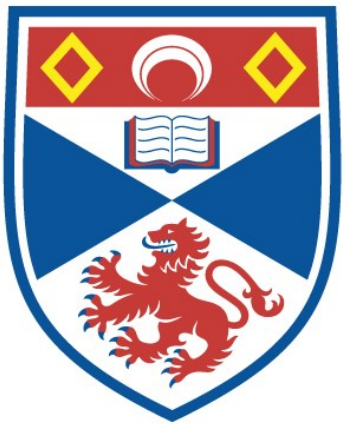
CHOLINERGIC STIMULATION OF THE SUBSTANTIA NEGRA

Graham Charles Parker

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Cholinergic stimulation of the substantia nigra

A thesis submitted to the University of St. Andrews

for the degree of Doctor of Philosophy

by

Graham Charles Parker

Department of Psychology

University of St. Andrews

January 1993

Declaration

a) I, Graham Charles Parker, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date 7 January 1993

b) I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 in October 1989, and as a candidate for the degree of Ph.D. in October 1989.

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This thesis is dedicated to my family: Mum, Dad, John, David and Anthony Parker; my friends past and present from University Hall, especially Dr Graham Smith, Laurence Fraval and Cindy Wesley; and to John Matheson and Ashok Desai.

Contents

Glossary

5-hydroxytryptamine, serotonin	5-HT
6-hydroxydopamine	6-OHDA
acetylcholine	ACh
acetylcholine esterase	AChE
caudate-putamen	CP
cholecystokinin	CCK
choline acetyltransferase	ChAT
CR lever	lever resulting in presentation of conditioned reinforcer
NCR lever	lever resulting in no programmed response
dopamine	DA
gamma-aminobutryic acid	GABA
high performance liquid chromatography	HPLC
lateral hypothalamic area	LHA
laterodorsal tegmental nucleus	LDTN
nucleus accumbens	NAcc
pars compacta, pars reticulata	PC, PR
pedunculopontine tegmental nucleus	PPTg
substance P	SP
substantia nigra	SN
ventral tegmental area of Tsai	VTA

Publications of work presented in this thesis

Papers

Parker, G.C., Rugg, E.L., Winn, P. (1991) Cholinergic stimulation of substantia nigra: abolition of carbachol-induced eating by unilateral 6-hydroxydopamine lesion of nigrostriatal dopamine neurones. Experimental Brain Research, 87, 597-603.

Parker, G.C. and Winn, P. (1992) An investigation of the relationship between nicotinic and muscarinic cholinergic stimulation of the anterior substantia nigra. NeuroReport: In press.

Parker, G.C., Inglis, W.L., and Winn, P. A comparison of behaviour following stimulation of the anterior substantia nigra by direct cholinergic agonists and anticholinesterases. Psychopharmacology: Accepted subject to revision.

Parker, G.C. and Winn, P. Cholinergic control of dopamine-containing neurones in the substantia nigra. Commissioned by Neuroscience.

Abstracts

Inglis, W., Parker, G. and Winn, P. (1991) Cholinergic stimulation of rat substantia nigra: comparison of carbachol, nicotine, neostigmine and effects of 6-OHDA lesions. Society for Neuroscience Abstracts 17 (1) p.451.

Parker, G.C. and Winn, P. (1992) Cholinergic stimulation of rat substantia nigra: interactions of nicotinic and muscarinic receptor activation on behavior. Society for Neuroscience Abstracts 18 (1) p.696.

Abstract

Convergent lines of research suggest there exists an excitatory cholinergic input to the substantia nigra from the pedunculopontine tegmental nucleus and possibly the laterodorsal tegmental nucleus. Previous work has suggested that microinjection of cholinergic agonists into substantia nigra elicits behaviours performed with a high frequency but with a low current rate (Winn 1991).

Experiments carried out during my PhD have demonstrated that:

Microinjection of cholinergic agonists to anterior substantia nigra (SN) elicited increased consumption of palatable food such as spaghetti but not rat maintenance diet in pre-satiated rats.

Stimulation of behaviour was achieved using direct agonists for either muscarinic or nicotinic cholinergic receptors (carbachol and nicotine respectively).

Stimulation of behaviour was also achieved using the indirect cholinergic agonist neostigmine which blocks the de-activation of endogenous acetylcholine by AChE.

Increased feeding elicited by cholinergic stimulation of the anterior SN was abolished by a selective lesion of ascending dopamine (DA) neurones which significantly depleted caudate DA levels but left accumbens DA levels unaltered.

A behaviourally potent dose of carbachol caused a significant increase in the response to different doses of nicotine suggesting an additive effect of muscarinic and nicotinic stimulation at the doses used.

Administration of cholinergic agonists to the VTA or SN caused indistinguishable effects on responding for conditioned reinforcement. Cholinergic stimulation caused increased responding for a conditioned reinforcer and also reinstated responding at the primary reward source.

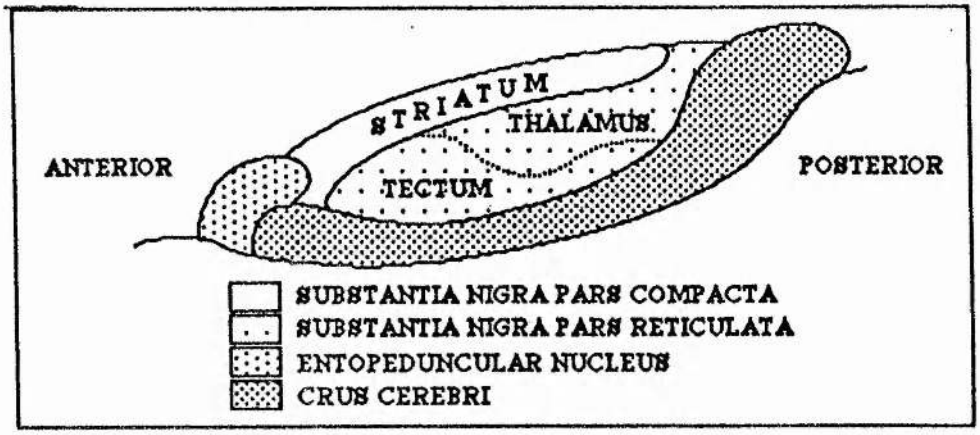
The functional significance of the cholinergic innervation of the DA-containing neurones of the substantia nigra is discussed with reference to its relationship to the neighbouring ventral tegmental area, and their innervation of the caudate-putamen and the nucleus accumbens. Cholinergic neurones in the PPTg and LDTN appear to exert a tonic control over the activity of midbrain DA-containing neurones. It is suggested that cholinergic control of midbrain DA-containing neurones facilitates the processing of information in the striatum and hence influence the selection of an appropriate behavioural response to a given situation.

1 The substantia nigra and midbrain dopamine systems

Substantia nigra

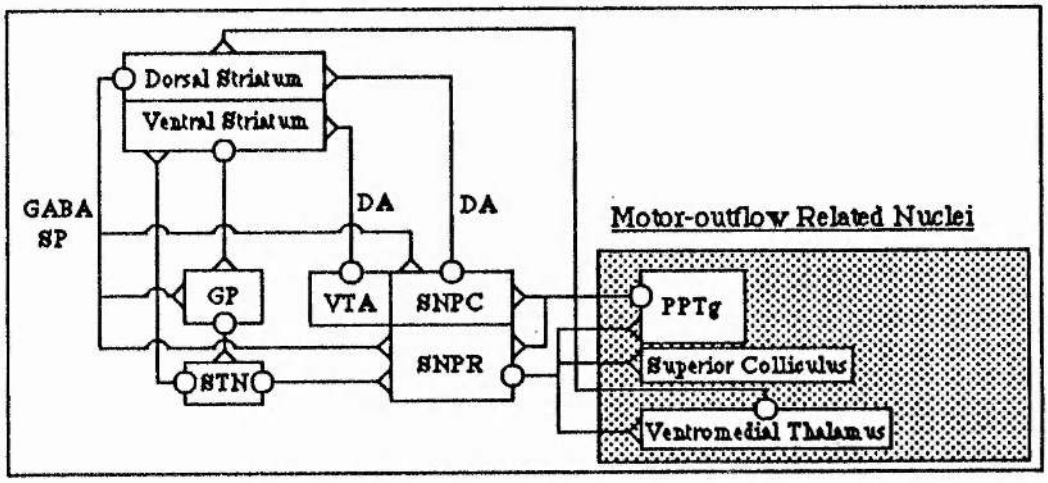
The substantia nigra (SN) is a mesencephalic structure which lies on the cerebral peduncle, ventral to the zona incerta and medial lemniscus, lateral to the ventral tegmental area of Tsai (VTA), caudal to the entopeduncular nucleus, and rostral to the pedunculopontine tegmental nucleus (PPTg). The SN can be differentiated into two main areas; pars compacta (SNPC) and pars reticulata (SNPR), and a third area generally considered to be a subdivision of SNPR, the pars lateralis (Figure 1). The two major parts may be distinguished cyto-architectonically: the PC is cell-dense while the PR, although having a greater area, has relatively few neurones. The majority of neurones of the SNPR are relatively large (25-40 μ) and have 3-5 large dendrites confined to the SNPR. In contrast, the SNPC is densely populated by medium-sized neurones (15-20 μ) having 4-6 smaller dendrites which mostly branch down into the SNPR. A third type of neurone described in both the SNPC and SNPR is a smaller neurone (8-12 μ) having 4-6 smaller dendrites that appear to branch arbitrarily (Gulley and Smithberg 1971, Gulley and Wood 1971). The close association between SNPC and SNPR due to the descending dendrites of the SNPC neurones has made functional dissociation of the two areas difficult. An injection aimed at anterior SN is more likely to effect SNPC neurones than one aimed at posterior SN. However, an injection that has been shown by histology to have been placed in SNPR may well have affected the dendrites of the SNPC neurones.

Figure 1: Saggital view of SN showing major projection areas



The importance of SN in motor control is suggested behaviourally by its involvement in Parkinson's disease (PD), and anatomically by its reciprocal connections within the basal ganglia and also with various sites known to be involved in motor outflow (Figure 2).

Figure 2: Schematic view of SN and projection sites involved in motor control



The major efferent pathway of the SNPC innervates the largest component of the basal ganglia, the neostriatum. This structure is also referred to as the dorsal striatum, or as it will be referred to here the caudate-putamen (CP). The nigrostriatal pathway projects rostral and medial to the SN to become part of the medial forebrain bundle (MFB) which projects up to the telencephalon. Also forming part of the MFB are the dopaminergic fibres of the mesolimbic DA system. It is thus called as its targets are associated with behaviours such as learning and memory, emotion and motivation. These are thought to be controlled by the limbic system which includes the hippocampus and amygdala. The cell bodies of this system lie in the VTA and innervate, among other sites, the nucleus accumbens (NAcc) and the olfactory tubercule. The NAcc is the most intensely investigated structure in paleo- or ventral striatum. The fibres from the SNPC mainly innervate the ipsilateral CP. The CP projects back to the SN - the striatonigral pathway - innervating the SNPC forming what is thought to be (in part) a negative feedback loop to the dopaminergic neurones of SNPC. SNPR receives projections from the CP, the globus pallidus (GP), the subthalamic nuclei (STN) and the raphe nuclei. All of these structures are implicated in motor control or the etiology of diseases which effect motor control. In turn the SNPR innervates various nuclei involved in motor outflow: the diencephalic ventromedial thalamus, the mesencephalic superior colliculus and the PPTg.

SNPC dopamine-containing neurones

The nigrostriatal neurones of the SNPC contain the catecholamine dopamine (DA). DA was recognized as being a neurotransmitter independent of its role as a precursor to noradrenaline (NA) in the mid-sixties due to the mismatch of its presence in certain tissues compared to that of NA. In the CP there is no measurable amount of NA but measurements of DA concentration had been made as long ago as the late 1950's.

Figure 3: Biosynthesis pathway of DA

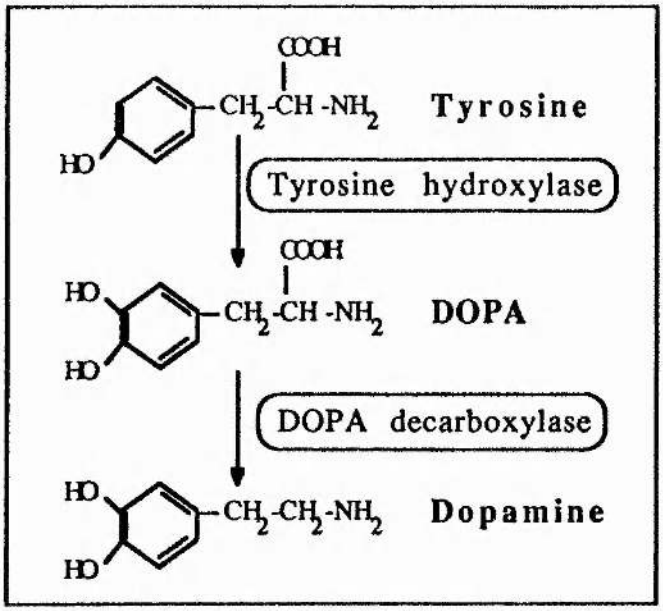


Figure 3 shows the steps of biosynthesis of DA. Tyrosine is one of the twenty amino acids that make up all mammalian proteins. As can be seen from Figure 3, the addition of an hydroxyl group (OH) converts it to DOPA. The enzyme which catalyses the hydroxylation of tyrosine is found only in catecholamine neurones. Tyrosine hydroxylase (TH) is extremely selective for tyrosine. The specificity of the enzyme and the localisation of the site to catecholamine neurones makes this the rate-limiting step of the process of catecholamine synthesis. The conversion of

DOPA to DA is not localized to catecholamine neurones and the enzyme responsible, DOPA decarboxylase, will catalyse the decarboxylation of the precursors of histamine and serotonin as well. Hence the levels of DOPA in neurones is usually very low. DA neurones do not contain DA-beta-hydroxylase so the chain of catecholamine synthesis is not taken to its noradrenergic conclusion.

DA is taken up by neurones by a high affinity system where it is broken down into metabolites by monoamine oxidase (MAO) and aldehyde dehydrogenase to DOPAC or by MAO catechol-O-methyl transferase and aldehyde dehydrogenase to HVA.

Electrical activity of DA neurones

Chiodo (1987) has described the electrophysiological characteristics of DA neurones. The firing pattern is biphasic or triphasic; the first phase always being positive, with an action potential duration of 2-5 msec with peak-to-peak amplitudes of 0.5-1.5 mV. The firing pattern is either irregular with a single spike or burst-firing of 2-8 spikes of increasing duration and decreasing amplitude; there is a sharp decrease on the initial positive phase of the potential called the initial segment-somatodendritic break. The second phase of the action potential depends on the striatonigral pathway causing inhibitory post synaptic potentials from the somatodendritic parts of the DA neurones. Hence antidromic stimulation of DA neurones will only stimulate the first phase of the action potential.

The action potential of DA neurones consists of a large inward sodium current and smaller transient potassium and calcium currents. The formation of sodium-dependent somatic action potentials is also to a certain extent dependent on dendritic calcium currents which carry the slow depolarizations.

There exists common to all DA neurones what Chiodo describes as 'pacemaker' activity. This is endogenous firing that occurs independently of any afferents to the neurones. The predominantly inhibitory afferents to the SNPC could then be seen to be restraining the DA neurones. The basal ganglia is described as being a system of inhibitory loops. Diseases associated with the basal ganglia have been seen as problems of disinhibition of the target zone of the particular pathway affected, for example, the DA nigrostriatal pathway in Parkinson's disease (PD) and the GABA striatonigral pathway in Huntington's chorea.

Striatal influence on nigrostriatal DA release

Various proteins effect the activity of DA neurones in the SNPC. The release of DA is also affected by proteins in the CP.

Giorguieff et al. (1977) used rat striatal slices to perform biochemical analysis of DA release in response to various drugs introduced into the perfusing medium. By introducing tritiated tyrosine they were able to measure the release of newly synthesized DA. Tetrodotoxin (TTX) blocks voltage-sensitive Na^+ channels thus precluding the possibility of release by action potentials. Perfusion with TTX or with a Ca^{2+}-free medium significantly reduced the release of [^{3}H]DA without affecting the rate of tyrosine hydroxylation. This shows that even without axonal action potentials, DA release is still dependent on normal neurotransmitter release mechanisms, i.e. is Na^+ channel and Ca^{2+} dependent. Also found to be Ca^{2+} dependent was release of DA in response to ACh. An increase of about 100% was seen in response to 10^{-5} M ACh. This increase could be reduced significantly using the nicotinic cholinergic antagonist pempidine (10^{-5}M) and also by the muscarinic cholinergic antagonist atropine (10^{-6}M). The reduction by using both blockers together was said by the authors "to approach significance". Neither

blocker significantly affected the spontaneous release of DA or its rate of synthesis when perfused without ACh. This would suggest that there are receptors on the presynaptic membranes of NSP DA neurones in the CP mediating cholinergic stimulation of DA release, and furthermore, that both nicotinic and muscarinic cholinergic receptors are present.

Gauchy et al. (1991) investigated the pre-synaptic control of DA release by ACh in the cat CP. The release of DA was increased by ACh and blocked by atropine suggesting it was mediated by activation of pre-synaptic muscarinic receptors on NSP DA terminals.

Many receptors have been shown to exist in various receptor subtypes. This has been done on the basis of pharmacological attributes and also by molecular analysis in cloning studies. This has led to a degree of confusion as to which category of subtype is being referred to for a given receptor, making the literature quite intimidating. Weiner et al. (1990) refer to muscarinic receptor sub-types M1, M2 and M3 on the basis of pharmacological data, and sub-types m1-m5 on the basis of molecular cloning studies. Weiner correlates them thus: M1 = m1, m4 and m5; M2 = m2; and M3 = m3. Weiner et al. (1990) attempted to map the distribution of muscarinic and DA receptors in the basal ganglia. They used probes for the mRNA of the 5 muscarinic sub-types defined by molecular cloning studies, and also probes for the D2 receptor. No probe for the D1 receptor was available. The localisation of the messenger RNA for a certain receptor subtype is suggestive that the receptor itself is present at that location. To do this Weiner et al. prepared oligodeoxynucleotide probes. A nucleotide is a 'sub-unit' of a nucleic acid of which RNA and DNA are two examples. The units link from their phosphate group to the next unit's deoxyribose group to make the chain. As oligo just means little, Weiner's probes identify which mRNA is present by characterising the links between the subunits of the mRNA.

This they do by linking, hybridizing, with the mRNA present in the tissue being analysed. The probes were radioactively labelled so they would show up on the slices when incubated against photographic film. Three muscarinic receptor sub-types (m1, m2 and m4) and the D2 receptor were found in the CP. The m1 mRNA was in >80% of striatal neurones, m2 was rarely observed and then only in relatively large neurones, and the m4 and D2 were present in 40-50%, one third of which expressed both m4 and D2 mRNAs. All of the neurones which expressed the D2 mRNA also expressed the m1 mRNA. The expression of mRNAs was observed to have gradients through the structure. D2 and m4 mRNAs were observed to have a medial to lateral gradient, while m1 had the reverse. Effectively all SNPC neurones expressed both the m5 and D2 mRNA. This is probably partly due to only the medial part of the structure being analysed. The only other structure in the basal ganglia to express muscarinic mRNAs was the STN which expressed m3 and m4. The entopeduncular nucleus did not express any of the receptor mRNA. The morphology of the neurones which expressed the m2 mRNA in the CP make it likely that they are cholinergic interneurones. As it was only the m5 mRNA expressed in the SNPC it seems likely that muscarinic cholinergic activation of SNPC neurones is mediated by m5 receptors. This further classification of muscarinic receptors may eventually explain its ability to have opposite effects on the same neuronal system. The antiparkinsonian drug trihexyphenidyl is reported by Weiner to have a stronger antagonistic effect on m1 and m4 receptors than on m2, m3 or m5. It seems likely that its action is in the CP rather than the SNPC where presumably it would inhibit DA release by antagonising m5 receptors on NSP neurones. However, m1 and m4 receptors are co-localized with D2 receptors in the CP, and D2 agonists decrease DA release. It would seem then that the anti-parkinsonian effects of

muscarinic antagonists have their effects on the CP in the same area as, and having opposing effects to, D2 receptors.

The excitatory amino acid glutamate innervates the CP from the overlying neocortex. Romo et al. (1986) have demonstrated that the terminals of NSP DA neurones release DA in response to stimulation by glutamate. Glutamate has a prescribed excitatory influence on seemingly all CNS neurones.

Dynorphin is an opioid peptide. It has various pharmacological effects depending on which of the three opioid receptor sub-types (delta, kappa or mu) it is activating. Gauchy et al. (1991) have produced evidence for the localisation of kappa opioid receptors presynaptically on NSP DA in the CP. Both dynorphin and a specific kappa agonist attenuated the release of NSP DA by ACh, and this effect was blocked by the dynorphin antagonist naxolene. TTX blocks the conduction of action potential in a neurone without affecting the resting membrane potential. Thus it effectively blocks any effects that may be caused indirectly by, for example, interneurones, as the interneurone membrane will not be able to achieve the increase in sodium conductance associated with axonal propagation of an action potential. The effects of dynorphin on ACh-evoked DA release were observed in the presence of TTX, therefore the site of action was probably the DA terminals of the NSP neurones.

Nigrostriatal DA influence on the caudate-putamen

The presence of the catabolic enzymes for ACh and DA in adjacent cells observed to make synaptic contact would be suggestive of a cholinergic-dopaminergic interaction. Kubota et al. (1987) used electron microscopy (EM) combined with choline acetyltransferase (ChAT) and TH immunoreactivity to investigate DA-ACh synaptic contact in the CP. 5-8% of striatal neurones were observed to be reacting positively to the

ChAT stain, the striatal cholinergic 'type I' neurones. When adjacent slices were stained for TH it was shown that the position of unstained cells adjacent to TH-positive cells correlated with 85% of the ChAT-positive cells. An interwoven network of smooth, unmyelinated TH-positive fibres were observed all throughout the CP. The membranes were seen using EM to have symmetrical synaptic specializations.

Pickel and Chan (1991) used ChAT immunohistochemistry and EM to determine how non-cholinergic neurones made contact with the ACh interneurones. As well as direct synaptic contacts between unlabelled terminals and ACh neurones they also observed 'appositions', contacts between ACh and non-ACh neuronal membranes, without the usual intervening astrocytes. These appositions showed electron densities associated with synaptic contact but without the ultrastructural features associated with classical synaptic contact. It is unclear from this study as to the neurotransmitter contained within the neurones contacting the ACh neurones. It is likely however that the non-ACh neurones would be DA, GABA, glutamate or a combination of afferent fibres.

It was suggested by Rolls et al. (1984) that the role of DA might be to increase the efficacy of other neurotransmitters in the striatum. This could explain the apparently contradictory actions of DA as being excitatory, inhibitory and in some papers, both. Chiodo and Berger (1986) iontophoretically introduced the excitatory amino acid glutamate and the inhibitory amino acid GABA to striatal type I neurones and then observed the effect of exogenous DA. DA on its own had an inhibitory effect on any neurones that responded. Glutamate increased cell firing. When applied at a relatively low current (small amount released), DA enhanced the increased firing in response to glutamate, but when applied using a higher current, response to glutamate was attenuated by 50%. Both of these effects were blocked by the DA receptor antagonist trifluoperizine.

GABA inhibited firing of all striatal type I neurones tested. At a concentration that by itself had no affect on the firing rate, DA enhanced the inhibition of GABA. When used at a higher concentration DA still enhanced the inhibition of GABA. These effects were also blocked by the DA antagonist trifluoperizine. It would appear that DA *does* enhance the effects of other neurotransmitters on type I neurones in the CP and that this enhancement is dose-dependent.

It appears that DA released by NSP terminals effects not the nature of other striatal afferents to the striatum, but does modulate their effect at their target type I neurones. That is to say, DA is affecting the 'bandwidth' of response by the striatal neurones rather than their 'tuning'. It follows then that an event that is supposed to increase NSP DA release, such as tail-pinch, should elicit a *modulation* of striatal type I neurone behaviour that is measurable using electrophysiological techniques.

De Keyser et al. (1988) used tritiated SCH 23390 and spiroperidol to label D1 and D2 receptors respectively in the human brain. They found high densities of both receptor sub-type in the caudate and putamen nucleus (the structures are physically separated in the human), and the NAcc. Both types of DA receptor were also present in the SN.

Characterisation of the relationship between the two sub-types of DA receptors has been made possible with the development of selective agonists. Kelland et al. (1988) used single-unit recording techniques to measure the effect of intravenous (i.v.) D1 agonist SKF 38393 on the inhibition of NSP DA neurones by i.v. D2 agonist quinpirole (LY 171555). They discovered that quinpirole was less effective at inhibiting 'fast' DA neurones than 'slow' DA neurones. This is thought to be due to there being less somatodendritic autoreceptors on 'fast' neurones. An autoreceptor is activated by the neurotransmitter that the neurone on which it is located releases. This differentiation was blocked by

pretreatment with SKF 38393 giving the slow population the same dose-response curve to quinpirole as the fast cells. Furthermore this effect of the D1 agonist could be blocked by administration of the D1 antagonist SCH 23390 or by transecting the forebrain and hence cutting the striatonigral pathway. Such a transection had no effect on the rate-dependent response of NSP DA neurones to quinpirole. This suggests that the effects of the D2 agonist are localized to SN rather than the CP, but the alteration of the effects of D2 stimulation are mediated by the striatonigral pathway and hence is mediated by D1 receptors in the CP. It is unclear which neuronal population is mediating the effects of D2 receptor stimulation on SN. It has no effect on the response of fast SNPC neurones to D1 agonists. In the same study they showed it also had no effect on the mesolimbic DA neurones of the VTA. It could be that the D2 receptive neurones of the CP are projecting selectively to the slow NSP DA neurones. It would be interesting to know in the light of Chiodo and Berger study (1986, see above) whether the effect of the D2 agonist would be to increase instead of decrease the difference between fast and slow NSP DA neurones if administered at a different dose. As the effect of the CP D2 receptor stimulation is to decrease the inhibitory effects of SNPC D1 receptor stimulation, NSP DA is effecting striatonigral neurones in such a manner as to increase the probability of firing of NSP DA neurones.

The release of DA in the striatum is causing the gating of slow SN DA neurones to be less responsive to inhibition by the release of DA by itself and its neighbouring cells. This puts a different perspective on the possible role of the lateral inhibition, or indeed, lateral stimulation with respect to the function of dendritic release of DA. Whether they release DA onto neighbouring neurones or not, they are not leaking onto receptors on a passive membrane, but a neurone whose responses depends

on its own state. It would appear we have not only lateral inhibition / excitation in the DA NSP neurones but potentially negative / positive feedback as well.

50 out 53 NSP DA neurones tested by Kelland et al. (1989) were inhibited by electrical stimulation of the sciatic nerve. This inhibition is still present following sectioning of the striatonigral pathway. Inhibition is increased by i.v. administration of the D1 agonist SKF 38393. This enhancement was reversed by the selective D1 antagonist SCH 23390, and blocked by sectioning the nigrostriatal pathway. The selective D2 antagonist l-sulpiride had no significant affect on response to sciatic nerve stimulation following administration of SKF 38393. This suggests that the NSP DA neurones are affected in a predictable manner by peripheral information. Furthermore the response of NSP DA neurones to such information is influenced by D1 receptors in the CP. The influence mediated by D1 receptors can increase or decrease NSP DA neurone response to peripheral information; the D1 antagonist reversed the effect of the D1 agonist, rather than just attenuate it.

There is obviously a body of evidence for a functional role for the D2 receptor in the CP and a functional role for D1 receptors in the SN. However, De Keyser et al. localized both type of DA receptors to both the CP and the SN in the human brain.

Bannon et al. (1989) were interested in the effect of DA receptor activation and the levels of enkephalin peptides in the CP. In particular they were interested in the response to an increase in the level of DA on the preproenkephalin (PPE) and preprotachykinin (PPT) mRNA levels. The MFB was stimulated at 10 Hz which is above normal for NSP neurones but of a level that does occur in individual neurones when highly stimulated (Chiodo 1988). DA release was seen to be significantly elevated in the ipsilateral olfactory tubercule as was levels of PPE mRNA

in the ipsilateral CP. Striatal levels of PPT mRNA were unaffected. Elevated levels of PPE mRNA were also produced by the systemic administration of indirect DA agonist methamphetamine and also by the selective D2 agonist quinpirole but not by the selective D1 agonist SKF 38393. Given that previous studies have indicated that D1 receptor agonists do affect enkephalin synthesis, Bannon et al. concluded that the D1 receptors are already saturated in this study, suggesting that it is the D2 receptor that is responsive to supranormal levels of DA in the CP. It could be that the D2 receptor *is* an index of supranormal stimulation for the CP but it is not obvious from this study.

From such studies it appears that NSP DA neurones are projecting to among other targets, cholinergic interneurones of the CP. Ajima et al. (1989) used microdialysis combined with HPLC and electrochemical detection techniques to monitor striatal ACh and DA release in free-moving rats. This technique also allowed them to introduce exogenous proteins into the area being studied. The D1 receptor agonist SKF 38393 dose-dependently increased striatal ACh release (dose range 10^{-4} M to 10^{-6} M). Highest increase was 150% of basal levels in response to 3 x 10^{-5} M SKF 38393. This increase appeared during the first hour and was still significant during the 3 hr post-injection period which was presented. DA levels quickly increased peaking at 500% of basal levels 1 hour after administration of 10^{-4} M SKF 38393. The D1 antagonist SCH 23390 dose-dependently increased (10^{-4} M) or decreased (10^{-3} M) ACh release. The D1 antagonist had the same effect on DA levels as the D1 agonist. Injection of both the agonist and the antagonist together produced no significant effect on ACh level, but produced additive effects on DA levels. The authors then used D2 receptor agonists and antagonists. Neither sulpiride (D2 antagonist) nor LY 171555 (D2 agonist) had a significant effect on ACh levels in the striatum. The D2 agonist did

attenuate the increase in ACh levels induced by the D1 agonist SKF 38393. On its own, LY 17155 significantly decreased DA release by 50%, but when administered with SKF 38393 DA levels were increased beyond that elicited by SKF 38393 alone.

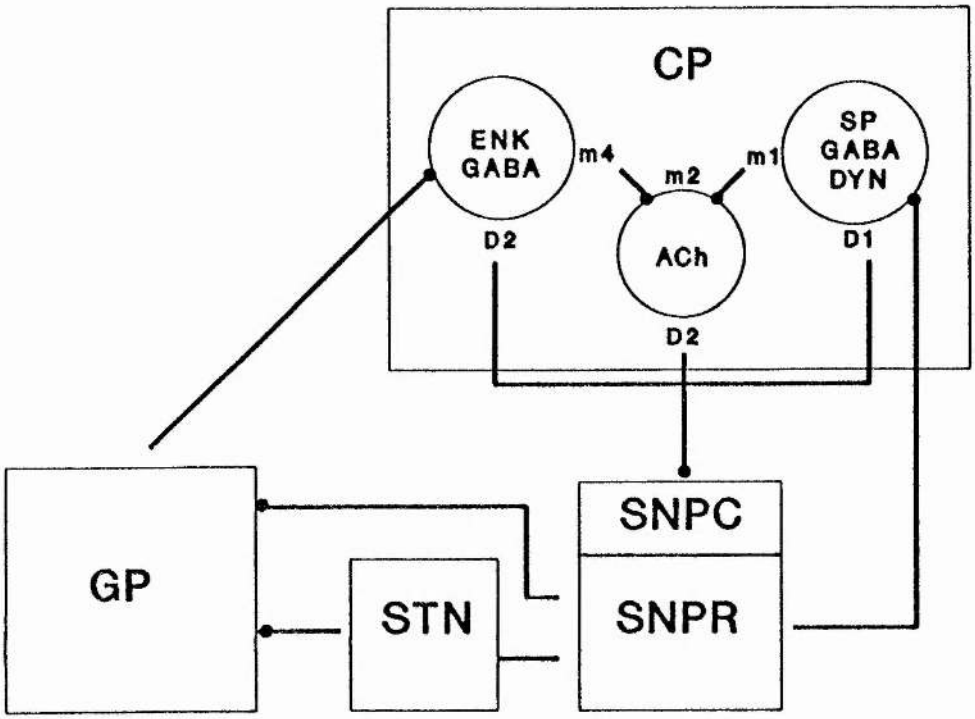
These data suggest that both D1 agonists and antagonists can elicit increased release of ACh from striatal neurones, though higher doses of the antagonist decreases striatal ACh. That both agonist and antagonist produce the same effect on a neurone, and that this same effect is blocked by their coadministration would appear to support a modulatory role for DA D1 receptors in the CP as discussed above (Chiodo and Berger 1986). Ajima et al. however, talk of effects on intervening interneurones to explain the effects. The role of the D2 receptor is still unclear. Its activation by agonists or antagonists had very little effect on ACh levels. Its lack of effect could have three not necessarily mutually exclusive possible explanations: firstly that D2 receptor activation does effect striatal ACh levels and the dose used was at the crossing point between excitation and inhibition of release, secondly that there are no post-synaptic D2 receptors on ACh neurones, or thirdly that the D2 receptor is tied to the D1 receptor for its effects and has no observable effects if activated independently. Ajima et al. report findings for only one dose of the D2 drugs. Ohno et al. (1987) have provided evidence for the presence of both D1 and D2 receptors on the same CP neurones though it has not been resolved as to whether this is the case for ACh striatal neurones. The D2 agonist certainly appears to have had an effect when used in conjunction with the D1 agonist SKF 38393. This might suggest that the third explanation of the D2 results is more likely.

The D2 agonist caused a significant decrease in striatal DA, arguing for a role as a pre-synaptic autoreceptor inhibiting DA terminals. It seems likely that the 'little' effect on ACh level by D2 activity alone is a result

of its effect on DA release from NSP terminals. If Bannon et al. (1989) (see above) are correct concerning their suggestion that the D2 receptors in the CP function when DA release is at a supranormal level where D1 receptors are saturated, this might explain why Ajima et al. have found little response to their doses of D2 drugs.

Using oligonucleotide probes in a technique similar to that described by Weiner et al. (1990, see above), and retrograde labelling by infusion of fluorogold into the SN, Gerfen et al. (1990) mapped the expression of the genes for D1 and D2 receptor subtypes in the CP. D1 and D2 mRNA were localised to the striatonigral and striatopallidal medium spiny GABA-containing neurones respectively that make up the majority of CP cells. 43% of CP neurones were striatonigral, and of these 149 out of 173 expressed D1 receptors while only 27 out of 184 cells that expressed D2 receptors were labelled as striatonigral cells. The authors assumed that the remaining cells were striatopallidal. Furthermore, the striatonigral neurones expressed the neuropeptides dynorphin and SP while the striatopallidal neurones expressed enkephalin. Surmeier et al. (1992) provided evidence that D2 receptors are also localised to striatonigral neurones. This appears to be in disagreement with Gerfen's broad conclusions but cannot be ruled out on the basis of his studies. Figure 4 provides a simplified summary of how DA and muscarinic receptors are thought to relate to striatal outflow neurones.

Figure 4: DA and muscarinic receptor sub-types on striatal neurones



The patches and matrices of the caudate-putamen

The existence and possible roles of DA receptors in the CP have been discussed with reference to their localisation within the CP. It has become apparent over the last decade that the afferent and efferent neurones of the CP are not homogeneous in their distribution. The CP has been subdivided into patch (or striosome, depending on the author) and matrix on the basis of the neurochemical content of these areas. The patches are known to have low levels of AChE (Graybiel and Ragsdale 1978) in the adult mammal and high density in infancy. The matrix areas show the opposite developmental histochemistry. DA receptors also display a heterogeneous distribution. D1 receptors show a higher binding in the patch than in the matrix (Besson et al. 1988), while post-synaptic D2 receptor binding is higher in the matrix.

In an investigation of glutamate NMDA receptors by Krebs et al. (1991) DA release from NSP DA terminals was increased by administration of 50 uM NMDA. The patches of the CP contain u-opiate receptors while the matrix areas do not. To compare the effects of NMDA on the two areas slices of the CP can be incubated with tritiated naxolene, a specific ligand for u-opiate receptors. Autoradiography then reveals which parts of the CP are patch and which matrix. DA release was significantly greater from the matrix than the patch area. TTX was used to investigate whether the increase in CP DA levels was due to direct activation of release from DA NSP terminals, or indirect through activation of neurones stimulating the NSP neurones at the SN. The NMDA-evoked release of DA from the matrix areas was significantly reduced, while release from patch areas was unaffected by local administration of TTX. There was no significant difference in the level of NMDA-evoked release of DA between the two CP compartments following TTX. It appears then that the greater release of DA from the matrix is due to other neurones, possibly striatonigral, synapsing onto DA NSP neurones.

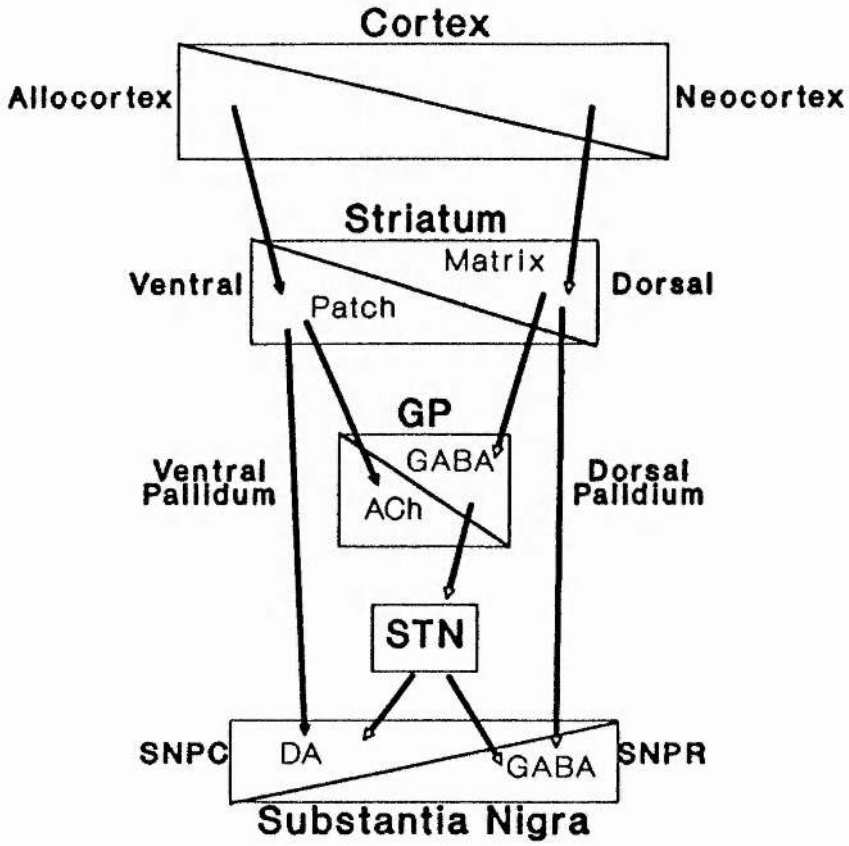
Gauchy et al. (1991) found that spontaneous release of DA was significantly higher in the matrix than in the patch areas of the CP. Furthermore, the release of DA was increased in both compartments by ACh. In the matrix area there was a significant inhibition of ACh-evoked DA release by TTX.

Using quantitative receptor autoradiography, Lowenstein et al. (1990) mapped the binding of tritiated pre-synaptic markers for dopaminergic and cholinergic neurones, and also M1 muscarinic receptors in the human brain. The binding site density for [^{3}H] hemicholinium-3, a ligand that marks the high-affinity sodium reuptake mechanism of DA neurones, showed significantly higher binding in the matrix areas of the caudate, the putamen and the NAcc. The same was generally true for the distribution

of [^{3}H] mazindol, the pre-synaptic cholinergic neurone marker, and also for [^{3}H] pirenzepine which labels M1 receptors. For all three markers, the density in the matrices was approximately twice that of the patches. This demonstrates a significantly higher incidence of cholinergic neurones, M1-cholinoceptive neurones and DA terminals in the matrices of the three main areas of the human striatum.

Gerfen (1992) has proposed a model of how limbic and non-limbic information may be distributed from allocortical and neocortical sites in relation to cortico-strio-pallido-nigral connections (Figure 5).

Figure 5: Limbic and non-limbic cortical outflow and the basal ganglia



It has been proposed by Gerfen (1989) that the patches of the dorsal striatum conveys 'limbic' information more normally associated with the ventral striatum (primarily the NAcc), and that the matrices of the ventral striatum conveys 'non-limbic' information more normally associated with dorsal striatal function. Furthermore Gerfen proposes that the limbic circuitry dominates in the ventral striatum, while the non-limbic circuitry dominates the dorsal striatum. Therefore direct administration of DA-ergic agonists into the NAcc will produce limbic behaviour, while DA-ergic stimulation of the CP will produce non-limbic behaviour.

Midbrain DA and behaviour

The innervation of the CP and NAcc by midbrain DA- containing neurones of the SNPC (A9) and the adjacent VTA (A10) respectively, has been extensively studied with respect to the initiation, selection and control of behavioural response. The methods employed include specific depletion of DA from the target structure and then ascription of function based on resultant behavioural abilities and inabilities, and more recently developed technology such as *in vivo* voltammetry whereby DA levels can be measured with a high temporal and neurochemical resolution in an unanaesthetised and hence behaving animal.

Mesolimbic DA and behaviour

Infusion of DA agonists directly into the NAcc has been shown to produce different behavioural under different test conditions. Costall and Naylor (1975) observed increased locomotion following infusion of DA into the NAcc. Increased locomotion following systemic administration of amphetamine was blocked by a 6-OHDA lesion of the NAcc but not a lesion of the CP (Kelly et al. 1975). Furthermore, the direct DA agonist apomorphine significantly increased locomotor activity in rats with an

NAcc DA depletion but not in those with a CP DA deletion. This is thought to be due to supersensitivity of the striatal DA receptors as a compensatory mechanism for the loss of DA terminals in that area.

Mesolimbic DA has also been intensely studied in the reinforcement of behaviours and as a neural substrate of reward (Fibiger and Phillips 1986). Electrical stimulation of certain sites in the brain reinforced the performance of a behaviour paired with such stimulation (Olds and Fobes 1981). These sites correlated well with the source and terminal fields of the midbrain DA systems including the VTA, SN, NAcc, CP, olfactory tubercule and prefrontal cortex. Both the mesolimbic and nigrostriatal pathway project rostrally forming part of the MFB. Electrical stimulation of this bundle will also reinforce behaviour. The reinforcing effect is attenuated by the local administration of a DA receptor antagonist into the NAcc (Stellar et al. 1983).

Fibiger et al. (1987) demonstrated that an animal trained to press a lever to self-administer electrical current to an electrode placed unilaterally in the VTA - intra-cranial self-stimulation (ICSS) - will cease pressing following an ipsilateral, but not contralateral, 6-OHDA lesion of the MFB causing a near total depletion of NAcc DA.

The rewarding effects of drugs of addiction such as cocaine and amphetamine are thought to be mediated by the mesolimbic DA system. Cocaine blocks the reuptake of DA hence prolonging its action at DA receptors. Amphetamine also blocks the reuptake of DA but also causes its release from DA-containing terminals. Roberts and Zito (1987) have shown that self-administration of cocaine or amphetamine is blocked by infusion of DA antagonists into the NAcc.

Carr and White (1983) were also interested in the neural substrates underlying reward. They used conditioned place preference (CPP), a technique whereby the subject, over a series of sessions, associates one

environment with the target drug and another with just saline. It was shown that when given a choice between the environment in which they had received amphetamine to another environment, those rats that received amphetamine injections into the NAcc displayed CPP but those having received amphetamine into the CP did not. The advantage of this paradigm is that the animal receives injections only during the training; in the test sessions the animal only has to express its preference for an environment. This removes any confounding effects of activational or stressful effects of the administration of a drug before a trial.

Taylor and Robbins (1984) demonstrated that a rat will find a previously neutral stimulus reinforcing if associated repeatedly with a primary reinforcer. They trained thirsty rats in operant chambers to associate the presentation of a compound stimulus (house light off, panel light on, and the sound of a server mechanism) with the presentation of water. The rats responding on a lever that would result in the presentation of the compound stimulus alone with no presentation of water, a second lever that had no programmed consequence, and responding on the water tray panel were recorded following bilateral infusions of doses of d-amphetamine to the NAcc, CP or the medio-dorsal thalamus. Taylor and Robbins observed clearly increased responding for the conditioned reinforcer following injections to the NAcc, ambiguous effects from the CP, and no effect from the medio-dorsal thalamus. The authors suggested that the results from CP injections may have been due to diffusion to the NAcc rather than due to a CP effect. In a second experiment in the same paper, Taylor and Robbins (1984) showed that the compound stimulus had to be correlated positively with the primary reward for there to be an increase in responding following NAcc injections of d-amphetamine. This they achieved by having three conditions during training: positive, whereby the compound stimulus immediately preceded the presentation of

water; negative whereby the compound stimulus came after the presentation; and random whereby the two events were uncorrelated.

In a follow-up study, Taylor and Robbins (1986) further delineated the reinforcing effects of DA-ergic agonists by showing attenuation of increased responding for a conditioned reinforcer following intra-NAcc d-amphetamine by 6-OHDA lesion of NAcc, but not by a similar depletion of CP DA. Furthermore, systemic administration of the direct DA agonist apomorphine increased responding in rats with an NAcc lesion but not those with a CP lesion. This led the authors to conclude that the increase in responding in those rats with an NAcc 6-OHDA lesion was due to the development of supersensitivity in the DA receptor sites following the loss of DA terminals.

Increased responding for conditioned reinforcement has also been demonstrated using food as a primary reward in hungry rats (Kelley and Delfs 1991). Similarly to the findings of Taylor and Robbins (1984), Kelley and Delfs found increased responding following injections of d-amphetamine into the NAcc, but ambiguous results from the CP.

A hungry rat that is allowed free access to water but only allowed one pellet of food every minute will drink much more water than a rat that is allowed the same amount of food without having to wait between pellets. Schedule-induced polydipsia (SIP) is believed to be an adjunctive behaviour, that is the rat is drinking because it is thwarted from its preferred activity, eating. Robbins and Koob (1980) demonstrated the relevance of mesolimbic DA to the acquisition of SIP by blocking it with a 6-OHDA lesion of the NAcc. The lesion did not impair the rat's ability to drink, nor does such a lesion impair the rat's ability to consume the pellets (Salamone 1988).

Although the evidence would seem to suggest a role for the ascending DA-containing neurones of the mesolimbic DA system as the neural

substrate for reward, Bielajew and Shizgal (1986) have shown that the reinforcing effects of electrical stimulation of the MFB is mediated by *descending* fibres, not ascending. Bielajew and Shizgal placed two electrodes in the rat brain, one in the MFB and one in the VTA. Each rat was allowed to, and did, lever press in order to receive electrical stimulation of the MFB and VTA. This demonstrated that the electrode sites could indeed support ICSS. They compared four conditions: using the MFB electrode as a cathode (-) with a skull screw or the VTA electrode as the anode (+); and the VTA electrode as the cathode with a skull screw or the MFB electrode as the anode. If an hyperpolarizing (inhibitory) current is applied to an axon an action potential will be attenuated and if the current is sufficiently large, blocked. They found that action potentials were blocked most effectively by using the MFB as the cathode rather than the VTA. As the ICSS was more efficiently blocked by the anodal hyperpolarization of the VTA, this suggests the action potential was running from the MFB toward the VTA. This suggests that the fibres being affected descend from MFB to VTA, rather than VTA to MFB. That the fibres that support ICSS are in fact descending rather than ascending means that the crucial fibres cannot be the ascending DA-containing neurones of the mesolimbic DA system. As discussed by Bielajew and Shizgal, their studies do not elucidate further the source nor the destination of these ICSS-supporting neurones further than their source being rostral or at the MFB electrode site and their terminals being at caudal or in the VTA. Although this study demonstrates that ICSS is not directly affecting mesolimbic DA neurones, there is still a great weight of evidence to support their involvement in reward-related behaviours.

The Bielajew and Shizgal study leaves unanswered whether the descending ICSS-supporting neurones might arise from the NAcc and

have their behaviour influenced by mesolimbic DA, or project to the VTA and influence the activity of DA-containing neurones in the VTA, or both, or indeed neither. What is does demonstrate is that ascribing psychological constructs such as reward to one pathway is to oversimplify the relationship between behaviour and brain function, and that a fuller picture of how the brain influences behaviour must be approached at a systems rather than a single structure level.

Nigrostriatal DA and behaviour

High doses of amphetamine (for example, 5 mg / kg) produce repetitive behaviours that seem to have no goal - stereotyped behaviour. Although clearly such a construct as stereotyped behaviour is problematic in its definition and hence its quantification, rating scales have been developed to try to characterise the behaviours involved. The rating scale of Creese and Iversen (1973) as used by Kelly et al. (1975) rates the intensity and location of stereotyped behaviour but does not clearly demonstrate what constitutes stereotypy.

Kelly et al. (1975) showed that 6-OHDA lesion of the CP but not the NAcc blocked stereotyped biting, licking and gnawing following systemic administration of a high dose of the indirect agonist amphetamine. Furthermore, the direct agonist apomorphine caused increased stereotyping in the rats with a CP lesion.

Kelley et al. (1988) demonstrated that specific orofacial stereotypies of licking, biting and self-injurious gnawing can be produced by injecting d-amphetamine into the ventrolateral CP, while the same doses injected to the posterior dorsal CP produced no effect on behaviour.

Lyon and Robbins (1975) proposed a theory characterising the effects of amphetamine on behaviour. The behaviour that a rat performed following administration of amphetamine was described as a function of the

complexity of the behaviour. With increasing dose, the chains of behaviours that could be performed become less complex (for example, eating > locomotion > sniffing) until with higher doses the animal can only perform behaviours that can be repeated rapidly and have a short time of completion.

The role of nigrostriatal DA in the control of behaviour is probably most clearly suggested by the debilitating effect of its absence in the progressive neurodegenerative disorder PD. The clinical relevance of the substantia nigra was realised during post-mortem inspection of the brains of people known to have been suffering from PD. The depigmentation of the tissue due to the loss of the melanised neurones was described and quantified by Hornykiewicz (1960) who estimated DA depletions in the region of 80 and 90% in the caudate and the SN, respectively. The difficulty in initiation and cessation of voluntary movements in PD is thought to result directly from the loss of the DA influence on the striatum. The tremor and rigidity that are the classic symptoms of PD are thought to not be a direct result of dysfunction in the NSP DA system *per se*. These symptoms are thought to be due to abnormal activity in the ventromedial thalamus, an output target for the CP known to be involved in motor control through its connections with the motor cortex and the basal ganglia. Indeed a selective lesion of this nucleus was the treatment of choice for PD, but the success of drug treatment aimed at the DA system, combined with an increasing nervousness concerning invasive brain techniques meant a decrease in its use that had little to do with its efficacy (Tasker et al. 1983).

The development of an animal model of the primary neurochemical deficit of PD, the loss of NSP DA has been made using the catecholamine neurotoxin 6-OHDA.

6-OHDA destroys adrenergic neurones in the peripheral nervous system producing a selective chemical sympathectomy. Being almost identical to the DA molecule (see Figure 6), it is taken up from extracellular space by adrenergic axons through their high affinity transport systems. When the molecule is metabolised within the cell, it oxidises to compounds that are cytotoxic (see Figure 7). In small doses there appears to be no adverse effect other than accumulations in the granular vesicles, presumably of metabolites that cannot be broken down further or disposed of by the cell. However, once the amount of 6-OHDA, or rather its cytotoxic metabolites, reach a threshold, the axon and terminals are destroyed. In the periphery, the somata will survive and eventually re-innervate their target structure. Systemic administration of 6-OHDA has little effect on the CNS. As with the DA and DOPA molecules, the 6-OHDA cannot easily pass the blood-brain barrier, though 6-OHDOPA can.

Figure 6: DA, NA and 6-OHDA

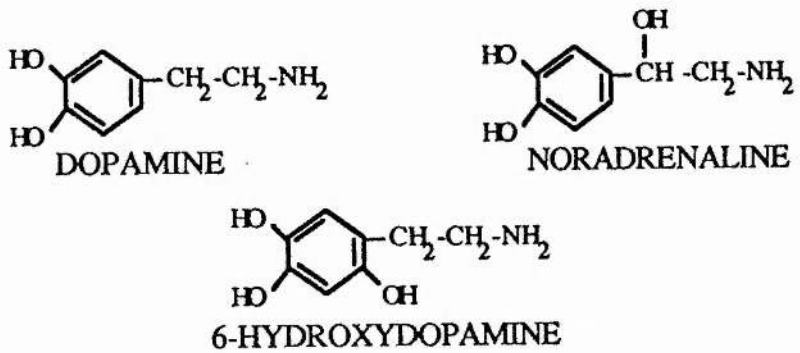
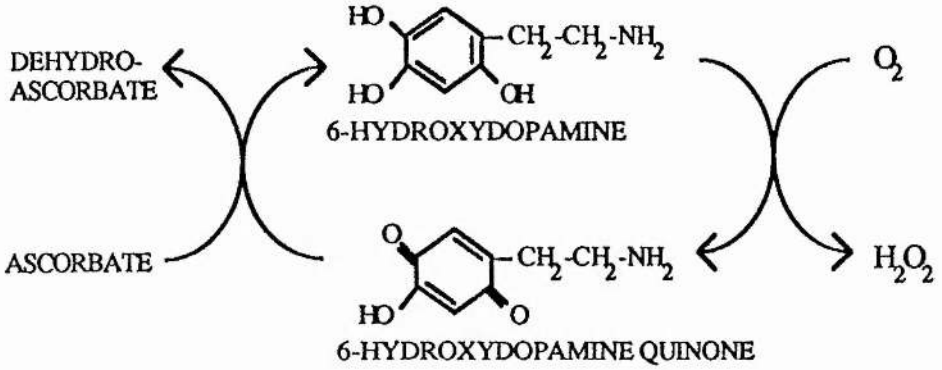


Figure 7: Metabolism of 6-OHDA to its cytotoxic metabolites



Uretsky and Iversen (1970) compared the effects on NA, DA, 5-HT and GABA levels in the rat brain following infusion of 6-OHDA into the ventricular CSF. Small doses significantly depleted NA with no observable effects on other neurotransmitter levels. A higher dose that caused >80% depletion of brain NA also depleted DA by 66% but had no effect on levels of 5-HT or GABA. Assays for the enzymes that catalyse the synthesis of DA and ultimately NA, TH and DOPA decarboxylase, were also significantly reduced in the striatum and the hypothalamus. Peripheral studies had previously shown only destruction of NA terminals by 6-OHDA. This study demonstrated that 6-OHDA was specifically a catecholamine neurotoxin, having no significant effect on the monoamine 5-HT or the amino acid GABA. In contrast to the periphery, there was no recovery of NA or DA levels even 142 days after administration of 6-OHDA.

Breese and Taylor (1971) administered 6-OHDA intracisternally, presumably to the cerebellomedullary cisterna as this is where cisternal punctures are tapped from. As above, small doses effected only NA, larger doses depleting DA as well. The hypotensive agent pargyline

inhibited the action of MAO-A and MOA-B by binding to the enzyme. By administering pargyline systemically 30 mins before the 6-OHDA, the efficacy of the depletion of DA was significantly increased. Pargyline is thought to have its effect by preventing the deactivation through deamination of the 6-OHDA by MAO, but if this is the only method of action it is surprising that it has no effect on NA depletion. TH activity in the CP was also seen to be significantly reduced indicating a greater reduction in DA terminals in the CP. The tricyclic antidepressants block uptake of amines through neuronal membrane transport systems. NA and 5-HT are strongly affected while DA neurones remain unaffected. Pretreatment of rats with imipramine or desipramine had no effect on 6-OHDA depletion of DA but blocked depletion of NA due to the blockade of 6-OHDA uptake.

To appreciate how valuable the selective neurotoxic properties of 6-OHDA in combination with desipramine and pargyline is, it should be compared with other lesion techniques. Javoy et al. (1976) observed similar depletions of CP DA levels following infusion of 2 μg 6-OHDA compared to electrolytic lesion of the SN. The damage to non-dopaminergic neurones by 6-OHDA was negligible while electrocoagulation caused destruction of all local neurones. Copper sulphate causes similarly restricted local cell loss to 6-OHDA but did not show a significant reduction in CP DA levels.

A specific lesion of DA-containing NSP neurones allows modelling of the primary neurochemical loss in the neurodegenerative disorder, PD. In parallel to PD, the loss of DA from the CP has to be almost total before any observable symptoms are present. Bilateral 6-OHDA lesions causing greater than 90% depletions have been observed to produce motor dysfunction; akinesia (an inability to produce motor actions without being

paralysed) and catalepsy (a suspension of voluntary movements associated with muscular rigidity). Physiological deficits that may be a direct result of the lesion or the motor dysfunction also occur. A bilateral lesion causes animals to become aphagic and adipsic and have to be maintained on a palatable diet. This might also be attributed to a psychological disorder such as loss of motivated behaviour or an inability to integrate sensory and motor information, both of which have been described in animals having a unilateral lesion.

The gross motor deficits induced by near total depletion of DA from the CP can be attenuated by replacing the DA. DOPA administered systemically can cross the blood-brain barrier, be hydrolysed to DA and raise striatal levels sufficiently to alleviate the gross symptoms. Keefe et al. (1989) have also shown a return of motor control in response to environment. When dropped into a water bath, a normally akinetic rat will for a few seconds produce normal control of musculature. Similarly, Apicella et al. (1990) observed co-ordinated control of musculature in response to a stressful stimulus in monkeys that had a unilateral 6-OHDA lesion and normally demonstrated hypokinesia on the contralateral side.

Chiodo (1988) has shown that a stressful or activating situation increases the release of DA from NSP neurones. Presumably the 6-OHDA rats and the PD patients have still some intact NSP neurones that respond to the situation by increasing DA release, allowing 'normal' control of musculature. That the relief from akinesia is only temporary might be due to a pharmacological limit of how much DA a neurone can catabolise and release over a sustained period, or due to a decrease in stimulation of DA release associated with recovery from the initial challenge.

It has been observed by Snyder et al. (1985) that rats with a partial striatal DA depletion while showing no obvious dysfunction demonstrate sensorimotor deficits when exposed to a stressor that has a long duration.

It is surprising that the largest component of the basal ganglia can tolerate a loss of up to 90% of DA with no apparent loss of function. Zigmond et al (1989) have proposed a model of how compensation for the loss of NSP DA may occur. A striatal neurone innervated by one NSP DA neurone's terminals will also receive DA released by neighbouring NSP neurones. This overlap means that the loss of a few DA neurones in a localised area may have little effect while others are still intact. DA is removed from the extracellular space by high affinity uptake systems located on the presynaptic terminal membrane. This reuptake serves as a source for future release or transmitter and also to preclude the DA having further effect on the post-synaptic membrane. Thus the loss of one axons terminals at a site would allow the DA molecules from outwith the site access to the post-synaptic membrane. This is possible as the astrocytes that normally enclose synaptic contact, effectively buffering from external influence, have been observed by Pickel and Chan (1991) to be absent on some striatal neurones innervated by NSP DA. Chiodo (1988) observed that there exists a proportion of NSP DA neurones that are 'quiescent', they exhibit a baseline firing rate but show no response to stimulation. Following 6-OHDA lesion, Zigmond et al. proposed that as the lesion increases so the proportion of quiescent DA neurones will decrease. Furthermore, there will be an increase in DA released from each terminal and an increase in the amount of DA synthesised by each neurone. Such an increase in local DA release may be due to reduced autoinihibition. Evidence to support such a scheme of recovery come from Onn et al. (1986). Bilateral lesions that do not affect gross motor

function are known to cause a decrease in the number of DA neurones and terminals in the SNPC and CP respectively. Following intraventricular administration of 6-OHDA, cell loss was observed in all parts of the rat CP but with a mediolateral gradient with most cell-sparing in the lateral CP. It would be expected that there would be a similar gradient of TH and DOPAC levels across the CP. This was not the case. Levels were decreased but less markedly than DA, and there was no concentration gradient across the CP. However such lesions do not decrease the number of active DA neurones. ~40% of SNPC DA neurones are quiescent but can be activated by application of the DA antagonist haloperidol. Zigmond et al. have also observed a much higher extracellular concentration of DA in the CP than would be expected following a 6-OHDA lesion.

Investigation of striatal function using bilateral lesion of NSP with 6-OHDA is confounded by its gross effect on motor function. The lack of response in an animal to a given task might be due to motor dysfunction, sensory dysfunction, a sensorimotor integration dysfunction or a motivational dysfunction however the animals physical inability to respond precludes observation of any other possible accompanying dysfunction. An animal with a unilateral lesion however, normally displays no behavioural difference to an unoperated animal. It is only when the animal is specifically challenged that the pharmacological deficit becomes behaviourally manifest.

Carli et al. (1985) demonstrated that such a unilateral lesion produced a deficit in making a contralateral response to a contralateral sensory event with no sensory or motor deficits. A rat was required to move its head towards or away from a laterally presented light. The rat responded correctly to stimuli presented ipsilaterally to the lesion, and when the rat

was required to respond ipsilaterally to a contralaterally presented stimulus. This led Carli et al. to propose a role for NSP DA in the integration of sensorimotor information.

Brown and Robbins (1991) investigated the role of CP DA in using advanced information in a visual spatial discrimination task. Rats were required to respond to the left or the right depending on the brightness of a bilaterally presented cue. This was presented either in advance of, or simultaneously with, an auditory stimulus indicating they should make their response. Hence, the task involved a test of simple reaction time (in trials with advance presentation of the discriminative cue), or choice reaction time where the rat had to make the discrimination and then respond. The delay between the onset of the trial and the presentation of the auditory stimulus was also a controlled variable. The rats were tested before and after a unilateral 6-OHDA lesion of the CP. Rats showed a bias in responding post-operatively towards the side of the lesion but retained faster responses on the simple than the choice reaction trials. Pre-operative trials showed a decrease in reaction time on both tasks with increased delay time between trial onset and auditory stimulus presentation. The authors suggested this was due to increasing probability of stimulus presentation as the period of delay increases. This effect was abolished post-operatively on trials requiring responding to the side contralateral to the lesion, but remained for ipsilateral response trials. Brown and Robbins concluded that their study did not support a role for the CP in the use of advance information to select particular behavioural response, but suggested that the abolition of the delay-dependent decrease in reaction time may imply a role for CP DA in preparing the rat to respond.

Chiodo et al. (1980) were interested in how the dopaminergic neurones of the SN respond to environmental stimuli. Using anaesthetized rats, first they identified the DA neurones by their electrophysiological characteristics; discharge rate of 1-9 Hz, biphasic action potentials with amplitudes of 0.4-1.5 mV and burst firing on discharge. All cells thus identified responded to stimuli that are 'activating' to an unanaesthetized animal; tail-pinch, air puffs to the face, olfactory stimuli (ammonia) and repeated light flash. On the basis of the cells' responses Chiodo et al. subdivided the dopaminergic neurones into Type A cells which increased their discharge by 2-3 times in response to tail-pinch, and Type B cells which decreased relative to baseline firing. Cells responded with a latency of 400 msecs with a waveform duration of 0.8-2.0 msec for Type A and 2.0-2.4 msec for Type B cells. There was also an initial segment, IS-spike, associated with Type B that was absent in Type A neurones. Similar response was found to the air puffs and ammonia. Latency of response to the repeated flash light was 1-2 s longer than in response to the other three stimuli and had similar effects on both Type A and B neurones irrespective of side of presentation, unilateral or bilateral. That all neurones respond to all the stimuli argues against the nigrostriatal pathway having a role in the identification of a certain stimuli nor in a specific behavioural response. In a previous study, Chiodo et al. (1979) found that intense pressure to the cervix of conscious female rats, inducing the immobilization associated with lordosis, had no effect on nigral DA neurones. The same pressure applied elsewhere on the body was 'activating' and increased nigral firing. It would appear the nigral DA neurones are behaving in manner consistent with the appropriate activational response to the environmental stimuli.

Further support for this idea comes from studies on primates. Schultz et al. (1983) observed an alteration in DA neurone firing preceding and

during forelimb movement in conscious monkeys. Furthermore, stimuli that did not normally elicit a behavioural response from the monkey did not activate DA neurones. However, normally activating stimuli caused DA neurones to increase firing even if the animal did not produce an obvious behavioural response (Ljungberg et al. 1992).

It is apparent that the information reaching the nigral DA neurones has already undergone processing that filters for the attribute 'activational'. A possible explanation could be that the neocortico-strio-nigral pathway could be providing this information. However Grace et al. (1980) demonstrated that destruction of the strionigral pathway had no effect on stimulation of SNPC neurones by environmental stimuli, although Kelland et al. (1989) produced evidence that nigral response to sciatic nerve stimulation was increased by a selective D1 receptor agonist (SKF 38393) administered i.v.. In agreement with Grace et al. transection of the strionigral pathway did not block nigral DA neuronal response to sciatic nerve stimulation, but did block the enhancement of the D1 agonist. It would therefore appear that the activation of DA neurones by environmental stimuli is mediated by pathways caudal to the DA-containing neurones of the midbrain.

2 Cholinergic control of SNPC and midbrain DA systems

Non-cholinergic influences on SNPC neurones

A behavioural study by Kelley and Iversen (1979) analysed the effects of SP infused bilaterally into the SN. It was found that stereotyped rearing and sniffing were induced after injections of SP (3 μg / nigra) into the SN but not after injections into the VTA. Sniffing and rearing were blocked by bilateral lesion of the CP with 6-OHDA, which caused an 80% depletion of DA from the caudate, but had no significant effect on NAcc DA levels.

Neurones of the striatonigral tract innervate SN with SP and the chemically similar neurokinin A (substance K). Herrera-Marchitz et al. (1986) have shown that nigral infusion of either causes increases in DA release in the ipsilateral striatum causing dose-dependent contralateral rotation. In a study comparing the actions of agonists and antagonists of SP and SK, Reid et al. (1990) have shown that the excitation is probably mediated by different receptors as the effects on striatal DA levels of SP could be blocked with a specific SP antagonist which had no effect on the effect of SK.

The type I striatal neurones innervate the GP and the SNPR with the inhibitory amino acid GABA. Arnt and Scheel-Kruger (1979) investigated the behavioural effects of injecting GABA into the SN. They found that contralateral turning following administration of the GABA agonist muscimol was unaffected by injection of the DA antagonist haloperidol into the SN, CP or NAcc. However, muscimol-induced turning increased following a 6-OHDA lesion of the MFB and decreased following systemic or intranigral injection of cholinergic agonists. The authors conclude that the effects of intranigral muscimol is not mediated by the DA systems, but that there is an interaction between GABA, DA and ACh in the SN. Intra-nigral muscimol has also been shown by Sperber et

al. (1989) to cause a significant increase in striatal DOPAC, a metabolite of DA, but no effect on levels of DA itself. Muscimol also produced repetitive head movements and contralateral turning.

Further evidence for such an interaction was provided by Redgrave et al. (1980) who demonstrated the involvement of the GABA-ergic nigrotectal pathway in the mediation of oral stereotypies produced by systemic administration of apomorphine. Experiments in the monkey on saccadic eye movements show similar responses by injecting the GABA agonist muscimol into SNPR as to injecting the GABA antagonist bicuculline into the superior colliculus (Hikosaka and Wurtz 1985). The authors suggest that the spontaneous contralateral saccades made by both manipulations are analogous to the contralateral turning produced by intra-nigral GABA in the rat in releasing the superior colliculus from the SNPR GABA-ergic inhibition.

Wirtshafter et al. (1987) demonstrated a serotonergic input to the SNPC DA neurones from the dorsal raphe nuclei. The receptors that are affected by serotonin have been shown by Fischette et al. (1987) to be 5-HT-1B receptors agonists of which have a small inhibitory influence on the firing of DA neurones (Sinton and Fallon 1988).

Kelland et al. (1990) have further specified that it is only the slow firing DA neurones of the SNPC that are inhibited by serotonergic agonists. The population of 'fast' DA cells of the SNPC do not respond to serotonin or its agonists.

Cholecystokinin C-terminal octapeptide (CCK8) is thought to be co-localized with the DA neurones of both the SNPC and the VTA, and i.v. administration of CCK will stimulate SNPC DA neurones that also contain CCK. If CCK is applied microiontophoretically onto such neurones there is a pronounced increase of firing and especially burst firing (Skirboll et al. 1981).

The DA neurones of the SN are known to release DA from their dendrites within the SN. It is not known whether this is to regulate the DA neurones themselves or perhaps neurones afferent to the SN. Groves and Linder (1983) discuss evidence for DA receptors on GABA terminals in the SN. Groves and Linder attempted to study the nature of dendritic synapses in the SN using the 'false' transmitter 5-hydroxydopamine. A false transmitter closely resembles in chemical form its 'true' transmitter, but only has weak effects on binding with the true transmitter's receptors. As its presence at the receptor complex denies access to the real transmitter molecule, the false transmitter effectively acts as antagonist. In this study, it was being used for its ability to be taken up by the DA neurones where its density could be observed in synaptic vesicles using EM. The dendrites can be differentiated from axons by their shape, size and cytoarchitecture. They discovered that although the dendrites in the SNPR only infrequently showed apposition to other dendrites, such appositions were observed over relatively long distances in the SNPC. twenty-nine dendritic associations were observed in the SNPC with no intervening glial processes, as opposed to zero in the SNPR. As discussed by Groves and Linder, such synaptic contacts only make up a relatively small proportion of the synaptic contacts that occur in SN compared to some thalamic nuclei where such appositions are frequent. Thus it appears that the DA released from the dendrites of DA neurones in SNPC is targeted at the dendrites of other DA neurones. Such an arrangement involving in some cases a single dendrite synapsing onto several other dendrites could well be a system for maintaining a tonic influence on neighbouring neurones. It is unclear what triggers release of DA from the dendrites, though it is probable that there is a constant release of DA in small quanta. It could be that dendritic release of DA is then accelerated, coupled to the propagation of an action potential to cause release at the

release at both sites was blocked by intra-nigral TTX, suggesting that DA release from the terminals in the CP and from dendrites in the SN is dependent on the firing of action potentials of nigral neurones. However, because the measurement of DA release in the SN was near the lowest level of detection by the technique employed, a DA uptake blocker nomifensine was used increasing DA output seven-fold in the SN. Although this is still evidence that DA release in the SN is tied to the propagation of action potentials by SNPC neurones, replication with a more sensitive technique precluding the need to use an uptake blocker would be preferable. The tying of dendritic DA release to the action potential is suggestive, but not conclusive evidence, that DA release in the SN is concurrent with DA release at the terminals in the CP. The release of DA from SN dendrites may still be an indirect result caused by striatal DA release influencing striatonigral efferents which in turn cause the dendritic release of DA.

Chiodo (1988) discusses evidence that the self-inhibition of SNPC neurones is mediated by D2 autoreceptors. These are present on the somatodendritic region of the neurones and are much more sensitive than DA receptors on type I CP neurones (Skirboll 1979). *In vitro* studies have shown that they produce K^+ currents using a mechanism that is dependent on the presence of the nucleotide guanine. Such autoreceptors are not present on the DA neurones of the VTA. It is clear that the DA neurones of the SNPC have many pharmacological influences on their post-synaptic membranes, both on their somata and dendrites in the PC and on their dendrites in the PR. Kelland et al. (1988) have shown that the inhibition of NSP DA neurones by the D2 agonist quinpirole was positively correlated with the baseline activity of the neurones. The 'fast' DA neurones were inhibited only by much higher doses of the agonist than the 'slow' population of neurones. Kelland et al. suggest this is due

to there being fewer somatodendritic autoreceptors on the 'fast' NSP DA neurones.

Most of the influences on SNPC DA neurones appeared for a long time to be inhibitory, GABA from the dorsal and ventral striatum mediated by $GABA_A$ receptors, serotonin from the dorsal raphe nuclei mediated by 5-HT-1B receptors and DA from the dendritic processes of the DA neurones themselves. Characterisation of excitatory influences on the DA neurones is on the increase. This probably reflects the advances in technology for the studying of peptides that proved harder to localize than the predominantly inhibitory catecholamines. SP and substance K (neurokinin A) all thought to be projected from the CP join ACh and possibly SP from the PPTg as excitatory influences on the SNPC DA neurones. Iontophoretic administration of glutamate has been reported to increase 'burst firing' in NSP DA neurones (Grace and Bunney 1984). This effect is blocked by the NMDA antagonist (+/-)-4-(3-phosphonopropyl)-2-piperazine carboxylic acid (Overton and Clark 1992). This is suggestive of an excitatory amino acid influence on NSP DA neurones mediated by NMDA glutamate receptors. Scarnati et al. (1986) have produced evidence that SNPC neurones are influenced by glutamate from neurones on the PPTg.

There is now considerable evidence from a number of disciplines to suggest an excitatory cholinergic influence on the DA-containing neurones of the SNPC. The rest of this chapter will describe acetylcholine, its action as a neurotransmitter, and the evidence for a functional cholinergic-dopaminergic interaction in the SN.

Acetylcholine as a neurotransmitter

Proteins vital for the synthesis of the neurotransmitter ACh, the amine base choline and the enzyme which catalyses the synthesis of ACh

(Cheney et al. 1975), ChAT as well as a certain amount of the neurotransmitter itself (Chubb et al. 1980) have been shown to be present in SN. ChAT is synthesized in the ribosomes of neuronal soma and transported to axonal terminals by axoplasmic flow. It is known to be located mostly in the axoplasm with some association with the membranes of synaptic vesicles. Choline exists in the extracellular fluid and can be transported into cells by a relatively low-affinity transport mechanism by non-cholinergic cells for its role in normal cell metabolism. In cholinergic axon terminals there is a high-affinity carrier-facilitated transport mechanism which allows cholinergic neurones to maintain a constant supply of choline for the synthesis of ACh. It is known that glucose, oxygen and sodium ions are vital for ACh synthesis. ACh is stored in vesicles (40-50 nm, containing 2000-40000 molecules of ACh) in the cholinergic terminals which migrate to the presynaptic cell wall, merge with it and rupture spilling its contents into the synaptic cleft. Even when neurones are not firing, ACh is constantly being released in small quanta. This process is massively accelerated when neurones fire and nerve impulses depolarize membranes. In the region of 250-400 vesicles will discharge releasing over 4 million molecules of ACh from one presynaptic membrane. The amount of ACh released will be limited if there is an insufficient concentration of Ca^{2+} in the extracellular fluid. It is thought they link the arrival of the nerve impulse to the release of the neurotransmitter. In contrast, magnesium ions will inhibit the release of ACh if in too great a concentration. ACh release at a terminal site can thus be seen, to some extent, to be a function of the concentration of Ca^{2+} and Mg^{2+} in the synaptic cleft. Massey and James (1978) have demonstrated the presence of both a sodium-dependent high affinity choline uptake mechanism and a calcium-dependent release mechanism in the SN.

Cholinergic neurotransmission is mediated by two main subclasses of receptor named after their classic agonists. Binding at nicotinic and muscarinic receptors produces different effects on the neuronal membrane (Figure 8). It is thought that ACh acts in its same preferred conformation at both receptor types, but different parts of the molecule form the link with each receptor complex. By comparing the structure of ACh itself with the structure of selective agonists for the two sub-types (Figure 9) it has been inferred that for nicotinic receptors it is the cationic head and the carbonyl oxygen atom, while for the muscarinic it is the cationic head, the ether oxygen and the terminal methyl group which are vital for the link. Muscarine, the classic muscarinic agonist, does not have a carbonyl group and so has little effect on nicotinic receptors, while the ether oxygen atom of nicotine is blocked from interacting with muscarinic receptors by the position of the alpha-methyl group of the molecule.

Figure 8: Effects of ACh mediated by nicotinic and muscarinic receptors on neuronal membranes

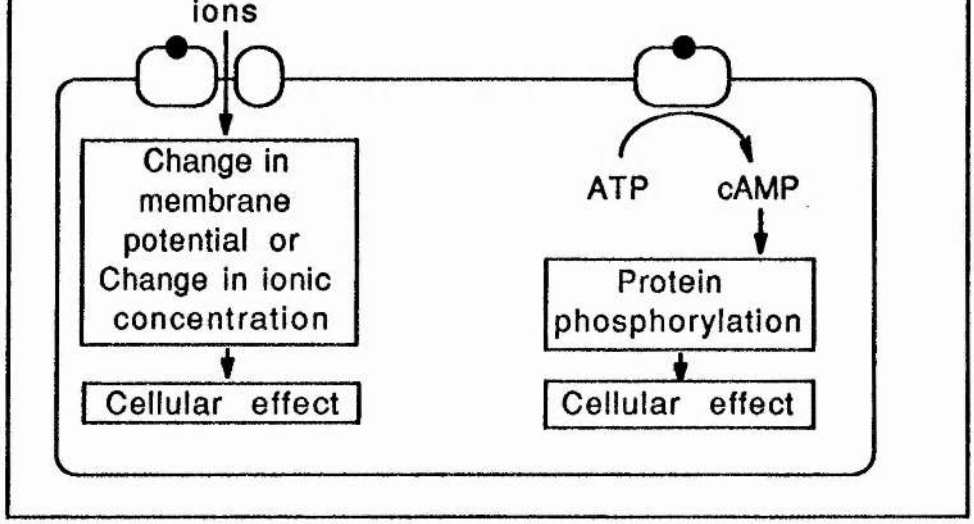
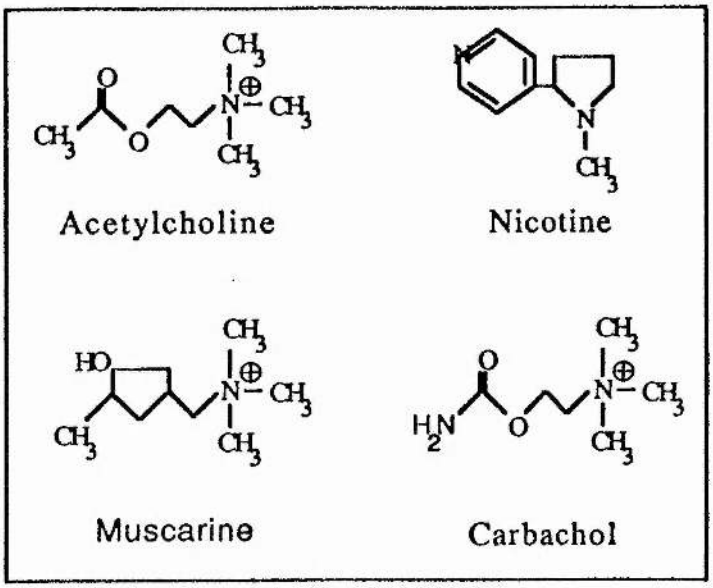


Figure 9: Chemical composition of ACh and nicotinic and muscarinic agonists



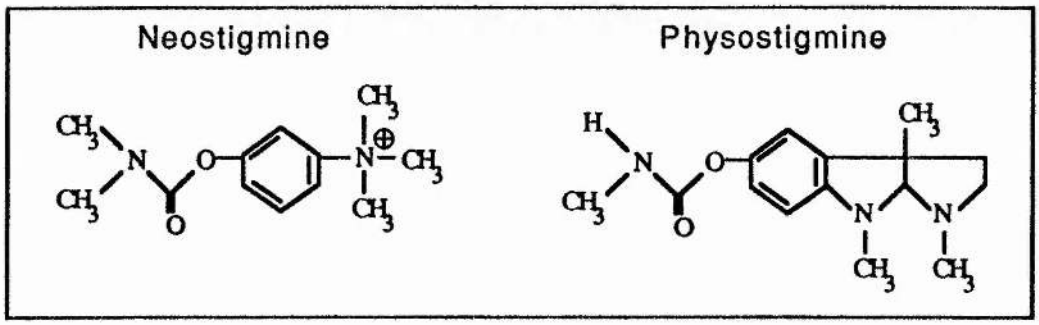
The binding of ACh with a cholinoceptive receptor complex will induce changes in the permeability of the membrane to Na^+, K^+ and Ca^{2+}. The Na^+ which is in a higher concentration outwith the cell flows inwards as does to a lesser extent Ca^{2+}, while K^+ which has a higher concentration inside the cell, flows outwards.

The neuropharmacological effects of ACh on the post-synaptic membrane cannot be classified simply as excitatory or inhibitory (Brown 1986). Indeed ACh can have an excitatory or inhibitory effect, or an effect more akin to potentiation. Potentiation is where the membrane shows a reaction to the binding of one ligand followed by another perhaps different ligand which is different to that of the sum of its reactions to the two ligands separately. Kemp (1977) has suggested that ACh acts as a 'fast' neurotransmitter (referring to its duration of binding with the receptor complex) when acting at nicotinic receptors, and as a 'slow' neurotransmitter when acting on muscarinic cholinergic receptors. When acting as a fast transmitter its effect appears to be consistently excitatory

on the post-synaptic membrane, but when acting as a slow transmitter it can be excitatory, inhibitory or potentiating.

After binding with the receptor complex ACh is deactivated by hydrolysis to its constituent choline and coenzyme A. There is a family of enzymes which catalyse the deactivation of choline esters. The one that deactivates ACh more potently than the rest is acetylcholinesterase (AChE). It is estimated (Bowman & Rand 1980, p. 10.33) that each active site of the enzyme can hydrolyse 4 x 10^5 ACh molecules per minute. Anticholinesterases such as neostigmine and physostigmine - also known as eserine - (see Figure 10), block the de-activation of ACh by AChE and hence act as cholinergic agonists.

Figure 10: Chemical composition of anticholinesterases



ACh and SNPC neurones

Muscarinic receptors and SNPC neurones

Cross and Waddington (1980) made unilateral lesions of the NSP using the catecholamine neurotoxin 6-OHDA. The nigrae were removed to compare *in vitro* the specific binding of a tritiated muscarinic receptor ligand, [^{3}H] quinuclidinyl benzylate. A 30% decrease in specific binding of [^{3}H]QNB in the homogenate of the nigra ipsilateral to the lesion compared to the control side was observed. This could be explained by either a decrease in the number of muscarinic receptors in response to the lesion, or a change in their affinity state. The latter would suggest that

muscarinic receptors were present in the SN but not localized to the DA neurones of the NSP and possessing a high degree of plasticity. This was resolved using a technique for analysing protein-binding data called Scatchard analysis which relates the concentration of the protein to the concentration of the protein bound to the receptor sites. The shape of the plot in response to various concentrations of the drug reveals the affinity state of the receptor, assuming homogeneity of receptor type. Such analysis suggested that the decrease in specific binding of [^{3}H]QNB was due to a decrease in the number of muscarinic receptors with minimal alteration to their affinity state and hence localisation of muscarinic receptors to DA NSP neurones.

Of the five types of muscarinic receptor that have been described on the basis of molecular structure (m1-m5), only the m5 receptor sub-type was found to be present on medial SNPC neurones (Weiner et al. 1990). The m5 receptor is thought to conform to the M1 receptor described by pharmacological studies (described as M1-M3, Buckley et al. 1989). M1 receptors stimulate phosphoinositide hydrolysis and inhibit cAMP production. The hydrolysis of triphosphoinositol (inositol 1,4,5-triphosphate) releases calcium from intracellular stores. M2 receptors are coupled to adenylate cyclase which increases cAMP levels. M2 receptors are also linked to K^+ channels. M3 receptors are coupled to arachidonic acid metabolism (Mei et al. 1987).

Lacey et al. (1990) identified electrophysiologically 114 DA neurones in the mesencephalon; 30 in the SNPC, 84 in the VTA. Muscarine (3-100 uM) increased the firing rate of active neurones by up to 400%, and caused firing of quiescent neurones (neurones that were not firing). There appeared to be no difference in response between the SNPC and VTA DA neurones. The effects of muscarine on the DA neurones was blocked by the M1 muscarinic antagonist pirenzepine.

Thus it would appear that cholinergic stimulation of the SNPC and VTA DA neurones when mediated by muscarinic receptors is through the m5 / M1 receptor sub-type as described by molecular / pharmacological nomenclature, respectively.

Nicotinic receptors and SNPC neurones

Autoradiographic evidence suggesting the localisation of nicotinic receptors to DA NSP neurones was provided by Clarke and Pert (1985) and Clarke et al. (1985). As in the previous study a unilateral lesion of the NSP with 6-OHDA was followed by labelling with a tritiated compound, in this case [^{3}H] nicotine. Unlike the assay procedure used in the previous study where the nigral tissue was homogenized and all cell differentiation lost, autoradiography is crucially dependent on the preservation of slices of the brain with the tritiated compound *in situ*, bound to the target receptor. The slice is incubated in a light-proof container against tritium-sensitive film and by localising the radiation, detection of where the tritiated compound is bound is possible, thus revealing where the nicotinic receptors are. They then used computer aided densitometry to asses how intense the radiation, and hence the binding, was. [^{3}H] nicotinic binding in the anterior SN was found to be ~45% of the control SN, suggesting a localisation of nicotinic receptors to the DA NSP neurones. In the same study, Clarke and Pert (1985) showed localisation of nicotinic receptors to mesolimbic neurones, that is, those neurones projecting from the VTA to the NAcc. These neurones increase their firing rate following microinjection of the cholinergic agonist nicotine (Grenhoff et al. 1986).

In an attempt to characterise the effects of cholinergic binding on SNPC DA neurones Lichtensteiger et al. (1982) administered nicotine to the SN using microiontophoresis. This electrophysiological technique uses a glass

micropipette (tip diameter ~2-6 u) attached to a recording electrode. The pipette is introduced into the brain and by studying the firing activity from the recording electrode it is possible to localise the tip onto or even pierce a target neurone. The positively ionized nicotine, held in the micropipette by a small negative current, can then be released by the passage of a small positive charge (~10 nA) down the pipette which repels the ionized nicotine. The only way of controlling the amount of fluid released is by the length of time the current is reversed for.

It was found that administration of nicotine or ACh accelerated the firing of the DA neurones. Furthermore, administration of the specific nicotinic antagonist dihydro-beta-erythroidine blocked the excitatory effect of nicotine and ACh, whereas the specific muscarinic antagonist atropine caused little or no blocking of the excitatory effect. This suggests that the effect of cholinergic binding on SNPC DA neurones was excitatory in nature and mediated by nicotinic but not muscarinic receptors. Having established a profile of nigrostriatal firing in response to nicotinic stimulation of SN, Lichtensteiger et al. then measured DA turnover rate in the striatum in response to electrical stimulation of the NSP at the same average frequency as produced by nicotine (~10 Hz). They found a similar increase in DA turnover as to nicotine but if they produced the same average frequency by using a higher frequency only intermittently, the change in DA turnover dropped to an insignificant level. This suggests the DA release was to some extent dependent on a pattern of stimulation caused by the nicotine rather than just responding non-specifically to a certain level of stimulation.

The pharmacological specificity of the cholinergic stimulation of SN neurones suggested by Lichtensteiger et al. is however questioned by other studies including that of Massey and James (1978). They found that infusion of ACh excited 105 and inhibited 20 out of 190 neurones tested;

nicotine excited 40 and inhibited 2 out of 70 tested; and the muscarinic agonist carbachol excited 15 and inhibited 1 out of 19 neurones tested. Pre-treatment with the muscarinic antagonist atropine blocked excitation by ACh in 2 out of 3 tested and blocked both of the nicotinic excitations that were tested. This admittedly small sample of results would tend to suggest that cholinergic binding to SNPC DA neurones can produce excitation when mediated by nicotinic and muscarinic receptors.

AChE and SNPC neurones

Shute and Lewis (1967) demonstrated a 'ventral tegmental pathway' arising from neurones in the SNPC and the VTA accumulated AChE caudal to the site of a radiofrequency lesion. That the accumulation was caudal, rather than rostral, to the lesion suggested that the neurones were ascending. Shute and Lewis mapped their innervation of, among others, limbic and basal ganglia sites including the CP.

Butcher and Talbot (1978) review the evidence accumulated from their studies that demonstrate that 80-90% of SNPC neurones contain AChE. Given that a similar percentage of SNPC neurones contained DA and demonstrated a similar histological profile, Butcher and Talbot concluded that most if not all SNPC DA neurones contained AChE and suggested a cholinergic-dopaminergic interaction at the level of the SNPC.

The presence of AChE in SN was demonstrated by Lehmann and Fibiger (1978) who showed it was AChE present in SNPC neurones and not one of the other enzymes of that family. This they did using three pharmacological criteria; substrate selectivity, pharmacokinetics and selective inhibition. Any enzyme will show maximal activity in the presence of a specific substrate. Therefore the identity of any enzyme may be inferred by characterising the target enzyme's activity in the presence of suitable substrates. Pharmacokinetics refers to the effect on

the cholinesterase of the environment it is in. More specifically, by studying the concentrations of putatively different cholinesterases (such as acetyl- and butyrylcholinesterases) in a particular neurochemical environment, they may be differentiated by their rates of chemical transformation in response to that medium. Selective inhibition is where the cholinesterase is displaced at a given site by a similar but inactive protein and hence the normal pharmacological action of the cholinesterase is blocked. As each cholinesterase has its own selective inhibitor, the identity of the target cholinesterase can be determined by comparing the action of several selective inhibitors of candidate cholinesterases. Having made a unilateral lesion of the NSP using 6-OHDA, the SN was removed for enzyme bioassays (the estimation of the amount of a given enzyme in a tissue extract) using the contralateral SN as control. The bioassay showed no difference in ChAT levels in the two nigrae suggesting the ChAT present in SN was not localized to the NSP DA neurones. However, the cholinesterase present was found to have decreased by ~43% compared to control. They then used the three criteria explained above to identify the cholinesterase. True AChE hydrolyses ACh faster than it hydrolyses acetyl-beta-methylcholine, which it in turn hydrolyses faster than butyrylcholine. The main pseudocholinesterase, butyrylcholinesterase, hydrolyses these three substrates with different velocities (BCh > ACh > A-beta-Ch). It was found that the cholinesterase from SN had a hydrolysing profile which conformed to that of true AChE. Pharmacokinetic velocity refers to the speed of the chemical transformation of a drug in the presence of a substrate. The velocity profile of nigral cholinesterase corresponded more closely to true AChE than that for BChE which showed less inhibition by higher concentrations of ACh. Finally, the responses of nigral ChE, true AChE and BChE to two AChE and one BChE selective inhibitors were

compared. The BChE inhibitor only affected the nigral ChE at concentrations that similarly affected true AChE. Both the nigral ChE and the true AChE gave a similar dose response curve to the AChE inhibitors while the curve for BChE was very different. Lehmann and Fibiger therefore provided strong evidence for the localisation of a cholinesterase to NSP DA neurones, and that the cholinesterase was AChE.

Henderson (1981) further characterised the distribution of AChE in the SN using a combination of EM, HRP labelling and AChE histochemistry. AChE was present in two types of cell in the SNPC; the majority of cells had a large cell body and were labelled with HRP injected into the CP; the smaller cell type were not labelled with HRP. AChE was observed not only in the cell bodies but also in the neuropil suggesting that AChE may be localised to the dendrites as well as the cell bodies of SNPC neurones.

Does AChE have other functions in SN ?

Although AChE will be present wherever ACh is present, it is also present in some areas in quantities far greater than would be needed to hydrolyse the ACh present - including the SN - and is therefore now seen as a bad marker for cholinergic activity. Its presence is necessary but not sufficient to indicate cholinergic transmission in a certain area. The synthesizing enzyme for ACh, ChAT is thought to be a much better marker. Levels of ChAT measured in structures correlate well with sites known to have cholinergic neurones or terminals.

That AChE may have functions in the CNS other than hydrolysing ACh is suggested by its topographical mismatch with the neurotransmitter. Its localization in many adrenergic tissues supports an hypothesis proposed by Burn & Rand (1959) that ACh has a role in facilitating noradrenaline release. From peripheral studies (Bowman and Rand 1980) of the superior cervical ganglion, the latency of firing of the neurones and knowledge of

the pharmacological conditions suggest that diffusion of ACh from the site alone could account for the cessation of cholinergic influence on the post-synaptic site. As it seems the AChE present is unnecessary for its classical role in the hydrolysis of normal levels of endogenous ACh, additional explanations might be sought for its presence at the post-synaptic site. Although this is at a peripheral site, similar instances may well be present in the CNS.

Greenfield has proposed many functions for AChE in the SN, in fact, every function other than the hydrolysis of ACh. There is a large amount of AChE in the cerebrospinal fluid. Greenfield and Smith (1979) have shown that the increase in CSF AChE following stimulation of the CP is blocked by a lesion of the ipsilateral SN. They conclude that the SN may be the source of CSF AChE. In an attempt to delineate further the AChE release, potassium was injected into SN. This caused an increase in nigral AChE, a decrease in ipsilateral striatal AChE, and had the reverse effects on DA levels at these two sites. If, as is thought, the AChE is released by dendrites of SN DA neurones, this means the AChE and DA are acting differently in response to the same signal. This suggests that AChE release in the SN is not a non-specific release, nor is it released sympathetically with DA. The release of AChE in SN might then have specific physiological functions. However the introduction of exogenous potassium ions can have varying effects on the neuronal membrane dependent on its concentration. Relatively small amounts will depolarize the membrane and increasing the probability of firing. However if a large amount is added depolarization will occur unevenly over the membrane causing repeated action potentials. Thus how physiologically relevant Greenfield's study of the effects of exogenous potassium on SN is questionable.

AChE has been demonstrated by Greenfield et al. (1988) to have a depressive effect on nigrostriatal neurones. This would be predicted if it was deactivating an excitatory cholinergic input to SN. However, this effect has been shown by Greenfield et al. (1988) even in the presence of soman, an irreversible inhibitor of the enzymatic activity of AChE. There exist two types of cholinesterase inhibitors which may be differentiated by their chemical composition and the stability of the complex they form with the enzyme. Reversible anticholinesterases like physostigmine and neostigmine have a chemical composition similar to ACh and form relatively short-lasting complexes with AChE. Irreversible anticholinesterases like soman form a complex with the AChE that is stable and bear no similarity to the ACh molecule. The enzyme is deactivated by the dissociation of the phosphoryl group from AChE by hydrolysis. This process may take months to complete, hence the appearance of the deactivation of the enzyme being irreversible. Such studies as Greenfield's are unfortunately confounded to a certain extent by the ability of anticholinesterases to stimulate release of ACh itself from the pre-synaptic terminal. This action is potentially quite independent of its action as an enzyme and hence could provide an alternative explanation for the modification of nigral activity by its infusion into the SN.

Taylor and Greenfield (1989) suggested that the release of AChE is not obviously related to nigral DA neurone firing, but may reflect instead the level of excitation of inputs to the SN, especially that from the CP. Unfortunately, other than AChE release following stimulation of the CP there is little evidence to support it, nor is it clear what predictions would follow were it true. If the level of AChE was acting as a barometer of input to the nigra, it might be predicted that exogenous AChE might increase behaviour associated with the SN. Potassium-evoked release of

AChE caused no visible change in behaviour (Taylor et al. 1990). Greenfield concludes that AChE release is instead a measure of sensorimotor events. It will be interesting to see what evidence is produced to support such an hypothesis, perhaps using a paradigm such as that of Carli et al. (1985) that seeks to dissociate motor, sensory and sensorimotor functions.

AChE has also been shown *in vitro* to hydrolyse the peptide neurotransmitter SP. SP is co-localized with ACh in several brain sites including the ascending reticular system (Vincent et al. 1983). It also makes up part of the projection to SN from the CP. Administration of nicotine to nigral slices inhibits release of SP (Torrens et al. 1981). SP has little effect directly on SNPC DA neurones (Chiodo et al. 1987) and although there is a relatively high concentration in the SN there appears to be few binding sites (Quirion et al. 1983). This leaves us with a surprisingly large amount of a neurotransmitter with nowhere to go, and a surprisingly large amount of an enzyme that could de-activate said neurotransmitter in the same area of the brain. This does not lead us to any functional explanation for their presence in the SN, but leaves the possibility that the two may be connected.

The pedunculopontine tegmental nucleus

The PPTg is a relatively small nucleus in the pontomesencephalic tegmentum. It lies rostral to the peribrachial nuclei, ventral to the lateral lemniscus, dorsal to the pontine reticular formation and lateral to the medial portion of the brachium conjunctivum at the level of the trochlear nucleus. At its most rostral, ectopic neurones are seen to abut on the SN. The rostro-caudal length of the group is 500-800 um and the mediolateral diameter is 400-500 um at the rostro-caudal middle of the cell group.

The PPTg is thought to be involved in the control of motor activity. It has been shown by Jackson and Crossman (1983) to be a projection area of the motor cortex areas 4 and 6. Locomotion-like movements can be elicited by electrical or chemical stimulation of the mesencephalic locomotor region, of which the PPTg is thought by some to be a part (Garcia-Rill et al. 1985), but a recent study has demonstrated that locomotion is associated with activity in the cuneiform nucleus rather than the PPTg (Shojania et al. 1992). PPTg neurones are also reported to be rhythmically active when an animal is moving (Garcia-Rill et al. 1983). Electrophysiological studies have shown that electrical stimulation of the PPTg evokes an excitatory response in SNPC and SNPR (Clarke et al. 1987, Scarnati et al. 1984).

In 1963 Kaebler demonstrated a resting tremor by making electrolytic lesions of the cat tegmentum. The animals also showed hyperphagia and a presumably associated increase in weight. Unfortunately, there was great variance in the extent of the lesions produced, and as such Kaebler could not delineate destruction of which tegmental structures were necessary or sufficient for the production of tremor. Kaebler suggested that the lesions were having their effect by destroying nigro-reticular pathways.

Clinical studies have noted abnormalities in the PPTg of patients who suffered from PD. Zweig (1987) reported a significant loss of neurones >20 um from the PPTg PC in three out of four PD patients and three out of four patients suffering from PD and Alzheimer's disease (AD). There were also Lewy bodies present in the PPTg PC; their presence in the SNPC is a well-documented feature of the Parkinsonian brain. Jellinger (1988) compared large cell loss from the PPTg PC in PD, AD and progressive supranuclear palsy (PSP) with that of control brains. PD brains ($n=14$) had roughly half the number and density of large neurones compared to controls, AD patients ($n=17$) lost 29% of large neurones

and PSP brains had lost 60% (n=2) of PPTg PC large neurones. These large neurones of the PPTg PC were presumed to be cholinergic. These data suggest that AD, although associated with loss of cholinergic innervation of the cerebral cortex from neurones in the nucleus basalis of Meynert, does not feature a severe loss of PPTg ACh neurones. However these two studies do suggest a PPTg cell loss in PD and, though with a small sample size, PSP. The cell loss from the PPTg in these two clinical conditions which feature motor dysfunction may not be causative of these conditions, but could cascade from the loss of neurones afferent to this area that are crucial to the clinical features of these conditions.

Karson et al. (1991) have produced evidence from a small subject sample suggesting that the number of cholinergic cells in the PPTg is greater in schizophrenic than non-schizophrenic human brains at post-mortem. Just as PD is classically associated with a loss of nigrostriatal DA, schizophrenia is associated with an over-activity in the DA system, although whether this is tied to DA release or receptor changes is unclear. However, if there is an increased number of cholinergic PPTg neurones, it is an attractive proposition that the positive symptoms of schizophrenia might be tied to their over-stimulation of the midbrain DA systems.

The PPTg and the basal ganglia

Anatomy

Probably the earliest demonstrations of a connection between the PPTg and the basal ganglia showed a labelling of the SNPC following the injection of amino acids labelled with tritium into the PPTg of the rat (Saper & Loewy 1982), and cat (Moon Edley & Graybiel 1983). This was followed by Jackson & Crossman (1983) using anterograde and retrograde transport of horseradish peroxidase (HRP, an enzyme transported in neurones axoplasmically).

The basal ganglia has historically been viewed as a collection of primarily inhibitory 'loop' systems, with the various nuclei seen as 'restraining' the firing of the nucleus/nuclei it projects to. The SNPC has inhibitory inputs from the CP, NAcc and also the medial raphe nuclei. McGeer and McGeer (1984) proposed that the SN received a probably excitatory cholinergic innervation from the PPTg. Following injection of the potent excitotoxin kainate into the PPTg PC, cell death and a loss of ChAT activity in the pars compacta of the SN was observed. McGeer and McGeer proposed that it may be overexcitation of the PPTg projection to the SN that induces necrosis of cholinoceptive DA neurones in SN in PD.

Sugimoto & Hattori (1984) define the pars compacta area of the PPTg as the area populated by large (2000-6000 um^3) neurones. To map the efferent projections areas of the PPTg, leucine labelled with a radioactive form of hydrogen, tritium, was injected into the PPTg PC. Sections were then cut and treated with photosensitive emulsion. Where the silver grains of the emulsion are observed using EM will show where the efferent projections of the PPTg PC have transported the tritiated leucine. Silver grains were observed in the VTA, SNPC, zona incerta and the lateral hypothalamic area (LHA), showing that fibre bundles project to or through these areas. Tritiated choline injections to the thalamus and STN produced retrograde labelling of the PPTg PC but no such labelling was observed following similar injections to SN. Interestingly, monosynaptic projections were observed from the PPTg PC to thalamostriatal neurones.

In 1986, Scarnati et al. used microiontophoresis to attempt to characterise the nature of the putative excitatory projection from the PPTg to SNPC. Using electrophysiological techniques, 120 nigral neurones were recognized as having PC DA neurone characteristics; slow firing rate, triphasic shape and long duration of action potentials. Short latency orthodromic excitation was observed in 30 of these cells following

electrical stimulation of the peribrachial area. ACh infused into the peribrachial area induced activation in 4 cells all of which were antagonized by atropine (50-200 nA) suggesting the cholinergic stimulation was perhaps mediated by muscarinic receptors. Glutamate (5-10 nA), however, excited all 30 neurones, and this excitation was antagonized by glutamate antagonists glutamic acid diethylester (GDEE) and D-alpha-aminoadipic acid (DAA). Atropine did not antagonise the excitatory effects of glutamate, nor did the glutamate antagonists antagonise the excitatory effects of ACh. Scarnati et al. concluded that although there was a cholinergic population in the PPTg, reports of its projection to the SNPC could be explained as fibres projecting through the SNPC *en route* to other structures.

Woolf and Butcher (1986) provided evidence for a cholinergic projection from PPTg to SNPC using ChAT immunohistochemistry (immunohistochemistry: the study of antibodies, antigens and their interactions when administered to histological sections), fluorescent tracer histology (whereby antibodies tagged with a fluorescent dye enable cellular components to be resolved using a fluorescent microscope), and AChE pharmohistochemistry. Fluorescent tracers injected into the SN produced labelled in the PPTg and LDTN. Neurones containing AChE were observed to innervate the SN from the PPTg and LDTN. However, although this suggests there is a projection from the pontine to the SN, ChAT immunohistochemistry suggested it was not cholinergic in nature.

In 1987 Scarnati et al. further characterized the PPTg-SN projection describing two types of neurones projecting to the SN, one having a relatively low firing rate of 0.5-8 spikes/s and a triphasic impulse with duration 3-4 ms, the second having a firing rate of 15-20 spikes/s with a biphasic impulse lasting less than 3 ms. There was no attempt to identify the neurotransmitter mediating the excitatory influence of both types of

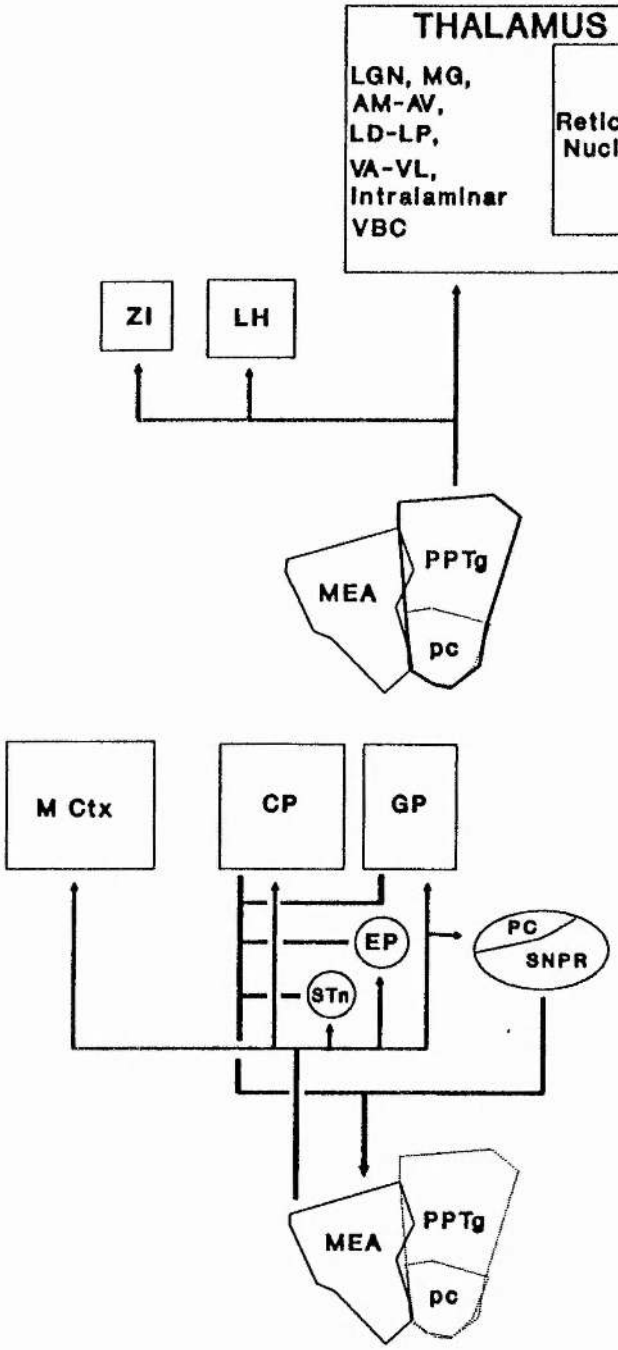
PPTg neurones on SN neurones. In an EM and Golgi study Scarnati et al. (1988) found degenerated terminals in the SN following electrolytic lesion of the PPTg. These were seen to make contact on dendritic processes of dendritic shafts. Post-synaptic high electron density was observed and was thought to represent a post-synaptic thickening. The PPTg contained 4 types of neurone based on morphology. Both large (20-40 μm), multiangular and medium angular shaped neurones appeared to have axons projecting outside the PPTg.

These reports show a projection from the PPTg to the SNPC, but equivocal evidence for it being cholinergic in nature. This view has been proposed probably most strongly by Wainer and his colleagues who believed that those few neurones that did project from the PPTg to SNPC were firstly non-cholinergic in nature, and secondly ectopic neurones of the midbrain extrapyramidal area (MEA, see Figure 11), (Lee et al. 1988).

Lee et al. used ChAT immunohistochemistry and retrograde tracing to map the projections to and from the MEA and PPTg. The MEA is defined as the non-cholinergic neurones medial to the cholinergic neurones of the PPTg. As is clear from the diagram, the thalamus is the major target of PPTg neurones, while the MEA appears to project to most of the basal ganglia, including the SNPC. The only significant retrograde labelling of PPTg following injections to SN were those where the injection had been shown to spread beyond SN.

However, using a similar approach to Wainer and his colleagues Beninato & Spencer (1987) found very different results. using simultaneous retrograde labelling by HRP and ChAT immunohistochemistry. HRP was injected into SN and subsequently localized on sections of the rat brain, followed by immunocytochemical localisation of ChAT. HRP labelling was found in the PPTg in proportion

Figure 11: Projection sites of the PPTg and midbrain extrapyramidal area (redrawn from Lee et al. 1988).



Abbr.: LGN lateral geniculate nuc.; MG medial geniculate nuc.; AM-AV anteromedial and anteroventral thal. nuc.; LD-LP laterodorsal and lateroposterior thal. nuc.; VA-VL ventrolateral thal. nuc.; VBC ventrobasal thal. nuc.; EP entopeducular nuc.; M Ctx motor cortex; ZI zona incerta.

to the size of the injection in SN. ChAT-immunoreactive neurones were found to exist in the PPTg and the LDTN. Double-labelled neurones, i.e. by HRP retrograde transport from SN and ChAT immunoreactivity, were found throughout the ipsilateral PPTg.

Although this suggests a cholinergic projection to SN, it is unclear whether the projection is to SNPC, SNPR, or both. Neuroanatomical and electrophysiological evidence for a cholinergic projection from PPTg to SNPC was provided by Clarke et al. (1987). First a double-labelling procedure was used to confirm that cholinergic neurones of the PPTg projected to SN. Then in the electrophysiological part of the study kainate was injected into the peribrachial area causing dose-dependent stimulation of SNPC neurones. The latency of the excitation of the SNPC neurones was thought to be too quick to have been caused by diffusion of kainate to SNPC to effect the neurones directly. It was also found that intraveneous administration of the nicotinic cholinergic antagonist mecamylamine blocked the stimulation at the same dose as blocks direct stimulation of SNPC neurones with nicotine. This suggests that there are nicotinic receptors on the SNPC DA neurones mediating cholinergic stimulation from the PPTg.

Tokuno et al. (1988) provided electron microscopical evidence that SNPC nigrostriatal neurones receive monosynaptic projection from the PPTg. An electrolytic lesion of the cat PPTg was made and HRP injected into the CP. EM revealed that degenerating axon terminals in SNPC made asymmetric contact with dendrites of neurones labelled with HRP. As in a control condition with no lesion to PPTg there were no degenerating axon terminals in SNPC, it is most probable that the terminals were from neurones in the PPTg, synapsing directly onto dopaminergic dendrites of the SNPC.

As a direct answer to the findings of Wainer and his colleagues, Gould, Woolf and Butcher (1989) used the same techniques but with a more complicated protocol for enhanced demonstration of ChAT-like immunoreactivity. Following infusions of the fluorescent tracers True Blue, propidium iodide or fluorogold to rat SNPC, neurones showing retrograde tracing from SNPC and ChAT-like immunoreactivity in the PPTg and LDTN were observed. Specifically, the cells were all larger than 15 um and found in the ipsilateral PPTg. This agrees with the description of PPTg-fugal neurones by Scarnati et al. (1988). Specific infusions of tracer into SNPC revealed 2-3 times as many neurones labelled in PPTg and LDTN than infusions into SNPR. Gould et al. also observed what they describe as 'bouton-like protuberances' in the SN following anterograde tracing with a similar distribution observed with ChAT immunoreactivity in SN. Woolf et al. (1990) similarly showed cholinergic projection from PPTg-LDTN to SNPC in the cat.

Martinez-Murillo et al. (1989) also used ChAT immunoreactivity to show asymmetric synaptic specialisations between cholinergic terminals and unlabelled dendrites in areas of the SNPC where there were no ChAT-positive neurones. The authors suggested that the surrounding glial cells were effectively isolating the points of synaptic contact between the cholinergic neurones and the SNPC DA neurones and that this implied a functional relationship between them.

Probably the clearest evidence for a cholinergic innervation of SNPC DA neurones is a double immunocytochemical study by Bolam et al. (1991) in the ferret. ChAT immunoreactivity and TH immunoreactivity were used to localise cholinergic terminals and dopaminergic neurones respectively. In the SNPR there were few TH positive cells, but many TH positive dendrites from neurones in the SNPC. However, it was in the SNPC that ChAT positive boutons were observed to be apposed to TH

positive dendrites most densely. Using both light and EM, cholinergic axons in the SNPR of the ferret were seen to make several synaptic contacts with TH positive dendrites. Many other non-ChAT immunoreactive boutons were seen to form synaptic appositions to SN neuronal dendrites demonstrating that the ACh input to the SNPC DA neurones represents only part of the input to SN neurones.

That a functional relationship does exist between the cholinergic nuerones of the PPTg and the DA-containing neurones of the SNPC was demonstrated by Blaha and Winn (In press). Using chronoamperometrometry, a technique which allows the *in vivo* measurement of dopamine release with a high temporal and neurochemical resolution when combined with a stearate-modified electrode, DA efflux in the CP was measured. Microinjection of the direct cholinergic agonists carbachol and nicotine and the cholinesterase inhibitor neostigmine increased DA release relative to baseline. A lesion of the neurones of the PPTg with quinolinic acid attenuated the response to neostigmine, but significantly increased the effect of intranigral nicotine. This suggests that the stimulatory effect on striatal DA efflux of intra-nigral neostigmine is caused by its prevention of breakdown of endogenous ACh released by terminals whose cell bodies are in the PPTg. Furthermore, because the lesion increased the effects of intra-nigral nicotine it is likely that the loss of ACh terminals in the SN has caused supersensitivity to develop in the cholinergic receptors known to exist on the DA-containing neurones of the SNPC.

Behaviour

Niijima and Yoshida (1988) investigated the behavioural effects of manipulation of the PPTg projection to the SNPC. They observed that 0.8 g of kainate caused ipsilateral body flexion and circling, peaking 30 min

post-injection (18 revs / min) which then decreased over the following 24 hr. To test whether this rotation was mediated by DA they used the DA antagonist haloperidol (0.1 mg/kg and 0.5 mg/kg) injected systemically: this attenuated the rotation (59% and 92% respectively). Alpha-methyltyrosine (AMPT) inhibits TH, an enzyme which is vital to the synthesis of DA. Systemic injection of AMPT 6 hr before kainate abolished rotation (200 mg / kg, -96%). Noradrenergic antagonists had no effect on rotation to kainate. The muscarinic antagonist atropine sulphate (10 μg) injected bilaterally into the VTA and SN reduced rotation to 32.7 / 30 min as compared to 128 / 30 min to saline before kainate. HPLC analysis allowed calculation of DA turnover in CP and NAcc from ratio of DA metabolites to DA; DOPAC + HVA / DA. DA turnover was increased in both the CP and the NAcc, and the increase was blocked by atropine sulphate in the NAcc but not the CP. The authors conclude that the kainate was transsynaptically stimulating the SNPC and VTA neurones.

Cholinergic control of midbrain DA-mediated behaviour

Although there is pharmacological and electrophysiological evidence for the presence of nicotinic and muscarinic receptors on the DA-containing neurones of the VTA (Clarke and Pert 1985, Grenhoff et al. 1986, Lacey et al. 1990), few studies have examined the behavioural effects of direct cholinergic stimulation of the mesolimbic DA system.

However, locomotion produced by administration of DA agonists into the NAcc can be replicated by systemic injections of nicotine in rats that have previous experience of the drug. Clarke et al. (1988) showed that increased locomotion was accompanied by an increase in DA utilization in the mesolimbic DA system as measured from tissue samples taken 30

min after nicotine administration. The increase in locomotion was blocked by a bilateral 6-OHDA lesion of the NAcc.

The involvement of the PPTg in behaviours associated with mesolimbic DA is suggested by the production and blocking of conditioned place preference by the microinjection of nicotine and lesion of the PPTg respectively (Bechara and van der Kooy 1989; Iwamoto et al. 1983).

Smelik & Ernst (1966), having demonstrated compulsive gnawing in response to crystalline DOPA and apomorphine implanted in the CP of the rat, attempted to produce the same results with implantation of crystalline physostigmine (a cholinesterase inhibitor) in the SN. Compulsive gnawing was observed with a latency of 30 min and duration of 2 hr following 30 μg of physostigmine. This could be blocked with systemic atropine (a muscarinic antagonist). The authors concluded that the SN had a behaviourally important link to the CP that could at least in part be manipulated by muscarinic cholinergic stimulation of the SN. It should be noted, however, that atropine in addition to its action as a muscarinic antagonist also has a direct effect on neurones depressing activity.

Wolfarth et al. (1974) found injections of the direct DA agonist apomorphine (2 μg) to rabbit SN decreased locomotor activity, which is the opposite of its effect in the CP. Nigral carbachol (2 μg) induced locomotion, sniffing, head turning, ipsilateral rotation and epileptiform EEG discharges. This suggests that Wolfarth was in fact 'overdosing' the animals. However, pre-injection with apomorphine attenuated the behavioural and abnormal EEG effects of carbachol. In a series of experiments the direct cholinergic agonists ACh and methacholine, and the indirect agonist neostigmine (a cholinesterase inhibitor), were injected bilaterally to SN. At 95 μg / nigra, ACh induced locomotion and EEG alerting for ~25 min. At higher doses (475 μg / nigra) this lasted over 40

min with epileptiform EEG discharges and body seizures. There was also sniffing and turning behaviour that was interpreted by the authors as stereotypic behaviour. Methacholine and neostigmine had similar effects except that there was a longer delay before the behavioural effects of neostigmine appeared (20 min as compared to 5 min for ACh). This is what would be expected as its main stimulatory action is thought to be by allowing the accumulation of endogenous ACh by blocking the actions of AChE. It should also be considered that neostigmine also has direct stimulant effects on cholinoceptive neurones. The effects of methacholine were more prolonged than with ACh and the dosage at least an order of magnitude less than that of ACh required to produce an effect. This was thought to be because methacholine is not as strongly affected by AChE as ACh. As with Smelik & Ernst (1966) pretreatment with atropine blocked the behavioural effects of cholinergic stimulation. It was found that similar behavioural effects to cholinergic agonists were found with systemic injections of the DA agonists amantadine and apomorphine. Also the behavioural effects of neostigmine were blocked by pretreatment with spiroperidol (spiperone) the most potent of the antipsychotic neuroleptic drugs. Given the massive dosages Wolfarth used it is possible that what Wolfarth interpreted as stereotypy were in fact convulsions; repeated jerking movements that would satisfy most criteria for stereotypy. Such convulsions are most probably mediated by the nigrotectal pathway rather than the nigrothalamic or nigrostriatal pathways. Wolfarth suggests that the effects of cholinergic substances injected to SN are mediated by two different pathways, nigrothalamic and nigrostriatal. Furthermore she suggests that the effects of carbachol has opposite effects on the CP depending on which pathway is stimulated. This she concluded after injections to lateral (nigrothalamic) and medial (nigrostriatal) SN, but no further evidence has been produced to support this claim.

Arnt and Scheel-Kruger (1979) were interested in the mechanisms mediating GABA-stimulated contralateral turning in the SN. As described earlier, the turning following intranigral muscimol was attenuated by large doses of carbachol injected into the SN. These doses of carbachol alone produced postural assymetries and infrequent ipsilateral turning when administered to the rostral SN. The doses that produced these effects, 5 and 10 μg carbachol, are larger than doses found to be behaviourally stimulating in studies in this laboratory, and indeed convulsions followed by death has been observed even at the 5 μg dose.

The NSP passes though the lateral hypothalamus, an area associated with the control of feeding. To investigate a possible role of the NSP in studies of feeding following manipulations of the lateral hypothalamus, Winn & Redgrave (1979) injected mixtures of ACh and physostigmine (2.5, 5.0 and 10.0 μg of each) into the rat SN. They found a dose-dependent increase in consumption of spaghetti compared to saline and a significant negative correlation between consumption response and proximity of the injection site to medial SNPC. Cholinergic stimulation had no significant effect on drinking. In a second experiment, 0.5, 1.0 and 5.0 μg of carbachol increased dose-dependent increase in lever pressing for a food reward. The higher two doses of carbachol produced some cases of stereotypic gnawing and biting. These latter findings were replicated by Taha & Redgrave (1980) who found they could block the oral stereotypy caused by 5.0 μg carbachol by s.c. pretreatment with the DA antagonist haloperidol (0.4 mg/kg). Consumption of spaghetti in response to 0.5 μg carbachol was also abolished by s.c. haloperidol. It is interesting to note that the haloperidol did not just block the increase in consumption following carbachol, but abolished all feeding. This casts doubt as to whether the dose of haloperidol was specifically blocking effects of nigral stimulation or having a general effect on the animals ability to respond in

a given situation, even though the dose of haloperidol used had no effect on locomotor behaviour. Winn and Redgrave (1981) observed dose dependent consumption of dry spaghetti with no effect on drinking using the indirect cholinergic agonist physostigmine sulphate injected into SN, and very similar results following systemic or microinjection to CP of low doses of d-amphetamine (Winn et al. 1982). It was also observed that doses of carbachol that did not induce locomotion in the presence of food, did so in its absence. These results taken overall suggested that stimulation of the nigrostriatal pathway might preferentially induce consumption of palatable food.

Winn et al. (1983) investigated this hypothesis in a series of experiments that also sought to discover whether cholinergic receptor blockade affected muscarinic cholinergic stimulation of SNPC. Behavioural analysis was made of eating, locomotion, sniffing, rearing and unconditioned responses to unilateral injection of carbachol to anterior SN. Having established a dose-dependent increase in consumption of dry spaghetti the experiment was repeated with wood chips instead of food. There was no significant change in any behaviour including gnawing. This suggests that the stimulation was not just preferentially but specifically consumption (as opposed to just gnawing). Finally, a similar increase in consumption was elicited using a mixture of ACh and eserine was blocked using the muscarinic antagonist atropine. This was interpreted as above to suggest that the cholinergic stimulation of SNPC is mediated by muscarinic receptors. Consumption of palatable food was also shown to be rate dependent in that increased feeding only occurred in those rats that had been allowed access to the food before injection. Rats that had not been exposed to the food pre-injection, and rats that been food-deprived did not show an increase in consumption following injection.

Further studies have demonstrated behaviours other than feeding can be elicited by cholinergic stimulation of the SNPC. Winn (1991) found that male sexual behaviour was affected in quite a subtle manner. Intranigral carbachol had no effect on latency to mount, first intromission or first ejaculation. However, intromission frequency; calculated as latency to ejaculate divided by number of intromissions, was significantly reduced following intranigral carbachol. As discussed by Winn the significance of this is not clear but does represent more effort on the part of the male rat for the same latency to ejaculate.

In the same series of experiments, Winn also demonstrated that rats that have been pre-exposed to saccharin and tap water will increase their consumption of the palatable fluid with no increase in consumption of tap water following an intranigral injection of carbachol. Note that saccharin although having a very sweet taste has no food value, and hence the rats will have been responding to its taste rather than any calorific intake. Winn (1991) suggested that cholinergic stimulation of the SNPC can increase the performance of behaviours which the animal has a pre-existing tendency to perform.

The evidence suggests that the DA-containing neurones of the SNPC respond to external, activating sensory stimuli and in some way influence the initiation or control of motor response to such stimuli. These neurones have both nicotinic and muscarinic cholinergic receptors and their activity is under a cholinergic influence from neurones whose cell bodies lie in the PPTg and perhaps also the LDTN. Previous studies have shown that microinjection of cholinergic agonists into the SN can influence behaviour in a manner that suggests there effects are mediated by the DA-containing neurones of the SNPC. The experiments presented in this thesis will investigate the functional significance of the cholinergic innervation of SN, demonstrate that the effects of this innervation are mediated by the

DA-containing neurones of the SNPC and investigate the role of cholinergic control of midbrain DA neurones in the control of behaviour.

Surgery

Rats had chronic intracranial guide cannulae placed in the brain to allow repeated access to the same injection site with a thinner micro-injection cannula 2 mm longer than the guide cannulae. The co-ordinates targetted for the actual site of injection were therefore 2 mm lower than the co-ordinates given for the placement of the guide cannulae in the two orientations described below.

Orientation of Level Skull

Placed in a stereotaxic frame with the incisor bar set for level skull. Using co-ordinates from Paxinos and Watson (1982): SN (interaural line (IAL) +3.8 mm, Lateral $\pm$2.2 mm, Vertical -5.5 mm); and the LHA (Bregma -2.3 mm, Lat. $\pm$2.0 mm, Vert. -5.5 mm).

Orientation of De Groot

Placed in a stereotaxic frame with the incisor bar 5.0 mm above the interaural line. The stereotaxic co-ordinates were SN: (Bregma -2.8 mm, Lat $\pm$2.0 mm, Vert -5.5 mm); VTA: (Bregma -2.6 mm, Lat $\pm$1.0 mm, Vert -5.5 mm).

Chronic cannulation

The rats were implanted with 11.5 mm stainless steel guide cannulae (23 ga), roughened beforehand to ensure purchase. These were secured using dental cement and skull screws. The cannulae were kept patent by the presence of removable 30 ga stylets.

Test procedure

The group of rats for testing were left for 1 hr in the test cages with weighed amounts of whatever was being consumed. Each animal was in

turn removed from its cage to receive a microinjection of drug or just vehicle solution depending on the condition. This was delivered by a 13.5 mm 30 ga stainless steel cannula connected to a 10 μl SGE syringe propelled by a Harvard infusion pump at a rate of 0.5 $\mu l.min^{-1}$ for 60 sec. The injection cannula was left in place for a further 30 sec to ensure the diffusion of the drug away from the cannula tip. The animal was then returned to its cage.

The order of administration of drugs was counterbalanced with a minimum of 48 hr between injections.

Direct cholinergic agonists used

Carbachol (carbamylcholine chloride) is a cholinergic agonist known to have agonistic effects at muscarinic receptor sites. It is now also known to have effects at nicotinic receptor sites but this is thought to be at a different dose range to its effects as a muscarinic agonist (Rang and Dale 1987, p.119). This agonist was used in this thesis as an extension of previous work which had used it as a muscarinic agonist (Winn et al. 1983). However, the possibility of its actions being also being mediated by nicotinic receptors does exist. In the last few years there has been an increasing literature on receptor sub-types and the specificity of agonists and antagonists. Carbachol should now be regarded as a useful cholinergic agonist, but not the ideal choice as a specific muscarinic agonist. There now exists specific M1 receptor agonists such as McN A 343, and it is likely that agonists specific for further muscarinic receptor sub-types will be available with further research.

Nicotine (nicotine hydrogen tartrate) is the classic nicotinic agonist and is thought to have no effect on muscarinic binding sites.

Statistical analysis

Analysis of Variance

Almost all of the data produced in this thesis were analysed parametrically using analysis of variance. The design of most of the experiments was of repeated measures. This being a special example of a multivariate ANOVA with the dependent variables being different levels of the same Within Subjects Factor, the analysis was performed on the PC version of SPSS using the MANOVA command with the WSFACTOR sub-command.

Post-hoc analysis

Statistically significant main effects and interactions were then further analysed using Newman-Keuls test for multiple comparisons.

Animal euthanasia

The animals used were killed by either stunning followed by decapitation or by perfusion. This consisted of an overdose of sodium pentobarbitone (0.4 $ml.kg^{-1}$) then perfused transcardially with saline followed by 300 ml fixative (4% paraformaldehyde/0.05% gluteraldehyde, in 0.1 M phosphate buffer). The brains were stored in fixative solution.

Histology

The brains having been stored in formalin for at least 24 hr were cut using a freezing microtome. The slices were 40 u thick and mounted on lumps of glass for subsequent staining with cresyl violet and studied under a Leitz 'Diaplan' microscope.

4 Direct and indirect cholinergic stimulation of anterior SN

Introduction

Microinjection of cholinergic substances into the rat anterior SN produces dose-dependent increases in activities for which the animal has both a low baseline rate and a positive predisposition (Winn et al. 1983; Winn 1991). Increased eating of spaghetti but not lab chow has been observed in satiated rats following microinjection of the muscarinic agonist carbachol (Winn et al. 1983), the AChE inhibitor eserine (Winn and Redgrave 1981) and mixtures of ACh and eserine (Winn and Redgrave 1979). This latter response was blocked by pre-injection into the SN of the muscarinic antagonist atropine, suggesting that it was mediated by muscarinic rather than nicotinic cholinergic receptors (Winn et al. 1983). Similarly, increased drinking of saccharin solution, but not water, has been shown in satiated rats following microinjection of carbachol, which has been shown also to affect sexual behaviour in male rats (Winn 1991). However, those doses of carbachol which affect food intake (0.1 and 0.5 μg/0.5 μl) appear to have no effect on other unconditioned activities such as gnawing, locomotion, grooming, sniffing or rearing (Winn et al. 1983; Winn 1991).

Previous psychopharmacological work has concentrated on cholinergic stimulation using the direct muscarinic agonist carbachol, but the presence of nicotinic receptors and cholinergic terminals suggests there may be differing effects of cholinergic activity depending upon which class of cholinergic receptor is stimulated. It has been suggested that ACh acts as a 'fast' neurotransmitter through the nicotinic receptor and as a 'slow' neurotransmitter through the muscarinic receptor (Kemp et al. 1977). Nicotinic cholinergic stimulation would be of a prescribed excitatory nature whereas muscarinic stimulation could be excitatory, inhibitory or it

could act to potentiate the neuronal membrane with respect to further binding by other molecules. It is therefore appropriate to make a direct comparison between microinjection of a direct muscarinic agonist, a direct nicotinic agonist and AChE inhibitors (which should reveal the effects of endogenous ACh) into the anterior SN. Hence, in the present experiments the actions of carbachol were compared with those of nicotine, eserine and neostigmine. The potency of action of neostigmine has not been well explored. There were therefore two ranges of doses used for this drug. Feeding and drinking were both measured, and a detailed assessment of other unconditioned activity including grooming, locomotion, gnawing and elements of exploration was carried out.

Methods

Animals

55 rats with mean body weight at the time of surgery 259.7 g (SE = 2.06) were used.

Stereotaxic surgery

Rats were anaesthetized with Avertin and placed in a stereotaxic frame in the orientation of De Groot. The rats were implanted unilaterally with cannulae terminating above the anterior SN.

Intracranial drug administration

The rats were arbitrarily assigned to one of five drug groups and received the following doses and the vehicle solution sterile saline: carbachol 0.1, 0.5, 5.0 μg; nicotine 0.1, 0.5, 5.0 μg; eserine 2.5, 5.0, 10.0 μg; neostigmine 0.1, 0.5, 1.0 μg; or neostigmine 1.25, 2.5, 5.0 μg.

Test procedure

Following recovery from surgery, the rats were habituated to the test cages and procedure. Rats were observed in individual cages with

sawdust-covered floors for 60 min prior to and 60 min following microinjection. During the habituation and test sessions rats had free access to weighed amounts of tap water, normal lab chow, dry macaroni (1470 Kj/100 g, 13.0 g protein/100 g) and polystyrene packing chips. Rats were transferred to fresh, provisioned cages every 30 min to monitor consumption during the test period before and after microinjection.

Behavioural analysis

Each rat's behaviour was quantified by the method of Fray et al. (1980). The observer, who was blind to which drug and dose the rat had received, recorded the presence of the following responses every 5 min in a 10 sec observation period:

(a) still/asleep: lying or sitting; not engaged in any activity.

(b) locomotion: all 4 legs moving for >3 sec.

(c) rearing: both forepaws raised from the cage floor.

(d) sniffing for >3 sec.

(e) grooming (forepaw): grooming of the face and head with the forepaws for >3 sec.

(f) grooming (other): grooming other than that in (e) for >3 sec.

(g) gnawing polystyrene chips for >3 sec.

Statistical analysis

Food and water intake data were analysed by analysis of variance (ANOVA). The data for each drug group was analysed separately with dose (saline and three doses of drug) and time (0-30 min and 30-60 min post-injection) being repeated measures. Post-hoc analysis was made with Newman-Keuls test for multiple comparisons. The remaining behavioural data, although collected by the observational method of Fray et al. (1980), could not be analysed using the method of those authors (the Information Statistic). This requires that all measures be independent, but

the data in the present study involved repeated measures, each animal serving as its own control. The control mean and standard deviation for each behavioural category was calculated from the saline data of each of the 5 drug groups independently for the first 30 min and first 60 min post-injection. The numbers of rats in the drug conditions scoring >1 SD and >2 SD above the control mean was tabulated.

Results

Histology

Cannula placements were verified post-mortem and Figure 12 presents representative sections showing these. Following histological examination, 46 rats were found to have injection sites in or immediately rostral to SN. The close association between SNPC and SNPR formed by the descending dendrites of PC DA neurones means that an injection site in PR will affect the dendrites of PC neurones. Those rats found by histological inspection to have misplaced cannulae - that is outside the SN - were excluded from the behavioural analyses and are shown on Figure 12 as misplaced cannulae. Although no formal examination was made, inspection of the histology suggests that there were no differences in cannulae placements between drug groups. Two rats not included in the analyses died following convulsions after the highest dose of neostigmine.

Eating and drinking in response to cholinergic stimulation

Analysis of the consumption of macaroni (see Figure 13) after injection of carbachol revealed a main effect of dose ($F=4.77$ $df=3,30$ $p<0.01$) but no effect of time ($F=1.98$ $df=1,10$) and no dose x time interaction ($F=2.59$ $df=3,30$). Similarly, nicotine produced a main effect of dose ($F=3.52$ $df=3,27$ $p<0.03$) but not time ($F=3.32$ $df=1,9$) and no dose x time interaction ($F=0.29$ $df=3,27$). The low dose range of neostigmine produced a main effect of dose that approached significance ($F=3.22$

Figure 12: Representative sections, redrawn from the atlas of Paxinos and Watson (1982) showing nigral (●) and misplaced (▲) cannulae. Figure A shows those injection sites for rats receiving carbachol and nicotine, Figure B (over) shows neostigmine and eserine. The placements of cannulae delivering the different drugs are shown separately, collapsed onto right or left hemispheres. The neostigmine placements show both high and low doses.

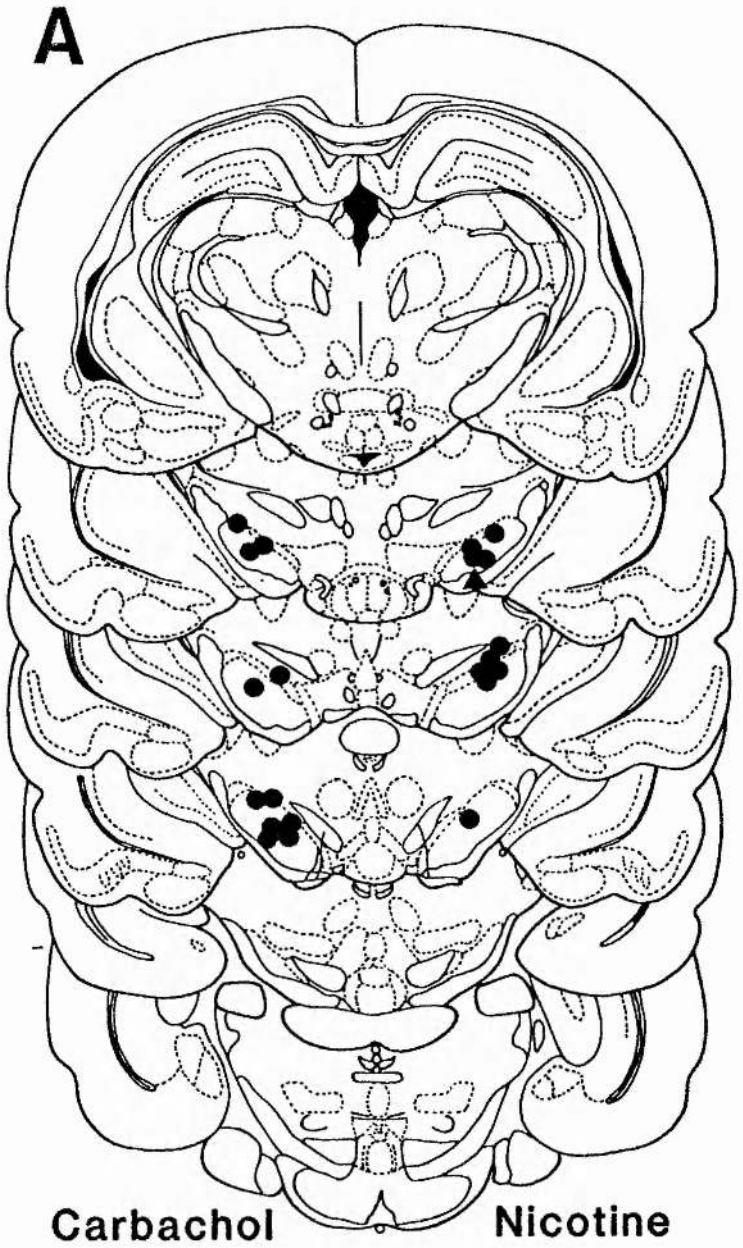
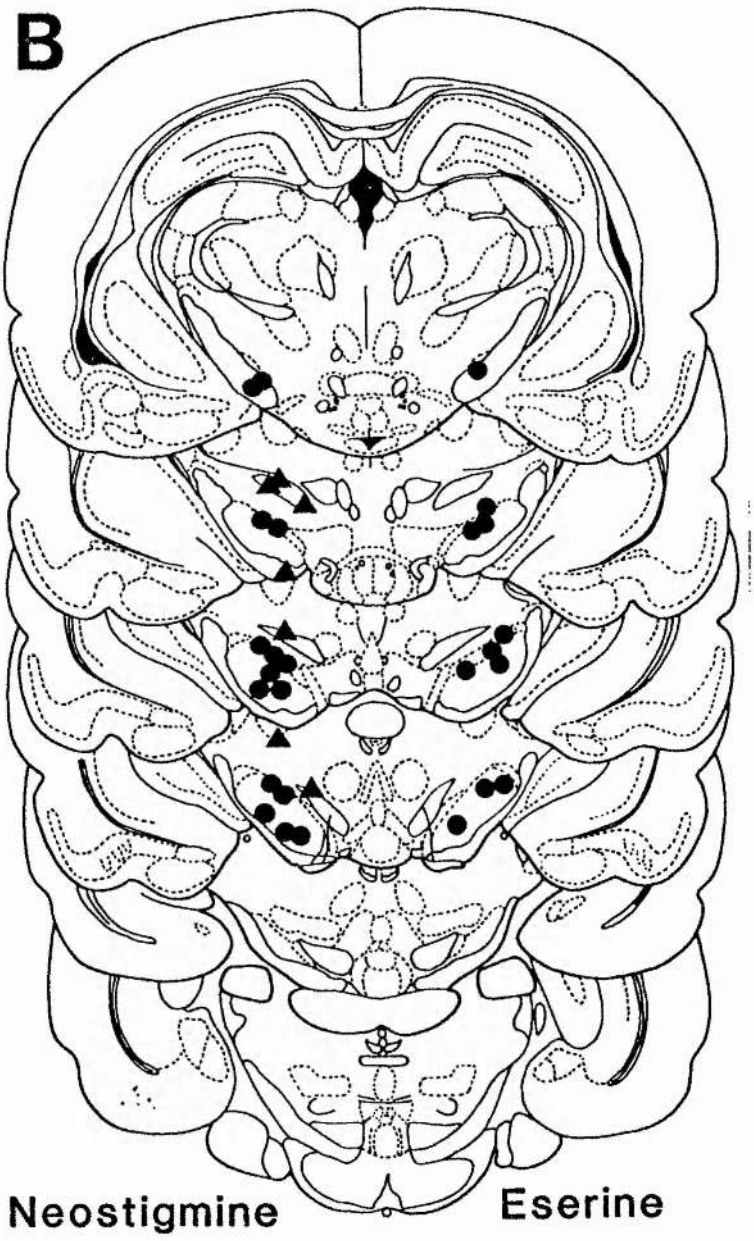


Figure 12: See legend on previous page for details

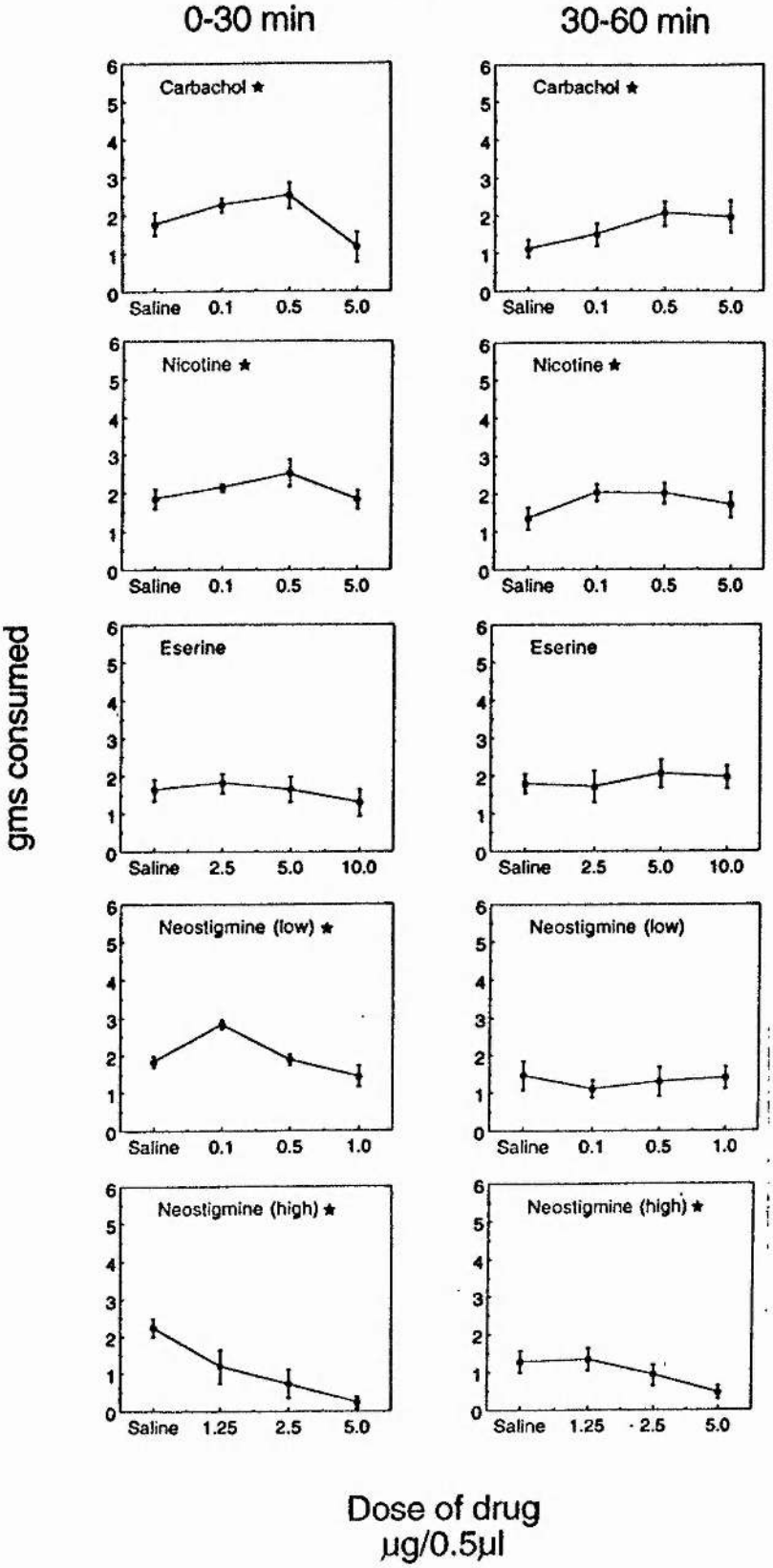


df=3,15 p=0.053), no main effect of time (F=5.60 df=1,5) but a dose x time interaction (F=5.12 df=3,15 $p<0.02$). Eserine showed no main effects of dose (F=0.38 df=3,30), time (F=1.09 df=1,10), or interaction (F=0.63 df=3,30). The high dose range of neostigmine showed a significant main effect of dose on macaroni consumption (F=6.02 df=3,21 $p<0.005$), no main effect of time (F=0.75 df=1,7), but a significant dose x time interaction (F=4.36 df=3,21 $p<0.02$).

Post-hoc analysis revealed significant effect of dose of carbachol collapsed over 0-60 min between saline and 0.5 μg ($p<0.01$). For nicotine, there were significant differences between saline and 0.5 μg ($p<0.05$). The dose x time interaction in response to low doses of neostigmine revealed a significant difference between saline and 0.1 μg over 0-30 min ($p<0.05$). The effect of 0.1 μg neostigmine was greater over the 0-30 min than 0.5 μg and 1.0 μg ($p<0.05$ and $p<0.01$ respectively), and greater than 0.1 μg over 30-60 min ($p<0.01$). Within the higher dose range of neostigmine, there was a significant decrease in eating after 5.0 μg neostigmine compared to saline ($p<0.05$) collapsed over 0-60 min. The significance of the dose x time interaction was between the control dose over 0-30 min compared to all other doses and time periods (all $p<0.01$).

Consumption of lab chow was negligible, occurring only in small quantities (< 0.2 g) in only 8 out of 456 tests, following stimulation by the highest doses of neostigmine and carbachol. Analysis of the consumption of tap water revealed no effect of dose for any drug group (carbachol: F=0.03 df=3,30; nicotine: F=0.94 df=3,27; low neostigmine: F=1.23 df=3,15; eserine: F=0.70 df=3,30; high neostigmine: F=0.53 df=3,21).

Figure 13: Mean (±SE) amount of food (dry macaroni) consumed in 0-30 and 30-60 min following unilateral microinjection of cholinergic agonists into the SN. (data in panel shows significant effect of dose $p < 0.05$)*



Behavioural analysis

Examination of still/asleep, locomotor, rearing, sniffing, gnawing of polystyrene chips and grooming responses was made (data not shown). The only differences between the drugs occurred with the highest dose of carbachol and the higher doses of neostigmine. No dose of nicotine, eserine or the lower doses of neostigmine had effects different to saline in any behavioural category. The high dose of carbachol increased rearing in 5 out of 11 rats and the highest doses of neostigmine increased rearing and sniffing in (at most) 6 out of 8 rats. (This proportion was not increased by relaxing the criterion level to > mean + 1 SD above control: 2 rats showed no difference in their responses to high doses of neostigmine and saline). Other forms of behaviour observed included ipsilateral turning, and scrabbling with the forepaws and cage-bar gnawing following high doses of neostigmine. Seizure-related activities (wet dog shaking and convulsions) were seen following 5.0 μg carbachol. Two rats not included in the analyses died following convulsions after 5.0 μg neostigmine.

Discussion

Microinjections of carbachol, nicotine and the low dose range of neostigmine all elicited dose-dependent increases in the consumption of palatable food when injected into the anterior substantia nigra. Carbachol and nicotine elicited significant dose-dependent increases in feeding over 0-60 min post-injection while neostigmine was significant only over the first 30 min; no dose of eserine increased feeding. The high dose range of neostigmine decreased feeding compared to saline. High doses of carbachol and neostigmine in some rats increased the frequency of sniffing and rearing, activities which had no apparent goal. Nicotine, and

the lower doses of carbachol and neostigmine, did not affect activity other than eating, while eserine was without clear effect.

The lowest dose of neostigmine, but no doses of eserine, increased feeding. Although the differences in chemical composition between the two molecules have been described, how such differences affect the pharmacological actions in the central nervous system are not well understood. The doses of eserine used here were taken from a previous study (Winn and Redgrave 1981) in which dose-dependent feeding was obtained following microinjection of eserine into the SN. The difference between those results and the present ones may be explained by the variable actions of eserine: in every rat tested here a dose of eserine was found which stimulated more eating than control but which dose and at what time post-injection varied considerably between rats, making mean levels of consumption difficult to discriminate. We have not attempted to analyse the eserine data by selecting those doses which appeared to work in individual rats and comparing them to control because the effects of no other cholinergic agent required such analysis. Presumably, the variable effects of eserine reflect some pharmacological difference between this and neostigmine. In the periphery, studies have shown that eserine but not neostigmine blocks ACh release (Alderdice 1979), which would be counter-productive in the present experiments, and that neostigmine has a direct nicotinic stimulatory effect which eserine does not (Bowman and Rand 1980 p. 10.21). The possibility that the effects of neostigmine were due to direct stimulation of nicotine receptors is however unlikely: it has been shown that lesions placed caudal to SN in the PPTg greatly attenuate the stimulatory effects of neostigmine but enhance the effects of nicotine (Blaha and Winn, In press).

The highest dose of neostigmine actually decreased consumption of macaroni compared to saline. Such a decrease in feeding with increasing

dose is also apparent with the direct cholinergic agonists carbachol and nicotine. That the highest doses of the direct cholinergic agonists did not produce a decrease in feeding compared to saline reflects differences in the range of doses used. Previous studies have shown that doses of direct agonists higher than those used here can attenuate feeding (Winn et al. 1983). The highest dose of neostigmine produced lethal convulsions in two of the subjects, as has also been observed in the past following high doses of carbachol.

The effects of nicotine and carbachol were, in this study, indistinguishable. Examination of other forms of behaviour may reveal differences between nicotinic and muscarinic activation, or different interactions between each of these and stimulation of other receptors present on SNPC neurones. The effects of neostigmine did not differ in any significant manner from carbachol. This is consistent with the hypothesis that neostigmine-induced blockade of AChE potentiated the action of endogenous ACh facilitating its action at cholinergic receptors in SNPC.

A body of evidence now suggests that the effects of cholinergic stimulation of the anterior SN are mediated by nigrostriatal DA-containing neurones. It has been thought that CP DA mediates many of the effects of amphetamine, including the behavioural stereotypies produced by higher doses of this drug. Such a conclusion is supported by lesion studies (Kelly et al. 1975) and by recent demonstrations of oral motor stereotypies following dopaminergic stimulation of the ventrolateral striatum (Delfs and Kelley 1990; Kelley et al. 1988). In agreement with previous studies however (Winn et al. 1983; Winn 1991) no stereotypy was observed following cholinergic stimulation of SN. The high doses of neostigmine did produce an increase in the frequency of sniffing and rearing, all but two rats showing frequencies greater than the saline mean

+ 2SD. However, the relationship between this and the stereotypy produced by amphetamine is not clear. Amphetamine treated rats tend to show sniffing, rearing and locomotion (3 on the Creese-Iversen scale, Kelly et al. 1975), these giving way at higher doses to "head down" sniffing maintained in one location, usually the corner of the cage. Neostigmine treated rats on the other hand, showed little increase in locomotion and no "head down" sniffing. Their behaviour may have had as much in common with the development of seizures as with stereotypies.

The size of the CP and its pattern of innervation by nigrostriatal DA make direct investigation of its role in striatal function difficult. The CP has a neurochemical and cytoarchitectonically heterogeneous structure. Lowenstein et al. (1990) found a greater number of DA terminals in the matrices than in the patches of the CP, but that D1 receptors are found predominantly in the patches (Besson et al. (1988). Administration of direct DA agonists into the CP will not correctly replicate the topographical distribution of DA release that occurs from nigrostriatal terminals. By stimulating DA release by injections to the SN one can be confident of releasing DA in both a physiologically and topographically relevant manner. It could be argued that the problems of administering direct agonists to the CP should also apply to the SN. However, our use of the indirect agonist neostigmine ensures that the normal topographical distribution of ACh stimulation, if not the normal physiological level, as released from ACh terminals is preserved.

Thus we have a powerful tool for investigating the dopaminergic influence of the SN on the CP and furthermore a means for bracketing this stimulation within the physiological limits set by its pontine tegmental afferents.

5 Behavioural specificity of cholinergic stimulation of anterior SN

Introduction

The previous experiment showed that cholinergic stimulation of SN produces a dose-dependent increase in feeding to palatable food to which the animal has been pre-exposed. The rats showed no increase in drinking to tap water. It had been suggested that cholinergic stimulation of SN might specifically stimulate eating behaviour (Winn et al. 1983). However, increased drinking behaviour has been observed following nigral stimulation in the presence of saccharin solution (Winn 1991). It is therefore appropriate to seek to replicate the findings of Winn (1991) using a different, but still palatable liquid. Rats will drink up to ten times as much 'Ribena' as water over an hour. This experiment therefore investigates whether cholinergic stimulation of SN can increase drinking to a palatable fluid to which the animal has been pre-exposed.

Methods

Animals

12 rats with mean body weight at the time of surgery 258.4 g (SE = 3.18) were used.

Stereotaxic surgery

Rats were anaesthetized with Avertin and placed in a stereotaxic frame in the orientation of Level Skull. The rats were implanted unilaterally with guide cannulae terminating above the anterior SN.

Intracranial drug administration

Each animal received a unilateral microinjection of 0.5 μg carbachol in 0.5 μl sterile saline, and saline alone, to the nigral cannula in a counterbalanced order.

Test procedure

Rats were placed in individual cages with weighed bottles of ribena made up to 20% with tap water. The group of animals for testing were left for 60 min to drink ribena before microinjection. Each bottle was then removed and its weight noted. The weight of each bottle was noted at 60 min post-injection.

Results

Histology

It was decided that any site showing less drinking to carbachol than to saline should be retested using a longer micro-injection cannula in an attempt to locate an 'active' site. This consisted of increasing the cannula length by 0.5 mm and repeating the testing as described above.

As can be seen from Figure 14, the sites of injection for each animal passed through SN to the crus cerebri below. As each animal was re-tested three times at progressively lower depths, 0.5 mm each time, the injection sites would have been in SN for at least one of the testing sessions.

Drinking in response to cholinergic stimulation

Figure 15 shows volume of ribena drunk in response to carbachol and saline. There was no difference in drinking ($T=0.23$, $df=1,11$ $p<0.82$) between the two conditions.

Figure 14: Representative sections showing deepest injection sites (▲) in the crus cerebri following repeated injections at progressively lower depths through SN.

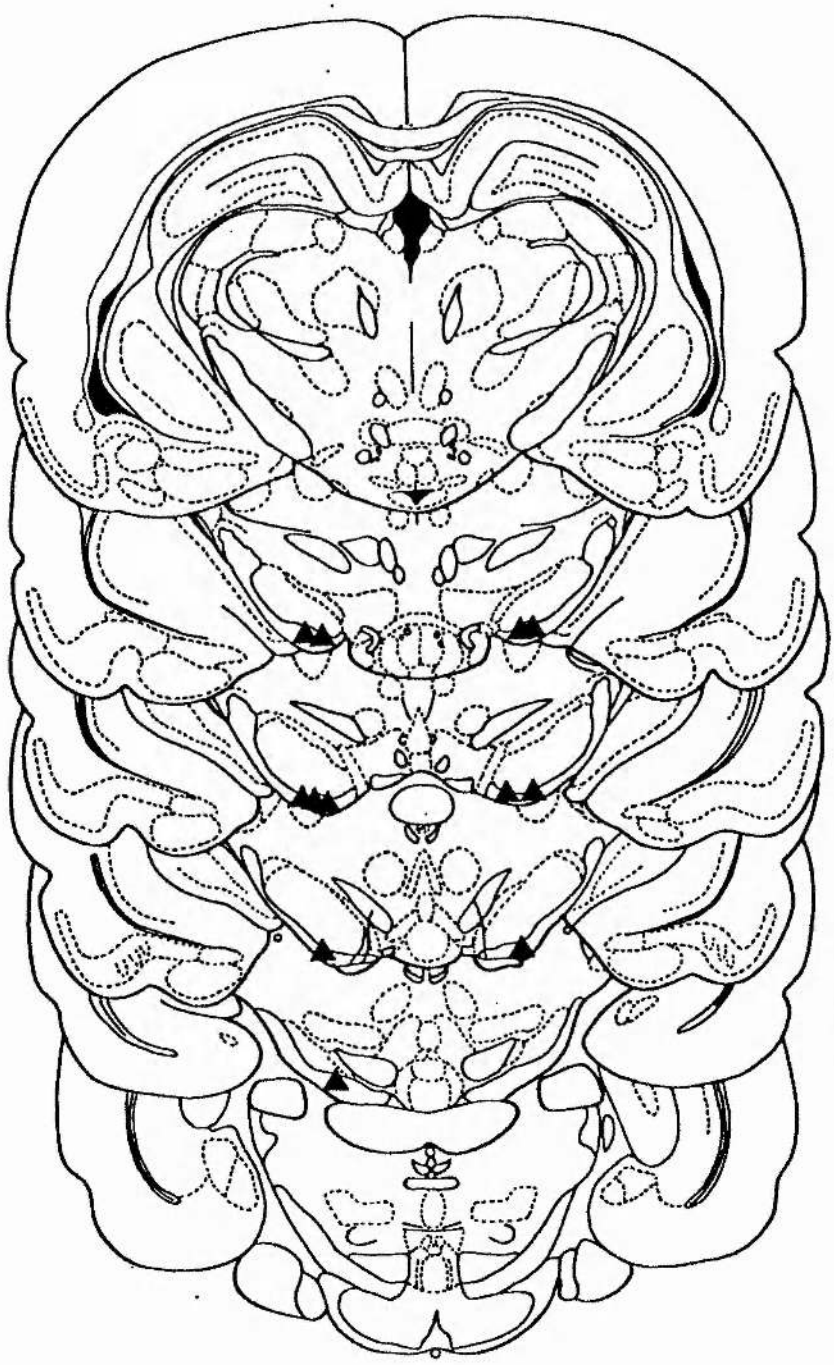
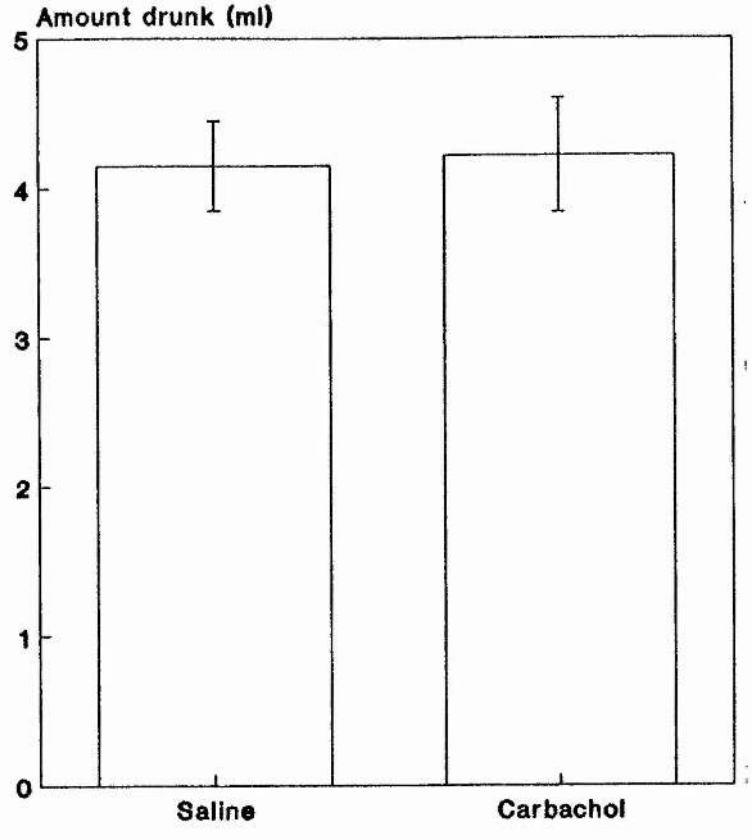


Figure 15: Mean (±SE) amount of ribena consumed (ml) in 60 min following injection of carbachol and saline into anterior SN



Discussion

As drinking to a palatable fluid such as ribena begins with a high frequency then gradually diminishes over time, it would be predicted that such behaviour could be stimulated by microinjection of cholinergic agonists into the SN (Winn 1991). Increased drinking has been shown previously to saccharin solution, and there is no doubt that the rats find the ribena palatable. In testing consumption of palatable food, there is a pattern to the response to carbachol as compared to vehicle solution as the site of injection moves downwards towards the nigra: when the site of injection is above the SN the level of feeding will be higher following saline than carbachol; then the same as carbachol; and when the site is in SN there will be an increase in feeding following carbachol compared to saline. Such a pattern did not occur in response to ribena. It is possible that the level of stimulation caused by a unilateral injection of 0.5 ug carbachol is not enough to produce the increase in consumption of palatable fluid. Although previous unilateral studies have shown increase consumption of palatable food, the Winn study using saccharin used bilateral injections.

Winn (1991) found increased drinking to a palatable fluid, saccharin. Saccharin, unlike ribena has no calorific value. It has been demonstrated that glucose has an effect on the metabolism of DA in the CP and it is possible that the actual behaviour being measured in response to cholinergic stimulation of DA release is itself having an effect on DA action in the CP independent of any rewarding properties the consumption of the palatable fluid might have. The effects of intranigral carbachol on feeding to palatable food was shown by Winn et al. (1983) to be rate dependent, food deprived or rats that had not been pre-exposed to the food did not demonstrate increased feeding to cholinergic stimulation. It

is possible that stimulation of consumption following intranigral carbachol is dependent on the activational potential of the behaviour in a manner analogous to the dependency of the effect on the baseline rate of eating. The previous study showed no increase in consumption of lab chow following carbachol. A stimulus that is highly activating may also be unaffected by the addition of stimulation caused by intranigral carbachol. Given that increases in firing rate of nigrostriatal DA neurones is associated with the presence of stimuli that are described as activating (Chiodo et al. 1980), it is possible that the presence of glucose is an extremely salient and powerful influence and that the additional stimulation caused by intranigral carbachol does not produce a behaviourally observable effect on nigrostriatal DA activity.

Alternatively, Ribena, while being extremely palatable, might not cause the feeling of satiation that occurs with the consumption of pasta in the previous experiment. Furthermore, the urination of unwanted fluid might make the condition of satiation less certain than in response to feeding.

Chiodo et al. (1980) showed that SN DA-containing neurones increased their firing rate following tail-pinch. As discussed by Robbins and Fray (1980), tail pinch can induce increased feeding, but not increased drinking of water. Although drinking of milk can follow tail-pinch, that could be argued to be ingestion of a food. It appears that the neurological systems underlying feeding and drinking are under different controls and influenced by differing factors and hence cholinergic stimulation of drinking may not be elicited in the same manner as feeding.

6 Muscarinic and nicotinic stimulation of anterior SN

Introduction

Behaviour for which there is a pre-existing tendency to perform at high frequency at first exposure, but is being performed currently at a low frequency, can be stimulated by administration of either nicotinic or muscarinic agonists to SNPC. It is of interest to know how stimulation of the two subtypes of cholinergic receptor interact, and to see whether such an interaction would produce discernible effects at the behavioural as well as pharmacological level. In chapter 4 there were no significant differences in effect on behaviour between nicotinic and muscarinic stimulation. The investigation of the cholinergic projection from the pons to the SNPC has yielded good evidence of a functional cholinergic innervation of SNPC DA neurones (Gould et al. 1989, Blaha and Winn, In press). But as yet no study has demonstrated the relationship between the cholinergic innervation and muscarinic and nicotinic receptors known to exist on the DA neurones. However, it seems likely that the target sites of ACh released by terminals in the SN would include both muscarinic and nicotinic cholinergic receptors.

There is presumably a functional reason as to why there should be two types of cholinergic receptor in the brain, and to this investigation especially, on the post-synaptic membranes of NSP DA neurones. It is now generally accepted that there is a cholinergic innervation of the SN from the pons. There is a clearer picture forming of the anatomical relationship between the pons and the mesencephalon, but little functional characterisation of the nature of this innervation. Given that previous experiments have shown a similar behavioural effect in response to both muscarinic and nicotinic stimulation, it is plausible that endogenous ACh, released from the terminals of pontine cholinergic neurones in the SNPC

can act at both types of receptor to change the state of SNPC DA neurones. The functional relationship between effects mediated at nicotinic and muscarinic receptors has not previously been investigated. This investigation therefore begins to characterise the relative contribution of the two sub-types of cholinergic receptor to the overall effect of cholinergic influence on NSP DA neurones. To investigate the relationship between nicotinic and muscarinic activation the present experiment will examine firstly the effects of carbachol and nicotine over a range of doses, and secondly the effect of adding the most effective dose of carbachol to the dose response curve to nicotine. This will allow us to begin to characterise the contribution to overall cholinergic stimulation made by each receptor type.

Methods

Animals

44 rats with mean body weight at the time of surgery 259.7 g (SE = 2.06) were used.

Stereotaxic surgery

Rats were anaesthetized with Xylazine and Ketamine and placed in a stereotaxic frame in the orientation of De Groot. The rats were implanted unilaterally with cannulae terminating above the anterior SN.

Intracranial drug administration

The rats were arbitrarily assigned to one of three drug groups and received the following doses: carbachol 0, 0.039, 0.1, 0.257 μg; nicotine, 0, 0.055, 0.1, 0.25 μg; most effective dose of carbachol + each of the above doses of nicotine.

Test procedure

Rats were placed in individual cages with free access to weighed amounts of tap water, normal lab chow, dry macaroni (1470 Kj/100 g, 13.0 g protein/100 g) and polystyrene packing chips for 60 min before and after administration of drug to the nigral cannula.

Statistical analysis

Food and water intake data were analysed without transformation by analysis of variance (ANOVA).

Results

Histology

Cannula placements were verified post-mortem and Figure 16 presents representative sections showing these. Following histological examination, 34 rats were found to have injection sites in or immediately rostral to substantia nigra.

Feeding to cholinergic stimulation

Figure 17 shows the mean intake of dry macaroni over 60 min following microinjection of cholinergic agonists: Figure 17 (A) shows the dose response to carbachol, Figure 17 (B) the effect of adding the most effective dose of carbachol, 0.1 ug, to the dose response to nicotine. Both carbachol (F=3.99, df 3,24, $p<0.02$) and nicotine (F=7.07, df 3,33 $p<0.01$) alone produced significant dose-dependent increases in consumption of macaroni.

Injections of carbachol + nicotine showed a significant main effect of dose (F=5.32, df 3,30, $p<0.005$). Comparison of carbachol + nicotine with nicotine alone showed a significant main effect of

Figure 16: Representative sections showing nigral (●) and misplaced (▲) cannulae

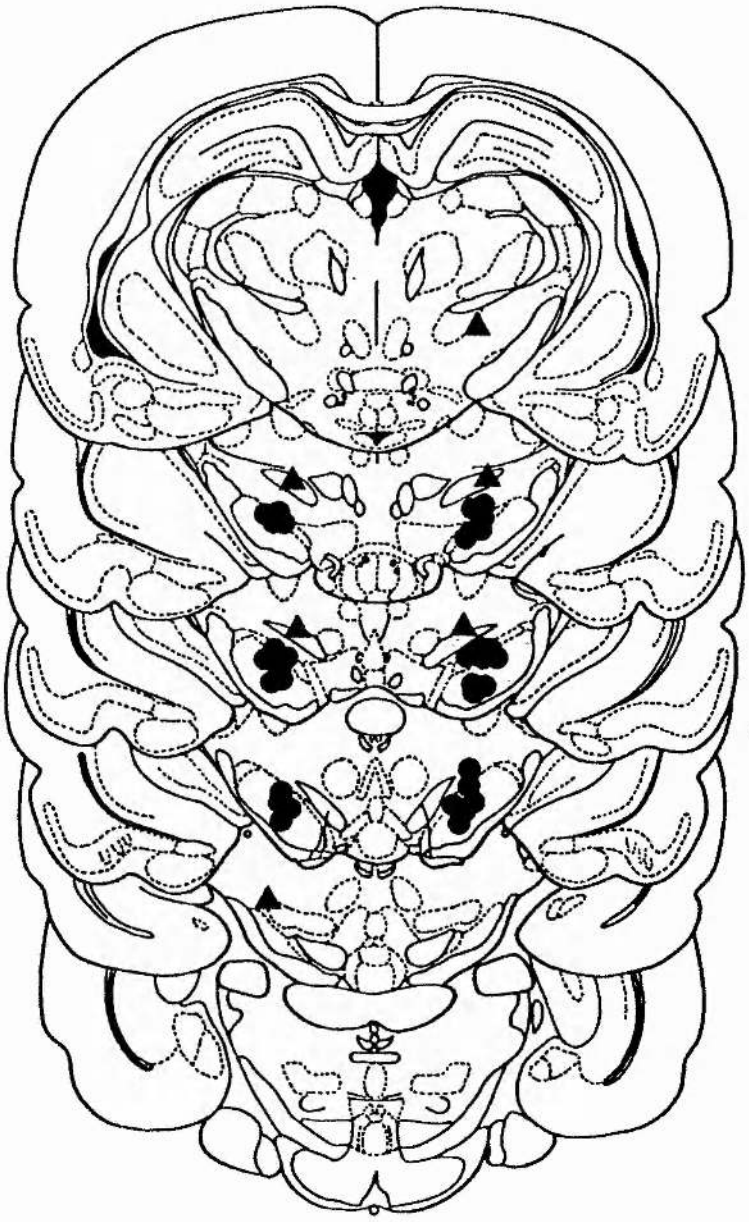
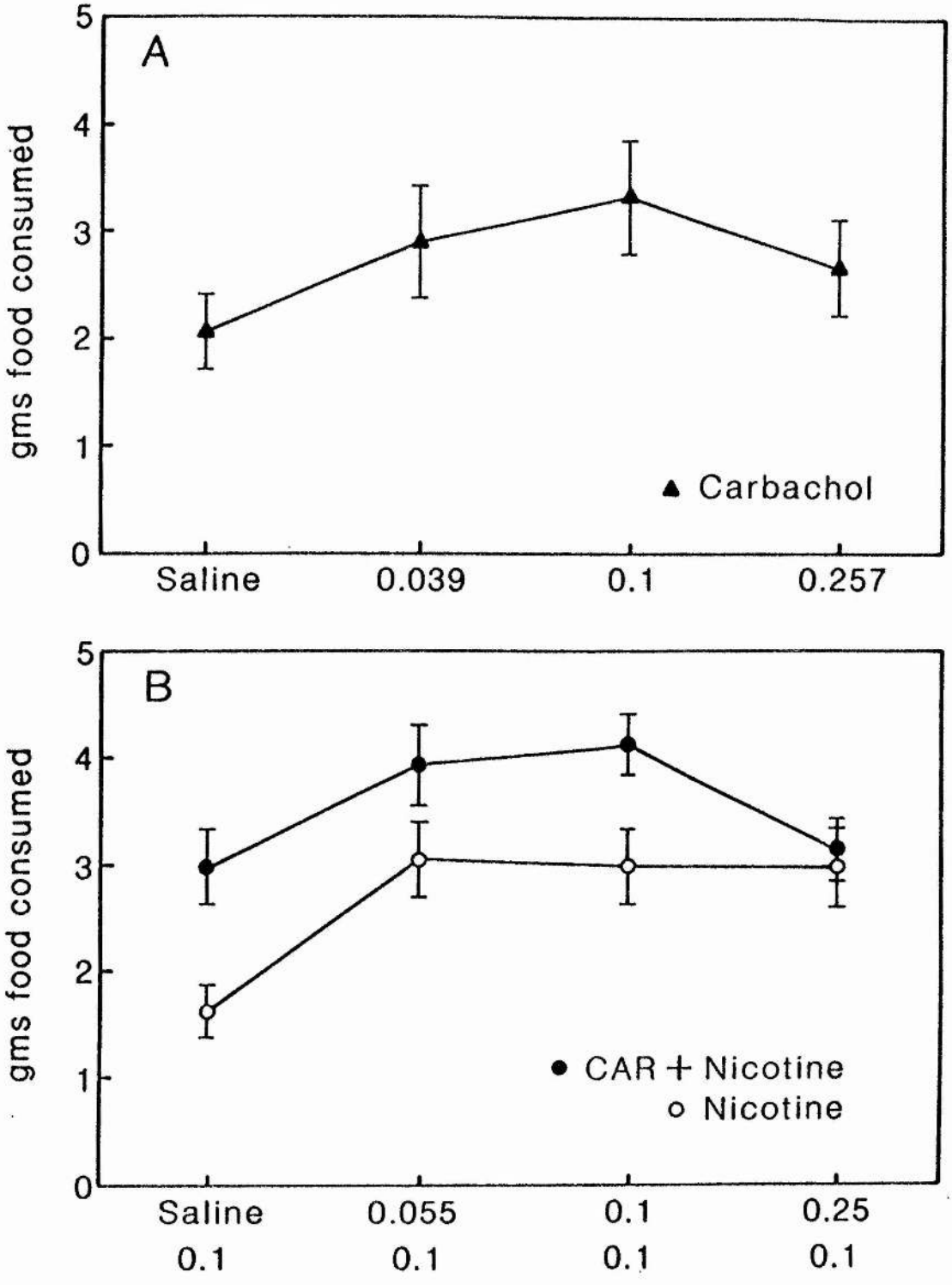


Figure 17: Mean (±SE) amounts of dry macaroni consumed in 60 min following injections of (A) carbachol, (B) nicotine, and carbachol + nicotine



drug (F=7.01, df 1,23, p<0.015) and dose (F=9.79, df 3,69, p<0.001) but no drug x dose interaction (F=1.97, df 3,69).

Consumption of lab chow and water

Consumption of lab chow occurred infrequently (24/136 tests) and in negligible quantities. Carbachol did not stimulate consumption of tap water (F=1.13, df 3,24) but drinking after administration of nicotine did approach (though not achieve) statistical significance (F=2.43, df 3,33 p<0.083). Addition of carbachol to nicotine also produced no main effect on drinking of drug (F=0.00, df 1,23), dose (F=2.07, df 3,69) or interaction (F=1.75, df 3,69). The amount drunk ranged from 1.44 to 2.5 ml.

Discussion

It is clear that stimulation with either a nicotinic or muscarinic agonist increased consumption of palatable food to which rats had been pre-exposed. Addition of a reliably stimulating dose of carbachol had an additive effect with nicotine. These data are consistent with the previous study that showed increased feeding in response to intranigral carbachol, nicotine and the anticholinesterase neostigmine but adds to that study by demonstrating that, at the doses used here, nicotinic and muscarinic stimulation can have additive effects. It should be noted that the dose response curve of consumption of palatable food following carbachol + nicotine showed a main effect of dose. This demonstrates that the response to nicotine was not overwhelmed by the addition of carbachol: the various doses of nicotine had discriminable effects following addition of carbachol. This suggests that the effects of muscarinic and nicotinic stimulation are truly additive, in that the nicotinic cholinergic stimulation produced dose dependent effects even in the presence of carbachol. The effect of adding carbachol was to elevate baseline responding.

Although these results are suggestive of an additivity of effect at the doses of carbachol and nicotine used, a more fuller investigation would include a dose response curve for carbachol with a fixed dose of nicotine added. This would allow a more stringent assessment of whether the two drugs can be truly be said to be additive in their effects. However, a more precise investigation of the relationship of the muscarinic and nicotinic stimulation of SN would more appropriately be carried out at a different level of analyisis from the behavioural level used in the present study. These results suggest that additivity is possible in the stimulation of both types of cholinergic receptors, but a more sensitive measure of the activity of SNPC DA-containing neurones would be required in a Schild analysis to demonstrate true additivity. Although the Schild analysis allows the measurement of biological responses to relate agonist-receptor effects, feeding behaviour would be a very noisy measure. Electrophysiological measurement of neurone activity, or even electrochemical detection of DA release in the CP would be more appropriate measures.

These results are consistent with the fact that both types of receptor are present on DA-containing neurones in the SNPC and that stimulation of each excites these neurones (Blaha and Winn, In press; Lacey et al. 1990), increasing DA efflux in the ipsilateral CP. Carbachol is now known to act over a certain dose range as an agonist at nicotinic as well as muscarinic cholinergic receptors (Norris et al. 1992). However it is clear from our results that the stimulation resulting from addition of carbachol to nicotine is significantly greater than could be achieved with any dose of nicotine. Were the effects of carbachol mediated by nicotine receptors alone we would expect to see similarly elevated levels of behavioural stimulation at the highest dose of nicotine.

The concept of 'fast' and 'slow' neurotransmitter has been used to differentiate the actions of those neurotransmitters opening ion-channels over a period of only msecs from others that act through second messenger systems over a period of secs or longer. Kemp et al. (1977) suggested that ACh acts as a fast neurotransmitter at nicotinic receptors and as a slow neurotransmitter at muscarinic cholinergic receptors. The binding of two molecules of ACh with the nicotinic receptor complex results in the opening of the ion channel associated with each receptor. This will induce changes in the permeability of the membrane to Na^+, K^+ and Ca^{2+}. The Na^+, which is in a higher concentration outwith the cell, flows inwards, as to a lesser extent does Ca^{2+}, while K^+ which has a higher concentration inside the cell flows outwards (Bowman and Rand 1980). Weiner et al. (1990) have used molecular cloning techniques to demonstrate that of the five types of muscarinic receptor that have been described on the basis of molecular structure, only the m5 receptor sub-type is present on SNPC neurones. The m5 receptor is thought to conform to the M1 receptor described by pharmacological studies (Buckley et al. 1989). Lacey et al. (1990) found that the effects of muscarine on SNPC DA neurones had the same effect as the M1 muscarinic agonist pirenzepine. The association of the M1 receptor subtype with SNPC DA-containing neurones was further confirmed by Nastuk and Graybiel (1991) who, also report M2 binding sites in the SN following autoradiographic analysis. M1 receptors stimulate phosphoinositide hydrolysis and inhibit cAMP production. The hydrolysis of triphosphoinositol (inositol 1,4,5-triphosphate) releases Ca^{2+} from intracellular stores that will depolarize the membrane and thus increase the probability of the threshold of excitation for that neurone being reached. Thus ACh has a chimeric ability to act as both a slow and a fast neurotransmitter on possibly the same DA neurones in the SNPC. One

conceivable functional reason for this could be that ACh has a straightforward stimulatory action on the SNPC DA neurones mediated by nicotine receptors, while the action at the muscarinic receptors not only stimulates increased firing of the neurones but also effects the membrane response and affinity to the binding of future ACh molecules and that of other neurotransmitters afferent to the SNPC.

Investigations of cholinergic-dopaminergic interactions in the basal ganglia have previously focussed on the striatum. However, pontine cholinergic neurones innervating midbrain DA-containing neurones are able clearly to increase the activity of nigrostriatal and also mesoaccumbens systems. This has implications for transplant studies using animal models of parkinsonism. These results might explain why there is only partial recovery of function produced by striatal implants of nigral cells into a rat model of hemiparkinsonism (Dunnett et al. 1981). The failure to reinstate normal feeding behaviour may well reflect the loss of appropriate control of striatal DA efflux associated with activating stimuli (Chiodo et al. 1979) and cholinergic influence on nigrostriatal DA activity.

7 The nigrostriatal pathway and cholinergic stimulation of SNPC

Introduction

The behavioural effects of microinjecting cholinergic drugs into the SN are thought to depend on stimulation of DA-containing neurones in the pars compacta. Four lines of evidence support this: (i) the strength of the behavioural response to cholinergic stimulation has been shown to be proportional to the proximity of the injection site to the SNPC (Winn and Redgrave 1979); (ii) microinjection of d-amphetamine into the CP, which causes efflux of DA from NSP terminals, causes increases in eating (Winn et al. 1982); (iii) systemic injections of non-sedative doses of the DA antagonist haloperidol block behaviour stimulated by injection of carbachol into SNPC (Taha and Redgrave 1980); (iv) both muscarinic (Cross and Waddington 1980) and nicotinic (Clarke and Pert 1985) cholinergic receptors have been localized to nigrostriatal DA neurones.

Although the evidence strongly suggests that cholinergic stimulation of the SN is mediated by the DA-containing neurones of the SNPC, this has never previously been demonstrated behaviourally. It is possible that cholinergic agonists injected into the SN are in fact effecting other nigral efferents, for example the nigrotectal or nigrothalamic pathways. The nigrotectal pathway has been demonstrated by Redgrave et al. (1980) to have a role in the mediation of the oral stereotypies induced by apomorphine. Furthermore, although Winn and Redgrave (1979) concluded that the stimulatory effect on eating of intranigral carbachol was inversely proportional to the distance of the injection site from the SNPC, the possibility cannot be excluded that the effects of intranigral carbachol may be in fact due to its diffusion to the VTA. The DA-containing neurones of the VTA increase firing in response to muscarinic

(Lacey et al. 1990) and nicotinic (Greenhoff et al 1986) agonists and has been associated with the control of eating (Koob et al 1978).

This experiment will attempt to demonstrate that increased feeding of a palatable food to which the rat has been pre-exposed by injection of carbachol into the SN can be blocked by a 6-OHDA lesion of the ascending DA-containing neurones of the SNPC. Rats will receive unilateral injections of carbachol and saline as previously described before and after a unilateral 6-OHDA lesion injected into the medial forebrain bundle as it passes through the LHA. This is preferable to trying to destroy the DA-containing cell bodies at source in the SN which has been less successful (Parker, Rugg and Winn, unpublished observations). Using a unilateral rather than bilateral study allows use of the unmanipulated hemisphere as a useful control for measurement of brain amines and also avoids the possible difficulties of interpretation associated with a bilateral 6-OHDA lesion that causes aphagia and adipsia if the depletion is severe enough (Ungerstedt 1971).

Methods

Animals

22 rats with mean body weight at the time of surgery 290.8 g ($\pm$ 8.65 SE) were used.

Stereotaxic surgery

Rats were anaesthetized with Avertin and placed in a stereotaxic frame in the orientation of level skull. The rats were implanted unilaterally with guide cannulae terminating above the anterior SN and the LHA. The LHA cannula allowed the subsequent injection with 6-OHDA or vehicle solution to the nigrostriatal DA neurones known to pass through this site.

Intracranial drug administration

During each test session each rat received a microinjection into the SN of 0.5 μg/0.5 μl carbachol or the vehicle solution saline.

Test procedure

Rats were habituated to the test procedure until they showed consecutive increase in spaghetti consumption. Rats were allowed access to spaghetti and tap water for 60 min before and after microinjection.

6-OHDA lesion

After each animal had been tested in response to both carbachol and saline, unilateral lesions were made by microinjecting 6-OHDA or the vehicle solution ascorbic saline into the LHA. Each animal was pre-treated at least 30 min before with i.p. injections of pargyline and DMI. 14 rats received 1.0 μl of an 8.0 $mg.ml^{-1}$ solution of 6-OHDA (calculated as free base) dissolved in 0.1 $mg.ml^{-1}$ ascorbic saline, 8 rats received the vehicle solution alone. Rats were unanaesthetised for this procedure and microinjected as described in chapter 3 except the cannulae left *in situ* for 120 sec. The rats were left for 10 days for the neurotoxin to take effect then retested with carbachol and saline as before.

Histological and biochemical analysis

Rats were killed by stunning and decapitation. The brains were removed onto ice where left and right CP and NAcc were dissected, wrapped in foil, placed in dry ice and stored at -22^{o}C. The remainder of the brain was post-fixed for further histological analysis as described in chapter 3. The concentrations of the neurotransmitters DA and 5-HT, and their metabolites DOPAC, HVA and 5-HIAA were determined from the CP and NAcc samples using HPLC. The method used was a modification of that developed by Mayer et al. (1983). A homogenising solution of 0.1 M

Perchloric acid containing approximately 0.25 pmols.ul^{-1} 3,4-Dihydroxybenzylamine (the external standard) was added to the tissue samples which were then given 10 to 15 bursts with a sonic probe ('Soniprobe', Lucas Dawes Ultrasonics) on a 50% cycle. The homogenate was placed in a Beckman Microfuge E for 30 sec then filtered through a 0.45 μm nylon 'acrodisc' (Gelman Sciences). The samples were then injected into a BAS 400 Liquid Chromatograph fitted with a 10 cm cartridge packed with 3 μm ODS2 spherisorb (Capital HPLC Specialists). The eluants were detected using a BAS amperometric detector (LC-4B), fitted with a glassy carbon working electrode maintained at +0.9 V. The biogenic amines were eluted with mobile phase containing 3% acetonitrile, 1.5% tetrahydrofluran, and 0.03% 1-octane sulphonic acid in 150 mM monochloroacetic acid pH 3.0 at a rate of 1 ml.min^{-1}.

Statistical analysis

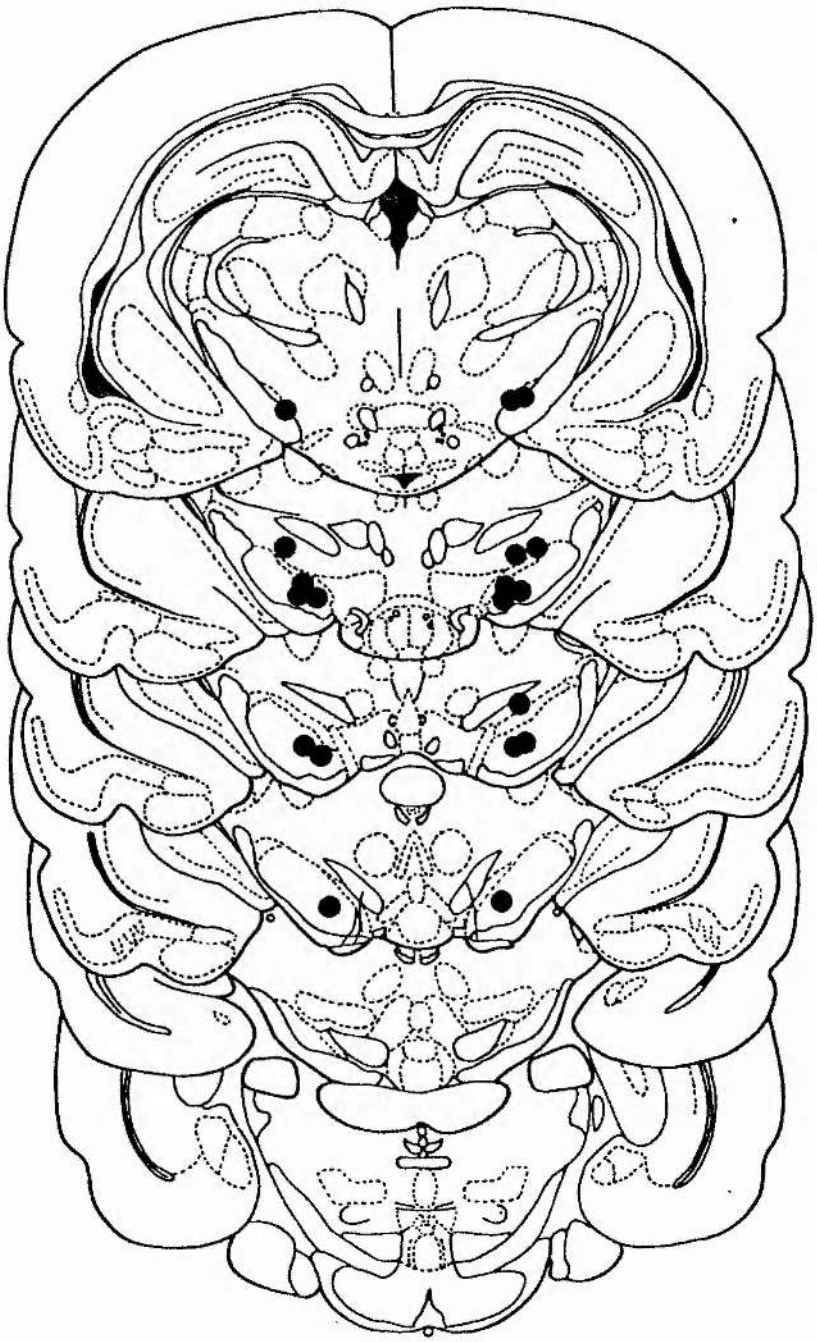
The feeding and HPLC data were analysed using ANOVA, followed by Tukey's method for multiple comparisons where appropriate. The data were analysed without transformation.

Results

Histology

Cannula placements were verified post-mortem and Figure 18 presents representative sections showing these. Following histological examination, one rat were found to have misplaced cannulae (in the ventroposteromedial thalamic nucleus) and was therefore discarded from the behavioural analyses. Two of the 8 control animals developed a gastric pathology ('bloat', in response to the surgical anaesthetic) and were destroyed.

Figure 18: Representative sections showing nigral cannulae



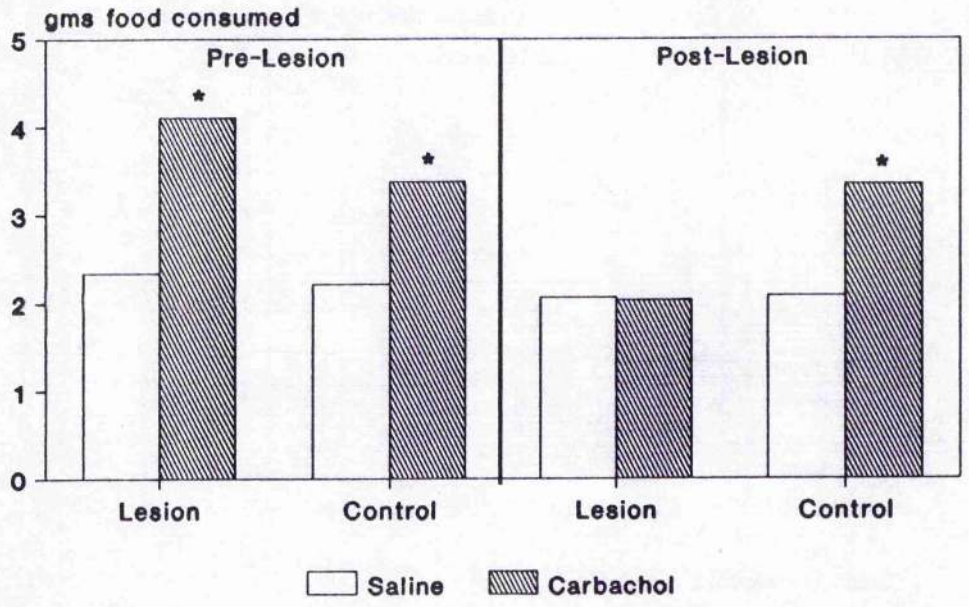
Eating behaviour

Figure 19 shows the mean amounts of dry spaghetti consumed in 60 min following microinjection of carbachol or saline to SN, before and after a lesion in the LHA with 6-OHDA or vehicle solution. There were significant main effects of drug (saline or carbachol) (F=32.94, df 1,14 $p<0.001$), but not lesion group (6-OHDA or sham) (F=0.26, df 1,14) or session (pre- or post-lesion) (F=2.62, df 1,14). However the three way interaction drug x lesion x session was significant (F=7.70, df 1,14 $p<0.03$). Post-hoc analysis revealed significant differences between carbachol and saline before lesion in both groups (6-OHDA $p<0.01$, sham $p<0.05$) but only in the sham group post-lesion (sham $p<0.05$). There was a significant difference in consumption to carbachol before and after lesion in the 6-OHDA group ($p<0.01$), but no difference in the sham group. There was no significant difference in feeding to saline in either group before or after lesion. In summary, carbachol increased eating compared to saline before and after the sham lesion, but the eating to carbachol was reduced to saline level by lesion with 6-OHDA.

HPLC analysis

The concentrations of DA, 5-HT, and their main metabolites in the CP and NAcc, are shown in Table 1 as a percentage of the concentrations found in the unmanipulated hemisphere. There was a significant effect of lesion group on DA levels in the CP (F=11.21, df 1,16 $p<0.005$) but not in the NAcc (F=1.28, df 1,16). Similarly, there was an effect of lesion group on levels of DOPAC (F=9.79, df 1,16 $p<0.007$) and HVA (F=5.14, df 1,16 $p<0.04$) in the CP, but not in the NAcc (DOPAC: F=0.43, df 1,16; HVA: F=4.32, df 1,16). Lesion group had no effect on levels of 5-HT or 5-HIAA in the CP (F=1.23, df 1,16; F=1.53, df 1,16) or the NAcc (F=1.23, df 1,16; F=0.21, df 1,16).

Figure 19: Mean amounts of dry spaghetti consumed in 60 min following injection of carbachol or saline, before and after a 6-OHDA or sham lesion of nigrostriatal DA neurones. Variance (SE) was always less than 11.92% of the mean. (indicates $p < 0.05$ of carbachol and saline being the same manipulation on this measure)*



Some measures of the turnover of DA and 5-HT were possible by calculating the ratio of neurotransmitter to metabolite present in the tissues. Table 1 shows the ratios of DA:DOPAC, DA:HVA and 5-HT:5-HIAA in the CP and NAcc as a percentage of the ratios found in the unmanipulated hemisphere. There was a significant effect of lesion group on the ratio of DA:DOPAC in the CP ($F=10.96$, df 1,16 $p<0.005$) but not in the NAcc ($F=1.05$, df 1,16). There was no effect on the ratio of DA:HVA in the CP ($F=3.50$, df 1,16) or the NAcc ($F=2.01$, df 1,16). The ratio of 5-HT:5-HIAA was also unaffected in the CP ($F=0.19$, df 1,16) and the NAcc ($F=1.14$, df 1,16). Table 2 shows the mean levels of DA, 5-HT and their metabolites as $ng.mg^{-1}$ of wet weight tissue in the CP and NAcc of the unmanipulated hemispheres of both lesion groups. In summary these data show a significant decrease in concentration of DA and its metabolites as a percentage of their unmanipulated hemispheres in the CP of the 6-OHDA lesion rats as compared to controls, but not in the NAcc. Furthermore, there is a significant increase in turnover of DA to DOPAC in the CP but not the NAcc. Levels of HVA, 5-HIAA and their ratio were not affected in the CP or the NAcc.

Table 1: Mean (±SE) levels of DA, 5-HT, their main metabolites, and ratios of monoamines to metabolites in the CP and NAcc of 6-OHDA and sham-lesioned rats calculated as percentage of unmanipulated hemisphere. (indicates $p < 0.05$ of lesion and control being the same population on that measure)*

	Caudate-putamen		Nucleus accumbens	
	Lesion	Control	Lesion	Control
DA	50.03* ±9.87	96.35 ±7.54	70.02 ±13.1	89.58 ±6.30
DOPAC	49.34* ±11.0	95.45 ±6.12	80.84 ±12.66	92.04 ±7.60
HVA	63.98* ±10.34	100.58 ±12.01	77.83 ±12.38	112.11 ±7.34
DA:DOPAC	50.72* ±9.84	96.24 ±7.43	72.33 ±12.85	89.71 ±6.05
DA:HVA	70.92 ±9.66	103.62 ±15.78	101.54 ±9.96	82.24 ±8.56
5-HT	85.12 ±12.30	103.59 ±7.41	81.00 ±14.76	94.26 ±6.84
5-HIAA	80.76 ±11.76	99.93 ±3.43	103.56 ±10.32	111.64 ±14.79
5-HT:5-HIAA	90.57 ±10.80	97.06 ±7.13	72.09 ±12.43	95.26 ±18.99

Table 2: Mean (+SE) amounts (ng.mg^{-1} wet weight tissue) of DA, 5-HT and metabolites in CP and NAcc of unmanipulated hemispheres of both lesion groups

	Caudate-putamen	Nucleus accumbens
DA	14.01	9.78
	±1.30	±1.05
DOPAC	1.81	1.85
	±0.26	±0.31
HVA	0.69	0.69
	±0.04	±0.06
5-HT	0.73	1.21
	±0.09	±0.17
5-HIAA	0.71	0.91
	±0.07	±0.13

Discussion

Increased feeding to palatable food following cholinergic stimulation of the SN was abolished by a unilateral 6-OHDA lesion of the ascending NSP DA neurones. The lesion caused a significant depletion of DA from the CP but not the NAcc. This suggests that it is nigrostriatal and not mesoaccumbens DA that is mediating the effects of cholinergic stimulation of the SN and furthermore that it is indeed the DA-containing neurones of the SNPC that are being stimulated rather than the source of the mesoaccumbens DA, the neighbouring VTA.

The depletion of DA from the CP was significant, but not total. The increased feeding to carbachol was abolished by reducing the DA content of CP tissue to 50% of the CP of the unmanipulated hemisphere. Such a level of depletion is known to promote weak rotation in response to amphetamine but much higher depletions of >90% are required to produce rotation to the direct agonist apomorphine (Hefti et al. 1980). This would suggest that a lesion of the scale reported here would reduce the ability of amphetamine to promote release of DA from terminals in the CP, but would not effect the post-synaptic sensitivity to DA. The present data suggest that significant behavioural effects can be observed with DA loss of only 50%, a level of loss not associated with significant compensatory post-synaptic changes (Marshall and Ungerstedt 1977).

HPLC analysis revealed that not only were the levels of DA and its main metabolite DOPAC reduced in the CP of the lesion group animals, but the ratio of DA:DOPAC was also reduced. This suggests that the turnover of DA in the CP was increased as a result of the lesion. This is consistent with the scheme proposed by Zigmond (1989, see Nigrostriatal DA and behaviour) of how partial recovery of function may occur following a partial loss of DA innervation of an area. The most

parsimonious explanation is that the DA neurones have increased their synthesis and release of DA from a reduced number of DA terminals such that the turnover of existing DA is increased. Clearly though, this increase is not sufficient to mask the effects of the lesion on the mediation of cholinergic stimulation by the NSP DA.

The depletion of NSP DA had no obvious effects on the normal behaviour of the animals. There was no obvious change in the ability of the animals to locomotor, eat or drink, as evinced by the lack of change in feeding to saline before and after the lesion. What was lost after the lesion was the *increase* in eating produced by intranigral carbachol. Previous studies have suggested that NSP DA is associated with the initiation of activity (Carli et al. 1985, Chiodo et al. 1979, 1980). It may be that intranigral cholinergic stimulation exerts its effects by releasing DA which in turn enhances the probability of certain behavioural procedures being initiated.

8 Conditioned reinforcement and cholinergic stimulation of midbrain DA systems

Introduction

Although the previous experiments have addressed the neuroanatomical and pharmacological, they do not address the psychological question of how cholinergic stimulation of the SN affects behaviour.

Mesolimbic DA has been intensely investigated with regard to its role in the reinforcement of behaviours. It is thought to be the site of action for several drugs of abuse such as amphetamine which releases DA from terminals within the NAcc, and cocaine which blocks the re-uptake of DA into terminals thus prolonging its action (Koob 1992). Systemic administration of nicotine causes increased DA efflux in the NAcc, but not the CP (Brazell et al. 1990).

Subjects will increase responding for a previously neutral stimuli if repeatedly associated with a primary reinforcer. Taylor and Robbins (1984) demonstrated increased responding for conditioned reinforcement following administration of the dopamine agonist amphetamine into the NAcc, but not the CP. Such studies suggest that reinforcement is mediated by mesolimbic rather than nigrostriatal DA. Similar studies using injections of amphetamine into a number of striatal sites have produced ambiguous results (Kelley and Delfs 1990). Infusions of substance P and other peptides into the VTA produced increased DA efflux of ~50%, stimulating locomotion but not increased responding for conditioned reinforcement (Kelley et al. 1985, Kelley and Delfs 1991).

There is evidence for a cholinergic innervation of the VTA and the neighbouring SNPC derived from cholinergic neurones in the pons: the Ch5 cell group in the PPTg and possibly the Ch6 cell group in the

LDTN. There is an increasing literature on the cholinergic control of SNPC dopamine neurones which suggests a role in the potentiation of behaviours for which there is a pre-existing tendency to perform but a low current frequency (Winn 1991). There is pharmacological evidence for the presence of nicotinic receptors and electrophysiological evidence for the presence of muscarinic receptors on both SNPC and VTA DA neurones (Clarke and Pert 1985; Lacey et al. 1990, resp.). Administration of nicotine to the SNPC increases DA efflux in the caudate-putamen (Blaha and Winn, In press) and increases firing rate of DA neurones innervating the NAcc, respectively (Grenhoff et al. 1986). Such stimulation can also be achieved by blocking the degradation, and thus prolonging the stimulatory action, of endogenous ACh in the SNPC (Blaha and Winn, In press; chapter 4). Given the ability of cholinergic terminals from pontine nuclei to stimulate DA release in the forebrain target sites of midbrain DA neurones, it is pertinent to ask whether the effects observed following administration of dopaminergic agonists into striatal areas can be replicated by stimulating DA efflux with cholinergic agonists microinjected to the cell bodies of dopamine neurones in the midbrain. Therefore this study will examine and compare the effects on responding in a conditioned reinforcement paradigm of cholinergic stimulation of VTA or SNPC. Given the results of previous studies using DA agonists injected into the CP and NAcc it is predicted that cholinergic stimulation of the VTA, but not the SN, will increase responding for conditioned reinforcement.

Methods

Animals

36 rats were used. To maintain the animals at 85% of free-feeding body weight, approximately 12 g of food (SDS maintenance diet no.1 chow

pellets) were given at least one hour after testing. Mean body weight at the time of surgery was 307.8 g (SE = 2.47).

Stereotaxic surgery

Rats were anaesthetized with xylazine and ketamine and placed in a stereotaxic frame in the orientation of De Groot. The rats were implanted bilaterally with guide cannulae terminating above the anterior SN or VTA.

Intracranial drug administration

During each test session each rat received a bilateral microinjection to the SN or VTA. The rats received a cholinergic cocktail containing the following doses of carbachol and nicotine: 0 + 0, 0.1 μg + 0.055 μg, 0.1 μg + 0.1 μg, 0.1 μg + 0.25 μg. Previous experiments (Chapter 6) have shown that these doses of cholinergic agonists have reliable stimulating effects in a free-feeding task.

Habituation and training

Following recovery from surgery, rats were habituated to the double-lever operant chamber over two 10 min sessions. In these habituation sessions the house lights were off, the food tray panel light on and 12 pellets (45 mg Dustless Precision Pellets, BioServ) were available in the food tray.

In the training phase the animals were trained to associate the dispensing of food into the food tray with a preceding compound stimulus. The compound stimulus consisted of: the house light going off, the food tray panel light coming on, and the auditory click of the food dispenser. The presentation of the stimulus followed by the dispensing of a food pellet was on a random-time schedule beginning with a random presentation time between 8 and 12 sec. Pushing open the food tray panel prematurely, i.e. before the compound stimulus presentation, was 'punished' by a 3-second 'time-out' before returning to the random-time

schedule of stimulus-food presentation; punishment was not cumulative. When subjects performed correctly (pressing the food tray panel only following the presentation of the compound stimulus) on at least 90% of the trials presented within a session, the random time schedule was increased by 5 sec. Each training session was 30 min in length and was repeated daily until the animals achieved criterion at a random time schedule between 23 and 27 seconds. For the rest of the experiment, there were no longer food pellets present in the food hopper.

One session was run for each animal where responding on one lever resulted in the presentation of the compound stimulus which henceforth will be referred to as the conditioned reinforcer lever (CR lever) but no food pellet. Responding on the other lever resulted in no programmed response (NCR lever, no conditioned reinforcer). Ascription of which lever (left or right) should be the CR lever was made as a function of counterbalancing equal L and R for the group and equal responding on L and R levers on the last training session. The session lasted until 10 correct responses (pressing the CR lever) were recorded.

Testing

24 h after the final training session, the rats began a series of 4 test sessions, separated from, each other by 48 h. Each animal received bilateral microinjections through the chronic cannulae before being placed in the operant chamber. The order of testing was counterbalanced for group and site. Responses on the CR and NCR levers and food tray panel presses were recorded for 30 min.

Statistical analysis

Responses on the CR and NCR levers were analysed by analysis of variance using site (SNPC and VTA), lever (CR lever and NCR lever) and dose (saline and three doses of drug) as main effects. Food tray panel pushes were analysed similarly for effects of site and dose. The data and

analysis are presented without transformation, but analysis of the data with a square-root transformation produced similar results. Post-hoc analysis was made with Newman-Keuls test for multiple comparisons.

Results

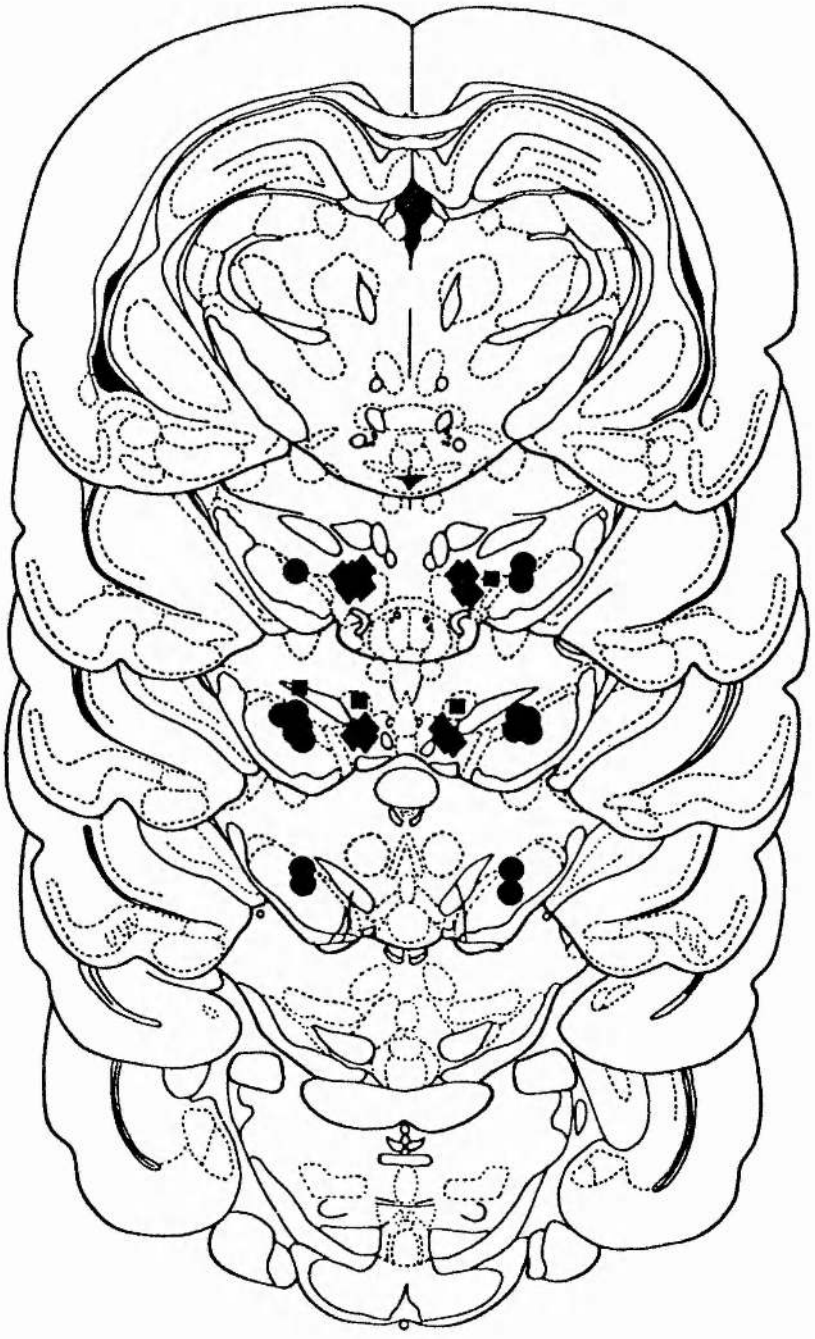
Histology

Cannula placements were verified post-mortem and Figure 20 presents representative sections showing these. Following histological examination, 16 rats were found to have bilateral injection sites in SNPC and 16 had bilateral sites in VTA. Three animals had injection sites dorsal to their target, and 1 animal had an ambiguous placement that may have effected SNPC or VTA neurones. These 4 rats were excluded from all analyses.

Responding for conditioned reinforcement

All of the animals achieved a criterion of pressing the NCR lever on less than 10% of trials. The animals took 24 training sessions to achieve this criterion. Analysis of responding over 30 min following microinjection revealed a main effect of lever ($F=7.82$ $df=1,30$ $p<0.01$) but no effect of site ($F=0.11$ $df=1,30$), dose ($F=0.41$ $df=3.90$) and no interactions (lever x site: $F=0.36$ $df=1,30$; dose x site: $F=0.54$ $df=3,90$; lever x dose: $F=0.29$ $df=3,90$; site x lever x dose: $F=0.29$ $df=3,90$). Analysis of panel pushing over the same time period revealed a significant main effect of dose ($F=3.81$ $df=3,90$ $p<0.02$) but no main effect of site ($F=0.36$ $df=1,30$) and no site x dose interaction ($F=0.65$ $df=3,90$). Post-hoc analysis of panel pushing revealed significant differences between the saline condition and 0.1 μg nicotine + 0.55 μg carbachol

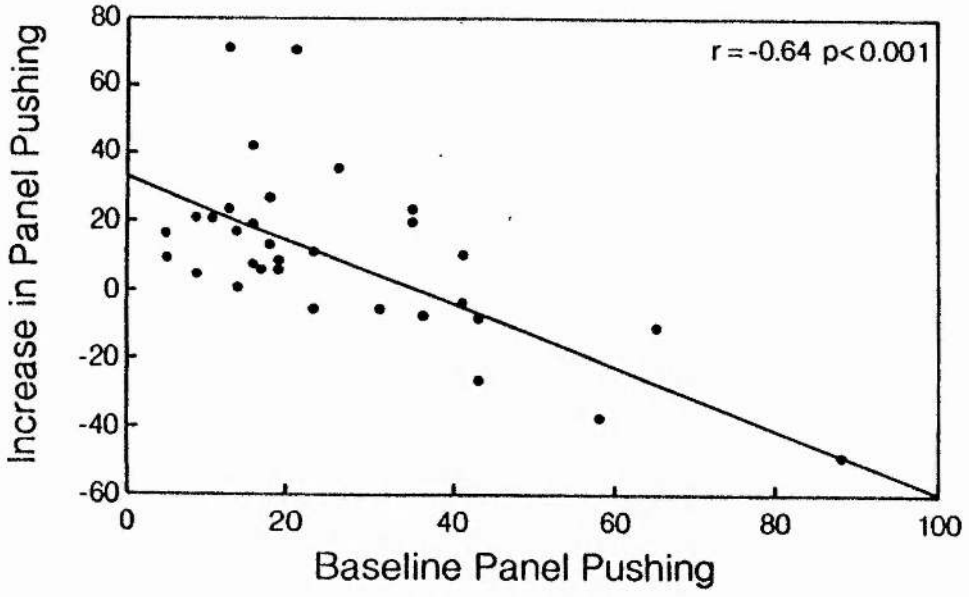
Figure 20: Representative sections showing SNPC (●), VTA (◆) and misplaced (■) cannulae. The placements of cannulae are shown here unilaterally for clarity, but represent bilateral injection sites. Misplaced cannulae were either outwith the SNPC and VTA or ambiguous in their placement



and between saline and 0.1 μg nicotine + 0.25 μg carbachol (both $p<0.05$).

An examination of the raw data for panel-pushing (Table 3) suggested that panel pushing in response to cholinergic stimulation might vary dependent on the baseline response to saline. As the ranking of responding shows, there was no effect of site on baseline responding. The Pearson correlation coefficient was calculated comparing panel pushing to saline with the increase in panel pushing to the cholinergic cocktail. This was calculated as the mean of the three doses minus the response to saline. There was a correlation of r=-.64, with a statistical significance of $p<0.001$ (Figure 21).

Figure 21: Scatterplot of panel pushing to saline vs increase in panel pushing to cholinergic stimulation (mean of three doses minus saline).



Given rate-dependency of panel pressing, the rats were divided into *low* and *high* baseline groups using a median-split (Figure 22). The *low* group, but not the *high* group, showed dose-dependent increased panel pressing (F=7.01 df 3,45 $p<0.001$, F=.62 df 3,39 resp.). Post hoc

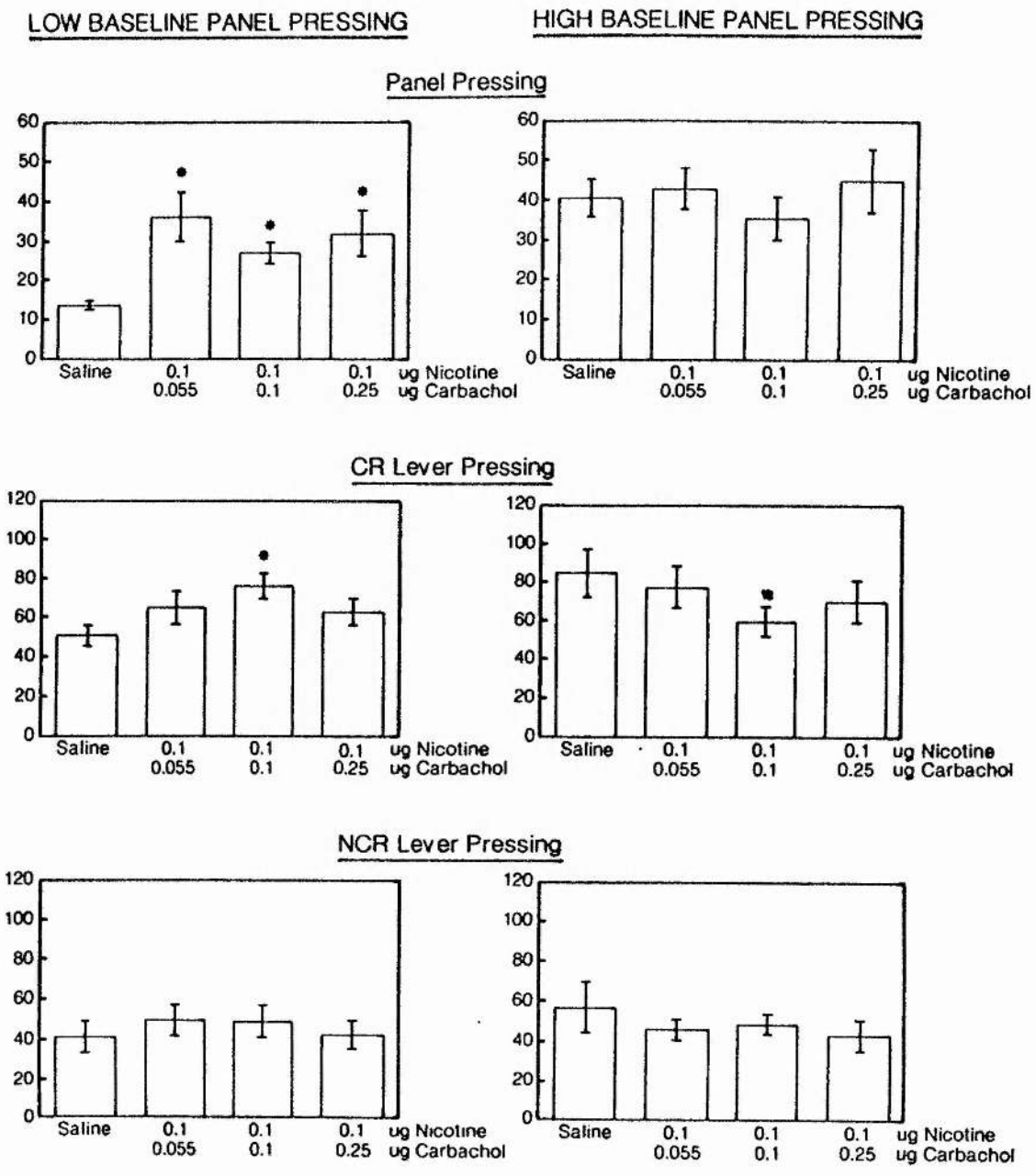
Table 3: Panel pressing following bilateral microinjection of cholinergic cocktail into SNPC or VTA (doses of carbachol + nicotine in 0.5 µl saline) ranked by response to saline measured over 30 min post-injection

Doses of Carbachol and Nicotine in saline	0 0	0.1 0.055	0.1 0.1	0.1 µg 0.25 µg
VTA	5	26	13	23
VTA	5	12	14	16
SNPC	9	28	31	29
VTA	9	14	13	14
VTA	11	52	25	17
VTA	13	120	42	89
SNPC	13	49	13	46
SNPC	14	18	18	5
SNPC	14	41	19	32
SNPC	16	30	43	31
VTA	16	38	17	13
SNPC	16	38	47	88
SNPC	17	28	30	11
SNPC	18	56	25	51
SNPC	18	19	37	36
VTA	19	24	30	21
VTA	19	21	40	21
VTA	21	88	81	103
VTA	23	40	30	31
VTA	23	5	22	25
SNPC	26	45	57	79
SNPC	31	33	11	32
SNPC	35	61	52	50
VTA	35	51	28	95
VTA	36	33	13	38
SNPC	41	40	71	42
SNPC	41	24	27	60
SNPC	43	48	20	35
VTA	43	24	22	4
VTA	58	34	25	3
SNPC	65	66	31	65
VTA	88	53	47	17

analysis of panel pressing in the *low* group revealed saline to be significantly lower than all three doses of cholinergic cocktail (all $p<0.01$). The lever pressing raw data was then re-analysed (ANOVA) with main effects: site (SNPC and VTA); baseline panel pressing (*low* and *high*); lever (CR and NCR); and dose (saline and three doses of cholinergic cocktail). There was no main effect of site ($F=.12$ *df* 1,28), baseline panel-pressing group ($F=.86$ *df* 1,28) or dose ($F=.48$ *df* 3,84) but there was a significant effect of lever ($F=9.20$ *df* 1,28 $p<0.005$), a panel-pressing group x dose interaction ($F=5.02$ *df* 3,84 $p<0.03$) and a panel-pressing group x lever x dose interaction ($F=2.86$ *df* 3,84 $p<0.05$). No other interactions were significant.

Post-hoc analysis revealed higher responding on both levers in response to saline in the *high* baseline panel-pressing than in the *low* panel-pressing group ($p<0.01$). Post-hoc analysis of the three-way interaction revealed higher responding on the CR lever to 0.1 μg carbachol + 0.1 μg nicotine than to saline in the *low* baseline panel-pressing group ($p<0.01$). Responding to CR lever in the *high* baseline panel-pressing group was significantly less in response to 0.1 μg carbachol + 0.1 μg nicotine than to saline ($p<0.01$). Responding was also significantly higher on the CR than the NCR lever in response to saline in the *high* baseline panel-pressing group ($p<0.05$).

Figure 22: Mean (±SE) panel pushes, CR and NCR lever presses in 30 min following bilateral microinjection of doses of carbachol + nicotine into the VTA and SNPC (data collapsed across site) in low and high baseline panel pressing groups. (significant effect of dose $p < 0.05$)*



Discussion

Cholinergic stimulation of midbrain DA neurones increased responding for conditioned reinforcement in animals with a low baseline rate of panel pressing. The rats with a high baseline rate of panel pressing showed a higher baseline rate of CR than NCR lever pressing but no increased CR responding following cholinergic stimulation. This suggests that learning not to press the hopper-panel is a better predictor of increased responding for conditioned reinforcement following cholinergic stimulation than baseline CR lever responding.

Rats with a high baseline rate of panel pressing showed no effect of dose on responding to the panel, while rats with a low baseline rate showed increased panel pressing following cholinergic stimulation. It could be suggested that this is due to a ceiling effect. However, it is unlikely that there would be such a strong correlation between baseline panel-pressing and increase in panel pressing following cholinergic stimulation. The decrease in responding on the CR lever with no difference in responding on the NCR lever also argue that we are observing an interesting effect rather than a non-specific effect on general behavioural responding. That low baseline panel pressing rats increase their CR lever pressing while high baseline panel pressing rats decreased their responding is analagous to behavioural effects seen before in manipulations of midbrain DA systems. Winn et al. (1983) observed that stimulation of increased feeding behaviour was dependent on the level of feeding prior to the injection. Such rate-dependency of effects of manipulating DA systems on behaviour has been more fully described by Lyon and Robbins (1975) in their investigation of the central effects of amphetamine.

Low baseline panel pressing animals showed increased panel pushing, and increased CR but not NCR lever pressing. They responded to the

conditioned reinforcer and the site of the primary reinforcer. One interpretation is that cholinergic stimulation has reinstated panel pressing, widening the behavioural repertoire to include behaviour that had previously been reinforcing. That the increase in panel pressing occurred in conjunction with an increase in CR lever responses suggests increased cholinergic stimulation of midbrain DA-containing neurones has influenced behavioural selection within a learnt response set, rather than caused a dissociation of the CR lever with reinforcement.

The experimental hypothesis that stimulation of the VTA but not the SNPC would increase responding for conditioned reinforcement was not supported by this experiment. Injections of cholinergic agonists produced no difference between the two sites. Both sites receive cholinergic innervation from the pons and respond by increasing the activity of DA-containing neurones projecting to their respective striatal sites. However, previous studies injecting DA agonists to the NAcc and CP have associated increased responding for conditioned reinforcement with mesolimbic but not nigrostriatal DA (Taylor and Robbins 1984). It is an obvious prediction to make that because direct manipulation of different striatal sites produces different behavioural effects, then stimulation of the sources of the DA innervation of these structures - the SNPC and the VTA - must also have different effects. However our results suggest that cholinergic stimulation of the SNPC and VTA produces no observable differences between sites in a conditioned reinforcement paradigm. There is evidence available that supports our data. Electrophysiological studies suggest that midbrain DA neurones in the monkey SNPC and VTA responded in a similar fashion in a conditioned reinforcement paradigm similar to that described in this paper (Ljungberg et al. 1992). Furthermore, Lacey et al. (1990) found that rat VTA and SNPC neurones

responded similarly to stimulation with M1 muscarinic cholinergic stimulation. As both sites receive cholinergic innervation from the pons, and this innervation is mediated at least in part by the same receptor sub-types, it is plausible that both mesencephalic sites are receiving and responding similarly to the same information. It could be that the results of studies using direct stimulation of the striatal sites reflect differences in the topographical innervation of the NAcc and the CP and the heterogeneity of DA receptor sub-type distribution in the striatum rather than a functional difference.

Administration of the peptide substance P to the VTA produced a statistically significant increase in responding on both the CR and NCR lever, suggesting a non-specific stimulation of motor output rather than a potentiation of responding for conditioned reinforcement (Kelley and Delfs 1991). However, the results of the present study following cholinergic stimulation demonstrate a dose-dependent increase in panel-pushing and increased responding on the CR but not NCR lever in low baseline panel pushing rats. Although the increase in responding for conditioned reinforcement was significant, it is not as strong as that produced by intra-NAcc amphetamine. This may be due to differences in levels of DA-ergic stimulation produced. In the present study DA efflux is stimulated by increasing the activity of midbrain DA-containing neurones to release DA within the physiological limits of normal neuronal activity. It has been suggested that increased responding for conditioned reinforcement using intra-NAcc amphetamine depends on massively increased efflux levels of DA compared to baseline (Hernandez and Hoebel 1988).

Increased DA efflux in the striatum has been interpreted as playing a variety of roles associated with a broad range of activities. These data fit well with the proposal by Robbins and Brown (1990) that one, if not the

major function, of the CP is to define a "response set". The spatio-temporal control of certain responses, that would necessarily be a subset of possible responses, will be reinforced by DA release in the CP. However, their discussion does not make explicit what effect DA release would have on the responses to a given situation. Ljungberg et al. (1992) described the firing of DA neurones as falling off with repetition of a task. He suggests that once learnt a task has less of an "expectation" component and becomes an automatic response. This would be in agreement with Robbins and Brown (1990) and the present experiment. To place this studies findings in the vernacular of Ljungberg et al., the rats expect to find food in the apparatus and DA neurones respond; they then learn that pressing a lever produces the presence of food in the hopper and DA neurones are still responding; over trials the behaviour becomes learnt and DA responding falls off with the lessening of an 'expectation' component; finally, in the test session the cholinergic stimulation of DA neurones signals that this situation is one requiring 'expectation'. However, the present studies results suggest this loosening of behavioural response to the situation only extends as far as behaviours that have some association with obtaining of food. Hence an increase in panel-pushing and no change in responding to the NCR lever. It might be expected that there would be a decrease in responding on the CR lever. That this is not the case supports the interpretation of an increase in behavioural repertoire within a learnt response set, rather than an unlearning of the association of the CR lever with reinforcement.

9 Discussion

This thesis has demonstrated behaviourally a cholinergic influence on midbrain DA-containing neurones. Administration of exogenous nicotinic and muscarinic cholinergic agonists into the anterior substantia nigra elicited increases in the consumption of palatable food but not standard lab chow in pre-satiated rats. Furthermore, this effect was also produced by blocking the de-activation of endogenous ACh using the AChE inhibitor neostigmine.

Both muscarinic and nicotinic receptors are present on midbrain DA-containing neurones. Stimulation with agonists for either produced increased consumption of palatable food in pre-satiated rats. Addition of a behaviourally stimulating dose of carbachol to a range of doses of nicotine produced a significant increase in feeding compared to nicotine alone. Furthermore, the nicotinic stimulation still produced dose dependent effects at the elevated level of feeding produced by carbachol suggesting a true additivity of effects. This study demonstrated that cholinergic stimulation of nicotinic and muscarinic receptors in the SN can produce a behavioural effect that is additive using a relatively coarse measure of behaviour. There is presumably a functional reason for having two sorts of cholinergic receptor in the SNPC, and indeed sub-types of muscarinic receptors. The effects mediated on the cell membrane by ACh at such sites need not necessarily always be additive. Although there is evidence for the presence of both types of receptor on DA-containing neurones of the SNPC and VTA, there is as yet no demonstration of (i) what proportion of neurones have nicotinic, muscarinic, and both types of receptor, (ii) how they relate on the somata and dendrites and (iii) how

the administration of specific agonists for each type effects the cholinergic stimulation at physiological and behavioural levels.

Although there was strong evidence for the effects of cholinergic stimulation of the SN being mediated by the DA-containing neurones of the SNPC, this had not previously been demonstrated directly. Increased consumption of palatable food elicited by cholinergic stimulation of the anterior SN was abolished by a selective 6-OHDA lesion of ascending DA containing neurones that significantly reduced DA concentration in the CP but left NAcc DA unaltered. Previous studies have described the need for near total depletions of CP DA levels for there to be obvious behavioural dysfunction in response to direct agonists, but unilateral lesions of the size described here will produce rotational behaviour in response to indirect DA agonist amphetamine (Hefti et al. 1980). The ~50% depletion of CP DA levels was sufficient to attenuate the increase in feeding caused by cholinergic stimulation of the SN. This level of depletion in a unilateral preparation did not effect normal eating behaviour as there was no difference in consumption following saline injection between the 6-OHDA and sham lesion groups. It would appear that the brain, whether it be rat, monkey or human, can sustain large depletions of CP DA without obvious effect on behaviour. That there is no significant post-synaptic compensatory response to the loss of DA until it reaches the order of 90% seems incredible. However, if the role of striatal DA is to facilitate the processing of cortico-strio-pallido-thalamo-cortico-reticular outflow, it is easier to see how such a neurochemical deficit might not be behaviourally obvious.

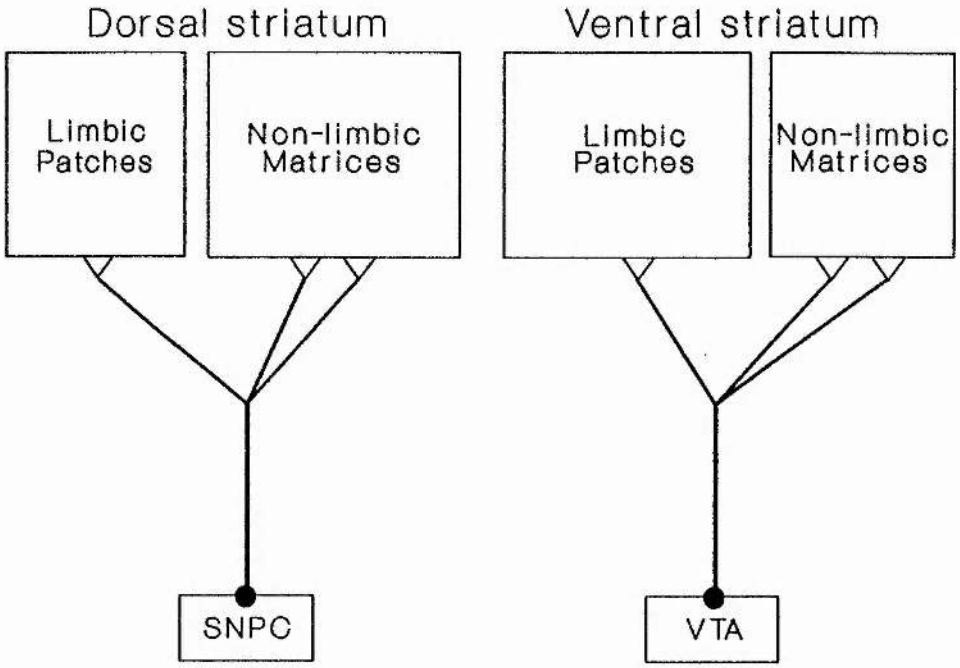
The mesencephalic DA-containing neurones of the SNPC and VTA innervate the CP and the NAcc. Studies injecting DA agonists directly into these forebrain sites have shown increased responding for

conditioned reinforcement from the NAcc but not the CP. Hence it was predicted that cholinergic stimulation of the VTA but not the SNPC would produce increased responding for conditioned reinforcement. Contrary to the experimental hypothesis, administration of cholinergic agonists to VTA or SN caused indistinguishable effects on responding in a conditioned reinforcement paradigm. Cholinergic stimulation of midbrain DA neurones increased responding for a conditioned reinforcer and reinstated responding at the primary reward source. Food was used as the primary reward associated with the previously neutral stimulus in the training phase of this experiment. However, in the test phase the rats were responding for the presentation of just the conditioned reinforcer. This study had shown that cholinergic stimulation of midbrain DA neurones can influence not only appetitive behaviour, but also the more abstract construct of responding for a reinforcing stimulus with the appetitive component removed.

There is still the question to be addressed of why cholinergic stimulation of the DA-containing neurones on the SNPC and VTA does not reflect the differences observed following administration of DA-ergic agonists into the CP and the NAcc. Gerfen's scheme (1992) of how cortico-strio-pallido-reticular connections relate (Figure 5) and Lowenstein's demonstration (1990) of consistently more DA terminals in the matrices than the patches of the CP *and* the NAcc - approximately 2:1 - may give us a clue. It has been proposed by Gerfen (1989) that the patches of the dorsal striatum conveys "limbic" information - motivation, learning and memory - normally associated with the ventral striatum, and that the matrices of the ventral striatum conveys "non-limbic" information - motor control and sensorimotor integration - more normally associated with dorsal striatal function. Furthermore Gerfen proposes that the limbic

circuitry dominates in the ventral striatum, while the non-limbic circuitry dominates the dorsal striatum. Therefore direct administration of DA-ergic agonists into the NAcc will favour "limbic behaviour", while DA-ergic stimulation of the CP will favour "non-limbic behaviour". However, if as is suggested by Lowenstein et al. (1990) the innervation of the matrices is two-fold greater than that that of the patches in both the dorsal and ventral striatum, stimulation of DA efflux by stimulating the SNPC or VTA would similarly favour the "non-limbic" matrices (see Figure 23).

Figure 23: Proposed model of DA-ergic innervation of striatum to explain the difference in effect of stimulation at striatal and midbrain sites



Thus administering DA agonists directly into the striatum would reveal the dorso-ventral gradient of "non-limbic" and "limbic" striatal targets, while promotion of DA efflux from the midbrain cell bodies would effect both the "non-limbic" matrices and "limbic" patches in a proportion that

consistently favours the "non-limbic" matrices. This could explain why cholinergic stimulation of the midbrain DA systems produced no site difference between the VTA and SNPC. It would also explain why the increase in responding for CRF was present, but not as clear as can be produced by intra-NAcc injections.

Leonard and Llinas (1990) have described three types of neurones in the PPTg and LDTN on the basis of their electrophysiology, morphology and hodology. It appears that type II and possibly type III neurones are cholinergic and are candidates for providing the cholinergic influence on midbrain DA-containing neurones. Type II neurones were observed to display tonic firing patterns, while type III neurones are characterised as being able to display tonic *or* phasic firing patterns. Cholinergic neurones displaying a tonic firing pattern would be in keeping with the view of the PPTg maintaining a tonic influence over the brain as part of an ascending reticular activating system (Garcia-Rill 1991). However, the possibility that there are also type III neurones innervating the midbrain from the PPTg make it tempting to speculate as to the purpose of having both muscarinic and nicotinic cholinergic receptors on the membranes of midbrain DA-containing neurones. That a phasic cholinergic influence might be mediated by nicotinic receptors while a tonic influence is maintained by muscarinic receptors provides us with the possibility that separate populations of cholinergic neurones in the pons are effecting the activity of midbrain DA-containing neurones in a tonic and a phasic manner. However, as it stands the evidence seems to favour a predominantly tonic effect of cholinergic neurones on midbrain DA-containing neurones. How the sub-types of cholinergic receptors are distributed on the DA-containing neurones and the pattern of cholinergic innervation from the PPTg and LDTN are questions that we can expect to be answered with existing techniques.

Cholinergic control of midbrain DA-containing neurones determines the efflux of DA at the respective forebrain sites of the SNPC and the VTA. It is tentatively suggested on the basis of the experiments described here and previous studies that the DA efflux is playing a facilitatory role on the processing of information from the limbic cortex in the NAcc and sensory and motor cortex in the CP. In so doing DA efflux is gating the processing of inputs that are presumably competing for influence over the output stations of the basal ganglia, the GP and the SNPR.

Increased DA release in the CP following cholinergic stimulation of SN is causing increased behavioural activity in a manner dependent on certain behavioural antecedents and environmental cues. Schultz and his colleagues (Ljungberg et al. 1992) state that "DA neurones...respond to stimuli that have the capacity to elicit behavioral reactions...stimuli which do not elicit attention or particular behavioral reactions from the subject, do not activate DA neurons". Furthermore, Schultz found that DA neurons increased firing if these conditions were satisfied, even if the animal did not then respond.

There exist neurones in the CP that appear to fire in a manner consistent with the expectation of a sensory cue or behavioural act that the animal should expect to find rewarding (Hikosaka et al. 1989). These neurones do not fire in response to fixation of a point, nor do they fire to the presentation of an unexpected reward. Indeed in studies using conditioned association of a neutral stimulus with a primary reward, once the association is learnt, the neurone will fire to the preceding conditioned stimulus and not the primary reward. Other CP neurones will fire in response to a novel stimulus, but such a stimulus might well provoke the expectation of reward in an animal high up the food chain. Such novelty to a rodent might well provoke an expectation of being lunch rather than finding it.

Behavioural responses have been observed following microinjections of cholinergic agonists into the SN. It might be predicted from the work of Hikosaka et al. (1989) and Ljungberg et al. (1992) that stimulation of DA firing in this way might elicit a behavioural response to any stimuli that might be presented to the animal. However, the experiments presented in this thesis show that this is not the case. It might be that a given stimulus must be 'plausible' as a target of behaviour. Whatever the construct is that guides the animals response or lack of response in a given situation, it does so such that an animal given no suitable or 'plausible' behaviour to perform following cholinergic stimulation of NSP DA neurones will perform as if no stimulation has occurred. The animal displays no visible signs that might be interpreted as thwarting, no increased locomotion, exploring to find a suitable outlet for said stimulation. It appears that some process has simply ignored or suppressed the information contained by the increased efflux of DA into the CP. Furthermore, if we now accept Hikosaka's views on caudate activity and Schultz's views on NSP DA firing, we might predict that stimulation of DA firing concomitant with the opportunity to perform a behaviour the animal would find rewarding should increase the probability of that behaviour being performed. If Schultz is correct, at a rat's first exposure to palatable food the DA neurones will be firing, responding to a stimulus that has the capacity to elicit a behavioural reaction. Administration of cholinergic agonists at this point would be attempting to cause an increase in firing by pharmacological means in neurones that are already firing as a consequence of a behavioural stimulus. It is not clear whether such a manipulation should be able to increase the behavioural response of the animal in response to, say, a mildly stimulating behavioural stimulus.

So what is happening such that the neurones are able to be stimulated once the animal has been pre-exposed? Is it that the DA neurones have

stopped firing after the first exposure and that the cholinergic stimulation is now able to stimulate increased behaviour now that the behavioural stimulus itself has lost its 'attraction' ?.

If such a behaviour can be seen as the culmination of a drive saying:

Expect a reward

and sensory feedback checking:

Is there a reward ?

and if Schultz is correct in his description of DA neurones responding to stimuli that could be expected to be rewarding, the cholinergic stimulation of DA neurones would be predicted to have a re-rewarding effect on any stimulus that previously had been rewarding but was not at the time of administration of cholinergic agonist to the SN. This would fit what is observed in the studies described here.

Blaha and Winn (In press) have demonstrated an elevated efflux of striatal DA following microinjection of cholinergic agonists or anticholinesterases into the SNPC of an anaesthetised rat. The time course of the effect on striatal DA levels following neostigmine is of the order of four hours which is consistent with its duration of pharmacological effect (Taylor 1980). Efflux of DA in response to nicotine is still elevated far longer than this. Why is it then that the behavioural responses observed following microinjection of the same doses into unanaesthetised rats produce last at most an hour? If a rat is left in a cage with nothing to do, microinjection of cholinergic agonists into the mesencephalon will have no observable behavioural effect. It would appear that there is a sensory component that must be satisfied for behaviour to occur. If there is no plausible opportunity for behaviour, then nothing occurs. If the animal is in the presence of palatable food, palatable fluid or an opportunity to copulate then there is a plausible behaviour for the animal to engage in. Blaha and Winn's measurement of DA efflux remained elevated for as

long as the drug's duration of effect possibly because there would be no cortico-striato-pallido-mesencephalic feedback in the anaesthetised rat to inhibit the firing of the DA neurones.

Both the VTA and the SNPC receive cholinergic innervation from the pons and respond, in a conditioned reinforcement paradigm and electrophysiological studies, in a similar manner to cholinergic stimulation. Given that their target striatal structures have other afferents and efferents that are not the same it is understandable that direct stimulation of these structures will produce varying effects on behaviour. But these results suggest that physiologically relevant activation of the DA-ergic afferents can produce the same effect on an appropriate behavioural test.

The innervation of these sites by pontine cholinergic neurones, although significant and clearly functionally important, is much smaller than the projection to various thalamic nuclei known to be involved in motor control, especially the ventrolateral thalamus (Lee et al. 1988). It could be that the cholinergic stimulation of the VTA and SNPC co-ordinates the *activation* of behaviour, with the *execution* being controlled by those areas of the thalamus receiving pontine efferents. Further research will characterise more fully how pontine cholinergic influence effects the relationship between the thalamus, the neocortex and the basal ganglia in the selection and control of behaviour.

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