

CHAPTER 3

RESULTS

3.4 THE OUTGROWTH OF DOPAMINERGIC NEURONS IN ORGANOTYPIC SLICE CULTURES OF CHICK MID-BRAIN

3.4 The outgrowth of dopaminergic neurons in organotypic slice cultures of chick mid-brain

An understanding of the mechanisms that influence the development and regeneration of the dopaminergic neurons of the nigro-striatal pathway is of particular importance since it is the death of these neurons that causes Parkinson's disease.

Over the last 10 years, a powerful *in vitro* technique has been developed, organotypic tissue culture (Gäwiler 1981), in which relatively large tissue sections are cultured so that they retain a high degree of their morphology (Østergaard 1993).

The primary aim of the current study was to determine whether chick mid-brain could be used as a suitable organotypic slice culture to study the outgrowth of dopaminergic neurons; Secondly, I also wanted to see whether the organotypic slice culture of a chick is different from that of a rat.

3.4.1 The distribution of tyrosine hydroxylase immunoreactive cells in the developing mid-brain of the chick

The basal ganglia system in avian consists of the paleostriatum augmentatum (PA), the lobus parolfactorius (LPO), the paleostriatum primitivum (PP) in the telencephalon (Anderson and Reiner 1990), the nucleus accumbens (Ac) in the telencephalon (Reiner et al. 1983) and the nucleus tegmenti pedunculo pontinus pars compacta (TPc) in the mesencephalon (Moons et al. 1994). The PA and LPO in avian is the equivalent to the corpus striatum of mammals and the Tpc is the equivalent of the substantia nigra of mammals (Moons et al. 1994), see figure 71.

In previous works, the vast majority of the DOPA and dopamine-immunoreactive cells are found in the mid-brain, particularly in the TPc and in the area ventralis of Tsai (AVT) (Moons et al. 1994). This study was also able to show TH-immunoreactive cell bodies and fibres in the mid-brain into the following brain regions: Substantia grisea centralis (GCT), AVT, locus coeruleus (LoC), TPc and nucleus subcoeruleus ventralis (Scv), see figure 72. From E18 on, what was seen as a strong TH staining of neurons and fibres was only seen as a weak TH staining at E16, and, at early stages, no immunoreactivity was seen.

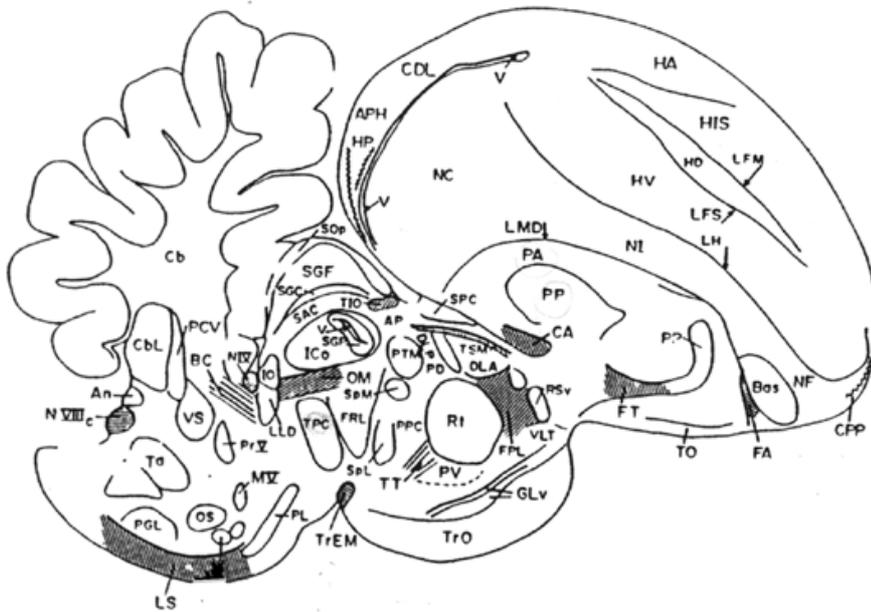


Figure 71: Schematic diagram showing the sagittal section of the chicken brain from the atlas of the chick brain of Kuenzel and Masson (1988). The point marked Cb refers to the cerebellum, and NC refers to the neostriatum caudale. The point marked TPC is the nucleus tegmenti pedunculopontinus, pars compacta, which is the equivalent of the substantia nigra of mammals.

In this study, TH positive neurons were observed in the developing chick mid-brain at different stages. TH-immunoreactive elements were stained with an antibody against TH which was kindly provided by Prof. H. Rohrer (*Max-Planck Institut für Hirnforschung*, Frankfurt/Main, an Institute for Cerebral Research). These sections were then incubated with a second biotinylated IgG and avidin-peroxidase. All sections were then stained with VIP. The coloured sections were examined and photographed. Nomenclature of brain areas was taken from the atlas of the chick brain of Kuenzel and Masson (1988).

TH immunoreactive neurons were not found until E16. At this stage, TH positive neurons in the substantia grisea centralis (GCt) and nucleus tegmenti pedunculo pontinus pars compacta (TPc) were weakly stained. On reaching developing stage E18 and onwards, neurons in the GCt and TPc were heavily stained, see figure 73. The distribution pattern of these immunoreactive neurons was the same at all positive-stained ages examined. As far as the localisation of the TH-immunoreactive system is concerned, the results in the chick midbrain are in complete agreement with previous publications in avian brain (Bailhache and Balthazart 1993, Bottjer 1993, Fraley and Kuenzel 1993). The localisation of the TH-immunoreactive elements shows many similarities with the distribution of L-DOPA- and dopamine-immunoreactive systems (Moons et al. 1994).

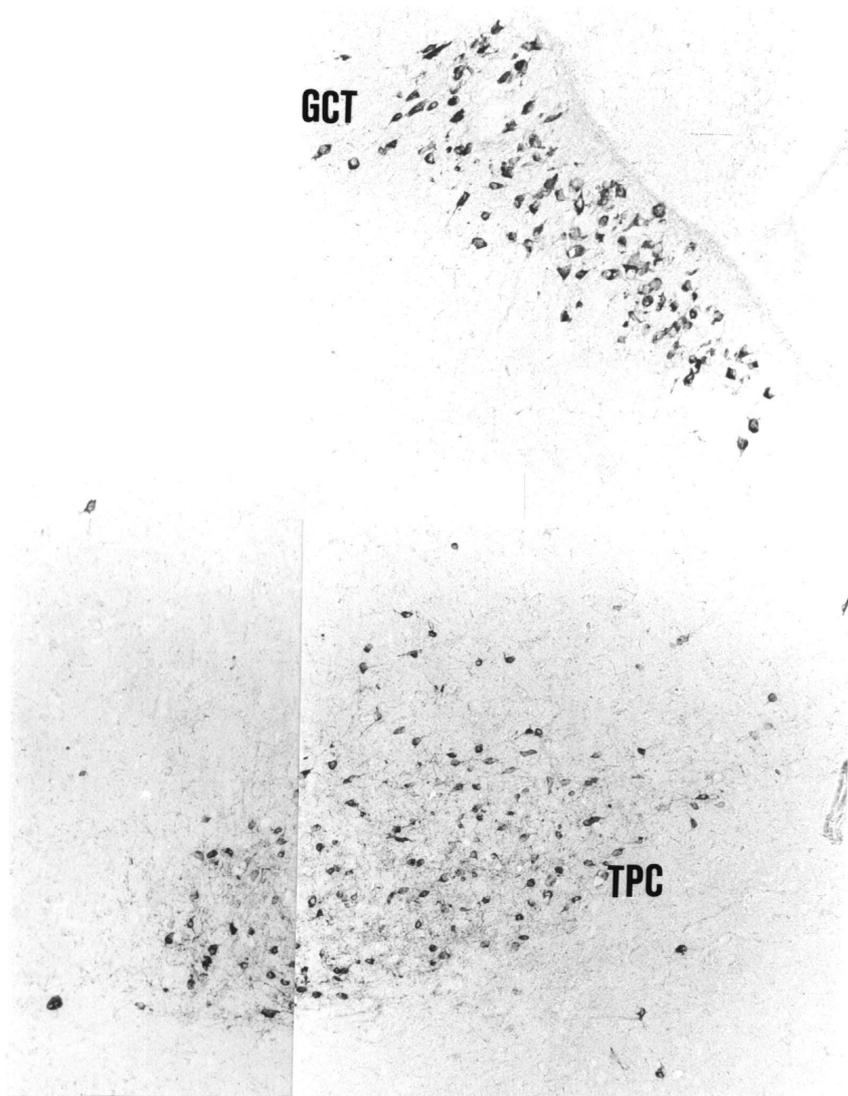


Figure 73: Photomicrograph revealing the tyrosine hydroxylase positive structures in a sagittal section of the chicken mesencephalon. Numerous cells are seen for tyrosine hydroxylase in the TPC, the avian homologue of the substantia nigra. The point marked GCT refers to the substantia grisea centralis. The point marked TPC is the nucleus tegmenti pedunculopontinus, pars compacta. Magnification x 200, scale bar = 50 μ m, visualising with SG.

3.4.2 Single ventral mesencephalic cultures

This report documents the first successful culturing of a chick's mesencephalon. This was accomplished by making several modifications to the well-adapted organotypic tissue culture. Cultures were prepared under sterile conditions using a lamina flow hood. Mesencephalon containing the nucleus tegmenti pedunculo pontinus pars compacta (TPc) was dissected from E18 old chicken embryos. This age point was chosen since a fair degree of the tissue-specific cytoarchitecture is already established and TH positive cells strongly appeared in the TPc, see section 3.4.1. Incubation times varied from 2-14 days. On completion of the incubation period, cultures were prepared to preserve the structure and tested for tyrosine hydroxylase immunohistochemistry using the biotin-avidin peroxidase method. Here, I used the specific monoclonal antibody m- α -TH (generous gift of Prof. H. Rohrer) and 3D10 (Tsim et al. 1988) to localise AChE. The stained cultures were observed using a light microscope.

TH positive neurons in the chick's organotypic cultures showed morphological characteristics similar to those found in the intact animal. During the culture period, the organotypic slice cultures thinned to a thickness of one or two cell-layers, allowing clear light-microscopic visualisation of individual, immunohistochemically-stained neurons. After 2 days incubation, the slices had not yet thinned to one cell layer (figure 74). After 4 days, the cultures began to flatten. The best visualisation of TH positive cells was reached after 8 or 9 days. At this stage, the culture had flattened to almost one cell thickness and long neurites extended into the surrounding glia tissue (figure 75). The cell bodies of TH positive neurons were not evenly distributed throughout the culture but were present in a specific region. The pars compacta and pars reticulata regions of the TP could not be identified. Neurites with extensive branching patterns extended from the TH immunoreactive cell bodies often reaching deeply into the plasma clot. Furthermore, high-power analysis of the cultures using a light-microscope revealed the presence of varicosities and growth cones on the TH positive neurites in organotypic cultures of the chick (figures 76, A and B). TH-immunoreactive cells were detectable in all organotypic cultures at the C8 and C9 stage of development. These cells displayed a variety in the shape of the somata including bipolar, pyramidal and multipolar forms (figure 77). In organotypic cultures taken from the chick, the dopaminergic neurons grew very well. It was seen as an imposing fibre growth (figure 78). In these cultures, it was found that the number of surviving TH positive neurons decreased as the culture became older. After fourteen days in culture, the tissue died and contracted to small rim-aligning

plasma ruptures. Furthermore, shaking was important for the surviving of the culture. Without shaking, each of the tissue regions degenerated on first day in culture to the extent that none survived. Mitosis inhibitors were added once for a short period to prevent major glia growth. Despite this, the glia tissue grew very rapidly so I thought it would be wise to incubate the tissue for a longer period with cytosine- β -D-arabinofuranosid and uridine. The modified-organotypic-slice-culture method allowed the chick neuronal tissue to be cultured for fourteen days. This resulted a great deal of axonal and dendritic growth.

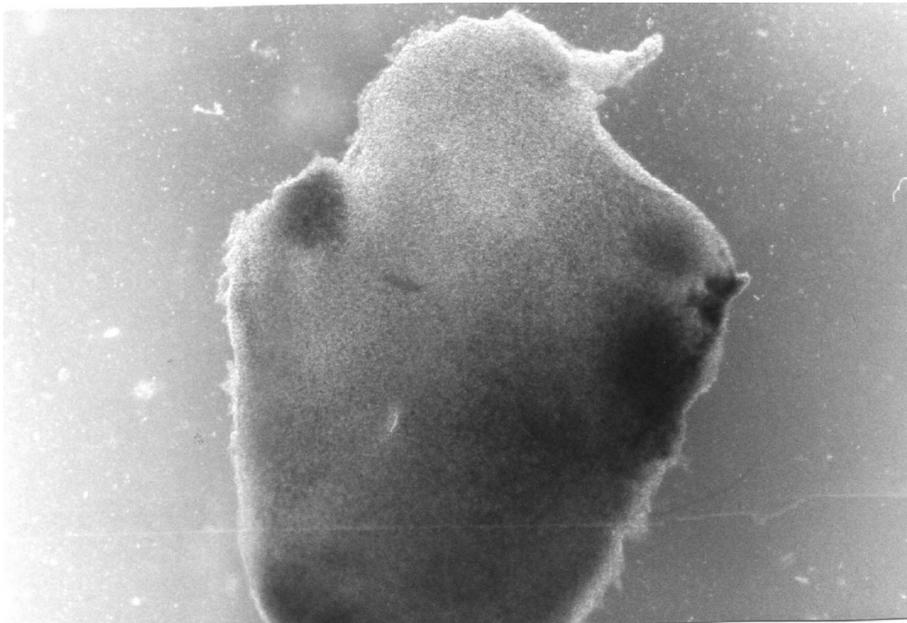


Figure 74: A single slice of chicken ventral mesencephalon, 200 μ m thick, after 2 days incubation in DMEM medium. The organotypic culture was immunohistochemically stained for TH. The slice had not yet thinned to one cell layer; magnification x 100, scale bar = 100 μ m, visualising with VIP.

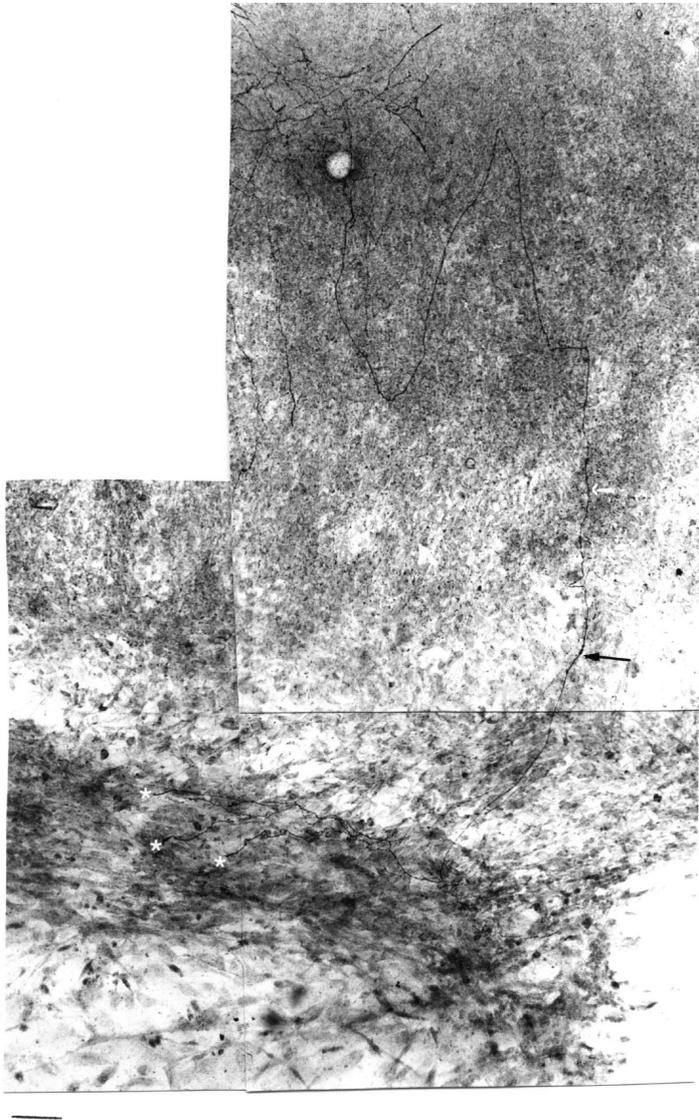
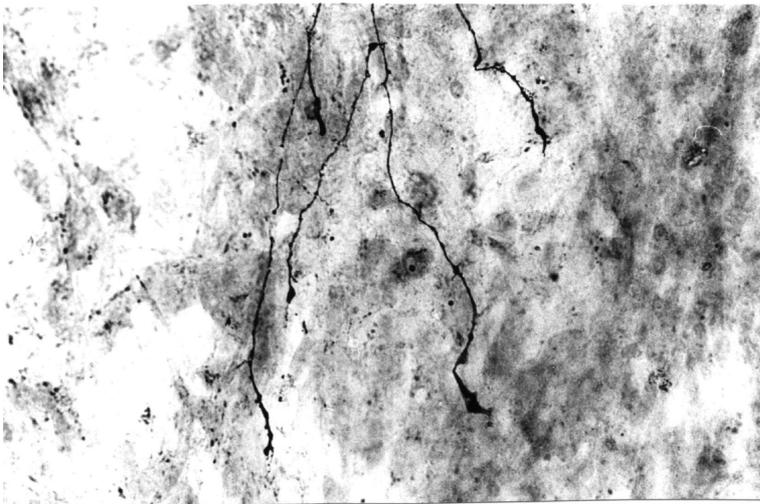
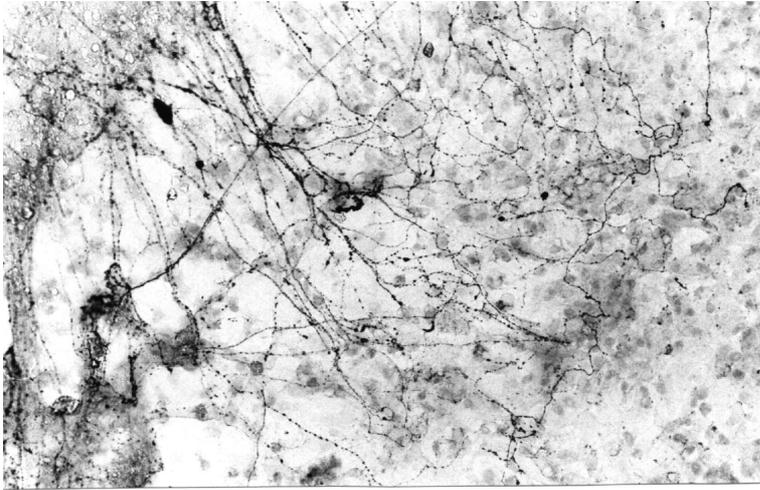


Figure 75: Organotypic culture of chicken ventral mesencephalon incubated in a DMEM medium for nine days. The culture was immunohistochemically stained for TH. TH+ neurites (arrow) and growth cones (stars) can be seen. Here, the slice has thinned to one or two cell-layers; magnification x 100, scale bar = 100 μ m, visualising with VIP.



(B)

Figure 76 (A) is a low-power photograph of a typical culture incubated in a DMEM medium for nine days. TH⁺ neurites project in all directions within the tissue and often extend into the surrounding glia tissue and plasma clot. (B) illustrates TH⁺ neurites growing within the glia tissue and plasma clot. TH⁺ axons showed numerous varicosities and growth cones at their terminals. The organotypic culture was immunohistochemically stained for TH; magnification x 200 for (A) and x 400 for (B), scale bar = 50 μ m, visualising with VIP.

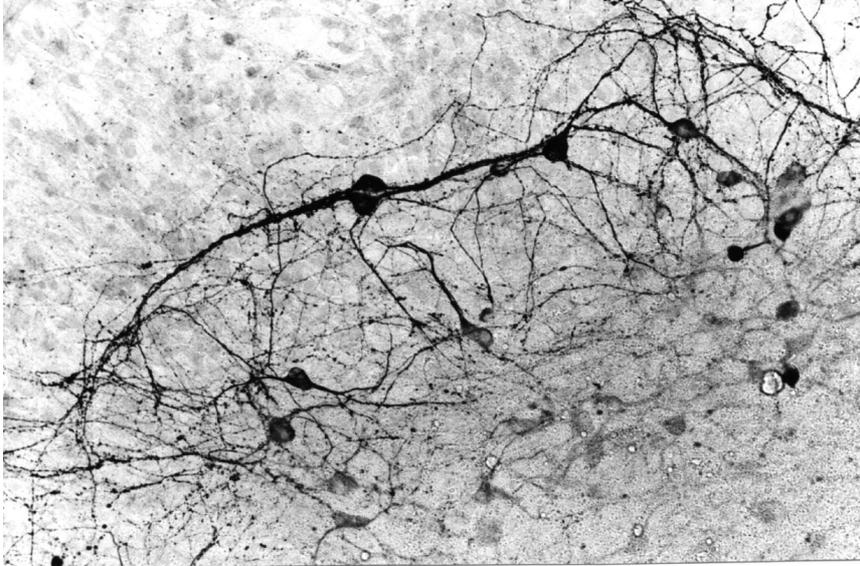


Figure 77: Each of the chicken's ventral mesencephalon cultures contained a variety of TH+ cell morphologies. The photograph of TH+ neurons shows multipolar, bipolar and pyramidal shaped neurons. The culture was immunohistochemically stained for TH; magnification x 200, scale bar = 50 μ m, visualising with VIP, culture duration nine days.

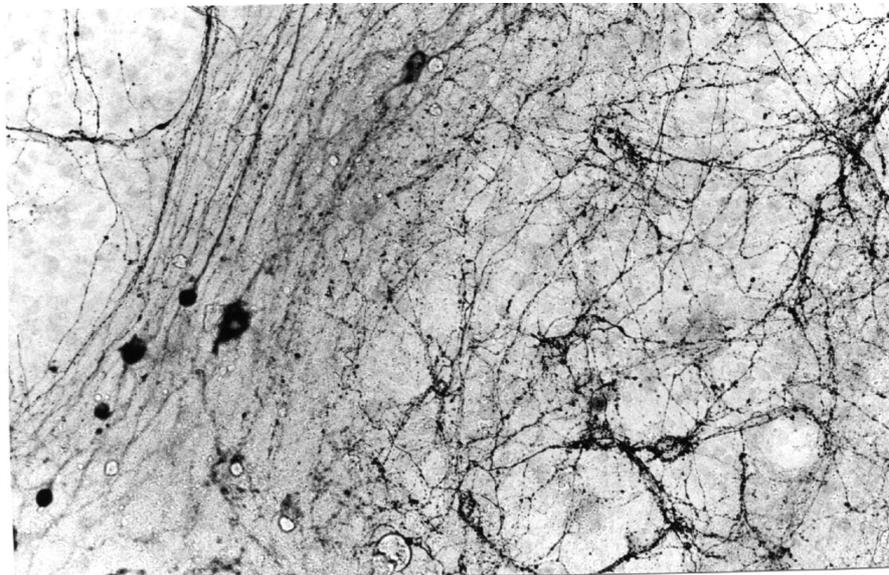


Figure 78: The TH positive neurites can be seen. The culture was immunohistochemically stained for TH. Culture duration nine days, magnification x 200, scale bar = 50 μ m, visualising with VIP.

3.4.3 TH positive neurons in organotypic cultures incubated with BW284c51 or AChE

When the cultures were incubated in BW284c51 at a concentration of 10^{-4} M, TH immunoreactive neurons were rudimentary and the tissue did show any sign of thinning (figure 79). However, the culture attached itself to the cover slip and did not come off like other tissue cultured over fourteen days. Despite continuous rotation of the culture, remains of the tissue were several hundred micrometers thick.

In another experiment, the medium was supplemented with 3U/ml AChE (Sigma). The culture produced by the organotypic slice taken from the chick developed very well after AChE treatment. After 4 days in culture, the tissue flattened and long neurites extended into the surrounding glia tissue and plasma clot. The organotypic slice culture incubated in DMEM medium supplemented with AChE did not differ from cultures which were not treated with AChE. The supplemented cultures showed the same pattern as far as the neurites, growth cones, varicosities and shape of the somata are concerned (figures 80 and 81).

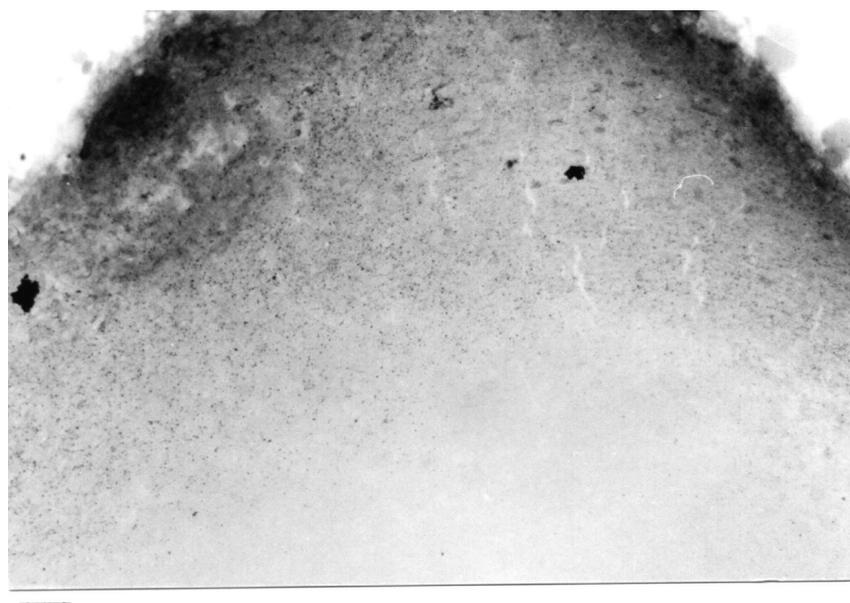


Figure 79: The organotypic culture of ventral mesencephalon incubated in a DMEM medium supplemented with 10^{-4} M BW284c51. The dopaminergic neurons were rudimentary and the tissue not thinned out by incubation in this medium. The culture was immunohistochemically stained for TH; magnification x 100, scale bar = 100 μ m, visualising with VIP.

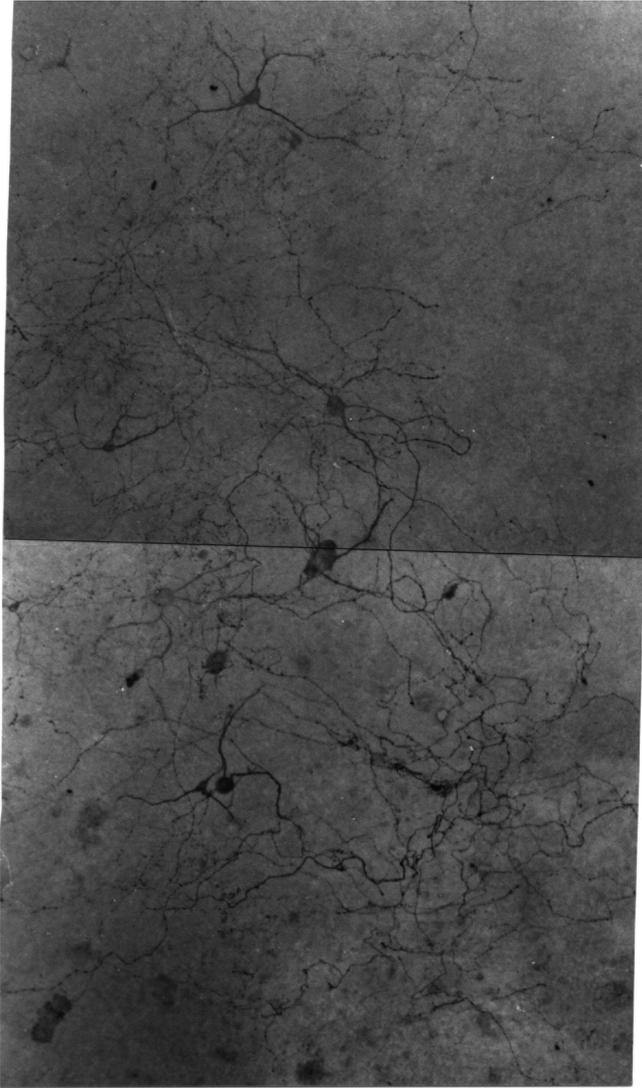


Figure 80: A single ventral mesencephalon culture incubated for nine days in a DMEM medium supplemented with 3U/ml AChE (Sigma). The tissue was immunohistochemically stained for TH. The organotypic slice culture did not differ from cultures without AChE. Visualising with VIP, magnification x 200, scale bar = 50 μ m.

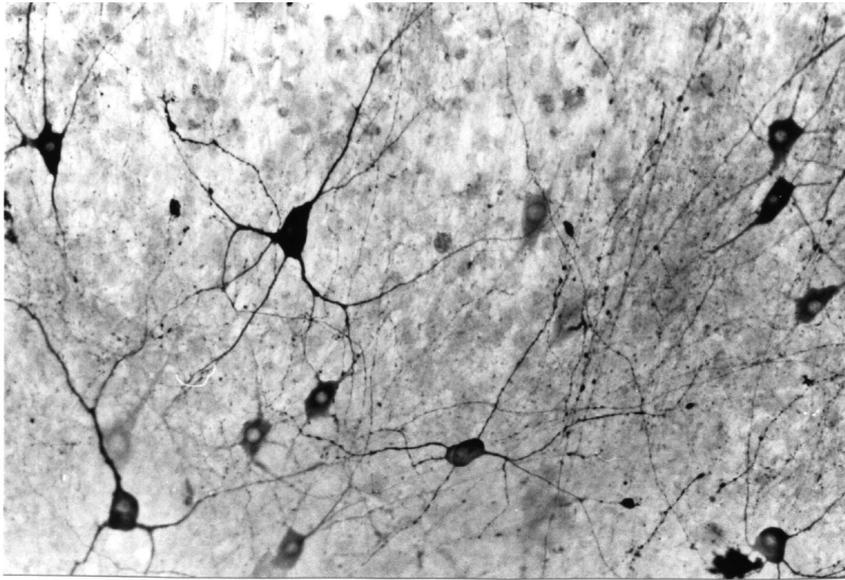


Figure 81: A photograph of TH⁺ neurons within the organotypic culture of ventral mesencephalon, incubated in a DMEM medium supplemented with 3U/ml AChE (Sigma). Culture duration eight days, immunohistochemically stained for TH, visualising with VIP, magnification x 200, scale bar = 50 μ m.

CHAPTER 4

4 DISCUSSION AND CONCLUSIONS

4 Discussion

This study has employed different approaches to investigate the role of AChE in conjunction with dopamine within the nigro-striatal pathway and developing retina. I examined the release of AChE in healthy and pathological nigro-striatal pathways stimulated both by amphetamine and other drugs. The organotypic slice culture of the chick was incubated to examine the regeneration of the nigro-striatal pathway of the chick. Since this thesis is divided clearly into several sections, the concluding chapter will consider the findings and their relevance separately.

The work detailed in this thesis deals mainly with the soluble form of AChE, which is released from the dendrites of the substantia nigra and developing retina and whose function is independent of its catalytic site. It should be appreciated that other forms of AChE exist at various stages of development and at various sites in the periphery and CNS.

4.1 General findings concerning the on-line experiments

4.1.1 The Push-pull cannula technique

The push-pull cannula technique was developed by Fox and Hilton (1958) and adapted by Gaddun (1961) to allow perfusion of a small region of brain tissue. Cannulae are constructed from two concentric tubes which can be implanted into the brain. The technique has the advantages that the exact site of release can be localised, molecules of any size can be assayed, and drugs can be applied to the tissue locally.

Artificial cerebrospinal fluid (ACSF) was continuously infused and simultaneously withdrawn at a constant rate via the cannula. The ACSF bathes a localised region of neurons and carries with it any released neurochemicals which are then assayed.

This technique has been used extensively to study the release of neurochemicals from the substantia nigra and striatum (Greenfield et al., 1980; Greenfield et al., 1983a, b; Greenfield and Shaw 1982; Nieoullon et al. 1977a,b; Weston and Greenfield 1986). The release of AChE

from the substantia nigra in anaesthetised guinea-pigs has also been studied using this technique (Taylor et al. 1988; Taylor and Greenfield 1989a). Any neurochemicals released in this region of the brain are deposited and transported out of the animal in the perfusate and can be collected for *in vitro* analysis using a protein assay (Ellman et al. 1961), or analysed *ex situ* as shown in this thesis using a procedure such as the on-line chemiluminescent technique (Taylor et al. 1989). Gaddum's technique was dependent upon hydrostatic pressure to maintain the flow of ACSF and it was not until the late 1970s that Nieoullon and colleagues (Nieoullon et al. 1977a, 1977b) modified this procedure by incorporating peristaltic pumps to maintain the flow. Over the last two decades, this system has been widely used to study the release of neurochemicals, including AChE, from the substantia nigra and striatum of a wide variety of animals such as cats (Greenfield et al. 1980), guinea-pigs (Taylor et al. 1989), rats (Weston and Greenfield 1984) and rabbits (Greenfield et al. 1981). Due to the large size of the soluble AChE molecule released from the substantia nigra (approximate molecular weight of 280-320 kDa, depending on species), a delivery system like the push-pull cannula is required to extract it from the brain region under investigation. It is therefore not surprising that this procedure is the most suitable for sampling AChE release from the substantia nigra.

During the research for this thesis, rats were the animals used. They are very agile and active creatures, so it was deduced that the push-pull cannula should be made as small as possible to minimise the exposed area of the cannula above the skull and reduce the chance of banging the cannula accidentally during the housing and experimentation periods.

4.1.2 Chemiluminescent assay

In 1981, Israel and Lesbats developed a chemiluminescent assay for determining the continuous release of acetylcholine from the torpedo electric organ synapse and synaptosomes. This assay was an adaptation of an existing choline oxidase enzymatic assay previously used by Takayama et al. (1977), in the examination of choline-containing phospholipids. The chemiluminescent reaction is dependent on a cascade of enzymatic reactions: From hydrolysis of acetylcholine, AChE produces acetate and choline, and the choline is then oxidised in the presence of choline-oxidase to create betaine and hydrogen peroxide. In the presence of hydrogen peroxide and peroxidase, luminol is oxidised and subsequently emits photons and light. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is a synthetic compound which

emits light (a non-thermal process) when oxidised by either peroxides or oxygen radicals. The reaction is catalysed by peroxidase enzymes or by appropriate inorganic, oxidative catalysts. The light signal produced from the reaction is then detected by a photomultiplier tube situated next to the reaction chamber. The molecules responsible for emitting the light absorb free energy released by the chemical reaction and become excited. In this state, some of the peripheral electrons of the molecule are raised to a higher energy level. When these electrons lose energy, they return to a lower energy level and the energy lost during this transition is emitted as photons, that is, light. When the electrons have lost all their absorbed energy, the molecule then returns to its stable, initial state once again. (Van Dyke 1985). The colour, and therefore the wavelength, of the emitted light depends on several factors. These include the type of molecule being excited, the pH of the liquid medium, the presence of metal ions, the type of catalyst, and the amount of free energy released by the chemical reaction. The energy needed to produce a quantum of light is such that an oxidising chemical reaction is required to excite the luminescent molecule. The intensity of light emitted depends on the reaction rate of the oxidoreduction step which, in turn, depends on the concentration of molecules involved in the reaction. Light intensity is therefore directly proportional to the concentration of reagents. This fact is of fundamental importance to luminescence analysis, where light emitted by a chemical reaction is measured to quantify the amount of a particular substance participating in the luminescent reaction. Bioluminescence naturally exists in light-emitting organisms such as fireflies, jellyfish, fungi, marine bacteria, etc. The oxidoreduction step of such reactions is catalysed by enzymes called luciferase (derived from the Latin word 'lucifer' meaning 'light-bringing') (Hastings and Tu 1981). The advantages that luminescence has over methods based on light absorption like colorimetry, spectrophotometry are a higher sensitivity, a wider measurement range and a smaller specimen sample.

Israel and Lesbats' main aim was to develop a simple assay for measuring acetylcholine-release induced by synaptic activity which could be easily reproduced. This chemiluminescent assay was adapted by Birman (1984) to allow the measurement of the quantity of AChE in synaptosomal fractions for the first time. In this assay, unlike that devised by Israel and Lesbats, the role of acetylcholine and AChE were reversed, so that exogenous acetylcholine was added to the system and the synaptosomal concentrations of AChE then determined. However, this procedure did not give any indication of the release of AChE in terms of real time, only the resultant AChE activity after several hours. The monitoring of AChE release in

real time was not achieved until 1987, when Llinás and Greenfield carried out the first on-line visualisation of dendritic release of AChE from mammalian substantia nigra neurons in vitro. The light emitted by the slice was detected using a light-cell connected to a photomultiplier tube. In order to quantify the signal, the resultant output from the photomultiplier tube was amplified and displayed on a chart recorder. This system was ideal for measuring AChE release in vitro. However, the next question that needed to be addressed was whether this procedure could be adapted to measure the release of AChE in a living animal. Taylor and colleagues (Taylor et al. 1989) combined the push-pull technique with the chemiluminescent reaction and incorporated both protocols into an assay system in which the AChE activity in push-pull cannula perfusate could be continuously monitored.

4.1.3 On-line monitoring

Up until the late 1980s, the majority of studies in which AchE-release was monitored involved the collection of aliquot samples over a period of several minutes; these samples were then analysed using a microfluorometric assay (Pavari et al., 1983) or a spectrophotometric assay (Ellman et al., 1961). However, after many years of research, it was apparent that if the physiological significance of AchE-release in the substantia nigra was to be assessed, then a new system needed to be developed with a time resolution closer to real time. The 'on-line' chemiluminescent technique was constructed and first reported by Taylor and colleagues (1989).

In the last six years, this assay has enabled the study of the properties of AChE in the substantia nigra and its interaction with the surrounding environment to be carried out with a time resolution vastly superior to previous assays (5 seconds as opposed to 10 minutes). The release of AChE in the substantia nigra has been shown to be pulse-like in fashion when the striatum is stimulated (Taylor and Greenfield, 1989) and this release has also been reported to occur following local application of potassium ions to the substantia nigra (Taylor et al., 1989, Dally and Greenfield 1994). In addition, it was also observed that the spontaneous release of AChE in conscious guinea-pigs was higher than in an anaesthetised state (Taylor D.Phil. thesis, 1989; Jones D.Phil. thesis, 1992).

The chemiluminescent assay is not specifically designed to measure AChE activity as it also measures non-specific cholinesterase activity. Bearing this in mind, the following question had to be addressed: How much of the cholinesterase in the perfusate analysed by the assay is actually AChE? When BW284c51 (a specific inhibitor of AChE) was applied to the perfusate withdrawn from the brain of a conscious and freely-moving animal, all phasic increases in the light signal stopped and the basal signal fell to a level that was approximately half that obtained from an anaesthetised animal (Taylor et al., 1990). It is therefore not unreasonable to assume that any increase in the light signal observed in these experiments was due to the release of AChE caused by the evoked release of BChE, something which has never been reported in any species or following any treatment (Greenfield et al., 1980; Greenfield et al., 1983a; Weston and Greenfield, 1986; Taylor et al., 1988).

The data presented in this thesis supports the indications that the 'on-line' chemiluminescent technique is a highly-powerful tool for measuring the *in vivo* release of AChE from the substantia nigra and also evaluating the significance of this phenomenon in relation to locomotor activity (Jones et al., 1994; Jones et al. 1991; Jones and Greenfield, 1991; Taylor et al., 1990) and local neurotransmitter systems (Dally and Greenfield, 1994; Dickie and Greenfield, 1994; Jones et al., 1994; Taylor et al., 1990).

The ability to measure AchE-release in the substantia nigra in terms of real time with respect to movement has broadened the horizons for research into this phenomenon. AChE has been shown to vary with movement (Jones et al., 1994; Jones et al., 1991; Jones and Greenfield, 1991). In this thesis, I was able to show that the rat can be used in a number of behavioural paradigms; perhaps one of the most significant opportunities was to examine AChE release in the rat substantia nigra during normal and abnormal locomotor activity.

4.1.4 Problems

The most common problems experienced were blockage of the cannulae (particularly the side-arms), blockage within the vinyl Manifold tubing (almost anywhere in the system), blockage of the light cell, and difficulty in handling treated animals. Unfortunately, as a result of these technical problems, not all experiments carried out during this thesis could be included in the final stage of data analysis. Every effort was made to ensure that the surgically-operated

animal did not dislodge or damage the cannula prior to and during the experiments. In an additional attempt to reduce environmental factors causing unnecessary stress to the animals, all experiments were performed in conditions as quiet as possible.

4.1.5 Does surgery and subsequent perfusion cause damage to brain tissue?

Due to the location of the substantia nigra at the base of the mid-brain, any implantation of the cannula to this region will cause damage to cortical and sub-cortical structures. The animal was allowed to recover from surgery for two days, and once recovered, it behaved exactly like an intact animal. Nevertheless, it is still possible that unknown or unseen damage was sustained. Histological examination of post-mortem tissue permits identification of any large scale damage that may have occurred as a result of the surgery or the experimentation. It is therefore imperative that these sections are studied following each experiment, not only to ensure correct cannula placement in the substantia nigra, but also to investigate any hidden damage that may be present.

4.1.6 Can endogenous compounds interfere with the chemiluminescent signal?

The chemiluminescent reaction is dependent on the production of hydrogen peroxide resulting from the oxidation of choline following the hydrolysis of acetylcholine. However, AChE is not the only naturally-occurring compound in the CNS that can hydrolyse acetylcholine: BChE can also do so. This potential problem has been addressed by Taylor et al. (1989), who applied 10 μ M BW284c51 (a specific inhibitor of AChE) to the perfusate obtained from the substantia nigra of a freely-moving animal connected to the system. These authors found that the addition of an inhibitor decreased the signal obtained from the chemiluminescent reaction to less than 50% of resting, basal levels.

However, as only one concentration of BW284c51 was used, this observation alone would imply that only about 50% of the total cholinesterase activity in the assay could be contributed to AChE. The hydrolysis of acetylcholine, and hence the chemiluminescent reaction, is only one third as sensitive for BChE as it is for AchE. Therefore, if BChE contributed to 50% of the signal produced, there would be a need for more BChE to be released into the substantia

nigra than AChE. Evidence suggests that this does not appear to be the case in the substantia nigra (Greenfield et al., 1983). In addition, the release of BChE as a result of this has never been shown to occur in the substantia nigra, despite many attempts to initiate it (Greenfield et al., 1980; Greenfield et al., 1983b; Taylor et al., 1988; Taylor and Greenfield, 1989). The release of nigral cholinesterase(s) has been shown to occur following striatal stimulation (illustrated by an enhanced signal). Yet when this procedure was repeated with BW 284c51 added to the system, a decrease in the chemiluminescent signal was observed. Hence all the cholinesterase registered would appear to be AChE (Taylor and Greenfield, 1989). It is therefore not unreasonable to assume that the vast majority of cholinesterase activity monitored in the perfusate from the substantia nigra is neither artefactual nor caused by variations in BChE activity (Jones D.Phil. thesis, 1992).

An additional problem with the assay is this; It has been observed that neurotransmitters within the substantia nigra, and certain drugs, can interfere with the chemiluminescent signal. The addition of 5-HT (Dickie and Greenfield 1995) or dopamine (Dallys D.Phil. thesis, 1996) to the system, at concentrations of $>10^{-6}$ M and $\geq 10^{-7}$ M respectively, produces a silencing/quenching effect on the signal. This effect is probably due to scavenging of the free radicals present in the system following the production of hydrogen peroxide. Under normal resting conditions, this effect is not observed, probably because basal levels of these neurochemicals within the substantia nigra are insufficient to cause an effect on the signal; however, it is possible that following stimulation of the substantia nigra, the increased release of dopamine in the striatum from nerve terminals may account for the quenching effect on the signal. Apomorphine (10^{-2} M) (see also Dally's D.Phil. thesis, 1995) and amphetamine (10^{-2} M and 10^{-3} M) infused into an freely-moving animal attached to the system resulted in an reduction of the trace-signal obtained from the perfusate of the substantia nigra. However, when a suitable control experiment was carried out in which these compounds were applied at the same concentration directly to the system without an animal attached, the same effect was produced. Hence, this compound interferes directly with the assay. This observation emphasises the necessity that in all cases where new compounds are added directly to the system or via the animal, the appropriate control measures must be taken to avoid misinterpretation of any data obtained.

4.1.7 Is there a regional distribution of AChE release within the substantia nigra?

The distribution of AChE release within the substantia nigra has been previously mapped by Dr. A. Jones during the course of her D.Phil. thesis. At first, it may appear almost impossible to determine any regional variations in AChE activity, particularly since the diameter of the push-pull cannula (0.8 mm) is large compared to the dimensions of the rat's substantia nigra. However, after careful histological verification of cannula placements, it was found that a locational variation in AChE release does exist in the substantia nigra of the guinea-pig (Jones D.Phil. thesis 1992) and rat (Hawkins and Greenfield 1996). Dr. Jones suggested that AChE-release may occur from a restricted number of neurones in the anterior region of the substantia nigra, and may not be indicative of the whole population of nigral neurones which release AChE.

Hawkins and Greenfield (1992a) observed differential behavioural effects in rats following AChE infusion into the animal's substantia nigra. These effects varied depending on the anterior-posterior site in which the cannula was placed. In addition, Webb and Greenfield (1992) have proposed that the anterior substantia nigra contains the majority of AChE-sensitive neurones. This would support the Jones' that the majority of AChE is released in the anterior half of the nigra.

4.1.8 Can AChE release in the substantia nigra be related to animal movement?

The temporal resolution of the 'on-line' chemiluminescent assay is now vastly improved compared to any existing technique of its kind. Using this technique, a five second pulse of AChE can be measured as opposed to ten minute pulses using existing procedures. Thus, unlike established techniques where the collection of AChE samples (over a period of minutes) is not comparable with animal movements (usually seconds), the 'on-line' chemiluminescent technique can be used to relate AChE release in the substantia nigra with animal movements (Jones et al., 1991; Jones and Greenfield, 1991).

It should be remembered that although AChE-release and movement can be related, this movement has to last for a greater period of time than the time resolution of the assay to allow

any meaningful data to be analysed: e.g. a rotational behaviour exhibited by an animal lasting longer than five seconds could, in theory, be related accurately to any changes in nigral-AChE-release. However, with sudden, sharp head movements lasting less than a second, it is impossible to accurately assess AChE-release in the substantia nigra.

In addition to any variation in the temporal resolution of this system, the exact placement of the cannula has to be considered when relating AChE release to movement. It has been previously shown that a regional distribution of AChE exists in the substantia nigra (Jones D.Phil. thesis, 1992; Hawkins and Greenfield, 1992a) and there is also evidence that AChE can diffuse throughout the extra-cellular space at a rate of 1-2 mm/hour (Kreutzfeld and Kaiya, 1974). Bearing these points in mind, it is possible that the local release of AChE at the cannula tip may be artificially raised at the time of cannula placement, due to AChE leaking from damaged cells into the surrounding tissue. Therefore, sufficient time was always be allowed prior to perfusion for the animal to recover and for levels of AChE to stabilise.

This study demonstrated that it was possible to correlate the enhanced release of AChE with specific rotating movements (Heiland and Greenfield 1999). Peaks of AChE-release occurred always in connection with enhanced movement, although the release of AChE in the substantia nigra was observed to occur in peaks and in a pulse-like fashion as previously shown (Jones D. Phil thesis 1992, Dally D. Phil thesis 1995).

4.2 The release of acetylcholinesterase in the healthy basal ganglion following amphetamine stimulation

The central nervous system (CNS) is a major focus of investigations into the causes of behaviour. In this context, causes may be an alteration in the brain's neuronal activity, or changes in the underlying neuronal mechanisms resulting in the appearance of specific behaviour.

The on-line system enables a comparison of the basal and evoked release of AChE and the animal's spontaneous and induced movements using a video recording system and an Antrak video-based animal-tracking system. The most studies to date which were carried out using the on-line chemiluminescent technique have used the guinea-pig as the animal under investigation (Dally and Greenfield 1994, Dickie and Greenfield 1994, Dickie et al. 1995, Jones et al. 1994, Jones et al. 1991, Jones and Greenfield 1991, Taylor and Greenfield 1989, Taylor et al. 1989, Taylor et al. 1990). The level of AChE released from the substantia nigra in the rat has also been examined in several studies (Greenfield et al. 1986, Greenfield et al. 1983b, Weston and Greenfield 1985, Weston and Greenfield 1986) using the greater temporal resolution of the on-line chemiluminescent technique (Dally 1995 thesis).

The spontaneous release of AChE of $0.11 \text{ mU} \pm 0.03$, $n=9$ and $0.25 \text{ mU} \pm 0.07$, $n=13$ was detected in the perfusate from the substantia nigra of the rat in this study. The basal levels of AChE released in the substantia nigra of the rat in this study appear to be comparable with those of past experiments (Dally et al. 1996, $0.08 \text{ mU} \pm 0.01$) and with those of the guinea-pig (Dally and Greenfield, 1994, $0.10 \text{ mU} \pm 0.02$). Henderson and Greenfield (1984) have shown that in the rat, AChE appears to be located mainly in the pars compacta of the substantia nigra.

I investigated the effects of local and systemic amphetamine treatment. Amphetamine mainly acts by blocking the dopamine transporter which, in turn, inhibits the re-uptake of dopamine and increases the concentration of dopamine at the synapse (Jaber et al. 1995). Amphetamine can cause the release of nigral-AChE in vivo when administered locally (Greenfield and Shaw 1982, rabbits, Heiland and Greenfield 1999, rats) or systemically (Taylor et al. 1990, guinea-pigs, Heiland and Greenfield 1999, rats).

In the present study, the application of 1mg/kg amphetamine resulted in a significant increase of approximately 40% in the spontaneous release of AChE in the rat substantia nigra compared to the control conditions and basal level. An increase in the release of AChE was associated with increased motor activity. Typically, after amphetamine injection, the animals become more active, moving around the entire box. This movement lasts for about one hour in either a contraversive or ipsiversive direction, with a bias varying from animal to animal. Pycock (1980) pointed out that rats and other rodents show a natural preference to one particular side, and that this preferred asymmetry can be revealed as turning behaviour following systemic administration of dopamine agonists such as amphetamine. A rise of released AChE leads to an increase in the distance moved and vice versa. However, amphetamine systemic stimulation as an emitter will stimulate not only the nigrostriatal pathway but other regions as well, such as the mesolimbic pathway, and will indeed influence other cerebral systems which are stimulated indirectly by dopamine. The increase in AChE release following systemic amphetamine administration was always subsequent to the onset of locomotor behaviour. Amphetamine inhibits the re-uptake of dopamine and, therefore, the increased concentration of dopamine caused locomotor behaviour and an increase in AChE release.

Direct infusion of amphetamine at concentrations 10^{-7} M to 10^{-4} M to one substantia nigra resulted in a significant increase of 22-35% in the spontaneous release of AChE compared to basal levels, reaching a plateau at 10^{-5} and 10^{-4} M. This enhanced release of AChE was correlated with a rise in circling behaviour which, nonetheless, remained at a minimal difference in intensity. A different type of circling was seen with 10^{-2} M in comparison to the weaker concentrations of amphetamine. This tighter type of rotation may be the result of stimulation of adjacent brain regions, (for example the VTA (ventral tegmental area)), which could be influenced by stronger concentrations of amphetamine through a process of passive diffusion. Circling behaviour was never seen without enhanced AChE-release and vice versa. Therefore, I conclude that AChE-release in the substantia nigra results from the action of amphetamine but circling behaviour results from the action of a higher concentration of AChE in the substantia nigra.

In both cases, (local and systemic stimulation with amphetamine), there is a correlation between AChE released and behaviour. The higher the animals' motor activity, the higher the release of AChE. I cannot say that enhanced mobility triggers AChE or an enhanced

concentration of AChE in the substantia nigra triggers mobility because both phenomena were observed at the same time (see Heiland and Greenfield 1999).

In the present study, a maximum increase of 35%-40% was observed in the spontaneous release of AChE. Taylor and colleagues (1990) observed an increase greater than 150% in an anaesthetised guinea-pig and Greenfield and Shaw (1982) an increase over 200% in the anaesthetised rabbit. Therefore the amphetamine-evoked release of AChE in the rat was less than the one observed in the guinea-pig or rabbit following both local and systemic application. Amphetamine caused an overall increase in activity of the animal's contraversive behaviour. Circling was seen following all amphetamine concentrations, but with a different pattern after administration of 10^{-2} M amphetamine. Taylor and colleagues (1990) and Dally and Greenfield (1994), who carried out similar experiments in guinea-pigs, and, Dally and colleagues (1996), who carried out similar experiments in rats, all failed to observe any significant changes in locomotor activity. Amphetamine administered locally in the substantia nigra increased locomotor activity in a dose-dependent manner. This effect has been shown to depend on amphetamine-induced dopamine release in this structure (Cador et al. 1995). Amphetamine produces a mixture of stereotyped behaviour and forward locomotion. It has been shown to increase the release of endogenous dopamine in the substantia nigra both in vivo and in vitro (Taylor et al. 1987). Dopamine and AChE-release in the substantia nigra possess similar properties; spontaneous release of each compound appears to be calcium-independent (Geffen et al., 1976; Greenfield et al., 1983a), whereas their evoked release is calcium-dependent and TTX insensitive (Greenfield, 1991). Evoked release of dopamine has been observed following the local and systemic application of amphetamine. The actions of this agent on AChE-release have been explored under normal and pathological conditions. Amphetamine, a sympathomimetic amine which acts indirectly, resembles noradrenaline/dopamine sufficiently enough to be transported into nerve terminals by re-uptake mechanisms. Once inside the nerve terminals, amphetamine can cause displacement of noradrenaline/dopamine from its vesicular store into the cytosol (Seiden et al., 1993). AChE and dopamine are both stored and released from dendrites within the substantia nigra. Using a sensitive chemiluminescent system to monitor AChE-release 'on-line', the effects of enhancing dopamine concentration within the substantia nigra upon behaviour can be investigated. In recent years the 'on-line' chemiluminescence technique has been used for the determination of AChE-activity in the guinea-pig substantia nigra (Jones et al., 1991; Jones

and Greenfield, 1991) and the rat (Dally et al., 1996). The assay has been shown to be sensitive and reliable for detection of the release of AChE in vivo (Taylor et al., 1989).

During the first experiments, the guinea-pig was used in conjunction with the chemiluminescent system (Dally and Greenfield, 1994; Dickie and Greenfield, 1994; Dickie et al., 1995; Jones et al., 1994; Jones et al., 1991; Jones and Greenfield, 1991; Taylor and Greenfield, 1989; Taylor et al., 1990; Taylor et al., 1989). The additional reasons for choosing this kind of animal were that they are docile in nature and thus easy to maintain and handle. Furthermore, guinea-pigs offer the largest surface area of brain for implanting multiple cannulae and studying physiological changes within a confined area (Taylor et al., 1989). The guinea-pig is not, however, a suitable animal for an experimental 'Parkinsonian' model (Perry et al., 1985; Dally D.Phil. thesis, 1995). Moreover, Henderson and Greenfield (1984) have demonstrated a variation in the localisation of AChE within the rat substantia nigra compared to that within the guinea-pig. Therefore, in order to explore whether this histological discrepancy is reflected in the release of AChE, and in an attempt to find a more appropriate model for future studies on Parkinsonian movement, the chemiluminescent system was adapted for the use with rats (Dally D.Phil. thesis, 1995). AChE-release from the substantia nigra and striatum has been examined in several studies using the rat (Bergun et al., 1985; Greenfield et al., 1986; Greenfield et al., 1983a; Weston and Greenfield, 1985; Weston and Greenfield, 1986); however, this phenomenon has never been previously examined using the greater temporal resolution of the 'on-line' chemiluminescent technique until Dally (1996). The rat is a well established animal model for all manner of behaviours including the rotational hemi-Parkinsonian model (Ungerstedt, 1971; Pycock 1980).

The accumulated evidence obtained from the 'on-line' chemiluminescent technique indicates that although there are differential responses in the guinea-pig compared to the rat, namely the lack of impaired movement following neurotoxin pre-treatment, there are several observations of similar physiological and pharmacological responses when these two species are compared. These similarities include: AMPT-induced reduction of nigral dendritic dopamine in the rat and guinea-pig (Leviel et al. 1977; Dally and Greenfield 1994); AChE and dopamine levels are both reduced in the substantia nigra and in the striatum of rats and guinea-pigs following 6-OHDA pre-treatment (Greenfield et al. 1986, Greenfield et al. 1983a, Dally D.Phil. thesis 1995). The induced release of AChE in the substantia nigra has been shown to be calcium-dependent in the rat and guinea-pig (Taylor D.Phil. thesis 1989, Weston and Greenfield 1986).

Guinea-pigs have been the sole animal model used in conjunction with the 'on-line' system since its assembly in 1989 (Taylor et al. 1989). The 'on-line' chemiluminescent system was devised whilst fundamental electrophysiological studies of the substantia nigra were being carried out in guinea-pigs (Greenfield et al., 1988; Greenfield et al., 1989; Last and Greenfield, 1987a, Last and Greenfield, 1987b; Llinás et al., 1984; Nedergaard et al., 1988a; Nedergaard et al., 1988b) and it was, therefore, not surprising that this animal model should be selected for use in parallel neurochemical experiments.

Hawkins and Greenfield (1992a) reported differential behavioural effects in rats following the local infusion of AChE into the animals' substantia nigra; these effects varied depending on the anterior-posterior placement site of the cannula. However, Hawkins and Greenfield (1996) have shown that in the rat substantia nigra, the actions of AChE may be particularly selective to the anterior tip of this region. These authors have observed that significant contraversive circling behaviour, (when compared to control animals), is only seen when AChE is applied in the anterior tip of the substantia nigra in the pars compacta region.

AChE and dopamine are both stored and released from dendrites within the substantia nigra. Using a sensitive chemiluminescent system to monitor AChE release 'on-line', the effects of enhancing dopamine concentration within the substantia nigra upon behaviour were investigated. In recent years, the 'on-line' chemiluminescence technique has been used for the determination of AChE activity in the guinea-pig substantia nigra (Jones et al., 1991; Jones and Greenfield, 1991) and rat (Dally et al. 1996). The assay has been shown to be sensitive and reliable for detection of the release of AChE *in vivo* (Taylor et al., 1989).

Stereotypical movements such as head movements, sniffing and gnawing increased in rats infused with AChE into the substantia nigra in comparison to rats infused with BChE or with the vehicle alone (Weston and Greenfield, 1985). Additional behavioural studies have also indicated that AChE has long term effects on circling behaviour in rats (Hawkins and Greenfield, 1992a; Hawkins and Greenfield, 1992b). In fact, these authors showed that infusion of AChE into one substantia nigra produced contraversive circling which was associated with an elevation in dopamine content within the striatum: this effect could not be reproduced with BChE. Microinjection of AChE (but not BChE) depresses the firing rate of substantia nigra pars compacta neurones as shown in electrophysiological studies in the rabbit substantia nigra (Greenfield et al., 1981); This study provided the first functional evidence for a novel role of AChE, as BChE (with a ten times more potent acetylcholine hydrolysing

activity) did not have an effect, whereas AChE did. In addition, despite pre-treatment with heat or exposure to Soman (an irreversible inhibitor of the protein), AChE applied exogenously to brain slices during *in vitro* electrophysiological studies results in hyperpolarisation of substantia nigra pars compacta neurones. Again, application of BChE did not reproduce these effects (Greenfield et al., 1989; Webb and Greenfield, 1992). Evidence accumulated over recent years may provide additional avenues to explore relating to a novel action of AChE in the substantia nigra. The main two approaches adopted to uncover this non-cholinergic function are: (a) the role of secretion of AChE and its actions and (b) the role of AChE in the development of the nervous system.

It is now well established that AChE and dopamine are both present in pars compacta cells of the substantia nigra (Butcher and Woolf, 1982; Chubb and Bornstein, 1985; Henderson and Greenfield, 1984; Lehmann and Fibiger, 1978). In addition, it has been shown that both these substances are present in the dendritic SER of these neurones (Henderson and Greenfield, 1984). *In vitro* electrophysiological studies have indicated that exogenous AChE can cause hyperpolarisation of substantia nigra pars compacta neurones; however, application of BChE to these cells failed to reproduce the effects observed with AChE (Greenfield et al., 1989; Webb and Greenfield, 1992). Evidence accumulated during recent years has indicated an apparent physiological association between AChE and dopamine in the substantia nigra. Although behavioural studies have indicated AChE can have a long term effect on the dopaminergic nigrostriatal pathway (Hawkins and Greenfield, 1992a; Hawkins and Greenfield, 1992b), application of a dopamine agonist to the substantia nigra has no effect on AChE release (Weston and Greenfield, 1986); however, these agents have not been shown to affect dopamine release in this region either (Nissbrandt and Hjorth, 1992).

Several observations have indicated that there may be a physiological association between AChE and dopamine in the substantia nigra. There are similarities in the release mechanisms of these two substances in that they are both tetrodotoxin resistant and require calcium entry into the cell (Llinás and Greenfield, 1987). Potassium-induced depolarisation has been shown to increase the local release of dopamine *in vivo* (Nieoullon et al., 1977) and also the release of AChE *in vivo* from the substantia nigra (Greenfield et al., 1980; Taylor et al., 1990).

Partial lesioning with 6-OHDA has been shown to cause a significant decrease in the levels of AChE in cerebrospinal fluid (Greenfield et al., 1986) and the substantia nigra (Greenfield et al., 1983b).

4.3 Release of acetylcholinesterase in the pathological basal ganglion in conjunction with amphetamine stimulation

The functions of the nigro-striatal pathway have been the focus of intense research since it is well known that deterioration of this pathway results in Parkinson's disease (Braak and Braak 2000). In order to gain further insight into the functions of the normal nigro-striatal pathway, many studies have investigated the behavioural effects of specific lesions of this pathway by 6-OHDA and MPTP (Grunblatt et al. 2000). Despite the findings of these investigations, it is always difficult to correlate deficits due to damage which may have been sustained by a specific brain region.

The severe and wide-ranging motor and sensory disturbances exhibited by Parkinsonian patients suggests that the normal nigro-striatal pathway plays a rather complex role in motor function. Deficits such as trembling and rigidity are believed to occur as a result of a loss of control over other brain regions whose unbridled activity then produce these 'positive' symptoms.

A resting tremor at a frequency of 4-6Hz occurs in about 70% of Parkinson's patients. In addition, the positive symptoms of rigidity in both flexor and extensor muscles initially occur only in the trunk region but then also extend to limb muscles. Individuals suffering from Parkinson's disease also exhibit a characteristic stooped posture and walk with small, unstable, shuffling steps. Moreover, patients have difficulty in initiating movement (akinesia) and display a reduction in spontaneous movement (hypokinesia). When patients do move, there is a delay in the onset, slowness in the execution (bradykinesia) and difficulty in the sequencing of movements, particularly during facial movements and skilled movements of the limbs. The motor disturbances exhibited by people suffering from Parkinson's disease may in part be due to deficits in sensorimotor processing since they produce more errors during sensorimotor tasks of the mouth than do normal controls (Schneider et al. 1986). Therefore, it has been suggested that destruction of the nigro-striatal pathway which occurs with Parkinson's disease is associated with a variety of motor deficits which, in turn, may be due to disturbances in using the appropriate sensory information for the execution of movement (Schneider 1987a, b).

Studies carried out on people suffering from Parkinson's disease, and on animals with experimentally induced Parkinson's disease, demonstrate that the nigro-striatal pathway is involved in both the initiation and the execution of movements.

Many attempts have been made to try to reproduce Parkinson's disease in animals, in an effort to try to understand the underlying mechanisms of the disease. Selective neurotoxins are widely used to destroy nerves while studying at least one of three following areas: (a) elucidation of the function or end consequence of the degeneration of a particular brain region in terms of physiological or behavioural function; (b) animal models of diseased state and (c) mapping of neuronal pathways.

The objective of the present study was to compare the physiological/behavioural effects of intra-cerebral application of the neurotoxin 6-OHDA on the *in vivo* release of AChE in the substantia nigra of the rat. The spontaneous and evoked release of AChE *in vivo* was detected and quantified using the on-line chemiluminescent system in conjunction with the Antrak video-based animal tracking system.

Destruction of the nigrostriatal dopamine system exceeding 95% produces a variety of motor cognitive and sensory deficiencies which result in a permanent loss of function; animals with an 80-95% destruction of the nigrostriatal pathway or striatal dopamine depletion (which results in a varying degree of loss of physiological function) eventually regain many of their behavioural capacities. Only following a partial lesion (<80% striatal dopamine levels) are behavioural deficits not so prominent and it is thus well established that less than 20% of total dopamine output in the striatum is necessary for maintaining normal functions.

The most widely used toxin-based animal model is the 6-hydroxydopamine hemi-lesioned rat, first used by Ungerstedt (1971a): this pre-treatment results in asymmetric circling behaviour. This behaviour results from unilateral degeneration of the substantia nigra following application of 6-OHDA to the median forebrain bundle on the ipsilateral side of the lesion.

The neurotoxin 6-OHDA was first shown to be able to destroy peripheral sympathetic nerve terminals by Tranzer and Theonen (1968). Since then, this compound has been widely used to screen novel compounds for anti-Parkinsonian activity (MacDonald and Sirviö, 1993). 6-OHDA is an extremely reactive and unstable compound as a result of the hydroxyl moiety inserted into the existing benzene ring of dopamine. In an attempt to prevent the premature oxidation of 6-OHDA, this compound is administered with ascorbic acid; however, it should be noted that ascorbic acid can also increase the effects of 6-OHDA *in vivo*. Oxidation of 6-OHDA results in the production of reactive intermediates such as p-quinones, which can attack

amino and sulphydryl groups of macromolecules. However, it has also been reported that following 6-OHDA administration, an uncoupling of oxidative phosphorylation of mitochondria (see Tipton and Singer, 1993) and the formation of reactive oxygen species occurs (see MacDonald and Sirviö, 1993). When considering the action of 6-OHDA, it is important to acknowledge that conversion of oxygen to these reactive oxygen species may lead to cellular hypoxia (Heikkila and Cohen, 1971) and thus would account for the highly toxic effects of 6-OHDA. A possible counter-measure against this damage may be the application of antioxidants such as vitamin E. Evidence suggests that the biochemical and behavioural abnormalities caused by free radicals following 6-OHDA application, are attenuated by pre-treatment with vitamin E (Cadet et al., 1989).

Autoxidation of 6-OHDA results in the production of reactive species which propagate the further breakdown of this compound. Oxygen radicals, such as the superoxide ion, are thought to be critical in aiding this form of oxidation (Cohen and Heikkila, 1974). It should also be noted that transition metals, notably ferric ions, can accelerate autoxidation of 6-OHDA; this may be particularly significant in the brain, as it has been shown that 6-OHDA can free iron from its storage protein, ferritin (Lode et al., 1990).

6-OHDA is relatively specific in its action, accumulating in dopaminergic and noradrenergic nerve terminals via pre-synaptic uptake mechanisms, with little (if any) penetration of this compound through the blood brain barrier. The specific nature of this compound can be further enhanced by applying uptake inhibitors for non-adrenergic neurones. In rats, 5-HT, acetylcholine and GABA, are only indirectly affected after a single dose of 6-OHDA. However, when multiple doses are applied, the specificity of 6-OHDA is lost due to the absence of uptake sites on noradrenertic/dopaminergic neurones (Kostrzewa and Jacobowitz, 1974). In neonatal rats, the action of 6-OHDA is slightly different, as the blood brain barrier is not fully developed and thus, peripheral administration of this compound can result in central effects. In addition, the peripheral effects observed can be more pronounced and even permanent, in comparison to those observed in the adult animal (MacDonald and Sirviö, 1993). The permanency of 6-OHDA lesions varies according to the dosage and period over which it is applied. In the periphery, a single dose of 6-OHDA (50 mg/kg) leads to the occurrence of a rapid and complete degeneration of nerve terminals with little (if any) effect on the cell bodies. However, this effect is almost completely reversed in rats after just 8 weeks (Jonsson and Sachs, 1970). In the CNS, the effect of 6-OHDA in rats is not so easily reversible, even after 2 years (Ungerstedt, 1971b). The most accurate way of localising the action of 6-OHDA is by

stereotactically applying this compound intracerebrally into an animal placed in stereotactic apparatus.

AChE is localised in regions of cholinergic transmission. Indeed, it has been known for a long time that AChE is present at neuromuscular junctions (Marney and Nachmansohn, 1938) and in the central nervous system (Mendel and Rudney, 1942; Shute and Lewis, 1967) where its function is to hydrolyse acetylcholine. Despite this, AChE is also present in regions which are not involved in cholinergic transmission - for example the surface of red blood cells (Mendel and Rudney, 1942). Also, in several regions of the brain, expression of AChE is surprisingly high in comparison to the levels of acetylcholine and its synthesising enzyme, choline acetyl transferase (ChAT). These regions include the hypothalamus, cerebellum, globus pallidus and substantia nigra (Henderson and Greenfield, 1984; Lloyd, 1975; Silver, 1974).

AChE can also be associated with catecholaminergic systems. In the adrenal medulla, AChE is both associated with splanchnic nerve terminals and catecholaminergic chromaffin cells (Somogyi et al., 1975). Similarly, AChE is present in the noradrenergic neurons of the locus coeruleus (Albanese and Butcher, 1979) and within the somata, dendrites, and axons of dopaminergic neurons in the substantia nigra (Butcher and Marchand, 1978; Henderson and Greenfield, 1984; Lehmann and Fibiger, 1978). AChE was ultrastructurally detected in the Golgi apparatus, the rough endoplasmic reticulum and plasma membrane of substantia nigra pars compacta neurons, and also within the smooth endoplasmic reticulum (SER) of their dendrites (Henderson and Greenfield, 1984).

Chubb and Smith (1975a) identified six isoenzymes of the G4 form of AChE, of which only one (AChE 5) was present in the extra-cellular space. Indeed, release of this form could be evoked by the application of depolarising concentrations of potassium ions. This release occurred in a calcium-dependent manner and in the same ratio as catecholamines which therefore suggests that AChE was released via exocytosis from chromaffin cells.

In 1976, Chubb et al. found that the AChE 5 form was also present in the cerebrospinal fluid (CSF) of the cisterna magna. Greenfield and Smith (1979) investigated the release of AChE during stimulation of specific brain regions. They found that stimulation of the caudate nucleus, substantia nigra, or hypothalamus led to an increase in the detectable activity of AChE in the CSF of rabbit cisterna magna. Following lesions of the substantia nigra, basal levels of AChE within the cisternal CSF dropped and no enhancement of AChE levels was detected following caudate nucleus stimulation.

4.3.1 Effects of 6-OHDA pre-treatment on behaviour

6-OHDA is a neurotoxin that selectively destroys catecholaminergic neurons. Infusion of the neurotoxin into the CSF of rodents results in a significant depletion of dopamine, norepinephrine, and their metabolites in various regions of the brain (Breese and Taylor 1970). However, its injection into the dopamine-rich substantia nigra pars compacta results in almost complete loss of dopamine cell bodies in the substantia nigra pars compacta and a concomitant depletion of dopamine in the rat striatum (Ungerstedt and Arbuthnott 1970, Zetterstrom et al. 1986). This loss of dopamine is accompanied by a specific behavioural pattern, namely dopamine-agonist-induced rotation-behaviour. Since its first description, this model of hemi-Parkinsonism has been the subject of a large number of behavioural and biochemical studies. This study may help to evaluate the response of the substantia nigra to an oxidative load which might have occurred some distance away.

The data from the current study indicates that the induction of a lesion with 6-OHDA significantly reduces the level of AChE-release in the substantia nigra, the dopamine content in the tissues of the striatum, and also changes the animal's behaviour. Unilateral damage results in pronounced behavioural asymmetries, including the turning behaviour characteristic of hemi-Parkinsonism as documented by Bracha and colleagues (Bracha et al. 1987), Pycock (Pycock 1980) and Ungerstedt (Ungerstedt 1971). Compared to control animals (sham-operated animals), abnormal changes in movement or rotational behaviour could be noted 3 weeks after neurotoxin treatment. Some animals appeared aggressive and gnashed their teeth, but all of them showed a high circling intensity of 0.89 ± 0.14 turns/min (local), 0.66 ± 0.04 turns/min (systemic). Both ipsiversive turning and contraversive turning was observed. Unilateral lesions produced an imbalance in the activity of the two parallel nigro-striatal pathways (Ungerstedt, 1971b). It could be possible that the implanted push-pull cannula and the permanent perfusion of this side with ACSF lead to a unspecific stimulation in itself. The same neurotoxic pre-treatment was carried out with guinea pigs, whereby 6-OHDA pre-treatment failed to produce any behavioural signs symptomatic of Parkinsonism (Dally et al. 1996). Thus the guinea pig does not seem appropriate as a suitable behavioural animal model for Parkinson's disease. As shown in this study, pre-treatment with 6-OHDA undoubtedly influences the nigro-striatal

pathway of the rat, a fact shown by the animals' changed behaviour. They are more restless and aggressive, exhibiting a turning syndrome.

In this study, animals pre-treated with neurotoxin and sham-operated animals were stimulated locally or systemically with amphetamine. Direct infusion of amphetamine (10^{-7} to 10^{-3} M) to the substantia nigra of lesioned animals lead to a decrease of the high turning behaviour, and the animals almost returned to the normal behaviour observed in naive animals.

I was able to observe predominantly contraversive circling. Hawkins and Greenfield (1992a) found that the direction of circling produced by infusion of AChE into the substantia nigra of rats depended on the side of infusion within the substantia nigra. The most common response to infusion of AChE was one of contraversive circling. The sites of infusion of this group were distributed throughout the substantia nigra. Despite this, the side of infusion in the majority of animals responding ipsiversively were located in the anterior substantia nigra at the levels of the mammillary body. Greenfield and colleagues (Greenfield et al. 1984) also found that infusion of AChE into the substantia nigra of rats caused contraversive circling. Following application of 10^{-2} M amphetamine to the substantia nigra, different behaviour was observed, resulting in a stimulation of adjacent brain regions. I was able to observe similar behaviour in sham-operated animals when compared to naive animals. Thus, it seems that injection of sterile saline with ascorbic acid in the median forebrain bundle had no influence on general health.

Amphetamine injected intraperitoneal caused ipsiversive (classical group) as well contraversive (paradoxical group) rotations. There was a significant difference between high-basal motor-activity and amphetamine-stimulated circling. Cadet and colleagues (Cadet et al. 1991) showed that an intrastriatal injection of 6-OHDA results in ipsilateral amphetamine-induced circling behaviour in rats. The greater availability of dopamine in the intact hemisphere relative to that of the lesioned hemisphere is the accepted basis for this amphetamine-induced rotational behaviour. According to the prevailing model of nigrostriatal asymmetry (originating from studies with rats with unilateral nigrostriatal lesions) rats rotate in a direction contralateral to the striatum displaying more dopamine or dopaminergic activity (Glick 1976, Ungerstedt 1971a, 1971b). However, several observations have been made both in normal and unilateral lesioned animals which were inconsistent with this model. The normal asymmetry in striatal dopamine levels is not as uniformly related to the direction of rotation in male rats as in female rats (Yamamoto 1984, Robinson 1980). In Robert's study (Robert 1972) amphetamine induced contralateral rotation was limited to the first week post-operative. A ipsiversive as

well contraversive circling behaviour in lesioned animals after amphetamine stimulation was also observed by Glick and colleagues (Glick et al. 1988) and Raymond and colleagues (Raymond et al. 1987). For both populations of rats, the amount of rotational behaviour is determined by the magnitude of the innervation asymmetry. Przedborski and colleagues (Przedborski et al. 1995) showed all of the animals that received 6-OHDA (1.25, 2.5 and 5 μ g/ μ l) rotated toward the lesioned side following amphetamine administration (3mg/kg) and away from the lesioned side following the administration of apomorphine (0.5mg/kg). They observed a 6-OHDA dose-related increase in the number of amphetamine-induced rotations.

4.3.2 Effects of 6-OHDA pre-treatment on the spontaneous release of AChE in the substantia nigra

In the present study, I detected a basal AChE-perfusate-value of 0.04 ± 0.01 mU following local pre-treatment with 6-OHDA (3-week lesion), and 0.04 ± 0.008 mU (systemic). This value was lower than that of naive or sham-operated animals. The spontaneous release of AChE into the substantia nigra was reduced significantly by 68%. In previous studies, it was shown that the spontaneous release of AChE in the substantia nigra of the guinea pig was reduced by 62%, (1-week lesion), and 55% (3-week lesion), following pre-treatment with 6-OHDA (Dally et al. 1996). These authors found that a significant reduction in the spontaneous release of AChE is accompanied by a corresponding decrease in evoked release of nigral AChE. A significant decrease in the levels of AChE were also found in cerebrospinal fluid (Greenfield et al. 1986) and in the release of AChE from the substantia nigra and the caudate nucleus (Greenfield et al. 1983a) following the application of 6-OHDA to rats. It is well established that 6-OHDA is a potent neurotoxin which destroys dopaminergic neurones (MacDonald and Sirviö 1993). In demented patients with Parkinson's disease, Konings and colleagues (Konings et al. 1995) and Hartikainen and colleagues (Hartikainen et al. 1992) demonstrated a decrease of CSF AChE activity. Demented Parkinson's patients showed lower AChE immunoreactivity in CSF, as it is suggested that cholinergic dysfunction in Parkinson's patients exists with cognitive impairment (Konings et al. 1995). This study supports the suggestion that AChE secreted from the brain of patients with dementia, or animals with 6-OHDA lesion, may be abnormal. The data from the current study indicates that an application of 6-OHDA to the median forebrain bundle resulted in damage of the nigrostriatal pathway

which reduced the level of AChE- release in the substantia nigra. If dopaminergic neurons in the substantia nigra pars compacta destroy and dopamine and AChE are present in the same pars compacta neurons, as proposed by Butcher and Marchand (1978), then AChE will be reduced. Similarly, Henderson and Greenfield (1984) found that AChE was located in the Golgi apparatus and SER of pars compacta neurons and their dendrites. Moreover, AChE was also located on the surface and surrounding extra-cellular spaces of pars compacta neurons. Secondly, in 1980, Greenfield et al. demonstrated that AChE can be released from the *in vivo* substantia nigra and this release can be induced by local application of potassium ions. Indeed, release of AChE occurs in a calcium dependent manner (Greenfield et al., 1983b). AChE is believed to be released primarily from dopaminergic neurons, since 6-OHDA lesions of the nigro-striatal pathway caused both a significant drop in dopamine content of the striatum and an 86% decrease in the release of AChE within the substantia nigra (Greenfield et al., 1983a). Furthermore, a 70% drop in the release of AChE in the striatum was detected. In addition to this, amphetamine, which displaces dopamine from its storage site and prevents its re-uptake, caused both the release of dopamine (Leviel et al., 1979; Nieoullon et al., 1977b) and AChE (Greenfield and Shaw 1982) within the substantia nigra. These results suggested that the release of both molecules could occur from the same neurons.

Weston and Greenfield (1986) also found that tetrodotoxin (a sodium channel blocker) and gamma hydroxybutyrate, which specifically blocks the impulse flow in dopamine neurons, caused a decrease in striatal release of AChE. However, tetrodotoxin did not alter the release of AChE in the substantia nigra, while gamma hydroxybutyrate caused a large calcium-dependent increase in the release of AChE. It was thus concluded that the release of AChE within the substantia nigra is not closely linked to the firing rate of nigro-striatal neurons and is not dependent on the potential for sodium-mediated action. Instead, it might be evoked, as previously suggested, by dendritic calcium conductances (Llinás et al., 1984). In 1988, Taylor et al. discovered that AChE was also released from the substantia nigra of guinea pigs and that this release could be evoked by 5-hydroxytryptamine, amphetamine or depolarising concentrations of potassium ions.

In the early 1980s, it was observed that AChE release, *in vivo*, could be evoked in the substantia nigra and that this release was calcium-dependent (Greenfield et al., 1980; Greenfield et al., 1983a). Parallel research using a neurotoxin (6-OHDA) to lesion the nigrostriatal pathway revealed an 86% decrease in the release of AChE within the substantia nigra and a 70% drop in the release of AChE in the striatum - in addition to a significant

decrease in striatal dopamine content (Greenfield et al., 1983b). However, unlike in the substantia nigra, it has been shown that an AChE release in the striatum can occur as a result of increased firing rates of nigrostriatal neurones (Burgun et al., 1985). In the substantia nigra, AChE release does not appear to be under the control of cholinergic receptors or directly related to the firing rate of nigrostriatal neurones (Weston and Greenfield, 1986). In addition, the same authors also showed that an application of the dopamine agonist, apomorphine, which decreases the firing rate of nigrostriatal neurones, produced no net change in AChE release.

More recent evidence (based upon existing knowledge on AChE release in the nigrostriatal pathway) has shown that AChE release in the substantia nigra can be evoked by 5-hydroxytryptamine (Dickie and Greenfield, 1994), amphetamine or depolarising concentrations of potassium ions (Taylor et al., 1989), and may also be regulated by dopamine systems in this region (Dally and Greenfield, 1994; Dally thesis, 1996). Perhaps one of the most important findings of recent years has been the discovery that in the substantia nigra, AChE can be removed/taken-up from the extra-cellular space into the dopaminergic cells. It was not until 1995 that AChE was shown to be taken-up into dopaminergic cells of the substantia nigra (Dickie et al., 1995). Furthermore, Dickie and colleagues (1995) showed that the re-uptake of AChE into dopaminergic cells of the substantia nigra appears to be dependent on temperature and energy, and also related to cell metabolism rather than just being passively trapped in the substantia nigra.

4.3.3 Does neurotoxic pre-treatment have any effect on the enhanced release of AChE resulting from local application of amphetamine?

Amphetamine (10^{-7} to 10^{-2} M) has been applied in this study to investigate whether or not the neurotoxic treatment affected the evoked release of nigral AChE. There was no significant percentage increase in the spontaneous release of nigral AChE in all groups following the application of amphetamine. For each concentration of amphetamine, a large variation in AChE was released. Amphetamine evoked the spontaneous release of 13-70.6% more nigral AChE. However, it should be noted that in comparison to sham-operated animals (and naive animals) the significant reduction in the spontaneous release of nigral AChE following the application of 6-OHDA is accompanied by a corresponding non-significant increase in the evoked release of AChE. Neurotoxin pre-treatment had an effect on the increased release of AChE (in percent)

following application of amphetamine, when the evoked data was expressed as a percentage of basal levels. These observations suggest that not all cells of the nigrostriatal pathway are intact as they indicate an overall decrease in striatal dopamine content. This observation appears to correspond with the findings of Greenfield and colleagues (Greenfield et al. 1983a), where it was noted that following a single injection of 6-OHDA into the rat, the evoked release of AChE was prevented. In this earlier study, animals were left for 8 weeks for the treatment to take effect. This treatment resulted in a decrease in the striatal dopamine content of more than 90% when compared to the untreated side of the brain. However, in the present study it is likely that enough neurones are still sufficiently intact to release detectable levels of AChE, as observed by Dally and colleagues (Dally et al. 1996), but the remaining cells are not in the position to release the level of AChE emitted by sham-operated animals and/or naive animals. It appears that the storage and release of AChE and dopamine are not exactly congruent. AChE is not stored exclusively in dopaminergic neurons within the substantia nigra. Henderson and Greenfield (1984) detected AChE in non-dopaminergic neurons in the substantia nigra, in addition to dopaminergic neurons. Lehman and Fibiger (1978) found that following 6-OHDA lesions, the level of TH staining in the substantia nigra was reduced to 10% of control values, whereby staining for AChE was reduced by only 30%. Furthermore, Greenfield and co-authors (Greenfield et al. 1983a) found that lesions of the nigro-striatal pathway reduce the dopamine content within the striatum by 93%, but reduce the release of AChE to a slightly smaller extent by 86% (8-week lesion). Therefore, it is possible that a small fraction of released AChE could occur from non-dopaminergic neurons. The efferent pathway of the substantia nigra which has been the focus of this study is the nigrostriatal pathway. This projection arises from axons of the dopaminergic A9 cell group of pars compacta neurons (Ungerstedt 1971a). This pathway is, in fact, neither exclusively dopaminergic nor does it arise exclusively from pars compacta neurons. Indeed, van der Kooy et al. (1981) found that 5% or less of nigro-striatal neurons are non-dopaminergic. Perfusion of amphetamine through one substantia nigra enhances the release of AChE and dopamine from this substantia nigra (Greenfield and Shaw 1982). The work in this thesis demonstrated that damaging the nigrostriatal pathway reduces the dopamine levels within the treated striatum by 76% and reduces the spontaneous release of AChE within the treated substantia nigra by 68% (3-week lesion). These results reflect the observation of Greenfield and colleagues.

Sham-operated animals show results similar to naive animals. A basal AChE- perfusate value of 0.21 ± 0.04 mU was observed. The release of AChE initiated during amphetamine

stimulation shows similar results as those achieved with naive animals. It seems that injection of sterile saline with ascorbic acid into the median forebrain bundle has no effect on nigrostriatal neurons and therefore the cells appear to be functioning in a fashion indistinguishable from naive animals.

A correlation between AChE-release in the substantia nigra and behaviour has only been observed in sham-operated animals.

4.3.4 Does neurotoxic pre-treatment have any effect on the intensified release of AChE resulting from systemic application of amphetamine?

A different approach used to investigate the action of neurotoxic pre-treatment on the enhanced release of AChE was stimulation through systemic application of amphetamine. The main finding of this study is that both the release of AChE and the level of turning behaviour increase greatly as a result of stimulation with amphetamine. Damage produced by 6-OHDA caused intense rotational behaviour in response to amphetamine. Studies performed by Taylor and colleagues (Taylor et al. 1990) demonstrated that the release of AChE was enhanced when systemic application of amphetamine increased locomotor activity (in the same manner as I described in section 3.1). Previous studies (Taylor et al. 1990) had shown that it is only the release of AChE which is enhanced in the moving animal, since on addition of the specific AChE inhibitor BW284c51, the light emitted signal for the chemiluminescent reaction was virtually extinguished. Amphetamine which was applied locally produced different reactions to amphetamine which was applied systemically. A significant rise of approximately 238% was seen in the spontaneous release of AChE in comparison to basal conditions. Amphetamine lead to an increase of turning behaviour in treated animals compared to the high basal circling of animals infected with Parkinson. Indeed, the increase in turning behaviour following amphetamine stimulation is higher than that observed in sham-operated or naive animals following systemic amphetamine stimulation. It seems that in one way or another, the loss of nigrostriatal neurons influences the effect of amphetamine. In contrast, sham-operated animals react like naive animals, which again confirms that sham-operation had no influence on the health or condition of the animal.

4.3.5 Does the extent of the damage affect the level of dopamine in the striatum?

The injection of 6-OHDA was found to decrease levels of the treated striatal dopamine by 76% versus the untreated side of the brain. Parkinson's disease is characterised by a loss of the darkly pigmented cell bodies in the substantia nigra pars compacta, resulting in the death of the nigrostriatal dopamine axons that innervate other parts of the basal ganglia, including the neostriatum. As a consequence, the levels of dopamine and the rate-limiting enzyme in its synthesis, tyrosine hydroxylase, decrease to less than 20% of normal levels in these innervated areas. Parkinson's disease is observed in approximately 1% of the population aged 60 or above (Lippert 1996). The major clinical symptoms, among them the inability of the afflicted person to initiate voluntary movements, can be partially relieved by palliative treatments (L-DOPA, dopaminergic grafts). By the time the first mild symptoms occur, striatal dopamine is already depleted by 70-80%, and the severe akinesia observed at later stages of the disease is commonly associated with a loss of 95% or more of this neurotransmitter (Javoy-Agid et al. 1986). In the present experiment, striatal dopamine was depleted by 76%. Thus, I was able to simulate experimental Parkinson's disease in treated animals. Dally and colleagues (Dally et al. 1996) observed a reduction of dopamine levels in the caudate-putamen of 76% and 59% following pre-treatment with 6-OHDA, after 1-week and 3-week lesions respectively. In an earlier study by Greenfield and colleagues (1983a), animals were left for 8 weeks for the lesion to take effect, this resulted in a greater than 90% decrease in striatal dopamine content compared to the untreated side of the brain. The sham-operated group received identical injections of ascorbate-saline solution alone. There was no significant difference in dopamine content between the treated and non-treated side of the brain.. However, there was a slight difference which was perhaps due to damage which occurred during surgery. Secondly, stereotaxic injection of ascorbate-saline solution might induce non-specific local neural damage.

4.3.6 Does the extent of the damage affect dopaminergic and cholinergic neurons in the substantia nigra?

Examination of coronal sections showed normal macroscopic morphology of the mid-brain. The level of TH and AChE-binding in the undamaged substantia nigra had not changed. The

staining would be even stronger if the animals were infused transcidentally with formaldehyde. Infusion was not possible because I required the striata for HPLC. In the damaged side of the brain, the number of dopaminergic and cholinergic neurons in the VTA and in the substantia nigra pars compacta were dramatically reduced. The HPLC results and immunohistological results suggest that intranigrostriatal injection of 6-OHDA causes a loss of both dopaminergic striatal nerve terminals and nigral cell bodies. With Parkinson's disease, the nigrostriatal pathway is more severely affected than the mesolimbic system (Agid et al. 1987). Establishing the loss of dopaminergic neurons requires concomitant loss of a dopamine marker (TH, the rate-limiting enzyme of catecholamine synthesis). I demonstrated the loss of dopamine neurons in the substantia nigra pars compacta and in the VTA in this model by fulfilling the above criteria. This observation agrees with the report produced by Przedborski and colleagues (Przedborski et al. 1995). In post-mortem brain samples from patients with Parkinson's disease (Kastner et al. 1993), and in animals with MPTP-induced Parkinsonism (Miller and Beninger 1991), the loss of TH protein and mRNA proportionately is greater than the loss of substantia nigra pars compacta neurons. This disproportionate loss may occur because some neurons in the substantia nigra pars compacta and the VTA are not dopaminergic (Van der Kooy et al. 1981).

4.4 The release of acetylcholinesterase in relation to other drug stimulations

The main area of investigation in this thesis is the substantia nigra, striatum, and the nigrostriatal pathway - which is one of the connection pathways between these two regions. It transports neurochemicals from the substantia nigra to the striatum and can also initiate responses in the striatum following biochemical manipulation of the substantia nigra.

On the one hand, the aim of the present study was to investigate the sensitivity of the nigrostriatal dopaminergic pathway to the excitatory amino-acid agonist NMDA, the D2 agonist quinpirole, and the mixed D1/D2 agonist apomorphine. On the other, my aim was to study the effect of other drugs on the dendritic release of AChE and concomitant behaviour.

The majority of dopaminergic neurons which form the nigrostriatal pathway originate from the A9 cell group of pars compacta neurones, and, in most cases, synapses on dendrites of striatal output neurons. Within the substantia nigra and the nigrostriatal pathway there are three types of dopaminergic receptors: D1, D2 and D3. At present, it is very important to gain some understanding of the role of D1 and D2 receptors and, in particular, how they interact. This is important because animal experiments indicate, that D1 and D2 receptors agonists have synergistic effects. Selective agonists and antagonists are already available for these receptors and something of the physiological and biochemical actions of drugs acting on these receptors is already known.

4.4.1 The effect of apomorphine on nigral AChE-release and concomitant behaviour

Dopamine is a major synaptic transmitter and modulator utilised by cells in various locations in the CNS. Dopamine interacts with G-protein-coupled receptors, through which it modulates the availability of cyclic AMP and other secondary messenger systems such as phospholipase C (Grandy and Cinelli 1992). Dopamine-receptors belong to two classes: D1-like, which are positively coupled to adenylate cyclase and lead to an increase in cAMP, and D2-like, which inhibit adenylate cyclase and lead to a decrease in cAMP accumulation or act through some other second messenger system. The application of molecular biological techniques has led to the identification of further dopamine receptor subtypes: D1 (Tiberi et al. 1991) and D5 (Sunahara et al. 1991) in the D1-family, and D2 (Bunzow et al. 1988), D3 (Sokoloff et al.

1992) and D4 (Van Tol et al. 1991) in the D2-family. These five receptors can all be distinguished by their topology, deduced primary structure, size of messenger RNA, chromosomal location, tissue distribution, and binding kinetics with specific ligands (Niznik and Van Tol 1992).

Naive animals and animals with a unilateral 6-OHDA lesion of the nigrostriatal pathway were treated systemically with the mixed dopaminergic agonist apomorphine (1mg/kg). A large fraction of the mesencephalic dopaminergic neurons are located in the substantia nigra pars compacta, which projects via the medial forebrain bundle to the neostriatum, where dopaminergic terminals synapse onto medium spiny neurons as well as onto interneurons (Groves et al. 1994, Smith and Bolam 1990). Apomorphine administered to naive rats caused both contraversive and ipsiversive rotation, ending with a ipsilateral preference. There was a significant difference between behaviour prior to treatment and following apomorphine treatment ($P < 0.05$). Apomorphine in nigrostriatal lesioned animals, which showed prominent basal circling in a contraversive as well as an ipsiversive direction, brought the circling behaviour to almost a complete halt. Apomorphine showed a significant drug-effect before and after apomorphine treatment ($P < 0.05$). According to the established mechanism for turning behaviour, the direction of circling reflects the functional imbalance of the dopaminergic activity between the two sides of the brain, with the animal rotating away from the side producing higher activity (Pycock 1980). Pioneering studies by Herrera-Marschitz and Ungerstedt (Herrera-Marschitz and Ungerstedt 1985) showed that the characteristics of the rotation exhibited by rats in the Ungerstedt model were dependent on whether the exciting-drug acted primarily on D1 or at D2 receptors. Turning produced by apomorphine was characterised by a two-peak pattern, with conspicuous gnawing and biting and a tight rotation during which the animal appeared to be chasing its tail. Apomorphine is a mixed D1/D2 agonist. Most importantly, the effect of apomorphine was completely eliminated by a kainic acid lesion to the substantia nigra, which had already been lesioned using 6-OHDA (Herrera-Marschitz and Ungerstedt 1984, Herrera-Marschitz and Ungerstedt 1985). This reinforces the contention that the substantia nigra is an important site for the action of dopamine agonists. In their seminal paper, Herrera-Marschitz and Ungerstedt (Herrera-Marschitz and Ungerstedt 1984) concluded that apomorphine has its main effect via a D1-dependent pathway going from striatum to the substantia nigra (the striatonigral pathway). It is now understood that information flows out from the striatum to the globus pallidus, to the thalamus, and then on to the cortex. In the present study, the systemic administration of the mixed dopaminergic agonist

apomorphine to naive animals activated the brain's dopaminergic system. There are three larger, separate dopaminergic pathways in the brain of the mammal: the nigrostriatal pathway, the mesolimbic and mesocortical pathway and the tuberoinfundibulare pathway (Elbert and Rockstroh 1990). Systemical apomorphine stimulation, as was the case here, may stimulate all three dopaminergic pathways, and other cerebral systems may also be indirectly stimulated by dopamine too. Pycock (1980) pointed out that rats and other rodents show a natural preference to one particular side, and that this preference asymmetry can be revealed as turning behaviour.

6-OHDA lesioned animals showed prominent basal circling. Systemic administration of apomorphine lead to a near complete stop of the circling behaviour. Unilateral ablation of the medial forebrain bundle cells interrupts the nigrostriatal outflow, and drug-induced ipsilateral rotation was expected. However, prediction of the direction of circling in line with the aforementioned model was not appropriate, while the dose of apomorphine produced almost a complete halt to rotation. The resulting behaviour may lie in the mechanism caused by apomorphine and a receptor affinity leading to distinct neural events. Electrophysiologic studies in intact rats reported an inconsistent, highly variable pattern of changes in substantia nigra pars reticulata cells firing after systemic administration of apomorphine where cells exhibited increases, decreases, no changes, or minute-to-minute fluctuations in firing (Waszszak et al. 1984). Other transmitter systems may be conceivably involved with nigrostriatal pathway lesions. Konitsiotis and colleagues (Konitsiotis et al. 1997) observed in the substantia nigra pars reticulata of rats which were first treated with ibotenic acid and then treated systemically with apomorphine. A weak contralateral rotation after 0.5mg/kg apomorphine was observed, in contrast to an intense ipsilateral rotation following administration of 1.5mg/kg of apomorphine. For instance, an i.p. dose of 0.5mg/kg of apomorphine given to rats produced a decrease in spontaneous motor-activity (also observed in this study) (Ljungberg and Ungerstedt 1976).

The impact of the findings on existing concepts of circling behaviour as an index of basal ganglia function, more specifically in relation to dopaminergic activity and control of movement, remains speculative. The unexpected ceasing of rotation which resulted following the administration of apomorphine suggests that the traditional view of basal ganglia control is oversimplified and that the direction of turning does not merely reflect greater dopaminergic stimulation on one side. The organisation of cellular elements within the substantia nigra, as well as the diverse afferent regulation and extensive and widespread efferent connections to

motor and non-motor areas, may contribute to these results. Laurie and colleagues (Laurie et al. 1991) found that the number of TH-immunoreactive neurons remaining in the lesioned substantia nigra pars compacta correlated significantly with the number of rotations induced by apomorphine. A similar correlation was noted between the percentage of dopaminergic neurons in the treated substantia nigra pars compacta and in the untreated substantia nigra pars compacta. Furthermore, the number of apomorphine-induced rotations was also similar. Previous neurochemical studies (Hefti et al. 1980, Heikkila et al. 1981) showed that only animals in which the substantia nigra pars compacta had been treated contained 10% or fewer TH-immunoreactive neurons than the intact side of the brain and only these animals rotated after the administration of apomorphine. In this work, anatomical analysis of post-mortem tissue of lesioned animals showed a substantial reduction of TH-immunoreactive neurons in the lesioned substantia nigra. Barnéoud and colleagues (Barnéoud et al. 1995) found that apomorphine induced a contralateral circling behaviour in 13 rats which had received a unilateral injection of 6-OHDA into the medial forebrain bundle, and, according to the present study, the remaining 16 lesioned rats did not exhibit rotational behaviour. They observed that rats which circle following systemic apomorphine administration showed a maximum loss of nerve cells in the striatum (99.8%), and a high level of loss in the substantia nigra (85%). The rats which did not rotate had suffered a smaller loss of nerve cells in both the striatum (72%) and the substantia nigra (56%). In the present study, the dopamine of the treated striatum was decreased by 76% versus the untreated side of the brain. Also, Hudson and colleagues (Hudson et al. 1993) showed that the rotations induced by apomorphine are seen only in animals which have maximal lesions of the striatum and substantia nigra. They also described partially-lesioned rats which did not rotate after receiving apomorphine. In the work of Przedborski and colleagues (Przedborski et al. 1995), all of the rats that received different doses of 6-OHDA into the medial forebrain bundle rotated away from the lesioned side following administration of apomorphine.

Yanai and colleagues (Yanai et al. 1995) developed an avian model for the study of neurological disorders of the CNS and their treatment by neural transplantation. Black Silkie chickens (*Gallus domesticus*) received 6-OHDA into the nucleus tegmenti pedunculo pontinus (TP) and were stimulated with amphetamine or apomorphine i.p.. Amphetamine failed to produce rotation. On the other hand, apomorphine induced strong contralateral rotation in 12 of 26 chickens injected with 6-OHDA (5.97 ± 0.58 turns per minute) but not in sham-lesioned chickens (0.02 ± 0.04 turns per minute). Amphetamine, at every dosage level administered (0-

40mg/kg), produced no effect on locomotion. After 6-OHDA injection, the paleostriatum augmentatum (PA) exhibited a decrease in density of TH positive fibres and varicosities. Yanai and colleagues (Yanai et al. 1995) were able to show a treatment of neurological disorder of the CNS by neural transplantation. In their study, all transplanted 6-OHDA treated animals ceased rotation after transplantation, while their respective controls continued to rotate. Axonal projections from the TP have been shown to terminate after a time and release dopamine in the PA (Lindenblatt and Delius 1988).

Lesioning with 6-OHDA elicited different profiles for amphetamine and apomorphine-induced rotations. In contrast, this study showed amphetamine to cause ipsiversive as well contraversive robust rotation in the rats with a unilateral 6-OHDA-induced lesion of the nigrostriatal pathway. 6-OHDA may cause postsynaptic dopaminergic changes in the lesioned striatum that impair apomorphine stimulation of dopamine receptors while preserving amphetamine-induced stimulations of dopamine receptors in the intact striatum. Alternatively, other structures involved in rotation may be affected. It appears that using amphetamine to induce rotations is more sensitive than using apomorphine.

During treatment with apomorphine, a nigral AChE-release was also observed with the powerful chemiluminescent system. Pre-treatment with 6-OHDA caused a significant reduction in the spontaneous release of AChE by approximately 68%. Application of apomorphine effected a rise in the spontaneous release of AChE of approximately 43% above basal conditions ($P < 0.01$). An increase of nigral AChE was connected with minor motor activity.

4.4.2 The effect of quinpirole on behaviour

Dopaminergic neurons of the substantia nigra can release dopamine at both the nerve terminal level in striatum and at the somatodendritic level in substantia nigra. The diversity of dopamine's role as a mediator of mammalian psychomotor behaviour is exemplified by the fact that the dysfunction of dopamine neurotransmission has been linked to a wide range of CNS disorders, including Parkinson's disease, schizophrenia and drug addiction. Such diversity is not surprising in the light of the highly complex neuroanatomical organisation of dopaminergic systems (Gerfen 1992, Graybiel 1990). Pioneering experiments primarily identified two dopamine receptor subtypes - a D1 receptor and a D2 receptor (Kebabian 1979). Advanced molecular cloning techniques have now identified at least three additional dopamine receptor

subtypes. Dopamine D1-like receptors include D1 and D5 subtypes, and D2-like receptors include D2, D3 and D4 (Seeman and Van Tol 1993, Sibley and Monsma 1992).

In this study, the D2 selective agonist quinpirole was infused intranigral into naive animals. Infusion of quinpirole into the substantia nigra induced weak, contraversive, circling behaviour. There was no significant difference ($P=0.422$) to modest basal ipsiversive circling. In the work of Paul and colleagues (Paul et al. 1995) quinpirole caused very low rates of contraversive turning in unilateral 6-OHDA lesioned rats when it was administered systemically. The phenomenon of dopamine receptor plasticity has been implicated in a wide range of dopamine related disorders. It is well established that selective activation of D2 dopamine receptors can effectively prime D1 receptor behavioural responses (Morelli et al. 1987). Paul and colleagues (Paul et al. 1995) were able to show that the (D2-like) quinpirole-mediated priming process appears to be critically dependent upon NMDA receptor activity. Different dopamine receptors subtypes have been described in the substantia nigra. D1 receptors are localised on GABA strationigral terminals and are thus found in the substantia nigra pars reticulata (Timmerman and Westerink 1995, Weick and Walters 1987). Dopamine receptors have also been described on dopamine dendrites, acting as D2 autoreceptors and thus controlling the synthesis, firing rate and release of dopamine in the cell-body area as well as in the terminal area. Therefore, they are localised pre-synaptically in the substantia nigra (Strange 1996).

In fact, the substantia nigra is one of the main output nuclei of basal ganglia and, as such, is of great importance. The different nuclei which constitute the basal ganglia are, however, complexly connected, which suggests that an even higher number of neurotransmitters might be involved.

4.4.3 The effect of NMDA on nigral AChE- release and concomitant behaviour

Over the past decade, neuroscience research has led to major insights into the structure and function of the basal ganglia and to the development of models of the normal and abnormal functions of these nuclei in movement disorders (Albin 1995, Chesselet and Delfs 1996, Brooks 1995). These models have gained considerable clinical relevance because of their importance in guiding drug development and new surgical approaches.

The motor circuit has received particular attention because of its relevance to movement disorders. It comprises pre-central and post-central sensorimotor fields, as well as motor areas

in the basal ganglia, the ventral anterior, and ventral lateral nuclei of the thalamus. Cortical projections mainly terminate in the putamen. Putamenal output is directed towards internal segment of the globus pallidus/substantia nigra pars reticulata via two pathways: A direct monosynaptic pathway and an indirect polysynaptic pathway that passes through the external pallidal segment and the subthalamic nucleus. There are also direct external segments of the internal globus pallidus that circumvent the subthalamic nucleus (Parent and Hazrati 1995). Basal ganglia output is directed towards both the ventral anterior nucleus of the thalamus/ventral lateral nucleus of the thalamus and the brain stem. With the exception of the excitatory (glutamatergic) efferents of the subthalamic nucleus, the intrinsic and output connections of the basal ganglia are inhibitory (GABAergic). Release of dopamine from terminals of the nigrostriatal projection appears to modulate the activity over the two pathways: transmission over the direct pathway is facilitated via D1 receptors, and transmission over the indirect pathway is inhibited via dopamine D2 receptors (Gerfen 1995). The overall effect of striatal dopamine release is to reduce basal ganglia output, leading to increased activity of thalamocortical projection neurons.

Glutamate, an excitatory amino acid, is an important excitatory neurotransmitter into the CNS and has 3 different receptors: The NMDA-receptor, kainate receptor and quisqualate receptor. In this study, the glutamate agonist NMDA were applied locally to the substantia nigra to investigate the effect both on the dendritic release of AChE and concomitant behaviour. Perfusion of 10^{-2} M NMDA into the substantia nigra lead to an expressive change in the animal's behaviour and significantly enhanced the number of turns per minute ($P < 0.05$). In most of the cases, the animals exhibited ipsiversive circling behaviour. These results are in line with the work of Jones and colleagues (Jones et al. 1994). On the other hand, the lower concentration of NMDA did not produce any noticeable changes in the animals' behaviour. Weissenborn and Winn (1992) also observed enhanced exploratory behaviour and spontaneous locomotor activity after bilateral infusion of NMDA into the nucleus accumbens. The causes of dopaminergic neuronal cell degeneration in Parkinson's disease are not known but it has been proposed that excitotoxicity due to over-activation of excitatory amino acid receptors may be a factor in this cell loss (Kopin 1993, Zeevalk et al. 1994). Glutamate receptors have been localised on the dopaminergic neurons of the substantia nigra pars compacta (Difazio et al. 1992), and experiments on cultured mesencephalic dopaminergic neurons have shown that activation of NMDA receptors on these neurons can cause toxicity (Kikuchi and Kim 1993). It

is widely acknowledged that nitric oxide (NO) is produced in response to activation of NMDA receptors (Garthwaite 1991).

Westerink and colleagues (Westerink et al. 1992) have demonstrated that the application of glutamate agonists to the substantia nigra induces a dendritic release of dopamine. Gauchy and colleagues (Gauchy et al. 1994) have demonstrated the NMDA-stimulated dendritic release of dopamine in the substantia nigra pars compacta and pars reticulata in vitro. Overton and Clarke (1992) have found that stimulation of NMDA receptors on dopamine neurones enhances both their spontaneous and burst-firing activity. Indeed, the dendritic release of dopamine and AChE can be evoked by the same treatments and may actually be interdependent (Greenfield 1985). Dickie and colleagues (Dickie et al. 1996) showed that NMDA (0.1 μM) may be trophic for TH positive neurons in organotypic slice culture of rat ventral mesencephalon, but increasing the concentration of NMDA (100 μM) caused cell death.

Only a higher concentration of NMDA perfused through the substantia nigra significantly enhanced the release of AChE. The lower concentration had no influence. Jones and colleagues (Jones et al. 1994) showed that the direct stimulation of nigral NMDA receptors caused the release of AChE. This effect is likely to be produced by calcium entry into the cell, which has previously been shown to be necessary during the evoked release of AChE (Greenfield et al. 1983). This release could be blocked by the NMDA antagonist AP5. In the presence of ketamine, an anaesthetic known to block NMDA receptor activation, perfusion of NMDA into the substantia nigra failed to stimulate the release of AChE. Webb and colleagues (Webb et al. 1996) suggested that the hyperpolarising effect of AChE on the dopaminergic neurons may involve the NMDA receptor. Sustained application of NMDA or AChE led to an eventual hyperpolarisation mediated by potassium ions and reversed by K-ATP channel blocker tolbutamide. A feed-forward action of NMDA which involves AChE was suggested: Activation of nigral NMDA receptors evokes the release of AChE (Jones et al. 1994). AChE may then enhance NMDA receptor activation causing increased calcium entry which, if excessive, may compromise mitochondrial function and thereby reduce ATP which would cause activation of K-ATP channels. Therefore, these authors propose that this mechanism of enhancement of calcium entry by AChE may play an important role in long-term actions of AChE such as those apparent from behavioural studies.

4.5 The outgrowth of dopaminergic neurons in an organotypic slice culture of chick mid-brain

4.5.1 Immunocytochemical localisation of tyrosine hydroxylase in the mid-brain of the chick

The basal ganglia system in avian consists of the paleostriatum augmentatum (PA), the lobus parolfactorius (LPO), the paleostriatum primitivum (PP) in the telencephalon (Anderson and Reiner 1990), the nucleus accumbens (Ac) in the telencephalon (Reiner et al. 1983) and the nucleus tegmenti pedunculo pontinus pars compacta (TPc) in the mesencephalon (Moons et al. 1994). The PA and LPO in avian is the equivalent to the corpus striatum of mammals. A rather dense distribution of L-DOPA and dopamine fibres can be found throughout the entire LPO, the highest concentration of fibres being present in a ventral position to the Ac, a dense fibre innervation is also found in the more caudal parts of the PA (Moons et al. 1994). At the transition point from the hypothalamus into the mid-brain, a large group of immunopositive somata appears in the area ventralis of Tsai (AVT). This nucleus, homologous to the ventral tegmental area of mammals, contains very large numbers of medium immunoreactive perikarya. This cell group expands into the TPc, the equivalent of the substantia nigra of mammals (Moons et al. 1994). Several authors report the distribution of TH in discrete bird brain regions (Anderson et al. 1991, Fraley and Kuenzel 1993). Moons and colleagues (Moons et al. 1994) were able to show a rich plexus of dopamine-immunoreactive axons and terminals throughout the LPO, Ac and PA of the paleostriatal complex, which corresponds to what has previously been described for the avian homologue of mammalian striatum. Connections between the telencephalic fibre terminal fields and the dopaminergic cell groups in the mesencephalon have been studied by anterograde and retrograde tracing (Moons et al. 1994) and this data is consistently existent in birds (and mammals), as are dopaminergic projections such as the nigrostriatal and the mesocorticolimbic pathway (Anderson and Reiner 1991).

In previous works, the vast majority of the DOPA and dopamine-immunoreactive cells are found in the mid-brain, particularly in the TPc and in the AVT (Moons et al. 1994). These two groups of L-DOPA and dopamine neurons therefore correspond to the dopaminergic A9 and A10 groups in the mammalian nomenclature (Björklund and Lindvall 1984). The distribution of these neurons in the chicken mesencephalon and metencephalon generally agree with those reported in the brain of other avian species (Bailhache and Balthazart 1993, Bottjer 1993). This study was also able to show TH-immunoreactive cell bodies and fibres in the mid-brain

into the following brain regions: Substantia grisea centralis (GCT), AVT, locus coeruleus (LoC), TPc and nucleus subcoeruleus ventralis (SCv). From E18 on, what was seen as a strong TH staining of neurons and fibres was only seen as a weak TH staining at E16, and, at early stages, no immunoreactivity was seen.

A comparison of the noradrenergic system in the chicken brain with the dopaminergic cell and fibre system shows that dopaminergic neurons clearly outnumber the noradrenergic cell bodies and have a much wider distribution pattern (Moons et al. 1995). The absence of noradrenergic perikarya in the mid-brain appears to be a common finding in vertebrate species. The theory put forward by Smeets and Steinbusch (Smeets and Steinbusch 1990) and the work of Moons and colleagues (Moons et al. 1995) using immunocytochemical fibre stainings against dopamine and noradrenaline and their synthesising enzymes TH and dopamine- β -hydroxylase, support the suggestion that antibodies against TH primarily demonstrate dopamine fibres and varicosities, whereas the distribution of dopamine- β -hydroxylase-immunoreactive fibres generally corresponds to that shown by antibodies against noradrenaline.

4.5.2 Ventral mesencephalic cultures

The *in vitro* tissue culture system of organotypic slice cultures (Gähwiler 1981, 1988) is the experimental model used in the second part of this thesis to investigate the survival and development of chick mid-brain dopaminergic and cholinergic neurons.

A major drawback of *in vivo* studies is the complexity and nature of the system. Additionally, further variables arise solely from the experimental procedures performed. For example, interactions of anaesthesia, interactions with other systems, access of test drugs to their site of action and drug metabolism are all factors that have to be taken into account. *In vitro* studies drastically reduce the complexity of the system, and, by rigorously controlling and manipulating the environment of the cells under study, allow the objective and systematic measurement of the impact of numerous factors on cell function and viability. Despite these advantages of *in vitro* systems, a degree of caution must be employed when using such experimental manipulations concerning the extent to which *in vitro* findings can be extrapolated from *in vivo* situations. It should be remembered that fundamental characteristics such as receptor sensitivities, afferent connectivity and release of neurotransmitters may change under *in vitro* conditions.

In the early 1900s, Harrison was among the first scientists to succeed in maintaining cells and tissue outside the living organism (Harrison 1907, 1910). Since these early studies, *in vitro* culture techniques have become widespread and invaluable research tools. The application of these systems during research of the nervous system has allowed the exact and highly-specific measurement of many fundamental biological processes isolated from complicating factors found *in vivo*. Moreover, such techniques offer a simplified system to help our understanding of the complex process of neuronal development. Today, many kinds of cell culture preparations exist including dissociated cultures, reaggregate cultures, organotypic explant cultures and organotypic slice cultures. The more physiological the culture technique, the greater the relevance to the *in vivo* situation.

The aim of the organotypic slice culture preparation is to provide a cell-culture system that combines the advantages of dissociated, reaggregate and explant cultures. Therefore, the organotypic slice culture technique is an adaptation of the explant culture technique that permits the long-term preservation of tissue structure within cultures that are highly accessible to observation and experimental manipulation. Dissociated cultures involve isolation of the brain region containing the cells under study, followed by dissociation of the tissue into a single cell suspension. The cells are then plated onto a surface they will bond with. Fawcett and colleagues (Fawcett et al. 1995) found that 87% of dopaminergic neurons in a dissociated monolayer culture system died - a percentage which was far greater than the cell death seen in a 3-dimensional culture system. Finally, an additional problem with the dissociated-culture technique is that the trauma experienced by the cells under study will be immense. Indeed, Fawcett and colleagues (Fawcett et al. 1995) found that dissociation of the VM caused the death of approximately 30% of dopaminergic neurons. Thus, dissociation of the tissue could account for the fact that the duration of the culture period for the dissociated-culture technique is limited, due to a gradual decline in health and survival of the cultures. For the reaggregate culture system, dissociated cell suspensions are maintained in rotating flasks. The major advantage of reaggregate cultures is that the cells develop in 3-dimensions and maintain cell/cell interactions. Additionally, this system allows the study of interactions between different cell populations dissociated from different brain regions. Again, the drawbacks are that dissociation causes cell death and damage. The fundamental difference between organotypic slice cultures and explant cultures, that contributes to the accessibility of the slice culture system to observation and experimental manipulation, is that the culture thins to a monolayer thickness due to constant rotation of the tissue. The preparation of organotypic

cultures that retain a high degree of their original morphology and characteristics, including electrophysiological characteristics and the formation of functional synaptic networks, has been described using source tissue from various brain regions. These include the VM (Jones et al. 1995, Dickie et al. 1996), hippocampus (Del Fio et al. 1991), cerebellum (Mouginot and Gähwiler 1995), retina (Feigenspan et al. 1993), striatum (Østergaard et al. 1995). Organotypic co-cultures of slices from two brain regions may also be prepared. Indeed, co-cultures of mesencephalon with striatum, hippocampus and cerebellum (Østergaard et al. 1990), mesencephalon and cortex and striatum (Plenz and Kitai 1996).

The use of the organotypic slice culture system to study the development and survival of dopaminergic neurons of the ventral mesencephalon has been described previously, but never with regard to the chick, with the exception of the organotypic culture system of chicken retina (Hoff et al. 1999). In many these studies, embryonic source tissue was used, and such tissue was also chosen for this thesis. At first, I tried to use the chick to obtain an organotypic slice culture to find out if the chick mid-brain could be used as a suitable organotypic slice culture to study the outgrowth of dopaminergic neurons. One advantage is the easy procurement of the eggs. Østergaard and colleagues (Østergaard et al. 1990) demonstrated that the morphology of dopaminergic neurons within organotypic slice cultures resemble that seen in the postnatal rat *in vivo*. They exhibit several somatic shapes including bipolar, pyramidal and multipolar. Electrophysiological characteristics of dopaminergic neurons in organotypic slice cultures of ventral mesencephalon have been shown to resemble, in some respects, those seen in other *in vitro* and *in vivo* preparations (Stenson et al. 1995). However, one fundamental difference between organotypic slice cultures and other *in vitro* preparations is the presence of spontaneous burst-firing activity in organotypic cultures (Stenson et al. 1995), which resembles a firing pattern characteristically only seen in the intact animal and is not present in brain slice preparations (Yung et al. 1991). As it has already been hypothesised that burst firing results from afferent inputs (Yung et al. 1991), it is possible that these afferents are present in organotypic cultures which, in turn, suggests that organotypic cultures are a more realistic representation of the intact animal.

The immunohistochemical identification of TH with m- α -TH (generous gift of Prof. H. Rohrer, Frankfurt/Main) in this study enabled the identification of dopaminergic neurons within the chick's ventral mesencephalon slice cultures. TH catalyses the rate-determining initial step in the biosynthesis of catecholamines such as dopamine, noradrenaline and adrenaline. Therefore, the antibody is used to stain dopaminergic and adrenergic neurons. Decavel and

colleagues (Decavel et al. 1987) demonstrated, using a monoclonal antibody against DA, that the substantia nigra and ventral tegmental area were rich in dopaminergic neurons. Moreover, no adrenergic neurons were detected in the substantia nigra by using an antibody to noradrenalin (Geffard et al. 1986). Therefore, TH immunoreactivity within the substantia nigra is widely accepted as a reliable marker of dopaminergic neurons. The organotypic slice culture system of the chick provides a highly accessible system for the study of developmental processes within neuronal tissue. TH positive neurons in organotypic cultures of mesencephalon of the chick showed morphological characteristics similar to those found in the intact animal. Neurites with extensive branching patterns extended from the TH positive cell bodies often reaching far into the plasma clot or surrounding glia tissue. Furthermore, a high-power light microscopic analysis of the cultures revealed the presence of varicosities and growth cones on the TH positive neurites in organotypic cultures. The organotypic slice culture of the chick is, when compared to the organotypic slice culture of the rat (Jones et al. 1994, Holmes et al. 1995), less equipped with TH positive cell bodies, but it does produce impressive fibre growth.

The organotypic slice culture technique has both advantages and disadvantages when compared to other culture systems. Due to the lack of dissociation, organotypic slice cultures retain a high degree of their original cytoarchitecture and cell/cell contacts. However, the extent of retention of *in vivo* characteristics and tissue organisation is dependent on both the age of the donor tissue and the tissue of origin.

The lack of dissociation of the tissue also means that less trauma is incurred during preparation of organotypic slice cultures. This decreased insult to the tissue could contribute to the fact that, typically, organotypic cultures survive for longer culture periods compared to dissociated cultures: Organotypic cultures are normally maintained for up to several weeks but are capable of surviving up to nine months in culture (Østergaard et al. 1991). Long-term isolation of cultures is advantageous, since it allows recovery from dissection trauma and adaptation to a new environment, while also presenting the opportunity for studies of chronic drug application. In this study, chick embryos at the E18 stage of development were chosen, since a fair degree of the tissue-specific cytoarchitecture is already established. In previous studies, organotypic cultures were prepared using the ventral mesencephalon of 1-day postnatal rats (Jones et al. 1995, Holmes et al. 1995). In contrast to an organotypic slice culture which could survive for weeks or months (Østergaard et al. 1991), the organotypic slice culture of the chick survived for a period similar to that observed in the work of Jones and colleagues (Jones et al. 1995),

where the culture duration was thirteen days. But I was able to determine that the TH positive neurons surviving in the organotypic slice culture of the chick decreased with increasing culture duration. After fourteen days in culture, the tissue contracted to a small rim-aligning plasma rupture. All the tissue regions degenerated and died. These observations are in agreement with previous work, where the number of TH positive neurons surviving decreased with increasing culture duration (Holmes D. Phil. thesis 1996). However, Holmes was able to show that the density of innervation significantly increased when the culture duration was extended.

During the culture period, the organotypic slice cultures thinned to the thickness of one or two cell layers. This flattening of the slice has been attributed to the continuous rotation of the culture, since explant cultures subjected to the same culture conditions but maintained in a petri dish remain several hundred micrometers thick (Gähwiler 1981). The mechanical action of the rotation was important for the thinning of the cultures. The culture conditions were also a contributing factor in the thinning of the cultures; cultures grown in horse serum and incubated are thinner than those kept in foetal calf serum (Gähwiler 1981) and cultures mounted on collagen spread and thin to a lesser extent than those mounted on a plasma clot (Jones D. Phil thesis 1992). The organotypic slice cultures of the chick were incubated in a petri dish (in a DMEM medium with foetal calf serum in an incubator) at 37°C, 70 rpm and 4% CO₂. The fact that the cultures were not incubated in a roller-drum with horse serum produced no disadvantage for the survival and development of dopaminergic neurons in the organotypic culture of the chick. Under these conditions the slice became flatter. The addition of cytostatic solutions prevent over-proliferation of non-neuronal cells. It is also apparent that different tissue sections flatten more than others, since cerebellar organotypic cultures reach a monolayer thickness more readily than spinal-cord cultures (Gähwiler 1981). The thinning of organotypic cultures has major advantages: The cultures may be easily visualised by conventional light microscopy, and pharmacological manipulation of the culture is facilitated since the diffusion barriers to drugs are weaker than in the thicker explant cultures. The differences of the organotypic slice culture of the chick compared to the organotypic slice culture of the rat in the experimental design are summarised in Table 1.

Table 1 Differences between organotypic culture of chick and rat in the experimental design

| | chick | rat |
|-------------------------|-------------------|------------------------------|
| donor age | E18 | P1 |
| coronal sections | medial to lateral | caudal to rostral |
| coverslip was placed in | a petri dish | a cap diagonal bottomed tube |
| serum | foetal calf serum | horse serum |
| rotation with | gyratory shaker | roller drum |

The main disadvantage of the organotypic slice culture technique used in this thesis was the variability seen, which impeded quantitative analysis. One reason for the increased variability of organotypic slice cultures compared to dissociated culture systems was the fact that unlike the dissociated culture system where the plating density can be calculated, the number of cells present in each slice at the start of the culture period was not known. A further factor adding to the variation seen was the fact that each slice contained dopaminergic neurons from a different level (medial to lateral). An organotypic slice culture does not mimic the *in vivo* environment exactly. During preparation of the slice cultures, all afferent and efferent pathways of the brain region are severed. Since the *in vitro* environment differs from the *in vivo* environment, it is also unlikely that all the factors which regulate innervation of the *in vivo* substantia nigra by dopaminergic neurons also regulate innervation in culture. Although these drawbacks should always be considered when interpreting results from studies employing organotypic slice culture, this approach has an advantage over *in vivo* studies in that it provides a simplified and controlled environment to study the dynamics of dopaminergic innervation of the substantia nigra.

Exogenously applied AChE has no influence on the dopaminergic neurons in the organotypic slice culture of the chick. In the study carried out by Jones and colleagues (Jones et al. 1995) the addition of a high concentration of purified AChE to the culture medium significantly enhanced TH positive neurite outgrowth. In the present study, only purchased AChE was available. Maybe this was the reason why no noticeable change was induced in the culture. In the study done by Layer and colleagues (Layer et al. 1993) anticholinesterases changed the morphology of retinal explant neurites when grown on a laminin stripe assay. There was an inhibition of neurite growth by BW284C51, an inhibitor of AChE, which prompted these authors to say that this observation strongly indicated that AChE can regulate neurite growth.

Perhaps the effect of AChE on neurite growth does not depend directly on the enzymatic activity, but may correlate in a different epitope of the AChE molecule. Similar AChE action has been further supported in recent studies. BW284C51 retarded neuritic outgrowth and neuronal migration of cultured dorsal root ganglion neurons (Dupree and Bigbee 1994). When dissociated chick brain or sympathetic neurons were grown on plates which had been pre-coated with purified AChE, neurite outgrowth was strongly stimulated (Small et al. 1996). Srivatsan and Peretz (1997) showed that AChE contributes significantly to the neurite-growth-promoting effects of hemolymph in *Apalysia*. The report on the effects of AChE on neurite growth in the embryonic neurons of the chick sympathetic ganglion culture (Small et al. 1995) also demonstrates that AChE promotes neurite growth. The extensive presence of AChE as a secreted protein at non-cholinergic sites, e.g. in extra-cellular space, plasma and CSF (Appleyard et al. 1987, Massoulié and Bon 1982, Massoulié et al. 1993) points to a non-catalytic function of this molecule. AChE is expressed at early stages of embryogenesis, before the formation of synapses in chicks (Layer et al. 1985). During development in chicks, migrating neural crest cells at cephalic and trunk levels show intense AChE activity suggesting that AChE has a morphogenetic function (Layer and Kaulich 1991). In the rat, Robertson (Robertson 1987, 1991) reported a transient expression of AChE in thalamo-cortical neurons between the first and third postnatal week, and suggested that AChE helped in target recognition and synapse formation. When the cultures in the present study were incubated in BW284C51 at a concentration of 10^{-4} M, the TH positive neurons became rudimentary. In the work of Jones and colleagues (Jones et al. 1995), TH positive neurons in the organotypic slice culture of the rat incubated under the same conditions decreased to 12%. They showed that incubation in 10^{-4} M BW284C51 results in a decrease of 12% of the cell body diameter of TH positive neurons and the scored density of neurite outgrowth was decreased to 32% of the control values. BW284C51 could have been toxic to TH positive neurons. A second possible mechanism by which BW284C51 could have produced such effects is by an effect on a secondary, non-enzymatic domain of AChE, which is involved in cell adhesion and neuritic outgrowth. Sussman and colleagues (Sussman et al. 1991) have demonstrated that the choline-binding site of AChE lies near the bottom of a deep and narrow gorge within the molecule (covered in aromatic residues) that produces many different ways and places for inhibitors to bind. Indeed, the binding of an inhibitor to AChE can produce structural changes in parts of the molecule distant from the inhibitor (Massoulié et al. 1993). BW284C51 is known to bind at a peripheral anionic site at the rim of the catalytic gorge, placing it in an ideal position to block

both the catalytic activity of the enzyme and also to modulate a postulated secondary, non-catalytic site in another part of the molecule. Layer and colleagues (Layer et al. 1993) have suggested that this secondary site could be the HNK-1 carbohydrate epitope which is present in electric eel AChE (Bon et al. 1987).

4.5.3 Clinical relevance of findings

The Parkinsonian model is used for the study of connection repair. Its symptoms can be induced by the degeneration of a single neuronal type, dopamine. Its motor pathways have been well studied. One treatment for Parkinson's disease which is becoming increasingly common is that concerning the transplantation of foetal tissue (Ugryumov 2000). Zhou and colleagues (Zhou et al. 1996) reported that a bridged mesencephalic transplant can anatomically, neurochemically and functionally reinstate a 6-OHDA-eradicated nigro-striatal pathway. At first glance, the findings of these studies may appear to be beneficial for the construction of a new nigrostriatal pathway in the Parkinsonian model. However, it should not be ignored that these experiments are the first to have ever been done. Further experiments to investigate the suitability of the organotypic slice culture of the chick when studying the survival and development of mid-brain dopaminergic neurons have to be carried out. More than a decade after neural transplantation was introduced, the concept of replenishing degenerated neurons has advanced from basic experimentation to clinical application (Lindvall and Odin 1994, Kordower et al. 1995). Progress has been made in all areas ranging from expanding sources of neuronal tissue to enhancing neuronal survival and nerve fibre outgrowth (Rosenstein 1993, Kordower and Sanberg 1995). To this date, the repair of a completely damaged pathway has not been achieved because of the inability to direct the outgrowth from a transplant. A major obstacle of the homotypic transplant, a graft placed in an ontogenic site, is the lack of outgrowth from the graft reaching to a distal ontogenic target region. The study carried out by Holmes (Holmes D. Phil thesis 1996) demonstrated (using co-cultures) that when the tissues were close together, innervation of striatum by TH positive neurons occurred by single axons, crossing into the striatum; but when the tissues were further than 1 mm apart, innervation occurred by what appeared to be a glial pathway. When the tissues were separated by more than 2.5 mm innervation nearly always failed to occur. However, the findings of this

thesis could have relevance in the research concerning the normal function of the basal ganglia, as well into present and future treatment for Parkinson's disease.

4.6 Concluding remarks

The aim of this project was to examine a selected aspect of central nervous systems - namely the nigrostriatal path in the rat and baby chick.

It is possible to examine various pharmaceutical aspects in this system using the sensitive chemiluminescence procedure. For the first time, it was also possible to extract a portion of this system from the chick and bring it to culture.

In the first part of this project it was possible to show that amphetamine had an effect upon the behaviour and nigral acetylcholinesterase. The introduction of amphetamine undoubtedly increased the number of nigral acetylcholinesterase and stimulated their behaviour.

The second phase of this project was able to demonstrate conclusively the difference between healthy subjects and those inflicted with Parkinson's. Amphetamine could actually lower the level of hyper-motoric activity of infected subjects, but could not increase the concentration of acetylcholinesterase.

In the third part of this experiment, it was shown that the alternative pharmaceuticals had no significant effect upon the nigral system, neither in healthy nor infected subjects.

The fourth phase of this study was successful in culturing dopaminergic cells of the substantia nigra of baby chicks. These cells and their cell growth were made visible and resulted in a thick web of cell multiplication.

In conclusion, it was shown that the relationship between acetylcholinesterase and behaviour could be effectively studied with the on-line system. From all of the pharmaceuticals tested, amphetamine proved the most effective. For the first time ever, it was also possible to grow and cultivate dopaminergic cells of the substantia nigra of baby chicks cultivated from a slice culture. The extent to which the conclusions and findings of this study will serve to help treat those afflicted with neuron-degenerative diseases remains to be seen.

CHAPTER 5

5 REFERENCES

5 References

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Affirmation

With this, I declare instead of an oath that I have drawn up the thesis presented here by myself and only by the aid of the mentioned resources.

Darmstadt, Winter 2001/02