DIE NICHT-CHOLINERGE FUNKTION DER AZETYLCHOLINESTERASE IN DOPAMINERGEN GEBIETEN DES ZNS IN GESUNDEN, PATHOLOGISCHEN UND SICH ENTWICKELNDEN SYSTEMEN

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THE NON-CHOLINERGIC FUNCTION OF ACETYLCHOLINESTERASE IN THE DOPAMINERGIC AREAS OF THE CNS IN HEALTHY, PATHOLOGICAL AND DEVELOPING SYSTEMS

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Preface

It is my belief that a few words of introduction are necessary in order to better understand this project. This project was undertaken using two separate research teams. One team was concerned with pharmacological in vivo experiments, mainly using rats and guinea-pigs, whereas the other research team concentrated on the development of chick retinas and worked in vitro.

Both teams, however, concentrated on the characteristics of acetylcholinesterase (AChE). In the case of the nigrostriatal system in the rat brain (when taken in conjunction with a neuron-degenerative central nervous system (CNS) disease), Parkinson's disease causes a great reduction in the neuron transmitter dopamine (DA) in a specific part of the brain - namely the substantia nigra (SN).

The first part of this study was conducted at the Department of Pharmacology in Oxford, England, while the second part was undertaken at the Institute for Zoology, Darmstadt, Germany. I proceeded as follows: First of all, I was interested in the behavioural effects of administering amphetamine to healthy animals and its effect upon the AChE in the SN. With the knowledge gained from those experiments, I wanted to examine the effect that an introduction of amphetamine would have upon sick animals (6-OHDA is a neurotoxin). The 6-OHDA in damaged animals is comparable to Parkinson's disease in humans. This experimental model was developed as it is not ethically permissible to conduct this type of research on humans.

Amphetamine was administered to substantially increase the concentration of DA at the synapses. To our advantage, we understand that in most cases of Parkinson's, the SN has been damaged consequently less DA will reach the basal ganglia. Using this method we are able to recognise the signs and symptoms of this disease. Present day treatment of Parkinson's is based on this theory, particularly the introduction of L-Dopa (an essential building block for DA, since DA cannot pass through the blood brain barrier) in order to replenish basal ganglia with a DA deficiency.

Amphetamine, however, is not the only drug to have an effect upon the DA system, thus my desire to test other pharmaceuticals. Apomorphine, a mixture of D1/D2 agonist, is a substance which binds and stimulates D1 and D2 receptors and thereby strengthens natural processes. Another substance was quinpirole, a selective D2 agonist. Glutamate receptors are found on dopaminergic neurons of the SN pars compacta. Glutamate is an important neuron transmitter stimulant and its agonist NMDA could facilitate the release of DA to the SN. The knowledge gained made me want to learn more about the effects of NMDA.

With the results of my experiments and the acquired knowledge, I returned to Germany where I pursued my interest in the basal ganglia and tried, with success, to cultivate the homologous structures of the SN and their DAergic neurons in baby chicks.

Kronberg/Schönberg Bettina Heiland

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ABSTRACT

The exact role and function of the CNS is extremely complex and, despite decades of research, still not clearly understood. Malfunction of the basal ganglia has been implicated in several diverse neurological disorders such as Alzheimer's disease, hemiballism and of most interest to this study, Parkinson's disease. The main topic of this thesis is the substantia nigra and, in particular, the apparent relationship between dopamine and acetylcholinesterase in this region. 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. Numerous neurotransmitter or neuromodulator substances are found in the CNS, the distribution, fibre connections and ultrastructure of the peptidergic system have been explored, though little is known about their function. Colocalisation of classical neurotransmitters and neuropeptides is a widely accepted feature of neurons in many parts of the CNS.

The materials and methods chapter provides a detailed account of the experimental procedures used in this thesis. Particular reference is made to the on-line chemiluminescent system, as this procedure is not used outside Prof. Dr. S.A. Greenfield's laboratory.

The experimental chapters presented in this thesis fall into three main sections. The first set of experiments determine whether a relationship exists between dopamine, the regulation of AChE- release in the substantia nigra of the rat and concomitant behaviour following different drug stimulations in naive and 6-OHDA lesioned rats. The experiments indicated that different drug stimulations in naive animals and in 6-OHDA animals have an influential effect on the release of AChE and behaviour. In addition, the local or systemic application of amphetamine significantly increased the spontaneous release of AChE in the substantia nigra

and concomitant behaviour in naive animals. Apomorphine, quinpirole and NMDA showed a lesser effect. Neurotoxin pre-treatment significantly reduced AChE-release in the substantia nigra and dopamine content of the ipslateral striatum. Amphetamine applied locally to lesioned animals showed no significant increase in the release of AchE, but did produce a significant return from robust basal circling to normal behaviour. In contrast to this, the systemic application of amphetamine increased the release of AChE and the level of both contraversive and ipsiversive circling behaviour. Sham-operated animals showed results similar to those achieved with the naive animals.

In the second study, a new animal model was established using (E18) embryonic chicks (Gallus gallus domesticus), the aim of which was to determine the development of mid-brain dopaminergic neurons in organotypic slice culture and the development and regeneration of chick dopaminergic neurons of the ventral mesencephalon. It was possible to culture single ventral mesencephalon slices of the chick. Cultures were stained for tyrosine hydroxylase. When the culture medium was supplemented with AChE, the level of growth in tyrosine hydroxylase-immunoreactive neurites was not changed. Addition of a specific inhibitor of AChE, BW284c51, caused cell death.

In the discussion chapter, the general findings and conclusions of this thesis are discussed in the light of previously published work. Furthermore, the possible role and function of the relationship between the dopaminergic and cholinergic systems are suggested, and future proposals are made regarding continued research which would both extend and clarify further the findings of this thesis.

Contents

5-HT	serotonin maleate
6-OHDA	6-hydroxydopamine
Ac	nucleus accumbens
ACh	acetylcholine
AChE	acetylcholinesterase
ACSF	artificial cerebrospinal fluid
ATP	adenosinetryphosphate
AVT	aria ventralis of Tsai
BChE	butyrylcholinesterase
BW284c51	1,5-bis-(4- allyldimethyl-ammoniumphenyl) pentan-3-one-dibromide
CNS	central nervous system
CSF	cerebrospinal fluid
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
DTAF	dichlorotriazinyl amino fluorescein
E14	embryonal day 14
GABA	gamma-aminobutyric acid
GBSS	Geys balanced salt solution
GCL	ganglio cell layer
GCT	substantia grisea centralis
HPLC	high performance liquide chromatography
INL	inner nuclear layer
ip	intraperitoneal
IPL	inner plexiform layer
LoC	locus coeruleus
LPO	lobus parlofactorius
MPTP	N-methyl-4-phenyl-1,2,5,6, tetrahydropyridine
NMDA	N-methyl-D-aspartate
OFL	optical fibre layer
ONL	outer nuclear layer
OPL	outer plexiform layer
P1	postnatal day 1
PA	paleostriatum augmentatum

PBS	phosphat bufferd saline
pc	pars compacta
PP	paleostriatum primitivum
pr	pars reticulata
RPM	revolutions per minute
SCv	nucleus subcoeruleus ventralis
SER	smooth endoplsmic reticulum
SN	substantia nigra
TH	tyrosine hydroxylase
TPc	nucleus tegmenti pedunculo pontinus pars compacta
TR	Texas Red
TTX	tetrodotoxin
VM	ventral mesencephalon
VTA	ventral tegmental area

CHAPTER 1

1 INTRODUCTION AND AIM OF THIS WORK

1 Introduction

In 1817, James Parkinson gave the first account of a disease which he described as `shaking palsy´ which is more commonly known today as Parkinson's disease (Parkinson, 1817). The disease is characterised by three major symptoms: a trembling of the limbs coupled with inferior movements and rigidity. These symptoms can also be accompanied by a stooped, shuffling walk and an inability to maintain an upright stature. The exact cause of the disease is unknown. However, various factors may be involved in the aetiology of the disease, including the environment, genetics, toxins, post-viral damage and free-radical formation. Moreover, a loss of dopaminergic neurones in the pars compacta region of the substantia nigra is present in all cases. This loss of neurones, particularly in the nigrostriatal pathway, leads to a lower release of dopamine in this projection and a reduction in the interaction of two major parts of the basal ganglia: the striatum and the substantia nigra. Despite extensive research in the field, the questions as to why the nigrostriatal pathway degenerates and why the nigral dopaminergic population of neurons is so vulnerable remain unanswered.

1.1 The basal ganglia

The role and function of the basal ganglia, which consists of the globus pallidus, the striatum, the subthalamic nucleus and the substantia nigra, is very complex. The exact role and function of this area is complicated and still not clearly understood. Malfunction of the basal ganglia has been implicated in several diverse neurological disorders such as Alzheimer's disease, hemiballism, and, of most significance to this study, Parkinson's disease. The basal ganglia is thought to be involved in the control of movements. However, there are no direct connections between any of its regions and the spinal cord, and, as a result, no direct control of muscle contraction. The basal ganglia contains multiple internal pathways involving a multitude of neurotransmitters (Trepel, 1999). The main reason for such extensive interest in the structure of these areas is the loss of function they experience when affected by several widespread, and, as yet incurable, neurological diseases. The main regions of the basal ganglia that are studied in this thesis are the striatum, substantia nigra and the nigrostriatal pathway. The **striatum**, which comprises the caudate nucleus and putamen, is the largest cell mass in the basal ganglia. This region receives projections from the cerebral cortex, the central median nucleus of the thalamus and from the substantia nigra (nigrostriatal pathway). Within the

striatum there are various levels of compartmental organisations, including a mosaic organisation of neurochemical systems related to neuroanatomical connections. Thus, the mosaic organisation into `patchwork´ and histologically-distinct `matrix´ compartments reflects the heterogeneous distribution of neuroanatomical markers. The striatum contains densely-packed neurons and, for this reason, the first level of compartmental organisation is divided into separate populations of neurones. A primate's **substantia nigra**, which literally means black substance, is characteristically black in colour because of the presence of a high concentration of neuromelanin pigment in the pars compacta region. The substantia nigra can be divided into three regions, pars lateralis, pars compacta (from where cells innervate the striatum) and pars reticulata (from which cells innervate the thalamus, superior colliculus and pedunculopontine nucleus). The pars compacta, the dorsal layer of the substantia nigra, contains neurones with no collaterals which project into the striatum. The vast majority of neurones are dopaminergic in nature. These dopaminergic neurones are densely-packed A9 neurones. In the ventral segment, dopaminergic dendrites exist which extend into the pars reticulata region. In the dorsal segment, dendrites of dopaminergic neurones extend to the mediolateral pars compacta. The dopaminergic neurones of the pars compacta continue into the striatum, on medium spiny output neurones which then project back to the substantia nigra. The pars reticulata, the ventral layer of the substantia nigra, is composed of efferent neuronal populations mixed with interneurones. In this region, the neurones are surrounded by more glial cells than those in the compacta region and the neurones are also less densely packed. The majority of the neurons project to structures outside of the substantia nigra, such as the superior colliculus, the thalamus and pedunculopontine nucleus. The majority of neurons in the pars reticulata are GABAergic. One of the connection pathways between substantia nigra and striatum is the **nigrostriatal pathway**. The nigrostriatal pathway transports neurochemicals from the substantia nigra to the striatum. The majority of dopaminergic neurones which form this pathway originate from the A9 cell group of pars compacta neurones, and, in most cases, synapse on dendrites of striatal output neurones. A selective loss or deficit in the substantia nigra pars compacta neurones produces clear deficit in locomotor activity, e.g. in Parkinson's disease and its equivalent animal models.

1.2 Dopamine in the nigrostriatal pathway

It has been known for more than 20 years that dopamine is spontaneously released from dendrites of dopaminergic nigrostriatal neurones. It was shown, in vitro, that the application of

depolarising concentrations of potassium caused a dendritic release of dopamine in a calcium-dependent manner (Geffen et al., 1976). In addition, in vivo studies showed that both amphetamine and depolarising concentrations of potassium could evoke a dendritic release of dopamine (Nieoullon et al., 1977). It has been suggested that dopamine is stored in the smooth endoplasmic reticulum (SER) (Henderson and Greenfield, 1984). Within the substantia nigra and the nigrostriatal pathway there are three types of dopaminergic receptors: D1 (pars reticulata), D2 and D3. Once released from within the substantia nigra, dopamine diffuses through the extra-cellular space and can act on these receptors on nigrostriatal neurones, whereby it is most potent on D3 receptors.

1.3 Acetylcholinesterase in the central nervous system

There are two different enzymes present in vertebrates that can hydrolyse acetylcholine; acetylcholinesterase (AChE, EC 3.1.1.7) is acknowledged as the enzyme that rapidly hydrolyses the neurotransmitter acetylcholine to acetate and choline. For this reason, it is absolutely necessary in order to maintain continuous synaptic transmission. The hydrolysis of acetylcholine by AChE takes place very quickly and is almost as fast as the maximal theoretical limit set by molecular diffusion of the substrate (Rosenberry, 1975). The existence of AChE was postulated by Dale in 1914 and demonstrated by Loewi and Navratil in 1926. In 1937, Marnay and Nachmansohn observed high concentrations of AChE at neuromuscular junctions and in the electric organs of Torpedo and Electrophorus (see Massoulié, 1993). The other form of cholinesterase is butyrylcholinesterase (BChE, EC 3.1.1.8), also called non-specific cholinesterase or pseudocholinesterase. It is present in serum and has no known physiological function. Although BChE can hydrolyse acetylcholine, (albeit three times less effective than AchE), it can also hydrolyse many other esters. AChE is predominant in muscles, in red blood cells, in the brain and in the peripheral nervous system, while BChE is mainly synthesised in the liver and secreted into the plasma, as well as being present in the brain. There is an additional variation between these two enzymes in that BChE exhibits maximum activity of acetylcholine hydrolysis in high concentrations of the transmitter acetylcholine; with these levels of acetlylcholine, AChE is inhibited by excess substrate (Massoulié and Bon, 1982). Evidence to date indicates that there is no correlation between the distribution of the two enzymes in various tissues; however, a lack of AChE is fatal. In contrast, certain human individuals can be deficient in BChE or even lack it, without any physiological consequences.

For many years, it was acknowledged that the sole function of the only form of AChE known at that time (the membrane-bound form) was the termination of cholinergic neurotransmission. Over the last forty years, these views have changed considerably, particularly with the detection of multiple forms of AChE and also with the discovery of soluble forms of AChE on the periphery and in the central nervous system.

AChE displays a very rich molecular polymorphism, for they exist as membrane-bound, basal lamina-anchored or soluble forms. They can exist as monomers or oligomers consisting of glycoproteic catalytic sub-units. Furthermore they can be distinguished on the basis of their quarternary structure; for example globular (G), containing one, two or four subunits (G1, G2 and G4 respectively), and asymmetric (A) forms, consisting of one to three globular tetramers (A4, A8 and A12) (Massoulié and Bon, 1982).
In the mammalian CNS, the predominant form of AChE is the G4 form. This can be further subdivided into the hydrophilic/soluble G4 (20% of total G4) and the membrane-bound G4 form (approximately 80% of total G4). Chubb and Smith (1975a) were the first to show secretion of the soluble G4 form of AChE in bovine adrenal medulla. In the CNS, AChE has been located within the cell body, dendrites (Henderson and Greenfield, 1984), axons of dopaminergic neurones in the substantia nigra, and within noradrenergic neurones in the locus corruleus. It was not until 1979 that AChE-release in the substantia nigra was first demonstrated (Greenfield and Smith, 1979). Further examination of this phenomenon revealed that the protein appears to be released from the dendrites of pars compacta dopaminergic neurones in this region (Greenfield et al., 1983b) in a similar way to dopamine (Greenfield, 1991) and the dendritic release of AChE has been shown to be evoked by a dendritic calcium conductance (Greenfield, 1985; Linás et al., 1984; Linás and Greenfield, 1987). Perhaps one of the greatest advances in the understanding of the relevance of dentritic release of AChE in the substantia nigra has come with the development of an `on-line´ chemiluminescent system which permits the continuous release of AChE in the region being monitored (Taylor et al., 1989). Recent studies using this system have indicated that the release of AChE can be correlated with motor activity and sensory stimulation (Jones et al., 1991, 1994).

In a similar fashion to other glycosylated membrane and secreted proteins, cholinesterases, in particular AChE, are synthesised in the rough endoplasmic reticulum and translocated to the lumen where signal peptides are cleaved. Histochemical studies carried out by Henderson and

Greenfield (1984) showed that AChE is in the SER and the Golgi apparatus of the dendrites in the substantia nigra. In addition, it was also reported that AChE was present in the extra-cellular space surrounding these pars compacta neurons.
In the early 1980s, it was observed that in vivo AChE release could be evoked in the substantia nigra and that this release was calcium-dependent (Greenfield et al., 1980; Greenfield et al., 1983a).

Over 20 years ago, a highly controversial theory proposed that cholinesterases may have non-classical functions (Silver, 1974). In the last two decades, this theory has slowly gained widespread acceptance. The key observation that led to the hypothesis of non-classical functions for AChE was that AChE may be present in areas unrelated to cholinergic transmission. Cholinesterases are widely distributed both in the central and peripheral nervous systems as well as in serum and other non-neuronal tissues. Such `non-cholinergic´ cholinesterases have been observed in non-neuronal structures (e.g. plasma, erythrocytes, lymphocytes, thymocytes, megakaryocytes), as well as neurons from various regions (e.g. cerebellum, locus coeruleus, dorsal raphé nucleus). Henderson and Greenfield (1987) showed that the substantia nigra is a brain region that contains disproportionately high amounts of AChE. Moreover, AChE within the substantia nigra is associated with the dopaminergic neurons of the pars compacta region (Henderson and Greenfield, 1984). Using electron microscopy, it was demonstrated that nigral AChE is localised within the Golgi apparatus, the smooth endoplasmic reticulum of dopaminergic pars compacta neurons and their dendrites, and to the surface and surrounding extra-cellular space of these cells (Henderson and Greenfield, 1984).
As soluble AChE is present in cerebrospinal fluid (CSF), Greenfield and Smith (1979) investigated release of AChE during stimulation of specific brain regions. Electrical stimulation of the caudate nucleus, substantia nigra or hypothalamus led to an increase in the detectable activity of AChE in CSF sampled from the cisterna magna of rabbits. Since this initial study, much data has been accumulated on the release of AChE from the substantia nigra. AChE release may be evoked by a depolarising concentration of potassium ions (Greenfield et al., 1983) and is independent of cholinergic receptor stimulation (Weston and Greenfield, 1986). Furthermore, stimulation of both the dorsal raphé nucleus (Dickie and Greenfield, 1994; Dickie and Greenfield, 1995) and the subthalamic nucleus (Jones et al., 1994) evoke nigral release of AChE. More recently, a relationship between AChE release and the synthesis of dopamine has

also been demonstrated (Dally and Greenfield, 1994; D. Phil. thesis Dally, 1996). Greenfield (1991) proposed that AChE release may be mediated by a special calcium conduction seen in the dendrites of dopaminergic pars compacta neurons. Since the release of a soluble form of AChE is not required by the molecule to carry out its classical role, this release could be a feature of the molecule's non-cholinergic functions in connection with dopaminergic nigral neurons.

Many lines of evidence have supported the hypothesis first suggested by Drews (1975) which put forward that an alternative role for AChE could be related to neuronal development. During development, it has been demonstrated that a temporal shift in expression of AChE forms exists. The shift is from the monomeric (G1) and dimeric (G2) forms, which have a lower molecular weight, to the tetrameric form (G4), which has a higher molecular weight. This shift has been shown to occur in several species including the chick (Layer et al., 1987), quail (Anselmet et al., 1994), mouse (Inestrosa et al., 1994), and rat (Muller et al., 1985).

The reasons for suggesting a morphogenic role for AChE have been based on the finding that expressions of AChE can change or disappear totally during the course of development. It has been proposed that this `embryonic´ AChE is non-cholinergic and does not change choline acetyltransferase levels, since choline acetyltransferase (the synthesising enzyme for acetylcholine) does not show similar transient expression patterns and lesions that inhibit AChE in projection fields (Robertson and Yu, 1993). Layer and colleagues found that the transient expression of AChE in the chick embryo corresponds to the period of increased differentiation and neurite extension. Interestingly, BChE expression precedes that of AChE and thus corresponds to periods of cellular proliferation (Layer, 1983; Layer and Sporns, 1987; Layer, 1991; Willbold and Layer, 1992). Since this group also found that BChE expression and AChE expression were mutually exclusive (Layer, 1983), they propose the following scheme: BChE has a role in the proliferation of cells but once levels of BChE start to drop i.e. at the end of the proliferative phase, levels of AChE rise. Thus, AChE could have a role in the control of neurite extension and synaptogenesis (Layer and Willbold, 1995).

More direct evidence that AChE has a morphogenic function was provided by Gupta and Bigbee (1992) who showed that the level of AChE activity in cultured dorsal root ganglion neurons varied according to the permissiveness of the substratum to neurite outgrowth. Layer

et al. (1993) also demonstrated that AChE is actively involved in neurite outgrowth since the extent and pattern of tectal and retinal neurites was altered by cholinesterase inhibitors (BW284c51 and the BChE inhibitors ethopropazine and bambuterol). Dupree and Bigbee (1994) found that treatment with a cholinesterase inhibitor (BW284c51) retarded neuritic outgrowth and neuronal migration of cultured basal root ganglion neurons. A stimulatory action of AChE has been further supported by recent studies. Small et al. (1996) showed that when dissociated chick brain or sympathetic neurons were grown on plates pre-coated with purified AChE and heparan sulfate proteoglycans, neurite outgrowth was strongly stimulated. Srivatsan and Peretz (1996) demonstrated that the stimulatory action of Apalysia hemolymph on cultured pedal dopaminergic neurons is mediated by AChE.

1.4 Monitoring of in vivo AChE release using the `on-line´ chemiluminescent technique

Neurochemical release can be measured by a wide variety of techniques, depending on the neurochemical under investigation and its location within the central nervous system. These techniques adopt one of two approaches, either measuring the release of the neurochemical into CSF (CSF sampling) or measurement of neuroactive substances released directly into the intercellular space (cortical cup method, microdialysis, in vivo voltammetry, push-pull technique). These techniques are very versatile and adaptable, but are all unsuitable for measuring and quantifying the in vivo release of AChE in the substantia nigra of freely moving animals. This problem has now been overcome with the development of the `on-line´ chemiluminescent technique (Taylor et al., 1989). The `on-line´ chemiluminescent system was the most fundamental procedure used in this thesis. This technique has been operational for less than ten years and is still classed as relatively new. This assay has been previously used solely to determine the activity of AChE released in the guinea-pig substantia nigra (Jones and Greenfield 1990, Jones et al. 1991, Jones et al. 1994, Dally and Greenfield 1994). However, more recently this assay has been adapted for use with the rat (Dally et al. 1996).

The `on-line´ chemiluminescent technique is actually a combination of two well established protocols, the push-pull perfusion method (first devised by Fox and Hilton, 1958) and the chemiluminescent assay developed by Israel and Lesbats (1981). In 1987, Linás and Greenfield carried out the first `on-line´ visualisation of dentritic release of AChE from mammalian substantia nigra neurones in vitro. In this system, the brain slice was placed in a bath so that it was immersed in a continuous flow of Ringer’s solution containing luminol, choline oxidase

and microperoxidase. The chemiluminescent reaction was initiated by the introduction of acetylcholine chloride to the system and the light emitted by the slice was detected using a `light cell´ connected to a photomultiplier tube. In order to quantify the signal, the output from the photomultiplier tube was amplified and displayed on a chart recorder. This system was ideal for measuring AChE release in vitro. To measure the release of AChE in living animals, Taylor et al. (1989) overcame some problems (i.e. the pH value was highly toxic for living animals, and the images of AChE-release in the substantia nigra could not be visualised because of the animal's skull) by devising a technique whereby the activity of the AChE released inside the animal could be analysed continuously and ex situ. Taylor et al. (1989) combined the push-pull technique with the chemiluminescent reaction and incorporated both protocols into an assay system in which AChE activity in the push-pull cannula perfusate could be continuously monitored. In the last six years, the `on-line´ chemiluminescent system has been adapted further. It is now possible to monitor AChE release in freely-moving animals and compare the spontaneous and evoked release of AChE into the substantia nigra (Jones and Greenfield, 1991; Jones et al., 1994; Taylor et al., 1990) or the striatum (Dally D, Phil. thesis, 1996) of moving animals. This technique also permits determination of the effect of drugs and toxins on the spontaneous release of AChE in these regions. Furthermore, it is possible to quantify any animal movement in parallel with AChE release in the substantia nigra.

Following the perfusion of the substantia nigra with ACSF, the perfusate containing neurochemicals within it was pumped out of the substantia nigra through the cannula. The outflow of the cannula was then introduced to the flow of the reagents required for the assay. The first exogenous AChE diluted in ACSF was exposed to the flow of a solution containing acetylcholine and choline oxidase independently. The hydrolysis of the acetylcholine by AChE and the resultant oxidation of choline produced from this reaction by choline oxidase produces hydrogen peroxide. The passage of this mixture of solution through a cold incubator preserves the hydrogen peroxide prior to its introduction into the flow of solution containing luminol, horseradish peroxidase and microperoxidase in a `light-cell´ located in front of a photomultiplier tube. In the presence of hydrogen peroxide, luminol was oxidised and light produced. The output signal was then amplified and displayed on a chart recorder and oscilloscope. A residual light signal was obtained from the spontaneous hydrolysis of acetylcholine. The assay was able to measure the continuous release of AChE and is sensitive enough to detect AChE activity to a level as low as 0.01 mU. A five-second pulse of AChE

was detected (Jones D. Phil. thesis, 1992). This system was extensively used to carry out the research for this thesis.

1.5 The aim of this work

Parkinson's disease cannot be cured, but as is the case with so many other disorders of the nervous systems, it can be controlled by therapy. This gave me the incentive to delve deeper into this area.

Parkinson's disease is an ailment exclusive to humans. Ethics forbid the use of human subjects, so it was of critical importance to find a substitute creature in the phylum vertebrata which could be artificially infected with the illness. The rat is an example of such mammals. Furthermore, I was also looking for another object in the phylum vertebrata for the sake of comparison. The nearest in the class was aves, and a publication using this bird already existed which related to my work. Last but not least, being a member of Professor Layer's working group gave me new possibilities to extend my studies further.

As time goes by, substantial evidence is being accumulated to support the idea of a non-cholinergic action of AChE. If AChE has a novel role in the substantia nigra, the question that has to be addressed is whether it involves any interaction with dopamine, the major transmitter in this system.

The purpose of this thesis is to provide an understanding of the development of dopaminergic neurones using chick brain, and to examine the normal and pathological nigrostriatal pathway of the rat brain in connection with a noncholinergic function of AChE.

CHAPTER 2

2 MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

reagent	source of supply
(-)-quinpirole HCl	RBI, USA
2-methyl-3-(3,4-dihydroxy-phenyl)-L-alanine (DOPA)	Sigma, Poole
3,3'-diaminobenzidine (DAB)	Sigma, Deisenhofen
3,4-dihydroxyphenylacetic acid (DOPAC)	Sigma, Poole
5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB)	Sigma, Poole
6-hydroxydopamine (6-OHDA)	Sigma, Poole
95% O_2/5% CO_2	BOC, UK
acetylcholine-chloride (ACh)	Sigma, Poole
acetylcholinesterase (AChE)	Sigma, Poole
acetylthiocholine iodide (ATC)	Sigma, Deisenhofen/Poole
alcohol	BDA, Poole
aluminia, for column chromatography	Sigma, Poole
ammonium nickel(II)sulfate hexahydrate	Sigma, Deisenhofen
amphetamine	Chris Webb, Oxford
apomorphine hydrochloride	Sigma, Poole
avidin-peroxidase	Sigma-Aldrich Chemie, Germany
boric acid (H_3BO_3)	Sigma, Poole
bovine serum albumine (BSA)	Sigma, Deisenhofen
calciumchloride ($CaCl_2$)	Sigma, Poole
choline oxidase	Sigma, Poole
chromic potassium sulphate ($CrK[SO_4]_2$)	Sigma, Deisenhofen
cicken serum (CS)	Gibco, Eggenstein
citric acid ($C_6H_8O_7$)	Sigma, Poole
cresyl violet acetate	Sigma, Poole

cupric sulfate (CuO_4S)	Sigma, Deisenhofen/Poole
cytosine-β-D-arabinofuranoside	Sigma, Deisenhofen
di-sodiumhydrogenorthophosphate (Na_2HPO_4)	BDA, Poole
dopamine (DA)	Sigma, Poole
DPX	Sigma, Poole
Dulbecco's modificated eagle minimal medium (DMEM)	Gibco, Eggenstein
ethylenediaminetetra-acetic acid disodium salt (EDTA)	Sigma, Poole
F12 medium	Gibco, Eggenstein
fetal calf serum (FCS)	Gibco, Eggenstein
Folin & Ciocalteu's phenol reagent	BDH, Poole
formaldehyde (solution 37%)	Sigma, Deisenhofen
gentamycin sulfate	BioWHITTAKER, Maryland
Geys balanced salt solution (GBSS)	Sigma, Deisenhofen
glutamine	BioWHITTAKER, Maryland
glutathione (GSH, $C_{10}H_{17}N_3O_6S$)	Sigma, Poole
halothane-M&B	Rhône Mérieux, UK
horseradish peroxidase (HRP)	Sigma, Poole
hydrogen peroxide, solution 35% (H_2O_2)	Merck, Darmstadt
Kaiser's glycerinegelatine	Merck, Darmstadt
l-ascorbic acid, free acid	Sigma, Poole
luminol ($C_8H_7N_3O_2$)	Sigma, Poole
magnesiumchloride ($MgCl_2$)	Sigma, Poole
maleic acid	Sigma, Deisenhofen
microperoxidase	Sigma, Poole
NMDA	Dr. S. Cragg, Oxford
P.E.P. powder	Intervet Laboratories LTD
penicilline/streptomycine	BioWHITTAKER, Maryland
perchloric acid ($HClO_4$)	Sigma, Poole
plama chicken	Sigma, Deisenhofen
plasma, chicken	Sigma-Aldrich Chemie, Germany
potassium hexacyanoferrate	Sigma, Poole
potassiumchloride (KCl)	BDA, Poole
potassiumdihydrogenorthophosphate (KH_2PO_4)	BDA, Poole

potassiumdihydrogenphosphate (KH_2PO_4)	Sigma, Poole
saccharose	Merck, Darmstadt
Sagatal (Pentobarbitone Sodium B.P.)	Rhône Mérieux, UK
simplex rapid: dental cement, dental acrylic	Astenal Dental products
sodium carbonate (Na_2CO_3)	Sigma, Poole
sodium chloride solution, 0.9%, sterile	Sigma-Aldrich Co., UK
sodium hydrogen carbonate ($NaHCO_3$)	Sigma, Poole
sodium hydroxide (NaOH)	Sigma, Poole
sodium potassium tartrate (Rochelle salt)	Sigma, Poole
sodium tetraborate (BORAX)	Sigma, Poole
sodiumchloride (NaCl)	BDA, Poole
tetraisopropylpyrophosphoramide (iso-OMPA)	Sigma, Deisenhofen
thrombin	Sigma, Deisenhofen
thrombin, bovine	Sigma-Aldrich Chemie, Germany
Tissue Tek	O.C.T.
trishydroxymethylaminomethane (Tris)	Sigma, Poole
Triton X-100	Sigma, Deisenhofen
Trizma BASE	Sigma, Poole
Trizma HCl	Sigma, Poole
uridine	Sigma, Deisenhofen
xylene	Sigma, Poole

2.1.2 Equipment

equipment	source of supply
bench centrifuge 112	Sigma
camera CCTV	Hitachi
centrifuge 3K10	Sigma
chartrecorder TE 850	Tekman
chromjet integrator SP 4400	Spectra Physics Analytical
CO_2-incubator B5060	Heraeus, Hanau
CO_2-Water-Jacketed incubator	Nuaire, USA

column apex octadecyl 5mm reverse phase	Jones Chromatography
computer RM NIMBUS	Research Machines Oxford
cotton buds	
cryostat HM500OM	Microm, Walldorf
cryotome	Leitz, Wetzlar
current-to-voltage amplifier/converter	purpose-built
digital timer	Smiths
dissecting equipment	neoLab
drill	Quayle Dental, Worthing
drill-heads	Kornet CARBIDE, Germany
egg-incubator	Jung
electrochemical detector Waters 460	Millipore
ento pins 38X40 mm	Asta, Tipton
FM/modulating/demodulating circuits	purpose-built
glass capillaries, borosilicate	World Precision Instruments, USA
gyratory shaker	purpose built
Hamilton syring 5.0µl	Hamilton Co. Reno. Nevada
hotplate B212	Bibby
injector	Rheodyne, California
lamina flow hood	BDK Luft-/Reinraumtechnik
laminar air flow gelaire	Gelman Instruments
laser printer HL-8e	Brother
metal sheets for rat cages	North Kent Plastic, Kent
micolance 3 0.8X40	Becton Dickinson
Micro Injection Unit model 5000	KOPF Tujunga, Ca. USA
microscope Diaplan	Leitz
modified videogram	purpose-built
monitor	Hitachi
needle printer LQ-850	Epson
oscilloscope DSO 420	Gould
ph-meter	
photomultiplier housing, containing photomultipier tube	purpose-built
photomultiplier tube power supply unit	purpose-built

pump LKB HPLC 2248	Pharmacia
pumps minipuls 2	Gilson
PVC Manifold tubing	Altec, Alton
razor blade tools	Gem
razor blades	
S/S Hypo needle tubing	
solenoid/relay controller	purpose-built
stereo tactic frame	
sterile filters (type AC)	sartorius, Göttingen
syring SGE	Australia
thermometer 303K	Levell
ultracentrifuge TL-400	Beckmann
UV analytical plate reader	Molecular Devices Corporation
vacuum pump	Whatman
videos AG-6200	Panasonic

2.1.3 Microscopes and photodocumentation

microscope/film	source of supply
microscope Axiophot	Zeiss
microscope Diaplan	Leitz
T-MAX 400 (black and white)	Kodak
ectachrome 400 (colour)	Kodak

2.1.4 Primary Antibodies

antibody	specification	source	dilution
mouse-anti-tyrosine	against tyrosine hydroxylase	Boehringer Mannheim	1:50
hydroxylase	of rat, chicken, quail, cow	and Prof. H. Rohrer	1:500
mouse-anti-AChE	against globular forms of	Tsim et al. 1988	1:100
mab 3D10	AChE		

rabbit-anti-AChE	polyclonal, against AChE	Dr. J. Grassi	1:50

2.1.5 Secondary Antibodies and detectionsystems

antibody	source	dilution
sheep-anti-mouse Ig-Biotin	Amersham,	1:100
donkey-anti-rabbit Ig-Biotin	Amersham	1:100
goat-anti-rabbit-DTAF	dianova	1:100
streptavidin-texas red	Amersham,	1:100
avidin-peroxidase	Sigma	1:100
Vector SG substrate kit for peroxidase		Vector Laboratories, USA
Vector VIP substrate kit for peroxidase		Vector Laboratories, USA

2.1.6 On-line chemiluminescent technique reagents

ACSF and all stock buffer solutions were made up monthly and stored at 4°C, and all other reagents used were made up just for the very day of the experiment. In all cases the solutions are made using de-ionised water. The chemicals used in the chemiluminescent assay were all purchased from Sigma Chemical Co., UK.

2.1.6.1 Artificial cerebrospinal fluid (NaCSF) stock solution (1litre)

NaCl	14.900g (255 mM)
KCl	0.444g (6 mM)
$NaHCO_3$	3.108g (37 mM)
KH_2PO_4	0.156g (1 mM)
Na_2HPO_4	0.142g (1 mM)

In order to make up 100 mls of the diluted solution, the following solutions compounds and solutions were mixed:

NaCSF stock	50 ml

De-ionised H_2O	30 ml
$CaCl_2$ (26 mM)	10 ml
$MgCl_2$ (8 mM)	10 ml
Glucose	90 mg

ACSF were always bubbled with 95% O_2 and 5% CO_2 before being used and during the hole experiment, this ensured that the correct pH was obtained.

2.1.6.2 Additional buffers

Each of the following buffer solutions were made up to 200 mls with de-ionised water.

Borax/EDTA buffer solution:

Borax	3.814g (50 mM)
EDTA	1.1167g (15 mM)

TRIS/EDTA buffer solution:

TRIS HCl	2.422g (100 mM)
EDTA	1.1167g (15 mM)

2.1.6.3 Chemiluminescent reaction reagents

Choline oxidase 100U vial was prepared by the addition of 2 ml of de-ionised water to each vial, mixing the resultant solution, and decanting out into either 0.4 ml or 0.8 ml aliquots into eppendorfs, which were then frozen at -20°C. On the day of the experiment, 1.6 ml of choline oxidase was defrostet and mixed with 6 ml of TRIS/EDTA buffer.

Acetylcholine chloride 150 mg vial was prepared on the day of experimentation by the addition of 125 ml of de-ionised water to one vial, mixing the resultant solution and altering the pH to pH 4.0 using 1 M hydrochloric acid.

Microperoxidase stock 10 mg vial was prepared by the addition of 1 ml of de-ionised water to each vial, mixing the resultant solution and decanting it out into 80 µl aliquots in eppendorts, which were the frozen at -20°C.

Horseradish Peroxidase stock was prepared by weighing out 40 mg of powder, to which was added 4 ml of de-ionised water. The resultant mixture was then mixed using a stirrer and 800 µl aliquots of the solution were decanted into eppendorfs. Any aliqute not intended for immediate use were frozen at -20°C.

Luminol/Peroxidase mixture was prepared on the day of experimentation. It contains 20 mg of luminol dissolved in 0.8 ml of 1 M NaOH. After ensuring that all the luminol had dissolved, 20 ml BORAX/EDTA buffer, 80 µl of microperoxidase stock and 800 µl of horseradish peroxidase stock were all added to this solution. After using a stirrer to mix these reagents, the pH of the resultant mixture was adjust to pH 11.4 using 5 M NaOH.

Acetylcholinesterase 500 U vial in order to prepare a stock solution (100 mU), 5 ml of de-ionised water was added to each vial and the solution was mixed. The resultant mixture was then decanted out into 0.5 ml aliquots in eppendorfs, which were then frozen at -20°C. On the day of the assay, in order to obtain a calibration plot, one aliquote was defrosted and serially diluted with ACSF, to achieve activities of 1, 2, 5 and10 mU/ml: where 1U of AChE will hydrolyse 1 µmol of acetylcholine per minute at pH 8 and 37°C.

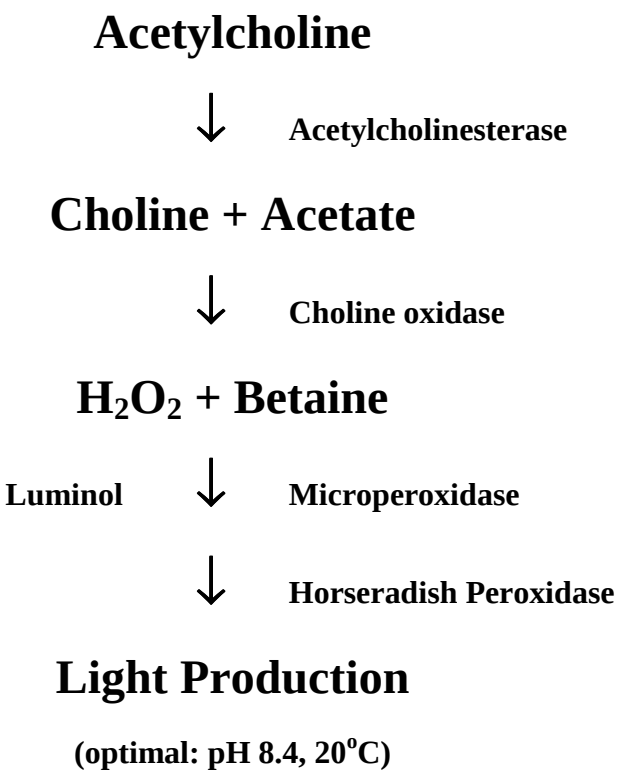


Figure 1 The chemiluminescent reaction used, to measure the release of AChE release from the substantia nigra of freely moving rats.

2.1.7 HPLC reagents

2.1.7.1 HPLC mobile phase

Citric Acid	15.760 g
KH_2PO_4	23.700 g
Octane Sulphonic Acid	2.340 g
EDTA	0.372 g

Make up about 5 litres. The pH of this mixture was adjusted to between 3.59-3.60 and 5 M NaOH was used. Once the correct pH was achieved, 15% of the volume was removed (750 ml in 5 litres) and replaced with methanol (HPLC Grade). Before it being used, de-gas mobile phase.

2.1.7.2 Additional buffers

Each of the following buffer solutions were made up to 100 mls with de-ionised water.

TRIS buffer solution (3 M, pH 8.6):	
TRIS BASE	27.90g (2.3 M)
TRIS HCl	10.98g (0.7 M)
Boric/Citric Acid:	
Boric Acid	1.5458g (0.250 mM)
Citric Acid	2.6260g (0.125 mM)

2.1.7.3 GSH/EDTA/$HClO_4$-solution

EDTA	80 mgs
perchloric acid	0.43 mls
de-ionesed water	5 mls
glutathione	24.6 mgs

make up to 50 mls with de-ionesed water.

2.1.7.4 Standards

α-Methyl Dopa	21.12 mg/10 mls of mobile phase is 10^{-2} M
Dopamine	18.96 mg/10 mls of mobile phase is 10^{-2} M
Dopac	16.81 mg/10 mls of mobile phase is 10^{-2} M

2.1.8 Equithesin

Sagatal	40.5 ml
$MgSO_4$	5.3 g
Ethanol	25 ml
Chloralhydrate	10.5 g
Propylene Glycol	99 ml

make up to 250 ml with distilled water, stir well.

2.1.9 6-Hydroxydopamine

Weight out 0.0080 g 6-Hydroxydopamine and 0.0040 g ascorbic acid in eppy, dissolve with 1 ml sterile saline. Store in dark and on ice.

2.1.10 Drugs

2.1.10.1 Amphetamine

Weight out 0.0184 g amphetamine and dissolve in 5 ml ACSF = 10^{-2} M.
Dillute each time 0.5 ml of the higher amphetamine solution with 4.5 ml ACSF to get different concentrations:
0.5 ml (10^{-2} M)) + 4.5 ml ACSF = 5 ml 10^{-3} M
0.5 ml (10^{-3} M)) + 4.5 ml ACSF = 5 ml 10^{-4} M
0.5 ml (10^{-4} M)) + 4.5 ml ACSF = 5 ml 10^{-5} M

0.5 ml (10^{-5} M)) + 4.5 ml ACSF = 5 ml 10^{-6} M

0.5 ml (10^{-6} M)) + 4.5 ml ACSF = 5 ml 10^{-7} M

For 1 mg amphetamine/kg bodyweigh(e.g. 0.3mg/300g) dissolve 2 mg amphetamine in 4 ml saline (e.g. 0.6 ml/300g).

2.1.10.2 Apomorphine

Weight out 0.01519 g apomorphine and dissolve in 5 ml ACSF = 10^{-2} M.

Dillute each time 0.5 ml of the higher apomorphine solution with 4.5 ml ACSF to get different concentrations:

0.5 ml (10^{-2} M)) + 4.5 ml ACSF = 5 ml 10^{-3} M

0.5 ml (10^{-3} M)) + 4.5 ml ACSF = 5 ml 10^{-4} M

0.5 ml (10^{-4} M)) + 4.5 ml ACSF = 5 ml 10^{-5} M

0.5 ml (10^{-5} M)) + 4.5 ml ACSF = 5 ml 10^{-6} M

0.5 ml (10^{-6} M)) + 4.5 ml ACSF = 5 ml 10^{-7} M

For 1 mg apomorphine/kg bodyweigh + 0.2 mg ascorbic acid/kg bodyweigh dissolve 2 mg apomorphine + 0.4 mg ascorbic acid in 4 ml saline.

2.1.10.3 Quinpirole

Weight out 0.0128 g quinpirole and dissolve in 5 ml ACSF = 10^{-2} M.

Dillute each time 0.5 ml of the higher quinpirole solution with 4.5 ml ACSF to get different concentrations:

0.5 ml (10^{-2} M)) + 4.5 ml ACSF = 5 ml 10^{-3} M

0.5 ml (10^{-3} M)) + 4.5 ml ACSF = 5 ml 10^{-4} M

0.5 ml (10^{-4} M)) + 4.5 ml ACSF = 5 ml 10^{-5} M

0.5 ml (10^{-5} M)) + 4.5 ml ACSF = 5 ml 10^{-6} M

0.5 ml (10^{-6} M)) + 4.5 ml ACSF = 5 ml 10^{-7} M

2.1.10.4 NMDA

Weight out NMDA and dissolve in 5 ml ACSF = 10^{-2} M.

Dillute each time 0.5 ml of the higher NMDA solution with 4.5 ml ACSF to get different concentrations:

0.5 ml (10^{-2} M)) + 4.5 ml ACSF = 5 ml 10^{-3} M

0.5 ml (10^{-3} M)) + 4.5 ml ACSF = 5 ml 10^{-4} M

0.5 ml (10^{-4} M)) + 4.5 ml ACSF = 5 ml 10^{-5} M

0.5 ml (10^{-5} M)) + 4.5 ml ACSF = 5 ml 10^{-6} M

0.5 ml (10^{-6} M)) + 4.5 ml ACSF = 5 ml 10^{-7} M

2.1.11 Ellman reagents

Make up 0.1 M KH_2PO_4 buffer pH 7.

Dissolve 31.7 mg 5,5'-dithio-bis(2-nitrobenzoic acid) and 12 mg sodium hydrogen carbonate together in 10 ml of the stock buffer (DTNB solution).

Dissolve 28.9 mg acetylthiocholine iodide in 10 ml of distilled water (ATC solution).

To make Ellman's reagent mix the KH_2PO_4 buffer, DTNB solution and ATC solution at a ratio of 5:1:1.

2.1.12 Lowry reagents

Dissolve 10 mg bovine serum albumin (BSA) in 10 ml of distilled water and make up BSA standards:

µl BSA +	µl H_2O =	µg/ml BSA
0	250	0
10	240	40
20	230	80
25	225	100
50	200	200
75	175	300
100	150	400

150	100	600
200	50	800
250	0	1000

Make a 1% copper sulphate solution (0.1g/10ml) and a 2% sodium potassium tartrate solution (0.2g/10ml). Mix equal volumes of these two solutions together and then add 1 ml to 50 ml 2% sodium carbonate/0.4% sodium hydroxide solution (2g and 0.4 g/100 ml respectively).
Dilut 2 N Folin-Ciocalteu's phenol reagent 1:1 with distilled water.

2.1.13 Culture medium

Mediums were stored at 4^{o}C, supplements at -20^{o}C. The culture medium was made up freshly and stored at 4^{o}C.

Mediums: 174.00 ml DMEM
174.00 ml F12

Supplements: 40.00 ml FCS (10%)
8.00 ml CS (2%)
4.00 ml L-glutamine (1%)
0.40 ml penicillin/streptomycin (0.1%)
0.15 ml gentamycine (0.02mg/ml)

2.2 Methods

2.2.1 Experimental animals

Male Wistar rats, weighing 200-250g were bought from OLAC, Harlan UK Limited, Shaw's Farm, Blackthorn, Bicester, Oxon, OX6 0TP. The animals were housed under standard conditions and fed with laboratory food. Chick embryos where also used, the given stages of development refer to the incubating time in days. Fertilized white Leghorn chicken eggs (Gallus gallus domesticus) were purchased from LSL-Rhein-Main (Geflügel-vermehrungsbetrieb GmbH, Dieburg). Until incubating the eggs were stored at 4°C. Brooding took place in an incubator at 37.8°C. The eggs were rotated automatically every 30 min. Atmospheric humidity was 92%.

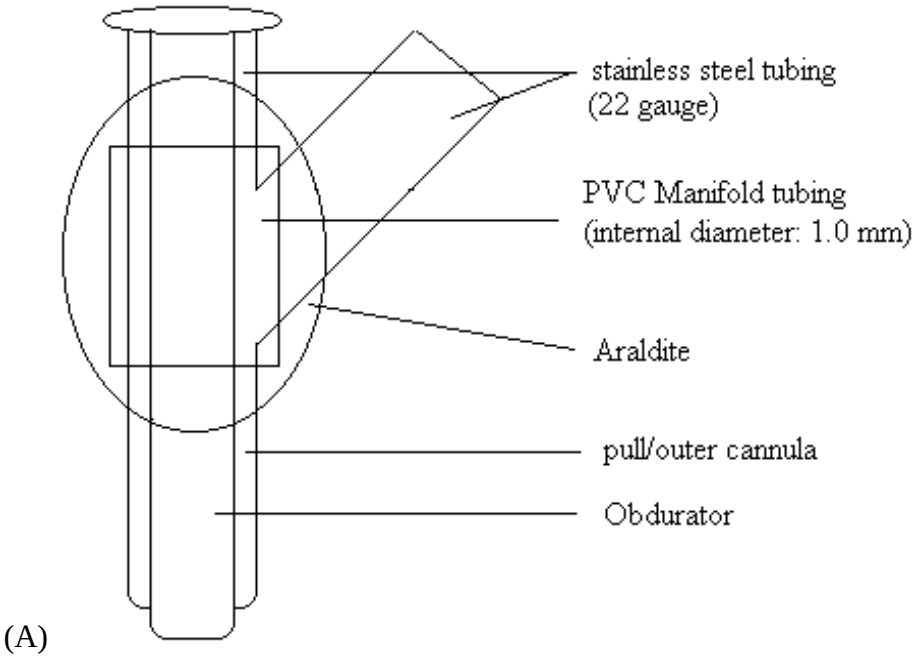
2.2.2 Construction of push-pull cannulae

Required materials: Microlance 3 sterile 22G 0.8X40 No2 T.W.P.M. Becton Dickson Dublin or Hypo needle tubing 22G 10-22gX30 cm S/S tubing, PVC Manifold Tubing Altec Alton Hampshire, Ento Pins 38X40 mm Asta Tipton, Araldite Rapid R2 Giba-Geigy Plastics Duxford Cambridge.

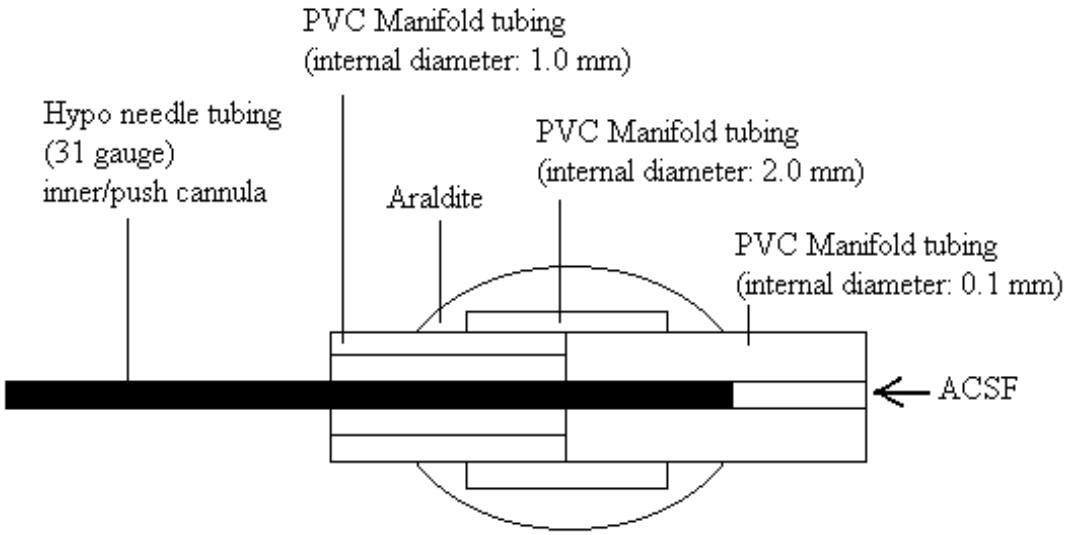
Push-pull cannulae were constructed from two concentric stainless-steel tubes (22 gauge,Microlance 3 or S/S Hypo needle tubing). An angled hole was filed out about half way along one side of one the tubes and a smaller length of tube (with one of its tips also filed) was fitted to it to make a `Y' shape (figure 2 A). The tubes were kept in place using vinyl tubing (1 mm internal diameter, PVC Manifold) and this was then sealed with araldite glue. The main shaft of the external cannula was kept patent by using an obdurator constructed from a 0.35 mm gauge insect pin (Ento Pin) which completely filled the bore and protruded about 0.5 mm from the bottom end of the cannula and prevent tissue blocking when the cannula is implanted. In all cases the cannula was made as small as possible to minimise the exposed area of the cannula above the skull and do reduce accidental shifting of the cannula during the housing and experimentation periods.

Required materials: Pin needle tubing, PVC Manifold Tubing Altec Alton Hampshire, Araldite Rapid R2 Giba-Geigy Plastics Duxford Cambridge.

An inner cannula was constructed using 31 gauge stainless-steel tubing (pin needle Tubing) and designed so as to protrude about 0.5 mm from the bottom of the vertical shaft of `Y' shaped cannula (figure 2B). The Hypo needle Tubing was held in PVC Manifold Tubing (0.1 mm internal diameter). In order for an air tight seal to be formed between the inner and outer cannulae, the 31 gauge tubing was threaded through Manifold Tubing 1.0 mm internal diameter and the construction supported by Manifold Tubing with an 2.0 mm internal diameter, this stucture was held together using araldite. Prior to experimentation the obdurator was removed and replaced by the inner cannula, which was connected to a continuous flow of ACSF.



(A)



(B)

Figure 2 Schematic diagram of the push-pull cannula used in this thesis (not to scale). The outer/pull cannula (A) was constructed from two concentric stainless steel tubes (22 gg) filed exactly to fit together in a Y-shape, this structure was then held in place using PVC Manifold tubing and araldite. An obdurator (0.35 mm gauge ento pin) completely filled the bore and protruded about 0.5 mm from the bottom end of the cannula. The inner/push cannula (B) was constructed using 31 gauge Hypo needle tubing held in PVC Manifold tubing (0.1 mm internal diameter) and designed so as to protrude to the same depth as the obdurator from the bottom of the vertical shaft of outer cannula.

2.2.3 Surgical procedure: implantation of push-pull cannula

Male Wistar rats, weighing 250-280g, were anaesthetised with Equithesin, 2 ml/kg (Hawkins and Greenfield, 1992a). This dosage produced a state of surgical anaesthesia for up to one hour. Following the establishment of deep anaesthesia, determined by an absence of a corneal and knee jerk reflexes, the hair on animal's head was shaved and the animal's head was fixed into a stereotaxic frame by ear bars and an incisor bar. When the animal was correctly positioned in the stereotaxic frame, an incision was made in the midline of the head to expose the skull and the periosteum pushed to side of the cranium. The head was levelled horizontally at the pionts bregma and lambda on the surface of the animals skull. A push-pull cannula was implanted unilaterally into the left or right substantia nigra at stereotaxic co-ordinates (modified from Paxinos and Watson, 1982): 5.3 mm anterio to bregma; 2.0 mm lateral or dorsal to the midline, and 8.0 mm below the skull. The exact position of the substantia nigra was marked on the animal's skull and a small hole drilled through the bone at this position. Four bone screws, implanted in a square pattern around the hole, are then carefully screwed into the skull around the proposed cannula site ; a square pattern is formed with the screws, which will provide a support structure for the dental cement (Astenal Dental Products) applied later. The dura mater was then carefully retracted using a needle tip and the cannula was lowered to the appropriate co-ordinates. While the cannula was still attached to the stereotaxic frame, dental cement was moulded around the base of the cannula and moulded into a crude cube shape supported by the four screws. Prior to detaching the cannula from the frame, a light emitting diode (LED) was implanted into the dental cement, for use with the ANTRAK system. When surgery was completed, a tetracycline-containing antibacterial powder (teramycin) was sprinkled in and around the existing cut on the animals head, in order to minimise any infection of this site. The animal was kept warm under a heat lamp until it had fully recovered from the anaesthetic. Each surgery-treated animal was then housed separately to minimise damage to the cannula-containing structure and allowed a minimum of 48 hours to recover from surgery, prior to the experiment. Food and water were available *ad libitum*. Again all surgically-operated animals were housed separately, unfortunately, it soon became apparent that the rats were knocking their cannula on the slanting roofs of these cages and thus an adaptation to existing rat-holding cages was required. By attaching a sheet of stainless steel (North Kent Plastics) to the base of the food tray, the animal cannot any longer enter the void space. Therefore, the chances of the animal damaging its cannula are decreased.

2.2.4 Surgical procedure: neurotoxintreatment

Male Wistar rats, weighing 250-280g, were anaesthetised with Equithesin, 2 ml/kg (Hawkins and Greenfield, 1992a). This dosage produced a state of surgical anaesthesia for up to one houre. Following the establishment of deep anaesthesia, determined by an absence of a corneal and knee jerk reflexes, the hair on animal's head was shaved and the animal's head was fixed into a stereotaxic frame by ear bars and an incisor bar. When the animal was correctly positioned in the stereotaxic frame, an incision was made in the midline of the head to expose the skull and the periosteum pushed to side of the cranium. The head was levelled horizontally at the pionts bregma and lambda on the surface of the animals skull. A fine-gauge needle was lowered stereotaxically into the medial forebrain bundle at coordinates 3.8 mm anterio to bregma, 1.8 mm lateral to the midline and 9.0 mm below the skull according to the atlas of Paxinos and Watson (Paxinos and Watson, 1982). The 6-OHDA was made up fresh on the day of use, in a concentration of 8 mg/ml in ascorbic acid 4 mg/ml in sterile saline and kept in ice. This solution was delivered from a Hamilton microsyringe (10 µl) via the fine needle at a rate of 0.25 µl/min for 4 mins (1.0µl of neurotoxinsolution), i.e. a total of 8 µg 6-OHDA was injected into the medial forebrain bundle. Following the injection the needle was kept in place for 2 mins to allow the neurotoxinsolution to diffuse from the needle tip into the surrounding tissue, then removed from the brain. The animals were left for at least 3 weeks for the degeneration to become complete. Another group of rats were sham-operated, i.e. subjected to the surgical procedure described above, but receiving a micro-infusion of sterile saline (9% v/w) with ascorbic acid only. After neurotoxin treatment the push-pull cannula was implanted unilaterally into the unlesioned substantia nigra as described above and a LED was implanted into the dental cement.

2.2.5 Perfusion via push-pull cannula

The substantia nigra was perfused with artificial cerebrospinal fluid (ACSF) at 37°C, gassed with 95% O_2, 5% CO_2, at a flow rat of 20 µl/ml. The ACSF contained (in mM): NaCl, 127; KCl, 3; $NaHCO_3$, 18.5; KH_2PO_4, 0.6; Na_2HPO_4, 0.5; $CaCl_2$, 2.5; $MgCl_2$, 0.8 and d-glucose, 5. A stock solution of d-amphetamine-sulphate (10^{-2} M) (generous gift of Chris Webb, Oxford), quinpirole (10^{-2} M) (RBI, USA) and NMDA (10^{-2} M) (generous gift of Dr. S. Cragg, Oxford)

was prepared in ACSF and diluted accordingly in ACSF prior to its introduction into the system. D-amphetamine, quinpirole and NMDA (10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M) was introduced for 5 min periods to the substantia nigra via the cannula and a 15 min recovery period was allowed between consecutive applications.

2.2.6 Flow circuit

A diagrammatic representation of the flow circuit used in this technique is illustrated in figure 3. In order to produce a continuous flow of ACSF into and out of the substantia nigra, Manifold and non-Manifold vinyl tubing (internal diameter between 0.15 and 0.5 mm) connected to Gilson `Minipuls 2´ peristaltic pumps was used in this circuit. Using a solenoid device, air bubbles were introduced into the flow of choline oxidase at a steady rate (every 5 seconds), see figure 4. Regularly spaced bubbles were introduced into the system in an attempt to restrict lateral diffusion of the AChE present in the ACSF perfusate. The closest site of integration of the bubbles into the flow of the perfusate was at the t-junction where the perfusate meets the flow of choline oxidase solution. Acetylcholine joined the mean stream just before the stream entered a cooled incubator. The two enzymatic cascades were completed at this point, and hydrogen peroxide was produced and preserved within this incubator. On entering the light cell, the hydrogen peroxide reacts with the cocktail of peroxidase enzymes and luminol, resulting in the oxidation of luminol, the emission of photons and hence the production of light.

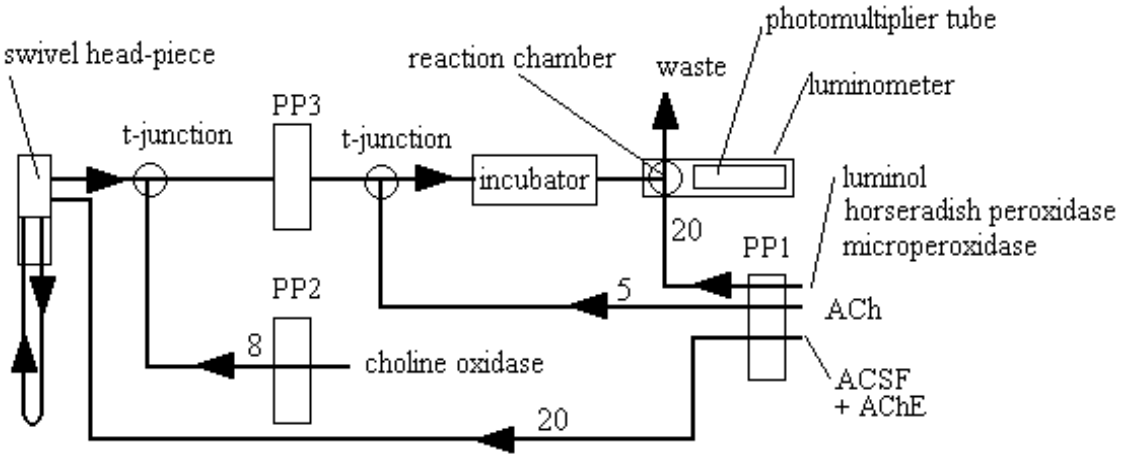


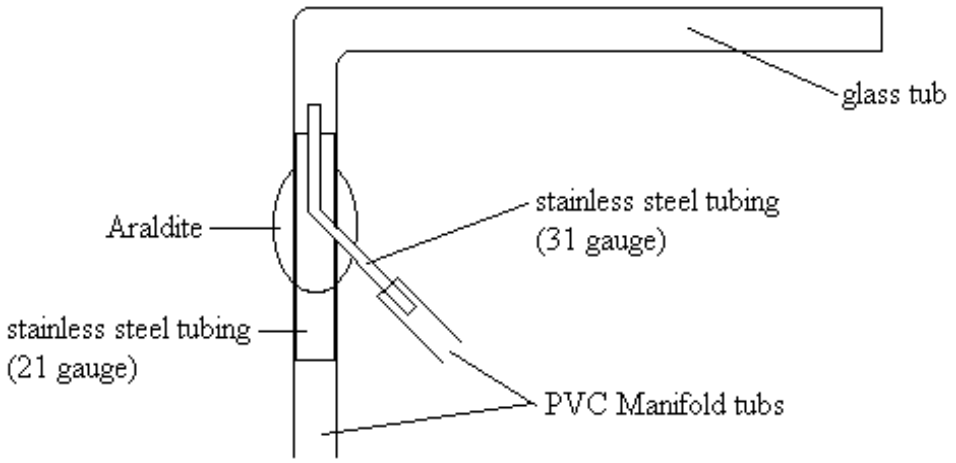
Figure 3 Schematic diagramm of the on-line circuit without an animal attached. Thick lines symbolize PVC Manifold tubs, arrows indicate direction of flow and numbers show flow rates (µl/minute). Exogenous AChE, diluted in ACSF, was introduced into the system via peristaltic pump (PP) 1 and pumped via the swivel head-piece to the light reaction chamber along with the reagents necessary for the chemiluminescent reaction. The light-derived signal was then displaced on an oscilloscope and chart recorder and recorded onto a video tape.

Figure 4 The injection system for introducing air bubbles into the flow of choline oxidase at a steady rate and at a low temperature (0 to 4^{o}C) consisting a solenoid device and a case to store ice.

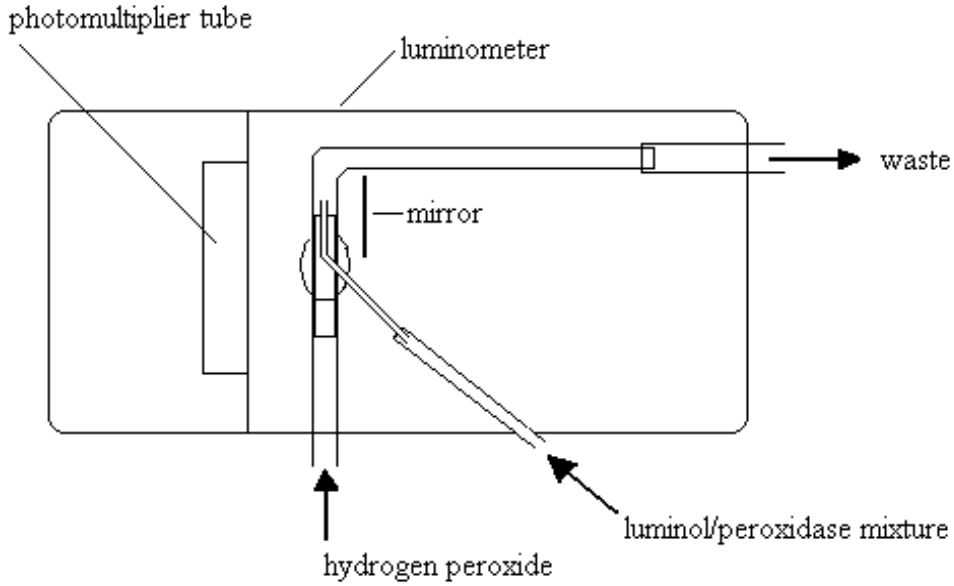
2.2.7 Light cell and luminometer

Required materials: Microlance 2 21G 0.8X40, Pin needle tubing 31G, Borosilicate Glass Capillars 1.4mm 4IN WPI Sarasota USA, PVC Manifold tubs, Araldite rapid Giba-Geigy.

The `light-cell´ reaction chamber was constructed from 21 and 31 gauge stainless steel tubing and a glass microtube (figure 5 A). In a section of 21 gauge tubing (about 30 mm in length) a small hole was filed out of the side of the tubing (about half way along). Through this hole, and thus through the lumen of the 21 gauge tubing, the 31 gauge was threaded so that it protruded about 0.5 mm from the tip of the wider tubing. The two-stainless steel tubes construction was then threaded into a thin glass microtube (internal diameter 1.5 mm) up to the filed hole and the structures were sealed using araldite.This structure was mounted in the luminometer in front of the photomultiplier tube and just behind a mirror positioned to reflect the light produced from the chemiluminescent reaction, back onto the photomultiplier tube (figure 5 B). The luminol/peroxidase mixture enters the chamber through the fine stainless steel tubing (31 gauge), while the perfusate/other reagents required for the reaction enter through the wider tubing (21 gauge). The chemiluminescent reaction occurs just above the protruding end of the thinner stainless steel tubing and is monitored by the photomultiplier tube. The reaction has to occur in the glass tube, not in a perspex tube, to avoid a loss of signal.



(A)



(B)

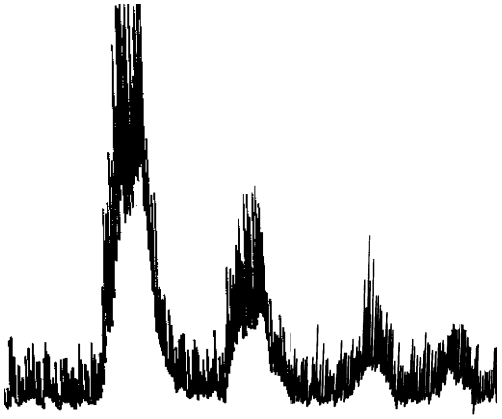
Figure 5 Diagrammatic representation of (A) the light cell reaction chamber, consisting of 21 and 31 gauge stainless steel tubs into a glass micotube. Figure (B) demonstrates the location of the light cell within the luminometer. The light cell reaction chamber was positioned immediately in front of the photomultiplier tube and a small mirror was mounted behind the light cell reaction chamber. The luminol/peroxidase solution entered the chamber through the thinner stainless steel tube and the ACSF solution containing hydrogen peroxide entered into the chamber via the wider stainless steel tube.

2.2.8 Calibration plot of actylcholinesterase activity

Prior to the start of each experiment a calibration plot of AChE activity was carried out to allow quantification of AChE released from the substantia nigra of the rat during the course of the experiment (see figure 6 A, B, C). Firstly, the system needs to be calibrated to insure the flow of ACSF into the system was equal to the removal of ACSF from the system. Connection of a Hamilton syringe, modified, so that it could be connected to the Manifolds vinyl tubing, to the system at the inflow and outflow tubing arround the swivel head piece (rotation-adapter), to allow the liquid flow in the tubing to be calibrated. This syringe can then be used to equalise the flow rates at 20 µl/min. Minor adjustment to the speed of the perfusion pumps was made until the meniscus in the Hamilton syringe remained stationary and therefore the flow rates were equal.

Once the inflow and outflow tubes were equalised, exogenous AChE(Sigma, Electric eel type VI-S), diluted accordingly with ACSF to activities of 1, 2, 5, 10 mU/ml, was added to the system (figure 6 A). Each sample was added to the system via outflow tube on the swivel into the flow of the choline oxidase solution (figure 3) for three minutes and then followed by a five minute period of ACSF. Since this system was based on an ex situ measurement of the activity of AChE released from the substantia nigra, a time delay occurs between the addition of the AChE sample and the resultant chemiluminescent reaction (usually about 15 minutes).

In order to calculate the time delay between sample application and chemiluminescent reaction, the end of the input tube was transferred from ACSF solution to ACSF solution containing AChE, and the exact time of the start of the appearance of the corresponding signal was noted. Thus, by carrying out a calibration plot on a daily basis, the exact time delay of the perfusate leaving the substantia nigra to it reaching the light cell can be calculated.



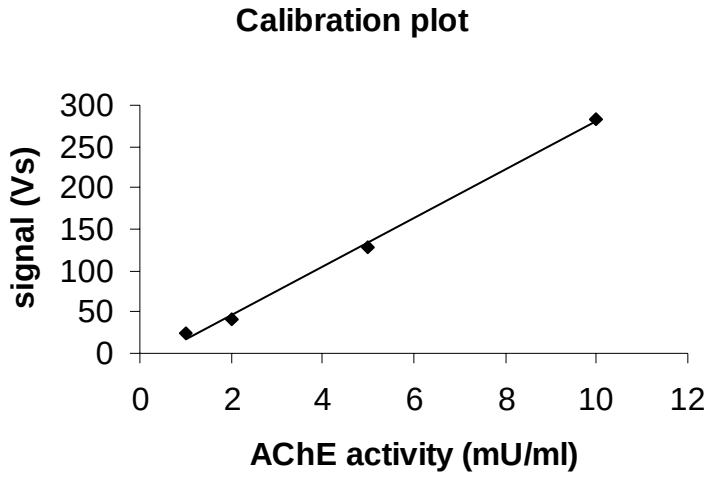
10 5 2 1

(0.4) (0.2) (0.08) (0.04)

(A)

mU	Vs
10	283,55
5	129,4
2	41
1	23,725

(B)



(C)

Figure 6 Typical plot of decreasing concentrations of exogenous AChE in mU/ml (Sigma, Electric eel type VI-S), added to the system prior to attachment on animal; absolute concentration mU, in brackets. Samples are added to the system for three minutes and then a period of five minutes of ACSF follows prior to the addition of the next sample. (B) Data got for example used in this thesis and (C) the calibration plot of exogenous AChE of this data. The areas under the various concentration curves were measured using a oscilloscope. Only the middle two minutes of the area under the curve were measured.

2.2.9 Connection of the animal to the system

Upon completion of the calibration plot, the animal under investigation was incorporated into the system, as shown in figure 7, and the perfusion of the substantia nigra commenced. ACSF was perfused into the substantia nigra through the inner cannula and removed through the side arm of the wider outer cannula (see figure 2 A, B). The perfusate was incorporated into the main flow circuit into the path of the flow of choline oxidase solution. A large signal peak was always obtained at the beginning of each infusion due to blood contamination/air when connecting the outflow tube to the side arm of the cannula. Thus, the signal should always be allowed to reach a constant/steady baseline value before any further experiments are carried out. Rats are quiet agile and active creatures compared to other experimental animals, e.g. guinea-pigs. This often led to entanglement of the inflow and outflow tubes connected to the cannula structure and necessitated detachment of the cannula from the tubing used in this system and thus unnecessary distress to the handler and animal. To overcome this problem a swivel head-piece was used (see figure 8).This swivel head-piece is clamped above the arena and the inflow and outflow tubes are attached to horizontal endports. Additional Manifold vinyl tubes can then be attached to the corresponding vertical endports, so as to create a continuous inflow - outflow system. The advantage of this system is that the Manifold vinyl tubes attached to the vertical endports can be threaded through the hollow cylindrical structure attached to the swivel head-piece and thus will never tangle. The animal is now free to move without any chance of entanglement of the inflow and outflow tubing.

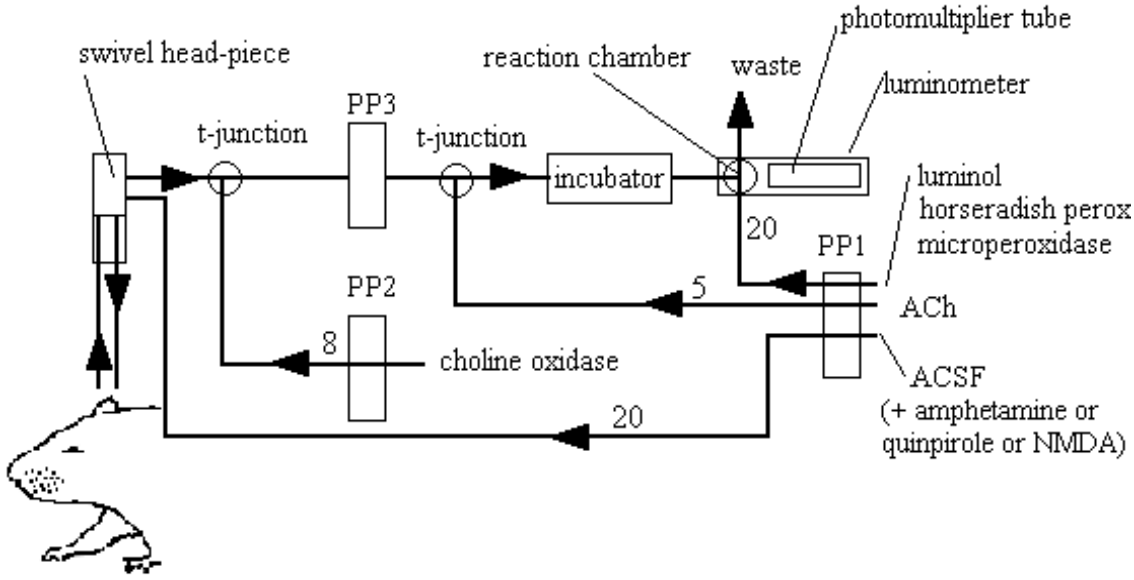


Figure 7 Diagrammatic representation of the chemiluminescent assay circuit for measuring endogenous release of AChE from the rat substantia nigra. Arrows indicate direction of flow and numbers show flow rate (µl/min) at points throughout the circuit. AChE secreted into the perfusate bathing the substantia nigra was delivered via peristaltic pump (PP) 1 to the light reaction chamber along with reagents necessary for the chemiluminescent reaction. The perfusate was continuously analysed ex situ and thus the signal occurs off-line (i.e. lagging approximately 15 minutes behind the event). The use of video analysis permits resynchronization of the signal trace, allowing the system to become essentially on-line. Thus any change in animal behaviour possibly related to AChE release can be determined.

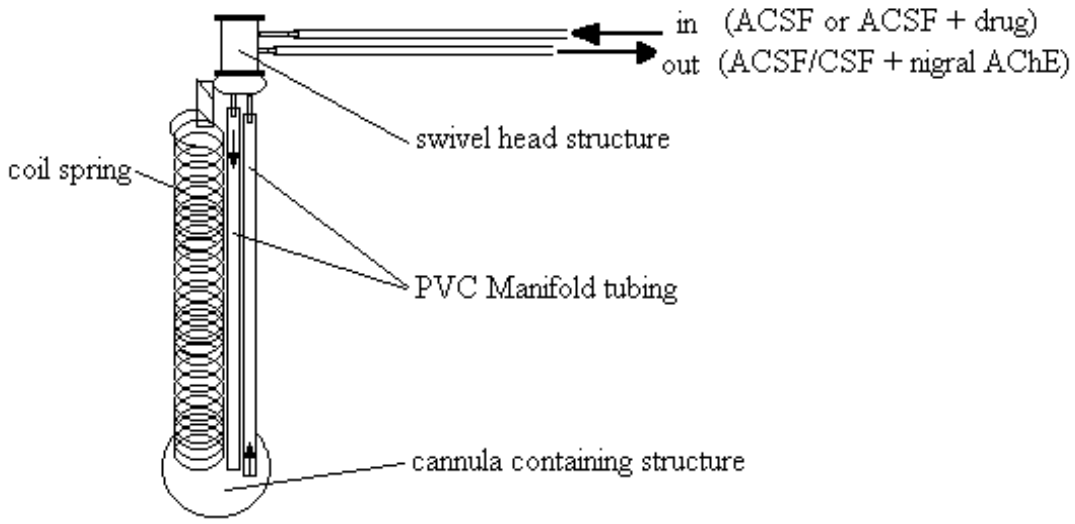
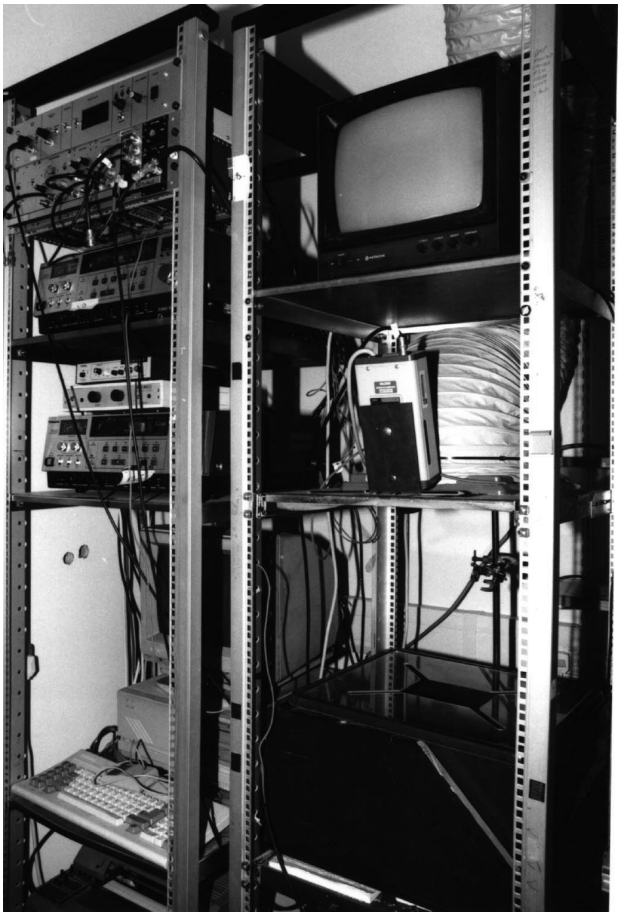


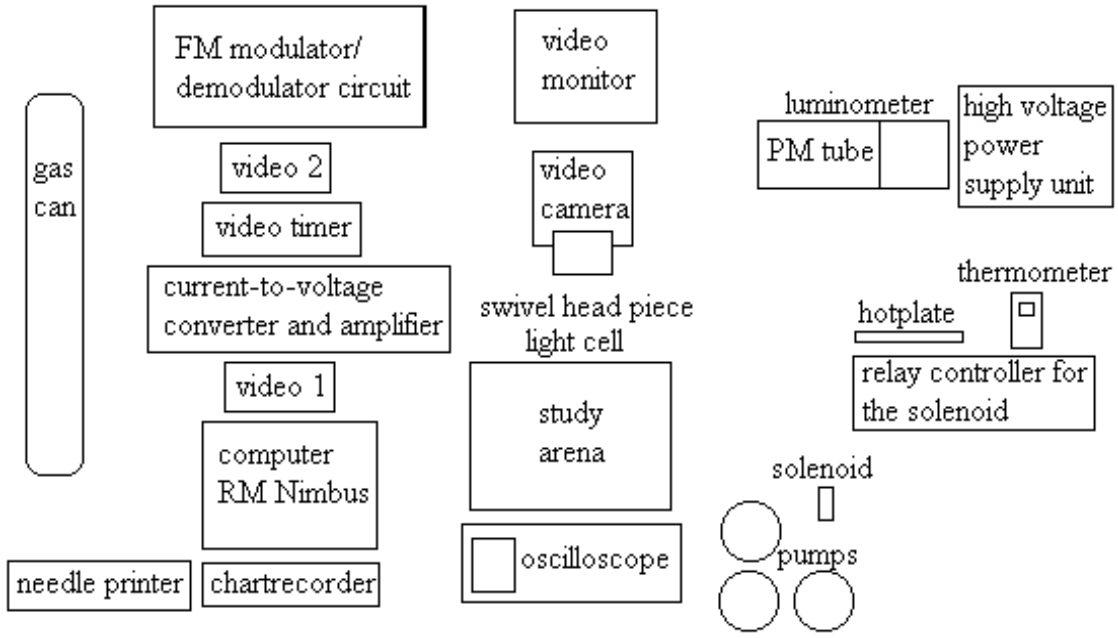
Figure 8 Diagrammatic representation of the swivel head piece. Rats are quiet agile creatures, when they receive drug/stimulatory agents which induce movement it soon becomes apparent that this result in problems like entanglement of the inflow and outflow tubes connected to the cannula structure. It necessitated detachment of the cannula from the tubing used in this system in an attempt to overcome this problem a swivel head piece was used. This swivel was clamped above the cage floor and the inflow and the outflow tubes are attached to horizontal endports. Additional Manifold tubes can then be attached to the corresponding vertical endports, so as to create a continous inflow - outflow system. The Manifold tubes attached to the vertical endports can be threaded through the hollow cylindrical structure attached to the swivel head piece in order to prevent them from being entangled.

2.2.10 Recording of acetylcholinesterase release and movement

The equipment used to monitor the continuous endogenous release of AChE and any animal movements is shown in figure 9 (A), (B). Light produced from the chemiluminescent reaction is first detected by the photomultiplier tube and the resultant signal is amplified and converted to a FM signal via frequency modulator. This signal was then recorded onto the audio channel of the VHS video recorder 1 and also displayed as a trace on the chart recorder. Two video recorders are used in this technique: one to record the FM signal and the video picture, the other just the video picture. Therefore, if any interesting observations occur with respect to AChE release and movement, the FM signal can be recorded onto the video picture in video recorder 2. A video timer can be used to start record the FM signal of one video tape onto the video picture of the other video tape, in order to give a FM signal/video picture in `real time´; thus the lag-time between release of enzyme and its detection is accounted for. The system now allows for the release of AChE to be observed in `real time´ with respect to movement.



(A)


FM modulator/
demodulator circuit
video
monitor
luminometer
high voltage
power
supply unit
gas
can
video 2
video
camera
PM tube
video timer
thermometer
current-to-voltage
converter and amplifier
swivel head piece
light cell
hotplate
video 1
relay controller for
the solenoid
study
arena
computer
RM Nimbus
solenoid
oscilloscope
pumps
needle printer
chartrecorder

(B)

Figure 9 Equipment used in the detection of in vivo AChE release from the substantia nigra and any animal movements. (A) photograph showing the equipment; 1. FM modulator/ demodulator circuit used to interconvert the voltage form of the AChE-derived light signal into an FM form (which could then be recorded onto video tape 2), 2. video recorder 2 used to record the AChE-derived light signal, 3. video timer used, ultimately, to allow the behaviour of the animal and the on-line signal of AChE release within the substantia nigra to be synchronized, 4. current-to-voltage converter and amplifier used to amplify and convert the current output signal of the photomultiplier tube into a voltage form, 5. video recorder 1 used to record the behaviour of the animal, 6. RM Nimbus computer used to analyse with the help of the Antrak-video based animal tracking system the behaviour of the animal, 7. needle printer used to print out the computer plotted picture of animal behaviour analysed with the Antrak system, 8. chartrecorder used to print out continual during the experiment the AChE release trace, 9. video monitor used to visualize the behaviour of the animal, 10. video camera used to monitor the behaviour of the animal during the experiment, 11. swivel head piece used to prevent entanglement of the tubes, 12. LED (light emitting diode) used to detect the animals behaviour in conjunction with the Antrak system, 13. housing box of the animal, 14. oscilloscope used to visualize the trace of AChE released, 14. luminometer containing the PM tube used to detect the light from the chemiluminescent reaction, 15. high voltage power supply unit for the PM, 16. hoteplate/thermometer used to controll the temperatur of ACSF, 17. relay controller for the solenoid used to indruce air bubbles into the main stream of the chemiluminescent assay flow circuit. (B) diagrammatic representation of the epuipment.

2.2.11 Determination of animal movement

Spontaneous or induced animal movements during the perfusion period, were monitored in addition to the video tape using an Antrak-video based animal tracking system (B. Reece Scientific Ltd., Newbury, UK), which was used in conjunction with the `on-line´ chemiluminescent technique. This system involves computerised tracking of a LED in and around a pre-set area within the study arena. The computer then plotted the animals movements within a pre-determined time period. It quantifies how far the animal moves and how long it spends in any area it has visited (figure 10). The animals movement were also count as a number of 360° turns. Animal rotation was measured as the number of 360° turns and using the Antrak-video based animal tracking system, motor activity was measured in terms of total distance moved.

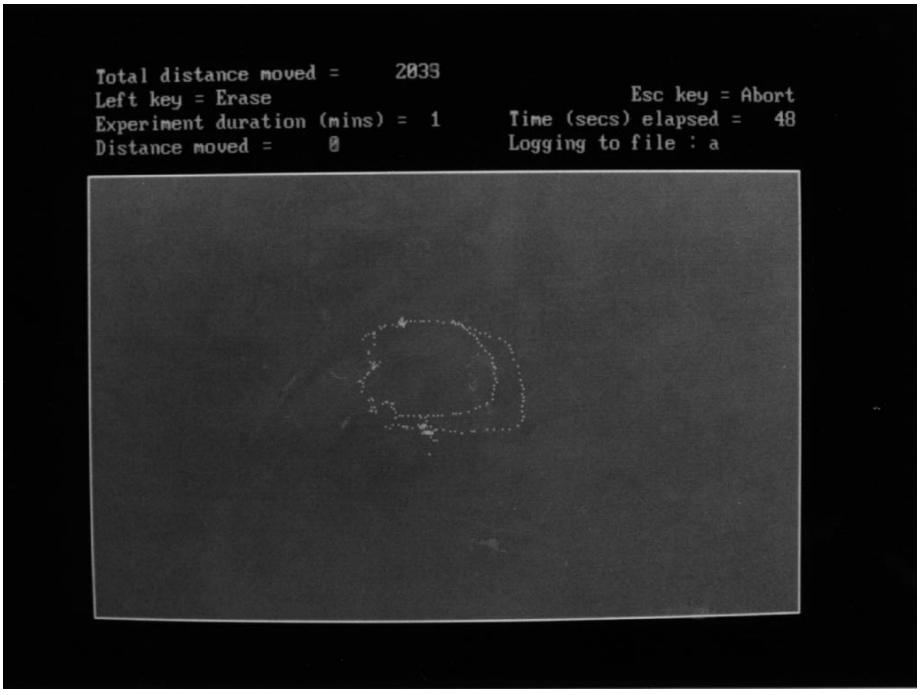


Figure 10 Animal movement monitored with an Antrak-video based animal tracking system, computer plotted picture.

2.2.12 Analysis of actylcholinesterase release in association with movement

Using the calibration plot carried out at the start of each experiment, the activity of AChE released from the substantia nigra can be quantified. The areas under the various concentration curves were measured using a Gould 420 oscilloscope, which had built-in measuring facilities. Although AChE was added to the system for three minutes, only the middle two minutes of the area under the curve were measured. This procedure was carried out to ensure that the air bubbles, which occur when transferring from ACSF to ACSF containing AChE and back again, did not interfere with the data recorded. Although the concentration of exogenous AChE applied were in mU/ml, all data is quantified in absolute units (mU). For example, if 1 mU/ml was applied to the system for two minutes at a flow rate of 20 µl/min, then the absolute activity of AChE = 0.04 mU (1mU/ml x 2 minutes x 20/1000 ml/minute). This permits a calibration plot of absolute activity (mU) versus the area under the curve to be constructed (figure 6 A, B, C).

Basal (spontaneous) release of AChE (in the absence of any drug) was calculated by taking three baseline readings and expressing the middle value as a percentage of the other two. Evoked release of AChE (following the application of drug) was similarly calculated, relative to a period immediately preceding and following the effect of the drug. In general, basal release and drug-evoked release of AChE in the substantia nigra were measured for a two minute perriod at the centre of each trace, in order to ensure continuity for data analysis. Basal release of AChE in both regions were expressed as mU of AChE activity (where 1 U of AChE will hydrolyse 1 µmole of acetylcholine per minute at pH 8 and 37°C), whereas the drug-evoked release of AChE were expressed as a percentage of the spontaneous release of AChE. Actual numbers of animals used in analysis are given in the respective figure legends. Some animals were not included in the final analysis due to technical problems, e.g. blocked push-pull cannula. Data were analysed working with Microsoft Exel using analysis of variance followed by paired t-test. All results are given as means $\pm$ SEM for experiments performed upon *n* animals. Statistical significance of the difference between means was estimated using paired t-test. The probability levels interpreted as statistically significant were $P<0.001$ (***), $P<0.01$ (**), $P<0.05$ (*).

2.2.13 Collection of CSF-samples

By stimulation with 10^{-3} M and 10^{-2} M amphetamine another group of animals was AChE release determined from 5 mins (200µl) aliquots collected during the experiment and kept on ice. AChE activity in perfusate samples obtained from the substantia nigra were determined using the Ellman technique (Ellman et al., 1961) and analysed spectrophotometrically.

2.2.14 Histology

At the end of each experiment, in order to assess cannula placements, dopamine concentration in striata, TH and AChE positive neurons in the substantia nigra, animals were deeply anesthetized with halothane (Rhône Mérieux Limited, Harlow Essex, UK) and decapitated. The cannula/dental cement structure was removed from the skull, great care was required when removing the implant. This was essential as the amount of damage caused by removing the cannula must be kept to a minimum to ensure that histological verification of the cannula tip was not compromised. The brains were carefully removed and stored in formaldehyde (4% v/v in phosphate buffered saline, pH 7.4, 4°C) for at least a week. Following fixation, brains were placed in cryoprotective solution (30% sucrose in PBS, pH 7.4) until they sink. 10 µm to 42 µm sections were cut on a freezing microtome or cryostat and each section was mounted on a gelatinisated glass slide and stained in different methods, whichever was appreciated. For each animal,prior fixation, both striata were removed and stored in a 0.1 M perchloric acid containing 1.6 mM reduced glutathione and 4.3 mM EDTA, prior to HPLC analysis.

2.2.15 High performance liquid chromatography and Lowry assay

HPLC is an ideal technique for analysing small molecules (molecular weight <1000). This procedure is an accurate and sensitive method of determining the catecholamine release and content in any brain region under investigation. The principle of this assay is based on the phenomenon of air-oxidation of catecholamine. Catecholamines are oxidised at the hydroxyl groups to produce an orthoquinone derivative with the release of two electrons.

In HPLC this reaction has been harnessed, i.e. a positive potential is applied to the electrode and electrons are transferred to the electrode. The current produced is directly proportional to the number of molecules oxidised. The oxidation occurs near the surface of a glassy carbon electrode. The potential is applied to the working electrode and maintained (via a reference electrode) by passing the required current through the working and auxiliary electrodes; hence the name - `three electrode system´. The reference electrode consists of an Ag/AgCl electrode, with 3 M KCl electrolyte, and functions to provide a stable potential to which the working electrode can be compared.

The HPLC system used in the current experiments consisted of a reverse-phase mode of separating samples. In reverse-phase chromatography, retention of compounds on the column is due to hydrophobic reactions between the solute and the hydrocarbaceous stationary phase (C18-octa-decyl-sulphate particles bound to a silica surface). The principle is that compounds are eluted in order of decreasing polarity/ increasing hydrophobicity, i.e. the greater the polarity of the compound, the less time it is retained by the non-polar/hydrophobic surface of the column and the quicker it is eluted. It should be noted, however, that care must always be taken with the pH of the mobile phase used with an octa-decyl-sulphate/silica stationary phase. If the pH of the mobile phase is > 7, the silica dissolves and the column is ruined.

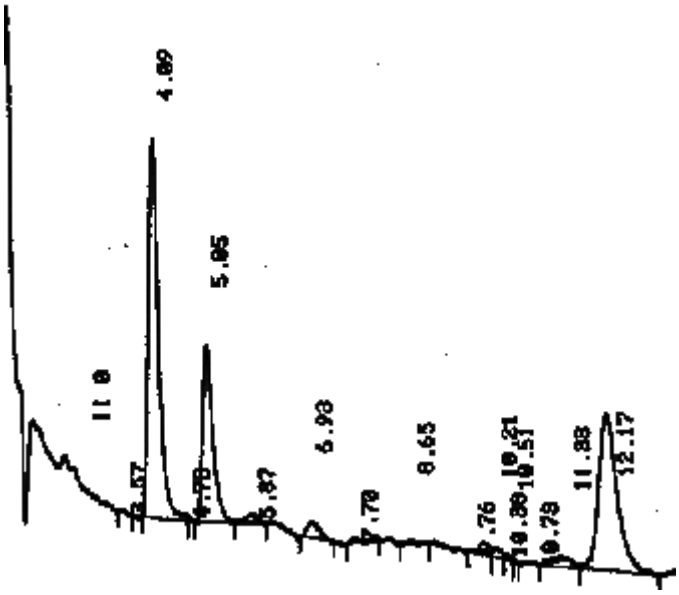
In order to evaluate the effect of neurotoxin pretreatment described in this thesis on tissue dopamine content, an HPLC system was used. Dopamine, DOPAC, noradrenaline, dihydroxyphenylalanine (dopa) and α-methyl dopa (internal standard) can be easily extracted following the method of Anton and Sayre (1962); this protocol is ideal for the requirements of the current studies, as it permits the removal of many unwanted neurochemicals such as soluble protein and other neurotransmitters and their metabolites, which normally mask desired peaks or interfere with the assay. There is one major drawback of this extraction method: the exclusion of homovanillic acid (HVA). Therefore, although dopamine content in the tissue under investigation can be accurately calculated, conclusions on dopamine turnover are severely limited.

The protein content of each sample is assayed using the standard Lowry procedure (Lowry et al., 1961). This now permits data representation as pmol/mg protein.

2.2.15.1 Determination of dopamine content in tissue samples using HPLC

Both striata were removed and stored at -20°C in perchloric acid-glutathione-EDTA solution. Dopamine and DOPAC were extracted using the method described by Anton and Sayre (1962) with minor modifications. Tissue samples were homogenised in 3 ml ice cold 0.1 M perchloric acid containing 1.6 mM reduced glutathione and 4.3 mM EDTA, and then centrifuged by 10000g at 4°C for 10 minutes. Two ml of supernatant, containing α-methyl-DOPA (internal standard: final concentration = 10^{-8}M), was added to 80 mg of alumina (Al_2O_3) and mixed with 2 ml of Tris buffer (pH 8.6). The resulting supernatant was removed after 10 minutes of mixing and the alumina washed twice with 2 ml water. Dopamine, DOPAC and α-methyl-DOPA were eluted with 400 µl of 0.25 M boric acid and 0.125 M citric acid solution. 50 µl aliquots of this solution were injected into the system.

DOPAC and dopamine were quantified by high performance liquid chromatography (C18 reverse phase column) with electrochemical detection (Waters 460; Millipore, U.K.) set at +700 mV with respect to the Ag/AgCl electrode (Felice et al., 1978). The mobile phase run at a flow rate of 2ml/minute. A typical trace showing the 3 peaks measured (figure 11 A) is shown below inclusive there area under the curve calculated by ChromJet integrator (figure 11 B).



(A)

PEAKS	AREA%	RT	AREA	BC
1	1.02	0.1	1700	01
2	0.179	3.57	167	01
3	39.245	4.09	86668	02
4	0.229	4.73	214	02
5	18.481	5.05	17265	08
6	1.115	5.87	1842	05
7	2.332	6.93	2169	01
8	0.492	7.7	460	01
9	0.152	8.65	142	01
10	0.686	9.76	641	02
11	0.886	10.21	828	02
12	0.895	10.38	369	02
13	0.875	10.51	70	03
14	0.493	10.73	461	02
15	2.585	11.88	2368	02
16	30.894	12.17	28861	03
TOTAL 100.			93428	

(B)

Figure 11 (A) A typical trace using HPLC showing the peaks measured of α-methyl dopa (retention time = 4.09 minutes), DOPAC (retention time = 5.05 minutes) and dopamine (retention time = 12.17 minutes). (B) shows the area under the curve calculated with a integrator.

2.2.15.2 Estimation of protein concentration by Lowry assay

Protein concentrations of the centrifuged pellet were quantified using the method of Lowry et al. (1951). The pellet was dissolved in 1ml 1M NaOH. A concentration of bovine serum albumin (BSA) standards ranging from 40 µg/ml to 1 mg/ml was required for each assay. This was achieved by adding 0-250 µl of 1 mg/ml BSA to individual tubes and adjusting the volume to 250 µl with de-ionised water. The protein content of the samples under investigation was calculated by diluting between 20-250 µl of the unknown protein solution with de-ionised water, ensuring that all solutions were adjusted to 250 µl in each tube. Therefore, by comparison of the spectophotometric data obtained from the samples with those obtained from BSA standards and accounting for any dilution factors, the protein content of unknown samples can be calculated.

The reagents for the Lowry assay consists of 0.01 % copper sulphate, 0.02 % sodium potassium tartrate, 2 % sodium carbonate and 0.4 % sodium hydroxide, made up to volume with de-ionised water. One ml of this solution was added to each tube containing unknown samples and mixed immediately. Once all the samples had been treated, 100 µl of a 1:1 dilution of 2 N Folin-Ciocalteu's reagent (diluted with de-ionised water) was added to each tube and again mixed immediately. Finally, after a period of 15 minutes, but not longer than two hours, 100 µl aliquots from each tube was added to a microtitre plate and the absorption read at 650 nm. Using spectrophotometric data obtained from BSA standard samples, the log absorbence is plotted against log standard protein concentration and the protein concentration of each sample calculated.

2.2.15.3 Analysis of data

Experimental data, obtained by HPLC, was analysed using standard calibration plots obtained for dopamine and DOPAC. These calibration plots can be made using standard solutions containing varying concentrations of dopamine and DOPAC, ranging from 10^{-7} to 10^{-10} M, with each sample being spiked with a constant concentration of α-methyl dopa (internal standard: final concentration = 10^{-8} M).

Dopamine or DOPAC concentration of the standard samples was plotted against:

$$\frac{\text{Area under the curve for the dopamine standard}}{\text{Peak height of internal standard in that sample}} \times \text{Mean peak height of internal standards in that run} =$$

$$\frac{a \times c}{b}$$

The function a/b was carried out for comparison of the unknown dopamine concentration with the known quantity of internal standard; however in order to standardise the results this function is then multiplied by `c´. Using the gradient obtained from each calibration plot, the concentration of dopamine and DOPAC in unknown sample could be calculated.

Data obtained from the Lowry assay is analysed using the `SOFTmax´ computer software which calculates the protein concentration of the unknown samples from a calibration plot derived from a set of protein standard concentrations which are incorporated into the assay at the time of running the assay.

Dopamine and dopac content of both striata obtained from contol and 6-OHDA treated animals was expressed as µg/mg protein in the pellet.

2.2.16 Determination of acetylcholinesterase activity in CSF samples using the Ellman assay

Rats stimulated local with amphetamine 10^{-3} M and 10^{-2} M respectively connected to the `on-line´ system CSF samples where collected every five minutes in eppendorf cups and kept on ice. After collecting the required samples AChE activity was measured immediately using the Ellman assay. The Ellman assay (Ellman et al., 1961) is a colour-changing enzymatic reaction, which quantifies non-specific cholinesterase activity over a specified time period. The substrate acetylthiocholine (ATC) is cleaved by AChE into thiocholine and acetate. The sulphydryl group of the thiocholine then reduces the S-S bond of Ellman's reagent DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)), releasing a yellow chromophore (5-thio-2-nitrobenzoate) which has an absorption maximum near 405 nm and so can be measured in microtiter plate reader. The increasing extinction was measured by the aid of a spectrometer (UV analytical plate reader) at 405 nm for 10 minutes. This procedure was carried out at room temperature (23^{o}C), with the stock solutions at approximately pH 7.

The 96 micro titer plate were prepared as follows: All samples were assayed in triplicate. 175 µl of a KH_2PO_4 solution containing ATC and DTNB was added to 25 µl of CSF contained in

cylindrical flat bottomed wells in a microtiter plate. Spontaneous hydrolysis of the substrate was corrected by a reagent blank incubated in the absence of enzyme.
Calculating AChE content: Using a Softmax computer programme (Molecular Devices Corporation) the quantity of cholinesterase activity in the sample (Ellman mU/ml) can be calculated. Taken the mean vmax value for each sample and multiply by any dilution factor, then multiply by 1.026 to give mU/ml.

2.2.17 Preparation of embryonic eyes

To get the eyes, chicken embryos of embryonic day (E) 5 to 20 , which ever was required, were used. Eggs were stored one hour prior preparation at 4°C. Than the eggshell was opened with a curved forceps and the embryo was taken out of the egg, stored into a Petri dish filled with PBS and decapitated. After this, the eyes of the embryo were removed with the curved forceps and rinsed three times in PBS for 10 mins.

2.2.18 Fixation of whole eyes

To keep the structure of the eyes have to be fixated with 4% formalin in PBS overnight at 4°C. Then the eyes were rinsed three times with PBS and transferred in a solution of 30% saccharose and rested at 4°C until they had sunk to the bottom. The saccharose as antifreeze protects the tissues against damages from the cryostat procedure.

2.2.19 Gelatinisation of microscope slides

Dissolve 5 g gelatine on heat stirrer in 500 ml distilled water and ad 0.3 g chromic potassium sulphate, filter and cool. Clean the slides with acid alkohol, 70% alcohol and 1% HCl (350 mls EtOH + 148 mls dH_2O + 2 mls HCl) and rub dry with lint free tissue and place into slide racks. Soak slides in 2% Decon, mixed with hot distilled water, over night. Wash slides in running tap water for 30 minutes. Than drain and soak in several changes of distilled water. Drain and soak in double distilled water. Dip each tray of slides in the cooled gelatin solution,

leave there for a few minutes and than drain onto filter paper. Dry racks in 60^{o}C oven over night. Remove slides from racks and replace into boxes, label and store at 4^{o}C.

2.2.20 Cutting with the cryostat

The box temperatur of the cryostat was about -25°C and the object temperature about -25°C. On the object holder of the cryostat a base plate of Tissue-Tek was frozen and later covered with a thin layer of saccharose solution. In order to prevent the eyes from cracking up in the saccharose solution, it was found necessary also to inject the solution through the fissure into the inside of the eye. The eyes were aligned with a forcep, so that the optic fissure rested on the base plate and the lens was on the front. The eye was then surrounded with the saccharose solution and frozen tightly to the base plate. Then now the 10 µm thin sections were cut. The sections were put onto gelatinisated microscope glass slides, which were dried overnight at room temperature and stored at -20°C until further use for staining.

2.2.21 Cresyl violet staining

Dissolve 1 g cresyl violet acetate in 1l distilled water, heat, while stirring until near boiling temperature. Cool. Add 5 ml 10% acetic acid. Slides were hydrated by passing through a cascade of solutions of decreasing alcohol content (100-50%), before finally being left in distilled water and stained in 1% cresyl violet solution for 30 secondes to 2 minutes. After staining slides were washed in distilled water, 70% alcohol and acid alcohol (70% alcohol + 1 N acetic acid). The stained slides were then dehydrated by passing through a cascade of solutions of increasing alcohol content (70-100%), before finally being left for 3 mins in xylene. Upon completion of this process the sections were covered with DePex mounting medium, onto which coverslips were placed. The slides were then studied under microscope to assess cannula tract.

2.2.22 Immunocytochemical identification of AChE

Frozen slides were dryed and adapted to room temperature. Then they were treated with hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. After icubating with Triton X-100, to make the cellmembrane permeable, and FCS, to prevent non-specific antibody binding, the sections were incubated with 3D10 (m-α-AChE) or r-α-AChE. After this, the sections were incubated with the secondary biotinylated antibody anti-mouse or anti-rabbit. To detecte the secondary antibody the sections were treated with avidin-peroxidase. The last step was the incubation with Vector VIP of SG (including H_2O_2) to visualise the peroxidase and therefore the AChE positive neurons.

Protocol:

- frozen slides drying and adapting to RT
- 3x15 mins washing of the slides with PBS
- incubating in peroxidase solution (10% methanol, 3% H_2O_2 in PBS) for at least 30 mins
- 2x15 mins washing with PBS
- 1x15 mins washing with PBS with Triton X-100 (1%)
- incubating in FCS (5% in PBS) for 30 mins
- transferring of the slides into a wet chamber
- incubating with primary antibody 3D10 or r-α-AChE (1:100/1:50 in PBS with 1% FCS and 0.1% Triton X-100) over night at 4°C
- 2x15 mins washing in PBS
- 1x15 mins washing in PBS with Triton X-100 (1%)
- incubating with secondary antibody α-m-Biotin or α-r-biotin (1:100 in PBS with 1% FCS and 0.1% Triton X-100) 2h
- 3x15 mins washing in PBS with Triton X-100 (1%)
- incubating with avidin-peroxidase (1:70 in PBS) 2h
- 3x15 mins washing in PBS
- incubating in Vector VIP or SG for 2 mins
- 3x15 mins washing in PBS
- rinsing slides in aqua dest. for 1-2 mins
- drying slides on heat plate (37^oC)
- covering up with Kaiser's glyceringelatine

2.2.23 Immunocytochemical identification of dopaminergic neurons in the substantia nigra

Tyrosine hydroxylase (TH) is the rate limiting enzyme of catecholamine synthesis and will therefore be present in adrenergic, noradrenergic and dopaminergic neurons. However, Decavel et al. (1987) demonstrated, by using a monoclonal antibody against dopamine, 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 noradrenaline (Geffard et al., 1986). Therefore, TH immunoreactivity within the substantia nigra is widely accepted as a reliable marker of dopaminergic neurons.

The brains were fixed in formaldehyde (4% v/v in phosphate buffered saline, pH 7.4, 4°C) for at least a week. Following fixation, brains were placed in cryoprotective solution (30% sucrose in PBS, pH 7.4) until they sink. 10 µm sections were cut on a freezing microtome and each section was mounted on a gelatinisated glass slide and dryed overnight. Then they were treated with hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. After icubating with Triton X-100, to make the cellmembrane permeable, and FCS, to prevent non-specific antibody binding, the sections were incubated with mouse anti-TH antiserum. After this, the sections were incubated with the secondary biotinylated antibody anti-mouse. To detecte the secondary antibody the sections were treated with avidin-peroxidase. The last step was the incubation with Vector VIP of SG (including H_2O_2) to visualise the peroxidase and therefore the TH positive neurons.

Protocol:

- frozen slides drying and adapting to RT
- 3x15 mins washing of the slides with PBS
- incubating in peroxidase solution (10% methanol, 3% H_2O_2 in PBS) for at least 30 mins
- 2x15 mins washing with PBS
- 1x15 mins washing with PBS with Triton X-100 (1%)
- incubating in FCS (5% in PBS) for 30 mins
- transferring of the slides into a wet chamber
- incubating with primary antibody m-α-TH (1:500 in PBS with 1% FCS and 0.1% Triton X-100) over night at 4°C
- 2x15 mins washing in PBS
- 1x15 mins washing in PBS with Triton X-100 (1%)
- incubating with secondary antibody α-m-Biotin (1:100 in PBS with 1% FCS and 0.1% Triton X-100) 2h

- 3x15 mins washing in PBS with Triton X-100 (1%)
- incubating with avidin-peroxidase (1:70 in PBS) 2h
- 3x15 mins washing in PBS
- incubating in Vector VIP or SG for 2 mins
- 3x15 mins washing in PBS
- rinsing slides in aqua dest. for 1-2 mins
- drying slides on heat plate (37^oC)
- covering up with Kaiser's glyceringelatine

2.2.24 Immunocytochemical identification of AChE or/and TH

Frozen slides were dryed and adapted to room temperature. They were then treated with Triton X-100, to make the cellmembrane permeable, and FCS, to prevent non-specific antibody binding, the sections were incubated with 3D10 (m-α-AChE) or r-α-AChE or m-α-TH. After this, the sections were incubated with the secondary biotinylated antibody anti-mouse or anti-rabbit or with the detectionssystem anti-rabbit-DTAF. To detecte the secondary antibody respectively AChE/TH positiv neurons the sections were treated with Streptavidin-Texas Red.

Protocol:

- frozen slides drying and adapting to RT
- 2x15 mins washing of the slides with PBS
- 1x15 mins washing with PBS with Triton X-100 (1%)
- incubating in FCS (5% in PBS) for 30 mins
- transferring of the slides into a wet chamber
- incubating with primary antibody 3D10 or r-α-AChE m-α-TH (1:100/1:50/1:500 in PBS with 1% FCS and 0.1% Triton X-100) over night at 4°C
- 2x15 mins washing in PBS
- 1x15 mins washing in PBS with Triton X-100 (1%)
- incubating with secondary antibody α-m-Biotin or α-r-Biotin (1:100 in PBS with 1% FCS and 0.1% Triton X-100) or with the detectionssystem α-r-DTAF 2h
- 3x15 mins washing in PBS
- incubating with steptavidin-texas red 2h

- 3x15 mins washing in PBS
- rinsing slides in aqua dest. for 1-2 mins
- drying slides on heat plate (37^{o}C)
- covering up with Kaiser's glyceringelatine

2.2.25 Organotypic slice cultures

Cultures were prepared under sterile conditions using a lamina flow hood. All dissecting equipment and required materials was sterilised. Solutions that were not obtained pre-sterilised were passed through a sterile filter.

Mesencephalon containing the nucleus tegmenti pedunculo pontinus pars compacta (TPc) was dissected from E18 old chicks. Throughout the dissection procedure, utmost care was taken in order to retain a maximum of histotypic architecture.

The eggshell was opened and the chick removed and quick decapitated by a scissor cut at the level of the foramen magnum. The skull was opened and the brain carfully removed in GBSS 4^{o}C and placed on the ventral surface on a sterile sartorius filter. The tissue on the filter was placed on a McIlwain tissue chopper and coronal sections (200µm) were cut and placed in fresh GBSS before being refrigerated for 1.5 hours. Leaving the slices in chilled GBSS for 1.5 hours allowed proteolytic enzymes to diffuse away from the tissue and for ruptured cell membranes to reseal.

The 200µm mesencephalon slices were attached to cleaned, sterile glass coverslips (diameter 2cm) by means of a plasma clot formed by mixing a solution of chicken plasma (lyophilised chicken plasma reconstituted in 5ml deionized water) with bovine thrombin (0.8mg/ml). The tissue section was placed in a 20µl drop of chicken plasma on the coverslip, and a 15µl drop of thrombin was then placed adjacent to the plasma drop. The two solutions were gently mixed until the plasma/thrombin clot covered the coverslip with the tissue section held in the centre. After mounting the sections, the coverslips were refrigerated for 1.5 hours to allow the clot to set.

Once the plasma clot had set, thus holding the isolated mesencephalon slice in place, the coverslip was placed in a culture dish (diameter 35mm). 2ml of serum-containing culture medium was then added to each culture dish. The culture dishes were then placed in a 145mm-culture dish within an incubator (37^0C, 4% CO_2, 70-75 rpm). Between the second and fourth day in vitro cytostatic solutions were added for 24 hours to prevent over-proliferation of non-neuronal cells. The anti-mitotic substances used were uridine and cytosine-β-D-arabinofuranoside, both at 10^{-3} M. Culture medium was changed weekly by carefully suck up the old medium and adding 2ml fresh medium.

2.2.26 Immunocytochemical identification of dopaminergic neurons

On completion of the incubation period, cultures were fixed and processed for tyrosine hydroxylase immunocytochemistry by the biotin-avidin peroxidase method.

Protocol:

- cultures were fixed in 4% formaldehyde for 30 mins
- 3x15 mins washing of the tissue with PBS
- incubating in peroxidase solution (10% methanol, 3% H_2O_2 in PBS) for at least 30 mins
- 2x15 mins washing with PBS
- 1x15 mins washing with PBS with Triton X-100 (1%)
- incubating in FCS (5% in PBS) for 30 mins
- transferring of the coverslip with the tissue into a wet chamber
- incubating with primary antibody m-α-TH (1:500 in PBS with 1% FCS and 0.1% Triton X-100) over night at 4°C
- 2x15 mins washing in PBS
- 1x15 mins washing in PBS with Triton X-100 (1%)
- incubating with secondary antibody α-m-Biotin (1:100 in PBS with 1% FCS and 0.1% Triton X-100) 2h
- 3x15 mins washing in PBS with Triton X-100 (1%)
- incubating with avidin-peroxidase (1:70 in PBS) 2h
- 3x15 mins washing in PBS
- incubating in Vector VIP or SG for 2 mins
- 3x15 mins washing in PBS

- rinsing tissue in aqua dest. for 1-2 mins
- drying coverslips on heat plate (37^{o}C)
- covering up with Kaiser’s glyceringelatine

CHAPTER 3

RESULTS

3.1 THE RELEASE OF ACETYLCHOLINESTERASE IN THE HEALTHY BASAL GANGLION FOLLOWING AMPHETAMINE STIMULATION

3 Results

A total of 112 animals were used during the `on-line´ study. The number of animals quoted initially does not necessarily correspond with the quantity used for statistical analysis, since some animals could not be included for technical reasons. For this reason, only the actual number of animals used in the analysis is noted in the key to the respective figure.

The placement of nigral cannulae was verified by examination of frozen, cut sections stained with cresyl violet.

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

The first aim of this study was to determine if a relationship exists between AChE-release in the substantia nigra and behaviour following amphetamine stimulation in naive animals.
First of all, I examined the effect which an increase in dopamine levels in the substantia nigra and the release of AChE in this region had on the behaviour of naive animals. The objective of this study was to determine if a relationship exists between dopamine, the regulation of AChE release in the substantia nigra and behaviour. If dopamine and AChE are functionally linked, then the enhancement of one may effect the other, as visualised in the behaviour patterns observed.
Figure 12 shows a typical trace of the chemiluminescent signal during different situations.

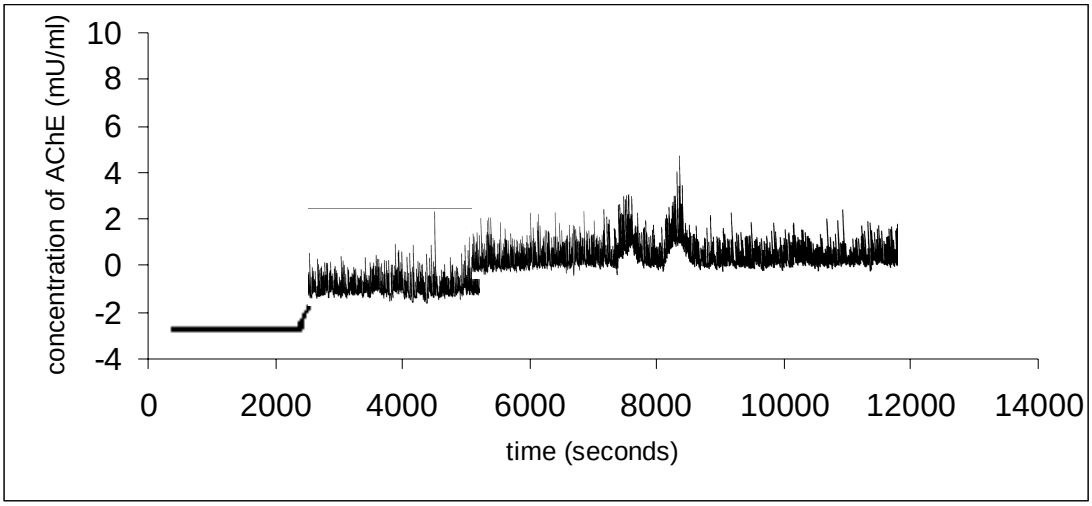


Figure 12: The data trace of a chemiluminescent signal, recorded with an oscillograph.

The x-axis represents time in seconds, while the y-axis represents the concentration of the AChE in mU/ml, in increments from -4 to 10. During the experiment, the trace was observed with an oscilloscope and recorded on the oscillograph. The converter transforms the light signals produced by chemiluminescent reactions into electrical impulses. The negative line of the trace describes no signal at all. Movement starts at 0 mU/ml AChE at 2000 seconds plus. This is a background value produced by the spontaneous disintegration of ACh. All values between 0 mU/ml AChE and 2 mU/ml are generated by a discharge of AChE from the animal's brain. These in vivo values are mostly in the region of 2 mU/ml and can be seen as basal in trials of this kind. However, if the concentration of AChE rose above normal basal levels, this was caused by medication which had been given to the animal a short time earlier. This concentration was produced by an increased discharge of enzyme into the CSF. The basal values are called 100% values and anything higher is therefore greater than 100%. The results can be observed in the following graphs. One thing worthy of note regarding the y axis: Calibration of the increments goes up to 10 mU/ml AChE, as this is seen as the highest concentration.

3.1.1 Amphetamine applied to the `on-line´-system without an animal connected

A stock solution of d-amphetamine-sulphate was prepared in ACSF and diluted accordingly in ACSF prior to its introduction into the system. D-amphetamine (10^{-7} to 10^{-2} M) was introduced for five-minute periods and a fifteen-minute recovery period was allowed between consecutive applications. A loss of the chemiluminescent signal was seen at 10^{-3} M and 10^{-2} M (see figure 13). This was also observed when these highest concentrations were tested separately (see figure 14).

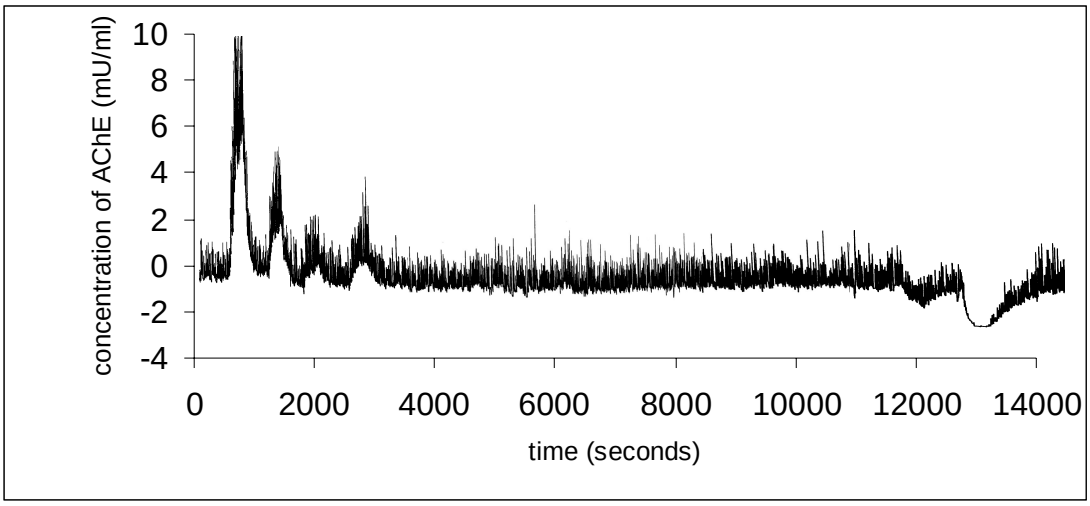


Figure 13: A typical trace showing continuous the chemiluminescent signal produced by the spontaneous hydrolysis of ACh in vitro. The first part shows the decreasing concentrations of exogenous AChE (Sigma) in mU/ml, added to the system prior to testing the amphetamine concentrations 10^{-7} M to 10^{-2} M. It shows a loss of the chemiluminescent signal by 10^{-3} M and 10^{-2} M amphetamine.

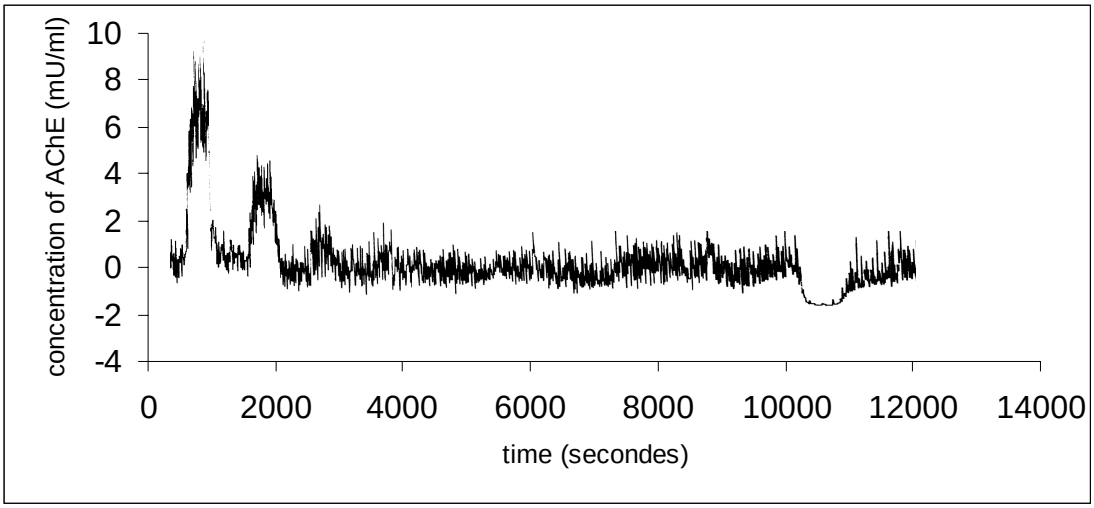


Figure 14: A typical trace showing the chemiluminescent signal produced by the spontaneous release of ACh in vitro. The first part shows decreasing concentrations of exogenous AChE (Sigma) in mU/ml, added to the system prior to testing the amphetamine concentrations 10^{-3} M and 10^{-2} M. It still shows a loss of the chemiluminescent signal by 10^{-3} M and 10^{-2} M amphetamine.

3.1.2 The basal release of acetylcholinesterase in the rat substantia nigra in vivo

The spontaneous hydrolysis of acetylcholinechloride yielded a signal prior to addition of AChE from the rat perfusate. However, subtraction of this background reading allowed the basal AChE-perfusate value to be determined. When the cannula was correctly implanted in the substantia nigra, a large light-signal was produced, attributable either to blood (in the early stage of perfusate extraction), air (when connecting the outflow tubing to the side arm of the cannula), or the excess release of AChE (resulting from the animal being handled while being connected to the system). Within approximately 20 minutes, this initial increase in the level of release gradually dropped to a level that was (in naive rats or sham-operated animals) still clearly above the baseline level prior to perfusion (see fig. 5). This signal represented a spontaneous release of AChE of 0.25 $\pm$ 0.07 mU (n=13 local), 0.11 $\pm$ 0.03 mU (n=9 systemic).

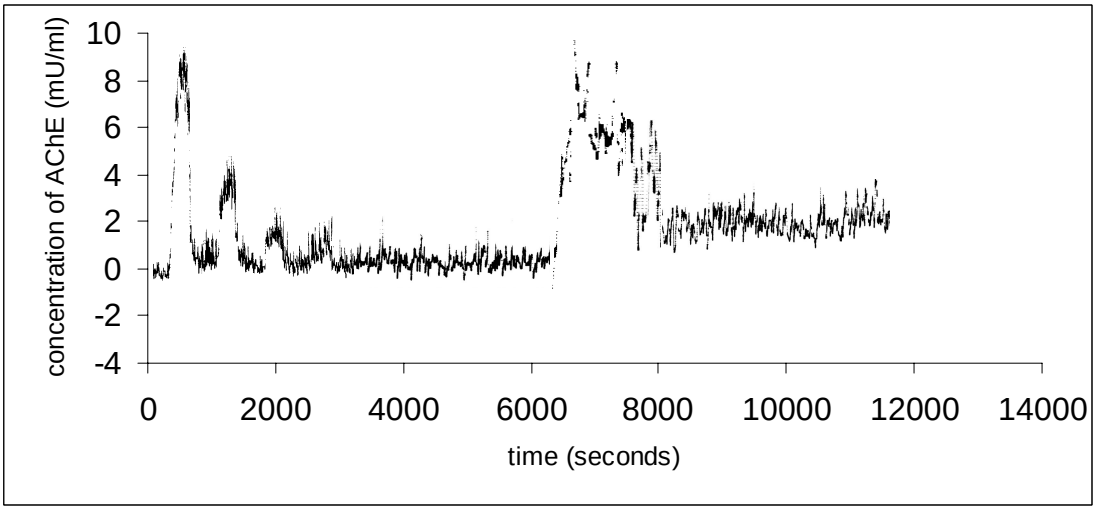


Figure 15: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) (0-3000 seconds) to the system prior to animal attachment. The spontaneous hydrolysis of ACh in vitro is shown as a background signal) (3000-6500 seconds). A large signal peak was observed when connecting the animal, due to blood/air contamination (6500-8200 sec.). On-line detection of release of AChE in vivo can be observed between 8200-11300 seconds.

3.1.2.1 Assessment of cannula placement

Animals which had traces of the push-pull cannula outside the substantia nigra and animals showing important gliosis were discarded.
A representative histological section of cannula placement in rats kept for the statistical analysis is depicted in figure 16.

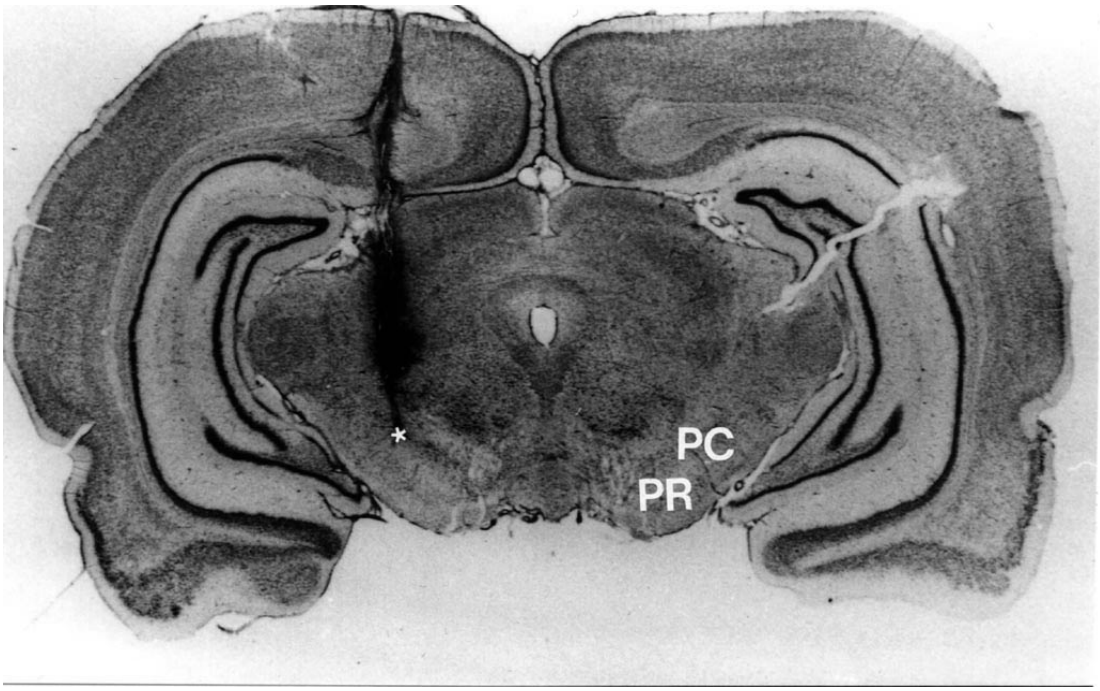


Figure 16: A section of the rat brain stained with cresyl violet (width: 42 µm), showing the tract of a push-pull cannula ending in the substantia nigra, as marked by an asterisk. Stereotaxic co-ordinates are stated in the text. The point marked PR refers to the substantia nigra pars reticulata. The point marked PC is the substantia nigra pars compacta.

3.1.3 The effect of local administration of amphetamine into the substantia nigra

3.1.3.1 Local administration of amphetamine and its effect on behavioural

Each concentration of amphetamine was infused over a period of five minutes in ACSF. In order to directly compare the effects of the different concentrations of amphetamine in the substantia nigra, all concentrations were infused under the same conditions: the circling behaviour was measured for 15 minutes from the point when amphetamine arrived in the substantia nigra to ensure the same conditions for every concentration. A paired t-test revealed a significant difference between a very modest basal circling, 0.062 $\pm$ 0.021 SEM turns/min and 10^{-7} M, 0.22 turns/min $\pm$ 0.05 SEM, ($P<0.05$); 10^{-6} M 0.28 turns/min $\pm$ 0.09 SEM ($P<0.05$); 10^{-5} M 0.22 turns/min $\pm$ 0.04 SEM ($P<0.01$); 10^{-4} M 0.25 turns/min $\pm$ 0.05 SEM ($P<0.01$); 10^{-3} M 0.23 turns/min $\pm$ 0.02 SEM ($P<0.001$) and 10^{-2} M 1.11 turns/min $\pm$ 0.31 SEM ($P<0.01$); see figure 17. The most intense circling was seen following 10^{-2} M amphetamine with 3.8 turns per minute. The number of turns per minute observed with this concentration are significantly different compared with the lower concentrations ranging from 10^{-7} M to 10^{-3} M amphetamine.

Local infusion of one substantia nigra with amphetamine resulted in a characteristic behaviour pattern, see figure 18; the animals often preferred one region of the box, displaying contraversive circling and exhibiting a very tight rotation around the axis of their body. Amphetamine caused an overall increase in the animal's activity, and contraversive behaviour circling was seen following all amphetamine concentrations. However, a different pattern was seen with 10^{-2} M amphetamine. With 10^{-2} M amphetamine, a high level of intense circling was seen, and, in some cases, animals showed a rapid and persistent shaking of the head. Some animals even showed an extreme twisting of the head, touching the rear flank and a rapid, continuous movement in one direction. Following amphetamine stimulation, after about 15 minutes, the circling behaviour and intense general activity ceased. This decline in activity coincided with a decrease in the release of AChE.

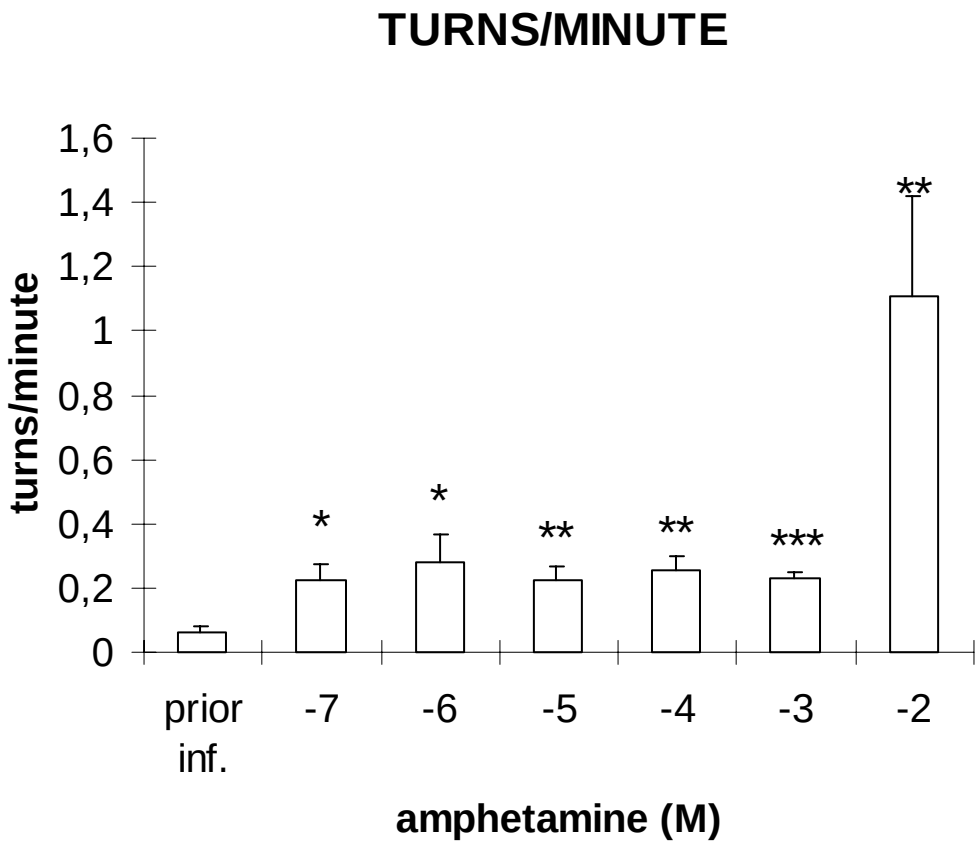
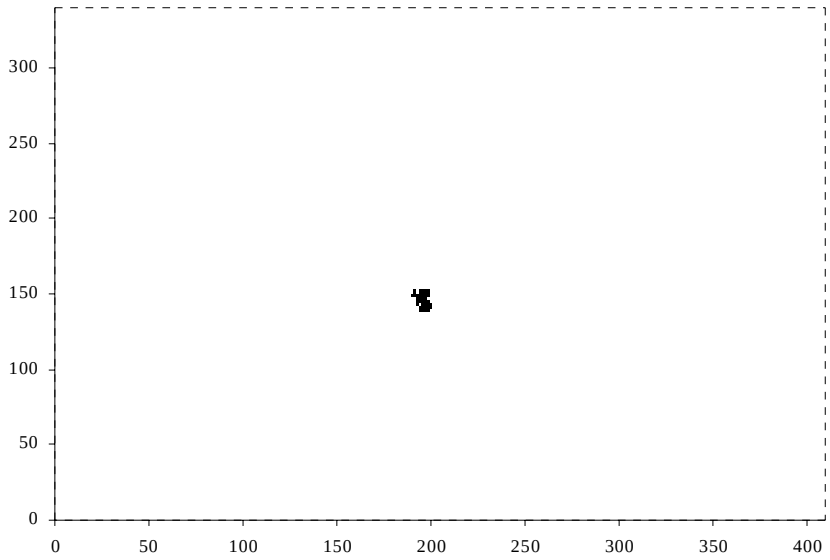
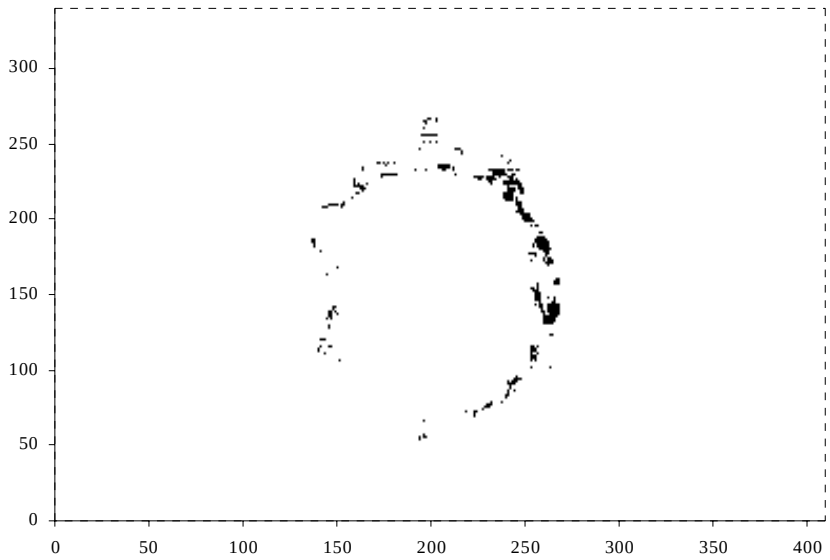


Figure 17: The number of turns per minute exhibited during the 15 minutes following infusion of each of the 6 consecutive amphetamine concentrations into the substantia nigra via the push-pull cannula, and prior to infusion with ACSF only. Results are expressed as means $\pm$ SEM; asterisks represent a significant difference compared to levels prior to amphetamine; * $P<0.05$, ** $P<0.01$, *** $P<0.001$; paired t-test. The number of animals used for each concentration was 20.



(A)



(B)

Figure 18: (A) Animal movement prior to infusion with amphetamine, monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 771 mm, time elapsed: 120 seconds.

(B) Animal movement induced by local stimulation with amphetamine (in the substantia nigra via the push-pull cannula), monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 4378 mm, time elapsed: 120 seconds, amphetamine dosage: 10^{-4} M.

3.1.3.2 The Local administration of amphetamine and its effect on AchE-release

The spontaneous hydrolysis of acetylcholinechloride yielded a signal prior to addition of AChE from the rat perfusate. However, subtraction of this background reading gave a basal AChE perfusate value of 0.25 $\pm$ 0.07 mU (n=13). On stimulating the animals with amphetamine (10^{-7} M to 10^{-2} M) administered locally to the substantia nigra, there was an increase in the level of AChE released (from 10^{-7} M to 10^{-4} M) with increasing concentrations of amphetamine. Using 10^{-5} M to 10^{-4} M, however, the increase in AchE-release reached a plateau - see figures 19 and 20. 10^{-7} M of amphetamine caused a significant enhancement of 22.87%, $P<0.01$, in the release of AChE; 10^{-6} M 35.34%, $P<0.01$; 10^{-5} M 32.44%, $P<0.05$ and 10^{-4} M 32.91%, $P<0.01$. The loss of the signal at 10^{-3} M and 10^{-2} M was not attributed to an accumulation of the serial amphetamine doses. These highest concentrations were also tested separately with and without the animal attached to the system, and a loss of the chemiluminescent signal was produced nonetheless. Hence, this reduction in light signal is not due to a physiological inhibition of AchE-release, but rather represents a direct chemical `quenching´ of the chemiluminescent signal. The same inhibitory effect has been reported with 10^{-6} M 5-HT (5.7-dihydroxytryptamine creatinine sulphate), α-methyl 5-HT (α-methylserotonin maleate) and 2-methyl 5-HT (2-methylserotonin maleate).

With a local administration of amphetamine, we were able to see a correlation between AChE release in the substantia nigra and behaviour measured in turns per minute - see figure 21. An increase in the turns per minute corresponded to a greater release of AChE. A higher concentration of amphetamine cannot influence turns per minute but does have an influence on AChE-release in the substantia nigra.

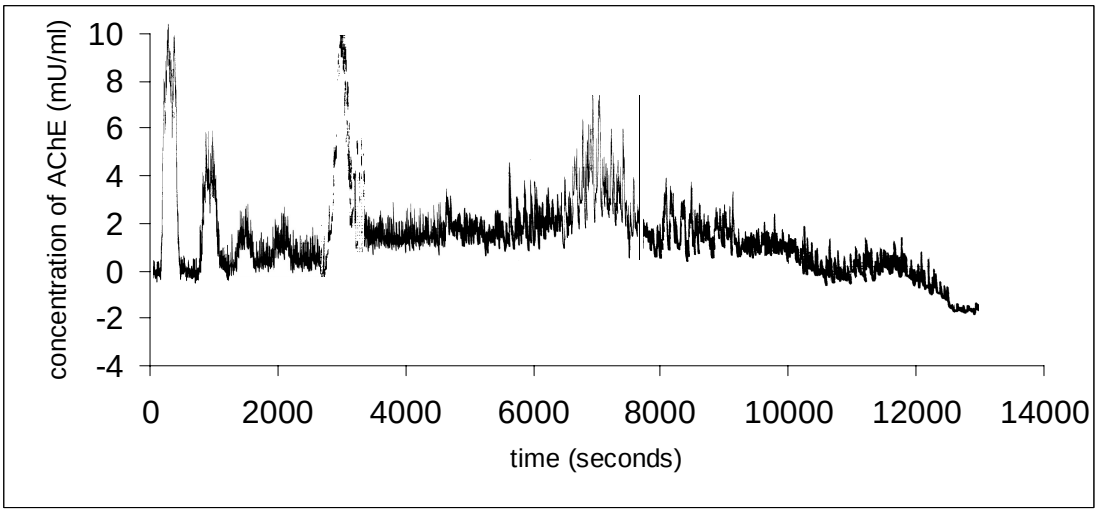


Figure 19: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) to the system prior to animal attachment. The spontaneous hydrolysis of ACh in vitro is shown as a background signal. A large signal peak was observed when connecting the animal due to blood/air contamination. This is followed by an on-line detection of release of AChE in vivo and the intranigral stimulation of AChE-release with amphetamine 10^{-7} M to 10^{-2} M.

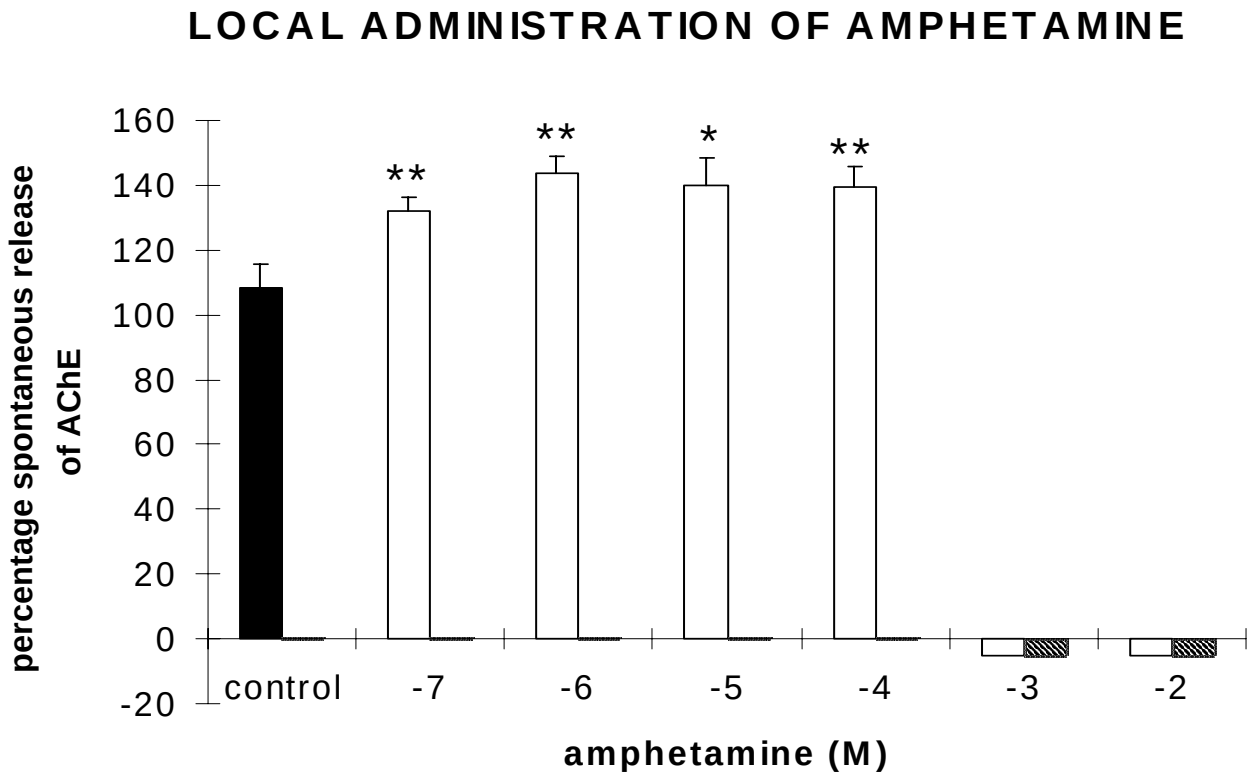


Figure 20: The spontaneous release of AChE in the substantia nigra of control and amphetamine-treated rats, expressed as a percentage. Results are shown as means $\pm$ SEM; whereby asterisks represent a significant difference from the drug-free control group. A paired t-test was used to calculate the areas in the diagram * $P<0.05$, **$P< 0.01$. A total of 13 animals were used. The black column represents the control group. The white column depicts the drug-treated animals. The striped column indicates the results obtained with no animal connected to the chemiluminescent system. Note that chemical inhibition of the chemiluminescent signal through 10^{-3} M and 10^{-2} M amphetamine is not due to a physiological inhibition of AChE-release, but rather represents a direct chemical `quenching´.

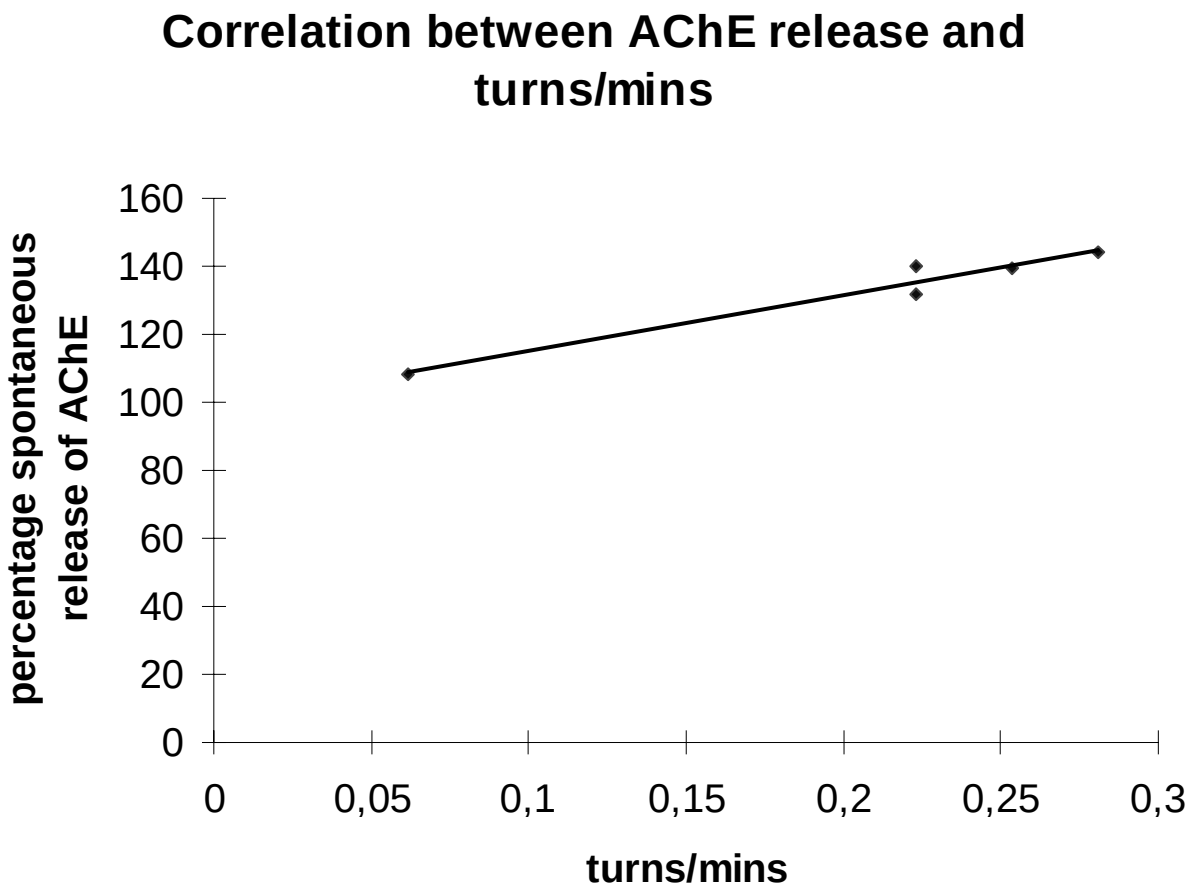


Figure 21 shows the correlation between AChE-release and turns per minute. The left-hand point represents the 13 control animals. They produced 108% AChE and 0.06 turns per minute. The animals which were infused with 10^{-7} M amphetamine produced 132% AChE and 0.22 turns per minute. Those which received 10^{-6} M amphetamine generated 143% AChE and rotated 0.28 times per minute. Animals which were given 10^{-5} M amphetamine generated 139% AChE and 0.22 turns per minute, while those which were infused with 10^{-4} M amphetamine produced 139% AChE and turned 0.25 times per minute.

3.1.4 The effect of systemic administration of amphetamine

3.1.4.1 The systemic administration of amphetamine and its behavioural effect

Amphetamine (1mg/kg in saline) was injected i.p. and the rats then tested immediately for enhanced motor-activity using the computer system to monitor the total distance moved in millimetres. The same procedure was carried out with control animals who were injected with a saline vehicle only. The computer system detected the movement with help of the LED in the animals headset (figure 22) and showed at which point (x and y) the animal travelled in terms of distance (mm) and time (in seconds). The sum of the distance travelled gave the total distance moved in a time period of 120 seconds. Paired t-tests showed a significant difference between control groups and amphetamine stimulation ($P<0.001$). The mean total distance moved was as follows: Treatment with saline only 2787 mm $\pm$ 322 SEM, treatment with amphetamine 7493 mm $\pm$ 406 SEM and after stimulation 4617 mm $\pm$ 785 SEM, see figure 23. In contrast, systemic stimulation of the animal with 1mg amphetamine/kg i.p. produced a different pattern of behaviour from that seen with local administration. Typically, approximately 5 minutes after amphetamine injection, the animals became more active, moving around the entire box. The activity lasted for about one hour with the animal moving either in a contraversive or ipsiversive direction. A bias varying from animal to animal was observed, see figure 24. This increased activity was associated with an increase in release of AChE.

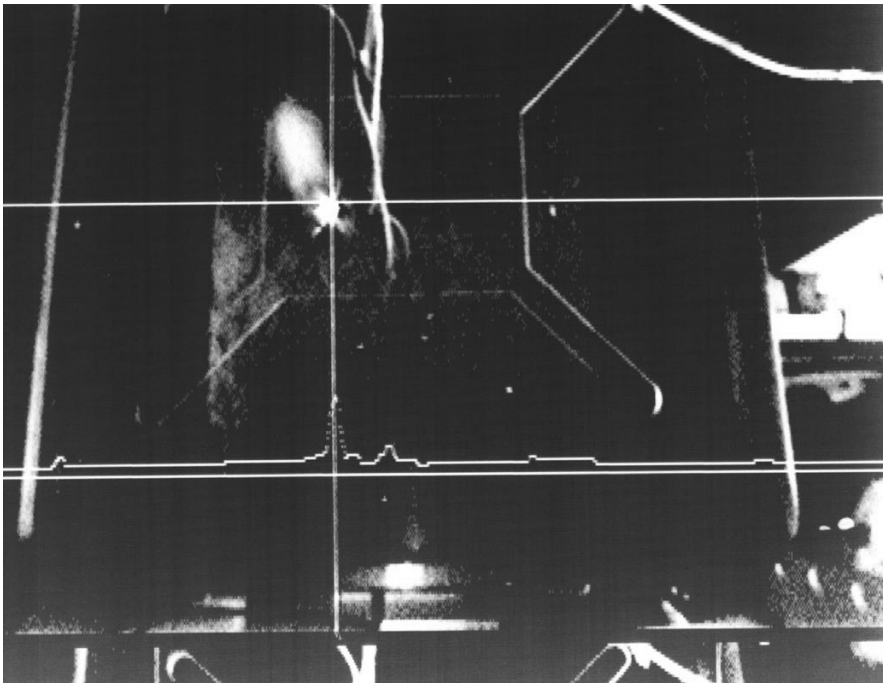


Figure 22: Animal movement monitored with an Antrak video-based animal tracking system with help of the LED in the animal's headset.

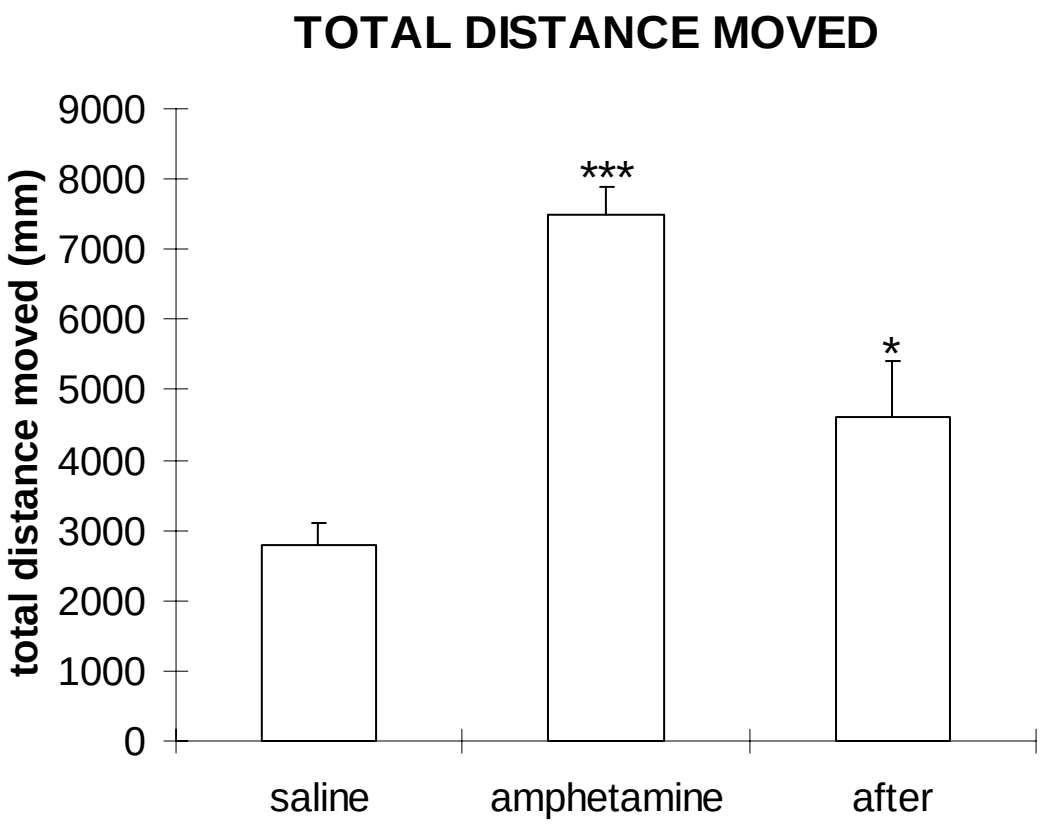
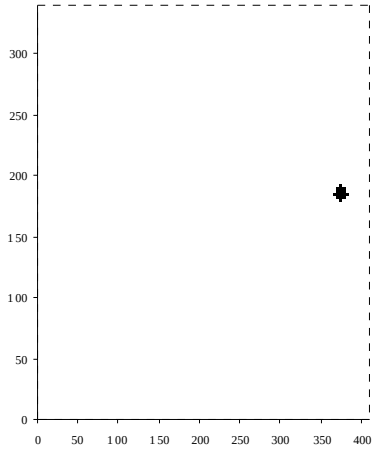


Figure 23: Total distance moved (mm) during systemic stimulation with amphetamine (1mg/kg) compared to control groups injected with saline only, and approximately one hour following amphetamine stimulation. Results are expressed as means $\pm$ SEM, asterisks represent a significant difference from drug-free control group, * $P<0.05$, *** $P<0.001$, paired t-test, n=10.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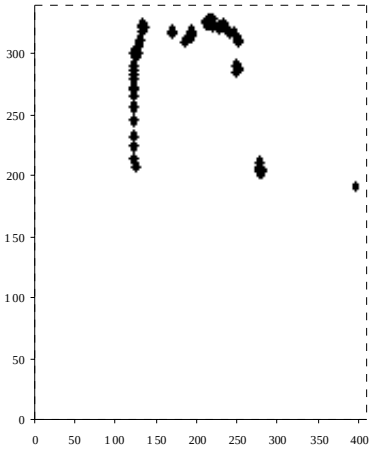
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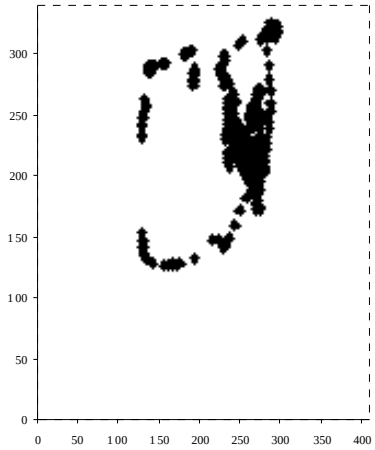
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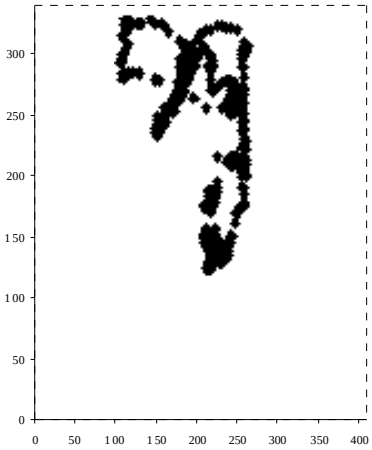
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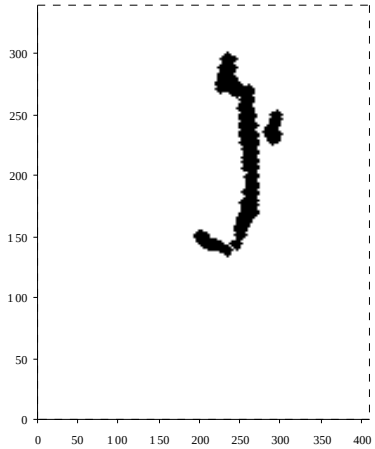
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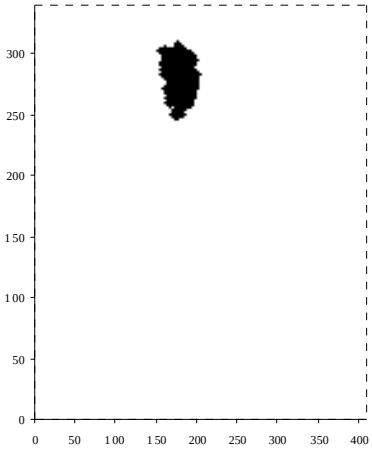
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Figure 24: Cumulative data showing motor activity following i.p. treatment with saline (A); with amphetamine (B)-(E), and approximately one hour after amphetamine treatment (F). The diagram shows behaviour evoked by amphetamine stimulation during the first 480 seconds and approximately one hour later. The animal movement induced by systemic stimulation with amphetamine is monitored with an Antrak video-based animal tracking system. Different computer plotted pictures are shown, each covering in a period of 120 seconds. Total distance moved (in mm): (A) 788, (B) 3572, (C) 5268, (D) 6484, (E) 3421, (F) 2129.

3.1.4.2 The systemic administration of amphetamine and its effect on the release of AChE

AChE-release was continuously monitored in relation to specific movements evoked by amphetamine stimulation (figure 26). The spontaneous release of AChE of 0.11 $\pm$ 0.03 mU (n=9) was detected in perfusate of the substantia nigra. An application of amphetamine (1mg/kg) caused an increase in the spontaneous release of AChE which exceeded control conditions by approximately 40% ($P<0.01$, paired t-test; see figure 27). There was no increase seen following the injection of saline only (figure 25). Increased motor activity was associated with an increase in release of AChE. The raised concentration of AChE-release lasted for approximately one hour at a steady level. The diminution in movements was associated with a decrease in AChE-release.

Even when amphetamine was administered systematically we were able to see a correlation between the level of AChE-release in the substantia nigra and behaviour measured in total distance moved - see figure 28. A increase in the level of AChE released led to an increase in the distance moved and vice versa.

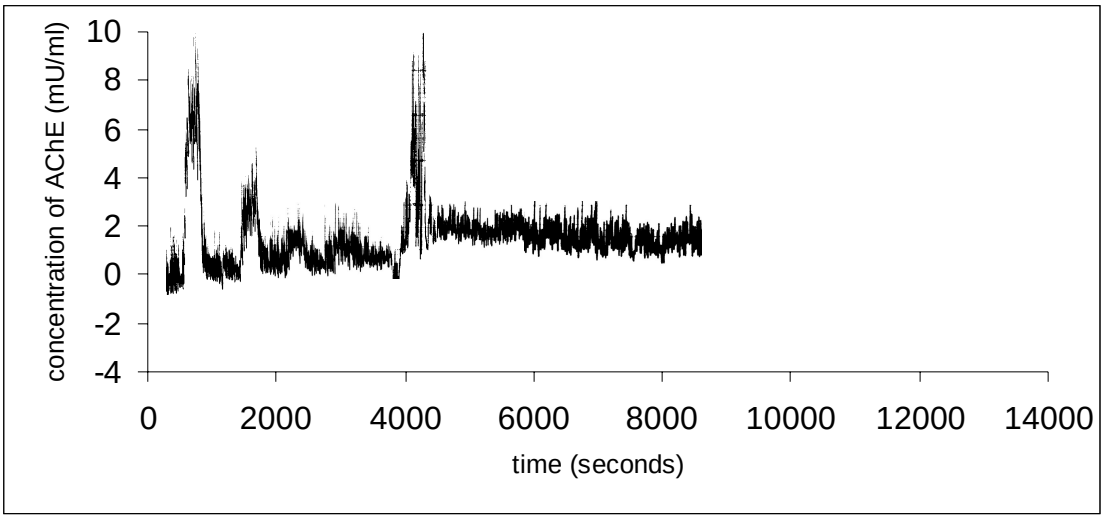


Figure 25: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) to the system prior to animal attachment. The left hand side of the graph represents the spontaneous hydrolysis of ACh in vitro (background signal). A large signal peak due to blood/air contamination was experienced when connecting the animal. On-line detection of release of AChE in vivo can be observed between 4500 – 5000 seconds. AChE-release was stimulated with sterile saline i.p..

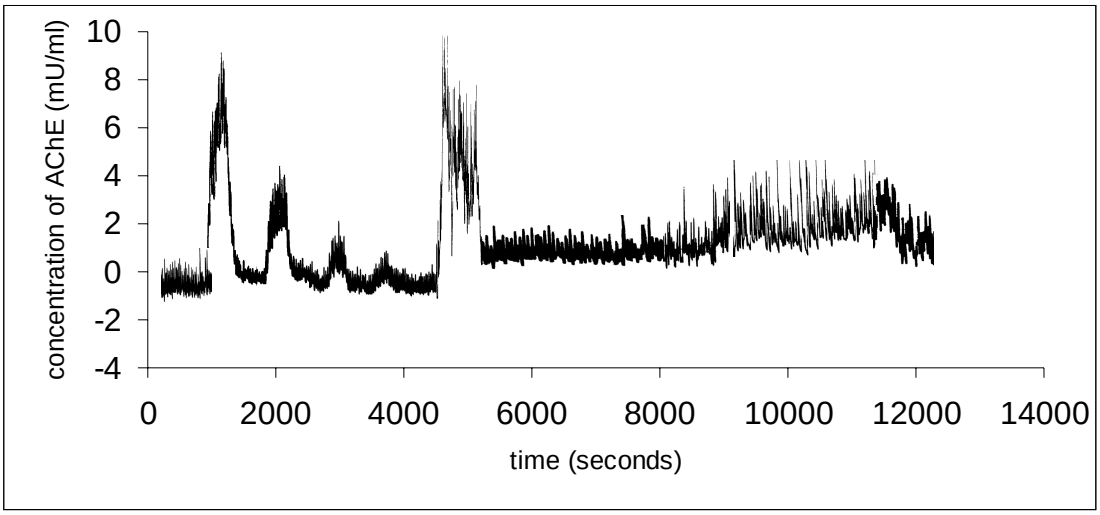


Figure 26: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) to the system prior to animal attachment. The left hand side of the graph represents the spontaneous hydrolysis of ACh in vitro (background signal). A large signal peak due to blood/air contamination was experienced when connecting the animal. On-line detection of release of AChE in vivo can be observed upon 5500 seconds. AChE-release was stimulated with amphetamine i.p..

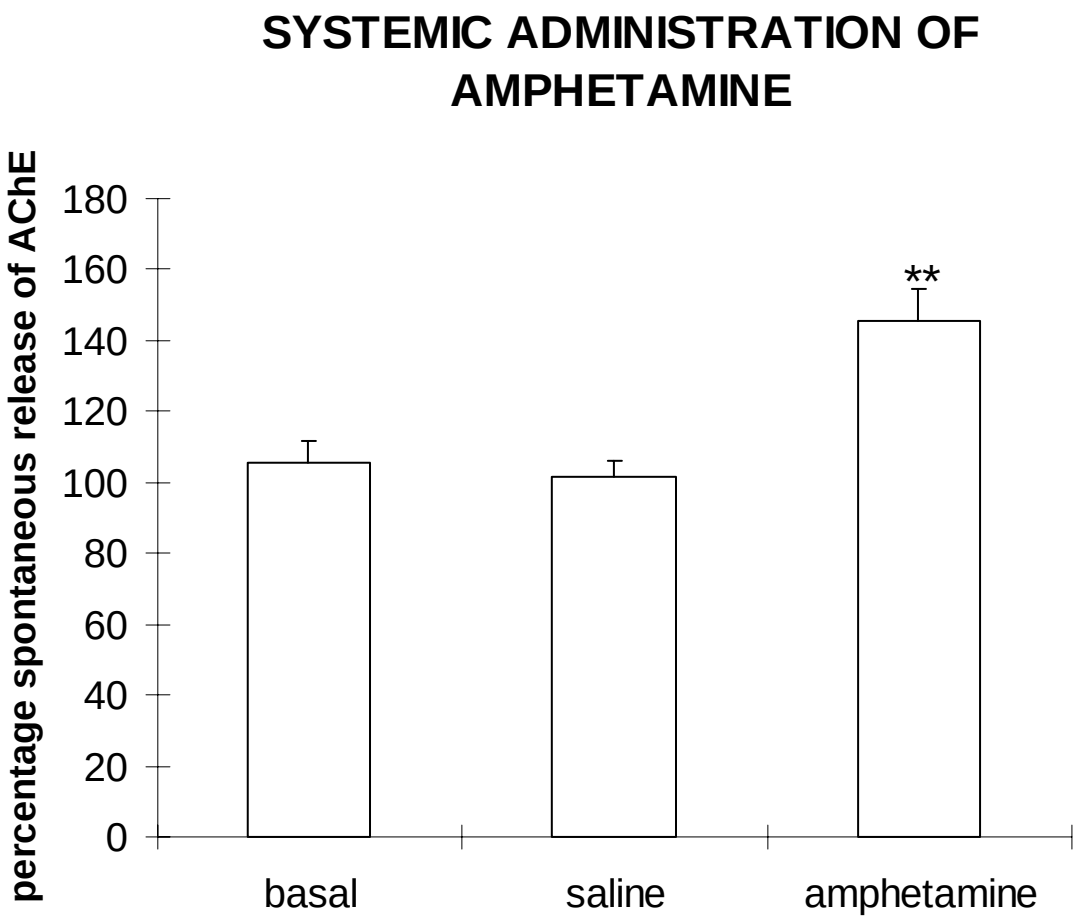


Figure 27: The spontaneous release of AChE from substantia nigra shown as a basal value. The other two columns represent the control animals and the animals treated with systemic amphetamine. Results are expressed as means $\pm$ SEM, asterisks represent a significant difference from the drug-free control group, ** $P<0.01$, paired t-test, n=9.

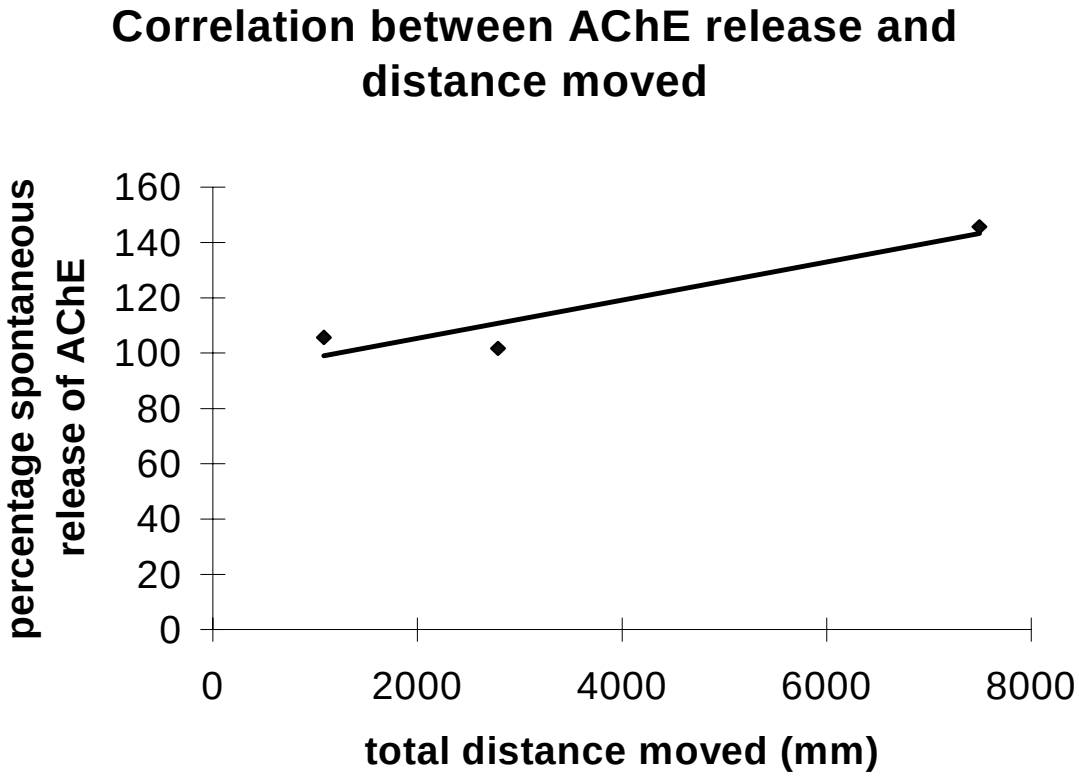


Figure 28: The correlation between AChE-release and total distance moved (mm). The basal value of 105% AChE was produced together with movement covering 1085 mm. The control animals produced 101% AChE and covered a distance of 2787 mm. Animals which were infused with amphetamine i.p. emitted 145% AChE and covered a distance of 7492 mm; r^2 =0.8878.

CHAPTER 3

RESULTS

3.2 THE RELEASE OF ACETYLCHOLINESTERASE IN THE PATHOLOGICAL BASAL GANGLION FOLLOWING AMPHETAMINE STIMULATION

3.2 The release of acetylcholinesterase in the pathological basal ganglion following amphetamine stimulation

The objectives of the present study were to show the physiological and behavioural effects of intracerebral application of the neurotoxin 6-OHDA on the in vivo release of AChE in the substantia nigra of the rat.

Parkinson's disease arises from a selective and progressive degeneration of neuromelanin containing dopaminergic neurons which project from the substantia nigra to the striatum.

In the present study, the neurotoxin 6-OHDA was used to disrupt/destroy the nigrostriatal pathway. This toxin was applied to one side of the brain. We expected to observe a marked gradient of nigrostriatal damage, including unilateral nigral and striatal dopamine depletion between the two sides of the animal's brain, and that stimulation of the neurotoxin-treated side would yield observable changes in movement.

6-OHDA toxicity is due to autoxidation. It is taken up by dopaminergic and noradrenergic neurones. It is well documented that this neurotoxin can induce a Parkinson-like motor-deficit in animal models (Kaakkola and Teräväinen1990).

The spontaneous and evoked release of AChE in vivo was detected and quantified using the on-line chemiluminescent system.

3.2.1 The histological verification of cannulae placements

Upon completion of each experiment, each animal was heavily anaesthetised, decapitated, and the brain removed and dissected. Verification of the placement of the nigral cannulae was achieved by an histological examination of 42 µm frozen cut sections stained with cresyl violet (figure 29).

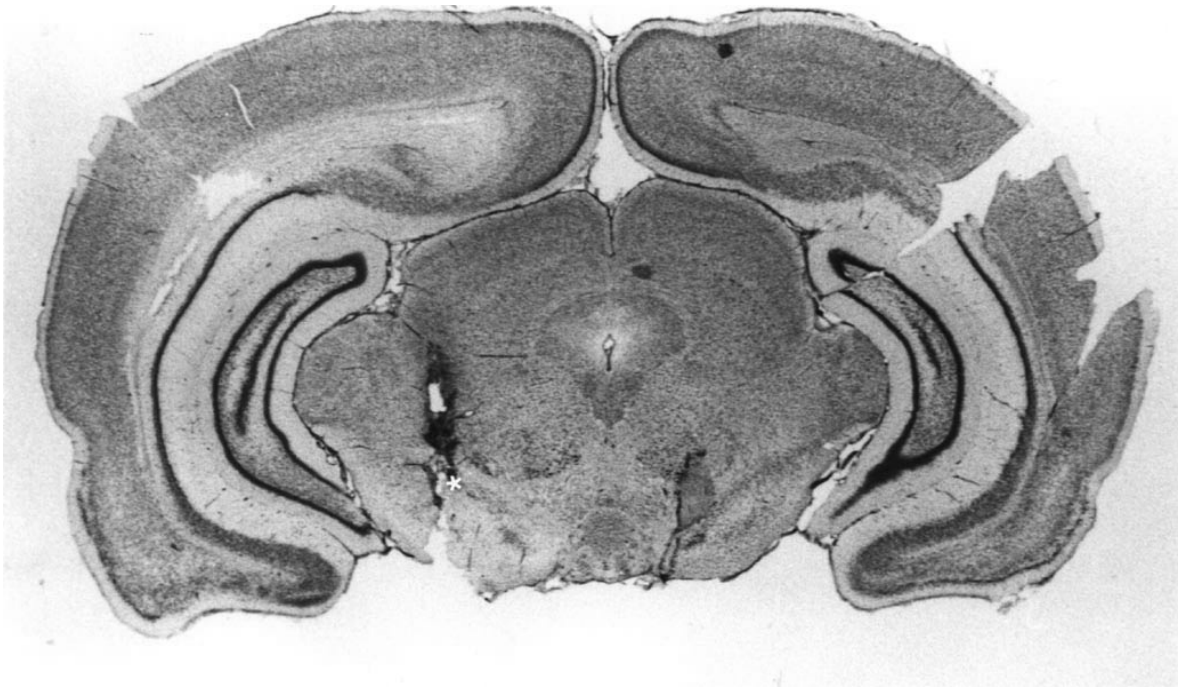


Figure 29: A section of the rat brain stained with cresyl violet (width: 42 µm), showing the tip of a push-pull cannula placement in the substantia nigra, as marked by an asterisk. Stereotaxic co-ordinates are given in the text.

3.2.2 The effect of a local administration of amphetamine

3.2.2.1 The local administration of amphetamine: Behavioural observations

The animals moved spontaneously once they had recovered from anaesthesia. Animals pre-treated with 6-OHDA or those which had been sham-operated did not appear to be any different to naive animals. No obvious changes in posture, general health/condition or movement were noted following surgery. However, abnormal changes in movement or rotational behaviour were noted 3 weeks after neurotoxin treatment. Some animals became aggressive and gnashed their teeth, and all displayed high circling movement. Sham-operated animals and those treated with neurotoxin were locally stimulated with amphetamine 10^{-7} M to 10^{-2} M. Each concentration of amphetamine was infused over a period of five minutes in ACSF. In order to directly compare the effects of the different concentration of amphetamine in the substantia nigra, each concentration was infused under the same conditions. Figure 30 shows the circling behaviour prior to amphetamine infusion and that observed during the infusion of different concentrations of amphetamine. A paired t-test revealed a significant difference between high basal circling, 0.89 $\pm$ 0.14 SEM turns/min and 10^{-7} M, 0.66 $\pm$ 0.08 SEM turns /min, ($P<0.05$); 10^{-6} M, 0.52 $\pm$ 0.09 SEM turns/min, ($P<0.01$); 10^{-5} M, 0.39 $\pm$ 0.09 SEM turns /min, ($P< 0.001$); 10^{-4} M, 0.21 $\pm$ 0.05 SEM turns/min, ($P<0.001$); 10^{-3} M, 0.17 $\pm$ 0.04 SEM turns/min, ($P<0.001$). The number of turns per minute decreased when the concentrations of amphetamine were increased, until a normal rotating behaviour was achieved similar to that of naive animals. Both ipsiversive turning (towards side of infusion) and contraversive turning (away from side of infusion) was observed following local amphetamine treatment (figure 31).

Different behaviour was seen following infusion of 10^{-2} M amphetamine. The resulting rate of turns per minute observed with this concentration was brought about by other biochemical reactions produced by the extremely high level of amphetamine. A large variation was observed in the intensity of circling of between 0.15 and 5.8 turns/min, mean $\pm$ SEM, 1.41 $\pm$ 0.65 turns/min ($P<0.42$).

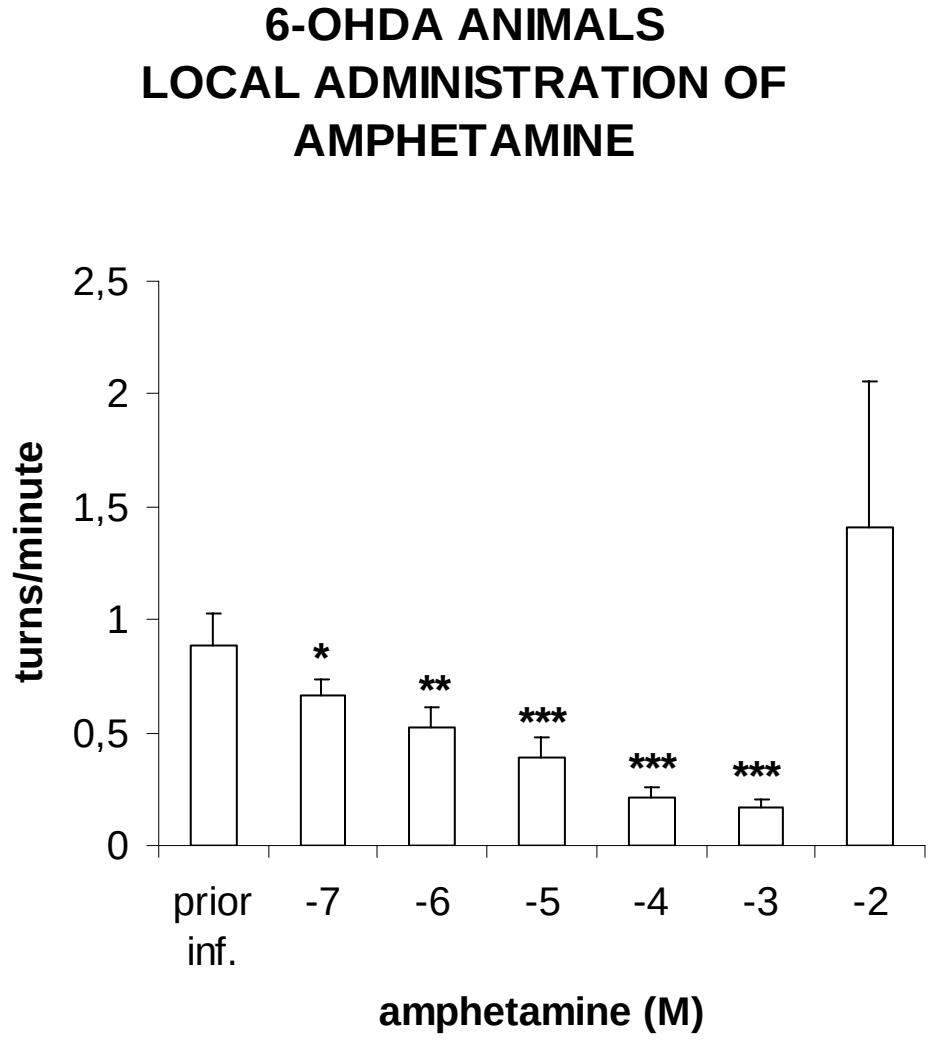
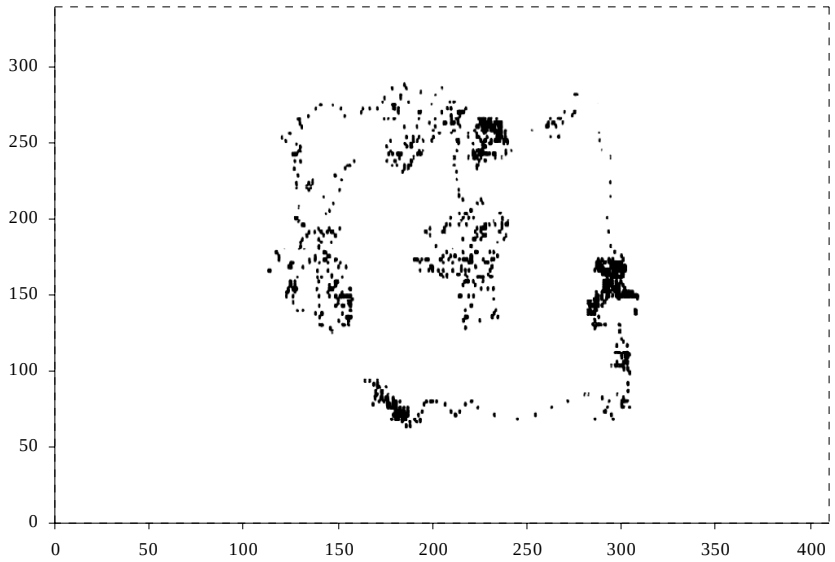


Figure 30: The number of turns per minute exhibited during the 15 minutes following each of the 6 consecutive amphetamine concentrations infused into the substantia nigra via the push-pull cannula, and prior to infusion with ACSF only. Results are expressed as means $\pm$ SEM; asterisks represent a significant difference compared to levels prior to amphetamine; * $P<0.05$, ** $P<0.01$, *** $P<0.001$; paired t-test. The number of animals used for each concentration was 10.



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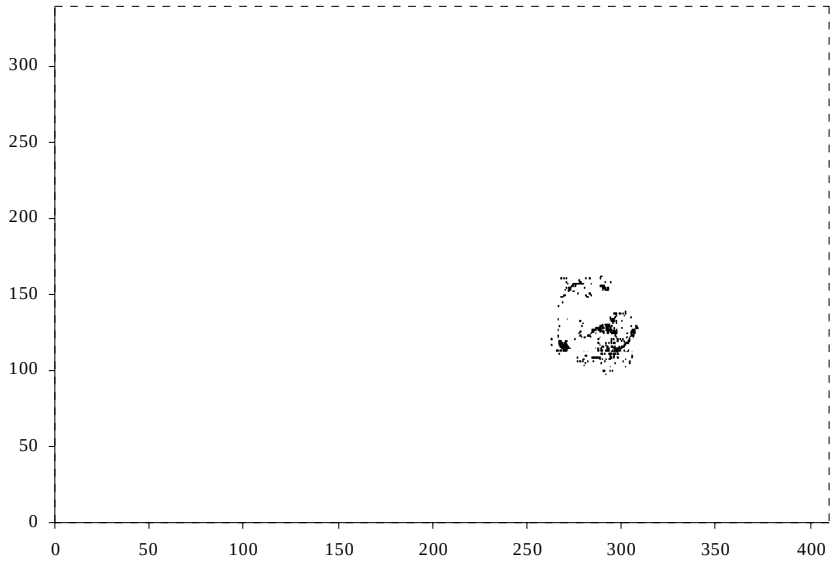


(B)

Figure 31: Animals pre-treated with 6-OHDA. (A) Animal movement prior to infusion with amphetamine, monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 8509 mm, time elapsed: 120 seconds. (B) Animal movement induced by local stimulation with amphetamine (in the substantia nigra via the push-pull cannula), monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 3791 mm, time elapsed: 120 seconds, amphetamine dosage: 10^{-3} M.

There was a different type of circling seen in sham-operated animals compared with animals pre-treated with 6-OHDA. Sham-operated animals showed a similar behaviour pattern to naive animals, see figures 32 and 33. The number of turns per minute was as follows: prior to infusion 0.24 turns/min $\pm$ 0.06 SEM; 10^{-7} M 0.32 turns/min $\pm$ 0.06 SEM ($P<0.36$); 10^{-6} M 0.5 turns/min $\pm$0.06 SEM ($P<0.05$); 10^{-5} M 0.5 turns/min $\pm$ 0.13 SEM ($P<0.22$); 10^{-4} M 0.43 turns/min $\pm$ 0.06 SEM ($P<0.06$); 10^{-3} M 0.33 turns/min $\pm$ 0.04 SEM ($P<0.27$); 10^{-2} M 2.39 turns/min $\pm$ 0.73 SEM ($P<0.10$). 6-OHDA pre-treated rats were more active, restless and aggressive, scoring more turns per minute before amphetamine stimulation. During stimulation with different concentrations of amphetamine (10^{-7} M to 10^{-2} M) the animals pre-treated with 6-OHDA became calmer, eventually reaching a normal pattern of behaviour.

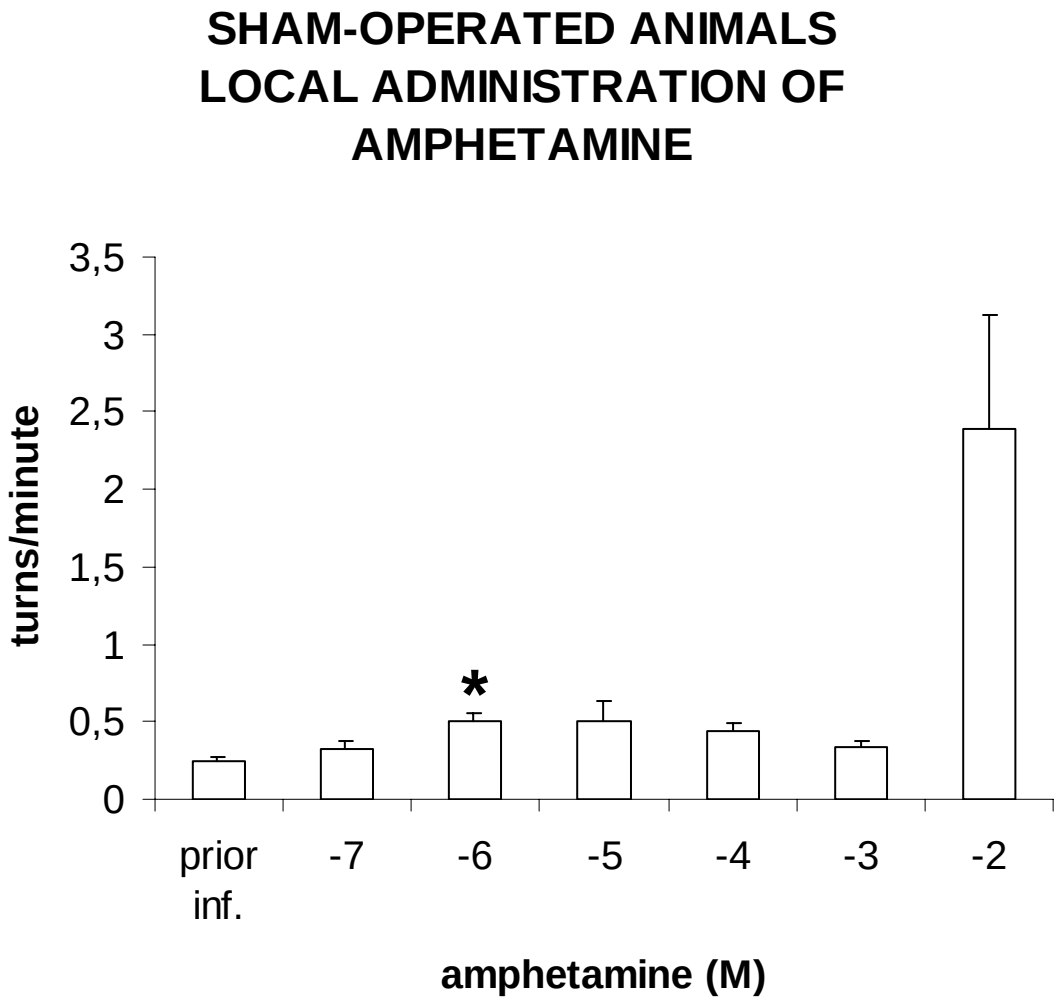
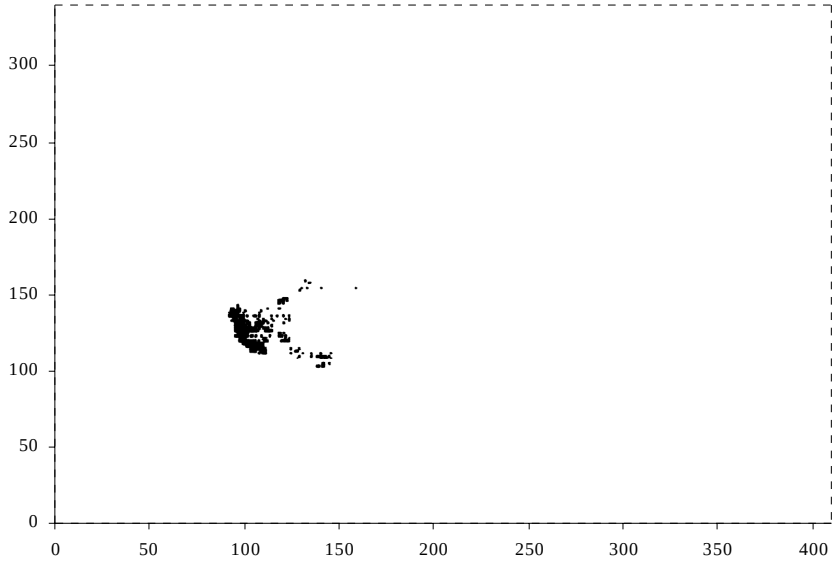


Figure 32: The number of turns per minute exhibited during the 15 minutes following each of the 6 consecutive amphetamine concentrations infused into the substantia nigra via the push-pull cannula, and prior to infusion with ACSF only. Results are expressed as means $\pm$ SEM; asterisks represent a significant difference compared to levels prior to amphetamine infusion; * $P<0.05$; paired t-test. The number of animals used for each concentration was 10.



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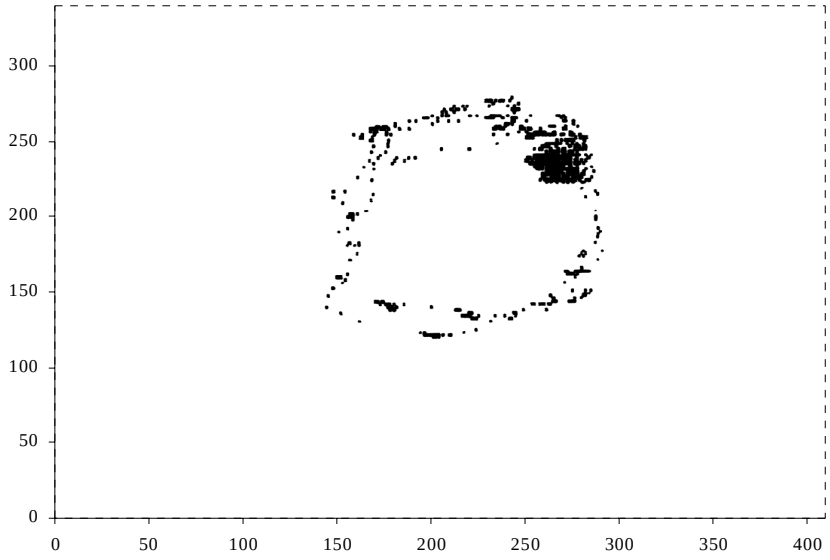


(B)

Figure 33: Sham-operated animals. (A) Animal movement prior to infusion with amphetamine monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 2896 mm, time elapsed: 120 seconds. (B) Animal movement induced by local stimulation with amphetamine (in the substantia nigra via a push-pull cannula), monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 6180 mm, time elapsed: 120 seconds, amphetamine dosage: 10^{-2} M.

3.2.2.2 The local administration of amphetamine and its effect on AChE-release

Figure 34 shows the spontaneous release of AChE in the substantia nigra of 3-week, 6-OHDA-treated animals compared to naive and sham-operated animals. The spontaneous release of AChE in the substantia nigra was significantly reduced by 68% following pre-treatment with 6-OHDA, 3-week lesion, $P<0.01$, t-test. The basal AChE perfusate value of 0.04 ± 0.01 mU (n=22) was detected.

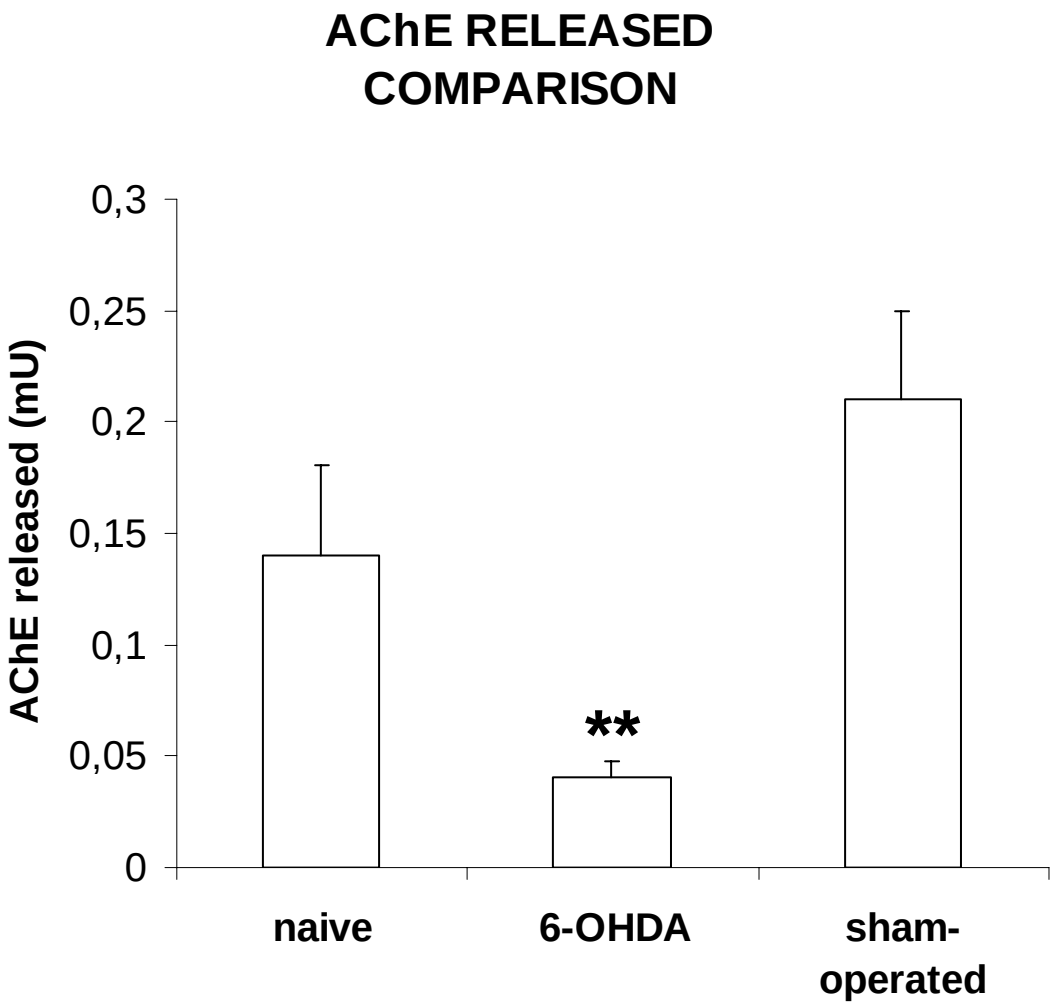


Figure 34 shows the spontaneous release of AChE in the substantia nigra of naive animals, 3-week 6-OHDA-treated animals, and sham-operated animals. Results are expressed as means $\pm$ SEM; n=10 for each group. Asterisks represent a significant difference from corresponding neurotoxin free control group; **P< 0.01, t-test.

The effect of local application of amphetamine on the spontaneous release of AChE into the substantia nigra on 3-week, 6-OHDA treated animals is shown in figure 35.

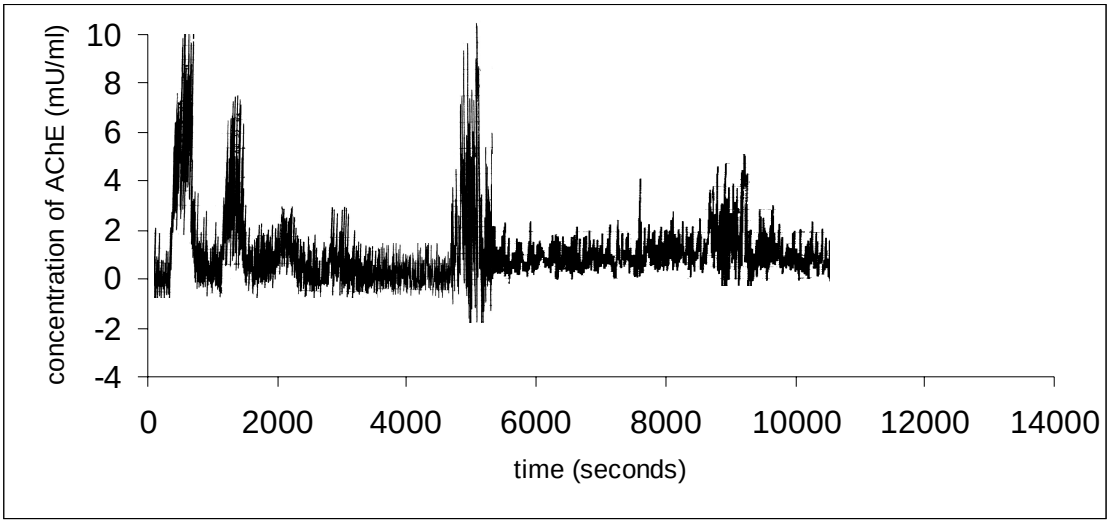


Figure 35: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) to the system prior to animal attachment. The left hand side of the graph represents the spontaneous hydrolysis of ACh in vitro (background signal). A large signal peak due to blood/air contamination was experienced when connecting the animal. On-line detection of release of AChE in vivo; AChE-release was stimulated intranigrally with 10^{-7} M to 10^{-4} M.

Figure 36 shows the effect of application of amphetamine 10^{-7} M to 10^{-4} M administered locally to the substantia nigra on the spontaneous release of AChE on 6-OHDA pre-treated animals. Release of nigral AChE, evoked in this way, increased 30.8%, $P<0.18$, 10^{-7} M; 70.6%, $P<0.28$, 10^{-6} M; 67.6%, $P<0.08$, 10^{-5} M; 13%, $P<0.74$, 10^{-4} M over basal conditions. There was no significant difference in the release of AChE between any of the experimental conditions. However, 10^{-4} M amphetamine treatment had no effect on the increase in the percentage release of AChE, when the evoked data was expressed as a percentage of basal levels. In addition, for each concentration of amphetamine was a large variation in AChE released.

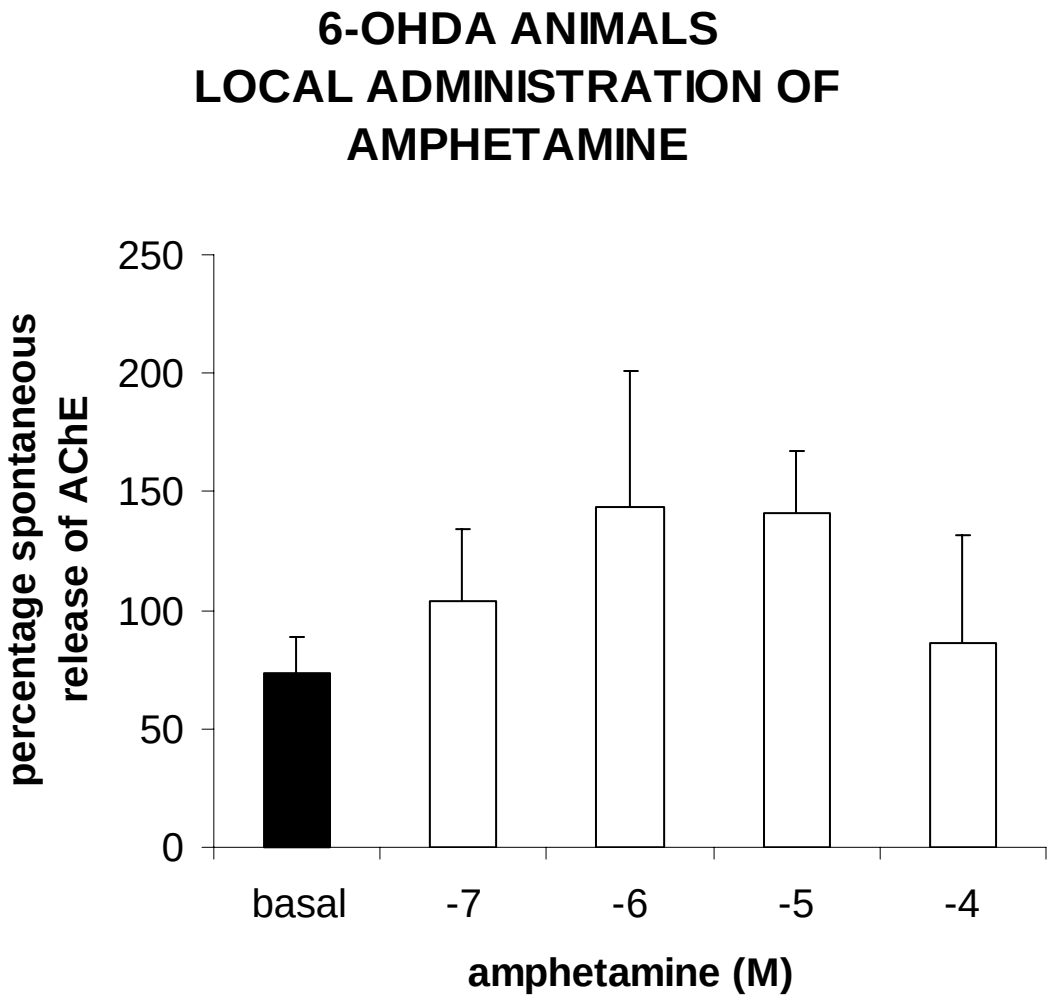


Figure 36: The spontaneous release of AChE in the substantia nigra of 3-week post-operative rats prepared with 6-OHDA. The spontaneous release is expressed as a percentage. Both the basal value and that achieved with amphetamine-stimulated animals are shown. Results are expressed as means $\pm$ SEM; paired t-test, n=10. The black column represents the 6-OHDA animals which were not treated with amphetamine. The white column represents the drug-treated 6-OHDA animals.

The effect of local application of amphetamine on the spontaneous release of AChE in the substantia nigra at sham-operated animals shows similar results to those achieved with naive animals (figure 37).

Mean $\pm$ SEM basal AChE perfusate value was 0.21 $\pm$ 0.04 mU (n=10). Amphetamine at 10^{-7} M caused significant enhancement of 27.5%, $P<0.05$, in the release of AChE; 10^{-6} M 51.5%, $P<0.05$; 10^{-5} M 55%, $P<0.05$ and 10^{-4} M 51.5%, $P<0.05$ (figure 38). The increase in the level of AChE released by sham-operated animals (also with 10^{-5} M to 10^{-4} M) reached a plateau, in the same way it did in naive animals.

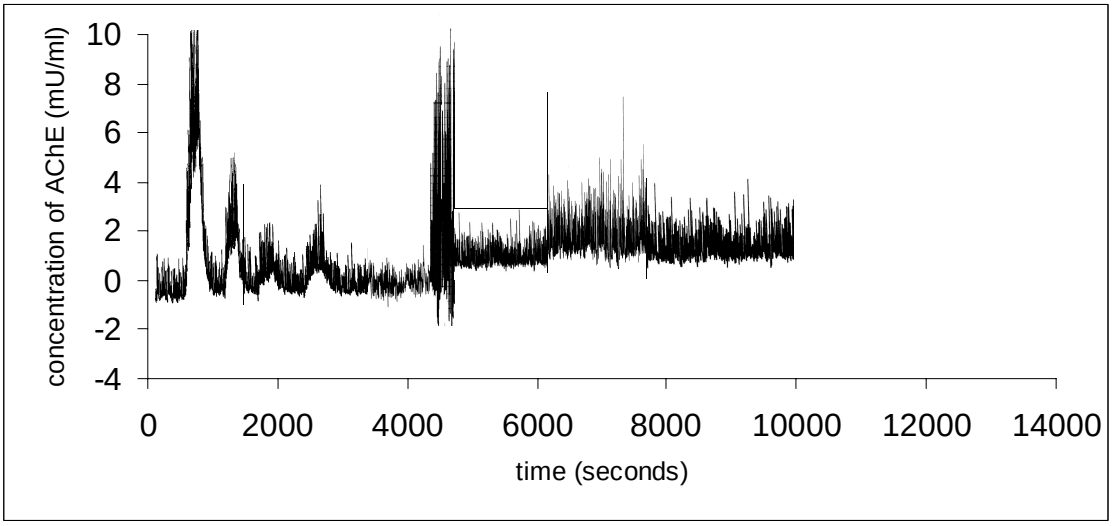


Figure 37: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) to the system prior to animal attachment. The left hand side of the graph represents the spontaneous hydrolysis of ACh in vitro (background signal). A large signal peak due to blood/air contamination was experienced when connecting the animal. On-line detection of release of AChE in vivo; AChE-release was stimulated intranigrally with 10^{-7} M to 10^{-4} M.

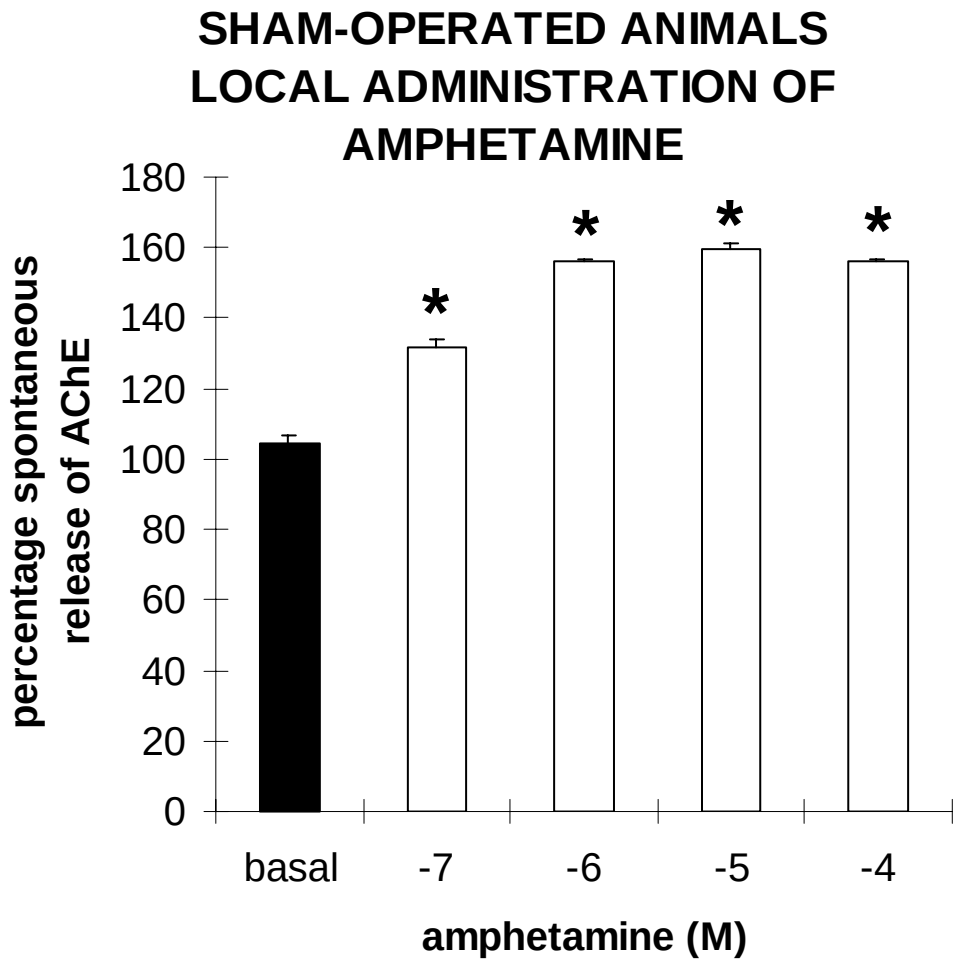


Figure 38: The spontaneous release of AChE in the substantia nigra of sham-operated animals (expressed as a percentage). The diagram shows basal values and those achieved following amphetamine stimulation. Results are expressed as means $\pm$ SEM; asterisks represent a significant difference from basal value; *P<0.05, paired t-test, n=10. The black column represents non-amphetamine-treated sham-operated animals, with the white column representing drug-treated sham-operated animals.

A correlation between the level of AChE released in the substantia nigra and the number of turns per minute was only seen by the sham-operated rats, see figure 39. A correlation existed between the level of AChE released in the substantia nigra and behaviour and vice versa. An increased number of turns per minute corresponded to an increase in the level of AChE released in the CSF, a higher concentration of AChE in the CSF correspond to more turns/min. However, higher concentration of amphetamine has an influence on AChE-release in the substantia nigra, but can influence the number of turns per minute.

There was no correlation seen between AChE-release and the number of turns per minute in neurotoxin treated animals, see figure 40.

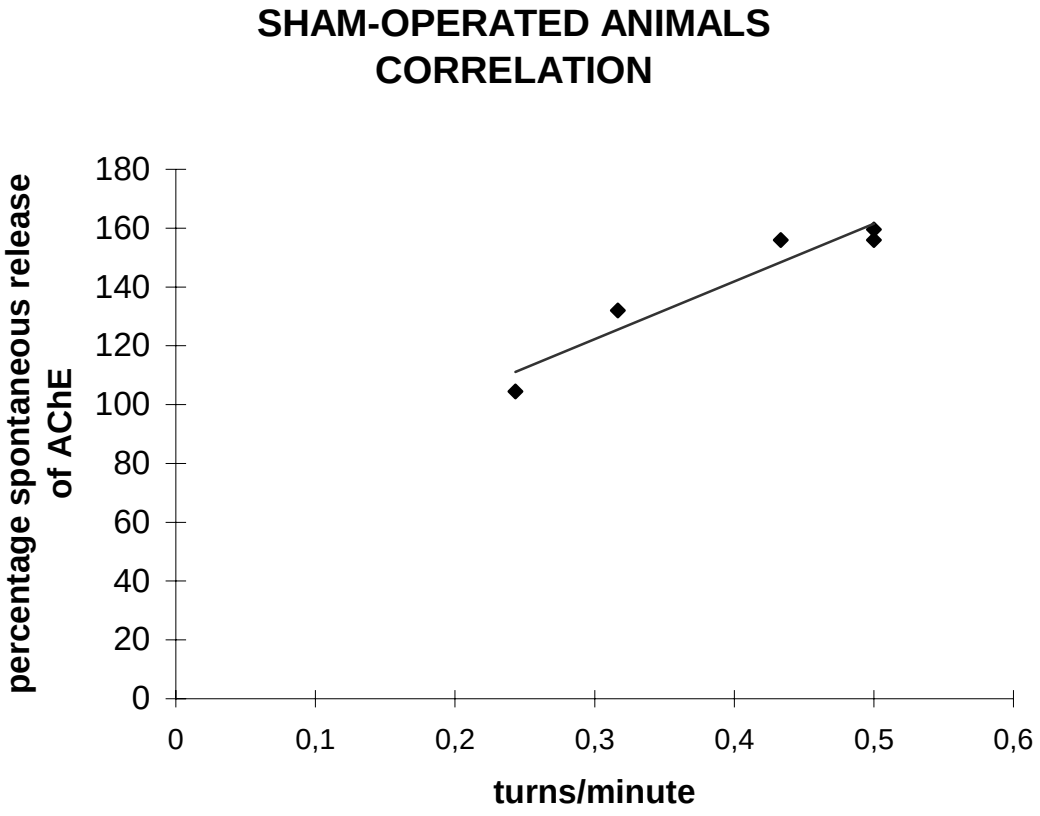


Figure 39: The correlation between AChE-release and the number of turns per minute. The basal values were 104.5% AChE with 0.24 turns per minute. Following infusion of 10^{-7} M amphetamine, 132% AChE was released with 0.32 turns per minute. With 10^{-6} M amphetamine, the level of AChE-release rose to 156% with 0.5 turns per minute. An infusion of 10^{-5} M amphetamine produced 159.5% AChE and 0.5 turns per minute, and, last but not least, 10^{-4} M amphetamine produced 156% AChE and 0.43 turns per minute; r^2=0.9194.

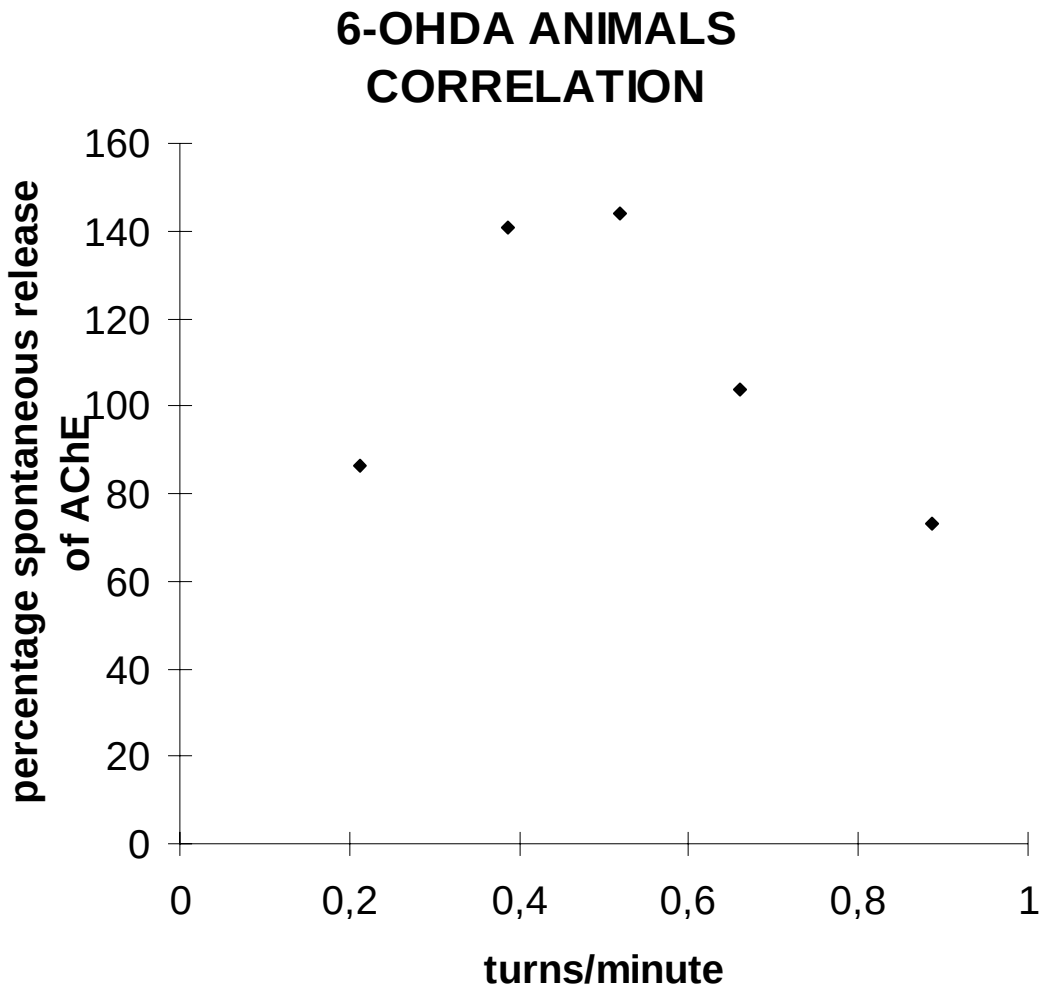


Figure 40: The correlation between AChE-release and the number of turns per minute. The basal values were 73.2% AChE with 0.89 turns per minute. Following infusion of 10^{-7} M amphetamine, 104% AChE was released with 0.66 turns per minute. With 10^{-6} M amphetamine, the level of AChE-release rose to 143.8% with 0.52 turns per minute. An infusion of 10^{-5} M amphetamine produced 140.8% AChE and 0.39 turns per minute, and, last but not least, 10^{-4} M amphetamine produced 86.2% AChE and 0.21 turns per minute.

3.2.3 The effect of systemic administration of amphetamine

3.2.3.1 Systemic administration of amphetamine: Behavioural observations

Any spontaneous or induced animal movements that occurred during the perfusion period were monitored using an Antrak video-based animal tracking system, which was used in conjunction with the on-line chemiluminescent system. This system involves a computerised tracking of a LED , in and around a pre-set area within the study-arena. The computer plotted the animal's movements within a pre-set time period. The animal's movements were also expressed as a number of 360^{o} turns.

Amphetamine (1mg/kg in saline) was injected intraperitoneally and the animals treated with 6-OHDA were tested immediately for changes in motor-activity. A paired t-test showed a significant difference of prior amphetamine stimulation and during the stimulation period ($P<0.01$). The mean number of turns per minute was as follows: Prior to amphetamine stimulation, I observed 0.66 turns per minute $\pm$ 0.04 SEM. Following treatment with amphetamine, the rate increased to 2.65 turns per minute $\pm$ 0.53 SEM, see figure 41.

Compared to naive animals, lesioned animals showed a high basal motor-activity, constantly changing between an ipsiversive and contraversive direction (figure 42).

Amphetamine caused ipsiversive as well contraversive rotation in the rats with unilateral 6-OHDA-induced lesions of the nigro-striatal dopamine pathway. The mean total distance moved reached 21278 mm $\pm$ 964 SEM (figure 43).

In comparison, sham-operated animals showed similar behaviour to naive animals (figures 44 and 45). Typically, the animals became more active approximately 5 minutes after amphetamine injection, rotating contraversively or ipsiversively. A paired t-test showed a significant difference between basal activity and amphetamine stimulation ($P<0.001$). The mean total distance moved prior to stimulation was 2885 mm $\pm$ 107 SEM, increasing to 6643 mm $\pm$ 191 SEM following treatment with amphetamine i.p..

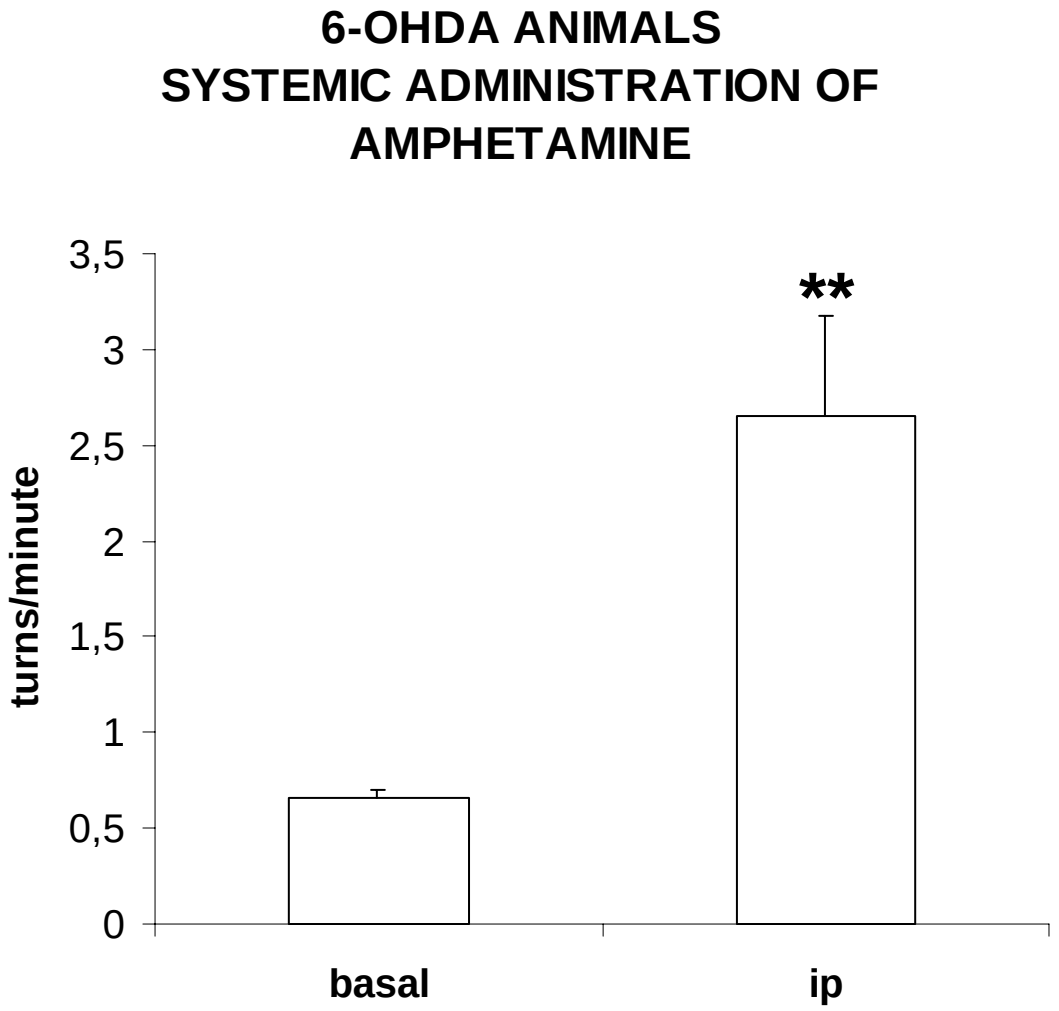


Figure 41: The number of turns per minute during systemic stimulation with amphetamine (1mg/kg) on operated animals, compared to prior i.p. stimulation. Results are expressed as means $\pm$ SEM, asterisks represent a significant difference from drug-free control group, **P<0.01, paired t-test, n=10.

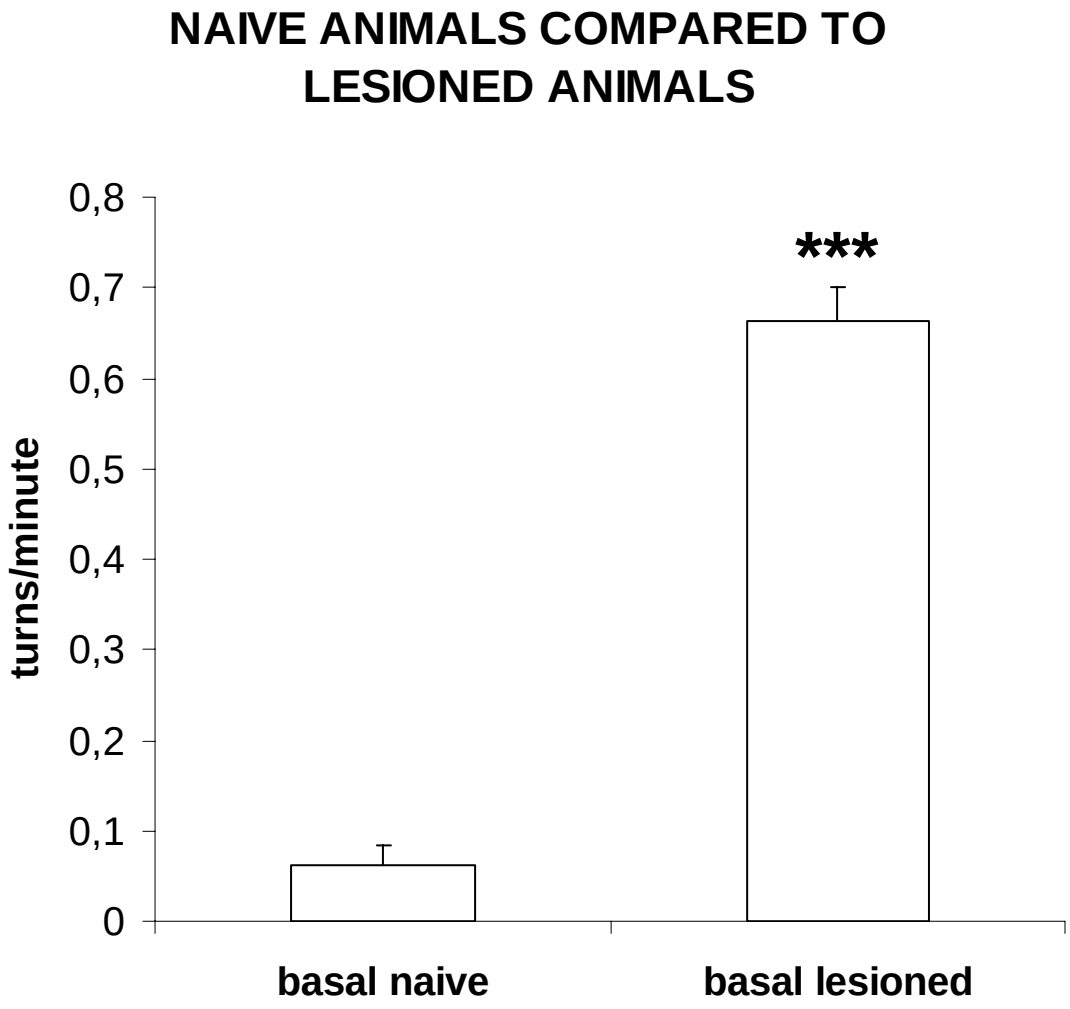
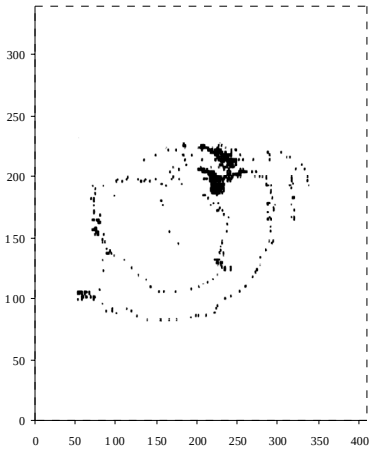


Figure 42: The number of turns per minute displayed by animals with a unilateral 6-OHDA-induced lesion of the nigro-striatal dopamine pathway, as compared to naive animals. Results are expressed as means ± SEM, asterisks represent a significant difference from naive animals, ***P<0,001, paired t-test, n=10.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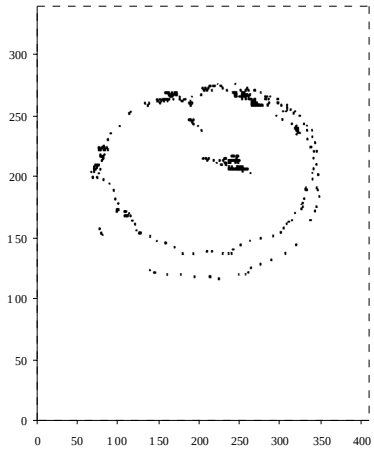
(a)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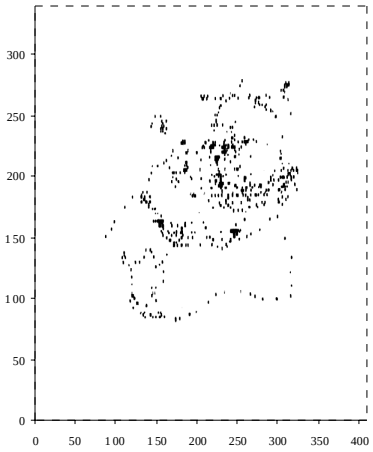
(b)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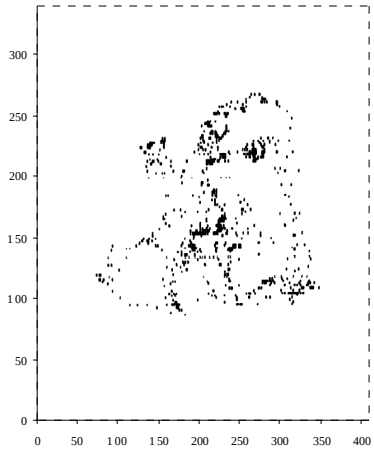
(c)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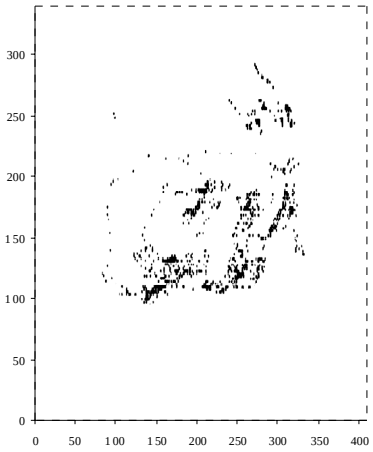
(d)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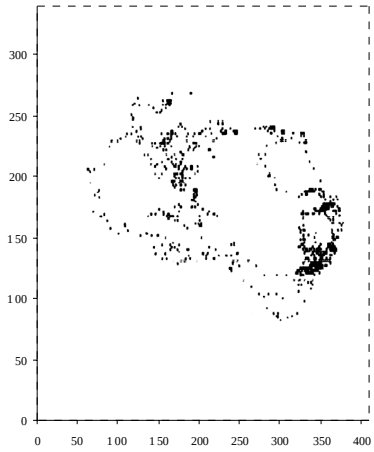
(e)


0 50 100 150 200 250 300 350 400
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(f)

Figure 43: 6-OHDA pretreated animals. Cumulative data showing motor-activity prior to amphetamine stimulation (a)-(b) and after i.p. treatment with amphetamine (c)-(f). The behaviour during the first 480 seconds evoked by amphetamine stimulation is shown. The animal movement was monitored with an Antrak video-based animal tracking system. The different computer-plotted pictures show periods of 120 seconds each. Total distance moved (in mm): (a) 8509, (b) 8134, (c) 21408, (d) 19470, (e) 20319, (f) 23913.

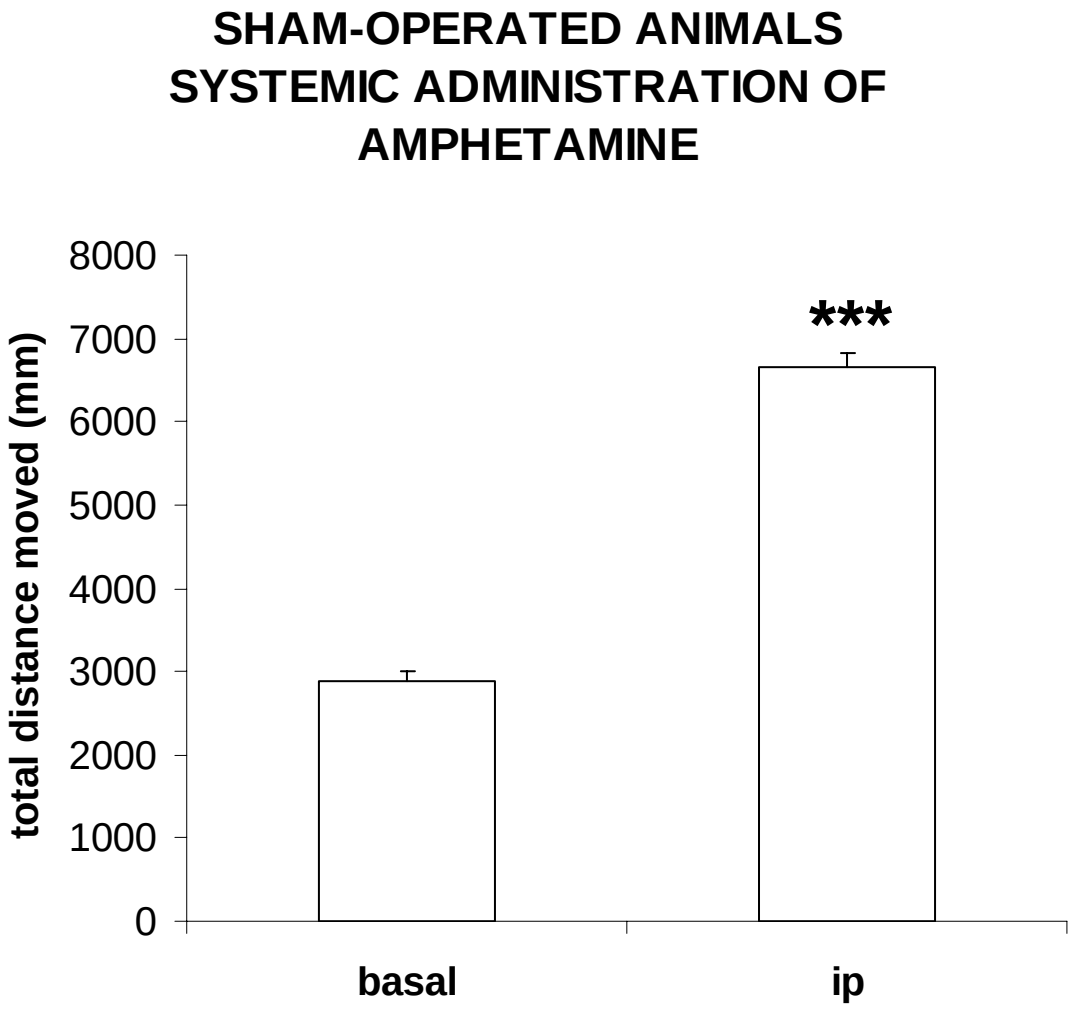
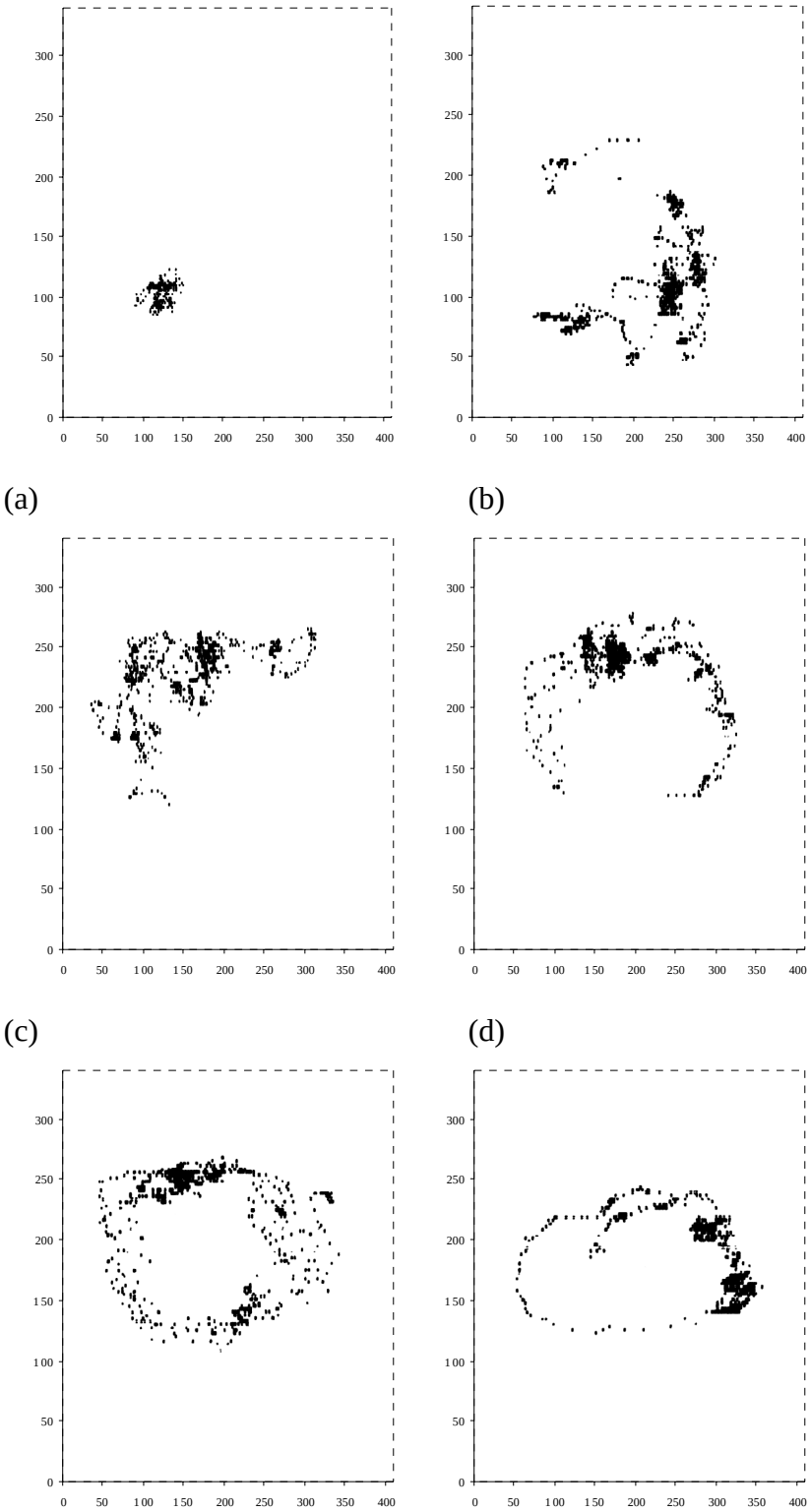


Figure 44: The total distance moved (in mm) during systemic stimulation with amphetamine (1mg/kg) of sham-operated animals, compared to their condition prior to i.p. stimulation. Results are expressed as means $\pm$ SEM, asterisks represent a significant difference from drug-free control group, ***P<0,001, paired t-test, n=10.



(a) (b)

(c) (d)

Figure 45: Sham-operated animals. Cumulative data showing motor-activity (a) prior to amphetamine stimulation and (b)-(f) after i.p. treatment with amphetamine. The behaviour during the first 600 seconds evoked by amphetamine stimulation is shown. The animal movement was monitored with an Antrak video-based animal tracking system. The different computer plotted pictures present periods of 120 seconds each. Total distance moved (in mm): (a) 1993, (b) 6454, (c) 5879,(d) 6243, (e) 6612, (f) 6186.

3.2.3.2 The systemic administration of amphetamine and its effect on the release of AChE

When the push-pull cannula was correctly implanted in the substantia nigra of the operated rat, the light signal produced is shown in figure 46.
Initially, a large signal was produced, attributable to either blood (in the early part of the perfusate) or air (when connecting the outflow tubing to the side arm of the cannula). This raised level of release gradually fell to a level that was lower than that of naive or sham-operated animals (figure 42). This signal represented a spontaneous release of AChE from the rat substantia nigra of 0,04 $\pm$ 0,008 mU (n=10).
A paired t-test showed that the application of amphetamine (1mg/kg) caused a significant rise in the spontaneous release of AChE of approximately 238% over basal conditions ($P<0,001$., see figure 47).

With the systemic administration of amphetamine, there was increase in the level of motor activity directly associated with an increase in the release of AChE. There was a correlation seen between AChE-release in the substantia nigra and behaviour measured in total distance moved, see figure 48.

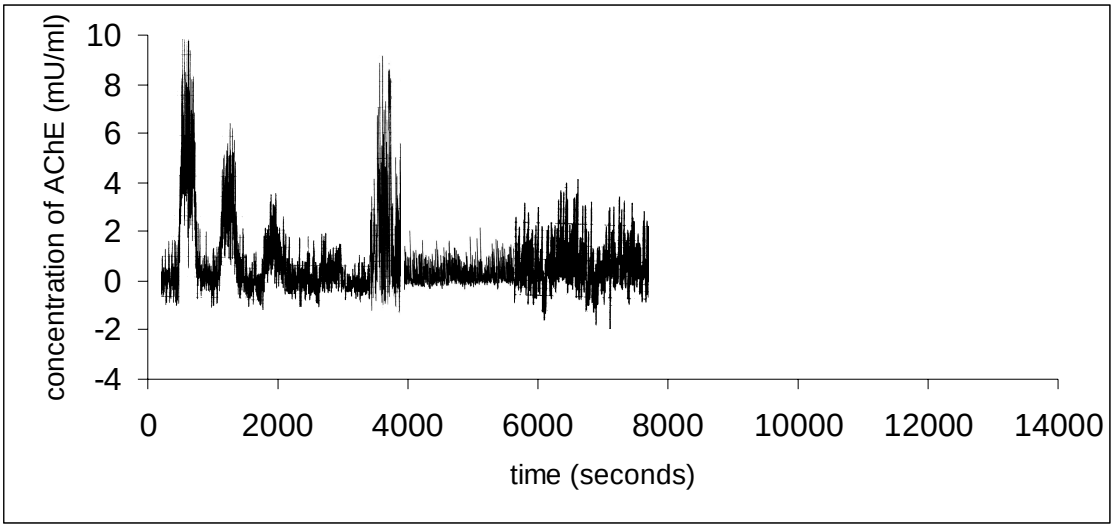


Figure 46: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) to the system prior to animal attachment. The left hand side of the graph represents the spontaneous hydrolysis of ACh in vitro (background signal). A large signal peak due to blood/air contamination was experienced when connecting the animal. On-line detection of release of AChE in vivo; AChE-release was stimulated with amphetamine i.p..

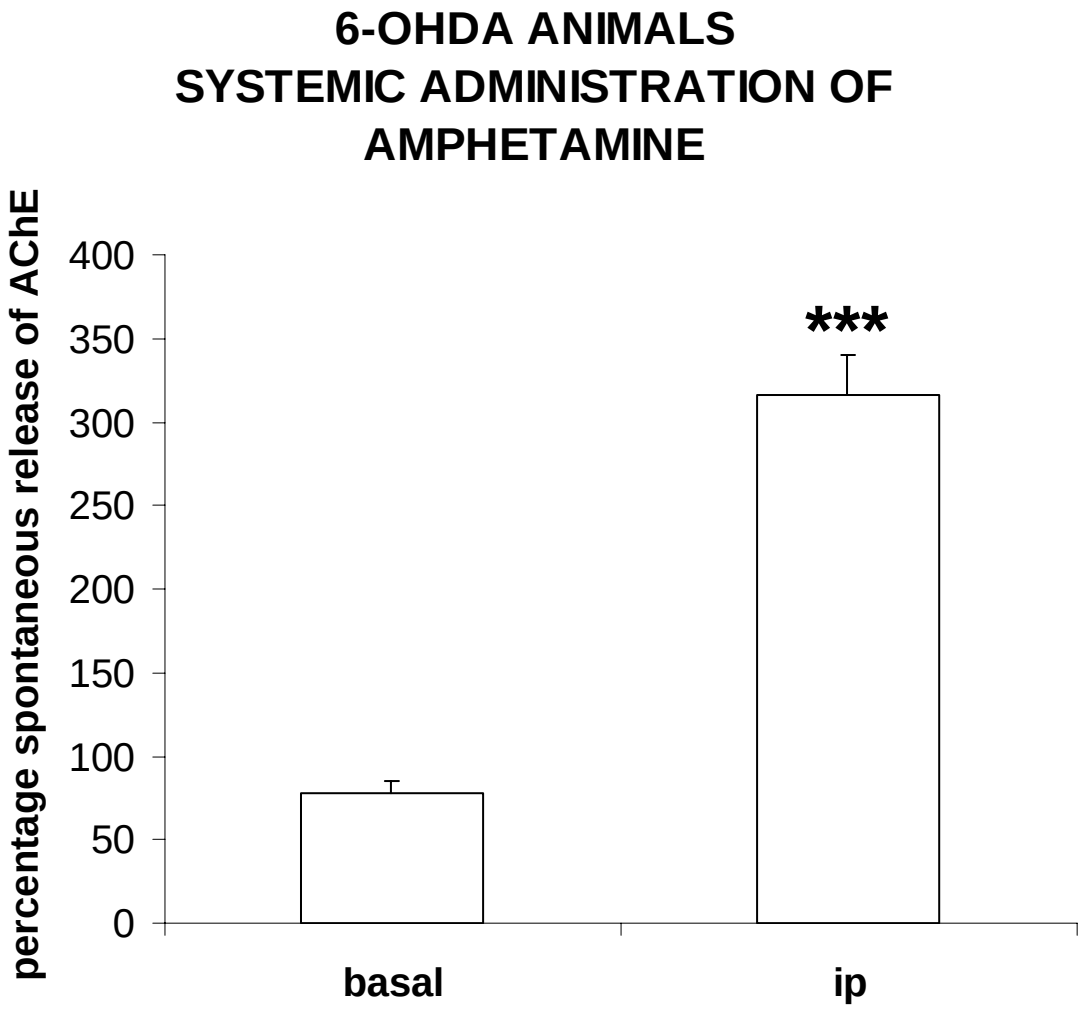


Figure 47: The spontaneous release of AChE from the substantia nigra of operated animals, shown as basal and treated with the systemic application of amphetamine. Results are expressed as means ± SEM, asterisks represent significant difference from the drug-free control group, ***P<0,001, paired t-test, n=10.

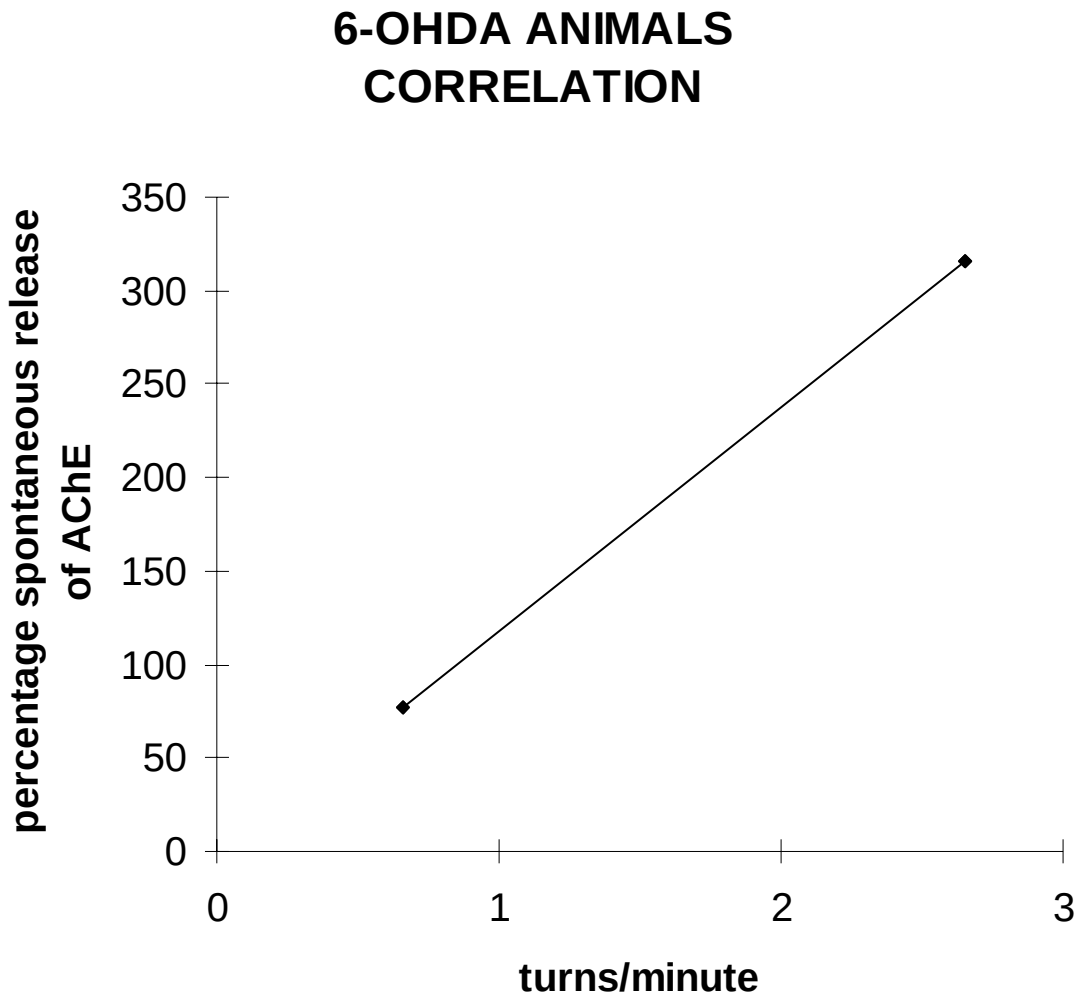


Figure 48: The correlation between AChE-release and the number of turns per minute. The basal values were 77.5% AChE with 0.66 turns per minute. Following application of amphetamine i.p. the values increased to 315.8% AChE with 2.65 turns per minute; r^2=1.

The effect of a systemic application of amphetamine on the spontaneous release of AChE in the substantia nigra of sham-operated animals showed 0,21 ± 0,04 mU (n=10) in the perfusate. Figure 49 shows the effect of application of amphetamine. The release of nigral-AChE evoked in this way increased 52% in comparison to basal levels (paired t-test, P<0,01, n=10), see figure 50.

A correlation between AChE release in the substantia nigra and behaviour was even observed in sham-operated animals, see figure 51.

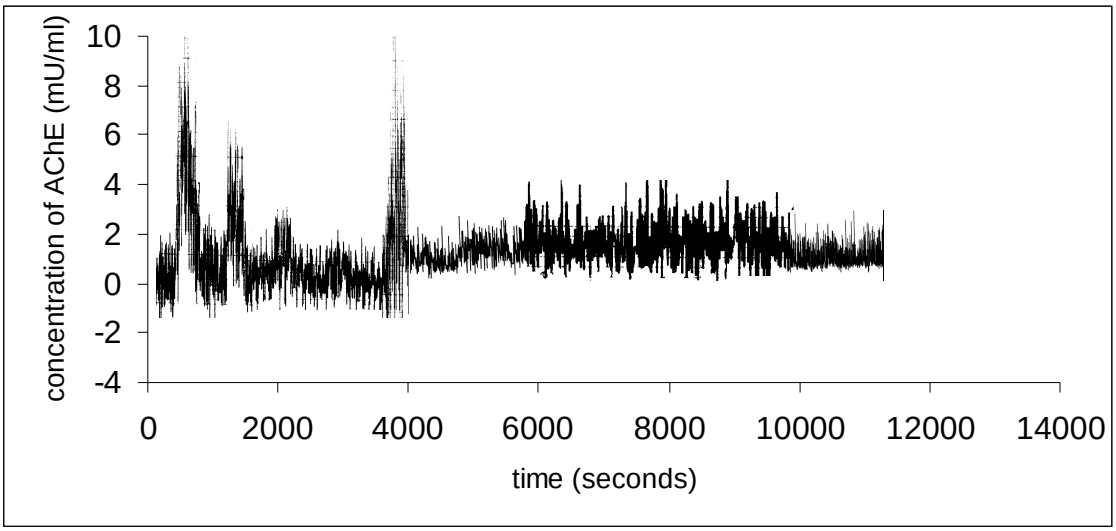


Figure 49: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) to the system prior to animal attachment. The left hand side of the graph represents the spontaneous hydrolysis of ACh in vitro (background signal). A large signal peak due to blood/air contamination was experienced when connecting the animal. On-line detection of release of AChE in vivo; AChE-release was stimulated with amphetamine i.p..

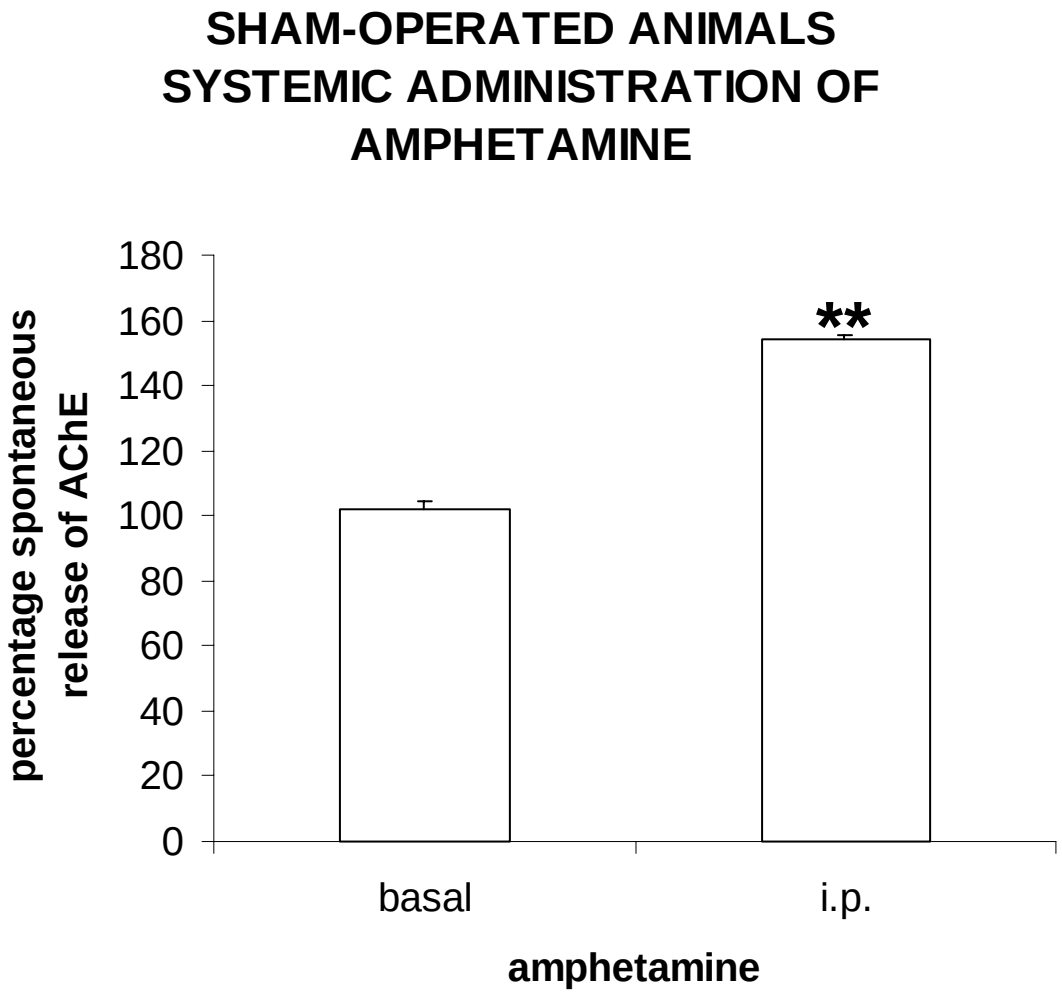


Figure 50: The spontaneous release of AChE from substantia nigra of sham-operated animals shown both as basal and in animals treated with systemic application of amphetamine. Results are expressed as means $\pm$ SEM, asterisks represent significant difference from the drug-free control group, **P<0,01, paired t-test, n=10.

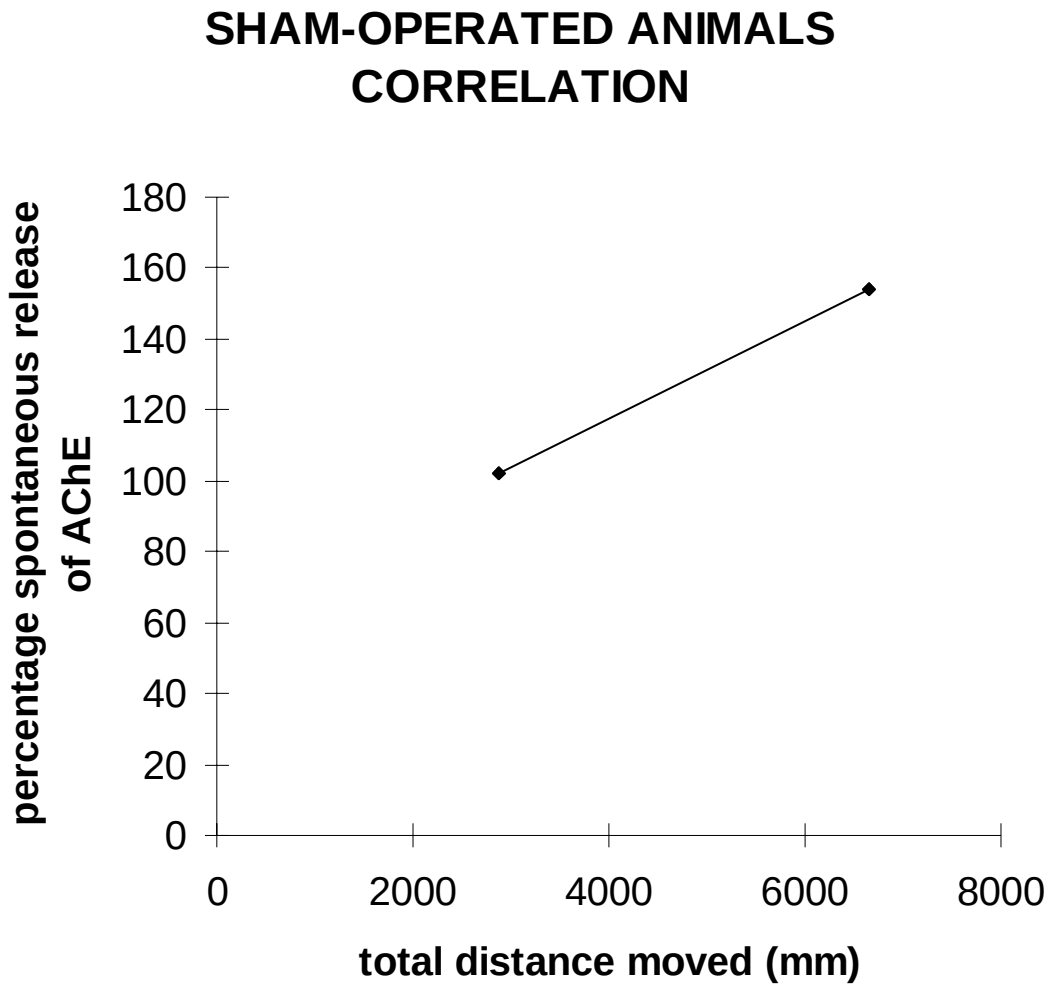


Figure 51: The correlation between AChE-release and the total distance moved (mm). The basal values observed were 102% AChE with total movement of 2885 mm. Following the application of amphetamine i.p., the level of AChE-release increased to 154% and the distance travelled to 6643 mm.

3.2.3.3 The dopamine content of tissue samples

Punches of tissue were removed from both striata. DOPAC (data not present) and dopamine were quantified by high performance liquid chromatography (HPLC: C_{18} reverse phase column).

Figure 52 shows the effect of the neurotoxic pre-treatment on the dopamine content of the striata versus the untreated side of the brain. The dopamine content of the treated striata is expressed relative to the respective non-treated striata (taken as approximately 100% $\pm$ SEM). Dopamine levels were significantly reduced by 76% following pre-treatment with 6-OHDA. A paired t-test revealed that in operated animals there was a significant decrease in dopamine content of the striata receiving the injection, compared to the untreated striata ($P<0,01$), see figure 52.

In addition, a paired t-test carried out on striatum samples taken from control animals (sham-operated) showed that there was no significant difference in dopamine content between the treated (sterile saline) and non-treated side, see figure 52.

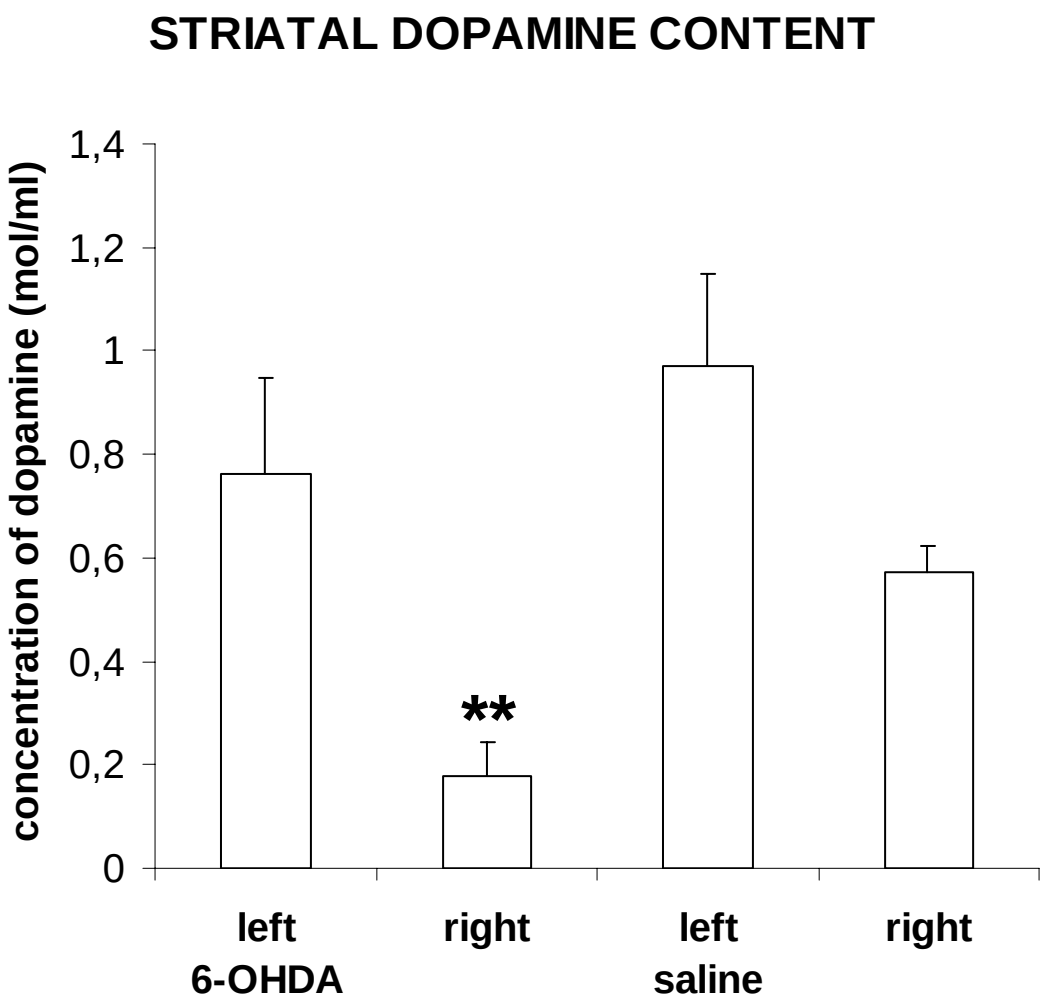


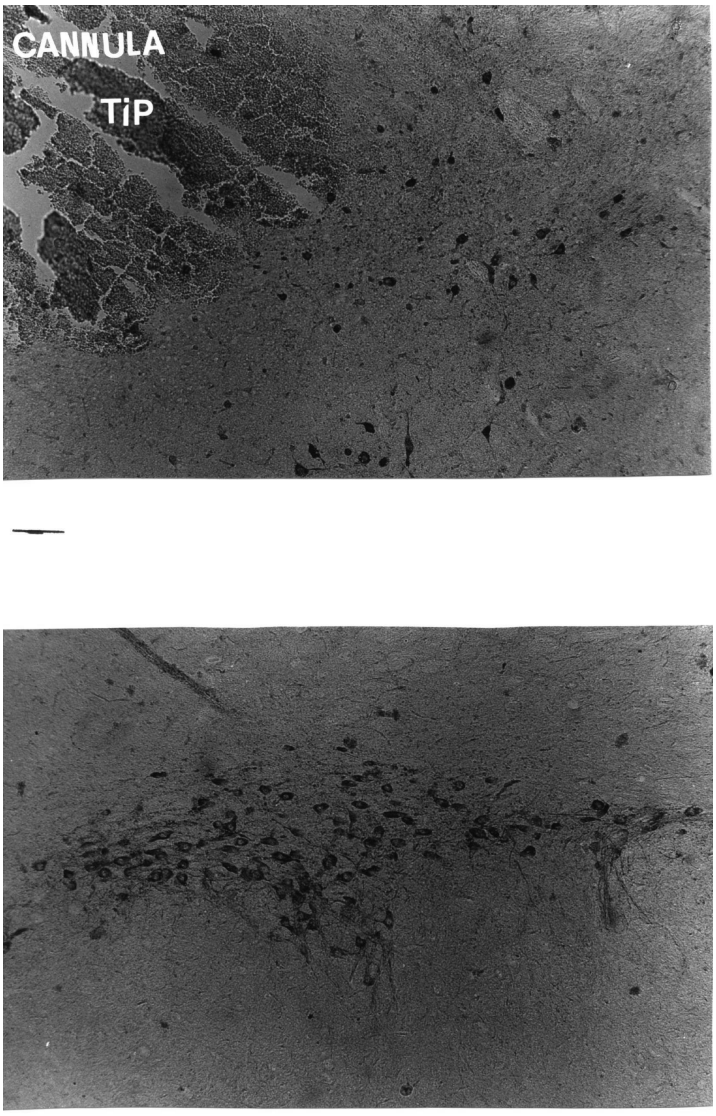
Figure 52: The dopamine content of striatal tissue samples from the non-treated side (left) and the treated side (right) of rats treated with 6-OHDA (3-week after operation), sterile saline and their respective drug-free controls groups. Results are expressed as mol/ml (means $\pm$ SEM); n=10 for each group. Asterisks represent a significant difference from the treated side; **P<0,01, paired t-test.

3.2.3.4 The location of TH immunreactivity in the substantia nigra

Dopaminergic neurons of the substantia nigra are well known to be pivotal in normal and pathological motor-control.

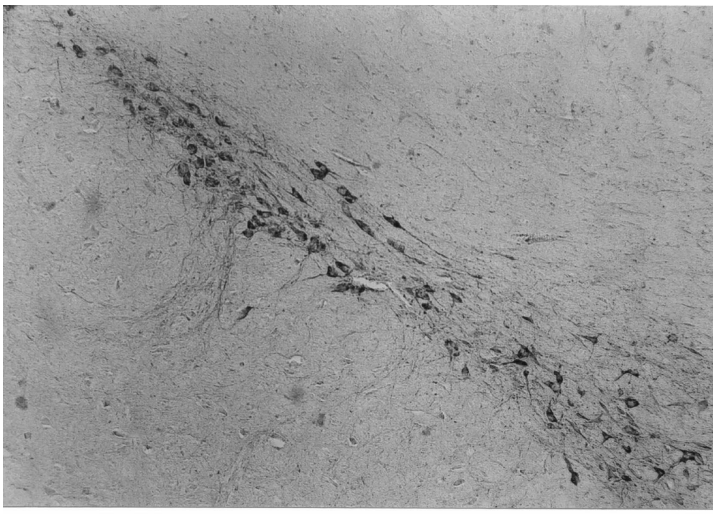
Anatomical analysis of post-mortem tissue revealed that the injection of 6-OHDA into the medial forebrain bundle resulted in substantial reduction of TH-immunoreactive neurons in the substantia nigra pars compacta and substantia nigra pars reticulata. This relative sparing of dopaminergic cells in the substantia nigra pars compacta reflects the pattern of cell loss in brains of patients with Parkinson's disease (German et al. 1989, Goto et al. 1989). Typically, animals showed 10% or fewer TH-immunreactive neurons in the treated SNpc than in the intact side of the brain (figure 53).

There was no obvious or marked difference in TH-staining when samples from both the treated and untreated brain-hemispheres of sham-operated animals were compared (figure 54).



(B)

Figure 53: TH+ neurons in a parasaggital section of the mesencephalon of the lesioned rat. Photograph (A) shows the lack of cell bodies in the substantia nigra following a partial 3-week treatment with 6-OHDA. Photograph (B) shows TH staining observed in a brain slice, from untreated side under magnification. (Magnification x 100, bar indicates 100 µm).

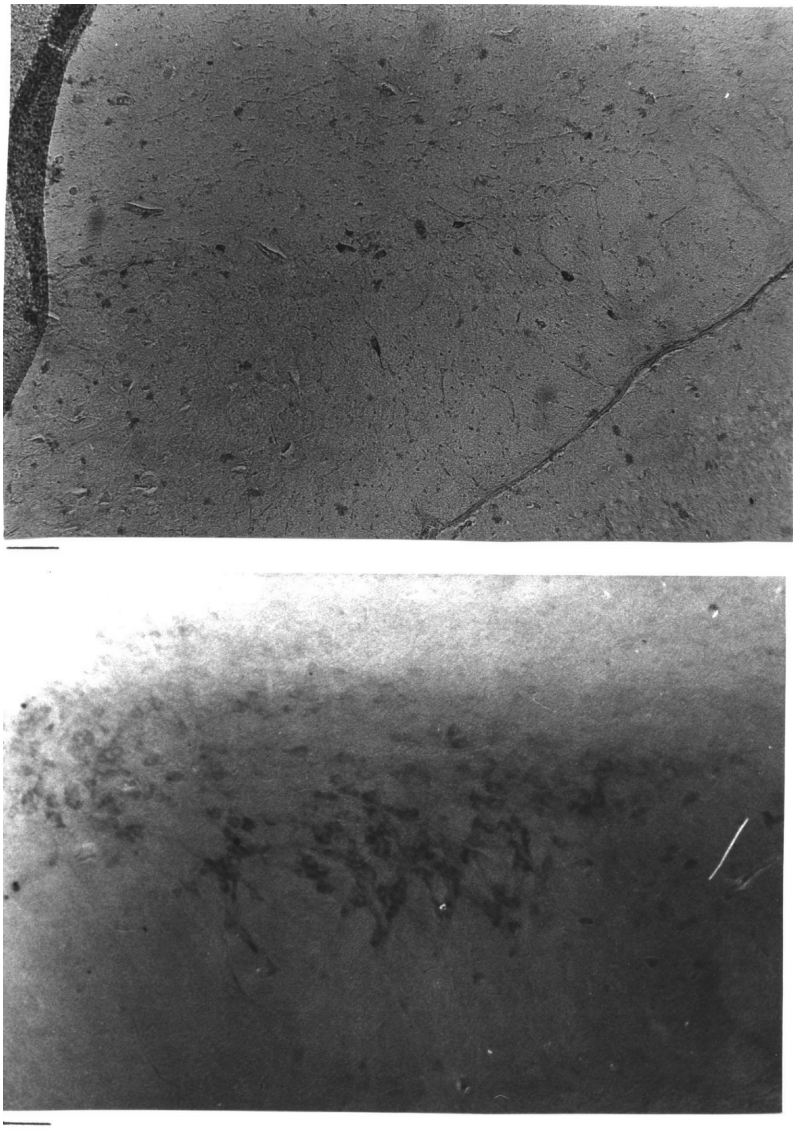


(B)

Figure 54: The anatomical features of (A) treated (sterile saline) substantia nigra and (B) untreated substantia nigra in sham-operated animals. The pictures show the immunhistochemical staining of TH+ cells; (magnification x 100, bar indicates 100 µm).

3.2.3.5 The location of AChE immunreactivity in the substantia nigra

Various studies suggest a relationship between AChE and dopamine in the substantia nigra.
Figure 55 shows the rat substantia nigra pars compacta stained for AChE in 6-OHDA pre-treated animals, following immunhistochemical staining with 3D10. These rat sections clearly show cell bodies staining for AChE.
We could see that an injection of 6-OHDA into the medial forebrain bundle resulted in a reduction of AChE-immunreactive neurons in the SNpars compacta. But there was no obvious or marked difference in AChE staining when samples from both treated (sterile saline) and untreated side of sham-operated animals were compared (data not shown).



(B)

Figure 55: Photograph of AChE immunhistochemical staining of a treated animal. (A) clearly shows the lack of cell bodies in the substantia nigra following a partial 3-week treatment with 6-OHDA. (B) shows AChE staining in a slice of the untreated side of the brain under magnification. (Magnification x 100, scale bar = 100 µm).

CHAPTER 3

RESULTS

3.3 THE RELEASE OF ACETYLCHOLINESTERASE IN RELATION TO OTHER DRUG STIMULATIONS

3.3 The release of acetylcholinesterase in relation to other drug stimulations

The objective of this study was to use drugs other than amphetamine to determine whether they had an influence on the intranigral AChE and on behaviour. The following compounds were used: apomorphine, quinpirole, NMDA.
In addition, an histological examination of all groups analysed indicated that cannulae placements were distributed evenly throughout the substantia nigra.

3.3.1 Apomorphine applied to the on-line system without an animal connected

It was found that apomorphine had a similar quenching effect to amphetamine (10^{-3} M to 10^{-2} M), 5-HT (10^{-6} M) and α-methyl 5-HT (10^{-6} M) on this system, when added locally (figure 56). Therefore, it was not possible to stimulate the animal locally using apomorphine.

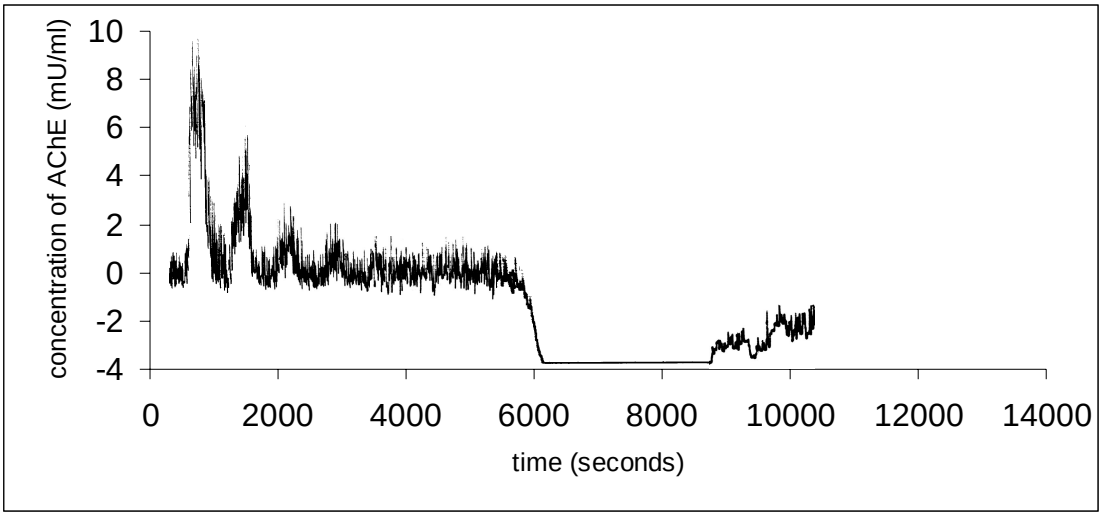


Figure 56: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) in mU/ml to the system prior to apomorphine testing (10^{-4} M). When apomorphine was added to the system, a loss of the chemiluminescent signal was observed.

3.3.2 The effect of the systemic administration of apomorphine

An additional set of experiments was carried out on naive animals and toxicated animals using apomorphine (1mg/kg in 0.2 mg/kg ascorbic acid). It was not possible to measure the nigral release of AChE in naive animals due to technical difficulties.

3.3.2.1 The systemic administration of apomorphine to naive animals

Apomorphine was injected intraperitoneally and the naive animals tested immediately for enhanced motor activity. A paired t-test showed a significant difference between the level of activity before and after i.p. stimulation with apomorphine ($P<0.05$). The mean total distance moved prior to treatment was 2490 mm $\pm$ 100 SEM. This increased to 3687 mm $\pm$ 114 SEM following treatment with apomorphine (figure 57).

The systemic administration of apomorphine resulted in a characteristic behaviour pattern. The animals often preferred to spread themselves out on the bedding, moving the head slowly, rotating firstly in a contraversive then in an ipsiversive direction, ending with permanent ipsiversive rotating behaviour which lasted for about 20 minutes (figure 58).

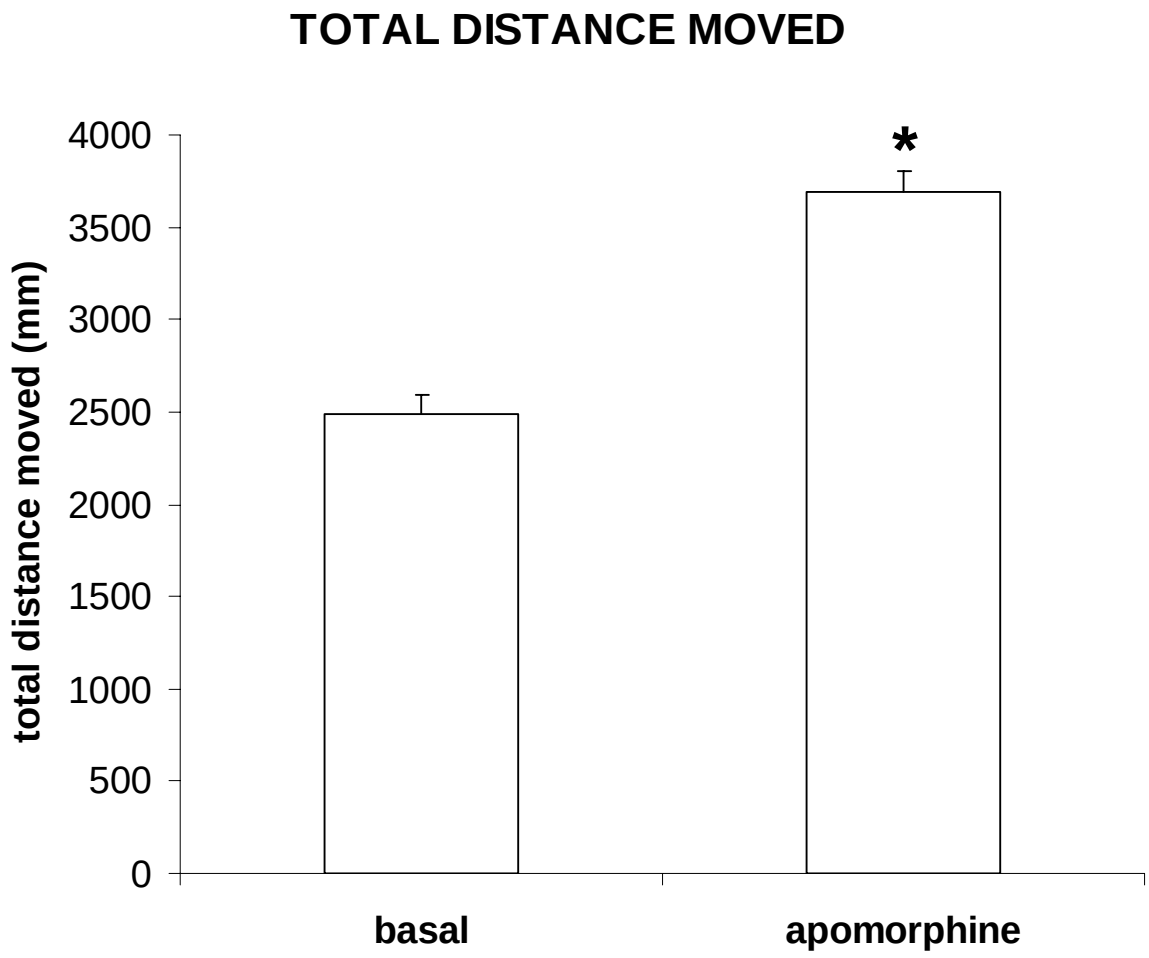
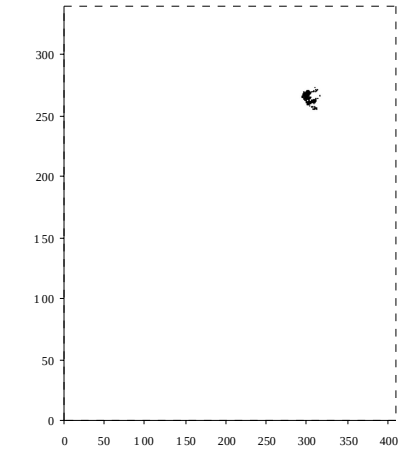


Figure 57: The total distance moved (mm) during systemic stimulation with apomorphine (1mg/kg), compared to the level of movement observed prior to stimulation. Results are expressed as means $\pm$ SEM, with asterisks representing a significant difference between the test animals and the drug-free control group, *$P<0.05$, paired t-test, n=3.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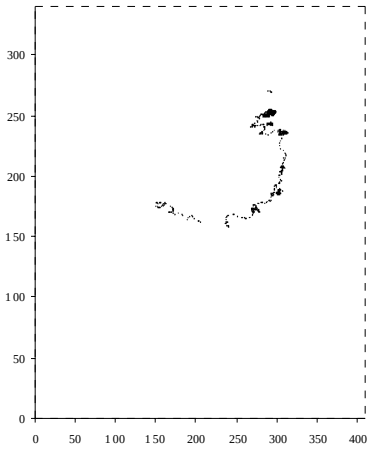
0 50 100 150 200 250 300

(a)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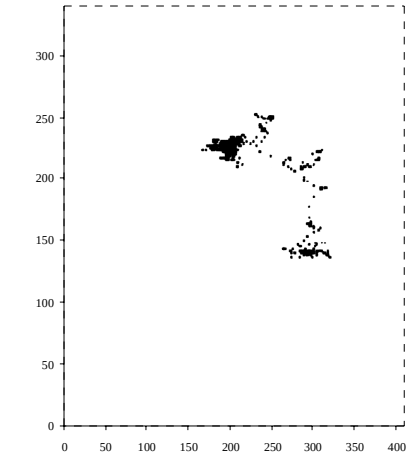
0 50 100 150 200 250 300

(b)


0 50 100 150 200 250 300 350 400
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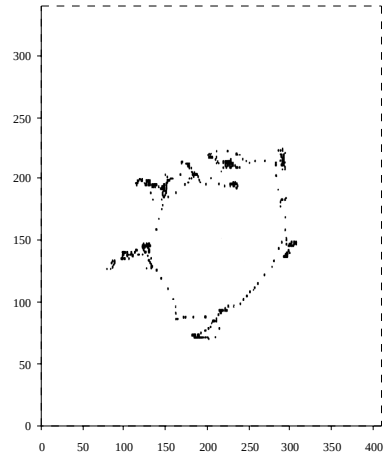
(c)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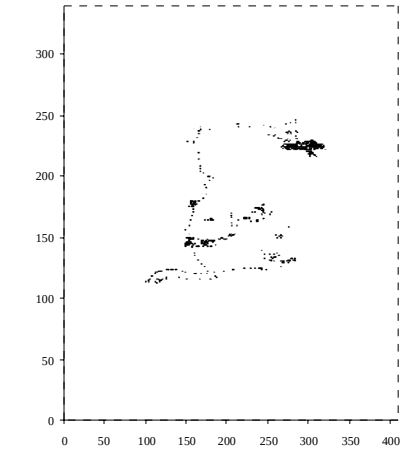
(d)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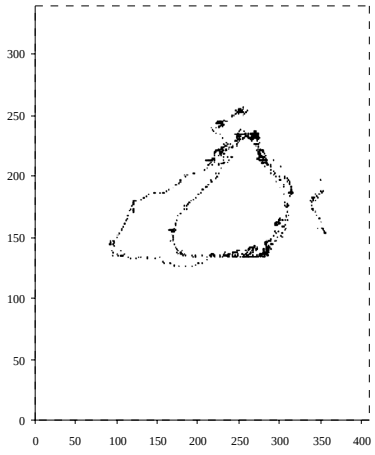
(e)

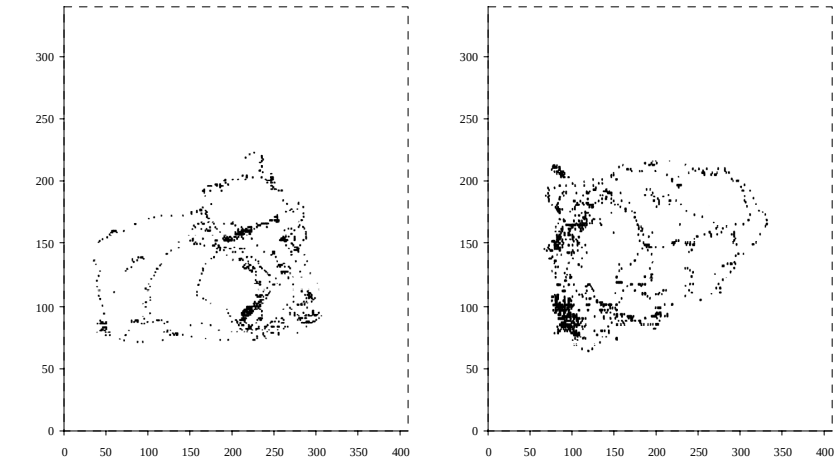

0 50 100 150 200 250 300 350 400
0 50 100 150 200 250 300

(f)

Figure 58: Cumulative data showing motor-activity prior to stimulation (a) and after i.p. treatment with apomorphine (b)-(f). The behaviour observed during the first 600 seconds following apomorphine stimulation is shown. The animal movement induced by systemic stimulation with apomorphine was monitored with an Antrak video-based animal tracking system. The different computer plotted pictures show periods of 120 seconds each. Total distance moved (in mm): (a) 1559, (b) 3177, (c) 3725, (d) 3081, (e) 3650, (f) 3542.

3.3.2.2 The systemic administration of apomorphine to 6-OHDA treated animals

Apomorphine was injected intraperitoneally and the treated rats were immediately observed for changes in motor activity using the computer system to monitor total distance moved. Furthermore, the number of 360^{o} degree turns completed by the animals were counted.
In comparison to naive animals, the treated animals showed a prominent basal circling in a contraversive as well as ipsiversive direction. Soon after apomorphine stimulation i.p., the circling behaviour ceased almost completely - see figure 59.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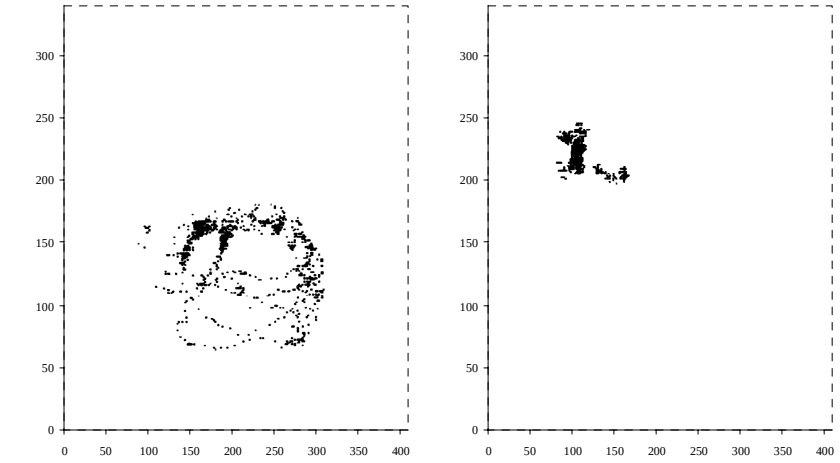
(a) (b)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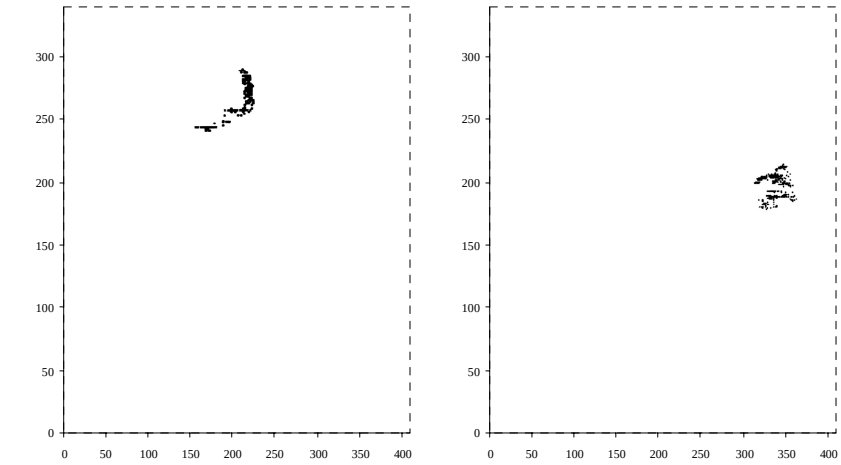
(c) (d)


0 50 100 150 200 250 300
0 50 100 150 200 250 300 350 400

(e) (f)

Figure 59: Cumulative data showing motor activity prior to stimulation (a)-(c) and after i.p. treatment with apomorphine (d)-(f). Animal movement was monitored with an Antrak video-based animal tracking system. The different computer-plotted pictures show periods of 120 seconds each. Total distance moved (mm): (a) 6824, (b)8965, (c) 6569, (d) 3248, (e) 1706, (f) 3182.

A paired t-test showed a significant difference between the basal circling and the circling caused by apomorphine ($P<0.05$). The mean number of turns per minute was as follows: The basal rate of circling was 0.40 turns per minute $\pm$ 0.04 SEM. This decreased to 0.07 turns per minute $\pm$ 0.07 SEM following treatment with apomorphine (figure 60).

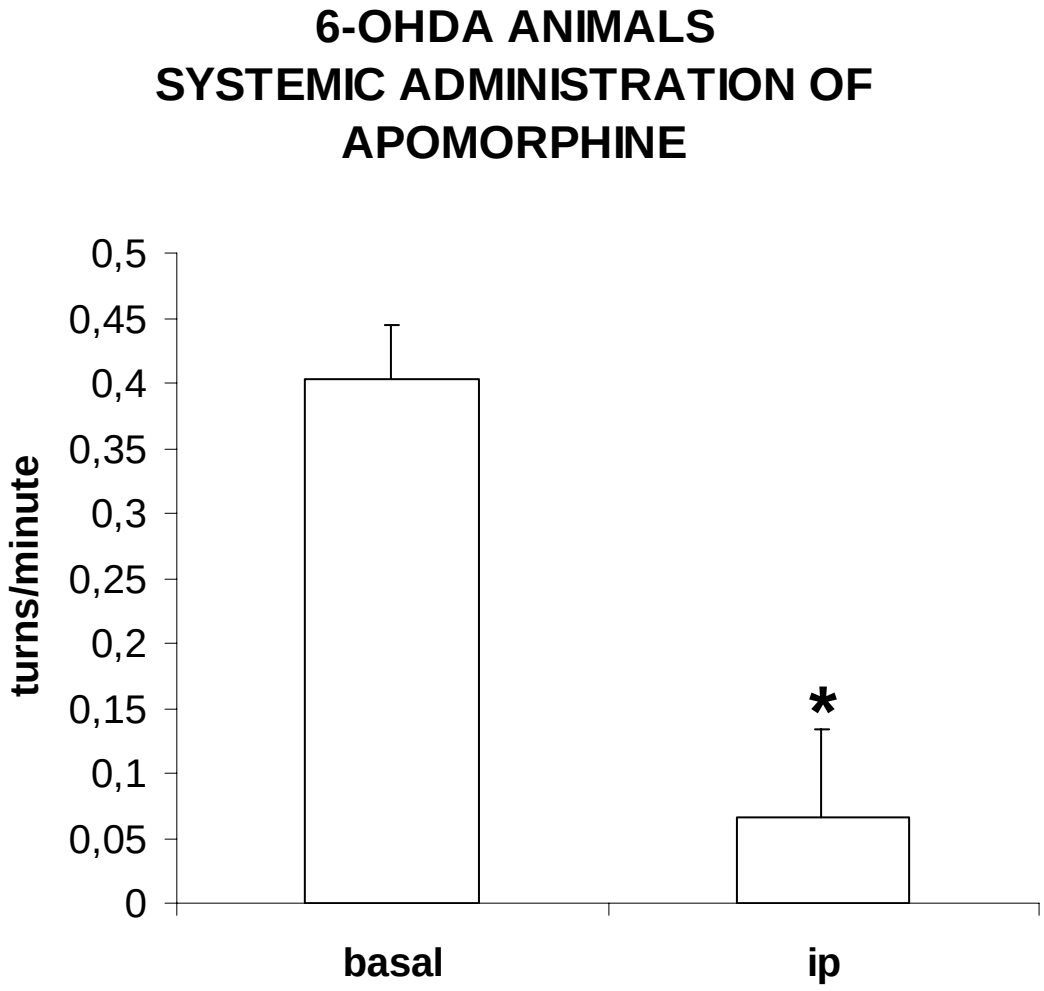


Figure 60: The number of turns per minute during systemic stimulation with apomorphine (1mg/kg) compared to the basal behaviour of toxicated animals. Results are expressed as means $\pm$ SEM, with asterisks representing a significant difference between the test animals and the drug-free group, *$P<0.05$, paired t-test, n=3.

AChE-release was permanently monitored (figure 61) to observe specific movements brought about by apomorphine stimulation. A paired t-test showed that neurotoxic pre-treatment significantly reduced the spontaneous release of AChE from the rat substantia nigra (P<0,01, n=22), see 3.2.2.2 fig. 34.

Pre-treatment with 6-OHDA led to a significant reduction of approximately 68% in the spontaneous release of AChE in free moving animals (P<0.01, t-test).

In contrast, application of apomorphine i.p. effected a rise in the spontaneous release of AChE of approximately 43% above basal conditions (P<0.01, paired t-test, see figure 62).

An increase of nigral AChE was seen in conjunction with a decrease of motor activity. The application of apomorphine resulted in a return to normal behaviour. The level of intranigral AChE increased to a level exceeding that normally observed (figure 63).

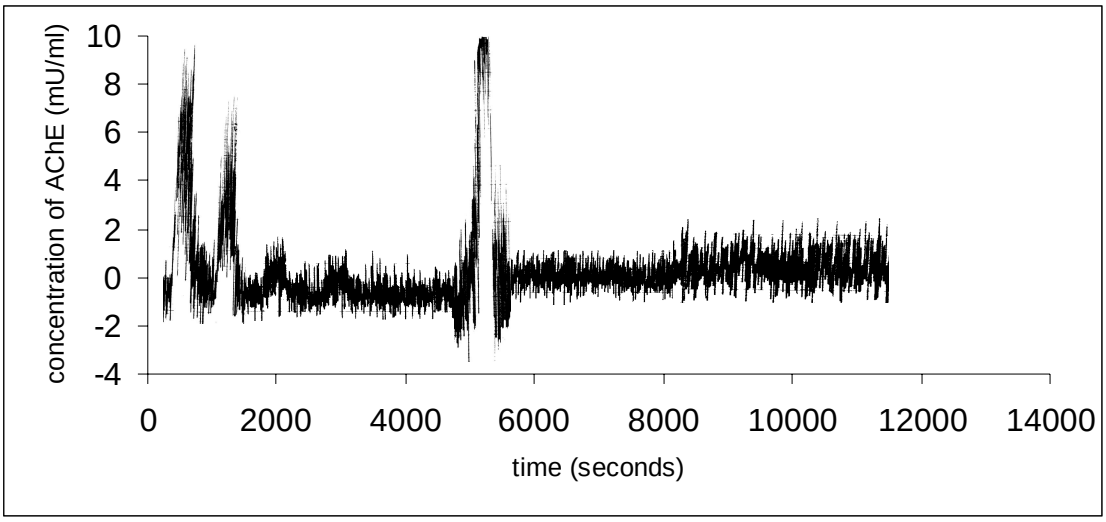


Figure 61: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) to the system prior to animal attachment. The left hand side of the graph represents the spontaneous hydrolysis of ACh in vitro (background signal). A large signal peak due to blood/air contamination was experienced when connecting the toxicated animal. This is followed by the on-line detection of AchE-release in vivo; AChE-release was stimulated with apomorphine i.p..

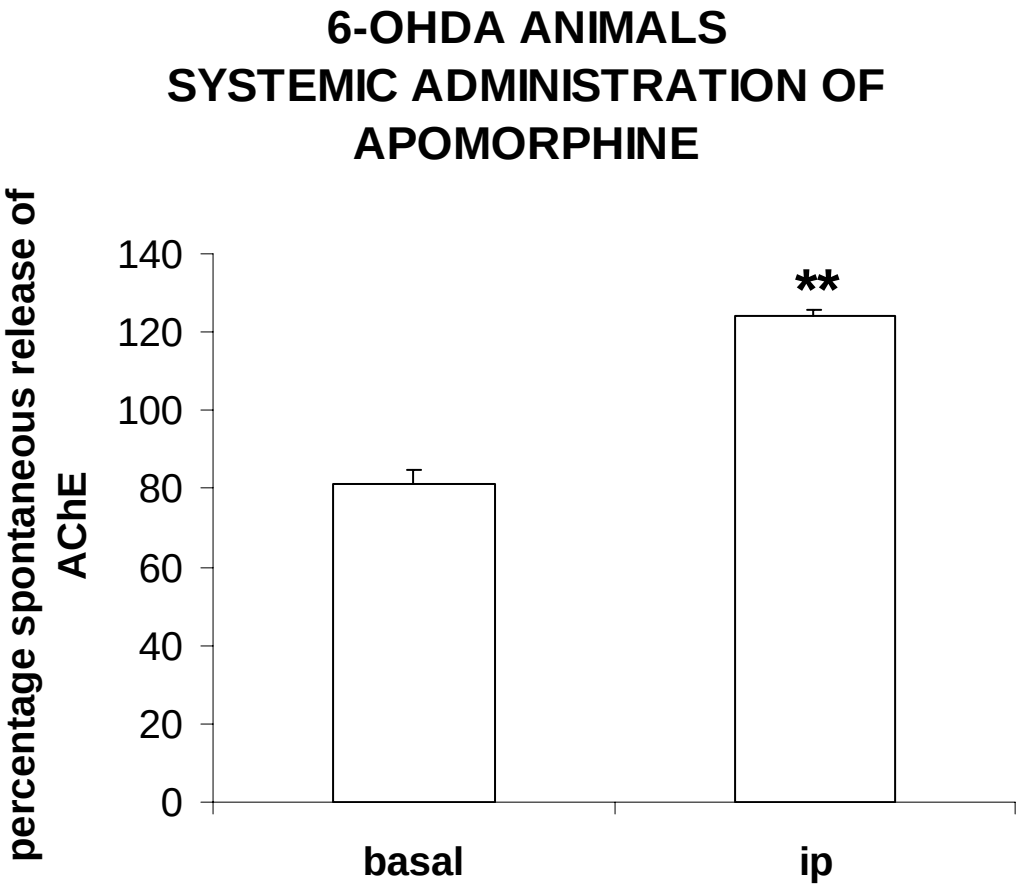


Figure 62: The spontaneous release of AChE in the substantia nigra after 3-weeks treatment in animals. The graph shows the basal values and those achieved following the systemic injection of apomorphine. Results are expressed as means $\pm$ SEM, with asterisks representing a significant difference between the test animals and the drug-free control group, **P<0.01, paired t-test, n=3.

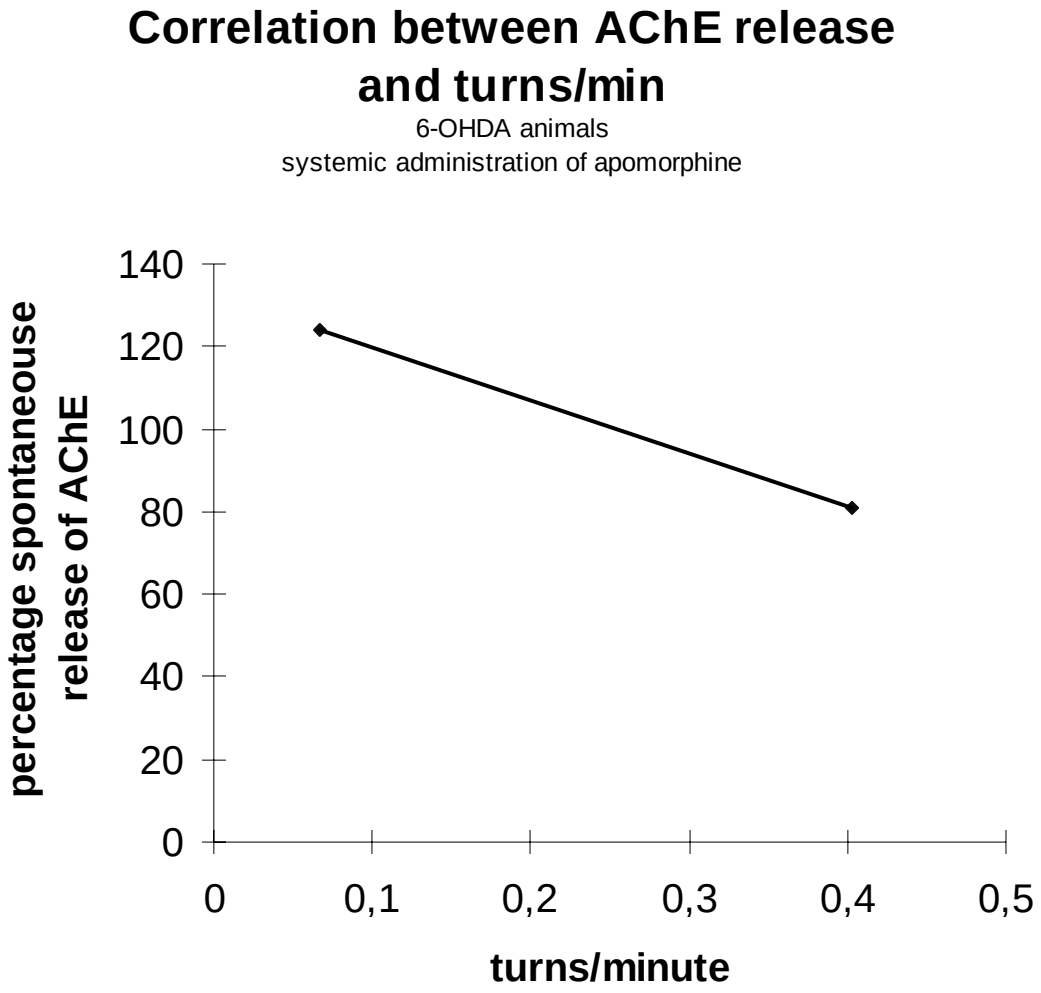


Figure 63: The correlation between AChE-release (%) and turns per minute. The basal values correspond to 81% AChE and 0.40 turns per minute. Following application of apomorphine i.p., values of 124% AChE and 0.07 turns per minute were observed.

For the dopamine content of tissue samples, see 3.2.3.3. For the location of TH immunreactivity, see 3.2.3.4.

3.3.3 The effect of quinpirole administration on naive animals

It was not possible to measure the nigral release of AChE in the substantia nigra due to technical difficulties.

Quinpirole (10^{-2} M in ACSF) was intranigrally infused into naive animals over a five-minute period. The circling behaviour was measured at the point when quinpirole arrived in the substantia nigra for 15 minutes.

Prior to infusion, the basal level of ipsiversive circling exhibited by the animals (n=3) was very modest. A paired t-test revealed no significant difference (P=0.422) between spontaneously circling behaviour, 0.13 $\pm$ 0.07 SEM turns/min and 10^{-2} M, 0.2 $\pm$ 0 SEM turns/min (figure 64).

No noticeable changes in animal behaviour or movement were observed following lower concentrations of quinpirole. Local infusion of 10^{-2} M quinpirole into one substantia nigra lead to a characteristic behaviour pattern (figure 65). The animals started contraversive low intensity circling, exhibiting a slight shaking of the head. They either fell onto their backs or came to rest on their side.

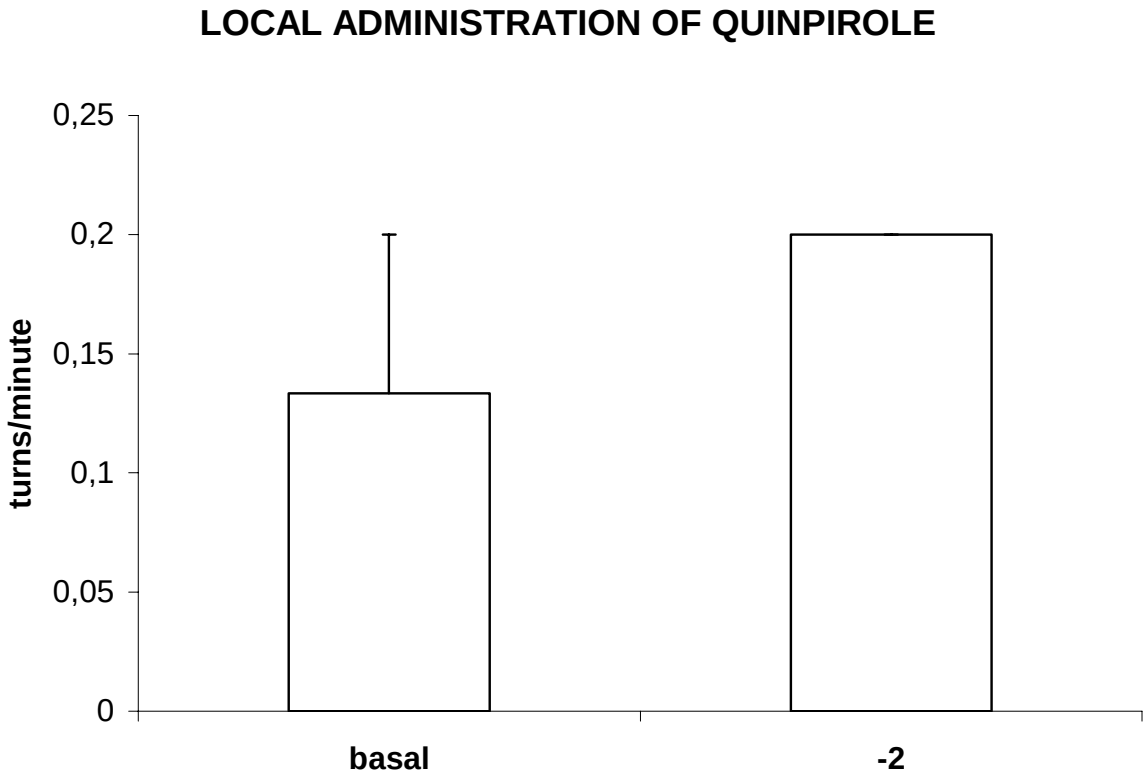
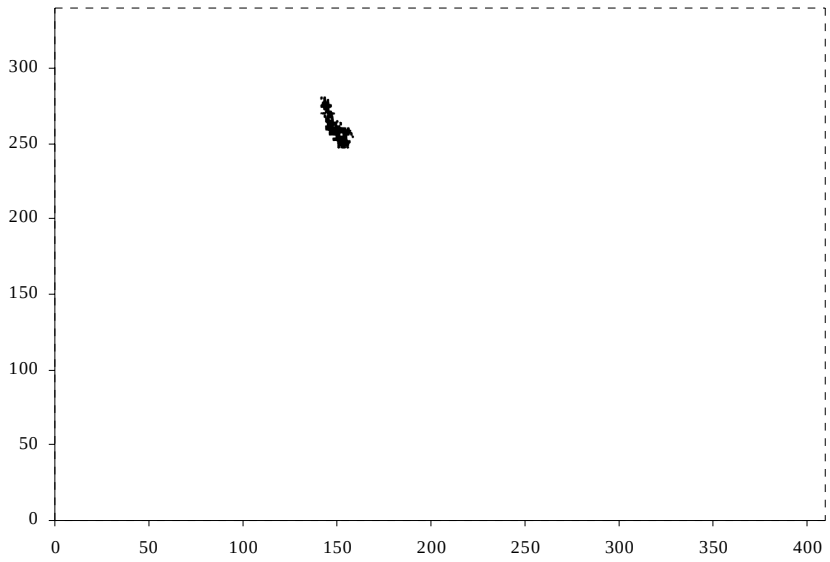
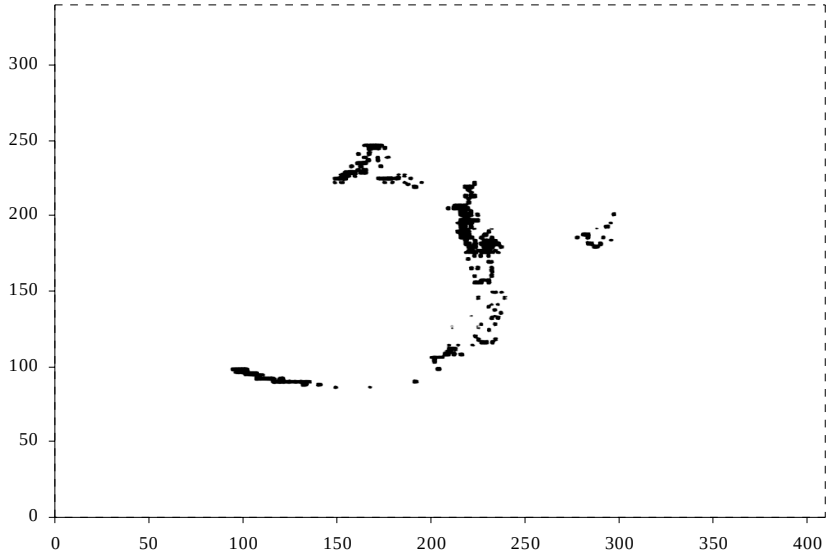


Figure 64 shows the number of turns per minute exhibited following infusion with ACSF, and during the 15 minutes following the infusion of 10^{-2} M quipirole into the substantia nigra via the push-pull cannula. Results are expressed as means $\pm$ SEM; no significant difference was seen between spontaneous circling behaviour and stimulated circling behaviour. P=0.422, paired t-test, n=3.



(A)



(B)

Figure 65 (A): Animal movement prior to infusion with quinpirole, monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 1639 mm, time elapsed: 120 seconds. Figure 65 (B): Animal movement induced by local stimulation with quinpirole, monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 2855 mm, time elapsed: 120 seconds, quinpirole dosage: 10^{-2} M.

3.3.4 The effect of NMDA administration on naive animals

Before the experiments were performed, the possibility that the introduction of NMDA into the chemiluminescent assay could change the light-emitted signal was investigated.

The light emitted signal was the same when 10^{-4} M or 10^{-2} M NMDA was subjected to the chemiluminescent reaction. Therefore, it was possible to study the effect of perfusion into the substantia nigra of NMDA on release of AChE without this substance having a direct effect on the light-emitted signal.

A stock solution of NMDA (10^{-2} M) was prepared in ACSF then diluted with ACSF (10^{-4} M). NMDA was introduced for five-minute periods to the substantia nigra via the push-pull cannula and a recovery period at least 15 minutes was allowed between consecutive applications. Both the behaviour of the animals and the release of AChE were monitored during NMDA treatment. Circling behaviour was measured in turns per minute.

There was a expressive change in the behaviour of the three animals when 10^{-2} M NMDA was perfused through the substantia nigra. Lower concentrations of NMDA showed no apparent effect. A paired t-test revealed a significant difference in the animals before and after infusion 0.22 $\pm$ 0.02 SEM turns/min and 10^{-2} M 5.62 $\pm$ 1.06 SEM turns/min ($P<0.05$), see figure 66. There was no significant difference at 10^{-4} M, 0.65 $\pm$ 0.193 SEM turns/min ($P=0.13$). Before infusion, the animals exhibited modest basal ipsiversive or contraversive circling, with the preference varying from animal to animal. Local infusion of one substantia nigra with NMDA resulted in excessive circling behaviour (figure 67). The first indications that NMDA had reached the substantia nigra were as follows: The animals sat up on their hind legs, fell onto one side and began to burrow through the bedding. Following this, they began circling rapidly. In most cases, the animals exhibited ipsiversive circling behaviour at high speed until the point of exhaustion.

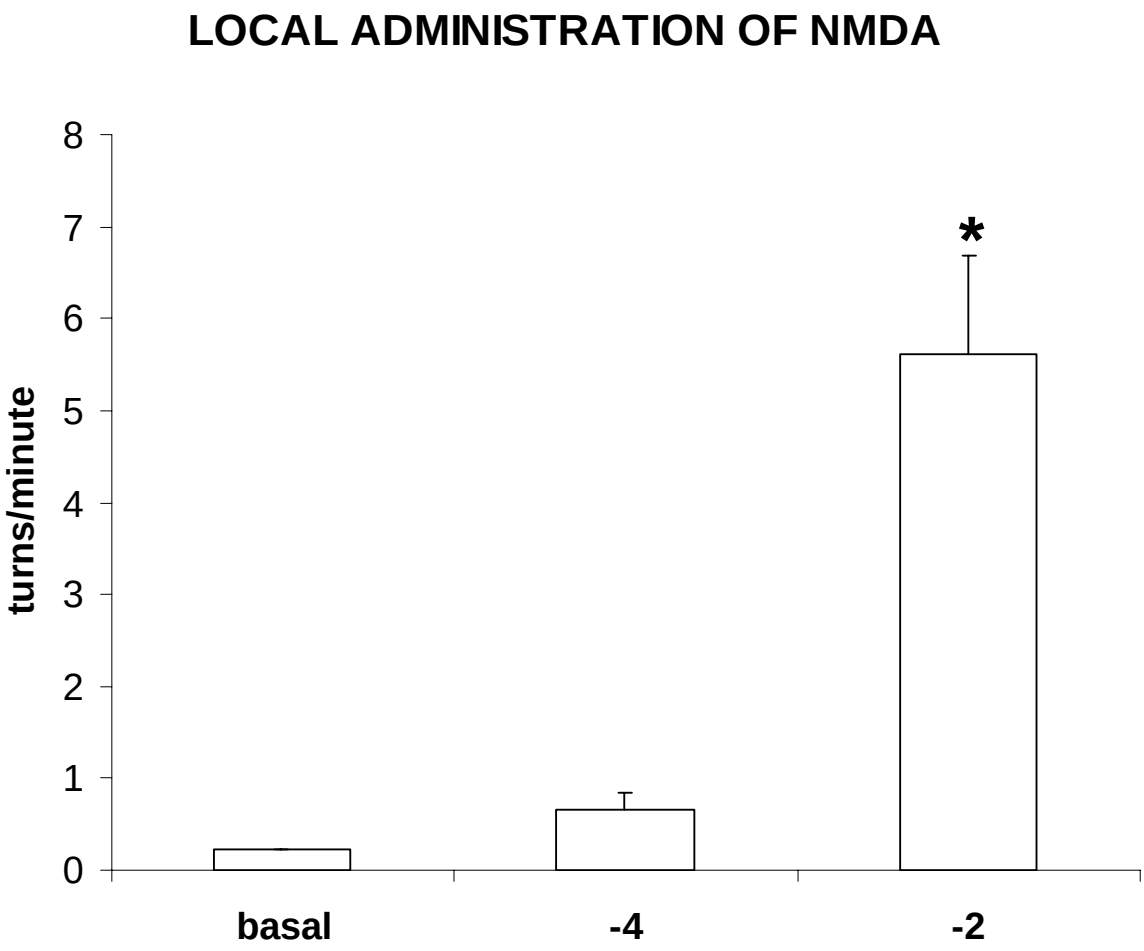
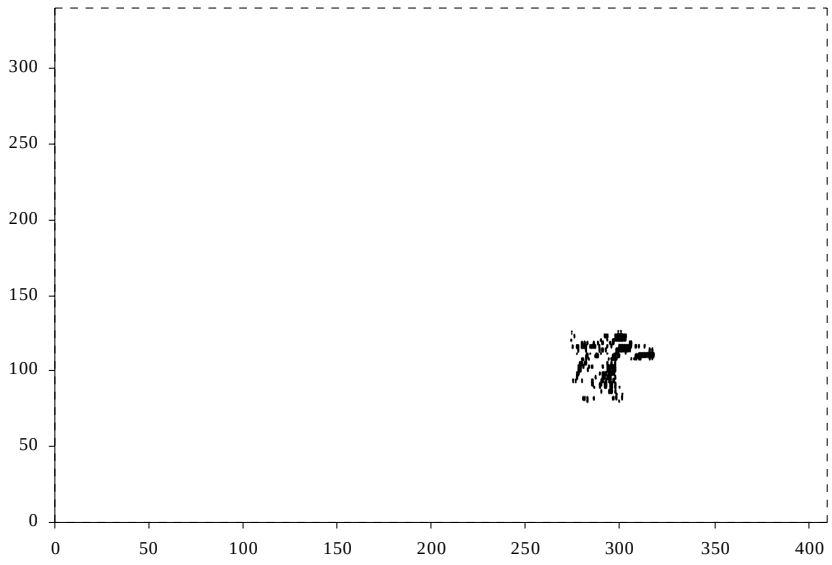
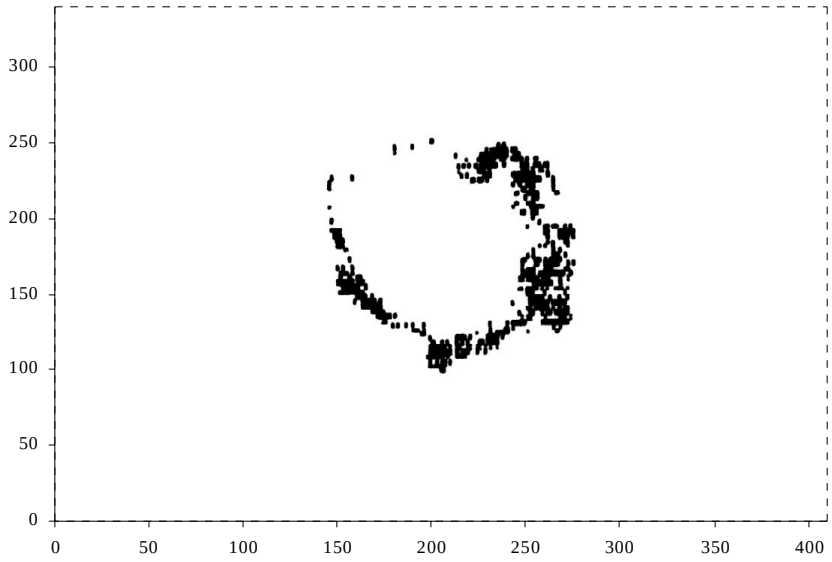


Figure 66: The number of turns per minute exhibited during the 15 minutes following each of the 2 consecutive NMDA concentrations infused into the substantia nigra via the push-pull cannula, and prior to infusion with ACSF only. Results are expressed as means $\pm$ SEM; with asterisks representing a significant difference compared to levels prior to NMDA infusion, *$P<0.05$, paired t-test. The number of animals used for each concentration was 3.



(A)



(B)

Figure 67 (A): Animal movement prior to infusion with NMDA, monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 2818 mm, time elapsed: 120 seconds. Figure 67 (B): Animal movement induced by local stimulation with NMDA, monitored with an Antrak video-based animal tracking system. Computer plotted picture, total distance moved: 9357 mm, time elapsed: 120 seconds, NMDA dosage: 10^{-2} M.

The baseline release of AChE was 0.16 ± 0.03 SEM mU, n=3. When the release of AChE was expressed as the percentage release of AChE in comparison to baseline release (which was taken as 100%), there was no significant difference (paired t-test, P=0.31) in the release of AChE between any of the experimental conditions (10^{-4} M, 108%) apart from 10^{-2} M (121%, P<0.05), see figures 68 and 69.

Following local administration of NMDA, a correlation was seen between AChE-release in the substantia nigra and the behaviour as measured in turns per minute, see figure 70. An increase in the number of turns per minute corresponds to an increased release of AChE. However, I was only able to observe an effect on the level of AChE-release when a very high dose of NMDA was administered.

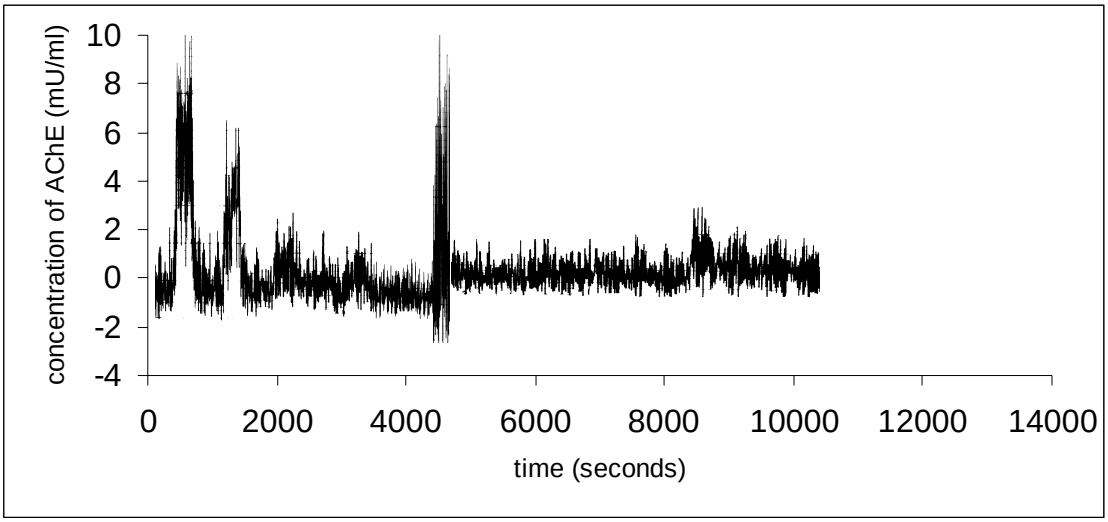


Figure 68: A typical trace showing the chemiluminescent signal produced by adding decreasing concentrations of exogenous AChE (Sigma) to the system prior to animal attachment (between 0 – 4000 seconds). The left hand side of the graph represents the spontaneous hydrolysis of ACh in vitro (background signal at 4500 seconds). A large signal peak due to blood/air contamination was experienced when connecting the animal. This is followed by the on-line detection of AChE-release in vivo (up to 7000 seconds). intranigral stimulation of AChE-release with NMDA 10^{-4} and 10^{-2} M.

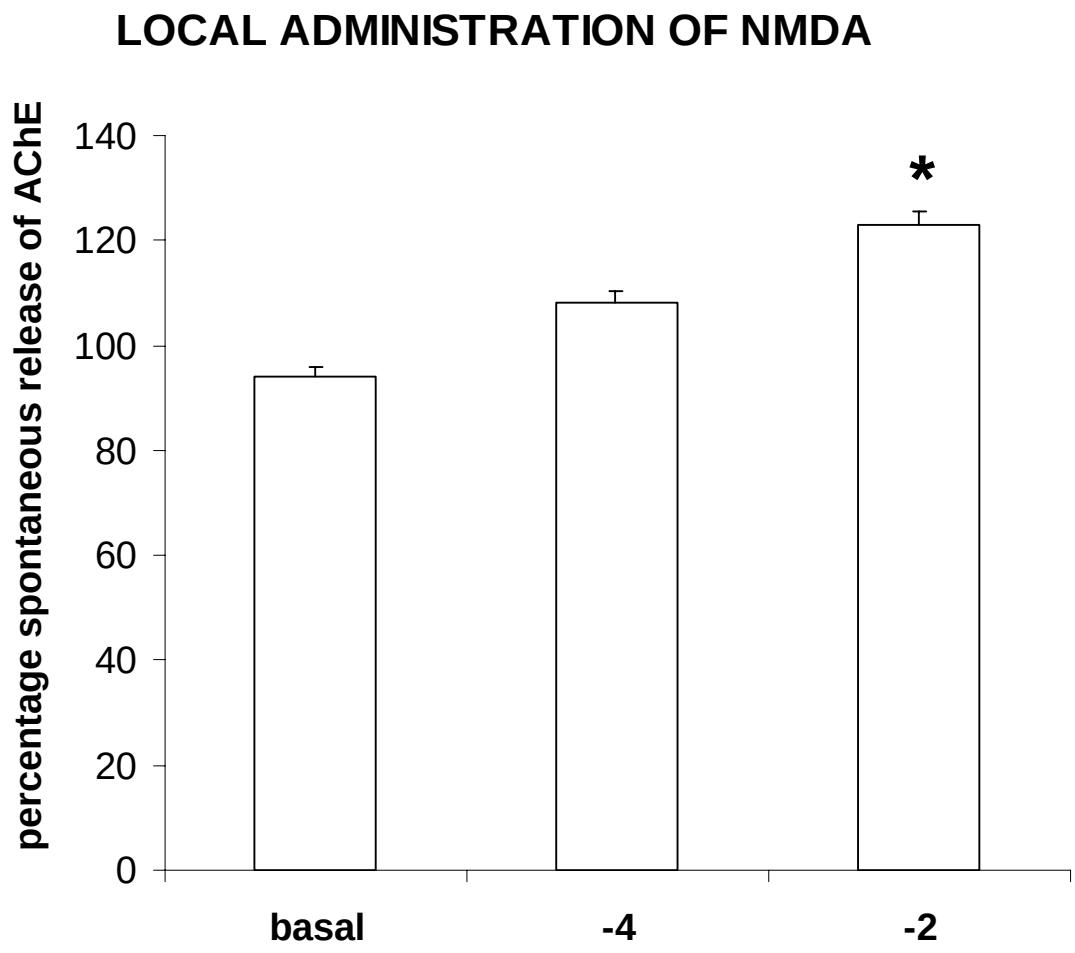


Figure 69: The spontaneous release of AChE in the substantia nigra of control and NMDA-treated rats, expressed as a percentage. Results are shown as means $\pm$ SEM; asterisks represent a significant difference between the test animals and the drug-free control group, *P<0.05, paired t-test, n=3.

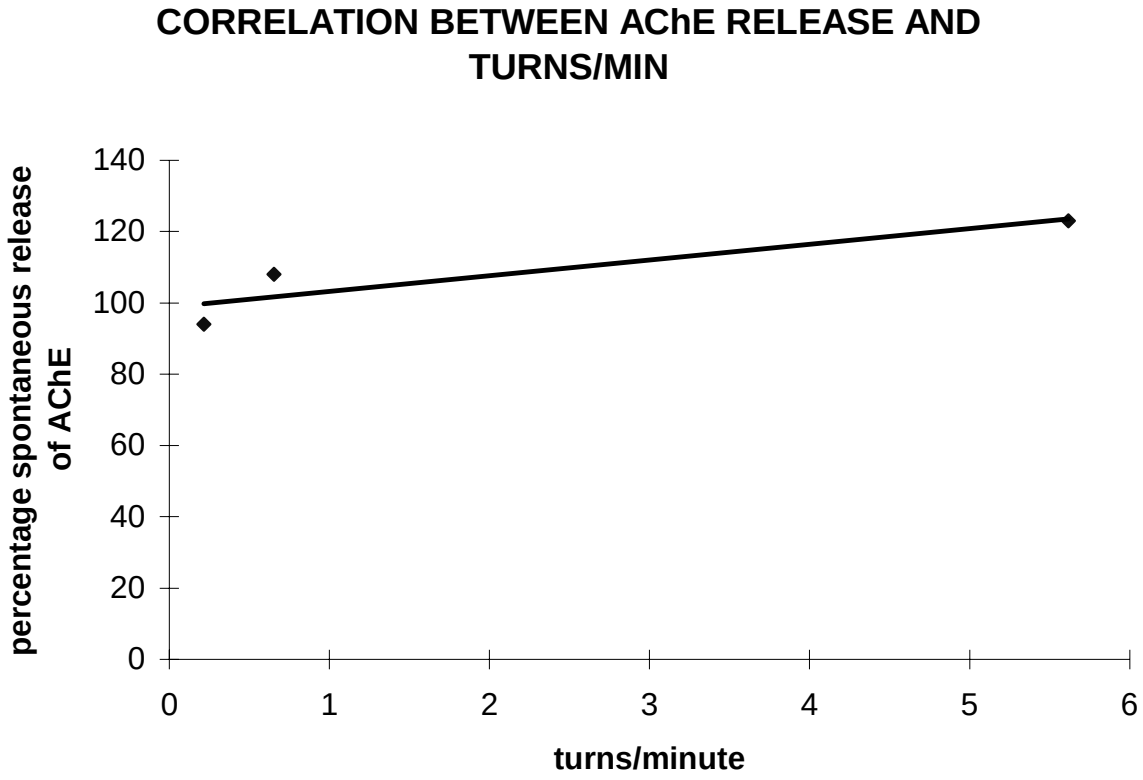


Figure 70: The correlation between AChE-release and turns per minute. The values produced by the control animals were 94% AChE and 0.22 turns per minute. The animals which received 10^{-4} M NMDA produced 108% AChE and 0.65 turns per minute. Those infused with 10^{-2} M NMDA emitted 123% AChE and produced 5.62 turns per minute; r^2=0.83, n=3.