1977; 1978; Fahn et al., 1971; Guldberg et al., 1967; Lloyd and Hornykiewicz, 1974; Lloyd, 1977). Other neurodegenerative disorders

related to Parkinson's disease, such as supranuclear palsy and multiple system atrophy, are also characterised by cell loss in a number of amine systems including 5-HT (Hedera and Whitehouse, 1994).

What is the effect of serotonin on locomotion in control and mutant rats?

The role of serotonin in locomotion can be studied by increasing and decreasing the availability of the 5-HT in the brain. In the present study, increasing 5-HT (by injection of 5-HTP) was found to cause more movement while depletion of 5-HT (by injection of PCPA) led to less movement as detected with infra-red apparatus. Open field studies have frequently been used to examine the role of 5-HT in locomotion. The results are similar to mine. Thus, administration of 5-HTP increased locomotion (Grahame-Smith, 1971; Magyar et al, 1978; Modigh, 1972; Modigh and Svensson, 1972; Schlosberg and Harvey, 1979; Stewart et al., 1976) whereas decreased movement followed PCPA administration (Marsden and Curzon, 1976; Matte and Tornow, 1978; Steigrad et al., 1978). Moreover, lesioning midbrain raphe nuclei leads to suppressed open-field activity (Bouhuys and Van den Hoofdakker, 1977; Dray et al., 1978; Jacobs et al., 1975; Kohler and Lorens, 1978; Lorens et al., 1976).

Both groups of rats (AS and AS/AGU) respond to 5-HT manipulation with similar changes in locomotor activity. This means that the remaining DRN cells in the AS/AGU are capable of mediating this change unless another 5-HT system is involved, for which there is no evidence.

How can a mutation in PKC-γ cause dysfunction and death of serotonergic neurons?

I am going to try answering this in three stages. Firstly, is there any role of the PKC family in general which may explain the dysfunction? Secondly, and specifically, is there any function of PKC- γ which could be linked to the dysfunction and, finally, is there any relation between PKC and 5-HT or PKC- γ and 5-HT.

The PKC family, as mentioned earlier in this thesis (chapter 2), has a wide variety of functions including modulation of ion channels (for review see Shearman et al., 1989) and

the desensitisation of receptors (Huganir and Greenyard, 1990). These may have indirect effects on the release of neurotransmitters where protein phosophorylation regulates the efficacy of synaptic transmission by modulating the release of neurotransmitter from the presynaptic nerve terminal and by modulating the sensitivity of receptors in the postsynaptic membrane. Perhaps the most interesting function of PKC in the context of the present study is the enhancement of neurotransmitter release (Malenka et al., 1986,1987).

Although there is a substantial literature on the PKC family, it is relatively rare to make distinctions between the different isoforms. The work on PKC- γ has shown involvement of this isoform in many disparate functions such as alcohol preference and tolerance (Bowers and Wehner, 2001) processing in the dorsal horn (Narita et al., 2001) postnatal reduction of cerebellar afferents (Ebralidze et al., 1996) and involvement in spinocerebellar ataxia (Chan, 2003; Chen et al., 2003). The work of our laboratory, showing locomotor abnormality plus a dysfunction of dopaminergic and, now, serotonergic inputs to the striatum, is quite different again. The recent demonstration of a role for PKC- γ in 5-HT secretion (Liu et al., 2003) supports our findings.

With dopamine there is a coherent hypothesis (unproved but reasonable) "DA must be free in the cytoplasm to allow such large amount of DOPAC/ HVA to be produced: Free DA is toxic to its own cells (forming free radicals, dopaquinones) therefore the cells become nonviable and die" (Payne et al., 2000).

Can a similar hypothesis be formulated for 5-HT? On the face of it the situation is the same (low 5-HT but high 5-HIAA), so

a) is free 5-HT auto-toxic? or

b) does the one mutation kill off 2 sets of cells by different means? or

c) is the DA death hypothesis wrong?

Indoleamines are often associated with the role of free radical scavengers. This is so not only for melatonin but also others including 5-HT (Poeggeler et al., 2002). However, it

also clear that 5-HT can be metabolised to toxic dimers and serotonin quinone-imines and that serotonin metabolism may lead to free radical formation (Perez-Reyes and Mason, 1981). This means that there is a possibility of serotonin acting as an auto-toxic agent to its own cells as has been proposed for DA (Payne et al., 2000).

My results show that the raphe-striatal serotonergic system is affected in AS/AGU rats. These findings together with previous studies on the mutant AS/AGU showing dysfunction of the nigrostriatal dopaminergic system, continue to demonstrate the potential importance of this model along side other neurodegenerative models in giving insight into human neurodegenerative conditions which involve abnormalities of aminergic systems, the basal ganglia and locomotion.

# 10 Chapter 9. References

1. Adell A, Casanovas JM, Artigas F. Comparative study in the rat of the actions of different types of stress on the release of 5-HT in raphe nuclei and forebrain areas. Neuropharmacology 1997;36(4-5):735-41.

2. Afsharpour S. Topographical projections of the cerebral-cortex to the subthalamic nucleus. J. Comp. Neurol 1985;236:14-28.

3. Aghajanian GK. Influence of drugs on the firing of serotonin-containing neurons in brain. Fed Proc 1972;31(1):91-6.

4. Aghajanian GK, Bloom FE. Localization of tritiated serotonin in rat brain by electron-microscopic autoradiography. J Pharmacol Exp Ther 1967;156(1):23-30.

5. Aghajanian GK, Foote WE, Sheard MH. Lysergic acid diethylamide: sensitive neuronal units in the midbrain raphe. Science 1968;161(842):706-8.

6. Aghajanian GK, Wang RY. Habenular and other midbrain raphe afferents demonstrated by a modified retrograde tracing technique. Brain Res 1977;122(2):229-42.

7. Agid Y, Javoy-Agid, F. and Ruberg, M, editor. Biochemistry of neurotransmitters in parkinson's disease. London: Butterworth; 1987.

8. Aitken AR, Tork I. Early development of serotonin-containing neurons and pathways as seen in wholemount preparations of the fetal rat brain. J Comp Neurol 1988;274(1):32-47.

9. Akers RF, Lovinger DM, Colley PA, Linden DJ, Routtenberg A. Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. Science 1986;231(4738):587-9.

10. Akiyama Y, Koshimura K, Ohue T, Lee K, Miwa S, Yamagata S, et al. Effects of hypoxia on the activity of the dopaminergic neuron system in the rat striatum as studied by in vivo brain microdialysis. J Neurochem 1991;57(3):997-1002.

11. Albin RL, Young, A. B. and Penney, J. B. The functional anatomy of basal ganglia disorders. Trends Neurosci 1989;12:366-375.

12. Alexander GEaC, M. D. Preparation for movement- Neural representations of intended direction in 3 motor areas of the monkey. J.neurophysiol 1990a;64:133-150.

13. Alexander GEaC, M. D. Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci 1990c;13:266-271.

14. Alexander GM, Schwartzman RJ, Brainard L, Gordon SW, Grothusen JR. Changes in brain catecholamines and dopamine uptake sites at different stages of MPTP parkinsonism in monkeys. Brain Res 1992;588(2):261-9.

15. Alxenander GE, De Long, M. R. and Strick, P. L. Parallel organisation of functionally segmented circuits linking basal ganglia and cortex. Annu. Rev. Neurosci 1986;9:357-381.

16. Alxenander GEaDL, M. R. Microstimulation of the primate neostriatum. 2. Somatotopic organisation of striatal microexcitable zones and their relation to neuronal response properties. J.neurophysiol 1985;53:1417-1430.

17. Anderson MEaH, F. B. influence of the globus pallidus on arm movements in monkeys .3. Timing of movement related information. J.neurophysiol 1985;54:433-448.

18. Anji A, Sullivan Hanley NR, Kumari M, Hensler JG. The role of protein kinase C in the regulation of serotonin-2A receptor expression. J Neurochem 2001;77(2):589-97.

19. Annett LE, Rogers DC, Hernandez TD, Dunnett SB. Behavioural analysis of unilateral monoamine depletion in the marmoset. Brain 1992;115 ( Pt 3):825-56.

20. Apparsundaram S, Schroeter S, Giovanetti E, Blakely RD. Acute regulation of norepinephrine transport: II. PKC-modulated surface expression of human norepinephrine transporter proteins. J Pharmacol Exp Ther 1998;287(2):744-51.

21. Arai R, Karasawa N, Geffard M, Nagatsu T, Nagatsu I. Immunohistochemical evidence that central serotonin neurons produce dopamine from exogenous L-DOPA in the rat, with reference to the involvement of aromatic L-amino acid decarboxylase. Brain Res 1994;667(2):295-9.

22. Arcusa MJ, Barcia-Salorio, J. L., Burguera, A. Chronic high frequency stimulation of the globus pallidus internus in parkinson's disease. Neurology 1996;46(2):490-495.

23. Arikuni T, Kubota K. The organization of prefrontocaudate projections and their laminar origin in the macaque monkey: a retrograde study using HRP-gel. J Comp Neurol 1986;244(4):492-510.

24. Artaiz I, Zazpe A, Del Rio J. Characterization of serotonergic mechanisms involved in the behavioural inhibition induced by 5-hydroxytryptophan in a modified light-dark test in mice. Behav Pharmacol 1998;9(2):103-12.

25. Ashkenazi R, Holman RB, Vogt M. Release of transmitters on stimulation of the nucleus linearis raphe in the cat. J Physiol 1972;223(1):255-9.

26. Aston-Jones G, Card JP. Use of pseudorabies virus to delineate multisynaptic circuits in brain: opportunities and limitations. J Neurosci Methods 2000;103(1):51-61.

27. Audet MA, Descarries L, Doucet G. Quantified regional and laminar distribution of the serotonin innervation in the anterior half of adult rat cerebral cortex. J Chem Neuroanat 1989;2(1):29-44.

28. Aziz TZ, Peggs, D., Sambrook, M. A. and Crossman, A. R. Lesion of the subthalamic nucleus for the alleviation of MPTP-inuced parkinsonism in the primate. Mov Disord 1991;6:288-292.

29. Azmitia EC, Segal M. An autoradiographic analysis of the differential ascending projections of the dorsal and median raphe nuclei in the rat. J Comp Neurol 1978;179(3):641-67.

30. Baev KV, Greene KA, Marciano FF, Samanta JE, Shetter AG, Smith KA, et al. Physiology and pathophysiology of cortico-basal ganglia-thalamocortical loops: theoretical and practical aspects. Prog Neuropsychopharmacol Biol Psychiatry 2002;26(4):771-804.

31. Baik JH, Picetti R, Saiardi A, Thiriet G, Dierich A, Depaulis A, et al. Parkinsonian-like locomotor impairment in mice lacking dopamine D2 receptors. Nature 1995;377(6548):424-8.

32. Bankiewicz KS, Oldfield, E. H., Chiueh, C. C., Doppman, J. L., Jacobowitz, D. M. and Kopin, I. J. Hemiparkinsonism in monkeys after unilateral internal carotid artery infusion of MPTP. Life Sci 1986;39:7-19.

33. Baraban JM, Aghajanian GK. Noradrenergic innervation of serotonergic neurons in the dorsal raphe: demonstration by electron microscopic autoradiography. Brain Res 1981;204(1):1-11.

34. Baraban JM, Snyder SH, Alger BE. Protein kinase C regulates ionic conductance in hippocampal pyramidal neurons: electrophysiological effects of phorbol esters. Proc Natl Acad Sci U S A 1985;82(8):2538-42.

35. Barbeito L, Girault JA, Godeheu G, Pittaluga A, Glowinski J, Cheramy A. Activation of the bilateral corticostriatal glutamatergic projection by infusion of GABA into thalamic motor nuclei in the cat: an in vivo release study. Neuroscience 1989;28(2):365-74.

36. Barrett RE, St Balch T. Uptake of catecholamines into serotonergic nerve cells as demonstrated by fluorescence histochemistry. Experientia 1971;27(6):663-4.

37. Bartholini G, Da Prada M, Pletscher A. Decrease of cerebral 5-hydroxytryptamine by 3,4-dihydroxyphenylalanine after inhibition of extracerebral decarboxylase. J Pharm Pharmacol 1968;20(3):228-9.

38. Basbaum AI, Menetrey D. Wheat germ agglutinin-apoHRP gold: a new retrograde tracer for light- and electron-microscopic single- and double-label studies. J Comp Neurol 1987;261(2):306-18.

39. Baumgarten HG, Bjorklund A. Neurotoxic indoleamines and monoamine neurons. Annu Rev Pharmacol Toxicol 1976;16:101-11.

40. Baumgarten HG, Klemm HP, Lachenmayer L, Bjorklund A, Lovenberg W, Schlossberger HG. Mode and mechanism of action of neurotoxic indoleamines: a review and a progress report. Ann N Y Acad Sci 1978;305:3-24.

41. Beal MF. Experimental models of Parkinson's disease. Nat Rev Neurosci 2001;2(5):325-34.

42. Beaudet A, Descarries L. Quantitative data on serotonin nerve terminals in adult rat neocortex. Brain Res 1976;111(2):301-9.

43. Beckstead RM. The thalamostriatal projection in the cat. J. Comp. Neurol 1984;223:313-346.

44. Benabid AL, Pollak, P., Gervason, C. Long term suppression of tremor by chronic stimulation of the ventral intermediate thalamic nucleus. Lancet 1991;337:403-406.

45. Benkirane S, Arbilla S, Langer SZ. A functional response to D1 dopamine receptor stimulation in the central nervous system: inhibition of the release of [3H]-serotonin from the rat substantia nigra. Naunyn Schmiedebergs Arch Pharmacol 1987;335(5):502-7.

46. Benloucif S, Galloway MP. Facilitation of dopamine release in vivo by serotonin agonists: studies with microdialysis. Eur J Pharmacol 1991;200(1):1-8.

47. Ben-Shlomo H, Sigmund O, Stabel S, Reiss N, Naor Z. Preferential release of catecholamine from permeabilized PC12 cells by alpha- and beta-type protein kinase C subspecies. Biochem J 1991;280 ( Pt 1):65-9.

48. Bergen C, Pollaner, M. S., Weyer, L., Black, S. E. and Lang, A. E. Unusual clinical presentations of cortical-basal ganglionic degeneration. Ann Neurol 1996;40:893-900.

49. Berger B, Verney C, Alvarez C, Vigny A, Helle KB. New dopaminergic terminal fields in the motor, visual (area 18b) and retrosplenial cortex in the young and adult rat. Immunocytochemical and catecholamine histochemical analyses. Neuroscience 1985;15(4):983-98.

50. Bergman H, Wichmann, T. and De Long, M. R. Reversal of experimental parkinsonism by lesion of the subthalamic nucleus. Science 1990;249:1436-1438.

51. Bernheimer H, Birkmayer, W., Hornykiewicz, O., Jellinger, K. and Stitelberger, F. Brain dopamine and the syndromes of parkinson and huntington. J.Neurol Sci 1973;20:415-455.

52. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000;3(12):1301-6.

53. Bjorklund A, Axelsson S, Falck B. Intraneuronal indolamines in the central nervous system. Adv Biochem Psychopharmacol 1976;15:87-94.

54. Bjorklund A, Baumgarten HG, Rensch A. 5,7-Dihydroxytryptamine: improvement of its selectivity for serotonin neurons in the CNS by pretreatment with desipramine. J Neurochem 1975;24(4):833-5.

55. Bjorklund A, Falck B, Stenevi U. Classification of monoamine neurones in the rat mesencephalon: distribution of a new monoamine neurone system. Brain Res 1971;32(2):269-85.

56. Bjugn R. The use of the optical disector to estimate the number of neurons, glial and endothelial cells in the spinal cord of the mouse--with a comparative note on the rat spinal cord. Brain Res 1993;627(1):25-33.

57. Blier P, Serrano A, Scatton B. Differential responsiveness of the rat dorsal and median raphe 5-HT systems to 5-HT1 receptor agonists and p-chloroamphetamine. Synapse 1990;5(2):120-33.

58. Bobillier P, Pettijean F, Salvert D, Ligier M, Seguin S. Differential projections of the nucleus raphe dorsalis and nucleus raphe centralis as revealed by autoradiography. Brain Res 1975;85(2):205-10.

59. Bobillier P, Seguin S, Degueurce A, Lewis BD, Pujol JF. The efferent connections of the nucleus raphe centralis superior in the rat as revealed by radioautography. Brain Res 1979;166(1):1-8.

60. Bobillier P, Seguin S, Petitjean F, Salvert D, Touret M, Jouvet M. The raphe nuclei of the cat brain stem: a topographical atlas of their efferent projections as revealed by autoradiography. Brain Res 1976;113(3):449-86.

61. Bolender RP, Charleston J, Mottet K, McCabe JT. Quantitative morphology of the nervous system: expanding horizons. Anat Rec 1991;231(4):407-15.

62. Borlongan CV, Freeman TB, Hauser RA, Cahill DW, Sanberg PR. Cyclosporine-A increases locomotor activity in rats with 6-hydroxydopamine-

induced hemiparkinsonism: relevance to neural transplantation. Surg Neurol 1996;46(4):384-8.

63. Bouhuys AL, Van Den Hoofdakker RH. Effects of midbrain raphe destruction on sleep and locomotor activity in rats. Physiol Behav 1977;19(4):535-41.

64. Bouyer JJ, Park DH, Joh TH, Pickel VM. Chemical and structural analysis of the relation between cortical inputs and tyrosine hydroxylase-containing terminals in rat neostriatum. Brain Res 1984;302(2):267-75.

65. Bowers BJ, Collins AC, Tritto T, Wehner JM. Mice lacking PKC gamma exhibit decreased anxiety. Behav Genet 2000;30(2):111-21.

66. Bowers BJ, Wehner JM. Ethanol consumption and behavioral impulsivity are increased in protein kinase Cgamma null mutant mice. J Neurosci 2001;21(21):RC180.

67. Bramwell GJ, Gonye T. Responses of midbrain neurones to microiontophoretically applied 5-hydroxytryptamine: comparison with the response to intravenously administered lysergic acid diethylamide. Neuropharmacology 1976;15(8):457-61.

68. Broderick PA, Phelix CF. I. Serotonin (5-HT) within dopamine reward circuits signals open-field behavior. II. Basis for 5-HT--DA interaction in cocaine dysfunctional behavior. Neurosci Biobehav Rev 1997;21(3):227-60.

69. Brodie MS, Bunney EB. Serotonin potentiates dopamine inhibition of ventral tegmental area neurons in vitro. J Neurophysiol 1996;76(3):2077-82.

70. Buhot MC, Rage P, Segu L. Changes in exploratory behaviour of hamsters following treatment with 8-hydroxy-2-(di-n-propylamino)tetralin. Behav Brain Res 1989;35(2):163-79.

71. Bunin MA, Prioleau C, Mailman RB, Wightman RM. Release and uptake rates of 5-hydroxytryptamine in the dorsal raphe and substantia nigra reticulata of the rat brain. J Neurochem 1998;70(3):1077-87.

72. Bunney BS, Aghajanian GK. Dopamine and norepinephrine innervated cells in the rat prefrontal cortex: pharmacological differentiation using microiontophoretic techniques. Life Sci 1976;19(11):1783-9.

73. Burns RS, Chiueh, C. C., Markey, S. P., Ebert, M. H., Jacobowitz, M. D. and Kopin, I. J. A primate model of parkinsonism-selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by MPTP. Proc Natl Acad Sci 1983;80:4546-4550.

74. Butcher L, Engel J, Fuxe K. L-dopa induced changes in central monoamine neurons after peripheral decarboxylase inhibition. J Pharm Pharmacol 1970;22(4):313-6.

75. Butcher LL, Hodge GK. Postnatal development of acetylcholinesterase in the caudate-putamen nucleus and substantia nigra of rats. Brain Res 1976;106(2):223-40.

76. Calas A, Alonso G, Arnauld E, Vincent JD. Demonstration of indolaminergic fibres in the media eminence of the duck, rat and monkey. Nature 1974;250(463):241-3.

77. Cameron DL, Wessendorf MW, Williams JT. A subset of ventral tegmental area neurons is inhibited by dopamine, 5-hydroxytryptamine and opioids. Neuroscience 1997;77(1):155-66.

78. Campbell JM, Gilmore DP, Russell D, Growney CA, Favor G, Kennedy AK, et al. Pharmacological analysis of extracellular dopamine and metabolites in the striatum of conscious as/agu rats, mutants with locomotor disorder. Neuroscience 2000;100(1):45-52.

79. Campbell JM, Gilmore DP, Russell D, Growney CA, Favor G, Weir J, et al. Extracellular levels of dopamine and its metabolite 3,4-dihydroxy-phenylacetic acid measured by microdialysis in the corpus striatum of conscious AS/AGU mutant rats. Neuroscience 1998;85(2):323-5.

80. Campbell JM, Payne AP, Gilmore DP, Byrne JE, Russell D, McGadey J, et al. Neostriatal dopamine depletion and locomotor abnormalities due to the Albino Swiss rat agu mutation. Neurosci Lett 1996;213(3):173-6.

81. Campbell JM, Payne AP, Gilmore DP, Russell D, McGadey J, Clarke DJ, et al. Age changes in dopamine levels in the corpus striatum of Albino Swiss (AS) and AS/AGU mutant rats. Neurosci Lett 1997;239(1):54-6.

82. Carboni E, Di Chiara G. Serotonin release estimated by transcortical dialysis in freely-moving rats. Neuroscience 1989;32(3):637-45.

83. Carlsson A, Lindqvist, M., Magnusson, T., and Waldeck, B. On the presence of 3-hydroxytyramine in brain. Science 1958;127:471.

84. Carlsson M, Carlsson A. Interactions between glutamatergic and monoaminergic systems within the basal ganglia--implications for schizophrenia and Parkinson's disease. Trends Neurosci 1990;13(7):272-6.

85. Casanovas JM, Lesourd M, Artigas F. The effect of the selective 5-HT1A agonists alnespirone (S-20499) and 8-OH-DPAT on extracellular 5-hydroxytryptamine in different regions of rat brain. Br J Pharmacol 1997;122(4):733-41.

86. Cattabeni F, Koslow SH, Costa E. Gas chromatographic-mass spectrometric assay of four indole alkylamines of rat pineal. Science 1972;178(57):166-8.

87. Cattabeni F, Koslow SH, Costa E. Gas chromatographic-mass spectrometric assay of four indole alkylamines of rat pineal. Science 1972;178(57):166-8.

88. Chan-Palay V. Indoleamine neurons and their processes in the normal rat brain and in chronic diet-induced thiamine deficiency demonstrated by uptake of 3H-serotonin. J Comp Neurol 1977;176(4):467-93.

89. Chazal G, Ralston HJ, 3rd. Serotonin-containing structures in the nucleus raphe dorsalis of the cat: an ultrastructural analysis of dendrites, presynaptic dendrites, and axon terminals. J Comp Neurol 1987;259(3):317-29.

90. Chen C, Kano M, Abeliovich A, Chen L, Bao S, Kim JJ, et al. Impaired motor coordination correlates with persistent multiple climbing fiber innervation in PKC gamma mutant mice. Cell 1995;83(7):1233-42.

91. Chen DH, Brkanac Z, Verlinde CL, Tan XJ, Bylenok L, Nochlin D, et al. Missense mutations in the regulatory domain of PKC gamma: a new mechanism for dominant nonepisodic cerebellar ataxia. Am J Hum Genet 2003;72(4):839-49.

92. Chen HY, Lin YP, Lee EH. Cholinergic and GABAergic mediations of the effects of apomorphine on serotonin neurons. Synapse 1992;10(1):34-43.

93. Chen S, Aston-Jones G. Evidence that cholera toxin B subunit (CTb) can be avidly taken up and transported by fibers of passage. Brain Res 1995;674(1):107-11.

94. Chen S, Yang M, Miselis RR, Aston-Jones G. Characterization of transsynaptic tracing with central application of pseudorabies virus. Brain Res 1999;838(1-2):171-83.

95. Chesselet MFaD, J. M. Basal ganglia and movement disorders: an update. Trends Neurosci 1996;19:417-422.

96. Chevalier G, Vacher, S., Deniau, J. M. and Desban, M. Disinhibition on a process in the expression of striatal functions. 1. The striato-nigral influence on tecto-spinal tectodiencephalic neurons. Brain Res 1985;334:215-226.

97. Chojnacka-Wojcik E. 5-Hydroxytryptamine in the central nervous system. Pol J Pharmacol 1995;47(3):219-35.

98. Clarke DJ, Payne AP. Neuroanatomical characterization of a new mutant rat with dopamine depletion in the substantia nigra. Eur J Neurosci 1994;6(5):885-8.

99. Clemens JA, Phebus LA. Dopamine depletion protects striatal neurons from ischemia-induced cell death. Life Sci 1988;42(6):707-13.

100. Clements JD. Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends Neurosci 1996;19(5):163-71.

101. Collins SJ, Ahlskog, J. E., Parisi, J. E. and Maraganore, D. M. Progressive supranuclear palsy: Neuropathologically based diagnostic clinical criteria. J.Neurol Neurosurg Psychiatry 1995;58:167-173.

102. Conrad LC, Leonard CM, Pfaff DW. Connections of the median and dorsal raphe nuclei in the rat: an autoradiographic and degeneration study. J Comp Neurol 1974;156(2):179-205.

103. Cooper RL, McNamara MC, Linnoila M. Catecholaminergic-serotonergic balance in the CNS and reproductive cycling in aging rats. Neurobiol Aging 1986;7(1):9-15.

104. Corvaja N, Doucet G, Bolam JP. Ultrastructure and synaptic targets of the raphe-nigral projection in the rat. Neuroscience 1993;55(2):417-27.

105. Coyle JT, Snyder SH. Catecholamine uptake by synaptosomes in homogenates of rat brain: stereospecificity in different areas. J Pharmacol Exp Ther 1969;170(2):221-31.

106. Craig NJ, Duran Alonso MB, Hawker KL, Shiels P, Glencorse TA, Campbell JM, et al. A candidate gene for human neurodegenerative disorders: a rat PKC gamma mutation causes a Parkinsonian syndrome. Nat Neurosci 2001;4(11):1061-2.

107. Crossman AR. Functional anatomy of movement disorders. J Anat 2000;196 ( Pt 4):519-25.

108. Crutcher. M. D. and De Long MR. Single cell studies of the primate putamen. 1. Functional organisation. Exp. Brain Res 1984;53:233-243.

109. Curzon G, Green AR. Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in small regions of rat brain. Br J Pharmacol 1970;39(3):653-5.

110. Curzon G, Green AR. Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in small regions of rat brain. Br J Pharmacol 1970;39(3):653-5.

111. Dahlstrom A, Fuxe K. Localization of monoamines in the lower brain stem. Experientia 1964;20(7):398-9.

112. Damsma G, Boisvert DP, Mudrick LA, Wenkstern D, Fibiger HC. Effects of transient forebrain ischemia and pargyline on extracellular concentrations of dopamine, serotonin, and their metabolites in the rat striatum as determined by in vivo microdialysis. J Neurochem 1990;54(3):801-8.

113. Davidson L, Lloyd K, Dankova J, Hornykiewicz O. L-DOPA treatment in Parkinson's disease: effect on dopamine and related substances in discrete brain regions. Experientia 1971;27(9):1048-9.

114. Davis M, Sheard MH. Habituation and sensitization of the rat startle response: effects of raphe lesions. Physiol Behav 1974;12(3):425-31.

115. De Vito JLaA, M. E. An autoradiographic study of efferent connections of the globus pallidus in macaca-mulatta. Exp. Brain Res 1982;46:107-117.

116. Descarries L, Audet MA, Doucet G, Garcia S, Oleskevich S, Seguela P, et al. Morphology of central serotonin neurons. Brief review of quantified aspects of

their distribution and ultrastructural relationships. Ann N Y Acad Sci 1990;600:81-92.

117. Descarries L, Lemay B, Doucet G, Berger B. Regional and laminar density of the dopamine innervation in adult rat cerebral cortex. Neuroscience 1987;21(3):807-24.

118. Dilts RP, Boadle-Biber MC. Differential activation of the 5-hydroxytryptamine-containing neurons of the midbrain raphe of the rat in response to randomly presented inescapable sound. Neurosci Lett 1995;199(1):78-80.

119. Doan VD, Grondin R, Hadj Tahar A, Gregoire L, Bedard PJ. Effect of the selective D1 antagonists SCH 23390 and NNC 01-0112 on the delay, duration, and improvement of behavioral responses to dopaminergic agents in MPTP-treated monkeys. Clin Neuropharmacol 1999;22(5):281-7.

120. Dogali M, Fazzini, E. Stereotaxic ventral pallidotomy for parkinson's disease. Neurology 1995;45:753-761.

121. Donoghue JP, Herkenham M. Neostriatal projections from individual cortical fields conform to histochemically distinct striatal compartments in the rat. Brain Res 1986;365(2):397-403.

122. Drago J, Gerfen CR, Westphal H, Steiner H. D1 dopamine receptor-deficient mouse: cocaine-induced regulation of immediate-early gene and substance P expression in the striatum. Neuroscience 1996;74(3):813-23.

123. Dray A. Serotonin in the basal ganglia: functions and interactions with other neuronal pathways. J Physiol (Paris) 1981;77(2-3):393-403.

124. Dray A, Davies J, Oakley NR, Tongroach P, Vellucci S. The dorsal and medial raphe projections to the substantia nigra in the rat: electrophysiological, biochemical and behavioural observations. Brain Res 1978;151(3):431-42.

125. Dunnett SB, Hernandez TD, Summerfield A, Jones GH, Arbuthnott G. Graft-derived recovery from 6-OHDA lesions: specificity of ventral mesencephalic graft tissues. Exp Brain Res 1988;71(2):411-24.

126. Eisenman LM, Gallagher E, Hawkes R. Regionalization defects in the weaver mouse cerebellum. J Comp Neurol 1998;394(4):431-44.

127. Everett GM, Borcherding JW. L-DOPA: effect on concentrations of dopamine, norepinephrine, and serotonin in brains of mice. Science 1970;168(933):847-50.

128. Falck B, Hillarp, N., Thieme, G., Torp, A. Fluorescence of catecholamines and related compounds with formaldehyde. J Histochem Cytochem 1962;10:348-354.

129. Fallon JHaL., E. S. Substantia nigra. In: Paxinos G, editor. The rat nervous system: Academic press; 1995.

130. Farnebo LO. Release of monoamines evoked by field stimulation--studies on some ionic and metabolic requirements. Acta Physiol Scand Suppl 1971;371:19-27.

131. Ferre S, Artigas F. Dopamine D2 receptor-mediated regulation of serotonin extracellular concentration in the dorsal raphe nucleus of freely moving rats. J Neurochem 1993;61(2):772-5.

132. Ferre S, Cortes R, Artigas F. Dopaminergic regulation of the serotonergic raphe-striatal pathway: microdialysis studies in freely moving rats. J Neurosci 1994;14(8):4839-46.

133. Filion M, Tremblay, L. and Bedard, P. J. Abnormal influences of passive limb movement on the activity of globus pallidus neurons in parkinsonian monkeys. Brain Res 1988;444:165-176.

134. Filion MaT, L. Abnormal spontaneous activity of globus pallidus neurons in monkey with MPTP-induced parkinsonism. Brain Res 1991;547:142-151.

135. Fishell G, van der Kooy D. Pattern formation in the striatum: developmental changes in the distribution of striatonigral neurons. J Neurosci 1987;7(7):1969-78.

136. Fredriksson A, Plaznik A, Sundstrom E, Jonsson G, Archer T. MPTP-induced hypoactivity in mice: reversal by L-dopa. Pharmacol Toxicol 1990;67(4):295-301.

137. Fujiwara H, Uemoto M, Tanaka C. Stimulation of the rat dorsal raphe in vivo releases labeled serotonin from the parietal cortex. Brain Res 1981;216(2):351-60.

138. Fuxe K, Jonsson G. A modification of the histochemical fluorescence method for the improved localization of 5-hydroxytryptamine. Histochemie 1967;11(2):161-6.

139. Fuxe K, Jonsson G. Further mapping of central 5-hydroxytryptamine neurons: studies with the neurotoxic dihydroxytryptamines. Adv Biochem Psychopharmacol 1974;10:1-12.

140. Gad SC. A neuromuscular screen for use in industrial toxicology. J Toxicol Environ Health 1982;9(5-6):691-704.

141. Garcia-Munoz M, Young SJ, Groves PM. Terminal excitability of the corticostriatal pathway. I. Regulation by dopamine receptor stimulation. Brain Res 1991;551(1-2):195-206.

142. Garris PA, Wightman RM. Different kinetics govern dopaminergic transmission in the amygdala, prefrontal cortex, and striatum: an in vivo voltammetric study. J Neurosci 1994;14(1):442-50.

143. Gartside SE, McQuade R, Sharp T. Effects of repeated administration of 3,4-methylenedioxymethamphetamine on 5-hydroxytryptamine neuronal activity and release in the rat brain in vivo. J Pharmacol Exp Ther 1996;279(1):277-83.

144. Georgopoulos AP, Crutcher, M. D. and Schwartz, A. A. Cognitive spatial motor processes .3. Motor cortical prediction of movement direction during an instructed delay period. Exp. Brain Res 1989;75:183-194.

145. Gerfen CR. The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems. Nature 1984;311(5985):461-4.

146. Gerfen CR. The neostriatal mosaic. I. Compartmental organization of projections from the striatum to the substantia nigra in the rat. J Comp Neurol 1985;236(4):454-76.

147. Gerfen CR. The neostriatal mosaic: striatal patch-matrix organization is related to cortical lamination. Science 1989;246(4928):385-8.

148. Gerfen CR. The neostriatal mosaic: multiple levels of compartmental organization in the basal ganglia. Annu Rev Neurosci 1992;15:285-320.

149. Geyer MA, Puerto A, Menkes DB, Segal DS, Mandell AJ. Behavioral studies following lesions of the mesolimbic and mesostriatal serotonergic pathways. Brain Res 1976;106(2):257-69.

150. Gill R, Foster AC, Woodruff GN. Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. J Neurosci 1987;7(10):3343-9.

151. Globus MY, Busto R, Dietrich WD, Martinez E, Valdes I, Ginsberg MD. Effect of ischemia on the in vivo release of striatal dopamine, glutamate, and gamma-aminobutyric acid studied by intracerebral microdialysis. J Neurochem 1988;51(5):1455-64.

152. Goicoechea C, Ormazabal MJ, Alfaro MJ, Martin MI. Age-related changes in nociception, behavior, and monoamine levels in rats. Gen Pharmacol 1997;28(2):331-6.

153. Goldman PSaN, W. J. H. An intricately patterned prefronto-Caudate projection in the rhesus monkey. J. Comp. Neurol 1977;171:369-386.

154. Goodnight JA, Mischak H, Kolch W, Mushinski JF. Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. Isoform-specific association with microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes. J Biol Chem 1995;270(17):9991-10001.

155. Goridis C, Rohrer H. Specification of catecholaminergic and serotonergic neurons. Nat Rev Neurosci 2002;3(7):531-41.

156. Gothert M, Weinheimer G. Extracellular 5-hydroxytryptamine inhibits 5-hydroxytryptamine release from rat brain cortex slices. Naunyn Schmiedebergs Arch Pharmacol 1979;310(1):93-6.

157. Graeff FG, Guimaraes FS, De Andrade TG, Deakin JF. Role of 5-HT in stress, anxiety, and depression. Pharmacol Biochem Behav 1996;54(1):129-41.

158. Graham JGaO, D. R. Orthostatic hypotension and nicotine sensitivy in a case of multiple system atrophy. J.Neurol Neurosurg Psychiatry 1969;32:28-34.

159. Grahame-Smith DG. Studies in vivo on the relationship between brain tryptophan, brain 5-HT synthesis and hyperactivity in rats treated with a monoamine oxidase inhibitor and L-tryptophan. J Neurochem 1971;18(6):1053-66.

160. Graybiel AM, Ragsdale CW, Jr. Histochemically distinct compartments in the striatum of human, monkeys, and cat demonstrated by acetylthiocholinesterase staining. Proc Natl Acad Sci U S A 1978;75(11):5723-6.

161. Green AR, Cross AJ, Goodwin GM. Review of the pharmacology and clinical pharmacology of 3,4-methylenedioxymethamphetamine (MDMA or "Ecstasy"). Psychopharmacology (Berl) 1995;119(3):247-60.

162. Grube D. Immunoperoxidase methods: increased efficiency using fluorescence microscopy for 3,3-diaminobenzidine (DAB) stained semithin sections. Histochemistry 1980;70(1):19-22.

163. Guillery R. Degeneration in the hypothalamic connexions of the albino rat. J Anat 1957;91:91-115.

164. Gundersen HJ. Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J Microsc 1986;143 ( Pt 1):3-45.

165. Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, et al. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. Apmis 1988;96(10):857-81.

166. Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. J Microsc 1987;147 ( Pt 3):229-63.

167. Gupta M, Felten DL, Ghetti B. Selective loss of monoaminergic neurons in weaver mutant mice--an immunocytochemical study. Brain Res 1987;402(2):379-82.

168. Guyton AC. Basic neuroscience, Anatomy and Physiology; 1991.

169. Hajos M, Gartside SE, Sharp T. Inhibition of median and dorsal raphe neurones following administration of the selective serotonin reuptake inhibitor paroxetine. Naunyn Schmiedebergs Arch Pharmacol 1995;351(6):624-9.

170. Hamlin KaF, F. Synthesis of 5-hydroxytryptamine. J Am Chem Soc 1951;73:5007-5008.

171. Hamon M, Bourgoin S, Jagger J, Glowinski J. Effects of LSD on synthesis and release of 5-HT in rat brain slices. Brain Res 1974;69(2):265-80.

172. Harvey JA, Schlosberg AJ, Yunger LM. Effect of p-chlorophenylalanine and brain lesions on pain sensitivity and morphine analgesia in the rat. Adv Biochem Psychopharmacol 1974;10:233-45.

173. Hashimoto T, Ase K, Sawamura S, Kikkawa U, Saito N, Tanaka C, et al. Postnatal development of a brain-specific subspecies of protein kinase C in rat. J Neurosci 1988;8(5):1678-83.

174. Hatzidimitriou G, McCann UD, Ricaurte GA. Altered serotonin innervation patterns in the forebrain of monkeys treated with (+/-)3,4-methylenedioxymethamphetamine seven years previously: factors influencing abnormal recovery. J Neurosci 1999;19(12):5096-107.

175. Hedera P, Whitehouse, P. J. Neurotransmitters in neurodegeneration. In: Calne DB, editor. Neurodegenerative disease; 1994.

176. Hedreen JCaF, S. E. Early loss of neostriatal striosome neurons in Huntington's disease. J. Neuropath. Exp. Neurol 1995;54:105-120.

177. Heller A, Harvey JA, Moore RY. A demonstration of a fall in brain serotonin following central nervous system lesions in the rat. Biochem Pharmacol 1962;11:859-66.

178. Hensler JG, Ferry RC, Labow DM, Kovachich GB, Frazer A. Quantitative autoradiography of the serotonin transporter to assess the distribution of serotonergic projections from the dorsal raphe nucleus. Synapse 1994;17(1):1-15.

179. Herkenham M, Pert CB. Mosaic distribution of opiate receptors, parafascicular projections and acetylcholinesterase in rat striatum. Nature 1981;291(5814):415-8.

180. Herve D, Pickel VM, Joh TH, Beaudet A. Serotonin axon terminals in the ventral tegmental area of the rat: fine structure and synaptic input to dopaminergic neurons. Brain Res 1987;435(1-2):71-83.

181. Hery F, Faudon M, Ternaux JP. In vivo release of serotonin in two raphe nuclei (raphe dorsalis and magnus) of the cat. Brain Res Bull 1982;8(2):123-9.

182. Hery F, Simonnet G, Bourgoin S, Soubrie P, Artaud F, Hamon M, et al. Effect of nerve activity on the in vivo release of [3H]serotonin continuously formed from L-[3H]tryptophan in the caudate nucleus of the cat. Brain Res 1979;169(2):317-34.

183. Hery F, Ternaux JP. Regulation of release processes in central serotoninergic neurons. J Physiol (Paris) 1981;77(2-3):287-301.

184. Hillegaart V. Effects of local application of 5-HT and 8-OH-DPAT into the dorsal and median raphe nuclei on motor activity in the rat. Physiol Behav 1990;48(1):143-8.

185. Hitri A, Hurd YL, Wyatt RJ, Deutsch SI. Molecular, functional and biochemical characteristics of the dopamine transporter: regional differences and clinical relevance. Clin Neuropharmacol 1994;17(1):1-22.

186. Horikawa K, Powell EW. Comparison of techniques for retrograde labeling using the rat's facial nucleus. J Neurosci Methods 1986;17(4):287-96.

187. Hornykiewicz O, editor. Metabolism of brain dopamine in human parkinsonism: neurochemical and clinical aspects. New York: Raven press; 1966.

188. Hornykiewicz O. Dopamine (3-hydroxytyramine) and brain function. Pharmacol Rev 1966;18(2):925-64.

189. Houchi H, Masserano JM, Weiner N. Bradykinin activates tyrosine hydroxylase in rat pheochromocytoma PC-12 cells. FEBS Lett 1988;235(1-2):137-40.

190. Hsu SM, Raine L, Fanger H. The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technics. Am J Clin Pathol 1981;75(6):816-21.

191. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981;29(4):577-80.

192. Huang FL, Arora PK, Hanna EE, Huang KP. Proteolytic degradation of protein kinase C in the phorbol ester-induced interleukin-2 secreting thymoma cells. Arch Biochem Biophys 1988;267(2):503-14.

193. Huganir RL, Greengard P. Regulation of neurotransmitter receptor desensitization by protein phosphorylation. Neuron 1990;5(5):555-67.

194. Imai H, Narabayashi, H., Sakata, E. 'Pure akinesia' and the later added supranuclear ophthalmoplegia. Adv Neurol 1986;45:207-212.

195. Imai H, Steindler DA, Kitai ST. The organization of divergent axonal projections from the midbrain raphe nuclei in the rat. J Comp Neurol 1986;243(3):363-80.

196. Insel TR, Winslow JT. Serotonin and neuropeptides in affiliative behaviors. Biol Psychiatry 1998;44(3):207-19.

197. Invernizzi R, Carli M, Di Clemente A, Samanin R. Administration of 8-hydroxy-2-(Di-n-propylamino)tetralin in raphe nuclei dorsalis and medianus reduces serotonin synthesis in the rat brain: differences in potency and regional sensitivity. J Neurochem 1991;56(1):243-7.

198. Iravani MM, Kruk ZL. Real-time measurement of stimulated 5-hydroxytryptamine release in rat substantia nigra pars reticulata brain slices. Synapse 1997;25(1):93-102.

199. Iravani MM, Kruk ZL. Real-time measurement of stimulated 5-hydroxytryptamine release in rat substantia nigra pars reticulata brain slices. Synapse 1997;25(1):93-102.

200. Isaacson JS, Nicoll RA. The uptake inhibitor L-trans-PDC enhances responses to glutamate but fails to alter the kinetics of excitatory synaptic currents in the hippocampus. J Neurophysiol 1993;70(5):2187-91.

201. Ishida Y, Hashiguchi H, Todaka K, Kuwahara I, Ishizuka Y, Nakane H, et al. Serotonergic activity in the rat striatum after intrastriatal transplantation of fetal nigra as measured by microdialysis. Brain Res 1998;788(1-2):207-14.

202. Ivens I. Neurotoxicity testing during long-term studies. Neurotoxicol Teratol 1990;12(6):637-41.

203. Iyer RN, Bradberry CW. Serotonin-mediated increase in prefrontal cortex dopamine release: pharmacological characterization. J Pharmacol Exp Ther 1996;277(1):40-7.

204. Jacobs BL, Azmitia EC. Structure and function of the brain serotonin system. Physiol Rev 1992;72(1):165-229.

205. Jacobs BL, Fornal CA. Activity of serotonergic neurons in behaving animals. Neuropsychopharmacology 1999;21(2 Suppl):9S-15S.

206. Jacobs BL, Simon SM, Ruimy DD, Trulson ME. A quantitative rotational model for studying serotonergic function in the rat. Brain Res 1977;124(2):271-81.

207. Jacobs BL, Trimbach C, Eubanks EE, Trulson M. Hippocampal mediation of raphe lesion- and PCPA-induced hyperactivity in the rat. Brain Res 1975;94(2):253-61.

208. Jellinger KA, editor. The pathology of parkinsonism; 1987.

209. Jellinger KA. Pathology of Parkinson's disease. Changes other than the nigrostriatal pathway. Mol Chem Neuropathol 1991;14(3):153-97.

210. Jenner PaM, editor. MPTP: an update on its relevance to the cause and treatment of Parkinson' s disease; 1992.

211. Joh TH, Park DH, Reis DJ. Direct phosphorylation of brain tyrosine hydroxylase by cyclic AMP-dependent protein kinase: mechanism of enzyme activation. Proc Natl Acad Sci U S A 1978;75(10):4744-8.

212. Johnson TNaR, H. E. Topographic projections of the globus pallidus and the substantia nigra of selectively placed lesions in the precommissural caudate nucleus and putamen in the monkey. Exp.Neurol 1971;33:584-596.

213. Jolicoeur FB, Rondeau DB, Hamel E, Butterworth RF, Barbeau A. Measurement of ataxia and related neurological signs in the laboratory rat. Can J Neurol Sci 1979;6(2):209-15.

214. Jonsson G, Einarsson P, Fuxe K, Hallman H. Microspectrofluorimetric analysis of the formaldehyde induced fluorescence in midbrain raphe neurons. Med Biol 1975;53(1):25-39.

215. Jouvet M. The role of monoamines and acetylcholine-containing neurons in the regulation of the sleep-waking cycle. Ergeb Physiol 1972;64:166-307.

216. Jussofie A, Lojeski, J. and Hiemke, C. Simultaneous automated determination of catecholamines, serotonin, and their metabolites in brain tissue by HPLC and electrochemical detection. J. Liquid Chromatography 1993;16:447-463.

217. Kalen P, Karlson M, Wiklund L. Possible excitatory amino acid afferents to nucleus raphe dorsalis of the rat investigated with retrograde wheat germ agglutinin and D-[3H]aspartate tracing. Brain Res 1985;360(1-2):285-97.

218. Kano M, Hashimoto K, Chen C, Abeliovich A, Aiba A, Kurihara H, et al. Impaired synapse elimination during cerebellar development in PKC gamma mutant mice. Cell 1995;83(7):1223-31.

219. Katz LC, Burkhalter A, Dreyer WJ. Fluorescent latex microspheres as a retrograde neuronal marker for in vivo and in vitro studies of visual cortex. Nature 1984;310(5977):498-500.

220. Kaufmann H. Multiple system atrophy. Curr Opin Neurol 1998;11:351-355.

221. Kelland MD, Freeman AS, Chiodo LA. Serotonergic afferent regulation of the basic physiology and pharmacological responsiveness of nigrostriatal dopamine neurons. J Pharmacol Exp Ther 1990;253(2):803-11.

222. Kinsky IA, Jouandet, M. L. and Goldman-Rakia, P. S. Organisation of nigrothalamocortical system in the rhesus monkey. J. Comp. Neurol 1985;236:315-330.

223. Knox JM. High performance liquid chromatography; 1978.

224. Koch DD, Kissinger PT. Current concepts: I. Liquid chromatography with pre-column sample enrichment and electrochemical detection. Regional determination of serotonin and 5-hydroxyindoleacetic acid in brain tissue. Life Sci 1980;26(14):1099-107.

225. Koe BK, Weissman A. p-Chlorophenylalanine: a specific depletor of brain serotonin. J Pharmacol Exp Ther 1966;154(3):499-516.

226. Kohler C, Chan-Palay V, Steinbusch H. The distribution and origin of serotonin-containing fibers in the septal area: a combined immunohistochemical and fluorescent retrograde tracing study in the rat. J Comp Neurol 1982;209(1):91-111.

227. Kohler C, Lorens SA. Open field activity and avoidance behavior following serotonin depletion: a comparison of the effects of parachlorophenylalanine and electrolytic midbrain raphe lesions. Pharmacol Biochem Behav 1978;8(3):223-33.

228. Kosofsky BE, Molliver ME. The serotoninergic innervation of cerebral cortex: different classes of axon terminals arise from dorsal and median raphe nuclei. Synapse 1987;1(2):153-68.

229. Kostowski W, Giacalone E, Garattini S, Valzelli L. Studies on behavioural and biochemical changes in rats after lesion of midbrain raphe. Eur J Pharmacol 1968;4(4):371-6.

230. Kovacs GG, Kloppel S, Fischer I, Dorner S, Lindeck-Pozza E, Birner P, et al. Nucleus-specific alteration of raphe neurons in human neurodegenerative disorders. Neuroreport 2003;14(1):73-6.

231. Kramer KL, Barnette JE, Yost HJ. PKCgamma regulates syndecan-2 inside-out signaling during xenopus left-right development. Cell 2002;111(7):981-90.

232. Kristensson K, Olsson Y. Retrograde axonal transport of protein. Brain Res 1971;29(2):363-5.

233. Kunzle H. Bilateral projections from precentral motor cortex to the putamen and other parts of the basal ganglia. An autoradiographic study in macaca fascicularis. Brain Res 1975;38:65-73.

234. Kunzle H. Projections from the primary somatosensory cortex to basal ganglia and thalamus in the monkey. Exp. Brain Res 1977;30:481-492.

235. Lackovic Z, Parenti M, Neff NH. Simultaneous determination of femtomole quantities of 5-hydroxytryptophan, serotonin and 5-hydroxyindoleacetic acid in brain using HPLC with electrochemical detection. Eur J Pharmacol 1981;69(3):347-52.

236. Lackovic Z, Parenti M, Neff NH. Simultaneous determination of femtomole quantities of 5-hydroxytryptophan, serotonin and 5-hydroxyindoleacetic acid in

brain using HPLC with electrochemical detection. Eur J Pharmacol 1981;69(3):347-52.

237. Lam AG, Campbell JM, Bennett NK, Payne AP, Davies RW, Sutcliffe RG, et al. Local cerebral glucose utilization in the AS/AGU rat: a mutant with movement disorders. Eur J Neurosci 1998;10(6):1963-7.

238. Lanciego JL, Wouterlood FG, Erro E, Arribas J, Gonzalo N, Urra X, et al. Complex brain circuits studied via simultaneous and permanent detection of three transported neuroanatomical tracers in the same histological section. J Neurosci Methods 2000;103(1):127-35.

239. Lane JD, Aprison MH. Calcium-dependent release of endogenous serotonin, dopamine and norepinephrine from nerve endings. Life Sci 1977;20(4):665-71.

240. Lang AE, and Lozano, A. M. Parkinsons disease (pt 1). N. Eng. J. Med 1998;339(15):1044-1053.

241. Langelier P, Roberge AG, Boucher R, Poirier LJ. Effects of chronically administered L-dopa in normal and lesioned cats. J Pharmacol Exp Ther 1973;187(1):15-26.

242. Lasley SM, Thurmond JB. Interaction of dietary tryptophan and social isolation on territorial aggression, motor activity, and neurochemistry in mice. Psychopharmacology (Berl) 1985;87(3):313-21.

243. LaVail JH, LaVail MM. Retrograde axonal transport in the central nervous system. Science 1972;176(42):1416-7.

244. Lehericy S, Gerardin E. Normal functional imaging of the basal ganglia. Epileptic Disord 2002;4 Suppl 3:S23-30.

245. Leonard BE. Serotonin receptors and their function in sleep, anxiety disorders and depression. Psychother Psychosom 1996;65(2):66-75.

246. Li JW, Watanabe M, Fujisawa Y, Shibuya T. Relation between age-related changes in hyper-emotionality and serotonergic neuronal activities in the rat limbic system. Nihon Shinkei Seishin Yakurigaku Zasshi 1995;15(3):231-8.

247. Lidbrink P, Jonsson G, Fuxe K. Selective reserpine-resistant accumulation of catecholamines in central dopamine neurones after DOPA administration. Brain Res 1974;67(3):439-56.

248. Lidov HG, Grzanna R, Molliver ME. The serotonin innervation of the cerebral cortex in the rat--an immunohistochemical analysis. Neuroscience 1980;5(2):207-27.

249. Lidov HG, Molliver ME. Immunohistochemical study of the development of serotonergic neurons in the rat CNS. Brain Res Bull 1982;9(1-6):559-604.

250. Lidov HG, Molliver ME. An immunohistochemical study of serotonin neuron development in the rat: ascending pathways and terminal fields. Brain Res Bull 1982;8(4):389-430.

251. Limousin P, Pollak, P., Benazzouz, A., Hoffmann, D., Libas, J. F., Broussolle, E., Perret, J. E. and Benabid, A. L. Effects on parkinsonian signs and symptoms of bilateral subthalamic nucleus stimulation. Lancet 1995;345:91-94.

252. Limousin P, Pollak P, Benazzouz A, Hoffmann D, Le Bas JF, Broussolle E, et al. Effect of parkinsonian signs and symptoms of bilateral subthalamic nucleus stimulation. Lancet 1995;345(8942):91-5.

253. Lindvall O, Bjorklund A. The glyoxylic acid fluorescence histochemical method: a detailed account of the methodology for the visualization of central catecholamine neurons. Histochemistry 1974;39(2):97-127.

254. Lindvall O, Bjorklund A. The organization of the ascending catecholamine neuron systems in the rat brain as revealed by the glyoxylic acid fluorescence method. Acta Physiol Scand Suppl 1974;412:1-48.

255. Lloyd KG, Davidson, L. and Hornykiewicz, O. The neurochemistry of parkinson's disease: Effects of L-dopa therapy. J. Pharmacol. Exp. Ther 1975;195:453-464.

256. Liposits Z, Gorcs T, Trombitas K. Ultrastructural analysis of central serotoninergic neurons immunolabeled by silver-gold-intensified diaminobenzidine chromogen. Completion of immunocytochemistry with X-ray microanalysis. J Histochem Cytochem 1985;33(6):604-10.

257. Lipska BK, Jaskiw GE, Arya A, Weinberger DR. Serotonin depletion causes long-term reduction of exploration in the rat. Pharmacol Biochem Behav 1992;43(4):1247-52.

258. Liu K, Hsiung S, Adlersberg M, Sacktor T, Gershon MD, Tamir H. Ca(2+)-evoked serotonin secretion by parafollicular cells: roles in signal transduction of phosphatidylinositol 3'-kinase, and the gamma and zeta isoforms of protein kinase C. J Neurosci 2000;20(4):1365-73.

259. Liu KP, Russo AF, Hsiung SC, Adlersberg M, Franke TF, Gershon MD, et al. Calcium receptor-induced serotonin secretion by parafollicular cells: role of phosphatidylinositol 3-kinase-dependent signal transduction pathways. J Neurosci 2003;23(6):2049-57.

260. Llewellyn-Smith IJ, Minson JB, Wright AP, Hodgson AJ. Cholera toxin B-gold, a retrograde tracer that can be used in light and electron microscopic immunocytochemical studies. J Comp Neurol 1990;294(2):179-91.

261. Loizou LA. The postnatal ontogeny of monoamine-containing neurones in the central nervous system of the albino rat. Brain Res 1972;40(2):395-418.

262. Loren I, Bjorklund A, Falck B, Lindvall O. [An improved histofluorescence procedure for freeze-dried paraffin-embedded tissue based on combined formaldehyde-glyoxylic acid perfusion with high magnesium content and acid pH.]. Histochemistry 1976;49(3):177-92.

263. Lorens SA, Guldberg HC, Hole K, Kohler C, Srebro B. Activity, avoidance learning and regional 5-hydroxytryptamine following intra-brain stem 5,7-dihydroxytryptamine and electrolytic midbrain raphe lesions in the rat. Brain Res 1976;108(1):97-113.

264. Lorens SA, Sorensen JP, Yunger LM. Behavioral and neurochemical effects of lesions in the raphe system of the rat. J Comp Physiol Psychol 1971;77(1):48-52.

265. Lorez HP, Saner A, Richards JG. Evidence against a neurotoxic action of halogenated amphetamines on serotoninergic B9 cells. A morphometric fluorescence histochemical study. Brain Res 1978;146(1):188-94.

266. Low WC, Triarhou LC, Kaseda Y, Norton J, Ghetti B. Functional innervation of the striatum by ventral mesencephalic grafts in mice with inherited nigrostriatal dopamine deficiency. Brain Res 1987;435(1-2):315-21.

267. Lowry OH, Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem 1951;193:265-275.

268. Lozano AM, Lang AE, Galvez-Jimenez N, Miyasaki J, Duff J, Hutchinson WD, et al. Effect of GPi pallidotomy on motor function in Parkinson's disease. Lancet 1995;346(8987):1383-7.

269. Luppi PH, Sakai K, Salvert D, Fort P, Jouvet M. Peptidergic hypothalamic afferents to the cat nucleus raphe pallidus as revealed by a double immunostaining technique using unconjugated cholera toxin as a retrograde tracer. Brain Res 1987;402(2):339-45.

270. Lyness WH, Friedle NM, Moore KE. Current concepts: II. Measurement of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in discrete brain nuclei using reverse phase liquid chromatography with electrochemical detection. Life Sci 1980;26(14):1109-14.

271. Lyness WH, Friedle NM, Moore KE. Current concepts: II. Measurement of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in discrete brain nuclei using reverse phase liquid chromatography with electrochemical detection. Life Sci 1980;26(14):1109-14.

272. Madison DV, Malenka RC, Nicoll RA. Phorbol esters block a voltage-sensitive chloride current in hippocampal pyramidal cells. Nature 1986;321(6071):695-7.

273. Magyar RL, Gillin JC, Wyatt RJ. Tolerance to the increased locomotor activity produced by L-5-hydroxytryptophan following peripheral decarboxylase inhibition in mice. Psychopharmacology (Berl) 1978;56(3):343-50.

274. Malenka RC, Ayoub GS, Nicoll RA. Phorbol esters enhance transmitter release in rat hippocampal slices. Brain Res 1987;403(1):198-203.

275. Malenka RC, Madison DV, Nicoll RA. Potentiation of synaptic transmission in the hippocampus by phorbol esters. Nature 1986;321(6066):175-7.

276. Maley BE, Engle MG, Humphreys S, Vascik DA, Howes KA, Newton BW, et al. Monoamine synaptic structure and localization in the central nervous system. J Electron Microsc Tech 1990;15(1):20-33.

277. Malleret G, Hen R, Guillou JL, Segu L, Buhot MC. 5-HT1B receptor knock-out mice exhibit increased exploratory activity and enhanced spatial memory performance in the Morris water maze. J Neurosci 1999;19(14):6157-68.

278. Mamounas LA, Molliver ME. Evidence for dual serotonergic projections to neocortex: axons from the dorsal and median raphe nuclei are differentially vulnerable to the neurotoxin p-chloroamphetamine (PCA). Exp Neurol 1988;102(1):23-36.

279. Mamounas LA, Mullen CA, O'Hearn E, Molliver ME. Dual serotoninergic projections to forebrain in the rat: morphologically distinct 5-HT axon terminals exhibit differential vulnerability to neurotoxic amphetamine derivatives. J Comp Neurol 1991;314(3):558-86.

280. Mann DM, Yates PO, Hawkes J. The pathology of the human locus ceruleus. Clin Neuropathol 1983;2(1):1-7.

281. Marsden CA, Curzon G. Effects of altered brain 5-hydroxytryptaminergic activity on brain tryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. Neuropharmacology 1976;15(11):703-8.

282. Maruyama W, Naoi M, Takahashi A, Watanabe H, Konagaya Y, Mokuno K, et al. The mechanism of perturbation in monoamine metabolism by L-dopa therapy: in vivo and in vitro studies. J Neural Transm Gen Sect 1992;90(3):183-97.

283. Matelli M, Luppino, G., Fogassi, L. and Rizzolatti, G. Thalamic input to inferior area-6 and area-4 in the macaque monkey. J. Comp. Neurol 1989;280:468-488.

284. Matsuo H, Takashima, H., Kishikawa, M. Pure akinesia: an atypical manifestation of progressive supernuclear palsy. J.Neurol Neurosurg Psychiatry 1991;54:397-400.

285. Matte AC, Tornow H. Parachlorophenylalanine produces dissociated effects on aggression "emotionality" and motor activity. Neuropharmacology 1978;17(8):555-8.

286. Mayeux R, Chen J, Mirabello E, Marder K, Bell K, Dooneief G, et al. An estimate of the incidence of dementia in idiopathic Parkinson's disease. Neurology 1990;40(10):1513-7.

287. Mayhew TM. A review of recent advances in stereology for quantifying neural structure. J Neurocytol 1992;21(5):313-28.

288. McIntyre TA, Souder MG, Hartl MW, Shibley IA. Ethanol-induced decrease of developmental PKC isoform expression in the embryonic chick brain. Brain Res Dev Brain Res 1999;117(2):191-7.

289. McNaught KS, Shashidharan P, Perl DP, Jenner P, Olanow CW. Aggresome-related biogenesis of Lewy bodies. Eur J Neurosci 2002;16(11):2136-48.

290. McQuade R, Sharp T. Functional mapping of dorsal and median raphe 5-hydroxytryptamine pathways in forebrain of the rat using microdialysis. J Neurochem 1997;69(2):791-6.

291. Meert TF. Ritanserin overcomes exploratory inhibition induced by cocaine withdrawal. Behav Pharmacol 1992;3(2):149-154.

292. Meert TF, Melis W, Aerts N, Clincke G. Antagonism of meta-chlorophenylpiperazine-induced inhibition of exploratory activity in an emergence procedure, the open field test, in rats. Behav Pharmacol 1997;8(4):353-63.

293. Mefford IN, Barchas JD. Determination of tryptophan and metabolites in rat brain and pineal tissue by reversed-phase high-performance liquid chromatography with electrochemical detection. J Chromatogr 1980;181(2):187-93.

294. Melamed E, Globus, M., Uzzan, A. and Roseenthal, J. Is dopamine formed from exogenous L-dopa stored within vesicles in striatal dopaminergic nerve terminals: Implications for L-dopa's mechanism of action in parkinson's disease. Neurology (suppl.) 1985;35:118.

295. Meltzer CC, Smith G, DeKosky ST, Pollock BG, Mathis CA, Moore RY, et al. Serotonin in aging, late-life depression, and Alzheimer's disease: the emerging role of functional imaging. Neuropsychopharmacology 1998;18(6):407-30.

296. Miller FP, Maickel RP. Fluorometric determination of indole derivatives. Life Sci I 1970;9(13):747-52.

297. Minabe Y, Emori K, Ashby CR, Jr. The depletion of brain serotonin levels by para-chlorophenylalanine administration significantly alters the activity of midbrain dopamine cells in rats: an extracellular single cell recording study. Synapse 1996;22(1):46-53.

298. Mitchell SJ, Richardson, R. T., Baker, F. H. and De Long, M. R. The primate globus pallidus-neuronal activity related direction of movement. Exp. Brain Res 1987;68:419-505.

299. Modigh K. Effects of chlorimipramine and protriptyline on the hyperactivity induced by 5-hydroxytryptophan after peripheral decarboxylase inhibition in mice. J Neural Transm 1973;34(2):101-9.

300. Modigh K, Svensson TH. On the role of central nervous system catecholamines and 5-hydroxytryptamine in the nialamide-induced behavioural syndrome. Br J Pharmacol 1972;46(1):32-45.

301. Moir AT, Eccleston D. The effects of precursor loading in the cerebral metabolism of 5-hydroxyindoles. J Neurochem 1968;15(10):1093-108.

302. Moore RY, Bhatnagar, R. K. and Heller, A. Anatomical and chemical studies of nigrostriatal projection in the cat. Brain Res 1971;30:119-135.

303. Moore RY, Halaris AE, Jones BE. Serotonin neurons of the midbrain raphe: ascending projections. J Comp Neurol 1978;180(3):417-38.

304. Morgan DG, May PC, Finch CE. Dopamine and serotonin systems in human and rodent brain: effects of age and neurodegenerative disease. J Am Geriatr Soc 1987;35(4):334-45.

305. Mosko SS, Haubrich D, Jacobs BL. Serotonergic afferents to the dorsal raphe nucleus: evdience from HRP and synaptosomal uptake studies. Brain Res 1977;119(2):269-90.

306. Moukhles H, Bosler O, Bolam JP, Vallee A, Umbriaco D, Geffard M, et al. Quantitative and morphometric data indicate precise cellular interactions between serotonin terminals and postsynaptic targets in rat substantia nigra. Neuroscience 1997;76(4):1159-71.

307. Mukhida K, Baker KA, Sadi D, Mendez I. Enhancement of sensorimotor behavioral recovery in hemiparkinsonian rats with intrastriatal, intranigral, and intrasubthalamic nucleus dopaminergic transplants. J Neurosci 2001;21(10):3521-30.

308. Mulder AH, van den Berg WB, Stoof JC. Calcium-dependent release of radiolabeled catecholamines and serotonin from rat brain synaptosomes in a superfusion system. Brain Res 1975;99(2):419-24.

309. Murphy DL, Li Q, Engel S, Wichems C, Andrews A, Lesch KP, et al. Genetic perspectives on the serotonin transporter. Brain Res Bull 2001;56(5):487-94.

310. Nakamura Y, Ohmaki M, Murakami K, Yoneda Y. Involvement of protein kinase C in glutamate release from cultured microglia. Brain Res 2003;962(1-2):122-8.

311. Nakanishi H, Kita, H. and Kitai, S. T. Intracellular study of rat substantia nigra pars reticulata neurons in an in vitro slice preparation: electrical membrane properties and response characteristics to subthalamic stimulation. Brain Res 1987;437:45-55.

312. Nakano I, Hirano A. Neuron loss in the nucleus basalis of Meynert in parkinsonism-dementia complex of Guam. Ann Neurol 1983;13(1):87-91.

313. Narita M, Mizoguchi H, Suzuki T, Dun NJ, Imai S, Yajima Y, et al. Enhanced mu-opioid responses in the spinal cord of mice lacking protein kinase Cgamma isoform. J Biol Chem 2001;276(18):15409-14.

314. Nedergaard S, Bolam JP, Greenfield SA. Facilitation of a dendritic calcium conductance by 5-hydroxytryptamine in the substantia nigra. Nature 1988;333(6169):174-7.

315. Nedergaard S, Engberg I, Flatman JA. The modulation of excitatory amino acid responses by serotonin in the cat neocortex in vitro. Cell Mol Neurobiol 1987;7(4):367-79.

316. Neef NH, Tozer TN, Brodie BB. Application of seady-state kinetics to studies of the transfer of 5-hydroxyindoleacetic acid from brain to plasma. J Pharmacol Exp Ther 1967;158(2):214-8.

317. Neff NH, Tozer TN. In vivo measurement of brain serotonin turnover. Adv Pharmacol 1968;6(Pt A):97-109.

318. Neill DB, Grant LD, Grossman SP. Selective potentiation of locomotor effects of amphetamine by midbrain raphe lesions. Physiol Behav 1972;9(4):655-7.

319. Ng KY, Chase TN, Kopin IJ. Drug-induced release of 3H-norepinephrine and 3H-serotonin from brain slices. Nature 1970;228(270):468-9.

320. Nishizuka Y. Turnover of inositol phospholipids and signal transduction. Science 1984;225(4668):1365-70.

321. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 1984;308(5961):693-8.

322. Nishizuka Y. Perspectives on the role of protein kinase C in stimulus-response coupling. J Natl Cancer Inst 1986;76(3):363-70.

323. Nowicki S, Kruse MS, Brismar H, Aperia A. Dopamine-induced translocation of protein kinase C isoforms visualized in renal epithelial cells. Am J Physiol Cell Physiol 2000;279(6):C1812-8.

324. Nuwayhid SJ, Werling LL. Sigma1 receptor agonist-mediated regulation of N-methyl-D-aspartate-stimulated [3H]dopamine release is dependent upon protein kinase C. J Pharmacol Exp Ther 2003;304(1):364-9.

325. Nygren LG, Olson L. Intracisternal neurotoxins and monoamine neurons innervating the spinal cord: acute and chronic effects on cell and axon counts and nerve terminal densities. Histochemistry 1977;52(4):281-306.

326. Obeso JA, Rodriguez-Oroz MC, Rodriguez M, Arbizu J, Gimenez-Amaya JM. The basal ganglia and disorders of movement: pathophysiological mechanisms. News Physiol Sci 2002;17:51-5.

327. Okada F, Saito Y, Fujieda T, Yamashita I. Monoamine changes in the brain of rats injected with L-5-hydroxytryptophan. Nature 1972;238(5363):355-6.

328. Olanow CWaT, W. G. Etiology and pathogenesis of parkinson's disease. Ann. Rev. Neurosci 1999;22:123-144.

329. Oorschot DE. Are you using neuronal densities, synaptic densities or neurochemical densities as your definitive data? There is a better way to go. Prog Neurobiol 1994;44(3):233-47.

330. Oorschot DE, Peterson DA, Jones DG. Neurite growth from, and neuronal survival within, cultured explants of the nervous system: a critical review of morphometric and stereological methods, and suggestions for the future. Prog Neurobiol 1991;37(6):525-46.

331. Orosz D, Bennett JP. Simultaneous microdialysis in striatum and substantia nigra suggests that the nigra is a major site of action of L-dihydroxyphenylalanine in the "hemiparkinsonian" rat. Exp Neurol 1992;115(3):388-93.

332. Osborne NN. Uptake, localization and release of serotonin in the chick retina. J Physiol 1982;331:469-79.

333. Pakkenberg B, Moller A, Gundersen HJ, Mouritzen Dam A, Pakkenberg H. The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method. J Neurol Neurosurg Psychiatry 1991;54(1):30-3.

334. Pan HS, Walters JR. Unilateral lesion of the nigrostriatal pathway decreases the firing rate and alters the firing pattern of globus pallidus neurons in the rat. Synapse 1988;2(6):650-6.

335. Papadopoulos GC, Parnavelas JG, Buijs RM. Light and electron microscopic immunocytochemical analysis of the dopamine innervation of the rat visual cortex. J Neurocytol 1989;18(3):303-10.

336. Parent A, Bouchard, C. and Smith, Y. The striatopallidal and striatonigral projections-2 distinct fibre system in primate. Brain Res 1984;303:385-390.

337. Parent A. Extrinsic connections of basal ganglia. Trends Neurosci 1990;13:254-258.

338. Parent A, Hazrati LN. Functional anatomy of the basal ganglia. I. The cortico-basal ganglia-thalamo-cortical loop. Brain Res Brain Res Rev 1995;20(1):91-127.

339. Parent A, Hazrati LN. Functional anatomy of the basal ganglia. II. The place of subthalamic nucleus and external pallidum in basal ganglia circuitry. Brain Res Brain Res Rev 1995;20(1):128-54.

340. Parsons LH, Justice JB, Jr. Perfusate serotonin increases extracellular dopamine in the nucleus accumbens as measured by in vivo microdialysis. Brain Res 1993;606(2):195-9.

341. Payne AP, Campbell JM, Russell D, Favor G, Sutcliffe RG, Bennett NK, et al. The AS/AGU rat: a spontaneous model of disruption and degeneration in the nigrostriatal dopaminergic system. J Anat 2000;196 ( Pt 4):629-33.

342. Payne AP, Sutcliffe RG, Campbell JM, Favor G, Russell D, Bennett NK, et al. Disordered locomotion in the AS/AGU mutant rat and the effects of L-dopa or fetal midbrain grafts. Mov Disord 1998;13(5):832-4.

343. Pazos A, Engel G, Palacios JM. beta-Adrenoceptor blocking agents recognize a subpopulation of serotonin receptors in brain. Brain Res 1985;343(2):403-8.

344. Pazos A, Probst A, Palacios JM. Serotonin receptors in the human brain--III. Autoradiographic mapping of serotonin-1 receptors. Neuroscience 1987;21(1):97-122.

345. Penney JBaY, A. B. GABA as the pallidothalamic neurotransmitter-implications for basal ganglia function. Brain Res 1981;207:195-199.

346. Pessia M, Jiang ZG, North RA, Johnson SW. Actions of 5-hydroxytryptamine on ventral tegmental area neurons of the rat in vitro. Brain Res 1994;654(2):324-30.

347. Peyron C, Petit JM, Rampon C, Jouvet M, Luppi PH. Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. Neuroscience 1998;82(2):443-68.

348. Pierce ET, Foote WE, Hobson JA. The efferent connection of the nucleus raphe dorsalis. Brain Res 1976;107(1):137-44.

349. Pletscher A, and Gey, K. F. Topographical differences in the cerebral metabolism of DL- 3,4-dihydroxyphenylalanine. Experientia 1962;18:512-513.

350. Poirier LJ, Singh P, Sourkes TL, Boucher R. Effect of amine precursors on the concentration of striatal dopamine and serotonin in cats with and without unilateral brain stem lesions. Brain Res 1967;6(4):654-66.

351. Poirier LJ, Singh P, Sourkes TL, Boucher R. Effect of amine precursors on the concentration of striatal dopamine and serotonin in cats with and without unilateral brain stem lesions. Brain Res 1967;6(4):654-66.

352. Pollack AE. Anatomy, physiology, and pharmacology of the basal ganglia. Neurol Clin 2001;19(3):523-34, v.

353. Porter RH, Greene, J. G., Higgins, D. S., Greenamyre, J. T. Polysynaptic regulation of glutamate receptors and mitochondrial enzyme activities in the basal ganglia of rats with unilateral dopamine depletion. Synapse 1994;2:650-656.

354. Prisco S, Pagannone S, Esposito E. Serotonin-dopamine interaction in the rat ventral tegmental area: an electrophysiological study in vivo. J Pharmacol Exp Ther 1994;271(1):83-90.

355. Raadsheer FC, Oorschot DE, Verwer RW, Tilders FJ, Swaab DF. Age-related increase in the total number of corticotropin-releasing hormone neurons in the human paraventricular nucleus in controls and Alzheimer's disease: comparison of the disector with an unfolding method. J Comp Neurol 1994;339(3):447-57.

356. Reader TA. The effects of dopamine, noradrenaline and serotonin in the visual cortex of the cat. Experientia 1978;34(12):1586-8.

357. Reader TA, Masse P, de Champlain J. The intracortical distribution of norepinephrine, dopamine and serotonin in the cerebral cortex of the cat. Brain Res 1979;177(3):499-513.

358. Reddy PH, William, M. and Tagle, D. A. Recent advances in understanding the pathogenesis of Huntington's disease. Trends Neurosci 1999;22:248-255.

359. Reiner A, Albin, R. L., Adenerson, K. D.., Damoto, C. J., Penney, J. B. and Young, A. B. Differential loss of striatal projection neurons in Huntington's disease. Proc Natl Acad Sci 1988;85:5733-5737.

360. Rinne UK, Sonninen V, Riekkinen P, Laaksonen H. Dopaminergic nervous transmission in Parkinson's disease. Med Biol 1974;52(3):208-17.

361. Rinne UK, Sonninen V, Sirtola T. Treatment of Parkinson's disease with L-DOPA and decarboxylase inhibitor. Z Neurol 1972;202(1):1-20.

362. Rocklin RD. Electrochemical detection. In: Parriot D, editor. A practical guide to HPLC detection; 1993.

363. Roffler-Tarlov S, Graybiel AM. Weaver mutation has differential effects on the dopamine-containing innervation of the limbic and nonlimbic striatum. Nature 1984;307(5946):62-6.

364. Ross RA, Joh TH, Reis DJ. Increase in the relative rate of synthesis of dopamine-beta-hydroxylase in the nucleus locus coeruleus elicited by reserpine. J Neurochem 1978;31(6):1491-500.

365. Rotman A. The effect of phospholipase C, phospholipase A2 and neuraminidase on the uptake of [3H]norepinephrine and [3H]serotonin by rat brain synaptosomes. J Neurochem 1977;28(6):1367-72.

366. Rouiller EM, Yu XH, Moret V, Tempini A, Wiesendanger M, Liang F. Dexterity in adult monkeys following early lesion of the motor cortical hand area: the role of cortex adjacent to the lesion. Eur J Neurosci 1998;10(2):729-40.

367. Routtenberg A. Protein kinase C activation leading to protein F1 phosphorylation may regulate synaptic plasticity by presynaptic terminal growth. Behav Neural Biol 1985;44(2):186-200.

368. Rozas G, Lopez-Martin E, Guerra MJ, Labandeira-Garcia JL. The overall rod performance test in the MPTP-treated-mouse model of Parkinsonism. J Neurosci Methods 1998;83(2):165-75.

369. Rubinstein M, Phillips TJ, Bunzow JR, Falzone TL, Dziewczapolski G, Zhang G, et al. Mice lacking dopamine D4 receptors are supersensitive to ethanol, cocaine, and methamphetamine. Cell 1997;90(6):991-1001.

370. Rueter LE, Jacobs BL. Changes in forebrain serotonin at the light-dark transition: correlation with behaviour. Neuroreport 1996;7(5):1107-11.

371. Russell D. The effects of age and L-Dopa replacement on locomotor deficits in the AS/AGU mutant rat [MSc]: of Glasgow; 2001.

372. Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. J Neurosci 2002;22(24):10690-8.

373. Saavedra JM, Brownstein M, Axelrod J. A specific and sensitive enzymatic-isotopic microassay for serotonin in tissues. J Pharmacol Exp Ther 1973;186(3):508-15.

374. Saavedra JM, Brownstein M, Axelrod J. A specific and sensitive enzymatic-isotopic microassay for serotonin in tissues. J Pharmacol Exp Ther 1973;186(3):508-15.

375. Saito N, Tsujino T, Fukuda K, Tanaka C. Alpha-, beta II- and gamma-subspecies of protein kinase C localized in the monkey hippocampus: pre- and post-synaptic localization of gamma-subspecies. Brain Res 1994;656(2):245-56.

376. Sakai K, Gash DM. Effect of bilateral 6-OHDA lesions of the substantia nigra on locomotor activity in the rat. Brain Res 1994;633(1-2):144-50.

377. Sauer H, Frodl EM, Kupsch A, ten Bruggencate G, Oertel WH. Cryopreservation, survival and function of intrastriatal fetal mesencephalic grafts in a rat model of Parkinson's disease. Exp Brain Res 1992;90(1):54-62.

378. Scatton B, Javoy-Agid F, Rouquier L, Dubois B, Agid Y. Reduction of cortical dopamine, noradrenaline, serotonin and their metabolites in Parkinson's disease. Brain Res 1983;275(2):321-8.

379. Schlosberg AJ, Harvey JA. Effects of L-dopa and L-5-hydroxytryptophan on locomotor activity of the rat after selective or combined destruction of central catecholamine and serotonin neurons. J Pharmacol Exp Ther 1979;211(2):296-304.

380. Schoen SW, Graybiel AM. 5'-nucleotidase: a new marker for striosomal organization in the rat caudoputamen. J Comp Neurol 1992;322(4):566-76.

381. Schwab ME, Suda K, Thoenen H. Selective retrograde transsynaptic transfer of a protein, tetanus toxin, subsequent to its retrograde axonal transport. J Cell Biol 1979;82(3):798-810.

382. Selemon LDaG-R, P. S. Longitudinal topography and interdigitation of corticostriatal projections in the rhesus monkey. J.Neurosci 1985;5:776-794.

383. Sharp T, Bramwell SR, Grahame-Smith DG. 5-HT1 agonists reduce 5-hydroxytryptamine release in rat hippocampus in vivo as determined by brain microdialysis. Br J Pharmacol 1989;96(2):283-90.

384. Sharp T, Hjorth S. Application of brain microdialysis to study the pharmacology of the 5-HT1A autoreceptor. J Neurosci Methods 1990;34(1-3):83-90.

385. Shearman MS, Sekiguchi K, Nishizuka Y. Modulation of ion channel activity: a key function of the protein kinase C enzyme family. Pharmacol Rev 1989;41(2):211-37.

386. Shimizu N, Duan SM, Hori T, Oomura Y. Glutamate modulates dopamine release in the striatum as measured by brain microdialysis. Brain Res Bull 1990;25(1):99-102.

387. Sibley DR. New insights into dopaminergic receptor function using antisense and genetically altered animals. Annu Rev Pharmacol Toxicol 1999;39:313-41.

388. Sinton CM, Fallon SL. Electrophysiological evidence for a functional differentiation between subtypes of the 5-HT1 receptor. Eur J Pharmacol 1988;157(2-3):173-81.

389. Smialowska M. Histofluorescence studies of central monoamines. Comparison of Falck, cryostat and SPG methods. Folia Histochem Cytochem (Krakow) 1979;17(3):215-24.

390. Smith YaP, A. Neurons of the subthalamic nucleus on primate display glutamate but not GABA immunoreactivity. Brain Res 1988;453:353-356.

391. Snyder SH, Axelrod J, Zweig M. A sensitive and specific fluorescence assay for tissue serotonin. Biochem Pharmacol 1965;14(5):831-5.

392. Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. Nat Rev Neurosci 2003;4(1):49-60.

393. Srebro B, Lorens SA. Behavioral effects of selective midbrain raphe lesions in the rat. Brain Res 1975;89(2):303-25.

394. Stanton GB, Goldberg, M. E. and Bruce, C. J. Frontal eye field efferents in the macaque monkey.1. Subcortical pathways and topography of striatal and thalamic terminal field. J. Comp. Neurol 1988;271:473-492.

395. Steigrad P, Tobler I, Waser PG, Borbely AA. Effect of p-chlorophenylalanine on cerebral serotonin binding, serotonin concentration and motor activity in the rat. Naunyn Schmiedebergs Arch Pharmacol 1978;305(2):143-8.

396. Steinbusch HW. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neuroscience 1981;6(4):557-618.

397. Steinbusch HW, Verhofstad AA, Joosten HW. Localization of serotonin in the central nervous system by immunohistochemistry: description of a specific and sensitive technique and some applications. Neuroscience 1978;3(9):811-9.

398. Steinbusch HW, Verhofstad AA, Penke B, Varga J, Joosten HW. Immunohistochemical characterization of monoamine-containing neurons in the central nervous system by antibodies to serotonin and noradrenalin. A study in the rat and the lamprey (Lampetra fluviatilis). Acta Histochem Suppl 1981;24:107-22.

399. Steinbusch HW, Verhofstad AA, Penke B, Varga J, Joosten HW. Immunohistochemical characterization of monoamine-containing neurons in the central nervous system by antibodies to serotonin and noradrenalin. A study in the rat and the lamprey (Lampetra fluviatilis). Acta Histochem Suppl 1981;24:107-22.

400. Steiner H, Fuchs S, Accili D. D3 dopamine receptor-deficient mouse: evidence for reduced anxiety. Physiol Behav 1997;63(1):137-41.

401. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 1984;134 ( Pt 2):127-36.

402. Sternau LL, Lust WD, Ricci AJ, Ratcheson R. Role for gamma-aminobutyric acid in selective vulnerability in gerbils. Stroke 1989;20(2):281-7.

403. Stewart RM, Growdon JH, Cancian D, Baldessarini RJ. 5-hydroxytryptophan-induced myoclonus: increased sensitivity to serotonin after intracranial 5,7-dihydroxytryptamine in the adult rat. Neuropharmacology 1976;15(8):449-55.

404. Stoeckel K, Schwab M, Thoenen H. Role of gangliosides in the uptake and retrograde axonal transport of cholera and tetanus toxin as compared to nerve growth factor and wheat germ agglutinin. Brain Res 1977;132(2):273-85.

405. Swaab DF, Uylings HB. Comments on review by Coleman and Flood, 'Neuron Numbers and Dendritic Extent in Normal Aging and Alzheimer's Disease.' Density measures: parameters to avoid. Neurobiol Aging 1987;8(6):574-6.

406. Takeuchi Y, Sawada T, Blunt S, Jenner P, Marsden CD. Effects of 6-hydroxydopamine lesions of the nigrostriatal pathway on striatal serotonin innervation in adult rats. Brain Res 1991;562(2):301-5.

407. Tanji JaK, K. Contrast in neuronal activity in supplementary and precentral motor cortex of monkeys .1. Responses to instructions determining motor responses to forthcoming signals of different modalities. J.neurophysiol 1985;53:129-141.

408. Teitler M, Herrick-Davis K. Multiple serotonin receptor subtypes: molecular cloning and functional expression. Crit Rev Neurobiol 1994;8(3):175-88.

409. Thierry AM, Blanc G, Sobel A, Stinus L, Golwinski J. Dopaminergic terminals in the rat cortex. Science 1973;182(4111):499-501.

410. Thierry AM, Stinus L, Blanc G, Glowinski J. Some evidence for the existence of dopaminergic neurons in the rat cortex. Brain Res 1973;50(1):230-4.

411. Tison F, Mons N, Geffard M, Henry P. The metabolism of exogenous L-dopa in the brain: an immunohistochemical study of its conversion to dopamine

in non-catecholaminergic cells of the rat brain. J Neural Transm Park Dis Dement Sect 1991;3(1):27-39.

412. Tobach E. Manipulation effects, open-field experience and digestive transit time in Wistar male and female rats. Psychol Rep 1966;19(2):375-8.

413. Tohgi H, Abe T, Takahashi S, Takahashi J, Hamato H. Concentrations of serotonin and its related substances in the cerebrospinal fluid of parkinsonian patients and their relations to the severity of symptoms. Neurosci Lett 1993;150(1):71-4.

414. Tork I. Anatomy of the serotonergic system. Ann N Y Acad Sci 1990;600:9-34; discussion 34-5.

415. Tork I, Turner S. Histochemical evidence for a catecholaminergic (presumably dopaminergic) projection from the ventral mesencephalic tegmentum to visual cortex in the cat. Neurosci Lett 1981;24(3):215-9.

416. Tremblay L, Filion, M. and . Bedard, P. J. Responses of pallidal neurones to striatal stimulation in intact waking monkeys. Brain Res 1989;498:17-33.

417. Trent F, Tepper JM. Dorsal raphe stimulation modifies striatal-evoked antidromic invasion of nigral dopaminergic neurons in vivo. Exp Brain Res 1991;84(3):620-30.

418. Triarhou LC, Ghetti B. Neuroanatomical substrate of behavioural impairment in weaver mutant mice. Exp Brain Res 1987;68(2):434-6.

419. Triarhou LC, Norton J, Ghetti B. Mesencephalic dopamine cell deficit involves areas A8, A9 and A10 in weaver mutant mice. Exp Brain Res 1988;70(2):256-65.

420. Trojanowski JQ, Gonatas JO, Gonatas NK. Horseradish peroxidase (HRP) conjugates of cholera toxin and lectins are more sensitive retrogradely transported markers than free HRP. Brain Res 1982;231(1):33-50.

421. Tsuji M, Takeda H, Matsumiya T. [Multiplicity of anxiety and serotonin nervous system]. Nippon Yakurigaku Zasshi 2000;115(1):29-38.

422. Tsujino T, Kose A, Saito N, Tanaka C. Light and electron microscopic localization of beta I-, beta II-, and gamma-subspecies of protein kinase C in rat cerebral neocortex. J Neurosci 1990;10(3):870-84.

423. Ungerstedt U. 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. Eur J Pharmacol 1968;5(1):107-10.

424. Ungerstedt U. Stereotaxic mapping of the monoamine pathways in the rat brain. Acta Physiol Scand Suppl 1971;367:1-48.

425. van der Kooy D, Hattori T. Dorsal raphe cells with collateral projections to the caudate-putamen and substantia nigra: a fluorescent retrograde double labeling study in the rat. Brain Res 1980;186(1):1-7.

426. Vergnes M, Mack G, Kempf E. [Behavioral and biochemical effects of lesions of the raphe on rat-mouse interspecific aggressive behavior]. Brain Res 1973;57(1):67-74.

427. Vergnes M, Mack G, Kempf E. [Inhibitory control of mouse-killing behaviour in the rat: serotonergic system of the raphe and olfactory input (author's transl)]. Brain Res 1974;70(3):481-91.

428. Waddington JL, Clifford JJ, McNamara FN, Tomiyama K, Koshikawa N, Croke DT. The psychopharmacology-molecular biology interface: exploring the

behavioural roles of dopamine receptor subtypes using targeted gene deletion ('knockout'). Prog Neuropsychopharmacol Biol Psychiatry 2001;25(4):925-64.

429. Wan XC, Trojanowski JQ, Gonatas JO. Cholera toxin and wheat germ agglutinin conjugates as neuroanatomical probes: their uptake and clearance, transganglionic and retrograde transport and sensitivity. Brain Res 1982;243(2):215-24.

430. Wardas J, Pietraszek M, Ossowska K, Wolfarth S. Specific involvement of striatal D1 and D2 dopamine receptors in the neuroleptic catalepsy in rats. Pol J Pharmacol 1995;47(4):349-53.

431. Watanabe Y. [Interactions of cerebral serotonin and catecholamines]. Nippon Yakurigaku Zasshi 1983;81(5):365-83.

432. Waters CM, Peck, R., Rossor, M., Reynolds, J. P. and Hunt, S. P. Immunocytochemical studies on the basal ganglia and substantia nigra in parkinson's disease and huntington's chorea. Neuroscience 1988;25:419-438.

433. Watts RL, Mirra, S. S. and Richardson, E. P, editor. Corticobasal ganglionic degeneration. London: Butterworths-Heinemann Ltd; 1995.

434. Weinberger J, Nieves-Rosa J, Cohen G. Nerve terminal damage in cerebral ischemia: protective effect of alpha-methyl-para-tyrosine. Stroke 1985;16(5):864-70.

435. Weissmann D, Belin MF, Aguera M, Meunier C, Maitre M, Cash CD, et al. Immunohistochemistry of tryptophan hydroxylase in the rat brain. Neuroscience 1987;23(1):291-304.

436. Wenning GK, Ben-Shlomo, Y., Magalhaes, M., Daniel, S. E., Quinn, N. P. Clinicopathological study of 35 cases of multiple system atrophy. J.Neurol Neurosurg Psychiatry 1995;58:160-166.

437. Wesemann W, Grote C, Clement HW, Block F, Sontag KH. Functional studies on monoaminergic transmitter release in parkinsonism. Prog Neuropsychopharmacol Biol Psychiatry 1993;17(3):487-99.

438. Westerink BH, Damsma G, Rollema H, De Vries JB, Horn AS. Scope and limitations of in vivo brain dialysis: a comparison of its application to various neurotransmitter systems. Life Sci 1987;41(15):1763-76.

439. Wichmann T, DeLong MR. Functional neuroanatomy of the basal ganglia in Parkinson's disease. Adv Neurol 2003;91:9-18.

440. Wiesendanger RaW, M. Thalamic connections with medial area-6 (supplementary motor cortex) in the monkey (Macaca fascicularis). Exp. Brain Res 1985;59:61-104.

441. Wiklund L, Leger L, Persson M. Monoamine cell distribution in the cat brain stem. A fluorescence histochemical study with quantification of indolaminergic and locus coeruleus cell groups. J Comp Neurol 1981;203(4):613-47.

442. Wilkinson LS, Humby T, Killcross S, Robbins TW, Everitt BJ. Dissociations in hippocampal 5-hydroxytryptamine release in the rat following Pavlovian aversive conditioning to discrete and contextual stimuli. Eur J Neurosci 1996;8(7):1479-87.

443. Wise SP, Weinrich, M. and Mauritz, K. H. Motor aspects of cue related neuronal activity in premotor cortex of the rhesus monkey. Brain Res 1983;260:301-305.

444. Wolf LW, LaRegina MC, Tolbert DL. A behavioral study of the development of hereditary cerebellar ataxia in the shaker rat mutant. Behav Brain Res 1996;75(1-2):67-81.

445. Wolfarth S, Ossowska K. Dopamine receptors--the present state of research and perspectives. Pol J Pharmacol 1995;47(3):207-18.

446. Wreford NG. Theory and practice of stereological techniques applied to the estimation of cell number and nuclear volume in the testis. Microsc Res Tech 1995;32(5):423-36.

447. Xu M, Hu XT, Cooper DC, Moratalla R, Graybiel AM, White FJ, et al. Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. Cell 1994;79(6):945-55.

448. Xu M, Moratalla R, Gold LH, Hiroi N, Koob GF, Graybiel AM, et al. Dopamine D1 receptor mutant mice are deficient in striatal expression of dynorphin and in dopamine-mediated behavioral responses. Cell 1994;79(4):729-42.

449. Yelnik J. Functional anatomy of the basal ganglia. Mov Disord 2002;17 Suppl 3:S15-21.

450. Yoshihara C, Saito N, Taniyama K, Tanaka C. Differential localization of four subspecies of protein kinase C in the rat striatum and substantia nigra. J Neurosci 1991;11(3):690-700.

451. Young AB, Bromberg, M. B. and Penney, J. B. Decreased glutamate uptake in subcortical areas deafferented by sensorimotor cortical ablation in the cat. J. Neurosci 1981;1:241-249.

452. Zhou J, Zhang A, Klass T, Johnson KM, Wang CZ, Ye YP, et al. Biaryl analogues of conformationally constrained tricyclic tropanes as potent and selective norepinephrine reuptake inhibitors: synthesis and evaluation of their uptake inhibition at monoamine transporter sites. J Med Chem 2003;46(10):1997-2007.

# Appendix 1

1- Cut and mount sections on slides coated with APES.

2- Deparaffinise sections and rehydrate to distilled water.

3- Place sections in 0.5% hydrogen peroxide/ $H_2O$ for 10 minutes.

4- Wash sections in tap water.

5- Heat 1500ml of the recommended unmasking solution (0.01M citrate buffer, pH 6.0 until boiling in stainless pressure cooker.

6- Position slides into metal staining racks and lower into pressure cooker ensuring slides are completely immersed in unmasking solution.

7- When the pressure cooker reaches operating temperature and pressure (after about 5 minutes) start a timer for 1 minute.

8- When the timer rings, remove pressure cooker from heat source and run under cold water with lid on.

9- Remove the slides and place immediately into a bath of tap water.

10- Place the sections in PBS.

11- Process for ICC.

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Solution

1- 0.01M citrate buffer (pH6)

Add 3.84 grams of citric acid (anhydrous) to 1.8 litres of distilled water. Adjust to pH6 using concentrated NaOH. Make up to 2 litres with distilled water.

# Appendix 2

Solution required for the HPLC procedure

**Internal standard:**

- 25mg DHBA/25ml 0.1M HCL(stock)
- 1ml stock / 99ml 0.1M HCL
- 1ml above / 99ml 0.1M HCL

This gave a final concentration of 1ng / 20µl

**Composite standard:**

- DOPAMINE (DA),
- DHBA,
- 5-hydroxyindoleacetic acid (5-HIAA),
- Serotonin (5-HT)

1ng / 20µl injection of each of the above.

**Mobile Phase:**

- 6.74g citric acid
- 4.81g sodium citrate
- 47mg ethylenediaminetetra acetic acid (EDTA)
- 200mg heptane sulphoric acid (HAS)
- 1.15ml glacial acetic acid
- 3ml tetrahydrofuran(THF)
- 25ml/L 2.5% methanol

The mobile phase was buffered to pH 4.9 with 10M NaOH and made up to 1 litre with HPLC de-ionized water. It was degassed for 10mins, every morning, with helium.