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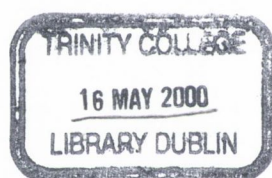
**Morphological Change in Nucleus Accumbens Neurons as a
Basis for the Development of Haloperidol-Induced
Movement Disorders**

By

Ian Ernesto João De Souza

This thesis is submitted in fulfilment of requirements for the degree of
Doctor in Philosophy to Trinity College, University of Dublin

Department of Zoology
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March 2000



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Declaration

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Summary

A syndrome of involuntary, hyperkinetic abnormal movements called tardive dyskinesia (TD) develop in predisposed individuals following treatment and withdrawal from neuroleptic drugs. These movements are primarily orofacial in nature, take several weeks to develop and persist even after drug withdrawal. Rats treated chronically with classical neuroleptic drugs such as haloperidol may develop a syndrome of vacuous chewing movements (VCMs) and these have been shown to be an animal model for TD. The neural basis for the development of TD or its rat equivalent remains elusive. However, it is known that chronic haloperidol treatment leads to slowly developing morphological changes in neurons. These morphological changes may then form the neural basis for the development of orofacial TD. Neuroleptics act by blocking dopamine receptors and one of their primary sites of action is in nucleus accumbens, a nucleus that forms part of the ventral striatum. Recent studies have shown that nucleus accumbens plays an important role in mediating orofacial movements in rats. Therefore, this study aimed to determine whether changes in the morphology of nucleus accumbens neurons provide the neural basis for the development of the rat equivalent of TD. From the data presented here it was found that rats, that develop a syndrome of vacuous chewing movements (VCM+ rats), have altered morphology compared to both vehicle-treated rats and neuroleptic-treated rats that did not develop the syndrome (VCM- rats). The development of a syndrome of VCMs in rats, that persists long after the drug is withdrawn, was associated with structural changes in neurons from both the shell and core territories of nucleus accumbens but was most evident in shell neurons. These cells have lost surface area along their dendrites, reduced their tortuosity, and yet increased their spine density. Such dramatic shifts in dendritic organisation may alter the physiological function of these cells and may provide the basis for the development of abnormal movements.

A reduced number of spines in distal core dendrites of VCM- rats compared to VCM+ rats is consistent with an excitotoxic mechanism at distal dendritic sites in this territory. In addition, they are most likely due to increased expression of the neuropeptide dynorphin in VCM+ rats. Excitotoxicity in VCM- rats may be mediated

by glutamate, since it is known that chronic haloperidol treatment elevates extracellular glutamate. It is demonstrated here that glutamate transport activity, important for terminating the action of glutamate by removing it from the synaptic cleft, is impaired following chronic haloperidol treatment and this may be important to both efficacious and undesirable effects of this drug. Glutamate is also important in regulating nitric oxide expression and data presented here shows that changes occur in the morphology of nitric oxide synthase immunoreactive (NOS-IR) neurons following chronic haloperidol treatment. In addition, neurons located in the core of VCM- rats are affected more than VCM+ rats. This is most likely due to differences in NMDA receptor expression between the two groups. However, the acute actions of haloperidol seem to be mediated by NMDA independent mechanisms in nucleus accumbens in contrast to the dorsal striatum where effects are NMDA dependent. In addition, the behavioural profile of VCM+ rats is consistent with increased activation of D₁ receptors compared to VCM- rats as evidenced by increases in grooming behaviour. Overall, it appears that selective activation of D₁ receptors in VCM+ rats results in morphological change in both the shell and core of nucleus accumbens that may be mediated through glutamate and the neuropeptide dynorphin. Thus, morphological change in the dendritic structure of projection neurons coupled with changes in NOS-IR interneurons may provide the neural basis for the development of vacuous chewing movement syndrome in rats treated chronically with haloperidol followed by withdrawal.

Acknowledgements

Even more challenging than any academic endeavour is to graciously acknowledge those who have been instrumental to its success. Therefore, first and foremost I must thank my parents and family both for their constant support and for instilling in me the desire to see out a project to fruition. To Eimear, I owe more than thanks for she has both embellished my soul and inspired my creative ability.

I must thank Gloria Meredith who has been instrumental in changing the morphology of my intellectual mind from that of a biochemist to one of a neurobiologist. She has advanced my ability to pose relevant scientific hypotheses and critically appraise relevant scientific literature.

I would like to thank Dr. Niamh Dawson for all her help and guidance in all aspects of this project.

I would like to express my sincerest thanks to Prof. Stanley Monkhouse and the staff of the Anatomy Department, RCSI for creating a friendly environment in which to conduct my research. I would also like to give special thanks to Prof. Barry Roberts and the staff and postgraduate students of the Zoology Department for their high spirits and constant encouragement.

Special thanks must be extended to Peter Kelleghan (Anatomy) and Peter Stafford (Zoology) for their expert problem solving abilities especially at times of ensuing project meltdown.

I would like to thank Dr. Gethin McBean and all at the Biochemistry Department UCD for all their help in carrying out the glutamate transport study.

I would also like to thank Prof. John Waddington and Dr. Gerry Clifford for their help with the behavioural aspects of this study.

I thank Gerry McCarthy, Derek Borwick and Martin Dunphy as well as Peter Nowlan and the staff of Bioresources for their expertise in animal care.

Thanks also to Ronan Conroy for help with all things statistical and to Joy Rice and Sinead Kelly for helping with the nitric oxide synthase study.

I would like to express my sincere gratitude to the Research Committee of the Royal College of Surgeons in Ireland and to Enterprise Ireland for generously funding this study.

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Abbreviations

Abbreviations

ABC	Avidin-biotin-peroxidase complex
AC	Anterior commissure
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CBP	Calcium-binding protein D _{28K}
CPu	Caudate/putamen
DAB	3-3'-diaminobenzidine
DAPI	4,6, diamidino-2-phenylindole
EDTA	Ethylene diamine tetra acetic acid
GABA	γ -aminobutyric acid
i.p.	Intraperitoneal
IR	Immunoreactivity
K_m	Michaelis-Menten constant
LV	Lateral ventricle
LY	Biotinylated lucifer yellow
MK801	Dizocilpine maleate
mRNA	Messenger ribonucleic acid
NHS	Normal horse serum
NSS	Normal swine serum
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthase
NSS	Normal swine serum
PBS	Phosphate buffered saline
PcRt	Parvicellular reticular formation
PFC	Prefrontal cortex

s.e.m.	Standard error of the mean
SNpc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
TD	Tardive dyskinesia
Tris	Tris(hydroxymethyl)methylamine
Tx	Triton X-100
VCM+	Positive for vacuous chewing movement syndrome
VCM-	Negative for vacuous chewing movement syndrome
VCMS	Vacuous chewing movements
V_{max}	Maximum rate of activity
VP	Ventral pallidum
VTa	Ventral tegmental area

Publications

The following publications are submitted in support of this Thesis:

De Souza, I.E.J., McBean, G.J., Meredith, G.E. (1999) Chronic haloperidol treatment impairs glutamate transport in the rat striatum. *Eur. J. Pharmacol.*, 382:139-142.

De Souza, I.E.J., Meredith, G.E. (1999) NMDA receptor blockade attenuates the haloperidol induction of Fos protein in the dorsal but not the ventral striatum. *Synapse* 32:243-253.

(see **Appendix**)

Chapter 1

General Introduction

Haloperidol is a neuroleptic drug used clinically in the treatment of psychoses such as schizophrenia (Ban et al., 1984) and acts primarily by blocking dopamine D₂ receptors. Blockade of these receptors occurs within hours, however, the therapeutic action takes several weeks to develop (Casey et al., 1960; Johnstone et al., 1978). As well as inducing a therapeutic effect, chronic blockade of dopamine receptors may cause abnormal hyperkinetic movement disorders that are primarily orofacial in nature and persist after the drug is withdrawn (Kane, 1986; Ellenbroek, 1993). The neuronal basis for the development of these disorders remains elusive. However, it is known that chronic haloperidol treatment leads to slowly developing morphological changes in neurons (Roberts et al., 1995; Mijster et al., 1996). These morphological changes may then form the neural basis for the development of orofacial tardive dyskinesia (TD). Assuming morphological change underlies the development of neuroleptic-induced orofacial dyskinesias, where would such changes be most likely to occur? The most obvious place is a brain region that is affected by neuroleptic treatment and also forms part of a circuit that is involved in controlling orofacial movements. In the rat, the nucleus accumbens fulfils these criteria.

Nucleus accumbens

Nucleus accumbens is a subcortical nucleus that makes up a large part of the ventral striatum (Heimer et al., 1985). It is considered to be part of the striatum (Heimer and Wilson, 1975), however it is set apart from it by its input from limbic regions (Groenewegen et al., 1991; Heimer et al., 1991). Its main function is to exert control over locomotor activity and motivational behaviour (Mogenson, 1987; Koob and Bloom, 1988). It is comprised of both projection and local circuit neurons that receive inputs from a large number of brain nuclei including the cortex, sub-cortex and brain stem (for review see Meredith and Totterdell, 1999).

Projection neurons, commonly referred to as medium-sized densely spiny neurons, account for about 90% of the total number of neurons in nucleus accumbens (Thomas et al., 1995). They vary in size from 9 to 20 μm in diameter and have a cross-sectional area of approximately 100 μm^2 (Chang and Kitai, 1985; Meredith et al., 1992). These neurons have three to eleven primary dendrites that are free of

spines proximally and become densely spiny further distally (Meredith et al., 1992; O'Donnell and Grace, 1993). Synaptic input to these neurons occurs on both proximal and distal dendrites as well as on spiny projections that are found on dendrites (Meredith and Totterdell, 1999). Diverse assortments of spines are found on dendrites throughout the central nervous system (Luksh et al., 1998) and these were originally described as simple protrusions of the dendrite. However, more recently they have been found to be more complex specialisations that contain high concentrations of receptors and second messenger systems (Segal, 1995; Harris, 1999). In addition, they have very diverse structures and may be stubby, thin, mushroom or branched shapes (Gray 1959; Peters and Kaiser-Abramof, 1970). In striatal and nucleus accumbens neurons, individual spines often receive an asymmetrical input at their head and the majority receives a symmetrical terminal at their neck (Wilson et al., 1983; Totterdell and Smith, 1989). In addition, the vast majority of synapses occur on dendrites and their associated spines, rather than on the perikaryal membrane (Meredith et al., 1990).

The cell soma of medium-sized densely spiny neurons is typically round with a single large nucleus and a perikaryon that is often surrounded by glial cells and has little synaptic input (Meredith et al., 1990). Projection neurons are found throughout nucleus accumbens and use γ -aminobutyric acid (GABA) as their primary neurotransmitter as well as a variety of neuropeptides and opioids (Meredith et al., 1993; Pickel et al., 1988; Zahm and Brog, 1992). These neurons are not homogenous in nucleus accumbens, they differ morphologically, in their density, distribution, receptors and neurotransmitters depending on what part of the nucleus accumbens they are located.

In addition to projection neurons, nucleus accumbens contains a number of populations of interneurons that may account for up to 10% of total accumbal neurons (Thomas et al., 1995). There is a great deal of heterogeneity in the structure of both their dendrites and cell soma. Their dendrites vary considerably in length, are generally aspiny and may be smooth or varicose (Meredith and Totterdell, 1999). Based on the neurotransmitters and proteins they contain, they can be divided into at least four distinct, nonoverlapping populations (Hussain et al., 1996). Three of these

neuronal populations contain GABA whereas the fourth contains acetylcholine. One of these groups of GABA containing interneurons is identified by the presence of the calcium binding protein, parvalbumin (Kawaguchi, 1993). Parvalbumin containing neurons have cell bodies with diameters ranging from 13 to 28 μm . These cells are found in highest density in the lateral nucleus accumbens at rostral levels and their density falls further caudally and medially (Bennett and Bolam, 1994). Another GABAergic population of interneurons contains the calcium binding protein calretinin. Their diameters vary between 5 and 15 μm . In contrast to the previous group, their density is highest medially and becomes lower laterally (Bennett and Bolam, 1993).

A third population of interneurons also contain GABA as well as the neuropeptides, somatostatin and neuropeptide Y and the neuronal form of nitric oxide synthase (Kawaguchi, 1993). These interneurons make up 1-2% of total nucleus accumbens neurons. Their cell bodies may be bipolar, fusiform or multipolar and their cell bodies vary in cross-sectional diameter from 8 to 18 μm (Kawaguchi, 1993). Their dendrites are long and usually unbranched containing distal portions that are highly twisted and varicose (Hussain et al., 1996). The final group of interneurons contain acetylcholine and are identified by the presence of the enzyme choline acetyltransferase. These are large neurons with a cell body having a long diameter of 15 to 35 μm . Their cell soma may be round, fusiform or triangular with one to three smooth, long, unbranched dendrites (Phelps and Vaughn, 1986).

Regions of nucleus accumbens

Nucleus accumbens is organised into two large, neurochemically and cytoarchitecturally distinct compartments. These divisions were first observed by Záborszky et al. (1985) on the basis of differential acetylcholinesterase activity, cholecystokinin immunoreactivity and Timm's staining. These markers divide the nucleus into an outer shell comprising medial, ventral and lateral parts of the nucleus and an inner core surrounding the anterior commissure. Later, this shell/core division was demonstrated using immunocytochemistry for calcium-binding protein D_{28K}

(CBP; Figure 1.1), substance P and other proteins and peptides (Zahm and Heimer, 1988; Meredith et al., 1989; Groenewegen et al., 1991).

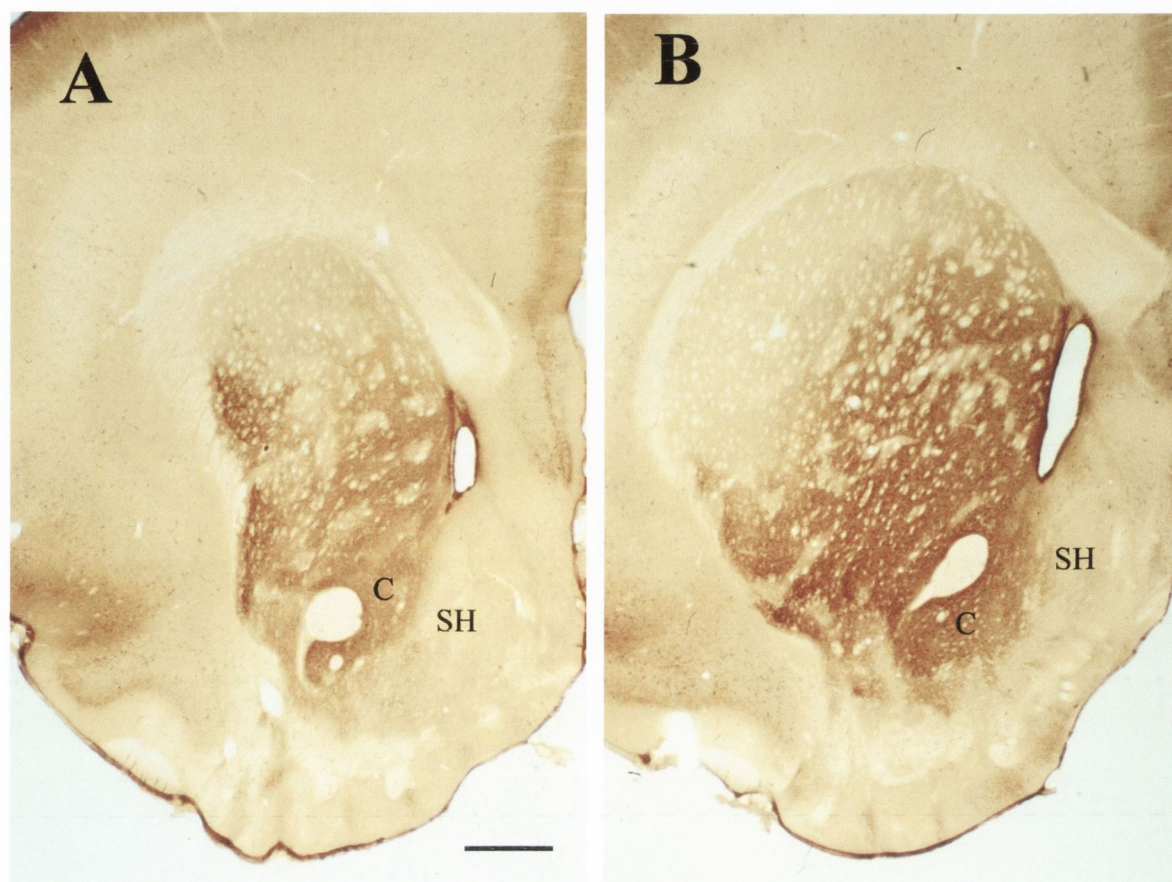


Figure 1.1 Photomicrographs of A: rostral and B: caudal sections through the rat striatum showing CBP immunoreactivity. Note in the core (C) immunoreactivity is strong whereas in the shell (SH) immunoreactivity is weak. Scale bar in A: also applies to B: and equals 500 μm .

The shell and core of nucleus accumbens differ in several respects. The structure of dendrites on accumbal projection neurons is markedly different both within the shell and between the shell and core territories. In the medial shell, neurons have fewer and more elongate dendritic arbours compared to both the lateral shell and the core (Meredith et al., 1992). Other morphological parameters such as spine density, dendritic length and number of dendritic branch points generally increase from medial to lateral parts of the shell whereas they remain relatively constant in the core (Meredith et al., 1992). There are also differences in the membrane properties of these projection neurons. Core neurons have more negative resting membrane potentials and lower mean input resistances than the medial shell (Meredith et al., 1993; O'Donnell and Grace, 1993). The density of dopamine receptors also differs in the two compartments (Bardo and Hammer, 1991).

As well as having morphologically distinct projection neurons the shell and core of nucleus accumbens have largely segregated inputs and outputs. Afferent input in nucleus accumbens is largely segregated between the shell and core (Groenewegen et al., 1989; Zahm and Heimer, 1990; Heimer et al., 1991; Zahm and Brog, 1992), however, this division of inputs is preferential since no projections are distributed exclusively in either compartment (Brog et al., 1993). The greatest contrast in input to the nucleus accumbens is illustrated by the contrasting afferent input to the core and the medial shell (Brog et al., 1993). The main asymmetrical synaptic inputs to the shell originate from the piriform, dorsal peduncular and infralimbic cortices, as well as the hippocampus, posterior amygdala and entorhinal cortex (Groenewegen et al., 1987; Berendse et al., 1992; Sesack and Pickel, 1992). These inputs are excitatory and presumably glutamatergic in nature. The shell also receives a dense dopaminergic projection from the ventral tegmental area (VTA) of the midbrain (Voorn et al., 1986). Excitatory glutamatergic input to the core arises primarily from the rostral agranular insular cortex as well as dorsal prelimbic and anterior cingulate areas of the prefrontal cortex (Berendse et al., 1992). In addition, the core receives dopaminergic projections from the substantia nigra pars compacta (SNpc) (Nauta et al., 1978; Zahm, 1991). In general, those areas that strongly innervate the shell tend to weakly innervate the core and vice-versa (Brog et al., 1993). However, while inputs to shell and core are largely segregated those inputs arising from the amygdala

and prefrontal area project to both territories (Meredith et al., 1993; Meredith and Totterdell, 1999). In addition, the spatial arrangement of synaptic input differs between accumbal territories. In the core, inputs from extrinsic sources primarily synapse on distal dendritic spines whereas those from intrinsic sources make contact with proximal dendrites or perikarya (Meredith and Totterdell, 1999). In the shell there is a much more even distribution of inputs from intrinsic and extrinsic sources throughout the dendritic arbour (Meredith, 1999).

The core and shell territories of nucleus accumbens also differ in their efferent projections (Zahm, 1989; Heimer et al., 1991). Neurons in the core primarily project to the dorsolateral ventral pallidum, substantia nigra pars reticulata (SNr), subthalamic nucleus and globus pallidus, whereas neurons in the shell output to the neurochemically distinct ventromedial ventral pallidum (Heimer et al., 1991). Overall nucleus accumbens is considered to be an integral but specialised part of the striatal complex. The core is largely similar to the caudate/putamen, indeed it is difficult to distinguish ventral caudate/putamen from the dorsal core (Heimer et al., 1991). The shell is both physiologically and morphologically quite different to the core (Meredith et al., 1992; Meredith et al., 1993). Shell neurons have fewer primary dendrites that branch less often and have lower spine densities than those in the core. In addition, neurons in the core have a much larger surface area for forming synapses (Meredith et al., 1993). Morphological differences are most marked between the medial shell and the core and become less in the lateral shell (Meredith et al., 1992). Indeed, the medial shell has been suggested to be a transition between the striatal complex and the extended amygdala (de Olmos, 1985; Alheid and Heimer, 1988; Heimer et al., 1991). This is based on neurochemical and structural studies (Záborszky et al., 1985; Alheid and Heimer, 1988; Meredith et al., 1989) that indicate segregation between the medial shell and more central and lateral parts. Therefore, any thorough investigation of structural change in nucleus accumbens neurons must examine the medial shell and the core separately.

The main excitatory inputs to nucleus accumbens form asymmetrical synapses (Groenewegen et al., 1987; Brog et al., 1993) that are presumably glutamatergic. Glutamate is the most abundant neurotransmitter in the central nervous system and approximately 40% of synapses in the brain are glutamatergic (Fonnum 1984;

Greenamyre, 1986). It is stored in synaptic vesicles at nerve terminals and is released after depolarization in a Ca^{2+} -dependent manner. The action of glutamate is terminated by removal from the synaptic cleft by specific, sodium-dependent, high-affinity transporters located on neurons and glial cells (Fonnum, 1984; Takahashi et al., 1997). Glutamate produces its physiological action by activating several classes of receptors that are located primarily on postsynaptic neurons. In nucleus accumbens, glutamate receptors are located almost exclusively on the heads of dendritic spines of projection neurons and on both the dendrites and cell soma of interneurons (see Meredith and Totterdell, 1999). Glutamate receptors have been classified pharmacologically into N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Watkins and Evans, 1981). The activation of these receptors opens ion channels. Activation of another class of glutamate receptors called metabotropic receptors that are linked to guanine nucleotide (G) proteins leads to changes in cyclic nucleotides and phosphoinositol metabolism (Nakanishi, 1992).

Of all the glutamate receptors, the NMDA receptor has been the most widely studied and appears to be selectively enriched on striatal projection neurons (Tallaksen-Greene et al., 1992). Activation of NMDA receptors leads to a massive intracellular influx of Ca^{2+} and Na^{+} (McBain and Mayer, 1994). Excessive stimulation of these receptors can lead to neuronal damage through excitotoxic mechanisms (Beal et al., 1986; Meldrum and Garthwaite, 1990). The AMPA receptor mediates most fast excitatory synaptic transmission and also acts as a ligand-gated ion channel. However, activation leads primarily to the influx of Na^{+} into the neuron with low permeability to Ca^{2+} . Receptors can be formed from four different subunits called GluR1-GluR4 (Hollmann et al., 1989) and these can form homomeric or heteromeric ion channels that have varying permeability to Ca^{2+} (Hollmann et al., 1991).

In neurons, Ca^{2+} ions are important in regulating several important intracellular processes including fast axonal transport of proteins, as well as synthesis and release of some neurotransmitters. Calcium binding proteins often act as mediators in the conversion of the Ca^{2+} message into an intracellular response

(Baimbridge et al., 1992; Heinzmann and Braun, 1992). These proteins vary widely in function and are involved in cytoskeletal reorganization, Ca^{2+} buffering and transport. In addition there are large numbers of calcium binding proteins with unknown functions. Some of these proteins such as parvalbumin, calretinin and CaBP are used as neuronal markers. These proteins are located in the cytoplasm and when stained immuno-histochemically can be used to aid studies of neuronal morphology.

It has recently been shown that spines located on dendrites of projection neurons act as individual compartments for Ca^{2+} (Müller and Connor, 1991) and its concentration can be raised to higher levels in spines than in their parent dendrite (Segal, 1995). This is due to the presence of relatively high concentrations of both ligand (e.g. NMDA receptors) and voltage gated Ca^{2+} channels. Spines also contain high concentrations of phosphatases and kinases that regulate Ca^{2+} -dependent protein phosphorylation and this has led to a suggested role in neuroprotection (Ouimet et al., 1995). However, sustained increases in Ca^{2+} within individual spines may lead to excitotoxicity and loss of that spine.

Apart from potential excitotoxicity in spines, activation of NMDA receptors on interneurons that contain NOS leads to the synthesis of nitric oxide, a free radical gas that acts as a neurotransmitter. Unlike classical neurotransmitters nitric oxide uses diffusion to affect postsynaptic targets (Dawson et al., 1992). It is highly reactive and has the ability to travel up to 1mm from its site of synthesis (Dawson et al., 1992). Nitric oxide production following the activation of NMDA receptors by glutamate, may be neurotoxic to surrounding neurons (Lipton et al., 1993) and this may be one of the mechanisms of glutamate excitotoxicity (Dawson et al., 1991).

As well as glutamatergic inputs, the nucleus accumbens receives dopaminergic projections from the VTA and the SNpc in the midbrain. This input is received onto the necks of spines and along dendritic shafts (Freund et al., 1984). Dopamine produces its physiological effects by acting on postsynaptic dopaminergic receptors and autoreceptors. These receptors belong to a large superfamily of neurotransmitter and hormone receptors that are coupled to G proteins (Kebabian and Calne, 1979). Dopamine receptors are broadly divided into D_1 and D_2 subtypes and both these families are found throughout nucleus accumbens (Bardo and Hammer, 1991). D_1

receptors have a much higher density in the shell than in the core. In contrast, D₂ receptor density is greater in the core than in the shell (Bardo and Hammer, 1991; Jongen-Rêlo, 1994).

Several studies have demonstrated the importance of dopamine in the maintenance of neuronal morphology in both nucleus accumbens and the dorsal striatum (Meredith et al., 1995; Ingham et al., 1991). In these studies, dopaminergic input to the striatum was abolished using a chemical lesion and this resulted in significant losses in spine density. One possibility is that these losses are directly due to loss of dopamine, however, glutamate may also play a role since excessive glutamate can be neurotoxic.

Structural changes in dendrites due to loss of dopamine or dopamine blockade may also be mediated by neuropeptides. The opioids enkephalin or dynorphin and substance-P are colocalised with GABA in medium-sized densely spiny neurons, and axon collaterals of these neurons provide another important source of symmetrical synaptic input in nucleus accumbens. Local terminals of enkephalinergic, dynorphinergic and substance-P positive neurons primarily contact dendrites, however they may also contact spines and perikarya of other medium-sized densely spiny neurons (Meredith, 1999). Consistent with the shell/core dichotomy in nucleus accumbens enkephalinergic terminals form many more axospinous contacts in the shell than in the core (Meredith et al., 1993). Changes in dopamine neurotransmission leads to alterations in the levels of the peptides enkephalin and dynorphin and their receptors. For example, chronic blockade of dopamine receptors upregulates enkephalin but has mixed effects on dynorphin (Egan et al., 1994; Meredith et al., 1997).

Classical neuroleptic drugs and dopamine receptor blockade

Blockade of dopamine D₂ receptors by haloperidol causes many changes in nucleus accumbens neurons. These changes can be divided into acute and chronic actions. Acute treatment immediately blocks dopamine receptors as evidenced by acute changes in prolactin and extrapyramidal side-effects (Johnstone et al., 1978). There is also an increase in the expression of *c-fos* throughout the brain following

acute haloperidol treatment (Dragunow et al., 1990; Robertson and Fibiger, 1992; Semba et al., 1996). Fos is the protein product of the immediate early gene *c-fos* and is postulated to be a marker of neuronal activation (Sheng and Greenberg, 1990; Morgan and Curran, 1991). In the striatum, many neurons that are normally silent become metabolically active following haloperidol treatment and there is a distinct anatomical distribution of these active neurons. They are found primarily in the dorsolateral striatum and medial shell of nucleus accumbens (Deutch et al., 1992; Robertson and Fibiger, 1992; Semba et al., 1996). This indicates that within hours of D₂ receptor blockade changes are taking place in these neurons.

In contrast to the acute effects and neurochemical changes, both the therapeutic effects and the development of TD only takes place following several months of treatment with haloperidol (Johnstone et al., 1978). Chronic haloperidol treatment leads to slowly developing morphological changes in nucleus accumbens projection neurons (Roberts et al., 1995; Meshul et al., 1994) including increases in spine density. Morphological change is believed to involve adaptations in brain neurotransmission and there is evidence that changes in synaptic structure are related to altered efficacy (Lisman and Harris, 1993; Pierce and Lewin, 1994). Moreover, neuroleptic drug treatment increases the numbers of perforated postsynaptic densities (PSD) in nucleus accumbens projection neurons (Meshul et al., 1996a). When asymmetrical synapses become perforated this indicates increased synaptic efficacy at these synapses that are most likely glutamatergic.

Orofacial dyskinesias that may develop following chronic neuroleptic drug treatment, persist even after the drug is withdrawn and are mimicked in an animal model (Waddington, 1990). In this model, vacuous chewing movements (VCMs) develop gradually during long term administration and are reminiscent of orobuccal movements in TD (Egan et al., 1994). VCMs that occur following chronic treatment are pharmacologically and neurochemically distinct from VCMs that occur after acute treatment (Egan et al., 1996b). Acute VCMs have been suggested to be a model of acute extrapyramidal symptoms (Rupniak et al. 1985, 1986), are made worse by administering more haloperidol and have similar features to parkinsonian and extrapyramidal symptoms seen in patients treated with neuroleptic drugs. On the other hand, late developing VCMs are similar to TD in that they can be temporarily

suppressed by increasing neuroleptic dose (Jeste et al., 1988; Egan et al., 1996b) and they persist after drug withdrawal (Kane, 1986).

Several theories have been postulated regarding the neuronal basis for the development of TD and the existence of a valid animal model allows us to investigate some of these. Original theories about the development of TD focused on the increase in density of dopamine D₂ receptors (Muller and Seeman, 1978) or an imbalance between dopamine D₁ and D₂ receptors (Rosengarten, 1983). The main problem with these theories are that upregulation of receptors occurs relatively quickly whereas TD takes several months to develop.

It has been suggested that TD may be due to disturbances in the mesolimbic dopamine system. Several studies have shown that the nucleus accumbens is involved in the control of oral behaviour (Bordi et al., 1989; Koshikawa et al., 1989, 1991). In addition manipulations of dopamine in the nucleus accumbens can induce vacuous chewing (Koene et al., 1993; Prinssen et al., 1994). In these studies, administration of either a D₁ or D₂ receptor agonist into nucleus accumbens induced oral behaviours in rats. Moreover, it was found that administration of a dopamine receptor agonist to the shell of nucleus accumbens but not the core resulted in orofacial dyskinesia in rats (Cools et al., 1995). This suggests that changes in dopamine neurotransmission such as those that occur following chronic haloperidol treatment in the nucleus accumbens especially the shell may mediate the development of VCMs. It is not surprising, therefore, that the shell of nucleus accumbens has also been recently implicated in the regulation of an important oral behaviour, feeding (Kelley and Swanson, 1997; Stratford and Kelley, 1997).

It has also been suggested that TD may result from cell loss in the striatum (Fibiger and Lloyd, 1984). This is based on studies that show striatal cell loss following chronic antipsychotic drug treatment in rats (Pakkenberg et al., 1973; Nielsen and Lyon, 1978). In addition, losses in spine density have been shown to occur in the striatum following chronic haloperidol treatment (Kelley et al., 1997) and in both the striatum and nucleus accumbens following a lesion of afferent dopaminergic inputs (Meredith et al., 1995). Loss of spines would result in a loss of both glutamatergic synapses on spine heads and dopaminergic synapses on spine

necks. This indicates that glutamate as well as dopamine may play an important role in the chronic action of haloperidol, since long-term treatment is known to elevate extracellular glutamate (See and Lynch, 1995; Yamamoto and Cooperman, 1994). Hence, excess glutamate may give rise to excitotoxicity in spines leading to their loss.

Overall evidence supports roles for dopamine and glutamate in neuroleptic-induced VCMs. This, coupled with the evidence that dopamine and glutamate are important in the maintenance of morphology suggests that the structure of projection neurons in nucleus accumbens will be altered following chronic neuroleptic drug treatment and this would be expected to alter functional circuits leading to abnormal movements.

Outputs of nucleus accumbens

It is important not to think of the nucleus accumbens in isolation but as part of a functional circuit. Neurons of the medial shell project to either the regions containing dopaminergic neurons of the VTA or the ventromedial ventral pallidum (Heimer et al., 1991). The ventromedial ventral pallidum then projects to the SNr in the midbrain. Similarly, areas of the core project directly to the SNr and through this may influence the input coming from the shell (Berendse et al., 1992; Zahm and Brog, 1992). In addition, core neurons project to the subthalamic nucleus and the globus pallidus. Hence, nucleus accumbens neurons send projections to midbrain structures with downstream connections and to parts of the midbrain that in turn send projections back to the nucleus accumbens or to their cortical inputs, forming both closed and open systems.

The projections that go downstream from the SNr project directly to the parvicellular reticular formation (PcRt) (Chronister et al., 1988). This nucleus has been shown to be important in the development of abnormal orofacial movements such as VCMs. Electrophysiological and tracing studies have shown that PcRt is a premotor area for the trigeminal and facial motor nuclei and can directly influence oral behaviour (Mogoseanu et al., 1993a, 1993b). Any structural change in either the morphology of core or shell neurons or both would change their targets and would be expected to lead to the development of abnormal movements.

This thesis tests the hypothesis that chronic treatment with haloperidol leads to changes in the dendritic morphology of medium-sized densely spiny projection neurons in distinct regions of nucleus accumbens and determines whether this forms the neural basis for the development of VCMs in rats. The aims are, therefore, to compare the morphology of nucleus accumbens projection neurons from VCM positive, VCM negative and control rats following chronic haloperidol treatment and withdrawal. Furthermore, this thesis investigates some of the potential mechanisms whereby haloperidol treatment may influence neuronal structure. Chapter two examines the profile of spontaneous behaviours of control rats, rats treated chronically with haloperidol that develop VCMs and treated rats that fail to develop VCMs. This will determine if other behaviours are altered specific to haloperidol treatment or to the development of VCMs. Chapter three compares the morphology of nucleus accumbens shell and core neurons from VCM+, VCM- and control rats in order to assess if there is a morphological basis for the development of orofacial TD in rats. Chapter four will compare the size and density of NOS-IR neurons in the shell and core of VCM+, VCM- and control rats. This will allow us to find out if nitric oxide producing neurons are altered following chronic treatment with haloperidol followed by withdrawal and if these changes are specific to development of VCMs. Chapter five will examine the activity of the glutamate transporter as a possible mechanism for the increase in extracellular glutamate that has been reported by others after chronic haloperidol treatment. Chapter six looks at the expression of Fos protein as a marker of neuronal activation following haloperidol administration and assesses the role played glutamate acting at its NMDA receptor in this action.

Chapter 2

The development of vacuous chewing movement syndrome in rats chronically treated with haloperidol is associated with several changes in behavioural profile

Introduction

Tardive dyskinesia (TD) is an adverse side effect of chronic neuroleptic drug treatment and comprises involuntary movements primarily of an orofacial nature (Kane, 1995). This syndrome takes several weeks to develop and may persist even after drug-withdrawal. It has been demonstrated that development of TD can be correlated to chronic neuroleptic treatment (Klawans and Rubovits, 1972). However, its pathophysiology is largely unknown.

When rats are treated chronically with haloperidol they may develop spontaneous VCMs (Iversen et al., 1980). This has been used as a rat model to investigate the neurobiology of TD (Egan et al., 1996b). Several aspects of this model demonstrate that it is analogous to TD. These include the delayed onset of VCMs (for review see Waddington, 1990), persistence after drug withdrawal and suppression by increasing neuroleptic dose (Egan et al., 1996b). It has previously been shown that acute neuroleptic treatment can give rise to VCMs (Rupniak et al., 1985, 1986). These acute movements have been shown to be neurochemically and pharmacologically distinct from tardive VCMs (Egan et al., 1996b) and their time course suggests they are a model of acute extrapyramidal syndrome or dystonia rather than TD (Rupniak et al., 1986). VCMs that develop over several weeks of neuroleptic treatment therefore appear to replicate TD and can be used to study some of the mechanisms of this disorder.

Manipulations of the dopamine system have been previously shown to affect classical dopaminergic behaviours such as sniffing, locomotion and rearing (Daly and Waddington, 1992). This evidence is coupled with the fact that chronic treatment with haloperidol is known to alter dopamine transmission (Creese, 1987). If VCMs develop as a result of changes in dopamine then these changes would also be expected to alter other behavioural parameters. A previous study has investigated the effects of chronic haloperidol treatment on several behaviours in rats (Andreassen and Jørgensen, 1995). This study found that chronic haloperidol treatment led to a significant increase in VCMs and jaw tremor compared to vehicle treated controls. Moreover, they found that the effects of chronic haloperidol treatment were restricted

to these behaviours and treatment had no effect on sitting moving, rearing or grooming compared to vehicle treated controls. However, this study did not examine differences in behaviour associated with the development of VCMs.

It is our hypothesis that rats treated chronically with haloperidol that develop VCMs will have altered profiles of spontaneous behaviour compared to treated rats that fail to develop VCMs or to control rats. We carried out a detailed characterisation of behavioural responses to chronic haloperidol using an ethologically-based, rapid time-sampling behavioural check-list procedure in order to examine the profile of spontaneous behaviour of rats treated chronically with haloperidol and control rats. We analysed behavioural counts to determine what behaviours are altered in conjunction with the development of vacuous chewing movement syndrome. Such an investigation should provide valuable insight into the behaviours that occur with or are dissociated from these abnormal orofacial movements. This will give us further understanding of VCMs as a model for TD and give us much needed information in elucidating its mechanisms and causes.

Materials and Methods

Thirty-five age matched male Wistar rats (Bioresources, Trinity College, Dublin) initially weighing 300-450g were housed in groups of five per cage (52x39x18 cm) with a wire grid top and with wood shavings as bedding material. Standard laboratory rat chow and water were available ad libitum. Animals were maintained at a constant temperature of 21 °C with a constant humidity of 45-65 units, on a 12 hour light/dark cycle (07:00 on; 19:00 off). All procedures for the treatment of these animals were in strict compliance with European Community Directive, 86/609/EC and Cruelty to Animals Act 1876, with protocols approved by the Research Committee of the Royal College of Surgeons in Ireland. Animals were randomly divided into two groups and received intramuscular haloperidol decanoate 28.5 mg/kg (Janssen Pharmaceutical Ltd., Ireland) the equivalent of 1 mg/kg per day of unconjugated haloperidol (n=23), every three weeks for 27 weeks, or the same volume of vehicle, sesame oil (n=8; Sigma-Aldrich, U.K.). Injections were administered to alternate hind legs every three

weeks during treatment. Animals were withdrawn from treatment for a further 18 weeks.

Behavioural assessments were carried out in a manner similar to that described previously (Clifford and Waddington, 1998). Observations were carried out in a randomized order by a person blind to the drug treatment each animal received and were performed between 11am and 3pm so as to reduce any effects that may be due to circadian rhythms. On days that behavioural observations were performed animals were removed from the home cage, weighed and placed in clear glass observation cages (36x29x20 cm). The observation cages had a wire top, like home cages, with wood shavings as bedding material. The observation room was adjacent to that in which the animals were housed and was similarly illuminated with artificial fluorescent light. A ventilation fan provided a constant level of background noise. Injections were given between 4pm and 5pm on the same day. Habituation periods were not employed as it has previously been shown that vacuous chewing movement ratings before and after habituation are highly correlated (Egan et al., 1995). Animals were assessed using a rapid time-sampling behavioural checklist. During each behavioural observation session each rat was observed individually in a glass cage (36x20x20 cm) for a period of 10 consecutive minutes (Figure 2.1). This 10-minute period was divided into 5-second windows and behaviour(s) that occurred in each of these observation windows was recorded using an extended, ethologically-based, behavioural checklist. This allowed the presence or absence of the behaviours, alone or in combination, to be determined in each 5-second period. The following behaviours (Table 2.1) were counted: stillness (motionless with no behaviour evident), sniffing, locomotion (coordinate movement of all four limbs resulting in a change in location), rearing free (front paws reaching upwards away from any cage wall while standing on hind limbs, rearing towards the cage wall (front paws reaching upwards on cage wall while standing on hind limbs), rearing in a sitting position (front paws reaching upwards with hind limbs on floor in sitting position), chewing (directed towards a physical material without consumption), eating (chewing with consumption), yawning, buccal tremor (tremulous movements of the face and mouth muscles), jaw tremor (tremulous movements of the jaw), sifting (sifting movements of the front paws through cage bedding material), licking, grooming and intense

grooming (grooming of the face with the forepaws progressing to vigorous grooming of the flanks with the snout) (Table 2.1).

Assessment of vacuous chewing (not directed onto any physical material and unrelated to grooming eating and gnawing) was carried out concurrently, in the same way as other behaviours, except that the number of episodes that occurred in each observation window was recorded. This resulted in a frequency of VCMs for the 10-minute period, for each observation period for each rat and a count of the number of 5-second windows a behaviour was present for all other behaviours examined. Observations were carried out every three weeks during both the treatment and withdrawal periods, giving a total of 15 observation sessions.

The mean number of VCMs was calculated for the control group for each observation session. Treated animals were assessed for each session and those that had vacuous chewing movement counts two standard deviations or more above the mean for the control group in seven or more observation sessions were classed as being positive for vacuous chewing movement syndrome (VCM+). Animals that had scores more than two standard deviations or more above the mean for the control group in three or fewer observation sessions were classed as negative for the development of vacuous chewing movement syndrome (VCM-). Using these criteria six rats were classed as VCM+ and eight as VCM-. A further seven treated rats had VCMs that were intermediate between VCM+ and VCM-. In addition, one control and two treated rats died during the experimental period and their data were eliminated from the analysis. One control rat consistently had VCM counts more than five standard deviations above the mean of the control group and was considered an outlier and eliminated from the analysis. This classification of rats into VCM+ and VCM- groups was confirmed by cluster analysis (Datadesk).

Analysis of behavioural measures was carried out with respect to both the treatment the animal received (haloperidol or vehicle) and its VCM classification (VCM+ or VCM-). For multiple observations, a repeated measures analysis of variance (rmANOVA) was used. Individual observation sessions were assessed for group effects using post hoc Fisher's protected least squares difference tests. A $p < 0.05$ was considered significant. In addition, individual behavioural counts were

added over all the observation sessions, yielding one value per animal for each category of behaviour and differences between groups were analysed with the Student's *t*-test. Separate analyses were carried out to compare haloperidol treated rats (VCM+, VCM- and intermediate) with vehicle treated controls, and controls, VCM+ and VCM- rats. There were no significant differences between the weights of control ($n=6$, 557 ± 22 g) and haloperidol-treated rats ($n=21$, 525 ± 14 g)



Figure 2.1 Photograph of rat in observation cage exhibiting sniffing and rearing behaviours.

Table 2.1

Behavioural checklist

Code	Behaviour	Description
Bt	Buccal Tremor	tremulous movements of the face and mouth muscles
Ch	Chewing	directed towards a physical material without consumption
E	Eating	eating (chewing with consumption)
Gr	Grooming	
Gri	Intense Grooming	grooming of the face with the forepaws progressing to vigorous grooming of the flanks with the snout
Jt	Jaw Tremor	jaw tremor (tremulous movements of the jaw)
Li	Licking	
L	Locomotion	Coordinate movement of all four limbs resulting in a change in location
Rf	Rear Free	Rearing free (front paws reaching upwards away from any cage wall while standing on hind limbs)
Rs	Rear Sitting	Rearing in a sitting position (front paws reaching upwards with hind limbs on floor in sitting position)
Rw	Rear Wall	rearing towards the cage wall (front paws reaching upwards on cage wall while standing on hind limbs)
S	Sifting	sifting movements of the front paws through cage bedding material
Sn	Sniffing	
St	Stillness	motionless with no behaviour evident
VCh	Vacuous Chewing	not directed onto any physical material and unrelated to grooming eating and gnawing
Y	Yawning	

Derived from Fray et al. (1980), as modified by Molloy and Waddington (1987), Murray and Waddington (1989) and Daly and Waddington (1992).

Results

Chronic treatment with haloperidol gave rise to a gradual increase in VCMs that remained elevated through the withdrawal period (treatment effect: $F=9.378$, $df=1$, $p=0.0053$; time effect: $F=9.496$, $df=14$, $p=0.0001$). VCMs in haloperidol-treated rats became significantly elevated over control values six weeks after treatment began and reached their peak following twenty-seven weeks of treatment (Figure 2.2). During the withdrawal period (Figure 2.2) VCMs in the haloperidol treated group remained significantly elevated up until the final observation session fifteen weeks after withdrawal. There were no significant differences in VCMs between control and VCM- rats (Figure 2.3).

A significant decrease in locomotion was observed due to treatment ($F=5.233$, $df=1$, $p=0.03$). Rearing towards a wall was also decreased ($F=9.41$, $df=1$, $p=0.005$) in haloperidol treated animals compared to controls (Figure 2.4). There were no significant differences due to treatment found in buccal tremor, jaw tremor, grooming, free rearing, rearing, sitting, sniffing, sniffing or stillness. Nor were there any treatment effects in chewing, eating, intense grooming, licking or yawning behaviour. The latter six behaviours occurred very infrequently and formed a small part of the behavioural profile of rats (Figure 2.4). However, there were significant effects due to time found in all behaviours examined with the exception of jaw tremor, chewing and eating.

A comparison of VCM+ and VCM- rats revealed significant differences between behaviours in the two groups (Figure 2.5). In rats that were VCM+ there were significant elevations in grooming (+42%; $p=0.013$), buccal tremor (+90%; $p=0.022$) and jaw tremor (+251%; $p=0.006$), as well as a reduction in stillness (-50%; $p=0.01$).

Figure 2.2 VCM ratings for haloperidol (n=21) and vehicle treated (n=6) groups. Session 0 is prior to treatment. Sessions T3 to T27 inclusive represent the treatment period and sessions W3 to W15 represent the withdrawal period. Rats were injected with haloperidol once every three weeks for 27 weeks followed by 18 weeks of withdrawal. * $p < 0.05$

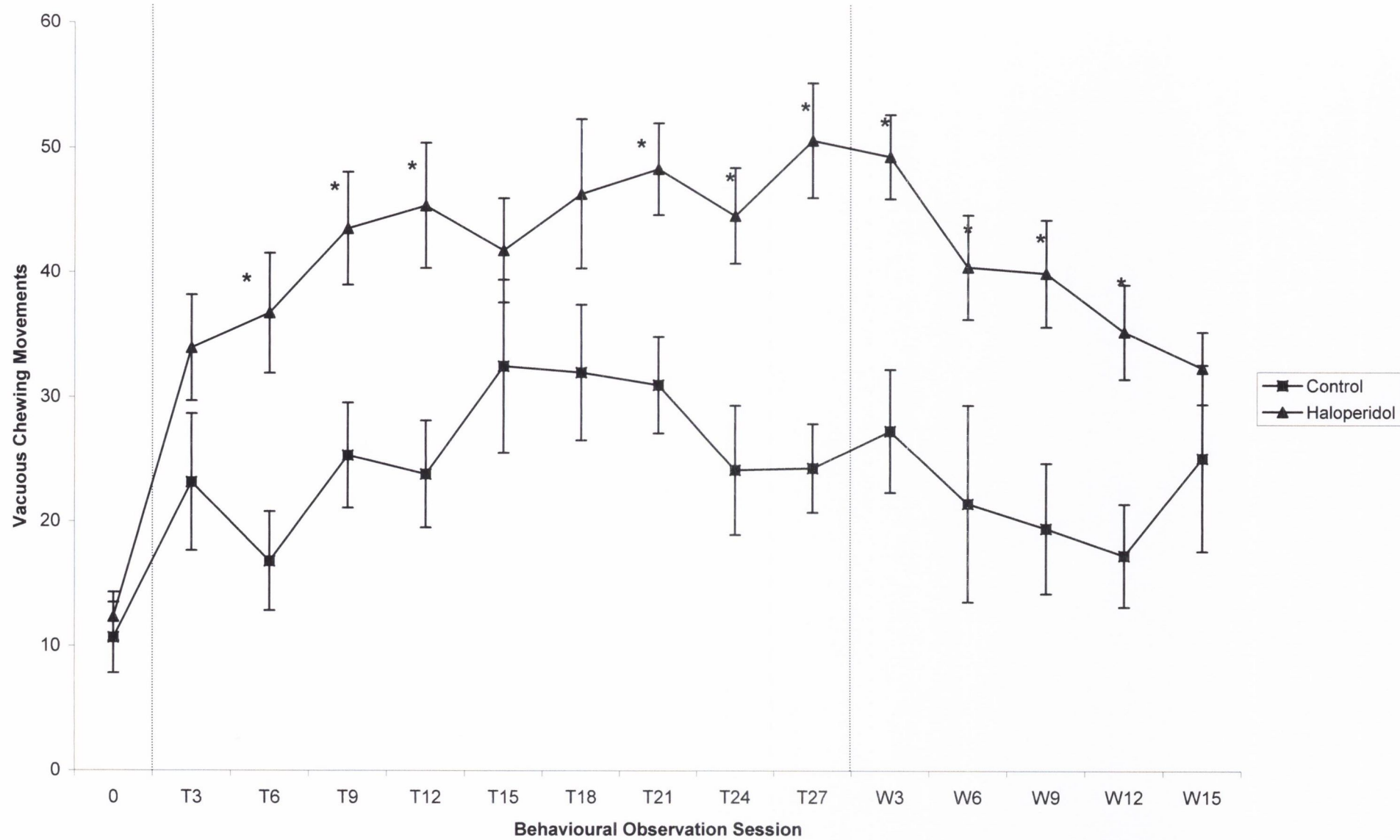


Figure 2.3 VCM rating for VCM+ (n=6), VCM- (n=8) and vehicle treated (n=6) groups. Session 0 is prior to treatment. Sessions T3 to T27 inclusive represent the treatment period and sessions W3 to W15 represent the withdrawal period. Rats were injected with haloperidol once every three weeks for 27 weeks followed by 18 weeks of withdrawal. * $p < 0.05$ differs significantly from vehicle treated group.

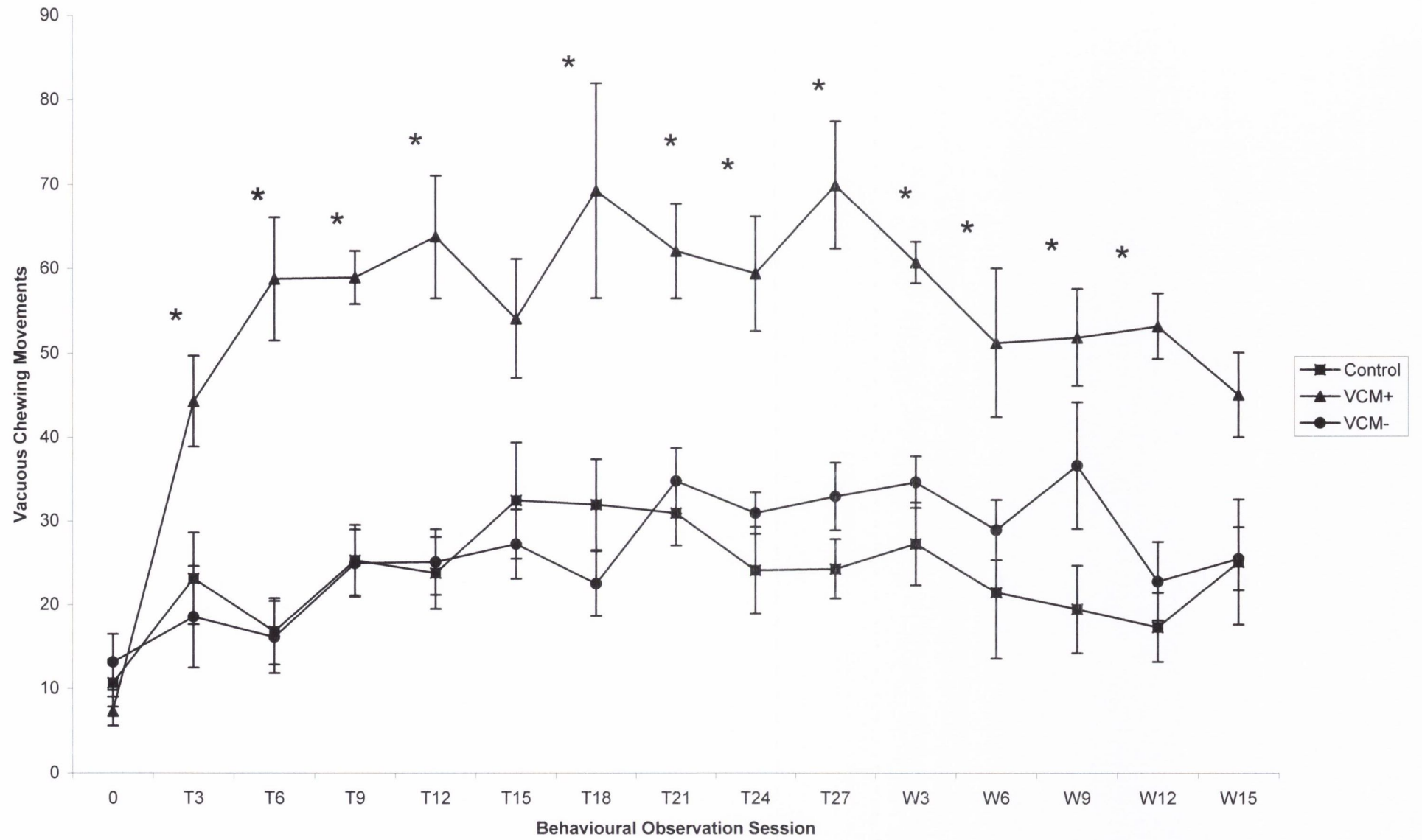


Figure 2.4 Behavioural counts for responses to chronic haloperidol treatment and withdrawal (n=21) compared to control rats (n=6). Data are means $\pm$ s.e.m..

* $p < 0.05$, ** $p < 0.01$ vs control.

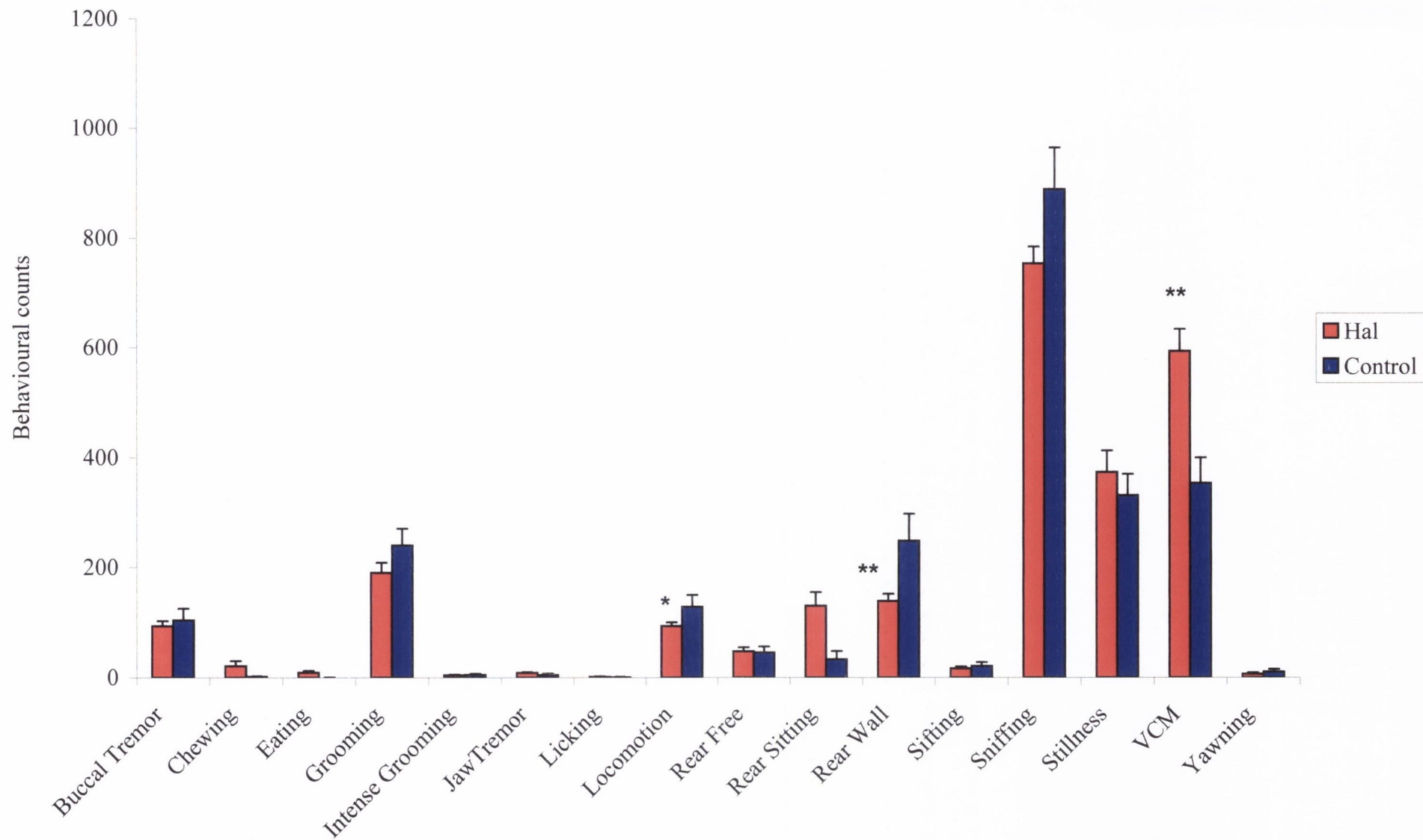


Figure 2.5 . Behavioural counts for responses to chronic haloperidol treatment and withdrawal in VCM+ (n=6) and VCM- (n=8) rats. Data are means $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$.

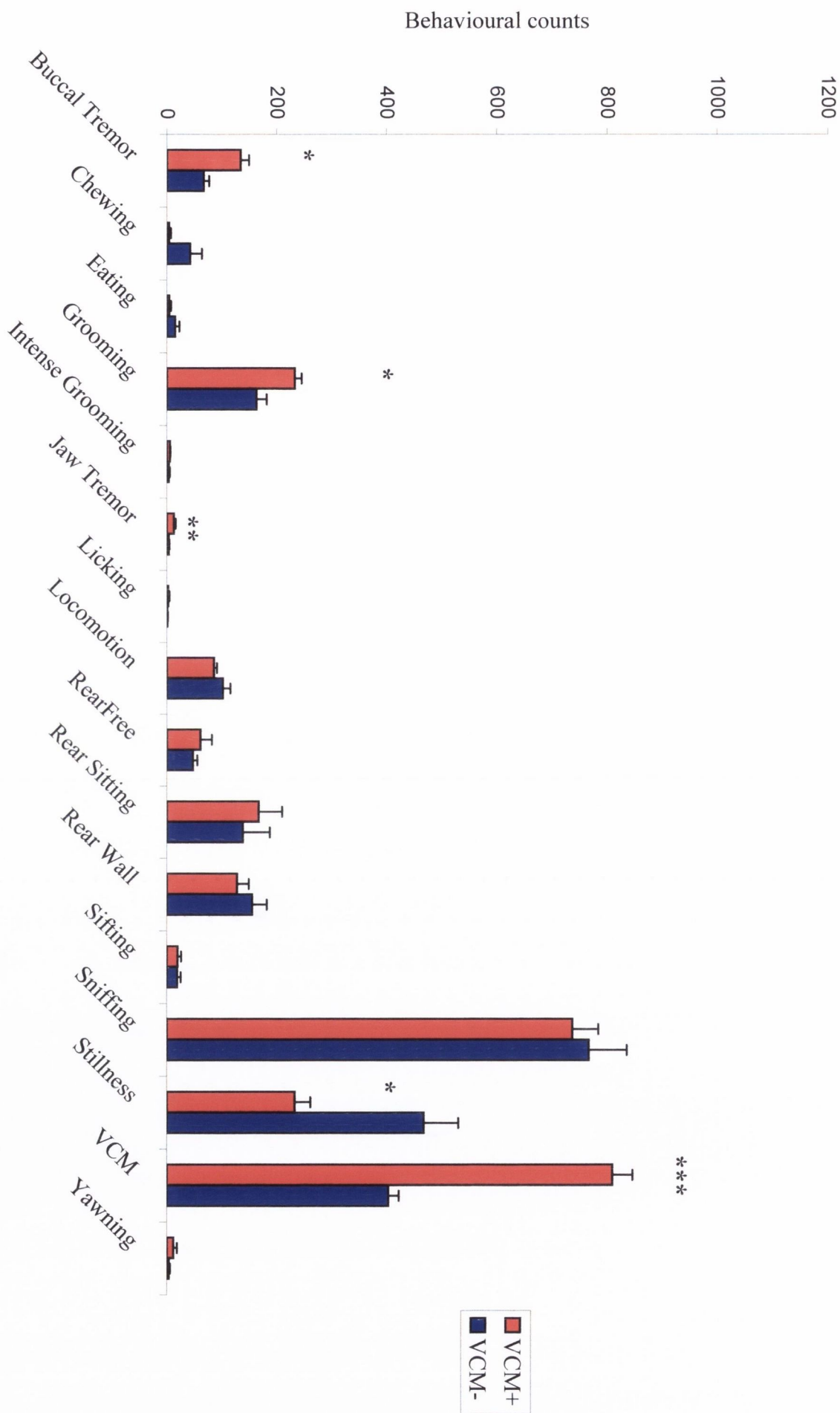
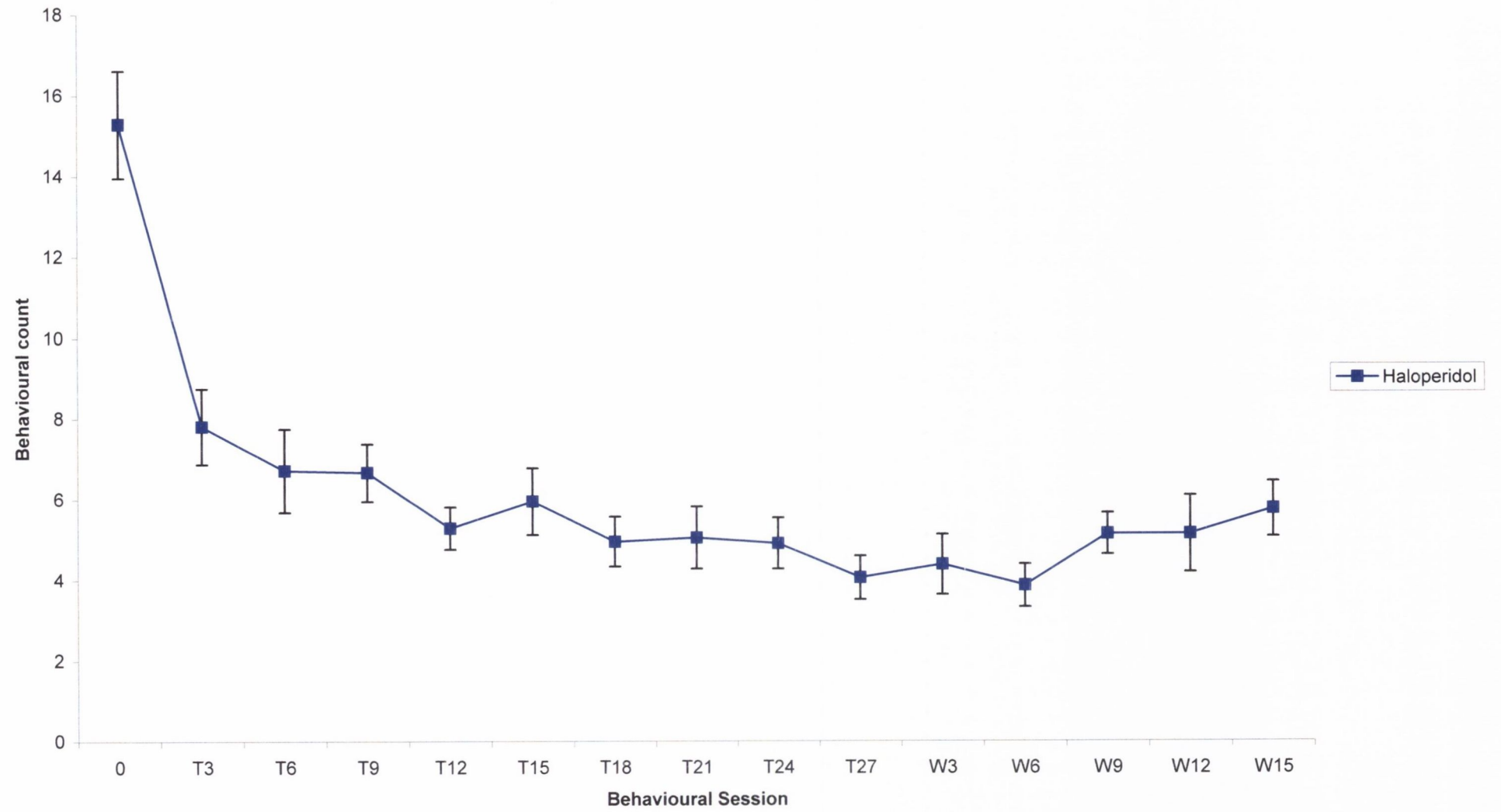


Figure 2.6 Locomotion ratings for haloperidol treatment followed by withdrawal (n=21). Session 0 is prior to treatment. Sessions T3 to T27 inclusive represent the treatment period and sessions W3 to W15 represent the withdrawal period. Rats were injected with haloperidol once every three weeks for 27 weeks followed by 18 weeks of withdrawal. * $p < 0.05$.

Haloperidol



Discussion

The results of this study showed that chronic haloperidol treatment gave rise to vacuous chewing movement syndrome that persisted even after drug withdrawal (Figure 2.2). The time course of this action was similar to that reported previously by Egan et al. (1996b). However, the persistence of VCMs was not as pronounced during the withdrawal period and began to fall toward control values at the end of the withdrawal period (Figure 2.2). This is not surprising, since Wistar rats were used in this study and a previous study has shown differences in VCM persistence between rat strains (Tamminga et al., 1990). In their work, they found that Sprague-Dawley rats exhibited much greater persistence of VCMs following withdrawal from chronic haloperidol treatment compared to Wistar or Long Evans rats.

Chronic haloperidol treatment has been previously shown to reduce locomotor activity in some studies (Bernardi and Palmero Neto, 1979; Ellison and See, 1990; See and Ellison, 1990) but not in others (Andreassen and Jørgensen, 1995). In this study we found chronic haloperidol treatment significantly reduces locomotion compared to controls (Figure 2.3). However unlike previous studies (Bernardi and Palmero Neto, 1979; See and Ellison, 1990) locomotion remained reduced even after withdrawal (Figure 2.6). Induction of locomotion is considered to be due to stimulation of both D₁ and D₂ dopamine receptors (Waddington, 1989). It has been hypothesised that the inverse relationship between oral movements and locomotion is an artifact, since VCMs would be easier to observe in the haloperidol-treated animals due to reduced locomotion (Levy et al., 1987 and see Waddington, 1990). However, the fact that there were highly significant differences between VCM counts in the VCM+ and VCM- animals and no significant difference in locomotion in these groups suggests that VCMs and locomotion are independent behaviours (Figure 2.5).

There were effects due to time seen in several of the behaviours examined. These occurred most prominently in the haloperidol treated animals and appeared to be due to a habituation effect. This is most evident in the exploratory behaviours in which counts follow a downward trend that is greatest between the first and second

observation period (Figure 2.6), most likely due to the observational cage environment being less novel over time.

A comparison of VCM+ from VCM- animals revealed several interesting findings. We found that grooming behaviour was present in VCM+ animals an average of 42% more often than in VCM- animals. It has been demonstrated previously that D₁-like dopamine receptor agonists induce grooming, and that this is a widely accepted behavioural index of D₁ receptor function in rats (Molloy and Waddington, 1984; Murray and Waddington, 1989; Deveney and Waddington, 1997). Rats that develop VCMs display increased grooming and this implies hyperactivity at D₁ dopamine receptors. Activity in nucleus accumbens has been shown to mediate orofacial behaviour in rats (Prinssen et al., 1994; Cools et al., 1995). In this nucleus an area called the medial shell contains a high density of D₁ dopamine receptors. This area has also been implicated in feeding behaviour (Stratford and Kelley, 1997) and is likely to play a role in the development of VCMs following chronic haloperidol treatment.

In summary, the ethogram of spontaneous behaviour in control, haloperidol-treated VCM+ and VCM- rats reveals important functional changes related to drug treatment as well as due to the development of vacuous chewing movement syndrome. Moreover changes specifically in grooming add to the evidence that the D₁ dopamine receptor may play a pivotal role in the development of VCMs.

Chapter 3

Differential modification of dendritic morphology in nucleus accumbens neurons following chronic haloperidol treatment provides the neural basis for the development of vacuous chewing movements

Introduction

Tardive dyskinesia (TD) is a syndrome of involuntary, hyperkinetic abnormal movements that occur in predisposed individuals following treatment and withdrawal from neuroleptic drugs (Casey, 1987). These movements are primarily orofacial in nature, take several weeks to develop and persist even after drug withdrawal. Rats treated chronically with classical neuroleptic drugs such as haloperidol may develop spontaneous vacuous chewing movements (VCMs) (Klawans and Rubovits, 1972; Tarsy and Baldessarini, 1974, 1975; Jeste and Wyatt, 1979; Iversen et al., 1980) and these have been shown to act as an animal model for TD (Waddington, 1990, Egan et al., 1995).

Neuroleptic drugs act primarily by blocking dopamine D₂ receptors (Carlsson and Lindquist, 1963; Creese et al., 1976) and consequently the development of TD was thought to be due to this blockade of dopamine receptors following neuroleptic drug treatment. However, a major problem with this theory is that dopamine receptor blockade occurs within hours of drug administration (Carr, 1983; Glovinski et al., 1992), whereas abnormal movements take several weeks to develop. It has, therefore, been suggested that persistent alterations in behaviour are probably mediated by structural modifications in neural circuitry especially alterations in synaptic connectivity (Robinson and Kolb, 1997; Greenough, 1984; Greenough and Bailey, 1988). If structural modification forms the basis for the development of the rat equivalent of TD, these modifications would be expected to occur in areas of the brain associated with oral behaviour. In rats, several studies have shown that nucleus accumbens plays an important role in mediating oral behaviour (Kelly and Swanson, 1997; Fletcher and Starr, 1987; Prinssen et al., 1994; Cools et al., 1995).

Nucleus accumbens receives glutamatergic afferent projections from the prefrontal cortex, the hippocampus and the amygdala (Brog et al., 1993; Fuller et al., 1987). In addition it receives dopaminergic inputs from the SNpc and VTA (Brog et al., 1993). Glutamatergic input synapses primarily on the heads of dendritic spines on nucleus accumbens projection neurons (Groenewegen et al., 1991). Dopamine input on the other hand, synapses primarily on spine necks and dendritic shafts and

plays a particularly important role in gating glutamatergic input (Groenewegen et al., 1991). In addition, blockade of presynaptic dopamine receptors leads to increased glutamate and dopamine release (Bardgett et al., 1993; Perry et al., 1979). Therefore, dopamine can exert control over glutamate release and, conversely, glutamate can control dopamine release.

Nucleus accumbens is not homogenous and can be divided into shell and core territories (Záborszky et al., 1985). In addition, there are significant differences between dendritic morphology of medium-sized densely spiny neurons located in the shell and core territories (Meredith et al., 1992). These territories not only receive dopaminergic and glutamatergic input from topographically distinct regions but they also differ in the ratios of D₁ and D₂ dopamine receptors (Bardo and Hammer, 1991). Dopaminergic input to the shell arises from the VTA and the retrorubral field (Deutch et al., 1988) and D₁ receptors predominate in this territory (Bardo and Hammer, 1991). The core of nucleus accumbens is innervated by mesostriatal input from the SNpc and mesolimbic fibers (Nauta and Domesick, 1984; Groenewegen et al., 1991) and contains primarily D₂ receptors (Bardo and Hammer, 1991).

Given that there are differences between shell and core territories it is not surprising that they respond differently to changes in dopamine neurotransmission. Dopamine plays an important role in the maintenance of dendritic morphology of medium-sized densely spiny neurons and dopamine depletion has been shown to alter the structure of dendrites in the nucleus accumbens (Meredith et al., 1995) and striatum (Ingham et al., 1991). In addition, dopamine depletion leads to spine loss in the core of nucleus accumbens while increasing dendritic tortuosity in the shell (Meredith et al., 1995). Chronic blockade of dopamine D₂ receptors with haloperidol has been shown to alter the density of synapses located on dendritic spines in the dorsal striatum. Ultrastructural studies revealed that haloperidol can increase the density of synapses on dendritic spines following subchronic treatment (Uranova et al., 1991). Conversely, the density of synapses forming on dendritic spines was decreased following chronic haloperidol treatment (Meshul and Casey, 1989; Meshul et al., 1994; Meshul et al., 1996b; Roberts et al., 1995; Uranova et al., 1991). It was shown in some studies that synaptic changes following drug withdrawal are persistent (Roberts et al., 1995). It is important that VCMs persist in this rat model (Egan et al.,

1996) since one of the problems with TD is the fact that it continues even after treatment has ceased. In addition, not all patients treated with antipsychotics develop TD (Casey, 1987).

While some studies have examined changes in dendritic morphology in nucleus accumbens as a basis for the development of vacuous chewing movement syndrome (Meshul et al., 1996a; Roberts et al., 1995) no study has examined the different accumbens territories. Moreover, while ultrastructural studies have the power to determine changes in the number of synapses as well as alterations in the size or density of spines they do not show whether changes take place at proximal or distal sites on the dendritic arbours. The main aims of this study were to determine the effects of chronic haloperidol treatment followed by withdrawal on the dendritic morphology of medium-sized densely spiny projection neurons located in the core or shell territories of nucleus accumbens and to investigate if changes in dendritic structure were associated with the development of the syndrome of VCMs. Moreover, further analyses examined proximal and distal dendritic segments separately, in order to determine the precise location of alterations in dendritic structure. These analyses will determine if changes in the structure of the dendritic arbours of nucleus accumbens core or shell projection neurons underlie the development of VCMs.

Materials and Methods

Male, Albino, Wistar rats (Bioresources, Trinity College, Dublin, Ireland) were used in these experiments. They were housed four per cage, with a constant temperature of 21°C, on a 12-h light and dark cycle with free access to food and water. They were divided into two groups: one received intramuscular injections of haloperidol decanoate (Janssen Pharmaceutical Ltd., Ireland) at a dose of 28.5 mg/kg the equivalent of 1 mg/kg per day of unconjugated haloperidol (n=23); the other (control), received same volume of vehicle sesame oil (n=8). The injections were administered to alternate hind legs every three weeks during the treatment period of 27 weeks. Animals were withdrawn from treatment for a further 18 weeks. These were the same rats that were used in the behavioural study in chapter two of this thesis.

During both the treatment and withdrawal periods all the rats were assessed for the development of VCMs every three weeks. Behavioural assessments were carried out in a manner similar to that described previously (Clifford and Waddington, 1998). During a behavioural session each rat was removed from its home cage and placed in a glass cage (36x20x20 cm) in a room adjacent to the home cage. Each observation session lasted for ten minutes and during this period the episodes of vacuous chewing movements were counted. Vacuous chewing was defined as chewing not directed onto any physical material and unrelated to grooming, eating and gnawing. Behavioural assessments were carried out in a randomized order by a person blind to the drug treatment each animal received. Assessments took place between 11 am and 3 pm and were followed by drug administration between 4 pm and 5 pm on the same day. Habituation periods were not employed as it has previously been shown that VCMs counted before and after habituation are highly correlated (Egan et al., 1995).

The mean number of VCMs was calculated for the control group for each observation session. Haloperidol-treated animals were classed as being positive for vacuous chewing movement syndrome (VCM+) if they had vacuous chewing movement counts two standard deviations or more above the mean for the control group in seven or more observation sessions. Haloperidol-treated animals that had

vacuous chewing movement counts more than two standard deviations above the control group in three or fewer sessions were classed as being negative for vacuous chewing movement syndrome (VCM-). Using these criteria six animals were classed as VCM+ and eight as VCM-. The seven remaining treated rats had vacuous chewing movement counts that were intermediate between VCM+ and VCM- and these intermediates were not analysed morphometrically.

Following the withdrawal period, the rats were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.) and perfused transcardially with saline containing 0.5% procaine, followed by fixative containing 4% paraformaldehyde, 15% saturated picric acid and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature. Each brain was removed; the forebrain blocked and transverse sections were cut at 150 μ m with a Vibraslice.

Intracellular injection of neurons was carried out by a procedure similar to that described previously by Meredith and Arbuthnott (1993). Sections were counter stained with the fluorescent marker 4,6 diamidino-2-phenylindole (DAPI) at a concentration of 1×10^{-7} M for 10 minutes in order to make the neuronal cell bodies visible. Each section was placed in a plastic dish containing 0.1M phosphate buffer, covered with a sheet of filter paper with a window of approximately 25 mm² cut into it and weighed down using stainless steel washers. The window was positioned to reveal the nucleus accumbens. The plastic dish containing buffer and the section of interest was secured on a fixed stage microscope (Figure 3.1). A circuit was formed by connecting the buffer with a wire to a constant current source (Digitimer Ltd, U.K) and another wire from the constant current source to the inside of an electrode containing a 4% solution of biotinylated Lucifer yellow (LY, Molecular Probes). Numerous neurons counterstained with the fluorescent marker DAPI were impaled in the nucleus accumbens of control, VCM+ and VCM- rats. The electrode containing LY was secured to a motorized micromanipulator (World Precision Instruments Inc., USA) and moved into position over the dish containing the section of interest. The electrode tip was positioned between the microscope lens and the window revealing the area of interest on the section. The section was observed by looking down the microscope and a neuronal cell body counterstained with DAPI was brought into

sharp focus. The electrode tip was lowered towards the cell body using a joystick driven motorized micromanipulator and the neuron impaled. A positive current of about 1nA was used to hold the LY in the electrode until the tip was inside the neuronal cell body. A negative current of 1-3 nA was used to inject LY into the neuron at an electrode resistance of 80-250 M Ω . Sections containing neurons filled with LY were then incubated in a solution of pre-prepared avidin-biotin peroxidase complex for 90 minutes at room temperature and rinsed three times in 0.05M Tris-HCl pH 7.4 (Tris). This was followed by reaction for approximately 10 minutes in a 0.05% 3,3'-diaminobenzidine (DAB) with 1% ammonium nickel sulphate in Tris. These sections were then mounted onto glass slides from a 0.2% gelatin solution, dehydrated and coverslipped. Slides were then coded to ensure that all analysis was unbiased.

LY filled neurons were analysed if they were medium-sized densely spiny neurons, their dendrites appeared completely filled and they could be unambiguously assigned to the shell or the core of nucleus accumbens. In total, thirty-two neurons were reconstructed and analysed morphometrically using a Nikon Labophot-2 microscope with a 100x oil-immersion objective and coupled to hardware (Micron Electronic Inc., USA) and software (Neurolucida, Microbrightfield Inc., Colchester, VT, USA) dedicated to neuron reconstruction (Figure 3.2). Each neuron was digitized in x-, y- and z- coordinates. Spines were recorded using special markers and the diameter of the neuron was recorded along its entire length. The analysis software (Neuroexplorer, Microbrightfield Inc. Colchester, VT, USA) calculated dendritic length, number of spines, spine density, dendritic surface area and dendritic tortuosity for each segment. The length of the first order dendritic segment is the distance from its starting point at the soma to the first branch point. Subsequent orders of dendritic segments are measured between branch points. The number of spines is the median number of spines per dendritic segment. Mean spine density per dendritic segment was calculated as described previously (Meredith et al., 1995). Briefly, mean spine density was first calculated for segments over 50 μ m in length. Segments that had spine density values within two standard deviations of the group mean for segments longer than 50 μ m were included in the analysis. To obtain this value the number of spines on each segment is divided by length of that segment. This yields a value of

spines per unit length of dendritic segment for each segment. Spine density is the mean value of spines/ μm for segments with valid densities. However, it is customary to express this value as spines/ 10 μm . This is achieved by multiplying spines/ μm by 10. In addition, post hoc analyses of the percentage of branched spines and mushroom-shaped spines were carried out by selecting a third order segment at random, that was at least thirty microns in length, from each cell. Branched spines and mushroom-shaped spines were counted and expressed as a percentage of the total number of spines. Values were then compared between control and haloperidol groups and also between control, VCM+ and VCM- rats. Branched spines were defined as spines with more than one head clearly connected to a common shaft (Comery et al. 1996, Robinson and Kolb, 1999). Spines were considered mushroom-shaped if their heads were larger than 0.3 microns in diameter.

The dendritic segment surface area is calculated using the formula for the surface area of a frustum of a cone for each portion of each dendritic segment.

$$\text{Surface Area} = \pi (R_1 + R_2) \sqrt{h^2 + (R_1 - R_2)^2}$$

Where R_1 is the radius at the start of the segment portion and R_2 is the radius at the end of a segment portion and h is the length of the portion. For comparisons, values are expressed as median surface area per dendritic segment. Surface area is an important parameter as it has previously been shown that the number of synapses that can form on a dendrite is proportional to its surface area (Harris and Kater, 1994). Dendritic segment tortuosity is defined as the length of a dendritic segment divided by its radial length. This means a straight line has a tortuosity ratio of one and the more twisted a dendritic segment, the higher its tortuosity. Slides were coded and all dendritic tracing was carried out blind to treatment conditions.

Differences between control, VCM+ and VCM-, in both the shell and core of nucleus accumbens were analysed statistically. Values were pooled and a Kolmogorov-Smirnov test was used to assess if the distribution of each data set was normal. Non-normal data sets were compared using a Kruskal-Wallis analysis of variance followed by post hoc non-parametric analysis using Mann-Whitney U tests. Normal data sets were compared using an analysis of variance followed by post hoc Scheffe's tests or Student's t-tests as appropriate. Values were expressed per dendritic segment as previous studies have demonstrated that this is the optimal unit

of measurement for studies of dendritic morphology (Meredith et al., 1992). Comparisons were made between control and haloperidol treated rats. Further analysis compared control, VCM+ and VCM- rats. In addition, further comparisons were made between groups with respect to distal and proximal branch orders in order to determine more precisely where morphological parameters take place.

Sholl analysis (Sholl, 1981) of ring intersections was used to estimate dendritic length for each cell and comparisons between control and haloperidol-treated rats groups were made using a Student's t-test and also between control, VCM+ and VCM- using a one way analysis of variance followed by post hoc Scheffe's tests. The mean number of branch points, dendritic segments and terminal branches were calculated and expressed per cell. Groups were then compared using Student's t-tests or a one way analysis of variance followed by post hoc Scheffe's tests in order to determine whether the degree of branching was altered. All statistical tests were carried out using dedicated computer software (SPSS Inc.) and $p < 0.05$ was considered significant.

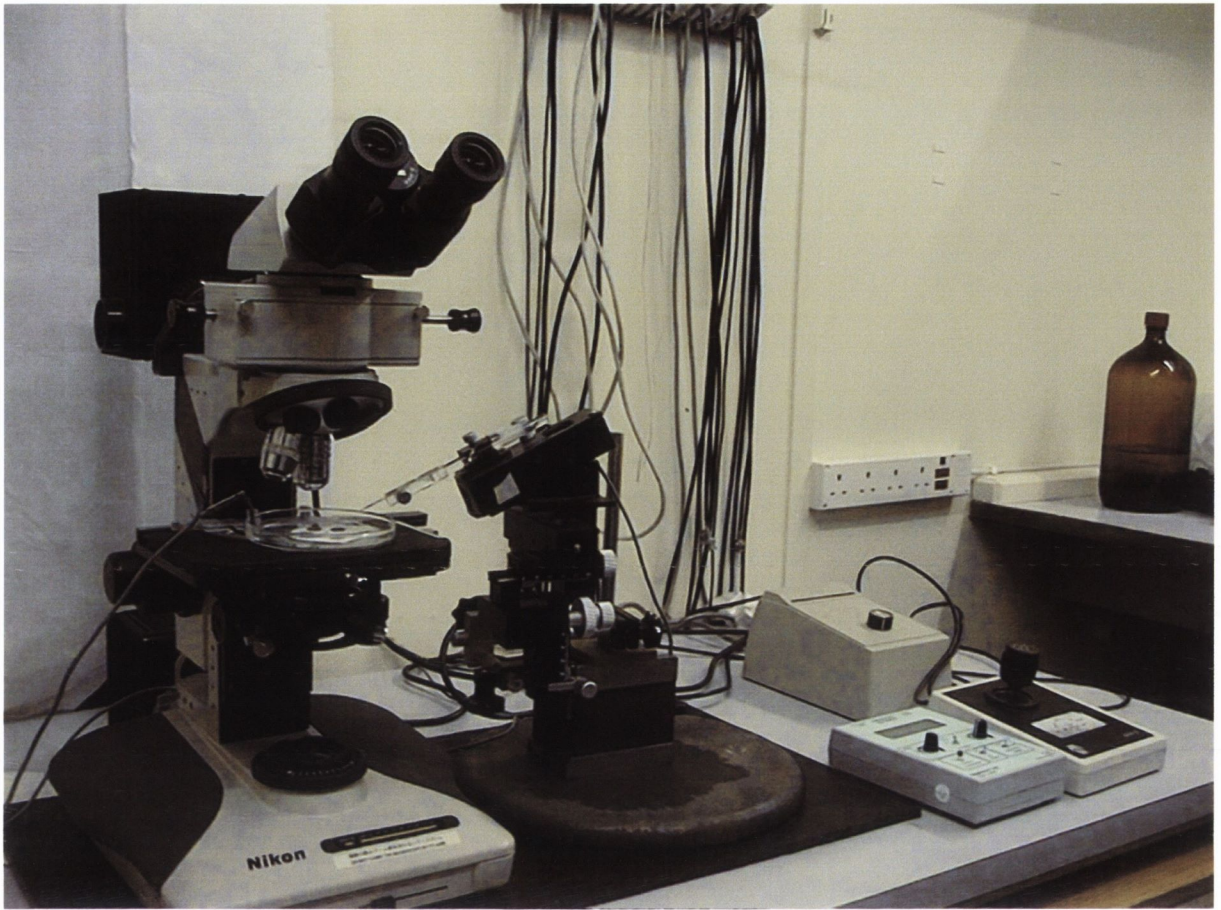


Figure 3.1 View of equipment used for intracellular filling of neurons. Note the micromanipulator to the right of the fixed-stage microscope. The constant current source and the joystick drive are on the far right.



Figure 3.2 Apparatus used for quantitative morphometric analysis of intracellularly filled medium-sized densely spiny striatal neurons.

Results

One control and two haloperidol-treated rats died during the experimental period. In addition, one control rat consistently had vacuous chewing movement counts more than five standard deviations above the mean of the control group and was considered an outlier. This rat was eliminated from the analysis.

Medium sized ensely spiny neurons were most commonly filled although a small number of aspiny cells and occasionally glial cells were also filled. Intracellularly filled medium-sized, densely-spiny neurons were selected for analysis provided that they were located at middle to caudal levels of nucleus accumbens between Bregma +0.7 mm and +1.2 mm (Paxinos and Watson, 1986) and they could be unambiguously assigned to the shell or core territories (Figure 3.3). Filled neurons from the core territory that were chosen for analysis were located close to the anterior commissure while those analysed in the shell territory were found primarily in its medial part (Figure 3.3). From a total of thirty-two reconstructed neurons, fifteen were located in the core territory while seventeen were located in the shell territory. Neurons from both control and haloperidol-treated rats had as many as six primary dendrites and each primary dendrite gave rise to an arbour consisting of as many as six branch orders in the core and seven in the shell. Dendrites were initially thick and free from spines but became spiny usually around the first branch point and became progressively thinner distally.

Dendritic segments from haloperidol-treated rats are less tortuous ($p<0.001$), have higher spine density ($p<0.001$) and reduced surface area ($p=0.03$) in comparison to those from control animals (Table 3.1 and Table 3.2). There were significant increases in mean spine density (+33.7%; $p<0.0001$), as well as significant reductions in tortuosity and dendritic surface area (-15.7%; $p<0.0001$, -27.7%; $p=0.0015$, respectively; Table 3.1) in dendritic segments of neurons located in the shell from haloperidol-treated rats. Similarly, mean spine density was increased (+30.2%; $p<0.001$; Table 3.2) and median surface area and dendritic tortuosity were reduced ($p<0.001$ and $p<0.0001$, respectively; Table 3.1) when shell segments from VCM+ rats were compared to those from control rats (compare Figure 3.4 to 3.6). A

comparison between shell segments from VCM- rats and those from controls rats revealed significantly reduced tortuosity ($p<0.001$) and increased mean spine density ($p<0.0001$) (compare Figure 3.4 to 3.5). In addition, animals that developed the vacuous chewing movement syndrome were found to have significantly reduced surface area (-28.4%; $p=0.013$; Table 3.1) when their shell dendritic segments were compared to those from animals that did not develop the syndrome (compare Figure 3.5 to 3.6 and 3.8 to 3.9).

Following initial comparisons, an analysis of proximal (1st-3rd order) and distal (4th-7th order) dendritic branches was carried out in order to determine if structural alterations were more prominent proximally or distally. Proximal shell segments from VCM+ rats were less tortuous, had reduced surface area and elevated spine density compared to controls (Table 3.3). Moreover, shell segments located distally had significantly higher spine density, numbers of spines and lower tortuosity than control rats. Proximal shell segments from VCM- rats were less tortuous and had elevated spine density compared to controls. However, the surface area of proximal shell segments from VCM- rats was not significantly different from controls and when tested statistically was found to be significantly increased compared to VCM+ rats ($p=0.006$). Distal shell segments from VCM- rats were less tortuous and had higher spine density than controls.

The Sholl analysis revealed a trend towards an increase in radial dendritic length for neurons located in the shell territory of haloperidol-treated rats compared to controls (+25.4%; $p=0.058$). In addition, there was a marked increase in branching in shell dendrites in haloperidol-treated animals as indicated by an increase of 47.4% in the mean number of branch points ($p=0.019$) and an increase of 36% in the number of terminal segments and ($p=0.025$). Neurons located in the shell had more 2nd (+51% $p=0.030$) 3rd (+52% $p=0.047$) and 5th (+234%; $p=0.029$) order dendritic segments per neuron in haloperidol-treated compared to control rats.

There was no significant difference in the frequency of branched spines and mushroom-shaped spines in the shell of nucleus accumbens between controls and haloperidol treated rats (8.56% vs. 7.83%, $p=0.557$ for branched spines and 13.58% vs. 8.89%, $p=0.140$ for mushroom-shaped spines). Further there were no significant

differences in these measures when control, VCM+ and VCM- rats were compared ($F=0.388$, $p=0.687$ for branched spines or $F=1.906$, $p=0.195$ for mushroom-shaped spines).

In contrast to shell segments, there were no significant differences between core dendritic segments from haloperidol-treated and control rats (Table 3.1). However, there was a trend towards decreased dendritic segment length in core neurons from haloperidol-treated animals (-21%; $p=0.074$; Table 3.1). Analysis of core dendritic segments from control, VCM+ and VCM- rats revealed that there were trends towards reduced segment length and numbers of spines in VCM- compared to control rats ($p=0.056$ and $p=0.088$, respectively; Table 3.1 and 3.2; compare figures 3.7, 3.8, 3.9). Conversely, core dendritic segments from VCM+ rats did not differ significantly from either control or VCM- rats in any morphological parameter examined (Table 3.1 and 3.2). However, distal dendritic segments located in the core were less tortuous in VCM+ rats (-6.1%) and significantly shorter (-50.7%) with reduced surface area (-60.6%) and reduced numbers of spines (-39.1%) in VCM-rats compared to control rats. In addition, distal core segments from VCM- rats were shorter (-25.4%; $p=0.044$) and had reduced surface area (-56.4%; $p=0.015$) as well as reduced numbers of spines (-7.1%; $p=0.048$) compared to VCM+ rats.

In haloperidol-treated rats the radial dendritic length of their core neurons was reduced by 27.8% ($p=0.007$). Comparing control, VCM+ and VCM- rats using an ANOVA revealed a reduction in length of core neurons ($F=4.792$, $df=2$, $p=0.030$). Post hoc tests showed significant reductions in both VCM+ rats (123 μm vs. 170 μm , $p=0.042$) and VCM-rats (122 μm vs. 170 μm , $p=0.044$) compared to control.

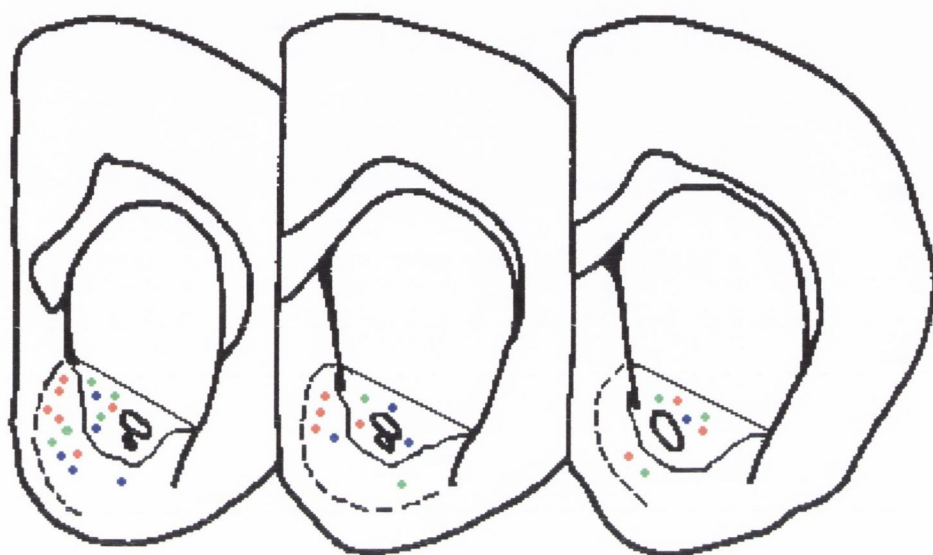


Figure 3.3 Diagrams showing the locations of neurons filled with Lucifer yellow coloured circles in sections through mid to caudal levels of the nucleus accumbens. Red circles show filled cells from control animals, green circles show cells from VCM+ animals and blue circles from VCM- animals.

Table 3.1 Median Values of length, tortuosity and surface area for dendritic segments in the shell and core of control, VCM+ and VCM- rats

Territory	Group	N	Length (μm)	Tortuosity ratio	Surface (μm^2)	Area
Accumbens	Control	318	40.4	1.76	32.74	
	Haloperidol	507	35.3	1.568 ***	28.29 **	
	VCM+	279	35.7	1.57 ***	27.91 **	
	VCM-	228	32.8	1.56 **	28.76	
Core	Control	142	44.3	1.54	28.34	
	Haloperidol	287	34.8	1.564	26.94	
	VCM+	169	35.5	1.57	30.03	
	VCM-	118	32.2	1.54	23.46	
Shell	Control	176	36.5	1.89	40.65	
	Haloperidol	220	36.0	1.594 ***	29.39 **	
	VCM+	110	37.7	1.61 ***	25.34****	
	VCM-	110	34.2	1.57 ***	35.40	

*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; significantly different to control values (Mann-Whitney U test)

Table 3.2 Mean spine density and median numbers of spines for dendritic segments in the shell and core of control, VCM+ and VCM- rats.

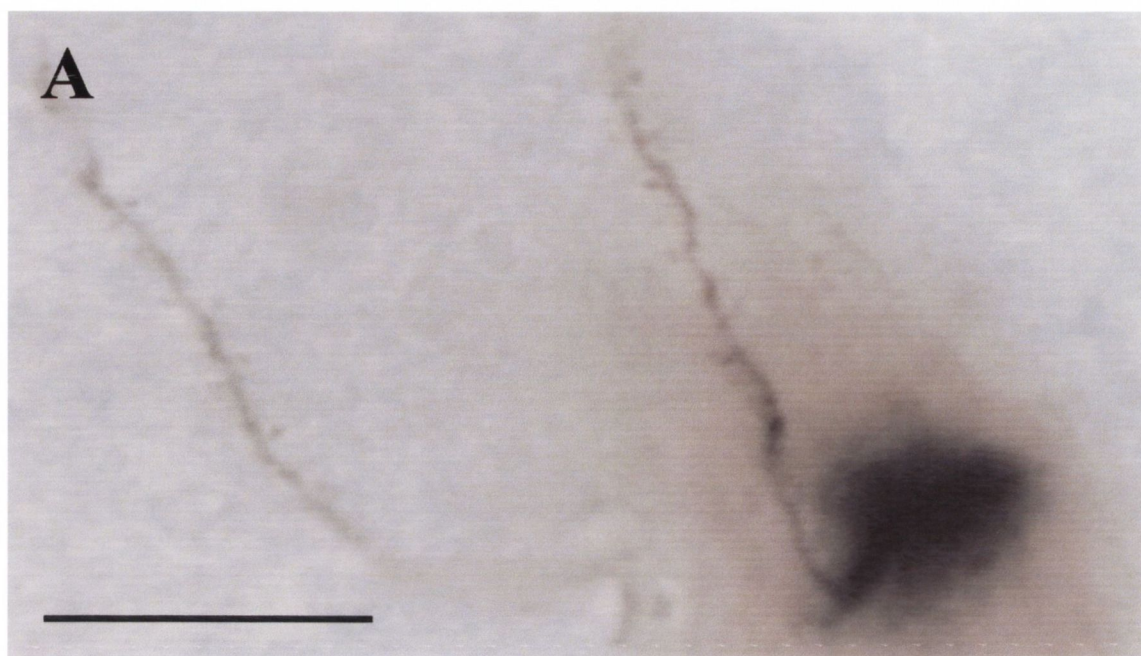
Territory	Group	N	Spine density spines per 10 µm	Number of spines per segment
Accumbens	Control	318	0.892	34
	Haloperidol	507	10.40 ***	31
	VCM+	279	10.37 ***	32
	VCM-	228	10.35 ***	31
Core	Control	142	10.43	44
	Haloperidol	287	10.55	32
	VCM+	169	10.71	32
	VCM-	118	10.24	32
Shell	Control	176	7.71	26
	Haloperidol	220	10.31 ***	31
	VCM+	110	10.04 ***	32
	VCM-	110	10.43 ***	29

*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; significantly different to control values (Mann-Whitney U test or Student's t test)

Table 3.3 Median values of the length, tortuosity, surface area and spines for proximal and distal dendritic segments in the core and shell of control VCM+ and VCM- rats.

	Shell	N	length	Tortuosity	Surface Area μm^2	spines	spine density per 10 μm
Proximal Dendrites	Control	119	29.6	1.85	49.38	23	5.76
	VCM+	80	32.2	1.62 **	28.04 ***	26	8.26 *
	VCM-	64	27.9	1.69 *	43.80 ++	23	8.03 *
Distal Dendrites	Control	57	43.7	1.94	22.60	30	8.11
	VCM+	30	48.2	1.59 **	19.60	54 *	12.26 ***
	VCM-	46	52.4	1.42 ***	26.94	48	11.57 ***
Core							
Proximal Dendrites	Control	93	30.6	1.50	28.74	30	9.29
	VCM+	111	34.8	1.54	34.68	28	9.09
	VCM-	88	36.1	1.54	31.26	31	9.30
Distal Dendrites	Control	49	61.3	1.68	27.75	64	10.86
	VCM+	58	40.5	1.58 *	25.14	42	11.55
	VCM-	30	30.2 ***, +	1.58	10.95 ***, +	39 ***, +	10.77

*, p<0.05, **, p<0.01, ***, p<0.001; significantly different with respect to control values. +, p<0.05, ++, p<0.01 significantly different with respect to VCM+ animals. (Mann-Whitney U tests)



B

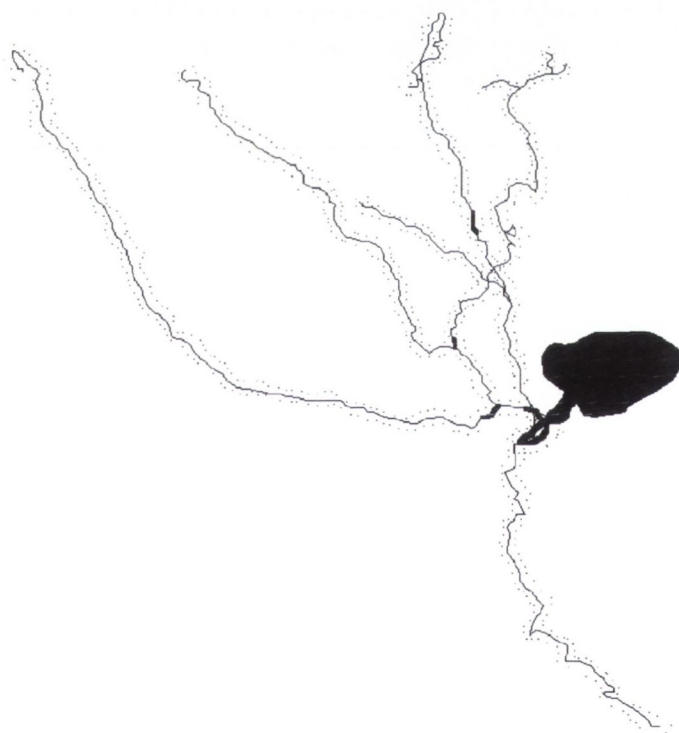


Figure 3.4 Photomicrograph A: and reconstruction B: of a filled neuron from the shell of nucleus accumbens in a control rat. Scale bars equal 25 μm .

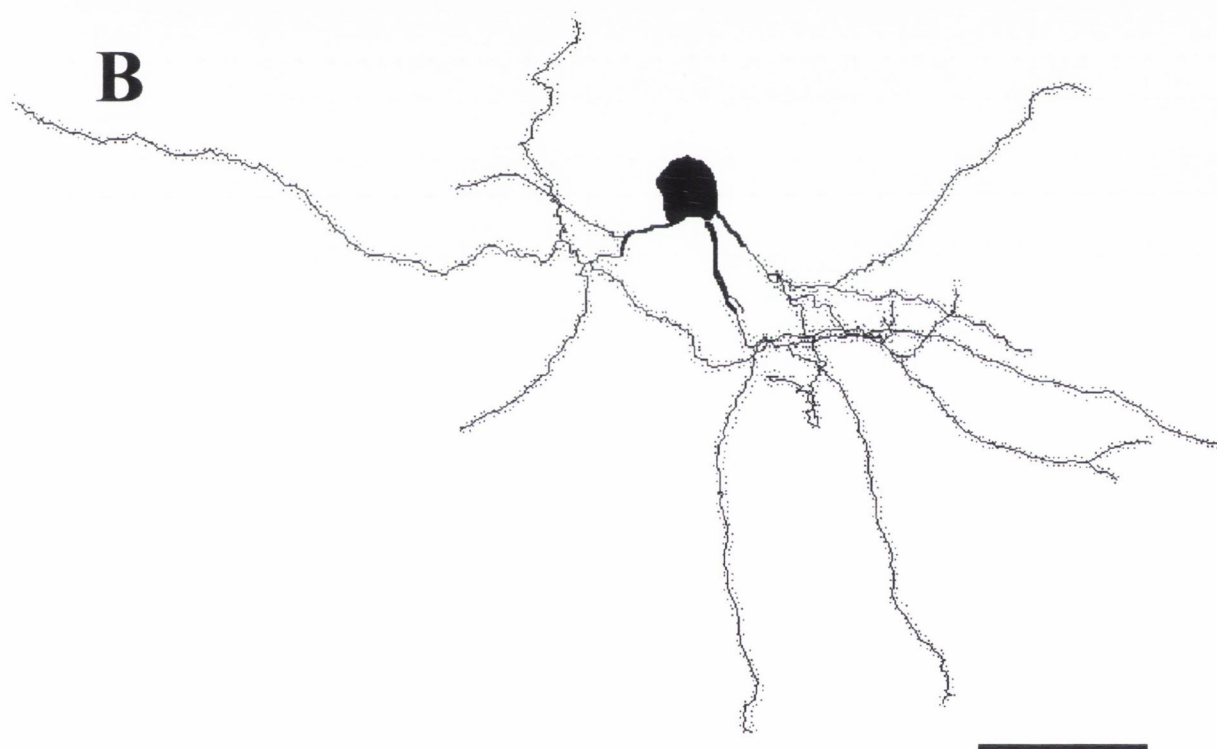
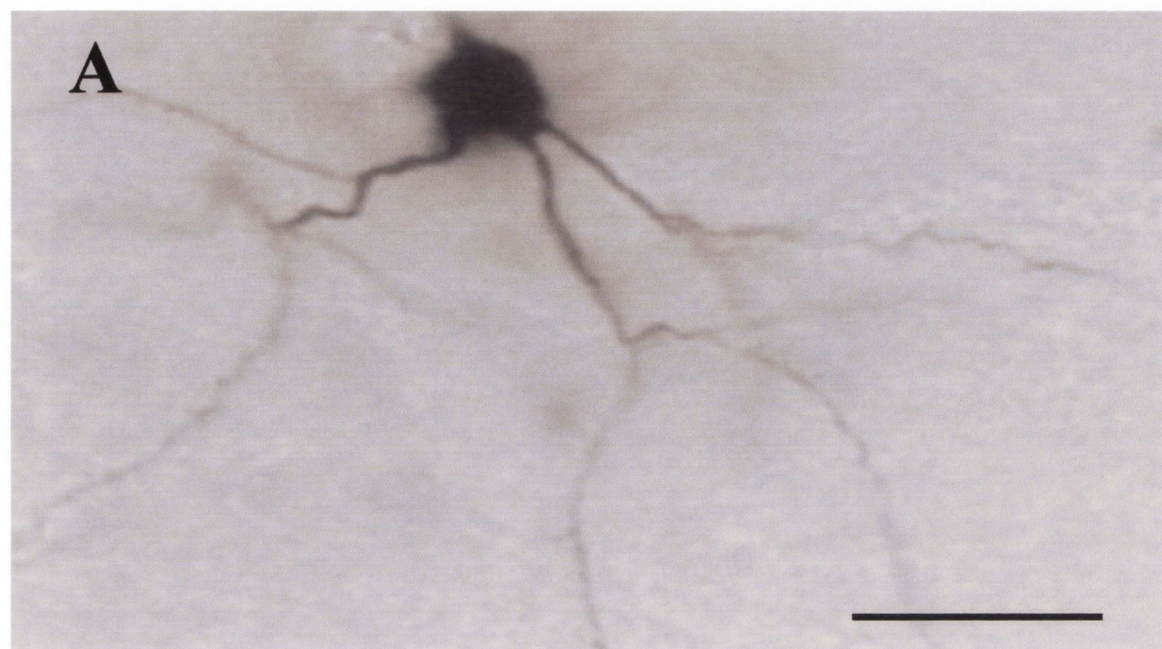


Figure 3.5 Photomicrograph A: and reconstruction B: of a neuron located in the shell territory of a VCM- rat. Note the dendritic segments more densely packed with spines compared to those in a control rat (figure 3.4). Scale bars equal 25 μm

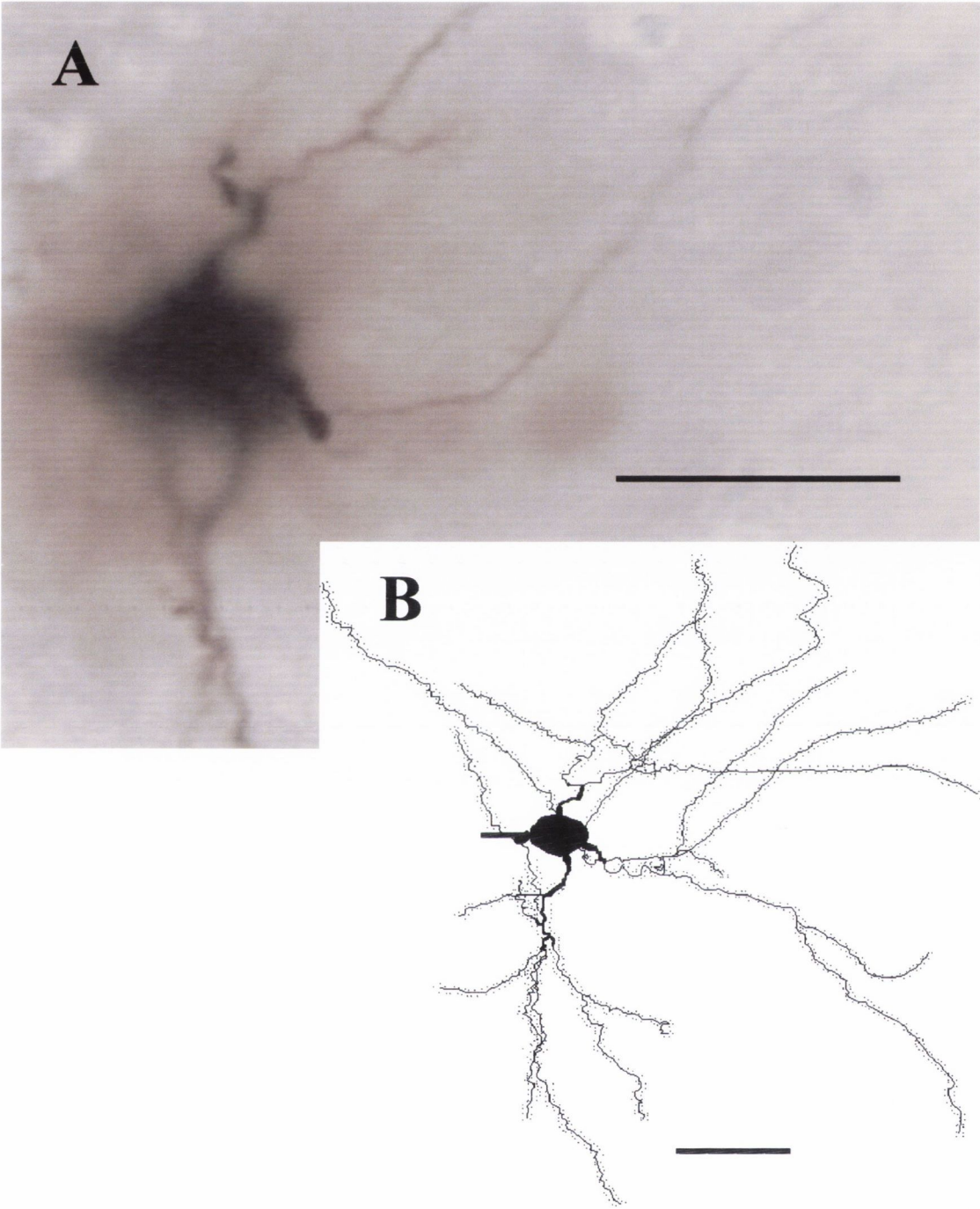


Figure 3.6 Photomicrograph A: and reconstruction B: of a filled neuron located in the shell territory of a VCM+ rat. Note the high spine density compared to a control rat (figure 3.4). Scale bars equal 25 μm .

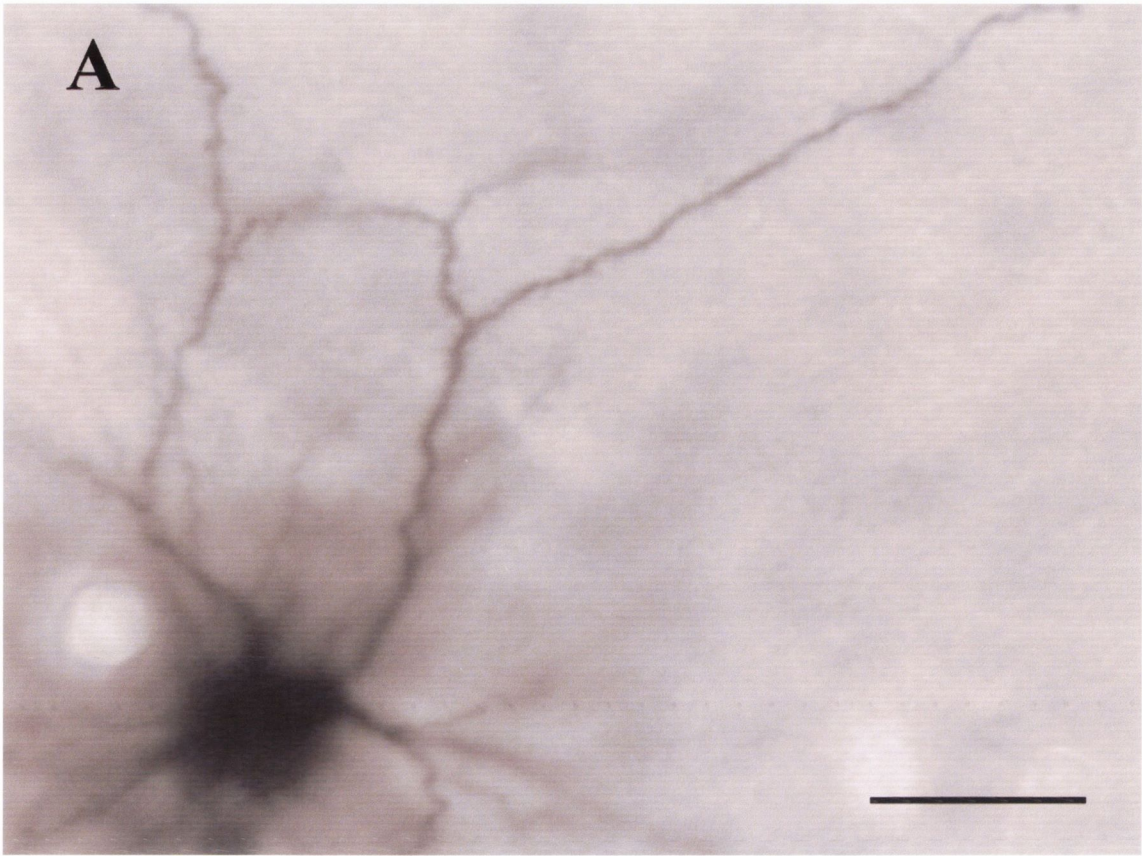


Figure 3.7 Photomicrograph A: and reconstruction B: of a filled neuron located in the core of nucleus accumbens of a control rat. Scale bars equal 25 μm .

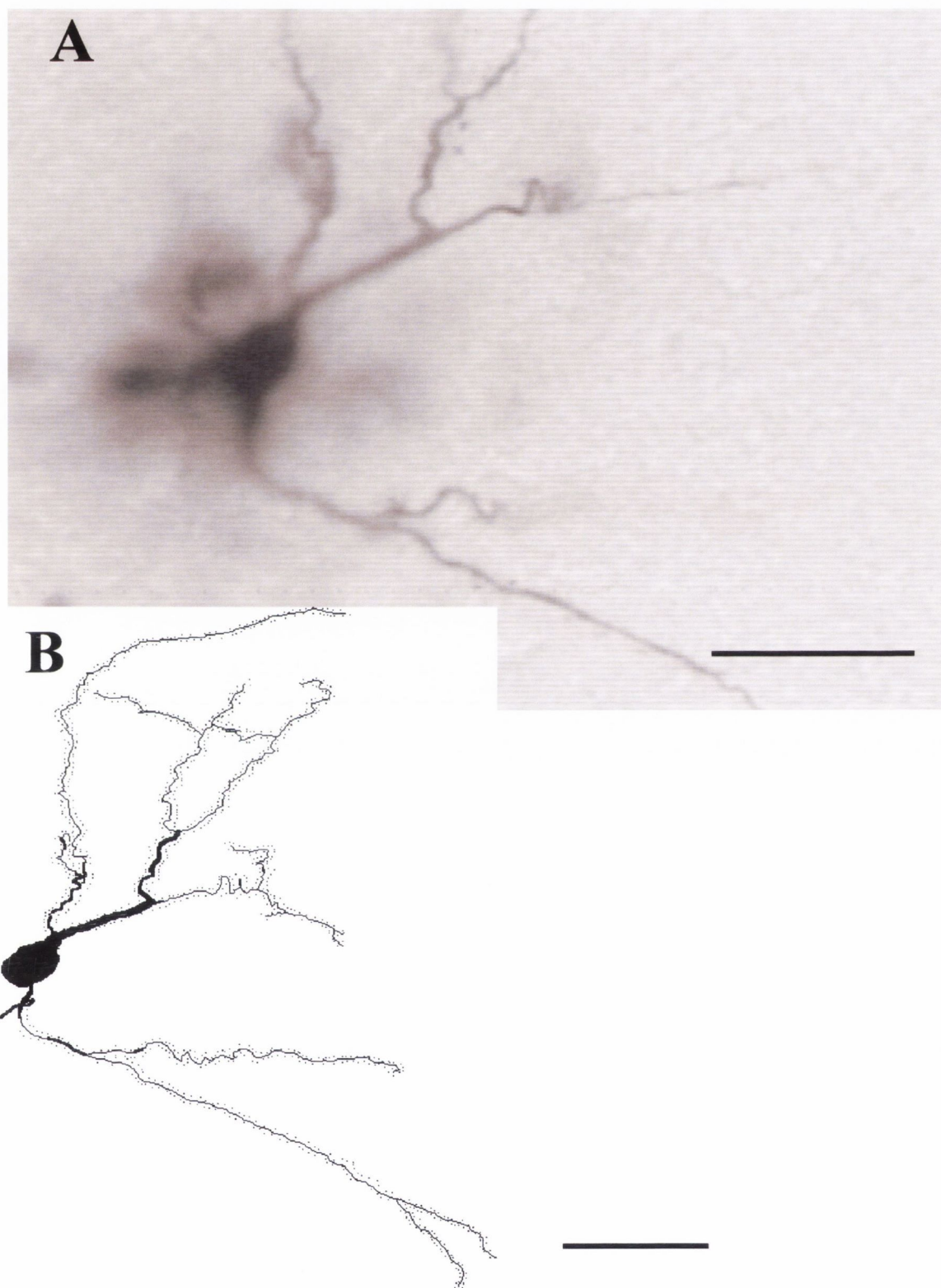


Figure 3.8 Photomicrograph A: and reconstruction B: of a neuron located in the core territory of a VCM- rat. Scale bars equal 25 μm .

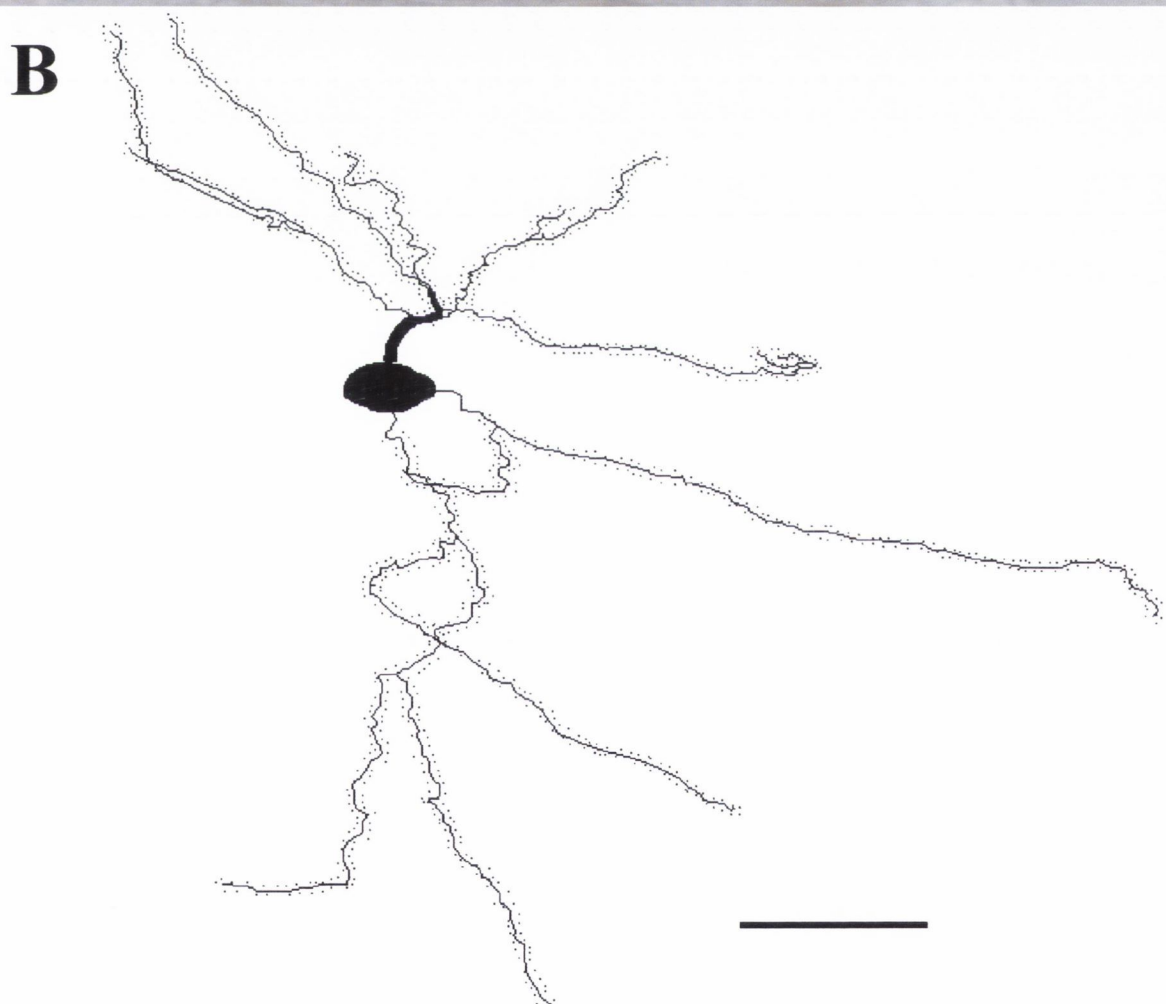
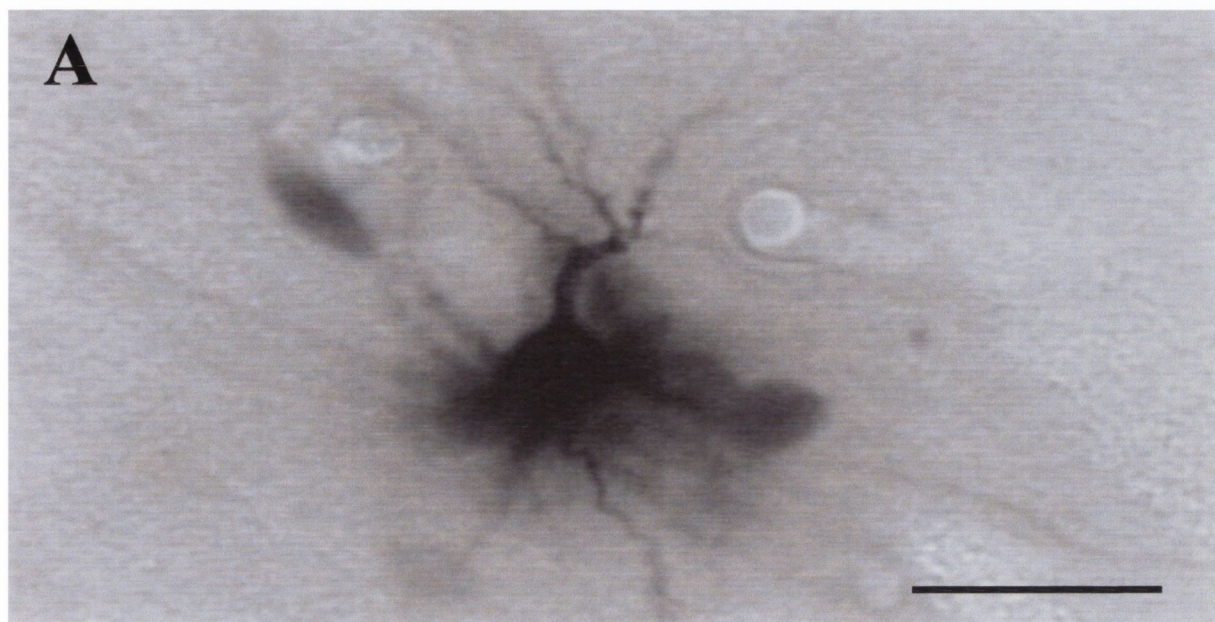


Figure 3.9 Photomicrograph A: and reconstruction B: of a filled neuron located in the core of nucleus accumbens of a VCM+ rat. Scale bar equals 25 μm .

Discussion

The development of a syndrome of VCMs in rats that persists long after the drug is withdrawn is associated with structural changes that are most evident in shell neurons. These cells have lost surface area along their dendrites, reduced their tortuosity, yet increased their spine density. Such dramatic shifts in dendritic organisation would alter the physiological function of these cells and may provide the basis for the development of abnormal movements. In addition, rats that fail to develop a syndrome of VCMs have changes in neurons located in the core. These neurons have reduced numbers of spines on their distal dendrites as well as shorter distal dendrites.

The numbers of spines counted are likely to be an underestimate of the true number due to spines protruding directly above or below the plane of the section being hidden from view. However, since analysis was carried out in an unbiased manner using coded slides and the proportion of spines hidden from view is likely to be equal in all medium sized densely spiny neurons this is unlikely to affect the differences that were found in spine density or numbers of spines. For spine density analyses dendritic segments were only used if their spine density fell within two standard deviations of the group mean for segments longer than 50 μm . This is due to the variable nature of dendritic segments that have been observed previously both in the dorsal striatum (Wilson et al., 1983) and nucleus accumbens (Meredith et al., 1992; Meredith et al., 1995). Dendrites may contain proximal segments that are aspyne as well as truncated ends that may have an unusually high or low spine density.

Extracellular glutamate concentration increases following chronic haloperidol treatment (See and Chapman, 1994; See and Lynch, 1995; Yamamoto and Cooperman, 1994). This treatment also increases perforated synapses (Meshul et al., 1996) and these are associated with increased synaptic activity (Geinisman, 1988, 1991). In the core of nucleus accumbens glutamatergic input is primarily onto spine heads (Sesack and Pickel, 1992) and the reduced numbers of spines in distal core dendrites of rats that do not develop a syndrome of VCMs is consistent with an excitotoxic mechanism. However, there was no spine loss in VCM+ rats. Differences

between spine numbers and the length of dendritic segments in neurons located in the core of VCM+ and VCM- rats are most likely due to increased expression of dynorphin in VCM+ rats. Egan et al. (1994) has previously shown that VCM+ rats express significantly more of this neuropeptide than VCM- rats. Although the core has primarily D₂ receptors it has been shown that up to 30 % of neurons expressing D₂ receptors also coexpress D₁ mRNA (Jongen-Rêlo, 1994). Therefore, activation of both D₁ and D₂ receptors in the core in VCM+ animals may be one of the mechanisms that they have increased expression of dynorphin. Dynorphin in the core of VCM+ animals would have the effect of reducing the potential excitotoxic effects through activation of kappa receptors that can selectively inhibit glutamate (Hill and Brotchie, 1995) and dopamine release (Steiner and Gerfen, 1996). Conversely, lack of neuropeptide upregulation in the core of VCM- rats may lead to atrophy in distal core dendrites. Therefore it appears that the role played by dopamine in maintaining dendritic morphology may be mediated by neuropeptides. Moreover, the loss in spine numbers on distal dendrites of VCM- rats would be expected to lead to loss of glutamatergic input to these spines and thereby prevent the development of VCMs in these animals.

The fact that morphological alteration occurs at distal sites implies that input from extrinsic sources is primarily altered since it has been demonstrated previously that synaptic input to distal sites on dendrites originates in cortical and thalamic areas whereas input received at more proximal dendrites originates from sources within the nucleus accumbens (Meredith and Totterdell, 1999). It is not surprising, therefore, that as well as spine loss there was a concurrent reduction in length and surface area in these neurons associated with distal dendrites. It has previously been hypothesised that the surface area of a dendrite is directly proportional to the number of synapses making contact with it (Harris and Kater, 1994). This implies that reduced surface area would result in a loss of input along dendritic shafts. The input onto distal core dendrites is primarily from dopaminergic terminals that arise from the SNpc (Groenewegen et al., 1991; Zahm, 1992). These dopaminergic contacts play a pivotal role in gating excitatory input from the cortex that is received on spine heads (Mogenson et al., 1993). Loss of dopamine inputs would result in loss of this gating and lead to unopposed excitatory input and increased activation of these core projection neurons (Mogenson, 1980). However, the spines with excitatory synapses

are capable of acting as individual compartments for calcium (Müller and Connor, 1991). Prolonged increased excitatory input may result in spine loss most likely through excitotoxic mechanisms (Beal et al., 1986; Meldrum and Garthwaithe, 1990). In addition, if spines are lost the glutamatergic input associated with spine heads is presumably lost as well (Meredith et al., 1993). The data presented in this study are in agreement with recent findings of Hamid et al. (1998) that show reduced NMDA receptor binding in the core of nucleus accumbens of VCM- but not VCM+ rats. Assuming that a loss in spines would lead to a loss in asymmetrical synapses, our findings are in agreement with previous ultrastructural studies (Roberts et al., 1995; Meshul et al., 1996a) that demonstrate reduced numbers of asymmetrical axospinous synapses following chronic haloperidol treatment that persists following drug withdrawal. However, unlike previous studies reduced numbers of spines were only observed in VCM- rats in our studies.

The increase in spine density and reduction in tortuosity in the shell found in neurons in treated animals, are diametrically opposed to the reduction in spine density and the increase in tortuosity previously reported by Meredith et al. (1995) following a 6-hydroxydopamine lesion. This lesion completely depletes dopamine while haloperidol acts mainly by its dopamine D₂ receptor antagonist properties. Therefore, since the shell has a high density of dopamine D₁ receptors (Bardo and Hammer, 1991) dopamine can still act on these. This would indicate that the morphological alterations observed in the shell are most likely due to activation of D₁ receptors. Dopaminergic activation of D₁ receptors is critical, not only increasing GABAergic activity but also in altering the neuropeptides dynorphin and substance P coexpressed in these neurons. It is interesting that Egan et al. (1994) observed an increase in dynorphin mRNA expression in VCM+ rats with no change in substance P. Assuming changes in mRNA reflect protein expression, what effects would increased dynorphin expression have? Dynorphin is an endogenous ligand of kappa opioid receptors that have inhibitory effects in the nervous system (Chavkin et al., 1982; Corbett et al., 1982). Increased dynorphin expression due to repeated D₁ receptor stimulation is a feedback mechanism altering the effects of dopamine (Steiner and Gerfen, 1998).

The findings presented here, coupled with increased activation of striatonigral circuits (Egan et al., 1994), supports a previously proposed model of hyperkinetic disorders (Albin et al., 1989; Crossman, 1990; DeLong, 1990). Moreover, there is accumulating evidence that shell circuitry plays a pivotal role in generating oral movements (Prinssen et al., 1994; Cools et al., 1995; Kelley and Swanson, 1997) and the mechanism appears to be through the D₁ receptor. Several lines of evidence support this, firstly it is known that stress leads to dopamine release preferentially in the shell of nucleus accumbens (Deutch and Cameron, 1992; Kalivas and Duffy, 1995) and stress exacerbates oral behaviours (Tarsy et al., 1983) presumably through activation of D₁ receptors. In addition, the shell contains one of the highest densities of this receptor (Bardo and Hammer, 1991) and both systemic and local infusions of D₁ agonists can induce vacuous chewing movements.

Increased dendritic branching and spine density in shell neurons has previously been shown following amphetamine or cocaine administration (Robinson and Kolb, 1997) and these changes are coupled with increases in branched spines and increased dendritic volume. Since the shell of nucleus accumbens contains predominantly D₁ receptors and the behavioural data presented in chapter two indicates activation of these receptors it may be expected that haloperidol treatment may lead to similar changes in the shell as occur following treatment with a dopamine agonist. The fact that increases in dendritic branching were found in this study following chronic haloperidol treatment appear to reinforce a similar mechanism of action to that of dopamine agonists, however, this was not associated with an increase in the percentage of branched spines or dendritic volume. Therefore increases in spine density and dendritic branching in the shell of nucleus accumbens due to chronic haloperidol treatment appear to occur via a different mechanism than those due to amphetamine. This is supported by the fact that haloperidol has been shown to block behavioural hypersensitivity associated with amphetamine.

In this study the use of pooled values from individual dendritic segments, coupled with separate analyses of proximal and distal sites, allowed the detection of structural alterations that may otherwise have gone undetected especially changes that occurred at distal core segments. This lends further support to the view that the dendritic segment is the optimal unit of comparison in examining changes in dendritic

morphology as has been demonstrated in previous studies (Meredith et al., 1992; Meredith et al., 1995). This is because of the great structural variability in nucleus accumbens projection neurons (Bayer, 1981; Rueda et al., 1986; Meredith et al., 1992; Meredith et al., 1995). Proximal segments were aspiny and spines only started to appear around the first branch point as has previously been described (Wilson et al., 1983; Meredith et al., 1992). In addition, dendrites were generally thicker at their most proximal segments and much thinner at distal segments.

Adequate functioning of neuronal circuitry relies on successful transmission of information across synapses and it has been shown previously that both glutamate and dopamine interactions are vital in this function in the striatum. Apart from these neurotransmitters a role for the neuropeptides cannot be overlooked. Accordingly, changes in any of these neurotransmitters would be expected to alter synapses and lead to structural changes in striatal circuits (Meredith et al., 1995). The structural alterations reported here support the theory that changes in dendritic morphology may underlie both the efficacious and undesirable effects of classical antipsychotic drug treatment (Arbuthnott and Ingham, 1993). Previous studies have suggested the shell of nucleus accumbens is a locus of antipsychotic drug action (Deutch et al., 1992; Merchant et al., 1994; Sebens et al., 1995) while the core is associated with motor side effects. However, the results presented here demonstrate a role for both the core and the shell in the development of abnormal orofacial movements. Animals that develop a syndrome of VCMs have dendrites in the shell territory that increased spine density and reduced surface area and no apparent change in the core territory. Rats that fail to develop this syndrome of VCMs have dendrites with increased spine density and normal surface area in the shell territory as well as distal dendrites in the core territory that are shorter and have less spines than those from VCM+ rats.

In summary, chronic haloperidol treatment leads to blockade of D₂ receptors and this would be expected to lead to a loss in the gating effect of dopamine in the core of both VCM+ and VCM-animals leaving the glutamatergic input unopposed. Initially this would be expected to increase activity of core projection neurons. However, behavioural data suggests increased dopamine D₁ receptor activation in VCM+ rats and this would protect core neurons of VCM+ rats through increased

dynorphin release and activation of kappa receptors (Hill and Brothie, 1995). In VCM- rats, spine loss and reduction in dendritic length occurs possibly through excitotoxic mechanisms. This putative mechanism is supported by data from Egan et al. (1994). Dynorphin is one of two main peptides coexpressed with GABA in core and shell neurons respectively and their expression would prevent excitotoxic consequences in VCM+ rats. Moreover, it has recently been shown that VCM- rats show reductions in NMDA receptors in the striatum and in particular in the core of nucleus accumbens whereas VCM+ rats do not (Hamid et al., 1998). It is also known that NMDA receptors are primarily localized at asymmetrical synapses on spine heads in nucleus accumbens (Gracy and Pickel, 1996). Taken together, it appears that selective activation of D₁ receptors in VCM+ rats results in morphological change in primarily in the shell of nucleus accumbens that is mediated through glutamate and the neuropeptide dynorphin may provide the neural basis for the development of vacuous chewing movement syndrome in rats. Moreover, morphological changes also occur in VCM- rats most notably at distal dendritic sites in the core territory and these changes are likely to form the basis for the lack of vacuous chewing movement syndrome in these animals.

Chapter 4

**Changes in nitric oxide synthase containing
neurons in nucleus accumbens of rats that
develop chronic haloperidol-induced vacuous
chewing movements**

Introduction

A group of neurons containing the neuronal isoform of the nitric oxide synthase (NOS), somatostatin and neuropeptide Y account for 1-2% of nucleus accumbens' neurons. These neurons have large perikarya that range in diameter from 12-25 μm with long smooth processes (Kawaguchi, 1993; Hussain et al., 1996). They are interneurons and their terminals are immunoreactive for GABA. In addition they are physiologically unique due to their ability to produce large and persistent plateau depolarizations and also Ca^{2+} -dependent low-threshold spikes in addition to fast spikes. Nitric oxide has been postulated to have at least two functions in the striatum. It primarily acts as a neurotransmitter (Garthwaite et al., 1988; Garthwaite, 1991) and can modulate both dopamine and glutamate release (Meffert et al., 1994). Therefore NOS immunoreactive (NOS-IR) neurons are likely to play an important role in striatal function. In addition nitric oxide can act directly on guanylate cyclase in vascular smooth muscle and is likely to control local blood flow in the striatum (Meyer et al., 1994).

Nitric oxide is a highly lipophilic gas and once synthesised it readily diffuses to adjacent cells where it acts by stimulating soluble guanylate cyclase, an enzyme that is enriched in medium sized densely spiny projection neurons (Ariano 1983). Moreover, the axons of NOS-IR neurons extend distances of up to 1mm, thus increasing the area that nitric oxide can exert an influence. In the striatum, nitric oxide is produced following the activation of glutamate NMDA receptors (Marin et al., 1993). Therefore, cortical input from the hippocampus, amygdala and prefrontal cortex that are excitatory to medium sized densely spiny neurons may also activate NMDA receptors on interneurons (O'Donnell et al., 1999). Therefore NOS-IR interneurons are in a key position to gate glutamatergic input.

Chronic treatment with the classical neuroleptic drug haloperidol leads to increases in extracellular glutamate (See and Chapman, 1994; See and Lynch, 1995; Yamamoto and Cooperman, 1994). Excess glutamate has the potential to be excitotoxic and may lead to the changes in morphology that occur following chronic haloperidol treatment that were demonstrated in chapter three of this thesis in both the shell and core of

nucleus accumbens. Moreover, these changes in the dendritic structure of medium spiny neurons may be mediated by nitric oxide, especially since nitric oxide is known to play a neuroprotective role in the striatum.

Chronic haloperidol treatment leads to the development of vacuous chewing movements in a percentage of rats treated with this drug and these abnormal movements persist following drug withdrawal (Kane et al., 1986; Ellenbroek, 1993). It has been postulated that vacuous chewing movements may be due to excitotoxic mechanisms that may occur due to increased activity of projection neurons in the striatum and nucleus accumbens (Egan et al., 1994; Hamid et al., 1998). However, given the important neuromodulatory role of nitric oxide it is important to examine if NOS-IR neurons are altered following chronic haloperidol treatment and withdrawal and especially whether changes in these neurons are associated with the development of vacuous chewing movements. Therefore, the aim of the present study was to determine if NOS-IR neurons are altered in number or cross-sectional area due to chronic haloperidol treatment. Moreover, we determined whether rats that developed vacuous chewing movements (VCMs) were differentially affected to those who failed to develop abnormal movements.

Materials and Methods

Thirty-five adult male Wistar rats (Bioresources, Trinity College Dublin, Ireland) initially weighing 200-250g were housed four per cage, with a constant temperature of 21°C, on a 12-h light and dark cycle with free access to food and water. Animals were divided into two groups and received intramuscular injections of haloperidol decanoate (Janssen Pharmaceutical Ltd., Ireland) at a dose of 28.5 mg/kg the equivalent of 1 mg/kg per day of unconjugated haloperidol (n=23), or the same volume of vehicle sesame oil (n=8). Injections were administered to alternate hind legs every three weeks during the treatment period of 27 weeks. Animals were withdrawn from treatment for a further 18 weeks.

During both the treatment and withdrawal periods each rat, in both the vehicle-treated and haloperidol treated groups, was assessed for the development of VCMs every three weeks, prior to receiving its injection. Behavioural assessments were carried out in a manner similar to that described previously (Clifford and Waddington 1998) and are described in detail in chapter 2 of this thesis.

Following the withdrawal period, the rats were anaesthetised with sodium pentobarbital (60 mg/kg, i.p.) and perfused transcardially with saline containing 0.5% procaine, followed by fixative containing 4% paraformaldehyde, 15% saturated picric acid and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature. Each brain was removed; the forebrain blocked and transverse sections were cut at 70 µm with a Vibraslice. Sections were then placed in an antifreeze solution containing 30% sucrose and 30% ethylene glycol in 0.1M PBS and stored at -20°C until processed for NOS immunohistochemistry.

Sections from control (n=6), VCM+ (n=6) and VCM- (n=6) rats were processed for NOS immunohistochemistry at the same time. These sections were removed from the freezer and rinsed thoroughly with 0.1 M PBS. Following rinsing, sections were incubated for 10 minutes in a 1 % solution of sodium borohydride made up in distilled water. This was followed by several rinses in 0.1M PBS until no bubbles remained. Following rinsing, sections were incubated in a 1:10,000 dilution

of sheep anti-NOS antiserum (kindly donated by Prof. Piers Emson, Babraham Institute, University of Cambridge, Cambridge, UK) in PBS containing 0.25% Tx, at 4°C for 24 h. Following multiple rinses, sections were incubated in a 1:200 dilution of biotinylated donkey anti-sheep IgG in PBS-Tx, at 4°C for 24 h. After rinsing, sections were incubated in a prepared avidin-biotin peroxidase complex (ABC reagent, Vector Laboratories, Peterborough, UK) for 90 min at room temperature. Sections were then rinsed three times in 0.05M Tris-HCl, pH 7.6, sections were reacted for 30 min in 0.05% DAB with hydrogen peroxide added at a final concentration of 0.01%. Sections were then rinsed and mounted onto slides from a 0.2% gelatin solution, dried, dehydrated, and coverslipped.

Analysis of the number of NOS-IR neurons per mm² and the crosssectional area of NOS-IR neurons was carried out using a dedicated computer (Micron Electronics Inc., USA) and software system Stereo Investigator (Microbrightfield Inc., USA). All measurements were carried out at a mid-caudal level (+ 1.0 mm Bregma; Paxinos and Watson 1986). The boundary between the shell and core territories of nucleus accumbens was traced referring to camera lucida sketches made from CBP immunoreacted sections. A grid of 100 µm x 100 µm squares was placed at random over the shell region. Each square contained a 50 µm x 50 µm counting frame located in its upper left corner. Cells were counted provided they were immunopositive for NOS and if any part of the cell body was inside the boundary of the counting frame and no part of the cell body touched the red “forbidden” boundary at the left hand and lower edge of the counting frame (Figure 4.1). Simultaneously an estimate of the cross sectional area of the NOS-IR neuron was estimated using a 2-D version of the nucleator (Gundersen, 1988; Larsen, 1998). From an approximate central point in the cell body the length of each test line from the centre to the edge of the cell was measured in five systematically random directions. The cross sectional area of the NOS-IR neuron was estimated using the formula: $a = \pi \bar{l}^2$. Where $\bar{l}$ is the mean length of line from the centre of the cell body to its edge (Figure 4.2). After counting and estimating the size of all the cells in a counting frame the stage was then moved automatically to the next site until all sites were sampled. The density of NOS-IR cells was then calculated and expressed as numbers of immunoreactive neurons per square millimeter. Analysis of NOS-IR neurons was carried out in a

similar manner for the core of nucleus accumbens. The Kolmogorov-Smirnoff test was used in order to test if the data was significantly different to a normal distribution. Normally distributed data was analysed using Student's t test and ANOVAs. Non-parametric data was analysed using a Mann-Whitney U tests and Kruskal Wallis tests. A p value of < 0.05 was considered significant.

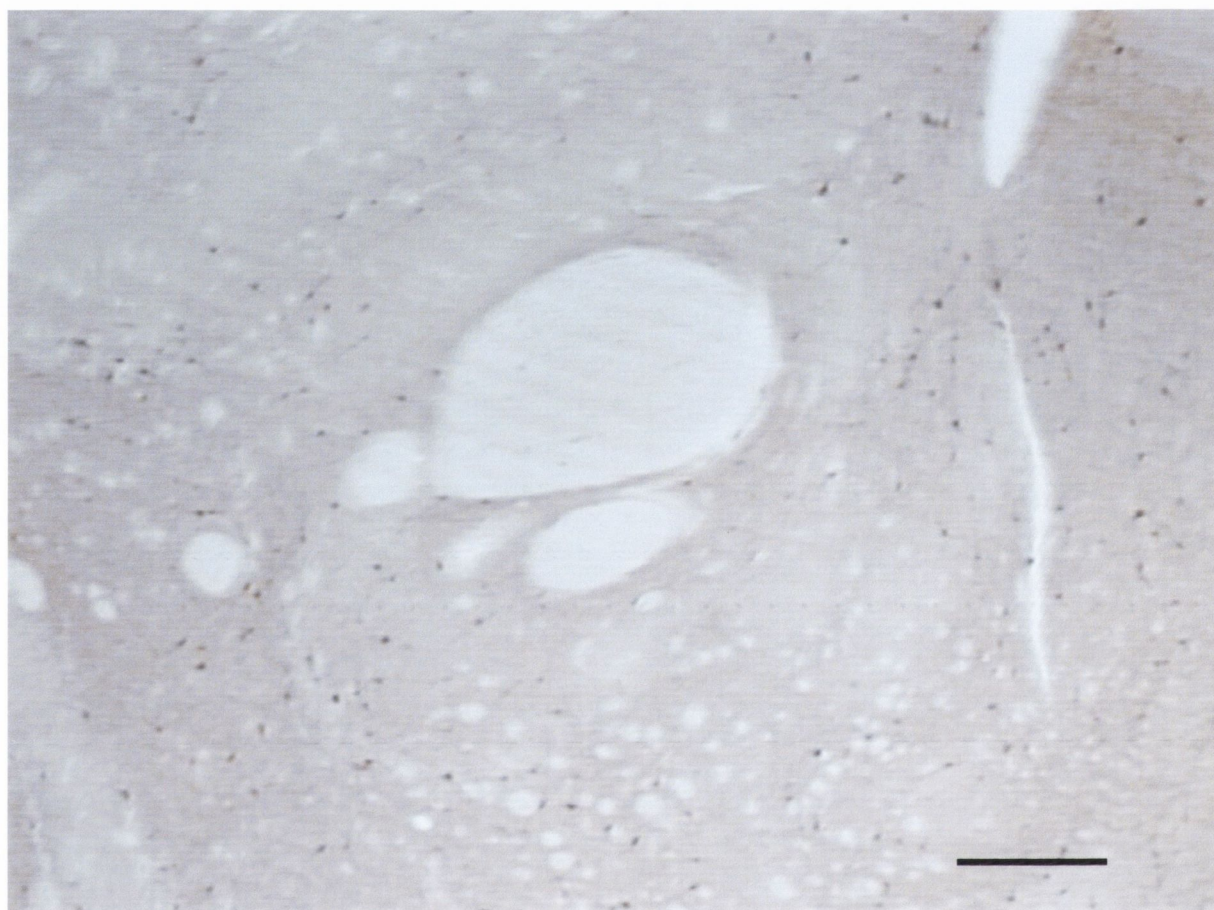


Figure 4.1 Photomicrograph of the distribution of NOS immunoreactive neurons in nucleus accumbens. Scale bar equals 100 μm .

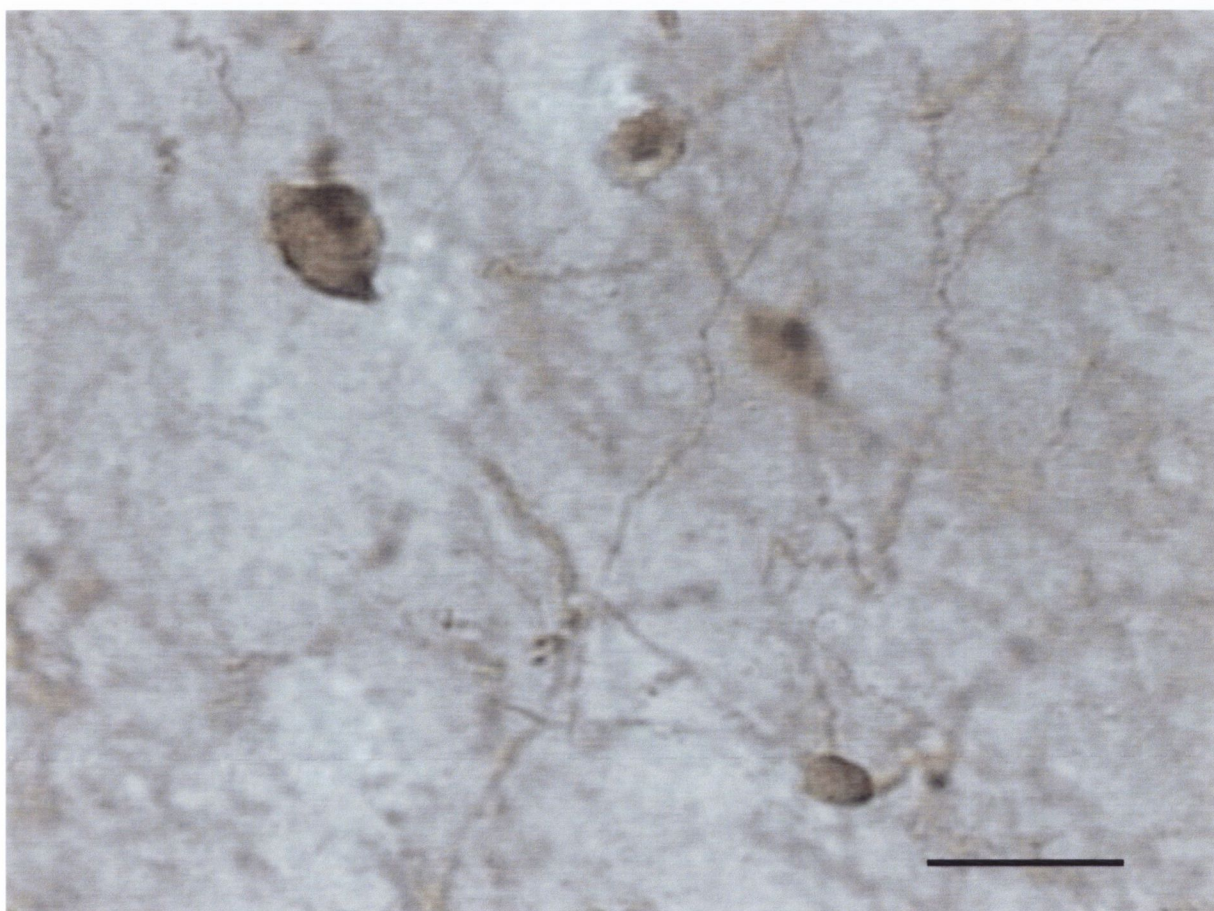


Figure 4.2 Photomicrograph of NOS-immunoreactive neurons in nucleus accumbens. Note the wide range of cell body sizes. Scale bar equals 25 μm .

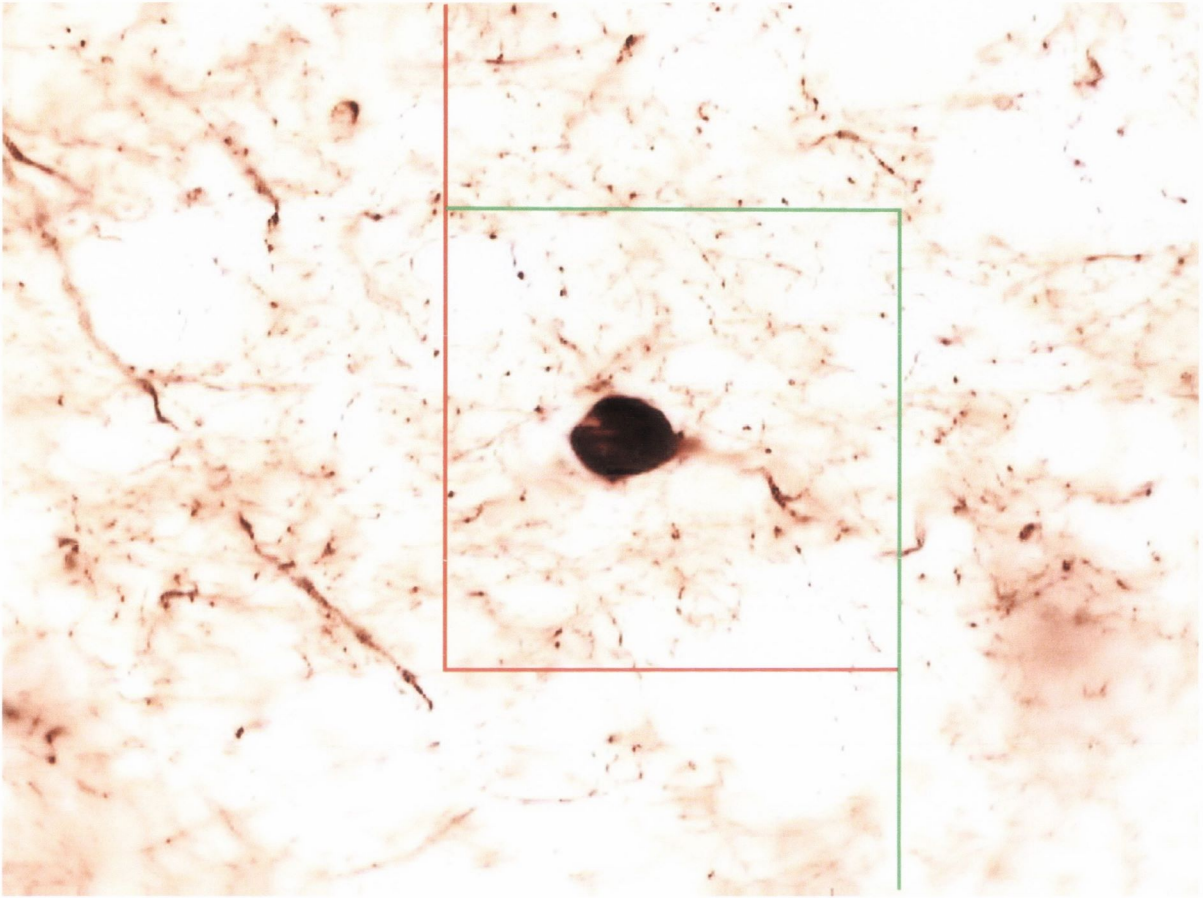


Figure 4.3 Photomicrograph of NOS-immunoreactive neuron in counting frame. The cell body does not touch the lower and left 'forbidden' lines and can be counted.

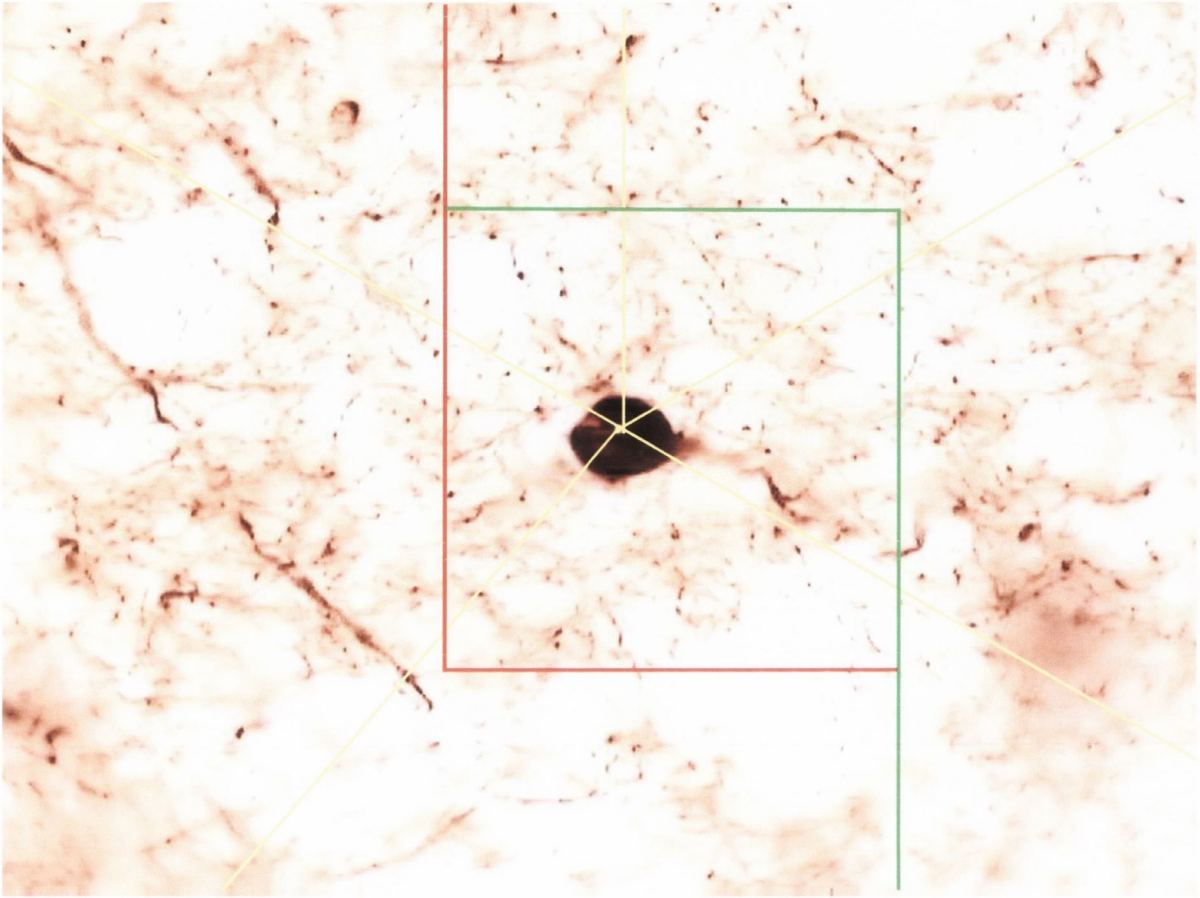


Figure 4.4 Photomicrograph of NOS-immunoreactive neuron in counting frame with a superimposed nucleator probe. The mean distance between the centre of the probe and the point at which each ray intersects the edge of the cell is used to estimate the cell area.

Results

In the present investigation we examined the regional distribution of NOS at a mid-caudal level of nucleus accumbens (Figure 4.1). The shell and core territories were delineated using sections immunoreacted for calcium binding protein D_{28K} (Figure 1.1). In both control and haloperidol treated groups NOS-IR cells were found widely distributed throughout the shell and core territories and there appeared to be no difference in the distribution of NOS-IR neurons between groups.

The density of NOS-IR neurons per mm² was calculated for the shell and core territories of both control and haloperidol treated rats and a significantly higher density of NOS-IR neurons was found in the shell territory compared to the core territory. This inter territorial difference in density occurred in both control and haloperidol treated rats. In control rats, the mean density of NOS-IR neurons was 91 cells mm⁻² in the shell territory compared to 73 cells mm⁻² in the core territory ($p=0.023$). Similarly in haloperidol-treated rats the mean density of NOS-IR neurons in the shell was 98 cells mm⁻² compared to 74 cells mm⁻² in the core ($p=0.020$). However, there were no significant intra territorial differences in the mean density of NOS-IR neurons in either the shell or core territories of control, VCM+ or VCM- rats.

NOS-IR neurons were found throughout the shell and core of nucleus accumbens at the mid-caudal level examined in this study. When we examined the area of these neurons located in the core territory it was found that the cross sectional area ranged from 25.2 μm^2 to 224.9 μm^2 in control rats and from 24.9 μm^2 to 213.3 μm^2 in haloperidol-treated rats. In the shell territory, the cross sectional area of NOS-IR neurons ranged from 24.15 μm^2 to 276.7 μm^2 in control rats and from 26.4 μm^2 to 252.1 μm^2 in haloperidol-treated rats.

Subsequent analysis revealed that NOS-IR neurons located in the shell territory had significantly reduced cross sectional area in both VCM+ and VCM- rats compared to control rats ($p<0.001$ and $p=0.002$, respectively; Table 4.1). In the core territory, the cross sectional area of NOS-IR neurons from VCM- rats were significantly smaller than NOS-IR neurons from control rats ($p=0.005$) and VCM+ rats ($p=0.037$; Table 4.1).

Table 4.1. Median cell size for NOS-IR neurons in the shell and core of control, VCM+ and VCM-rats.

Territory	Group	N	Cross sectional area mm ²
Accumbens Core	Control	171	104.20
	Haloperidol	355	95.74 *
	VCM+	201	98.98
	VCM-	154	93.08 **, +
Accumbens Shell	Control	360	102.29
	Haloperidol	797	93.20 **
	VCM+	397	93.72 ****
	VCM-	400	91.85 **

*, p<0.05, **,p<0.01, ***, p<0.001; significantly different to control values
+, p<0.05 significantly different to VCM+ animals(Mann-Whitney U test).

Discussion

In the present investigation we have shown for the first time that the cross sectional area of NOS-IR neurons is significantly reduced in both the shell and core territories at a mid-caudal level of nucleus accumbens following treatment and withdrawal from haloperidol. Moreover, it was found that of NOS-IR neurons located in the core territory had significantly reduced cross sectional area only in animals that failed to develop a syndrome of vacuous chewing movements. In addition, the density of NOS-IR neurons differed between the shell and core territories.

As has been described previously, NOS-IR neurons in the striatum are local circuit neurons, they are GABAergic and are also immunopositive for the neuropeptides somatostatin and neuropeptide Y (Kawaguchi and Kubota, 1995). The neurons that reacted for NOS in this study had similar morphology to those characterised previously using NADPH-diaphorase immunohistochemistry (Kawaguchi, 1993; Hussain et al., 1996). It has been reported previously that there is no overall difference in the distribution of these neurons in nucleus accumbens. However, our inter-territorial comparisons revealed that the shell had a much higher density of NOS-IR neurons than the core.

We were highly selective in the rostro-caudal level we chose for our analysis. This level was also used based on previous evidence that suggests the caudomedial nucleus accumbens is a locus of antipsychotic drug action (Deutch et al., 1992) based on studies using fos as a marker for neuronal activation. Therefore, the most pronounced effects of chronic haloperidol treatment would be expected to occur in this level of nucleus accumbens. In addition, the shell and core territories were examined separately as these regions have different inputs and outputs (see Chapters 1, 3 and 7). It has been shown previously that nitric oxide plays an important role in synaptic plasticity (Bohme et al., 1991) and the differences in numbers of NOS-IR neurons between the shell and core territories at this rostro-caudal level may indicate another mechanism of differential control of plasticity between these accumbens regions.

Reduced cross sectional area of NOS-IR neurons in haloperidol treated rats would be expected to lead to reduced distances over which nitric oxide could diffuse from the cell body (Philippides et al., 2000). Moreover, reductions in neuronal size in the striatum have been correlated to reductions in the amount mRNA produced by neurons (Gerfen et al., 1991). In addition, a recent study (Iwahashi et al., 1996) demonstrates that haloperidol inhibits nNOS activity as well as nitric oxide release. Release of nitric oxide has been shown to inhibit glutamate transmission through its NMDA receptor thereby preventing increases in intracellular Ca^{2+} concentration (Mazoni et al., 1992; Mazoni and Bockaert, 1993) and this may be the mechanism for the neuroprotective effects of nitric oxide in striatal cells. A reduction in cross sectional area of NOS-IR neurons from VCM- rats would be expected to lead to both reduced production of nitric oxide as well as a reduced distance over which nitric oxide could diffuse (Philippides et al., 2000). Therefore, if nitric oxide released by interneurons diffuses a lesser distance in the core of VCM- rats this would be expected to result in reduced inhibition of glutamate transmission via the NMDA receptor in medium-sized densely spiny neurons at sites where nitric oxide normally acted. Increased glutamate could then lead to hyperexcitability at individual spines and loss of spines in medium-sized densely spiny neurons in the core territory in VCM- rats. This would concur with results presented in chapter 3 of this thesis where the distal dendrites of medium sized densely-spiny neurons had significantly reduced numbers of spines.

In summary, reductions in the cross sectional area of NOS-IR neurons would be expected to lead to less nitric oxide being produced as well as a reduced distance over which this transmitter could diffuse and act. Reduced effects of nitric oxide would result in spines of medium-spiny neurons becoming more susceptible to glutamate-induced hyperexcitability and loss of spines through excitotoxic mechanisms. Therefore haloperidol may have excitotoxic actions in the core of nucleus accumbens that are mediated by nitric oxide. Paradoxically, excitotoxicity especially if it occurs in spines may lead to an overall beneficial effect by protecting the animal from developing abnormal movement side effects.

It must be noted that the present study only takes into account changes that occur in cell body size. It is also known that nitric oxide can be produced at synapses

where it is postulated to act as a retrograde messenger. In addition, the neuropeptides and neurotransmitters that are also contained in the NOS-IR neurons may also play a role. Nevertheless the fact that NOS-IR neurons are significantly altered following haloperidol treatment and withdrawal indicates the potential for fundamental changes in the degree of neuromodulation by nitric oxide and therefore changes in neuronal circuitry. It has been shown that the activity of NOS-IR neurons in the striatum is subject to a tonic, NMDA receptor-mediated excitatory influence from the corticostriatal pathway (Carlsson and Carlsson, 1990). Treatment with haloperidol results in inhibition of both NOS activity and release of nitric oxide (Iwahashi et al., 1996). Therefore, not only is the gating effect of dopamine lost following chronic haloperidol treatment but the neuromodulatory effect of nitric oxide is also compromised.

Chapter 5

Glutamate transport is impaired in the rat striatum following chronic haloperidol treatment

Introduction

Haloperidol, a classical antipsychotic drug, is believed to exert its effects by blocking D₂ dopamine receptors (Seeman and Lee, 1975; Creese et al., 1976). The highest densities of both D₁ and D₂ dopamine receptors are found in the striatum (caudate/putamen and nucleus accumbens). These nuclei are generally considered to be sites of both efficacious and undesirable effects of antipsychotic drug treatment (Crow et al., 1975; Deutch et al., 1992). The entire striatum receives major glutamatergic projections from the cortex in addition to dopaminergic inputs from the SNpc and VTA. It has previously been shown that chronic haloperidol treatment raises the concentration of extracellular glutamate (See and Chapman, 1994; Yamamoto and Cooperman, 1994; See and Lynch, 1995). However, the basis for this elevation is presently unknown.

A family of high-affinity sodium-dependent glutamate transporters are essential for terminating the postsynaptic action of glutamate by rapidly removing it from the synaptic cleft (Fonnum, 1984; Takahashi et al., 1997). Within this family, the GLT-1 glutamate transporter, which is located on glial cells, accounts for approximately 70% of the total glutamate transported (Rothstein, 1996). Recent work by Schneider et al. (1998) has shown that GLT-1 mRNA in the striatum was significantly reduced following chronic haloperidol treatment. However, it was not determined whether the changes in these mRNA levels alter the functional activity of the glutamate transporters. In the present study, we have measured glutamate transporter activity and show that administration of haloperidol for 27 weeks significantly impairs the capacity of these transporters to take up glutamate in the striatum. This investigation was carried out in order to elucidate the mechanism of action of haloperidol. It was originally envisioned that separate analyses would be carried out of glutamate transport in the dorsal and ventral striatum, however, due to difficulties in accurately separating the two regions in wet tissue samples and the quantity of tissue required our final analysis utilised synaptosomes prepared from the entire striatum.

Materials and Methods

Male, Albino, Wistar rats (Bioresources, Trinity College, Dublin, Ireland) initially weighing 300-350g were housed four per cage, with a constant temperature of 21⁰C, on a 12-h light and dark cycle with free access to food and water. Animals were divided into two groups. The control group (n=4) was treated with sesame oil (Sigma, Poole, Dorset, UK) as vehicle while the haloperidol group (n=4) received the depot neuroleptic drug, haloperidol decanoate (Janssen Pharmaceutical Limited, Little Island, Cork, Ireland) at a dose of 28.5 mg kg⁻¹, the equivalent of 1 mg kg⁻¹day⁻¹ of unconjugated haloperidol. Injections were given intramuscularly every 3 weeks for 27 weeks in a volume of 1 ml kg⁻¹.

Following the treatment period, rats were killed by stunning and cervical dislocation. Each brain was removed and the paired striata, including caudate/putamen and nucleus accumbens, were dissected out. Striata from two brains were pooled for each assay, homogenised in ice-cold gradient medium, which was composed of 0.32 M sucrose, 1 mM EDTA and 0.25 mM dithiothreitol, pH 7.4. The homogenate was centrifuged for 10 min at 1000 g at 4⁰C (Sorvall centrifuge, SS-34 rotor) and the supernatants were collected and the synaptosomes were purified using a modification of the method of Dunkley et al. (1988). Briefly, Percoll diluted in gradient medium was layered into 10-ml polycarbonate tubes using a peristaltic pump at a flow rate of 1 ml min⁻¹, starting with the most dense (23% v/v), followed by, in order, 15% v/v, 10% v/v, and 3% v/v. A 2 ml sample of the supernatant was gently layered, using a Pasteur pipette, onto the top of the gradients and centrifuged at 32,500 g for exactly 5 min at 4⁰C. Synaptosomes in the 15%/23% Percoll interfacial fraction (layer 4) were carefully removed using a Pasteur pipette. They were washed twice in sodium-free Krebs' bicarbonate medium (choline chloride 116.8 mM, KCl 4.72 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, Tris 8.1 mM, glucose 11 mM and CaCl₂ 2.5mM) pH 7.4 and resuspended in a final volume of 1.25 ml of sodium-free Krebs' medium. An aliquot was removed for protein determination by the method of Markwell et al. (1978) and the remaining synaptosomes used in the transport assay. They were incubated for 4 minutes at 25⁰C in normal Krebs' bicarbonate medium (NaCl 109.6 mM, KCl 4.72 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 25

mM, glucose 11 mM and CaCl₂ 2.5 mM) pH 7.4 buffer, containing D-[³H]aspartate ($4.6 \times 10^{-5} - 1.15 \times 10^{-4}$ MBq mmol⁻¹ specific activity). The reaction was stopped by the addition of 200 µl of ice-cold 1 mM D-aspartate followed immediately by centrifugation for 10 min at 13,000 rpm at 4⁰C. The pellet was retained, washed twice with ice-cold gradient buffer and solubilised overnight in a 2% sodium dodecyl sulphate solution. The quantity of radioactivity in each sample was determined by liquid scintillation spectroscopy, and the rate of transport was plotted as a function of substrate concentration.

The transport assay was performed at 25⁰C, rather than 37⁰C, in order to slow down the activity of the transporters to a measurable rate *in vitro* and previous studies have determined that the transport of D-aspartate was linear between 0-20 min at 25⁰C (McBean, 1994). D-aspartate was used in preference to L-glutamate in these experiments because it is a non-metabolisable substrate for the glutamate transporters (Davies and Johnston, 1976; Fyske et al., 1992). In a separate set of experiments, synaptosomes were prepared from untreated male, albino Wistar rats (n=6). Haloperidol (Sigma, Poole Dorset, UK) was dissolved in a minimal amount of glacial acetic acid, diluted with distilled water (final pH 7.4) and added to the transport assay at a final concentration of 4 nM and 40 nM to determine if haloperidol had any direct effect on glutamate transport.

The experimental data were analysed by non-linear regression using Graphpad 'Prism' software (San Diego, CA, USA). Differences between control and treated rats were evaluated with the two tailed Student's *t*-test (*p* < 0.05 was considered significant). Data are expressed as mean values ± s.e.m..

Results

The rate of transport of D-[³H]aspartate into rat striatal synaptosomes was determined over a concentration range of 0.16 to 40 μ M in both control and haloperidol-treated animals (Figure 5.1). Calculated values for $V_{\max}$ decreased significantly from 0.73 ± 0.08 nmol mg protein⁻¹min⁻¹ for controls to 0.27 ± 0.03 nmol mg protein⁻¹min⁻¹ in chronic haloperidol treated rats ($P < 0.01$). At a substrate concentration of 40 μ M D-aspartate, the rate of transport was reduced to 42% of the control level. There was no significant change in the K_m value (3.21 ± 1.41 for control vs. 2.29 ± 0.92 for haloperidol-treated rats).

The inclusion of haloperidol, at concentrations as high as 40 nM in the transport assay, caused no significant change in the rate of transport of D-[³H]aspartate. The $V_{\max}$ for transport in the controls was 1.10 ± 0.11 nmol mg protein⁻¹ min⁻¹, whereas with 40 nM haloperidol it was 1.06 ± 0.40 nmol mg protein⁻¹ min⁻¹. In addition, there were no significant differences in the body weight, health or striatal protein content between treated and control animals.

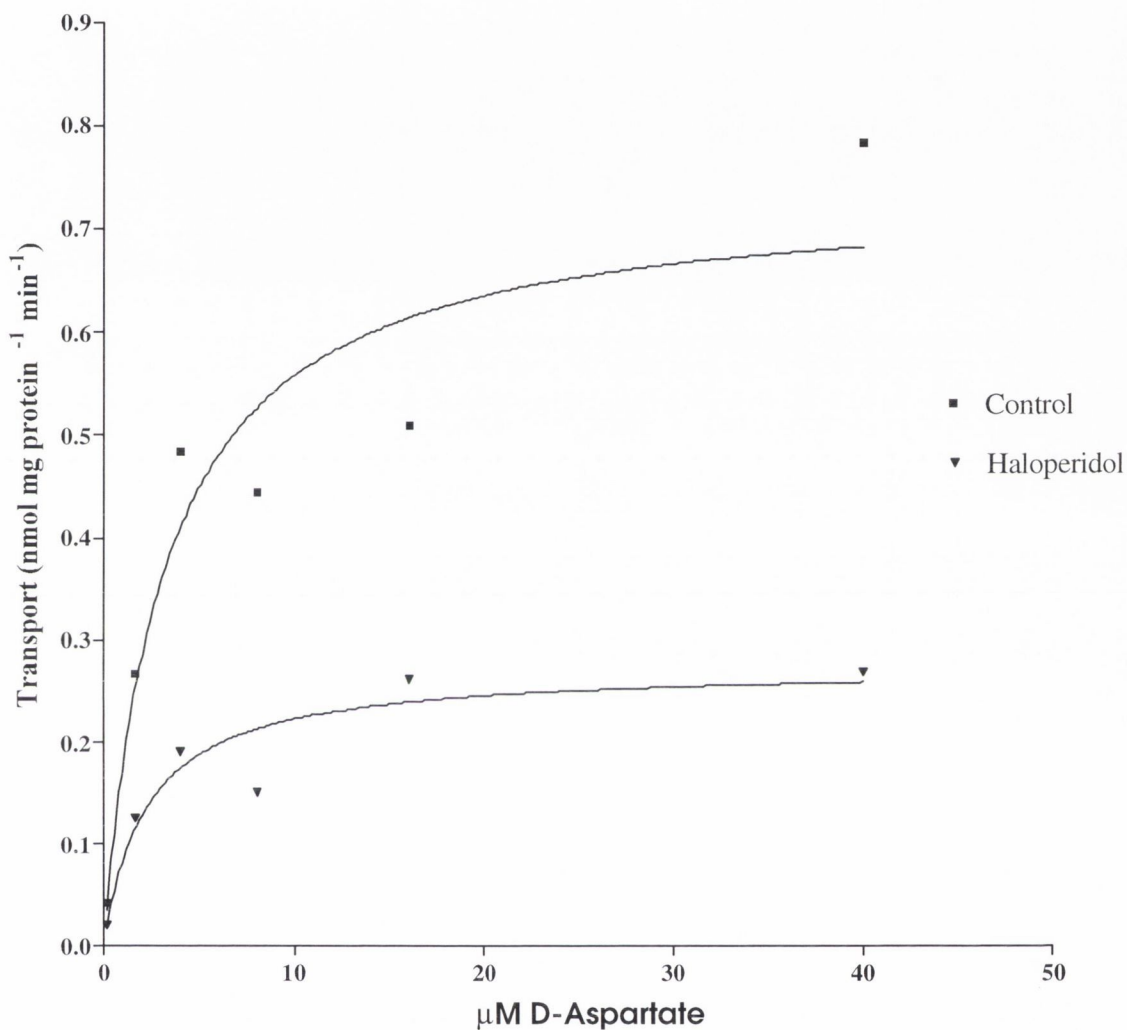


Figure 5.1. Effect of chronic haloperidol treatment on kinetic parameters of glutamate transport. Synaptosomes were incubated at 25 °C for 4 min and uptake of D-[³H]aspartate at the specified concentrations was measured (n= 2 individual experiments, each performed in triplicate). The data were analysed by non-linear regression analysis and show a significant decrease in $V_{\max}$ (0.73 ± 0.08 nmol mg protein⁻¹min⁻¹ for controls and 0.27 ± 0.03 nmol mg protein⁻¹min⁻¹ in chronic haloperidol-treated rats ($p < 0.01$)).

Discussion

These findings demonstrate that chronic haloperidol treatment significantly impairs the activity of the high-affinity, sodium-dependent glutamate transporters in the striatum. Although D-aspartate was used in place of L-glutamate in these experiments, one can assume that L-glutamate transport would be similarly affected. Thus, at physiologically relevant (low micromolar) concentrations of L-glutamate, the ability of the transporters to take up glutamate would be significantly reduced following long-term neuroleptic treatment. See and Lynch (1995) have previously shown that extracellular glutamate is elevated following chronic neuroleptic drug treatment. In their work they treated rats for 24 weeks with haloperidol. Following treatment, glutamate was released by high K^+ infusion and the extracellular glutamate concentration was significantly elevated in the haloperidol-treated rats following infusion compared to controls. Our results suggest that this increase in extracellular glutamate may be due to reduced activity of glutamate transporters.

The fact that the $V_{\max}$ is significantly reduced with no change in the K_m implies that non-competitive inhibition is taking place. This suggests a reduced number of transport sites rather than a change in the ability of individual transporters to take up glutamate. This implies that 27 weeks of haloperidol treatment reduces the number of glutamate transport sites and therefore impairs transport. These results are reinforced by the results of Schneider et al. (1998) who observed a decrease in the expression of mRNA for GLT-1 glutamate transporters following 28 days of haloperidol treatment in the rat.

While a reduced number of glutamate transporters may be the most likely explanation for impaired transport, a number of other mechanisms are possible. Since neuroleptic drug treatment increases dopamine efflux in the striatum (Santiago and Westerink, 1991), dopamine oxidation products could inhibit glutamate transport in synaptosomes (Berman and Hastings, 1997). Another mechanism could involve metabolites of haloperidol. One of these metabolites, haloperidol pyridinium, has recently been shown to impair the dopamine transporter and lead to high levels of dopamine in the synapse (Wright et al., 1998) that could then block high-affinity

glutamate uptake (Kerkerian et al., 1987). However, in light of our own results that show non-competitive inhibition and no change in glutamate transport with the addition of haloperidol to the transport assay, these possibilities are unlikely to occur.

Chronic blockade of D₂ dopamine receptors increases synaptic release of glutamate in the striatum (Bardgett et al., 1993; Perry et al., 1979). In addition, reduced glutamate transport activity, as demonstrated in this study, would maintain a high concentration of glutamate in the synaptic cleft for longer than in controls. However, the technical limitations of this study must be addressed. In the present investigation we examined glutamate transport in synaptosomes prepared by the method of Dunkey et al. 1988 and their study showed that synaptosomes comprise of both presynaptic and postsynaptic elements with many containing mitochondria located at terminals. Glial cells located close to the terminals are notably absent from preparations and it has been shown recently (Rothstein et al., 1996) that glutamate uptake at neurons through the EAAC1 transporter accounts for 20-40 % of glutamate uptake whereas transport by the glial located GLT1 and GLAST transporters is much greater. Nevertheless, the fact that we have demonstrated impaired glutamate transport in synaptosomes coupled with evidence from a previous study that GLT-1 mRNA is reduced following chronic haloperidol treatment implies that this antipsychotic drug alters glutamate transport in the striatum.

Therefore, impaired glutamate transport coupled with increased glutamate release could result in increased glutamate neurotransmission and this may be the mechanism involved in the therapeutic action of haloperidol. Moreover, increased glutamatergic transmission may also lead to excitotoxic effects due to prolonged activation especially at N-methyl-D-aspartic acid receptors (Olney, 1990) and this may play a role in side effects of chronic neuroleptic drug treatment such as TD (Andreassen and Jørgensen, 1994; De Keyser, 1991; Meshul et al., 1996a).

Chapter 6

**Attenuation of the haloperidol induction of Fos
immunoreactivity in the dorsal but not the
ventral striatum following
N-methyl-D-aspartate receptor blockade**

Introduction

Haloperidol, a classical antipsychotic drug, is believed to exert its effects by blocking dopamine D₂ receptors. Some studies suggest that the efficacious actions of this drug involves nucleus accumbens (Crow et al., 1975; Blaha and Lane, 1987; Moghaddam and Bunney, 1990; Goldstein and Deutch, 1992), whereas the induction of undesirable side effects is due to action in sensorimotor parts of the caudate-putamen (CPu, Blaha and Lane, 1987; Meshul et al., 1996a). Other reports, however, question whether D₂ blockade at the level of nucleus accumbens is responsible for controlling psychosis (Laruelle et al., 1992) and in the dorsal striatum for producing abnormal movements (Koshikawa et al., 1989; Koene et al., 1993; Egan et al., 1994). Certainly, acute and chronic haloperidol treatment alters dorsal striatal dopamine neurotransmission and upregulates D₂ receptors while leaving the D₁ receptors unchanged (Bunney and Aghajanian, 1975; Moghaddam and Bunney, 1990; Laruelle et al., 1992; Debonnel et al., 1990; Egan et al., 1993; Egan et al., 1996). In nucleus accumbens, where territorial divisions are well-defined anatomically and functionally (see Alheid and Heimer, 1988; Zahm and Brog, 1992; Meredith et al., 1993), haloperidol seems to augment dopamine turnover and modulate cell coupling differentially in the shell and core (Deutch and Cameron, 1992; O'Donnell and Grace, 1995).

In addition to and perhaps as a result of influencing dopaminergic action, antipsychotic drug administration affects other neurotransmitter systems (Johnson et al., 1994; See and Chapman, 1994; Meshul and Tan, 1994; Meshul et al., 1994). Recent work has shown that chronic haloperidol treatment elevates extracellular glutamate (See and Chapman, 1994; See and Lynch, 1995) and increases the number of perforated glutamatergic synapses in the CPu (Meshul et al., 1996b). The mechanism responsible for the interaction between neuroleptic drugs and glutamate is not known, but the reduced stimulation of D₂ receptors increases activity at N-methyl-D-aspartate (NMDA) glutamate receptors. Excessive stimulation of these receptors raises intracellular levels of calcium and can produce neuronal damage through excitotoxic mechanisms (Beal et al., 1986; Meldrum and Garthwaite, 1990). NMDA antagonists seem to protect the striatum from such damage (Weiloch, 1985;

Simon et al., 1986), and lesioning the corticostriatal pathway ameliorates excitotoxin-induced injury (Weiloch, 1985; Linden et al., 1987). Thus, elevation of glutamate after haloperidol administration could alter striatal neurons in a deleterious manner.

Fos protein is an early immediate gene product which is postulated to be a marker of neuronal activity because of its rapid and intense induction (Sheng and Greenberg, 1990; Morgan and Curran, 1991) and its presence may be used to define neuroanatomical sites of drug action (Nakajima et al., 1989; Presley et al., 1990; Deutch et al., 1992). In the CPu, the basal level of Fos and its mRNA are very low but increase dramatically following haloperidol administration (Dragunow et al., 1990; Robertson and Fibiger, 1992; Semba et al., 1996). In nucleus accumbens, however, the action of haloperidol is less clear, since acute treatment increases the overall density of Fos-immunoreactive (IR) cells (Robertson and Fibiger, 1992; Robertson and Jian, 1995), while inducing *c-fos* mRNA in only one of its territories (Semba et al., 1996). There are data to suggest that in the CPu the potent non-competitive NMDA antagonist, dizocilpine maleate (MK801) can prevent haloperidol-induced elevations of *c-fos* mRNA (Ziółkowska and Höllt, 1993) but not the increased density of Fos-IR cells, unless administered at a very high dose (Dragunow et al., 1990). It is not known whether NMDA antagonism affects haloperidol-induced changes in Fos in nucleus accumbens. In light of regional differences in NMDA receptor distribution and activation in the ventral striatum (Pennartz et al., 1990; Gracy and Pickel, 1996), antagonism of these receptors may not be homogeneous; thus, the effects of haloperidol-induced glutamate elevations could also be regionally restricted. If so, this would have important implications for linking the action of haloperidol to specific sites. The aim of the present study therefore is to examine whether glutamate, acting via the NMDA receptor, contributes to the action of haloperidol in the dorsal or ventral parts of the striatum and if so, where this might take place.

Materials and methods

Adult, male, Wistar rats (Bioresources, Trinity College Dublin, Ireland) weighing 200-300g, were housed four to a cage, and maintained on a 12 hour, light/dark schedule, at a constant temperature of 21° C with food and water provided ad libitum. All animals were handled on a daily basis for two days before drug administration in order to prevent any increase in the basal level of Fos by stress (Asanuma et al., 1992; Helton and McGinty, 1993).

The effects of MK801 (Research Biochemicals Inc., Natick, MA) on the haloperidol (Baker Norton, Harlow, Essex, U.K) induction of Fos were examined in four experiments. All drugs were administered intraperitoneally in a volume of 1 ml/kg, and the two injections in each experiment were given 30 min apart. Animals were left quietly for three hours after their final injection. The same dosage of haloperidol (0.5 mg/kg) was used in all four experiments but MK801 was administered at 5 mg/kg (MK5) in the first three experiments. This dose was selected on the basis of its ability to inhibit, maximally, the induction of *c-fos* mRNA by haloperidol as measured by Ziolkowska and Höllt (1993) using Northern Blot analysis. A higher dose (10 mg/kg, MK10) was also used in the final experiment. From a total of sixteen, four rats were randomly assigned to one of four injection protocols for each experimental cohort: saline-saline (SAL-SAL, n = 4), saline-haloperidol (SAL-HAL, n = 4), MK801-saline (MK5-SAL, n = 3; MK10-SAL, n = 1), MK801-haloperidol (MK5-HAL, n = 3; MK10-HAL, n = 1).

Three hours after receiving the second injection, each rat was anesthetized with sodium pentobarbitone (60 mg/kg) and perfused transcardially with physiological saline followed by a fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer [PB], pH 7.4) at room temperature. The four animals of each cohort were injected, anesthetized and perfused at the same time, and their brains processed using incubation buffers prepared in sufficient volume to handle the tissue from all four brains (cohort design, Mijster et al., 1996).

The brain of each rat was removed and postfixed for an hour at room temperature in the same fixative. Each forebrain was blocked and cut into transverse sections (70 μ m) on a Vibratome. Alternate sections were incubated in 10% normal swine serum (NSS) in 0.01M phosphate buffered saline with 0.25% Triton X-100 (PBS-Tx) for 30 min at room temperature with gentle shaking. After 3 rinses, sections were incubated in a 1:1000 dilution of sheep anti-Fos antiserum (Genosys Biotechnologies Inc., Cambridge, U.K.) in PBS-Tx with 1% NSS added, at 4° C for 36 hours. Following multiple rinses, sections were incubated in a 1:500 dilution of biotinylated donkey anti-sheep IgG in PBS-Tx with 1% NSS added, for 2 hours at room temperature. After rinsing, sections were incubated in a preprepared ABC reagent (Vector Laboratories, Bretton, Peterborough, U.K.) for 2 hours at room temperature. Following 3 rinses in 0.05M Tris-HCl, pH 7.6, sections were reacted for 25-30 min in DAB with hydrogen peroxide added at a final concentration of 0.01%. Sections were then rinsed and mounted onto slides from a 0.2% gelatin solution, dried, dehydrated and coverslipped.

Alternate sections were processed for CBP immunocytochemistry. These sections were washed with 0.05M Tris Buffered Saline, pH 7.6, with 0.5% Triton X-100 added (TBS-Tx) and then incubated in 5% normal horse serum (NHS) in TBS-Tx at room temperature for 30 min. Sections were then incubated in a 1:8000 dilution of mouse anti-CBP antiserum (Sigma-Aldrich, Dorset, U.K.) in TBS-Tx with 1% NHS added, at 4° C for 36 hours. Sections were rinsed and incubated in a 1:300 dilution of biotinylated horse anti-mouse IgG in TBS-Tx for 2 hours with gentle agitation. After rinsing in TBS-Tx, sections were incubated in ABC for 2 hours at room temperature. Sections were then reacted in DAB (0.05M Tris-HCl, pH 7.6) with added hydrogen peroxide at a final concentration of 0.01% for 5-8 min. Sections were rinsed and mounted onto slides from a 0.2% gelatin solution, dried, dehydrated and coverslipped.

To determine the distribution of Fos-IR neurons, sections through the striatum were drawn and cells were plotted onto these drawings with the aid of a camera lucida attached to a Nikon microscope. In order to demarcate the shell and core of nucleus accumbens, separate drawings of adjacent CBP-immunoreacted sections

(Figures 6.1A-B) were traced for each animal onto clear acetate sheets. As noted by others (Gerfen et al., 1985; Groenewegen et al., 1991), the dorsolateral CPu consistently immunostained weakly for CBP and in nucleus accumbens, the shell exhibited weak to moderate immunoreactivity, whereas the core was densely immunostained.

The most rostral quarter of nucleus accumbens is regarded as a distinct territory termed the rostral pole (Zahm and Brog, 1992; Zahm and Heimer, 1993). The dorsolateral quadrant of the CPu was isolated by drawing two lines at right angles to each other onto an acetate sheet. The length of the horizontal line was calculated to be one-third the distance along a straight line between the lateral edge of the corpus callosum to the most dorsal point of the lateral ventricle; the vertical line was drawn at a right angle to the horizontal line and was calculated to be one-fourth the distance from the corpus callosum to the base of the brain. To control for plane of section and shrinkage, a set of lines delineating the quadrant was fitted separately for each brain section that was analyzed (see Figure 6.1B).

Acetate sheets delineating territorial divisions in the striatum were placed over the line drawings of the plotted sections, using blood vessels, prominent fibre bundles and/or the lateral ventricle as alignment guides. The distribution of cells in each territory was digitized using a tablet attached to a computer. A software program (Macstereology, Ranfurly Microsystems, Renfrewshire, U.K.) was used to estimate the cross-sectional areas of the entire striatum and its restricted regions, i.e. the dorsolateral quadrant of the CPu and the shell, core and rostral pole of nucleus accumbens (Figures 6.1A-B, 6.2), and to analyse the density of Fos-IR cells. The Fos-IR cells along the entire rostro-caudal axis of nucleus accumbens and adjacent CPu (Bregma +2.7mm to +0.2mm; Paxinos and Watson, 1986) were also reconstructed from a minimum of twelve sections. To achieve this, sections were aligned and rotated to produce a 3-dimensional image of Fos-IR cells throughout the striatum.

Counts of Fos-IR cells, which were recorded at rostral and caudal levels of both dorsal and ventral parts of the striatum (Figure 6.1A,B), were expressed as cells/500 μm^2 for each region (dorsolateral CPu, remainder CPu, shell and core of

nucleus accumbens). A single central level at the rostral pole was also selected for analysis. For statistical tests, data were transformed by taking the log of the values before analysis (Matthews and Farewell, 1996). This resulted in a good correlation with the expected normal distribution ($r = 0.983$). To distinguish effects due to drug treatment (SAL-HAL versus MK5-HAL), region and perfusion/immunohistochemical batches (cohorts), the data were analyzed using a general linear model design with repeated measures. In this way, values from individual animals were nested in treatment by cohort and region was entered as a random factor.

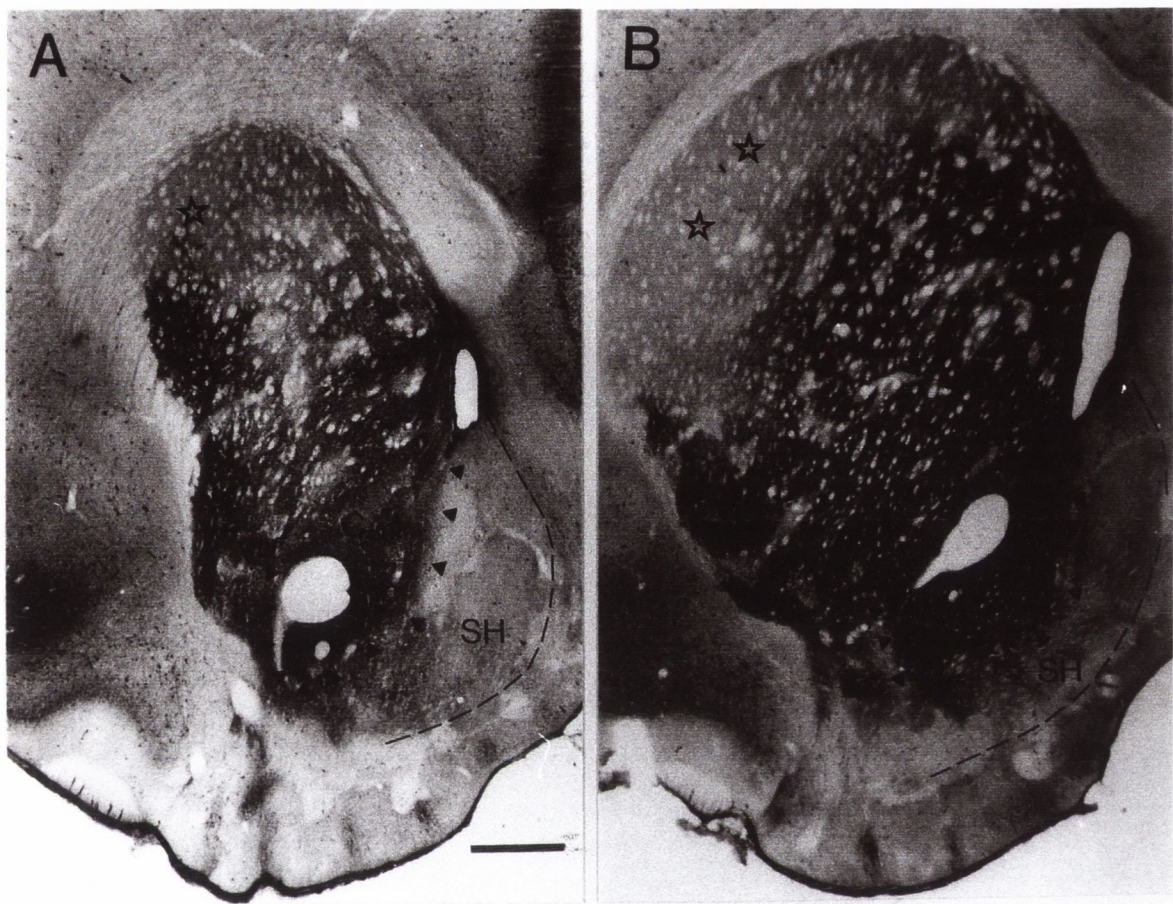


Figure 6.1 Photomicrograph of A: rostral and B: caudal sections through the rat striatum showing CBP immunoreactivity. The boundary between the core (C) and the shell (SH) in niucleus accumbens is marked by filled arrowheads. The medial and ventral borders of this nucleus are outlined by dashed lines. The dorsolateral part of the striatum is marked by stars. Scale bar in A: which also applies to B: is equal to 500 μ m.

Results

In the present study we examined the regional distribution of Fos-IR cells in the CPu (dorsolateral quadrant and remainder) and nucleus accumbens (rostral pole, shell and core). We used sections immunoreacted for CBP that were adjacent to Fos-IR sections, to demarcate nucleus accumbens' territories (Figure 6.1A,B). In control animals treated with saline (SAL-SAL), a few Fos-IR cells were found in the dorsomedial part of the CPu (Figure 6.2A) and scattered in nucleus accumbens, especially at the rostral pole (Figure 6.3A). In rats treated with MK801 (MK5-SAL), cells appeared denser in dorsolateral (Figure 6.2B) than in dorsomedial or ventral parts of the CPu and were sparse in the core and shell of nucleus accumbens. Fos-IR cells appeared to be densest at the rostral pole (Figure 6.3B).

In rats treated with haloperidol (SAL-HAL) or with MK801 followed by haloperidol administration (MK5-HAL), Fos-IR cells were distributed densely but heterogeneously throughout dorsal and ventral parts of the striatum (Figure 6.4 A-D). The reconstructions revealed patterns of cell distributions that differed between treatments but not between animals receiving the same treatment. The Fos-IR cells in the CPu appeared less dense in MK5-HAL animals (Figure 6.4 B,D) than in SAL-HAL brains (Figure. 6.4 A,C); this difference was particularly evident in the dorsomedial part of the CPu (Figure 6.5 A-B). In nucleus accumbens, however, changes in density were less evident, except in the caudal core where cells appeared less dense in MK5-HAL (Figure 6.4D) than in SAL-HAL (Figure 6.4C) treated animals and in the medial shell where immunopositive cells appeared as dense rostrally (Figure 6.4 A,B) as caudally (Figure 6.4 C,D).

Densities of Fos-IR cells were calculated for both CPu and nucleus accumbens for all rats treated with SAL-HAL and MK5-HAL. In the CPu, measurements were taken at three rostro-caudal levels and in the dorsolateral quadrant, densities were calculated at two levels (Table 6.1). In nucleus accumbens, density measurements were carried out at the rostral pole and at one rostral and one caudal level of the shell and of the core (Table 6.2). Statistical comparisons showed no significant effect due to animal for the CPu ($p = 0.97$) or for nucleus accumbens (p

= 0.4). However, there was a significant regional effect in the CPu ($F = 16.275$, $df = 4,19$, $p < 0.0001$; for region designations, see Table 6.1). With haloperidol treatment, Fos-IR cell density was found to be greatest at rostral levels of the CPu but declined by approximately 35% further caudally (Table 6.1). However, the cell density in the dorsolateral quadrant of SAL-HAL-treated animals did not decrease between rostral and caudal levels (Table 6.1 and compare Figure. 6.4A with 6.4C). With MK5-HAL treatment, the density of Fos-IR cells decreased at caudal as compared to rostral levels of all regions in the CPu (Table 6.1).

There was also a marked effect due to region in nucleus accumbens ($F = 21.335$, $df = 4,19$, $P < 0.0001$; for region designations, see Table 6.2). Rostral cell density declines with haloperidol treatment by 50% in the caudal core and by 40% in the caudal shell (Table 6.2). With MK5-HAL treatment, the density of Fos-IR cells also decreased at caudal as compared to rostral levels of nucleus accumbens' territories. The decrease was particularly evident in the core, where the density declined by 73% over the rostro-caudal axis (Table 6.2).

Statistical tests showed that there was a marked effect due to treatment in the CPu (Figures. 6.5A-B), in that Fos-IR cell density was significantly decreased with MK5-HAL treatment as compared to that with SAL-HAL ($F = 761.31$, $df = 1,19$, $p = 0.0013$; Table 6.1). However, this difference was not evident for any one region, although a trend towards a treatment/region interaction between the three principal levels of the CPu ($p < 0.08$) but not for the two levels of the dorsolateral quadrant ($p = 0.2$), was found. In nucleus accumbens, there was no effect due to treatment; the density of Fos-IR cells was not reduced significantly by MK801 pretreatment for any territory (Table 6.2). There was, however, a trend towards an interaction between region and treatment ($p < 0.07$). Fos-IR cell density at the rostral pole was increased by 24 percent in MK5-HAL as compared to SAL-HAL-treated rats (Table 6.2; compare Figure. 6.3C and 6.3D). The cell density in the rostral core of pretreated rats was also elevated over that in SAL-HAL rats. No change was evident in the shell. In the caudal core, the mean Fos-IR cell density decreased, but not significantly, in pretreated as compared to SAL-HAL rats (Table 6.2).

In striatal sections taken from animals treated with the higher dose of MK801 (10mg/kg; MK10-HAL), neither the pattern nor the density of Fos-IR cells differed from those found in animals injected with the low dose (5mg/kg; MK5-HAL).

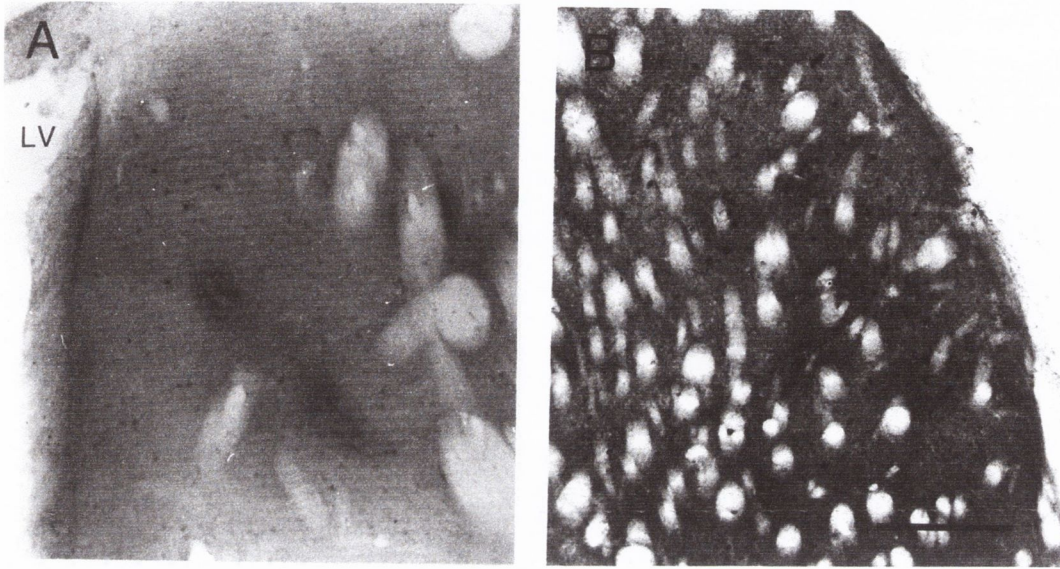


Figure 6.2 Photomicrographs taken at the same level of the CPu. A: the distribution of Fos-IR cells in the dorsomedial quadrant after saline injection (SAL-SAL). B: Fos-IR cells in the dorsolateral quadrant in a rat treated with MK801 (MK5-SAL). Abbreviation: LV, lateral ventricle. Scale bar in B: which also applies to A:, is equal to 200 μm .

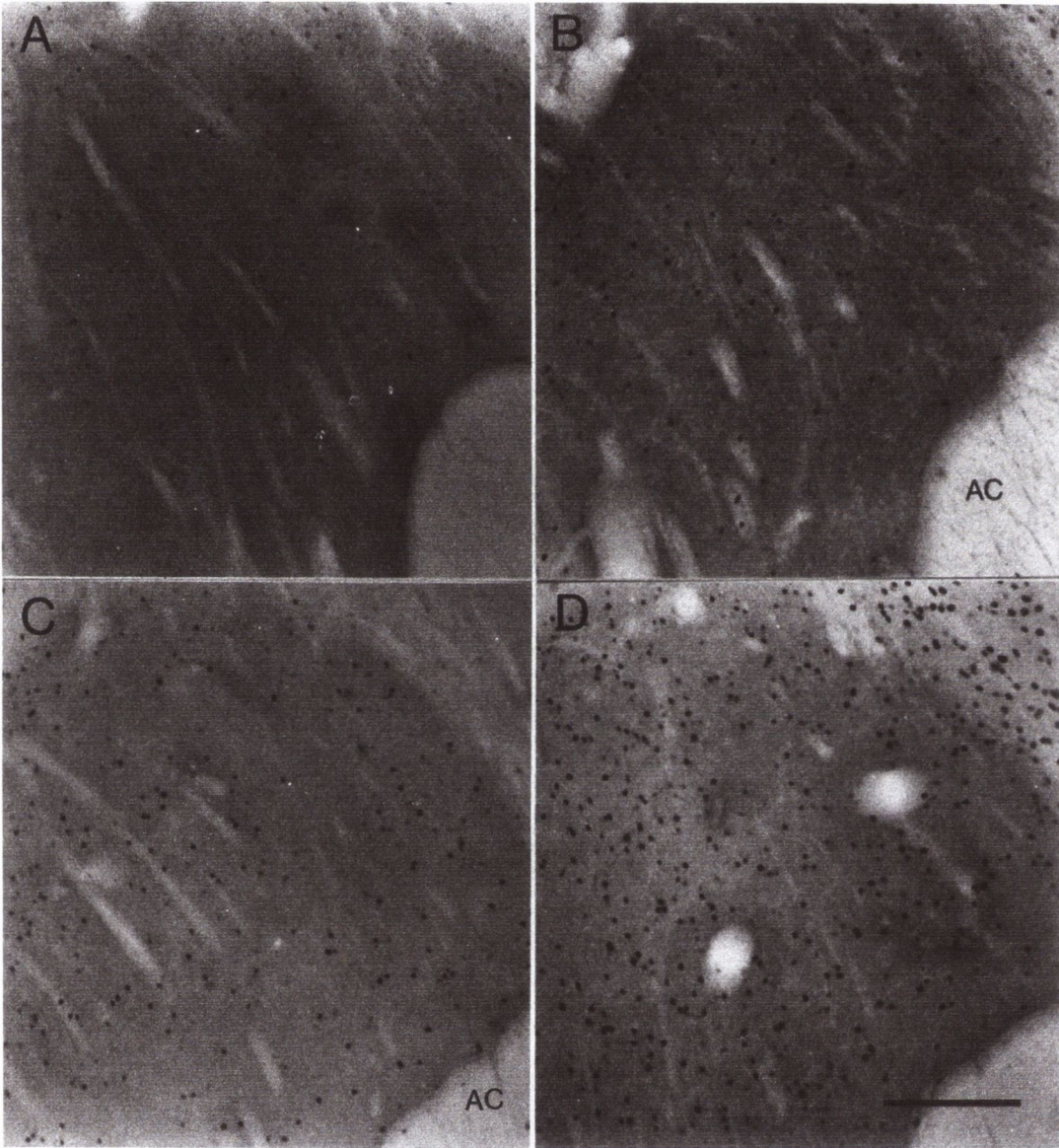


Figure 6.3 Photomicrographs of Fos-IR cells in the rostral pole of nucleus accumbens in rats treated with A: saline (SAL-SAL), B: MK801 (MK5-HAL). A comparison of C: with D: illustrates that Fos-IR cells are increased in number in rats pretreated with MK801. Abbreviation: AC, anterior commissure. Scale bar in D: which also applies to A,B,C, is equal to 200 μ m.

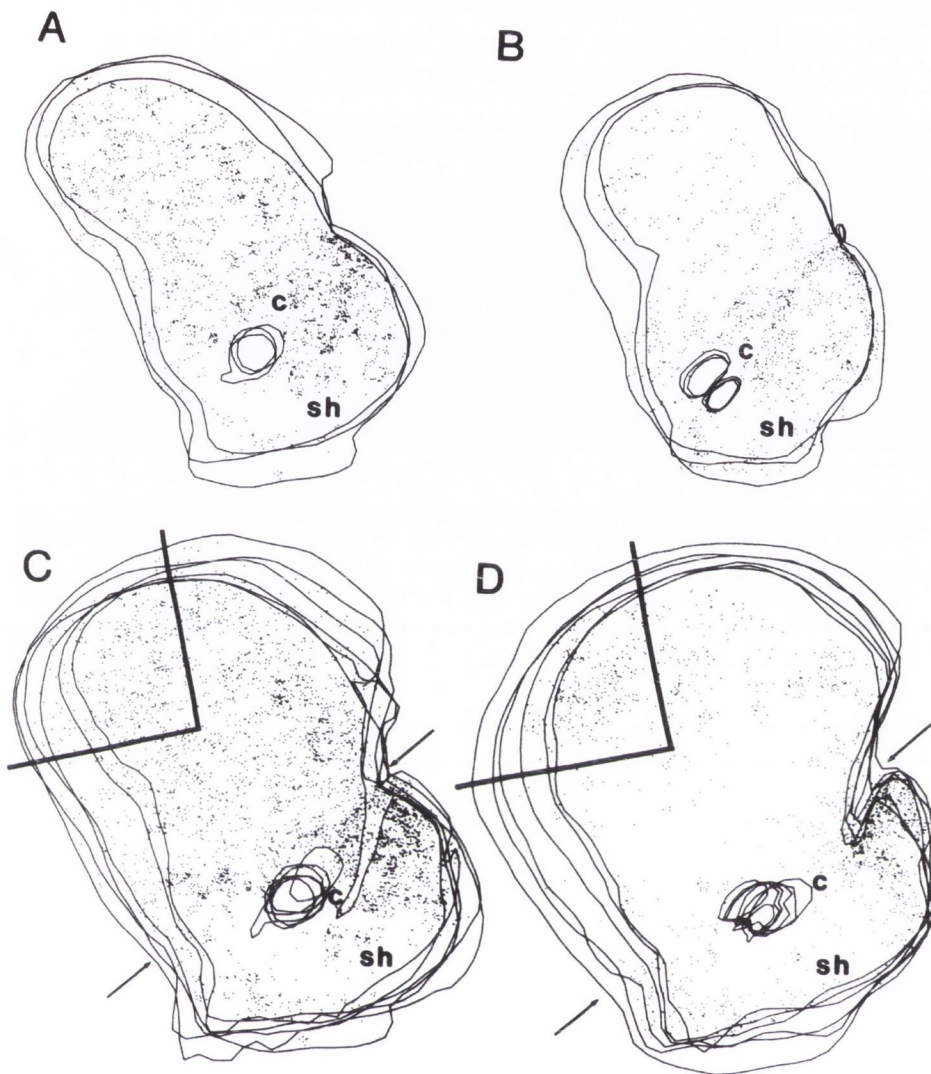


Figure 6.4 Four digitized reconstructions of alternate sections, 70 μ m apart showing rostral and caudal striatal levels (half brains, midline on the right) with Fos-IR cells (dots) plotted onto superimposed sections. A: and B: show 3 superimposed, rostral sections each and C: and D: 6 serial, caudal sections each. The cell plots in A: and C: are in a haloperidol-treated rat and in B: and D: in a rat treated with MK5-HAL. Note the sparser distribution of Fos-IR cells in MK5-HAL treatment (compare B: to A:) and note, that at more caudal levels, Fos-IR cells are reduced in number throughout the CPu (compare D: to C:) but not in nucleus accumbens. Abbreviations: C, core of nucleus accumbens; SH shell of nucleus accumbens. Long arrows delineate the approximate boundary between nucleus accumbens and CPu. Thick black lines outline the borders of the dorsolateral quadrant of the CPu.

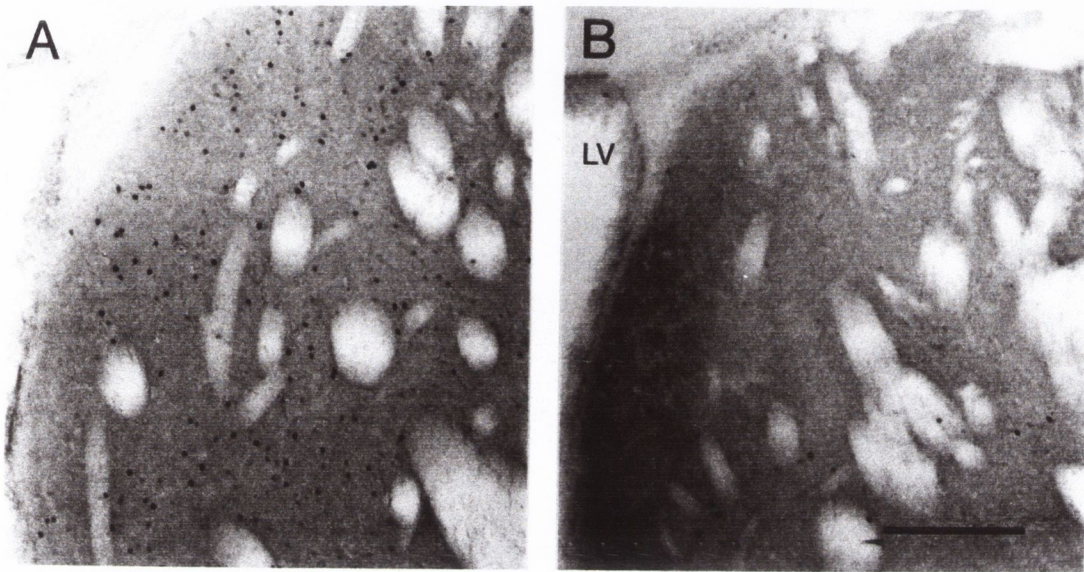


Figure 6.5 Photomicrographs taken at the same level of the dorsomedial striatum demonstrating Fos-IR cells: A: after haloperidol treatment (SAL-HAL) and B: pretreatment with MK801 followed by haloperidol treatment (MK5-HAL). A comparison of A: with B: shows that that Fos-IR cells are reduced in density in pretreated rats. Abbreviation: LV, lateral ventricle. Scale bar in B: which also applies to A:, is equal to 200 μm .

Table 6.1 Effects of MK801 pretreatment on the haloperidol induction of Fos-IR cells in caudate-putamen (mean cell density $\pm$ s.e.m. / 500 μ m²).

Region	Saline	Haloperidol	MK801/haloperidol
caudate-putamen	1.83 $\pm$ 0.35	58.68 $\pm$ 8.14 +++	33.05 $\pm$ 4.33*, +++
rostral dorsolateral quadrant	1.84 $\pm$ 1.57	73.18 $\pm$ 26.37	37.43 $\pm$ 8.56 +
caudal dorsolateral quadrant	0.62 $\pm$ 0.50	77.01 $\pm$ 23.5	37.8 $\pm$ 6.73 +
rostral CPu (Bregma +2.2mm)	1.89 $\pm$ 0.84	61.06 $\pm$ 15.61	46.56 $\pm$ 13.42
midrostral CPu (Bregma +1.2mm)	1.83 $\pm$ 0.52	43.20 $\pm$ 12.43	24.33 $\pm$ 8.31
midcaudal CPu (Bregma +0.2mm)	2.30 $\pm$ 0.46	38.97 $\pm$ 9.25	19.33 $\pm$ 7.14

* p < 0.05; significantly different from haloperidol treatment;
+ p<0.05, ++ p<0.01, +++ p<0.001; significantly different from saline controls

Table 6.2 Effects of MK801 pretreatment on the haloperidol induction of Fos-IR cells in nucleus accumbens (mean cell density $\pm$ s.e.m./ 500 μ m²).

Region	Saline	Haloperidol	MK801/haloperidol
nucleus accumbens	5.72 $\pm$ 0.87	49.39 $\pm$ 6.8 ++	55.25 $\pm$ 8.68 ++
rostral pole (Bregma +2.7mm)	9.18 $\pm$ 3.07	60.11 $\pm$ 17.31	74.65 $\pm$ 18.86
rostral core (Bregma +1.7mm)	5.09 $\pm$ 0.81	70.54 $\pm$ 21.62	88.9 $\pm$ 23.63
caudal core (Bregma +1.0mm)	4.13 $\pm$ 1.86	34.9 $\pm$ 9.67	24.33 $\pm$ 10.87
rostral shell (Bregma +1.7mm)	6.72 $\pm$ 1.12	51.35 $\pm$ 12.01	53.49 $\pm$ 11.39
caudal shell (Bregma +1.0mm)	3.48 $\pm$ 1.03	30.06 $\pm$ 6.06	34.86 $\pm$ 5.69 +

+ p<0.05, ++ p<0.01; significantly different from saline controls

Discussion

In the present study we found that treating animals with haloperidol resulted in large increases in Fos-IR cells in the CPu and in all parts of nucleus accumbens. When we administered the non-competitive NMDA receptor antagonist, MK801, before haloperidol, we found the density of Fos-IR cells to be significantly reduced in the CPu but not in any territory of nucleus accumbens. These findings clarify and extend previous observations for the CPu (Dragunow et al., 1990; Ziolkowska and Höllt, 1993), but more importantly, they illustrate for the first time that the haloperidol induction of Fos is dependent upon the activation of NMDA receptors in a regionally-specific manner.

By reconstructing Fos-IR cell distributions along the rostro-caudal axis of the striatum, we consistently found the same pattern of cell distributions across experiments. When we compared rostral and caudal levels for each treatment, however, a regional pattern emerged. With statistical comparisons, we found a marked effect for SAL-HAL and MK5-HAL groups due to treatment and region but not to animal. The latter presumably means that with the cohort design (Mijnster et al., 1996), we controlled adequately for individual animal differences. The significant regional effects are not surprising in light of heterogenous density of D₂ and NMDA receptors in dorsal and ventral striata (Dubois and Scatton, 1985; Boyson et al., 1986; Maragos et al., 1988; Bardo and Hammer, 1991; Jongen-Rêlo, 1994). We know for example that the higher numbers of Fos-IR cells following haloperidol administration at rostral as compared to caudal levels of the CPu correlate well with the density of D₂ receptors (Joyce et al., 1985; Robertson and Fibiger, 1992), as presumably does the rostro-caudal decline in cell density that we found in nucleus accumbens (Bardo and Hammer, 1991; Jongen-Rêlo, 1994). Moreover, the decline in Fos-IR cells along the rostro-caudal axis of both dorsal and ventral striata in MK5-HAL-treated animals could also reflect the decrease in Fos protein evident with D₂ blockade but may be related, at least in part, to NMDA receptor distribution (see below).

In relation to SAL-HAL treatment, there is some question as to whether haloperidol treatment elevates Fos in all territories of nucleus accumbens (Semba et al., 1996). While this drug reportedly increases the numbers of Fos-IR neurons throughout the nucleus (Robertson and Fibiger, 1992 and present results), the *c-fos* gene seems to be upregulated only in the shell (Semba et al., 1996). The reason for this discrepancy is unclear. It could lie in the difference between *in situ* hybridization and immunohistochemical studies, but a more likely explanation relates to how the data were gathered. We calculated cell density for the entire core at rostral and caudal levels, whereas Semba et al. (1996) measured changes in a very restricted ventrolateral part of the core, a region where we too found few cells with treatment (unpublished observations). Since the induction of Fos appears to be dependent upon the location and density of D₂ receptors, it seems important to examine Fos-IR cell densities for entire regions (present results) or to sample areas widely (see Chapman and Zahm, 1996) rather than to select a single, small zone for analysis (Semba et al., 1996).

The present results show that MK801 administration did significantly decrease the density of Fos-IR neurons induced by haloperidol treatment, in the CPu. These data contrast with those of Dragunow et al. (1990), who showed that Fos-IR cell density in the CPu could not be attenuated with MK801 unless a very high dose (10 mg/kg) of MK801 was administered. Such a dose may have been toxic, since maximal effects are reached at a much lower dosage (Ziolkowska and Höllt, 1993). The reason for the discrepancy between our results and those of Dragunow et al. (1990) is unclear but could be reflected in the regional rather than restricted manner, respectively, that these analyses were conducted. Although insignificant in tests, the present work showed an interaction between treatment and region suggesting that the selection of rostro-caudal level to be investigated may be important. Furthermore, the inhomogeneous distribution of NMDA receptors in the striatum (Maragos et al., 1988) could be directly related to the sites at which these receptors can modulate Fos expression. The fact that MK801 is able to attenuate the haloperidol induction of Fos in the CPu, however, suggests that the actions of haloperidol are dependent, at least in part, upon glutamate acting at the NMDA receptor (Tarazi et al., 1996). The blockade of D₂ receptors presynaptically on corticostriatal terminals is known to stimulate

glutamate efflux (Mitchell and Doggett, 1980; Garside et al., 1996) and chronic haloperidol treatment raises levels of extracellular glutamate (Yamamoto and Cooperman, 1994; See and Chapman, 1994). Therefore, early antagonism of the NMDA receptor may be important for preventing such increases, at least in the CPu.

In the nucleus accumbens, MK801 failed to block the haloperidol-induced increase in Fos. Indeed, NMDA antagonism preceding haloperidol administration here brought about an increase, although insignificant in tests, in the mean number of Fos-IR cells at the rostral pole and rostral core but little change in other parts. This may mean that elevations in glutamate with neuroleptic blockade do not involve the NMDA receptor in the ventral striatum or that these receptors are located on different cells from those with D2 receptors. Nevertheless, NMDA receptors are thought to be involved in dopaminergic neurotoxicity brought about by methamphetamine (Ohmori et al., 1993). Nucleus accumbens is innervated by glutamatergic fibers originating in the hippocampus, amygdala, prefrontal cortex and thalamus (Groenewegen et al., 1987; Berendse et al., 1992; Berendse and Groenewegen, 1990; Zahm and Brog, 1992; Brog et al., 1993; Wright and Groenewegen, 1996) but glutamate neurotransmission is mediated primarily by AMPA /kainate receptors (Pennartz, 1990; Meredith et al., 1993). Under conditions of decreased inhibition, however, NMDA receptors are activated (Pennartz, 1990). Classical neuroleptic drugs seem to increase rather than decrease inhibition in nucleus accumbens by elevating GABA transmission (Johnson et al., 1994), which may explain why NMDA antagonism here has little effect on the haloperidol-induction of Fos.

There is a greater density of NMDA receptors in nucleus accumbens as compared to the CPu (Maragos et al., 1988). This may explain why pretreatment with MK801 elevates rather than decreases the number of Fos-IR cells over haloperidol treatment at the rostral pole and in the rostral core. The fact that MK801 pretreatment does not reduce Fos anywhere in nucleus accumbens could reflect NMDA receptor localization, i.e. receptors are not located where they can be influenced by D₂ blockade. In the CPu, the glutamatergic input to striatal principal neurons is primarily onto the heads of spines and the dopaminergic input onto their necks (Freund et al., 1984). This arrangement is a potential site for balanced interactions between the two inputs and for enhanced glutamate release if presynaptic

D₂ receptors on nigrostriatal terminals are blocked. In nucleus accumbens, however, other relationships between these two inputs have been described, especially in the shell (Zahm, 1992; Totterdell and Smith, 1989; Meredith et al., 1993). The rostral pole has not been examined. Recent work by Gracy and Pickel (1996) found the R1 subunit of the NMDA receptor to be colocalized with tyrosine hydroxylase - presumably dopaminergic - axons in the shell. Although there is no anatomical evidence for presynaptic contacts in any part of the striatum, there is pharmacological evidence for such action. If NMDA receptors are located presynaptically on dopaminergic terminals as Gracy and Pickel (1996) find, they will not be in a position to influence the effects of D₂ blockade. The arrangement in the core or at the rostral pole have yet to be investigated. Certainly, additional studies are needed to ascertain where D₂ and NMDA receptors interact within cells in the ventral striatum.

Differences in the dependence of the NMDA receptor in the haloperidol activation of Fos may have functional consequences for both the desirable and the undesirable effects of antipsychotic drug treatment. Extrapyramidal syndromes develop with chronic administration of classical neuroleptics (Jaber et al., 1996), and Meshul et al. (1996a) and Mijster et al. (1996) have hypothesized that morphological change may underlie these abnormal movements. If these syndromes are brought about by the deleterious effects of glutamate on striatal neurons (Meshul et al., 1996b), then it is important to locate the site(s) where these effects may occur. The dorsolateral CPu, important for motor control, has been suggested as a likely place for the development of these syndromes (Roberts et al., 1995; Meshul et al., 1996a), but others (Prinssen et al., 1994; Egan et al., 1994) have suggested that nucleus accumbens is important. The present results point to the CPu as a likely site for glutamatergic stimulation of NMDA receptors, data that are in agreement with earlier work by Meshul and colleagues (1994), who showed that NMDA antagonists can attenuate haloperidol-induced synaptic changes in the dorsolateral striatum. Although NMDA channels permit rapid rises in calcium which can alter striatal neurons (Rothman et al., 1987; Choi, 1988), calcium-permeable AMPA receptors have an even greater potential to be neurotoxic if they are activated for prolonged periods (Choi, 1992; Lu et al., 1996). The latter receptors are readily activated in nucleus accumbens (Meredith et al., 1993) and it seems important, therefore, to assess their

role in haloperidol's action in ventral striatal regions where antipsychotic action appears to be NMDA independent.

Chapter 7

Summary and General Discussion

The primary aim of this thesis was to determine whether there is a morphological basis for the development of haloperidol-induced TD using a rat model of this syndrome. A further aim was to investigate some of the mechanisms of action of haloperidol treatment that may underlie any morphological change.

The classical neuroleptic drug, haloperidol is used clinically in the treatment of psychotic illnesses for example, schizophrenia (Johnstone et al., 1978; Ban et al., 1984). The therapeutic effects of neuroleptic drugs have been correlated with their ability to block dopamine D₂ receptors (Creese et al., 1976). Chronic neuroleptic drug treatment can cause TD in 20-40% of patients and this disorder is characterised by several involuntary movements including orofacial dyskinesia (Casey et al. 1988). Haloperidol induces a syndrome involuntary, spontaneous, VCMs in about 20 to 40% of rats treated chronically with this drug and this syndrome has been used as a rat model of TD (Egan et al., 1995). There is a good deal of evidence that suggests the syndrome of VCMs is a valid model of TD. It has been demonstrated that this syndrome takes several weeks to develop (Gunne and Growdon, 1982; Gunne and Haggstrom, 1983; Waddington et al., 1985).

In chapter 2 of this thesis it was confirmed that haloperidol induces a syndrome of vacuous chewing movements in about 30% of rats treated chronically with haloperidol, a similar percentage to that reported in previous studies (Egan et al., 1995; Hashimoto et al., 1998). This is also similar to the percentage of neuroleptic treated patients that develop TD (Casey, 1987). In order to increase our understanding of the mechanism of action of haloperidol we compared the ethological profile of haloperidol-treated and controls rats. In addition, to further elucidate the syndrome of vacuous chewing movements we compared the behavioural profile of VCM+ and VCM- rats. Rats that were treated chronically with haloperidol followed by a withdrawal period had altered behavioural profiles in which, locomotion and rearing directed towards the cage wall were significantly reduced. It is concluded that haloperidol treatment reduces levels of exploratory activity. In particular, the behaviours affected include those mediated by synergistic actions of dopamine D₁ and D₂ receptors (Molloy et al. 1986; Waddington, 1986; Clark and White, 1987). This is in agreement with studies that show the D₁ receptor has a permissive role in the

manifestation of D₂ mediated behaviours (Clark and White, 1987; Waddington, 1986).

Development of the syndrome of VCMs was associated with significantly increased grooming, buccal tremor and jaw tremor and reduced levels of stillness. It has been demonstrated that both grooming and oral behaviours are mediated by dopamine D₁ receptor agonists (Arnt, 1985; Molloy and Waddington, 1987). Therefore, development of the syndrome of VCMs appears to be associated with an increase in dopamine D₁ receptor activation. In favour of this, some investigators have observed VCMs following D₁ agonist administration (Koshiwaga et al. 1991; Rosengarten et al. 1983) while others have observed them only with D₁ agonist and D₂ antagonist administration (Waddington, 1989).

This behavioural study suggests a role for dopamine D₁ receptor activation in the development of the syndrome of VCMs. However, many underlying mechanisms of TD and its rat equivalent have been proposed that focus primarily on the dopamine receptor due to the fact that neuroleptic drugs are potent dopamine antagonists. It was originally proposed that chronic dopamine D₂ receptor blockade leads to disuse hypersensitivity (Carlsson et al., 1973; Klawans, 1973) and upregulation of these receptors (Muller and Seeman, 1978; Rupniak et al., 1985) resulting in TD. However, there is a temporal problem with this hypothesis in that dopamine receptors become hypersensitive within two weeks whereas, development of the rat equivalent of TD takes several weeks.

With the advent of selective D₁ and D₂ receptor agonists and antagonists, a role for the D₁ receptor in TD has been proposed. This is based on studies by Rosengarten et al. (1983) that demonstrate induction of VCMs using D₁ agonists or D₂ antagonists. This led to the suggestion that development of TD or their rat equivalent is due to an imbalance between D₁ and D₂ receptor stimulation. This would most likely be due to selective stimulation of D₁ receptors during D₂ blockade. However, this mechanism fails to account for the fact that only about 20-40% of patients or rats treated develops abnormal movement side effects, while D₂ receptor upregulation occurs in all rats treated with haloperidol (Shirakawa and Tamminga, 1994). It has also been hypothesised that TD may be due to disturbances in the

mesolimbic dopamine system based on studies showing activation of dopamine receptors in nucleus accumbens leads to orofacial dyskinesias in the rat (Koene et al., 1993; Prinssen et al., 1994). Recently it has been shown that the shell of nucleus accumbens plays an important role in mediating orofacial behaviours (Cools et al., 1995).

Projection neurons of nucleus accumbens contain GABA as their neurotransmitter. In neurons located in the core, the neuropeptide enkephalin is primarily co-localized with GABA and these neurons also contain primarily D₂ receptors. In neurons located in the shell, substance P and dynorphin are co-localized with GABA and these neurons contain predominantly D₁ receptors. Since dopamine is known to exert a regulatory role over several neurotransmitters and neuropeptides it is not surprising that there were different structural alterations to the dendrites of shell and core neurons. What was particularly interesting was that within the core there were significant differences between the dendritic morphology of neurons in animals that developed a syndrome of VCMs and those that did not.

Several investigators have found increased synthesis and release of enkephalin in striatal neurons following both acute and chronic neuroleptic-drug treatment (Hong et al., 1978; Romano et al., 1987; Mijster et al., 1996; Le Moine et al., 1990). However, recently it has been shown that the development of the rat equivalent of TD following chronic haloperidol treatment is associated with increased enkephalin and dynorphin mRNA with no change in those animals that fail to develop these abnormal movements (Egan et al., 1994). Chronic haloperidol treatment has also been associated with increased substance P, GABA as well as increased activity of glutamic acid decarboxylase, the rate limiting enzyme for GABA synthesis, in the striatum. However, these changes have not been found in nucleus accumbens of VCM+ or VCM- rats (Egan et al., 1994).

As well as neuropeptide alterations chronic haloperidol treatment is associated with alterations in dopamine. Acute administration leads to an increase in the number and firing rate of spontaneously active dopaminergic cells in the SNpc (Bunney and Grace, 1978; Chiodo and Bunney, 1983) as well as increased dopamine concentration in the striatum (Moghaddam and Bunney, 1990). However, chronic administration

eventually leads to a reduction in numbers of spontaneously active dopaminergic neurons in a phenomenon known as depolarization inactivation (see Grace, 1992). Although the exact mechanisms of dopamine-glutamate interactions in the striatum have not been fully elucidated, it has been shown that glutamate release is under the control of presynaptic dopamine receptors. Blockade of D₂ receptors with haloperidol increases glutamate transmission throughout the striatum. Certain levels of glutamate are critical for synaptogenesis and plasticity as has been postulated to be a structural basis for learning and memory (Harris, 1999). However, excess glutamate leads to detrimental effects through excitotoxic mechanisms. In addition, dopamine has been shown to be important in maintenance of morphology in the core of nucleus accumbens. In addition, morphological changes have been shown in enkephalinergic bouton in the striatum as a result of subchronic haloperidol treatment. Therefore, it appears that both the shell and core of nucleus accumbens may play a role in the development of the syndrome of VCMs in rats treated chronically with haloperidol.

There is a large amount of data based on neurochemical, structural and connectional evidence, that the caudomedial shell is distinct from more lateral parts. It has been noted that neuroleptic drugs have powerful effects in the caudomedial shell and this region is often considered to be a transitional zone of the extended amygdala. Nucleus accumbens was chosen as the region of interest as this is one of the primary sites of action of haloperidol (Deutch et al., 1992). Therefore, our morphological studies in the shell were concentrated in this caudomedial region.

Chapter 3 examined the morphology of dendrites on medium spiny neurons following chronic haloperidol treatment and withdrawal. A comparison was made between the structure of dendritic segments from haloperidol-treated rats that developed vacuous chewing movements and those that failed to develop these abnormal movements. Results of this study show that morphological change is correlated with the development of vacuous chewing movements in rats. Dendritic segments located in the shell have reduced surface area in VCM+ compared to VCM- rats. In addition, distal dendritic segments located in the nucleus accumbens' core from VCM- rats were significantly shorter and had reduced numbers of spines compared to those from VCM+ rats.

Reduced surface area of dendrites in the shell of VCM+ has important consequences for the normal functioning of circuits through this part of nucleus accumbens. It has been shown that the surface area of a dendrite is directly proportional to the number of synapses that can be formed along it (Harris and Kater, 1994). Therefore, shell dendrites from VCM+ would, due to lower surface area, have less room for axodendritic synapses. This would lead to two possibilities, firstly axodendritic synapses may be lost or secondly they may move from the dendritic shaft onto the dendritic spines thereby increasing the number of axospinous inputs. In support of the former, one study found significant decreases in the density of symmetric axodendritic synapses in VCM+ rats (Roberts et al. 1995), however this did not persist after drug withdrawal. It is noteworthy that this previous ultrastructural study was carried out on the dorsal striatum. A recent study (Meredith et al. 1997) examined changes in the shell of nucleus accumbens and found the number of dynorphinergic axospinous contacts increased in the shell of VCM+ rats. This suggests that these contacts may be moving from dendritic shafts onto spines perhaps due to new spine formation.

The increase in spine density on dendritic segments located in the shell may be related to the mechanism of action of haloperidol. Our current understanding of factors controlling spine density is largely based on studies carried out in the hippocampus. However, general underlying principles would be expected to be the same as those for the shell of nucleus accumbens. Recent electrophysiological studies have demonstrated that an optimal level of synaptic input in neurons is maintained by increasing synaptic strength when activation is low and decreasing synaptic strength when activation is high (Turrigiano et al. 1998). This is coupled with evidence that shows increased numbers of spines in slices compared to perfusion fixed tissue in the hippocampus (Kirov et al., 1999). However, it has also been demonstrated recently that miniature synaptic events maintain dendritic spines through the activation of AMPA receptors (McKinney et al., 1999). Therefore the increase in spine density found in this study suggests increased synaptic input to the dendritic spines of neurons located in the shell, however, the exact mechanism by which this takes place remains unknown.

The structural modifications found in this study have functional implications for the development of the rat equivalent of TD. A previous study (Egan et al., 1994) suggested that vacuous chewing movement syndrome is associated with increased activation of both striatonigral and striatopallidal pathways. In addition, the most prevalent changes in the striatum appear to occur in nucleus accumbens. There is also strong support that chewing movements are associated with dopamine D₁ receptor stimulation and that the nucleus accumbens is important in their regulation (Fletcher and Starr, 1987; Koene et al., 1993; Koshikawa et al. 1991).

The primary morphological findings of this study suggest that major synaptic reorganisation occurs on the dendrites and spines of projection neurons located in the shell and core territories of rats that develop the syndrome of VCMs that would be expected to alter several circuits (Figure 7.1). There are two main projections from the shell of nucleus accumbens, one to the VTA of the midbrain and the other to the ventromedial ventral pallidum (Heimer et al., 1991). The ventromedial ventral pallidum in turn projects to the SNr and neurons here then project to the parvocellular reticular formation (Bevan et al., 1996). In addition, the parvocellular reticular formation region receives a heavy direct projection arising from the face and jaw areas of the primary motor cortex. Thus any change in the morphology of the neurons of the shell would be expected to cause changes in circuitry which would eventually lead to dysregulation of the facial motor nucleus and lead to the development of orofacial dyskinesia.

Projection neurons in the core also project to the SNr and since they contain GABA as their neurotransmitter increased activation would lead to inhibition of SNr neurons which in turn would result in an overall disinhibition of the thalamus and lead to increased excitation of the areas in the cortex that the thalamus projects to. Alternatively, SNr neurons also project directly to the parvocellular reticular formation (von Krosigk and Smith, 1991; Yasui et al., 1992). It has also been demonstrated that neurons in the PcRt form direct synaptic contact with the motoneurons in the trigeminal motor nucleus that innervate the muscles of mastication (Mogoseanu et al., 1993). In addition, PcRt neurons project to the facial motor nucleus (Ter Horst et al. 1991) and form direct synaptic input on facial motoneurons (Mogoseanu et al. 1993a).

Therefore, reduced activity of SNr would also result in reduced inhibition of the PcRt and lead to an increase in orofacial behaviour.

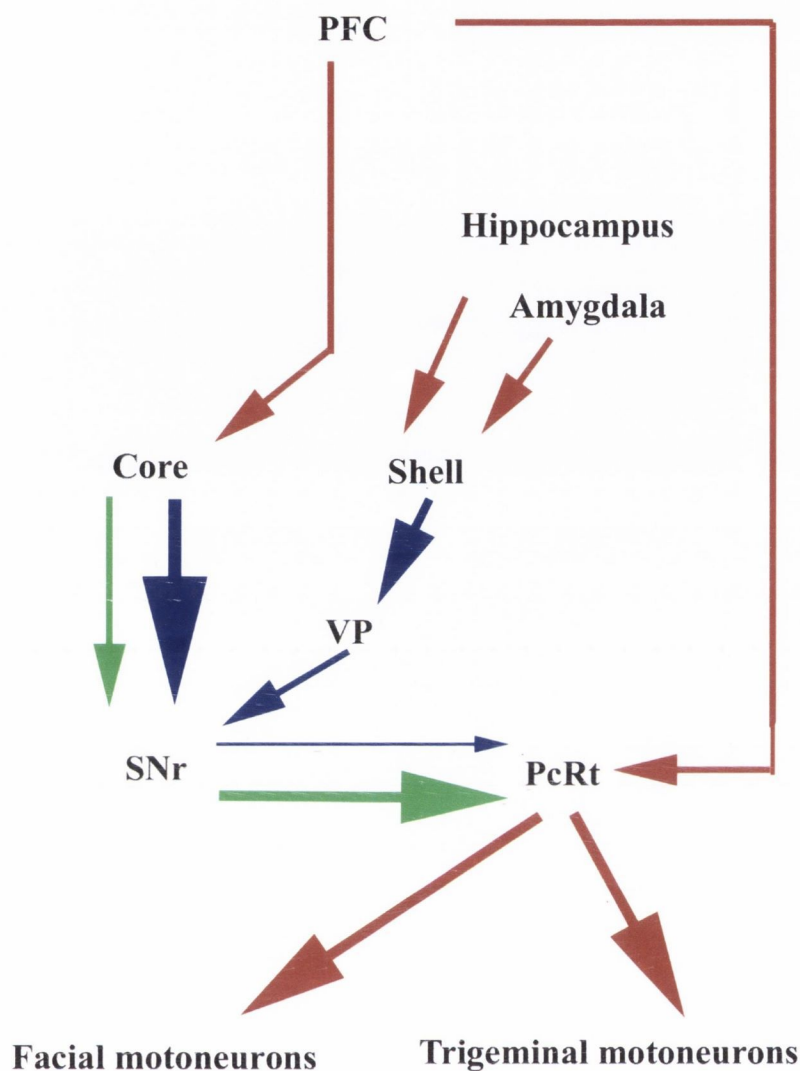


Figure 7.1 Diagram showing the main circuits through the shell and core of nucleus accumbens that are likely to be involved in the development of VCMs. In VCM+ rats output from both the shell and core is increased (large blue arrows) resulting in inhibition of the SNr and disinhibition of the PcRt (thin blue arrow). This would result in increased excitation of projections from PcRt to the facial and trigeminal motoneurons and development of VCMs. In VCM- rats loss of spines and reduced length of distal dendrites would lead to reduced output from the core (thin green arrow) and disinhibition of the SNr. This would result in increased inhibition of PcRt (large green arrow) and failure to excite facial and trigeminal motoneurons resulting in failure to develop VCMs. Red arrows represent excitatory projections and green and blue arrows represent inhibitory projections. Abbreviations: PcRt, parvocellular reticular formation; PFC, prefrontal cortex; SNr, substantia nigra pars reticulata; VP, ventral pallidum.

One could quite easily envisage a situation where increased activity of projection neurons from the core and shell lead to inhibition of the SNr, this in turn leads to disinhibition of both the PcRt and the thalamus. Increased thalamus activity, which would excite both cortical neurons and striatal neurons. Increased activity of the cortex would in turn excite nucleus accumbens neurons and also directly activate the PcRt. Therefore, evidence seems to suggest that several open and closed circuits could be affected as a result of the morphological changes that are reported here in nucleus accumbens and dysregulation in any or all of these has the potential to cause vacuous chewing movement syndrome. It also appears that animals that fail to develop this syndrome have structural changes in distal dendrites in the core territory of nucleus accumbens that suggest excitotoxicity may be occurring at distal dendritic sites and preventing the development of VCMs. This is supported by evidence from a recent study (Hamid et al., 1998) where a reduced number of NMDA receptors were found in the core of nucleus accumbens in animals that failed to develop VCMs.

As well as morphological modifications that could be assigned to the development of vacuous chewing movement syndrome, there were many structural alterations in dendritic segments due to haloperidol treatment. These changes were especially prevalent in the shell where chronic haloperidol treatment resulted in increases in spine density and reductions in tortuosity. Changes in spine density are generally thought to reflect changes in synaptic efficacy and this may be important in the efficacious actions of haloperidol. In addition, reduced tortuosity like reduced surface area would mean that there would be less room for synapses to be made.

It is interesting that the dendritic structure of core and shell were differentially affected by chronic haloperidol treatment. Increases in dynorphin mRNA in the shell may account for these differences. It has been shown that dynorphin inhibits neuronal adenylate cyclase and reduces calcium currents through activation of G proteins (Gross et al., 1990). In addition, the release of glutamate may be inhibited through activation of k-opioid receptors located presynaptically on glutamatergic axons in the striatum (Mc Ginty et al., 1995) due to the presence of increased dynorphin. Hence the ability to achieve morphological plasticity may be central to preventing excitotoxicity in the shell.

In so far as the core is considered a striatal-like region findings in the striatum may be generally applied to this territory. Our results in the core are consistent with the findings of previous studies indicating oxidative damage and excitotoxicity in the striatum following chronic neuroleptic drug treatment. Presynaptic dopamine D₂ receptors inhibit the release of glutamate from excitatory corticostriatal projections (Carlsson and Carlsson, 1990). Blockade of these receptors with neuroleptic drugs results in an increase in glutamate release in the striatum (Bardgett et al. 1993; Perry et al., 1979). Normal striatal functioning appears to be dependent on maintaining a balance between dopamine and glutamate release (Carlsson and Carlsson, 1990). Alterations in glutamate neurotransmission have been proposed to play a role in the pathophysiology of TD (Hamid et al., 1998). In addition, prolonged activation of glutamate receptors, especially the NMDA receptor may raise intracellular levels of calcium and induce neuronal damage through excitotoxic mechanisms (Beal et al., 1986; Meldrum and Garthwaite, 1990). Two early studies have suggested a role for cell loss in the striatum in the development of abnormal movements in rats (Pakkenberg et al., 1973; Nielsen and Lyon, 1978). Glutamate NMDA receptors are also located on a group of nucleus accumbens' interneurons and activation of these receptors leads to the synthesis of nitric oxide. Therefore, nitric oxide is likely to play an important role in mediating morphological change in nucleus accumbens.

In chapter 4 the size and density of NOS-IR neurons was compared in nucleus accumbens in controls, VCM+ and VCM- rats. Results of this study showed that NOS-IR neurons in the shell were smaller in both VCM+ and VCM- rats. In the core, NOS-IR neurons were significantly smaller in VCM- rats but not VCM+ rats. In addition, NOS-IR neurons located in the core territory were significantly smaller in VCM- compared to VCM+ rats. In some respects these results parallel the morphological changes found in medium-sized densely spiny neurons where effects in the shell appear to be due to haloperidol action since they affect both VCM+ and VCM- rats. However, in the core changes are correlated with the development of vacuous chewing movements and this suggest nitric oxide may play a role in the morphological changes we found in distal dendrites of medium spiny neurons in this territory. Recently (O'Donnell and Grace, 1997) have demonstrated that release of nitric oxide leads to an increase in gap junction permeability and is a mechanism by which strong cortical activation can override the gating effect of the hippocampus on

nucleus accumbens neurons. Therefore, reduced size of NOS-IR neurons in VCM-rats suggests less nitric oxide would be produced as well as diffusing over a lesser distance (Philippides et al., 2000) possibly resulting in a reduced effect of nitric oxide on projections neurons in the core territory of VCM- rats. Hence changes in NOS-IR in VCM- rats may be an important factor in their failure to develop abnormal movements.

In chapter 5 of this thesis we investigated changes in glutamate transport as a potential mechanism of increased extracellular glutamate concentrations that occur following chronic haloperidol treatment. Our results indicate that chronic haloperidol treatment leads to reduction of up to 63% in glutamate transporter activity. Given the importance of these receptors in rapidly removing glutamate from the synaptic cleft following its release, it can be concluded that reduced glutamate transporter activity is likely to play an important role in the haloperidol-induced elevation of extracellular glutamate. Previous ultrastructural studies have found reduced levels of glutamate in the presynaptic membranes of assymetrical synapses following chronic haloperidol treatment (Meshul et al., 1996b). These results could be explained by reductions in the activity of the glutamate transporters, if less glutamate is reuptaken following release this would result in an increase in the synaptic cleft and a reduction in the terminal.

Increased extracellular glutamate concentrations have the potential to be excitotoxic especially if they activate the NMDA receptors. Therefore, in chapter 6, our aim was to examine whether glutamate acting via the NMDA receptor played a role in the action of haloperidol in the dorsal or ventral striatum. Our results indicate that haloperidol acts by an NMDA-dependent mechanism in the caudate-putamen but by an NMDA-independent mechanism in nucleus accumbens. This provides further evidence for a different mechanism of action of haloperidol in the ventral striatum compared with the dorsal striatum and also raises important questions about the nature of the structural changes we found in nucleus accumbens. Given that there is raised extracellular glutamate this has the potential to act via AMPA or metabotropic glutamate receptors and lead to excitotoxic damage.

A great deal of further work will be required to elucidate the exact mechanism for the development of haloperidol-induced syndrome of VCMs. Nevertheless, the results presented here indicate an important role for morphological change in the dendritic structure of both the shell and core territories of nucleus accumbens in the development of this syndrome. The main focus of this study has been on alterations that take place in nucleus accumbens neurons and their associated dendrites and spines. It has been demonstrated here that morphological changes take place in nucleus accumbens following chronic haloperidol treatment followed by withdrawal. Some morphological changes especially the reduction in spines and dendritic segment length of distal dendrites as well as the reduced size of NOS-IR were changes that took place in the core territory and were correlated with the development of orofacial movements. It is important to view the present results in the context of functional circuits. For example, recent studies have shown that the paraventricular nucleus of the thalamus may also be a novel locus of antipsychotic drug action (Deutch et al., 1995; Cohen and Wan, 1996). Therefore, while nucleus accumbens can be considered to play a central role in the circuitry involved in the development of abnormal orofacial movements other nuclei that form part of the cortical-striatal-thalamus loops or part of the downstream circuits that terminate on face and jaw motoneurons may also be altered. In short, morphological change is rarely restricted to one portion of a circuit. It is not surprising then that changes have been reported in target areas of nucleus accumbens (ventral pallidum and substantia nigra pars reticulata) as well as the prefrontal cortex that projects to nucleus accumbens.

A thorough analysis of the sequence of morphological change in all the nuclei associated with orofacial behaviour, as well as physiological, pharmacological, biochemical and molecular biological characterisation of these changes are required in order to fully elucidate the mechanisms of TD or its rat equivalent. Nevertheless, it is clear from the results presented in this thesis that neuroleptic-induced movement disorders are associated with irreversible changes in the dendritic morphology of neurons located in both the shell and core of nucleus accumbens.

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Appendix

Short communication

Chronic haloperidol treatment impairs glutamate transport in the rat striatum

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Received 1 July 1999; received in revised form 6 August 1999; accepted 10 August 1999

Abstract

High-affinity, Na⁺-dependent transport of glutamate into neurons and glial cells maintains the extracellular concentration of this neurotransmitter at a sub-toxic level. Chronic blockade of dopamine D₂ receptors with haloperidol elevates extracellular glutamate levels in the striatum. The present study examines the effect of long-term haloperidol treatment on glutamate transporter activity using an assay based on measuring the uptake of D-[³H]aspartate in striatal synaptosomes prepared from male Wistar rats. The maximal rate of glutamate transport in the striatum is reduced by 63% following 27 weeks of haloperidol treatment. This impairment of glutamate transport may be important in chronic neuroleptic drug action. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Glutamate; Haloperidol; Transport; Striatum; Synaptosome

1. Introduction

Haloperidol, a classical antipsychotic drug, is believed to exert its effects by blocking dopamine D₂ receptors (Seeman and Lee, 1975; Creese et al., 1976). The highest densities of both dopamine D₁ and D₂ receptors are found in the striatum (caudate/putamen and nucleus accumbens). These nuclei are generally considered to be sites of both efficacious and undesirable effects of antipsychotic drug treatment (Crow et al., 1975; Deutch et al., 1992). The entire striatum receives major glutamatergic projections from the cortex in addition to dopaminergic inputs from the substantia nigra pars compacta and ventral tegmental area. It has previously been shown that chronic haloperidol treatment raises the concentration of extracellular glutamate (See and Chapman, 1994; See and Lynch, 1995; Yamamoto and Cooperman, 1994). However, the basis for this elevation is presently unknown.

A family of high-affinity Na⁺-dependent glutamate transporters are essential for terminating the postsynaptic action of glutamate by rapidly removing it from the synaptic cleft (Fonnum, 1984; Takahashi et al., 1997). Within this family, the GLT-1 glutamate transporter, which is

located on glial cells, accounts for approximately 70% of the total glutamate transported (Rothstein et al., 1996). Recent work by Schneider et al. (1998) has shown that GLT-1 mRNA in the striatum was significantly reduced following chronic haloperidol treatment. However, it was not determined whether the changes in these mRNA levels alter the functional activity of the glutamate transporters. In the present study, we have measured glutamate transporter activity and show that administration of haloperidol for 27 weeks significantly impairs the capacity of these transporters to take up glutamate in the striatum.

2. Materials and methods

Male, albino, Wistar rats (Bioresources, Trinity College, Dublin, Ireland) initially weighing 300–350 g were housed four per cage, with a constant temperature of 21°C, on a 12-h light and dark cycle with free access to food and water. Animals were divided into two groups. The control group (*n* = 4) was treated with sesame (Sigma, Poole, Dorset, UK) oil as vehicle while the haloperidol group (*n* = 4) received the depot neuroleptic drug, haloperidol decanoate (Janssen Pharmaceutical, Little Island, Cork, Ireland) at a dose of 28.5 mg kg⁻¹, the equivalent of 1 mg kg⁻¹ day⁻¹ of unconjugated haloperidol. Injections were

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given intramuscularly every 3 weeks for 27 weeks in a volume of 1 ml kg⁻¹. All procedures for the treatment of these animals were in strict compliance with European Community Directive, 86/609/EC and Cruelty to Animals Act 1876, with protocols approved by the Research Committee of the Royal College of Surgeons in Ireland.

Following the treatment period, rats were killed by stunning and cervical dislocation. Each brain was removed and the paired striata, including caudate/putamen and nucleus accumbens, were dissected out, and homogenised in ice-cold gradient medium, which was composed of 0.32 M sucrose, 1 mM EDTA and 0.25 mM dithiothreitol, pH 7.4. The homogenate was centrifuged for 10 min at 1000 g at 4°C (Sorvall centrifuge, SS-34 rotor) and the supernatants were collected and the synaptosomes were purified using a modification of the method of Dunkley et al. (1988). Briefly, Percoll diluted in gradient medium was layered into 10-ml polycarbonate tubes using a peristaltic

pump and a flow rate of 1 ml min⁻¹, starting with the most dense (23% v/v), followed by, in order, 15% v/v, 10% v/v, and 3% v/v. A 2-ml sample of the supernatant was gently layered, using a Pasteur pipette, onto the top of the gradients and centrifuged at 32,500 g for exactly 5 min at 4°C. Synaptosomes in the 15%/23% Percoll interfacial fraction (layer 4) were carefully removed using a Pasteur pipette. They were washed twice in sodium-free Krebs' bicarbonate medium (choline chloride 116.8 mM, KCl 4.72 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, Tris 8.1 mM, glucose 11 mM and CaCl₂ 2.5 mM) pH 7.4 and resuspended in a final volume of 1.25 ml of sodium-free Krebs' medium. An aliquot was removed for protein determination by the method of Markwell et al. (1978) and the remaining synaptosomes used in the transport assay. They were incubated for 4 min at 25°C in normal Krebs' bicarbonate medium (NaCl 109.6 mM, KCl 4.72 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 25 mM, glucose 11

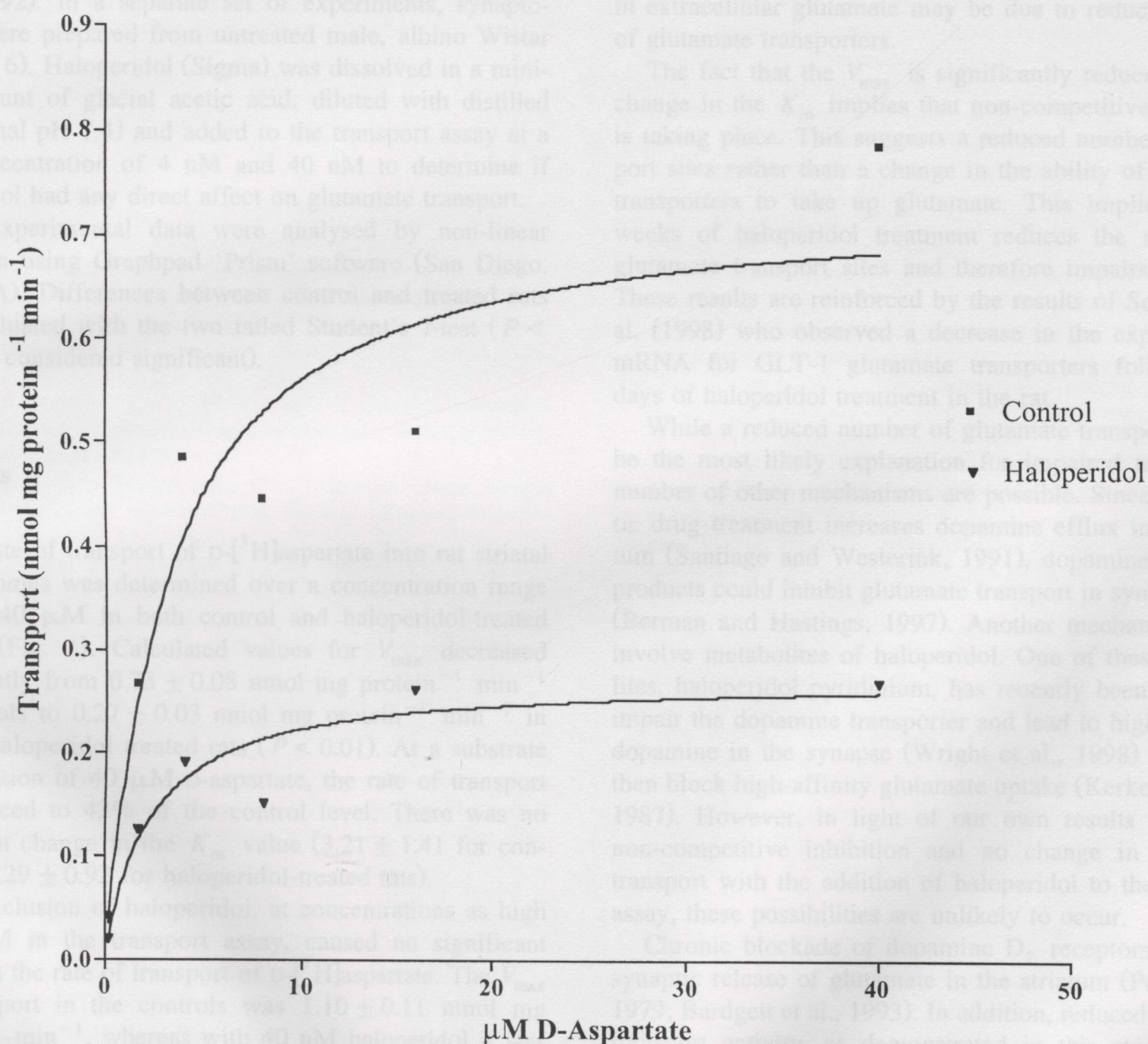


Fig. 1. Effect of chronic haloperidol treatment on kinetic parameters of glutamate transport. Synaptosomes were incubated at 25°C for 4 min and uptake of D-[³H]aspartate at the specified concentrations was measured ($n = 2$ individual experiments, each performed in triplicate). The data were analysed by non-linear regression analysis and show a significant decrease in $V_{\max}$ (0.73 ± 0.08 nmol mg protein⁻¹ min⁻¹ for controls and 0.27 ± 0.03 nmol mg protein⁻¹ min⁻¹ in chronic haloperidol-treated rats ($P < 0.01$)).

mM and CaCl_2 2.5 mM) pH 7.4 buffer, containing D- $[\text{}^3\text{H}]$ aspartate (4.6×10^{-5} – 1.15×10^{-4} MBq mmol^{-1} specific activity). The reaction was stopped by the addition of 200 μl of ice-cold 1 mM D-aspartate followed immediately by centrifugation for 10 min at 13,000 rpm at 4°C . The pellet was retained, washed twice with ice-cold gradient buffer and solubilised overnight in a 2% sodium dodecyl sulphate solution. The quantity of radioactivity in each sample was determined by liquid scintillation spectroscopy, and the rate of transport was plotted as a function of substrate concentration.

The transport assay was performed at 25°C , rather than 37°C , in order to slow down the activity of the transporters to a measurable rate in vitro and previous studies have determined that the transport of D-aspartate was linear between 0 and 20 min at 25°C (McBean, 1994). D-aspartate was used in preference to L-glutamate in these experiments because it is a non-metabolisable substrate for the glutamate transporters (Davies and Johnston, 1976; Fyske et al. 1992). In a separate set of experiments, synaptosomes were prepared from untreated male, albino Wistar rats ($n = 6$). Haloperidol (Sigma) was dissolved in a minimal amount of glacial acetic acid, diluted with distilled water (final pH 7.4) and added to the transport assay at a final concentration of 4 nM and 40 nM to determine if haloperidol had any direct affect on glutamate transport.

The experimental data were analysed by non-linear regression using Graphpad 'Prism' software (San Diego, CA, USA). Differences between control and treated rats were evaluated with the two tailed Student's *t*-test ($P < 0.05$ was considered significant).

3. Results

The rate of transport of D- $[\text{}^3\text{H}]$ aspartate into rat striatal synaptosomes was determined over a concentration range of 0.16–40 μM in both control and haloperidol-treated animals (Fig. 1). Calculated values for V_{max} decreased significantly from 0.73 ± 0.08 nmol mg protein $^{-1}$ min $^{-1}$ for controls to 0.27 ± 0.03 nmol mg protein $^{-1}$ min $^{-1}$ in chronic haloperidol treated rats ($P < 0.01$). At a substrate concentration of 40 μM D-aspartate, the rate of transport was reduced to 42% of the control level. There was no significant change in the K_m value (3.21 ± 1.41 for control vs. 2.29 ± 0.92 for haloperidol-treated rats).

The inclusion of haloperidol, at concentrations as high as 40 nM in the transport assay, caused no significant change in the rate of transport of D- $[\text{}^3\text{H}]$ aspartate. The V_{max} for transport in the controls was 1.10 ± 0.11 nmol mg protein $^{-1}$ min $^{-1}$, whereas with 40 nM haloperidol it was 1.06 ± 0.40 nmol mg protein $^{-1}$ min $^{-1}$ (results not shown). In addition, there were no significant differences in the body weight, health or striatal protein content between treated and control animals.

4. Discussion

These findings demonstrate that chronic haloperidol treatment significantly impairs the activity of the high-affinity, sodium-dependent glutamate transporters in the striatum. Although D-aspartate was used in place of L-glutamate in these experiments, one can assume that L-glutamate transport would be similarly affected. Thus, at physiologically relevant (low micromolar) concentrations of L-glutamate, the ability of the transporters to take up glutamate would be significantly reduced following long-term neuroleptic treatment. See and Lynch (1995) have previously shown that extracellular glutamate is elevated following chronic neuroleptic drug treatment. In their work they treated rats for 24 weeks with haloperidol. Following treatment, glutamate was released by high K^+ infusion and the extracellular glutamate concentration was significantly elevated in the haloperidol-treated rats following infusion compared to controls. Our results suggest that this increase in extracellular glutamate may be due to reduced activity of glutamate transporters.

The fact that the V_{max} is significantly reduced with no change in the K_m implies that non-competitive inhibition is taking place. This suggests a reduced number of transport sites rather than a change in the ability of individual transporters to take up glutamate. This implies that 27 weeks of haloperidol treatment reduces the number of glutamate transport sites and therefore impairs transport. These results are reinforced by the results of Schneider et al. (1998) who observed a decrease in the expression of mRNA for GLT-1 glutamate transporters following 28 days of haloperidol treatment in the rat.

While a reduced number of glutamate transporters may be the most likely explanation for impaired transport, a number of other mechanisms are possible. Since neuroleptic drug treatment increases dopamine efflux in the striatum (Santiago and Westerink, 1991), dopamine oxidation products could inhibit glutamate transport in synaptosomes (Berman and Hastings, 1997). Another mechanism could involve metabolites of haloperidol. One of these metabolites, haloperidol pyridinium, has recently been shown to impair the dopamine transporter and lead to high levels of dopamine in the synapse (Wright et al., 1998) that could then block high-affinity glutamate uptake (Kerkerian et al., 1987). However, in light of our own results that show non-competitive inhibition and no change in glutamate transport with the addition of haloperidol to the transport assay, these possibilities are unlikely to occur.

Chronic blockade of dopamine D_2 receptors increases synaptic release of glutamate in the striatum (Perry et al., 1979; Bardgett et al., 1993). In addition, reduced glutamate transport activity, as demonstrated in this study, would maintain a high concentration of glutamate in the synaptic cleft for longer than in controls. Therefore, impaired glutamate transport coupled with increased glutamate release could result in increased glutamate neurotransmission and

this may be the mechanism involved in the therapeutic action of haloperidol. Moreover, increased glutamatergic transmission may also lead to excitotoxic effects due to prolonged activation especially at NMDA receptors (Olney, 1990), and this may play a role in side effects of chronic neuroleptic drug treatment such as tardive dyskinesia (De Keyser, 1991; Andreassen and Jørgensen, 1994; Meshul et al., 1996).

In conclusion, our results indicate that chronic haloperidol treatment inhibits glutamate transport in the rat striatum and this is most likely due to a reduced number of glutamate transport sites. Further studies are required to establish where and how this takes place.

Acknowledgements

This study was supported by the Research Committee of the Royal College of Surgeons in Ireland. We thank Mr. Paul Rooney for technical assistance.

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NMDA Receptor Blockade Attenuates the Haloperidol Induction of Fos Protein in the Dorsal But Not the Ventral Striatum

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KEY WORDS dizocilpine maleate; caudate-putamen; rostral pole; core; shell; nucleus accumbens

ABSTRACT Neuroleptic blockade of dopamine receptors is known to produce an increase in the expression of Fos. This increase may be related to elevations in glutamate transmission which in turn activates N-methyl-D-aspartate (NMDA) receptors. In the present study, we examine the role of these receptors in the haloperidol-induced augmentation of Fos in the caudate-putamen and nucleus accumbens of Wistar rats. Animals were divided into four groups for each experiment and each was injected either with saline; a noncompetitive NMDA antagonist, dizocilpine maleate (MK801, 5 mg/kg); haloperidol (0.5 mg/kg); or MK801 followed by an injection of haloperidol. Fos-immunoreactive cells appear in large numbers in all parts of the striatum 3 h after the administration of haloperidol. Pretreatment with MK801 attenuates the haloperidol-induced increase in Fos in the caudate-putamen. However, antagonism of the NMDA receptor does not significantly reduce the density of Fos-immunoreactive cells in any territory of nucleus accumbens, i.e., shell, core, or rostral pole. These data suggest that haloperidol acts in an NMDA-dependent manner in the caudate-putamen, but independently in parts of nucleus accumbens traditionally considered to be targets of antipsychotic drugs. **Synapse 32:243–253, 1999.** © 1999 Wiley-Liss, Inc.

INTRODUCTION

Haloperidol, a classical antipsychotic drug, is believed to exert its effects by blocking dopamine D2 receptors. Some studies suggest that the efficacious actions of this drug involves nucleus accumbens (Crow et al., 1975; Blaha and Lane, 1987; Moghaddam and Bunney, 1990; Goldstein and Deutch, 1992), whereas the induction of undesirable side effects is due to action in sensorimotor parts of the caudate-putamen (CPu) (Blaha and Lane, 1987; Meshul et al., 1996a). Other reports, however, question whether D2 blockade at the level of nucleus accumbens is responsible for controlling psychosis (Laruelle et al., 1992) and in the dorsal striatum for producing abnormal movements (Koshikawa et al., 1989; Koene et al., 1993; Egan et al., 1994). Certainly, acute and chronic haloperidol treatment alters dorsal striatal dopamine neurotransmission and upregulates D2 receptors, while leaving the D1 receptors unchanged (Bunney and Aghajanian, 1975; Moghaddam and Bunney, 1990; Laruelle et al., 1992; Debonnel et al., 1990; Egan et al., 1993, 1996). In nucleus accumbens, where territorial divisions are well defined anatomically and functionally (see Alheid and

Heimer, 1988; Zahm and Brog, 1992; Meredith et al., 1993), haloperidol seems to augment dopamine turnover and modulate cell coupling differentially in the shell and core (Deutch and Cameron, 1992; O'Donnell and Grace, 1993).

In addition to and perhaps as a result of influencing dopaminergic action, antipsychotic drug administration affects other neurotransmitter systems (Johnson et al., 1994; See and Chapman, 1994; Meshul and Tan, 1994; Meshul et al., 1994). Recent work has shown that chronic haloperidol treatment elevates extracellular glutamate (See and Chapman, 1994; See and Lynch, 1995) and increases the number of perforated glutamatergic synapses in the CPu (Meshul et al., 1996b). The mechanism responsible for the interaction between neuroleptic drugs and glutamate is not known, but the reduced stimulation of D2 receptors increases activity

Contract grant sponsor: the Research Committee of the Royal College of Surgeons in Ireland.

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Received 26 May 1998; Accepted 3 August 1998

at N-methyl-D-aspartate (NMDA) glutamate receptors. Excessive stimulation of these receptors raises intracellular levels of calcium and can produce neuronal damage through excitotoxic mechanisms (Beal et al., 1986; Meldrum and Garthwaite, 1990). NMDA antagonists seem to protect the striatum from such damage (Weiloch, 1985; Simon et al., 1986), and lesioning the corticostriatal pathway ameliorates excitotoxin-induced injury (Weiloch, 1985; Linden et al., 1987). Thus, elevation of glutamate after haloperidol administration could alter striatal neurons in a deleterious manner.

Fos protein is an early immediate gene product, postulated to be a marker of neuronal activity because of its rapid and intense induction (Sheng and Greenberg, 1990; Morgan and Curran, 1991); its presence may be used to define neuroanatomical sites of drug action (Nakajima et al., 1989; Presley et al., 1990; Deutch et al., 1992). In the CPu, the basal level of Fos and its mRNA are very low, but increase dramatically following haloperidol administration (Dragunow et al., 1990; Robertson and Fibiger, 1992; Semba et al., 1996). In nucleus accumbens, however, the action of haloperidol is less clear, since acute treatment increases the overall density of Fos-immunoreactive (IR) cells (Robertson and Fibiger, 1992; Robertson and Jian, 1995), while inducing *c-fos* mRNA in only one of its territories (Semba et al., 1996). There are data to suggest that in the CPu, the potent noncompetitive NMDA antagonist dizocilpine maleate (MK801) can prevent haloperidol-induced elevations of *c-fos* mRNA (Ziółkowska and Höllt, 1993), but not the increased density of Fos-IR cells, unless administered at a very high dose (Dragunow et al., 1990). It is not known whether NMDA antagonism affects haloperidol-induced changes in Fos in nucleus accumbens. In light of regional differences in NMDA receptor distribution and activation in the ventral striatum (Pennartz et al., 1990; Gracy and Pickel, 1996), antagonism of these receptors may not be homogeneous; thus, the effects of haloperidol-induced glutamate elevations could also be regionally restricted. If so, this would have important implications for linking the action of haloperidol to specific sites. The aim of the present study, therefore, is to examine whether glutamate, acting via the NMDA receptor, contributes to the action of haloperidol in the dorsal or ventral parts of the striatum and, if so, where this might take place.

MATERIALS AND METHODS

Animals and drug treatment

Adult, male Wistar rats (Bioresources, Trinity College, Dublin, Ireland) weighing 200–300 g were housed four to a cage and maintained on a 12-h light/dark schedule, at a constant temperature of 21°C with food and water provided ad libitum. All animals were handled on a daily basis for 2 days before drug administration in order to prevent any increase in the basal level of Fos by

stress (Asanuma et al., 1992; Helton and McGinty, 1993).

The effects of MK801 (Research Biochemicals Inc., Natick, MA) on the haloperidol (Baker Norton, Harlow, Essex, UK) induction of Fos were examined in four experiments. All drugs were administered intraperitoneally in a volume of 1 ml/kg; the two injections in each experiment were given 30 min apart. Animals were left quietly for 3 h after their final injection. The same dosage of haloperidol (0.5 mg/kg) was used in all four experiments but MK801 was administered at 5 mg/kg (MK5) in the first three experiments. This dose was selected on the basis of its ability to inhibit, maximally, the induction of *c-fos* mRNA by haloperidol as measured by Ziółkowska and Höllt (1993) using Northern blot analysis. A higher dose (10 mg/kg, MK10) was also used in the final experiment. From a total of 16, four rats were randomly assigned to one of four injection protocols for each experimental cohort: saline-saline (SAL-SAL, $n = 4$), saline-haloperidol (SAL-HAL, $n = 4$), MK801-saline (MK5-SAL, $n = 3$; MK10-SAL, $n = 1$), MK801-haloperidol (MK5-HAL, $n = 3$; MK10-HAL, $n = 1$).

Three hours after receiving the second injection, each rat was anesthetized with sodium pentobarbitone (60 mg/kg) and perfused transcardially with physiological saline followed by a fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer [PB], pH 7.4) at room temperature. The four animals of each cohort were injected, anesthetized, and perfused at the same time, and their brains processed using incubation buffers prepared in sufficient volume to handle the tissue from all four brains (cohort design, Mijster et al., 1996).

Immunocytochemistry

The brain of each rat was removed and postfixed for 1 h at room temperature in the same fixative. Each forebrain was blocked and cut into transverse sections (70 μ m) on a Vibratome. Alternate sections were incubated in 10% normal swine serum (NSS) in 0.01 M phosphate buffered saline with 0.25% Triton X-100 (PBS-Tx) for 30 min at room temperature with gentle shaking. After three rinses, sections were incubated in a 1:1,000 dilution of sheep anti-Fos antiserum (Genosys Biotechnologies Inc., Cambridge, UK) in PBS-Tx with 1% NSS added, at 4°C for 36 h. Following multiple rinses, sections were incubated in a 1:500 dilution of biotinylated donkey anti-sheep IgG in PBS-Tx with 1% NSS added for 2 h at room temperature. After rinsing, sections were incubated in a prepared avidin-biotin peroxidase complex (ABC reagent, Vector Laboratories, Bretton, Peterborough, UK) for 2 h at room temperature. Following three rinses in 0.05 M Tris-HCl, pH 7.6, sections were reacted for 25–30 min in 0.05% 3–3'-diaminobenzidine (DAB) with hydrogen peroxide added at a final concentration of 0.01%. Sections were then rinsed

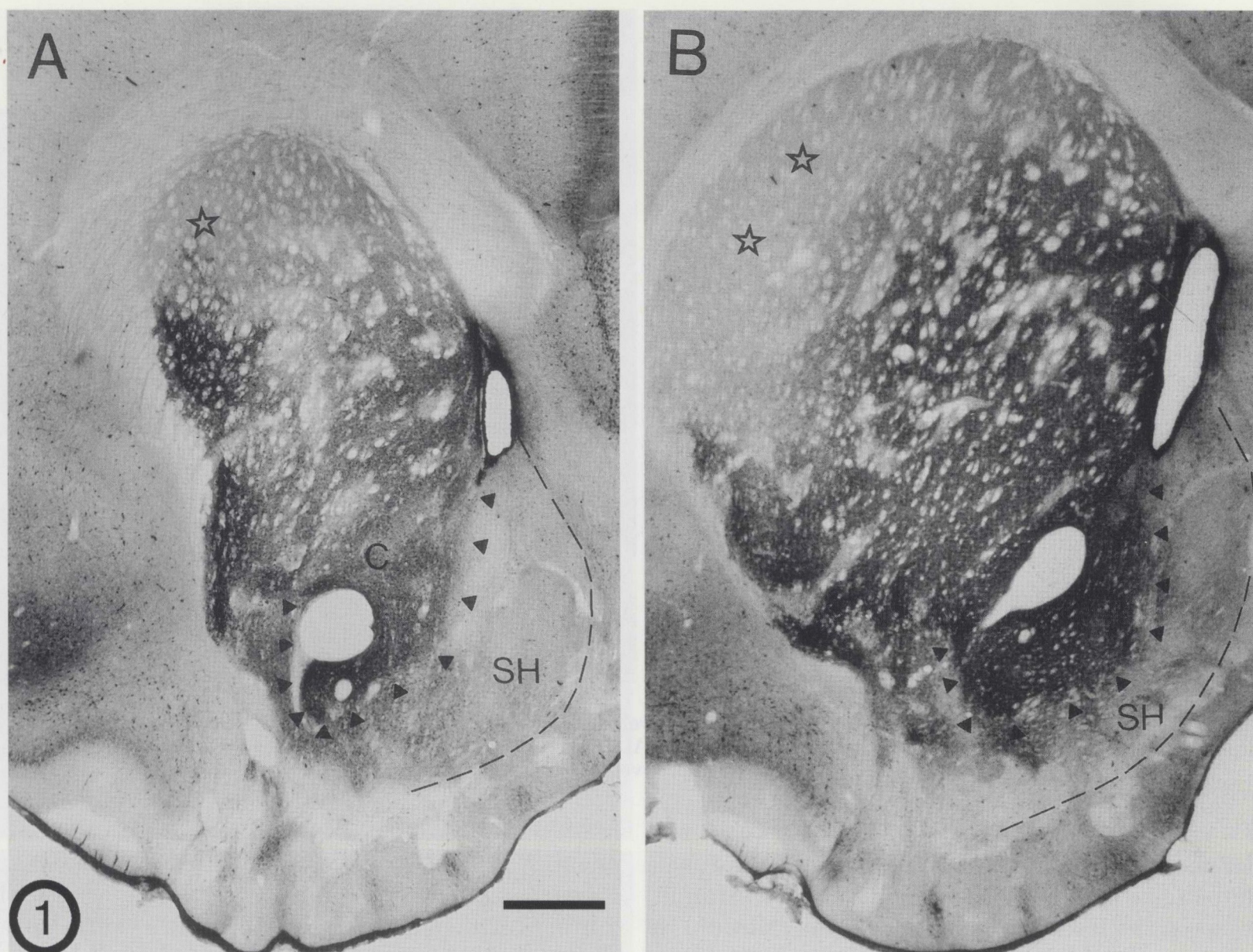


Fig. 1. Photomicrographs of (A) rostral and (B) caudal sections through the rat striatum showing CBP immunoreactivity. The boundary between the core (C) and the shell (SH) in nucleus accumbens is marked by filled arrowheads. The medial and ventral borders of this nucleus are outlined by dashed lines. The dorsolateral part of the striatum is marked by stars. Scale bar in A applies to B as well, and = 500 μ m.

and mounted onto slides from a 0.2% gelatin solution, dried, dehydrated, and coverslipped.

Alternate sections were processed for calcium binding protein D28k (CBP) immunocytochemistry. These sections were washed with 0.05M TBS, pH 7.6, with 0.5% Triton X-100 added (TBS-Tx), then incubated in 5% normal horse serum (NHS) in TBS-Tx at room temperature for 30 min. Sections were then incubated in a 1:8,000 dilution of mouse anti-CBP antiserum (Sigma-Aldrich, Dorset, UK) in TBS-Tx with 1% NHS added at 4°C for 36 h. Sections were rinsed and incubated in a 1:300 dilution of biotinylated horse anti-mouse IgG in TBS-Tx for 2 h with gentle agitation. After rinsing in TBS-Tx, sections were incubated in ABC for 2 h at room temperature. Sections were then reacted in DAB (0.05 M Tris-HCl, pH 7.6) with added hydrogen peroxide at a final concentration of 0.01% for 5–8 min. Sections were rinsed and mounted onto slides from a 0.2% gelatin solution, dried, dehydrated, and coverslipped.

Data analysis

To determine the distribution of Fos-IR neurons, sections through the striatum were drawn and cells were plotted onto these drawings with the aid of a camera lucida attached to a Nikon microscope. In order to demarcate the shell and core of nucleus accumbens, separate drawings of adjacent CBP-immunoreacted sections (Fig. 1A,B) were traced for each animal onto clear acetate sheets. As noted by others (Gerfen et al., 1985; Groenewegen et al., 1991), the dorsolateral CPu consistently immunostained weakly for CBP, and in nucleus accumbens the shell exhibited weak to moderate immunoreactivity, whereas the core was densely immunostained.

The most rostral quarter of nucleus accumbens is regarded as a distinct territory termed the rostral pole (Zahm and Brog, 1992; Zahm and Heimer, 1993). The dorsolateral quadrant of the CPu was isolated by drawing two lines at right angles to each other onto an

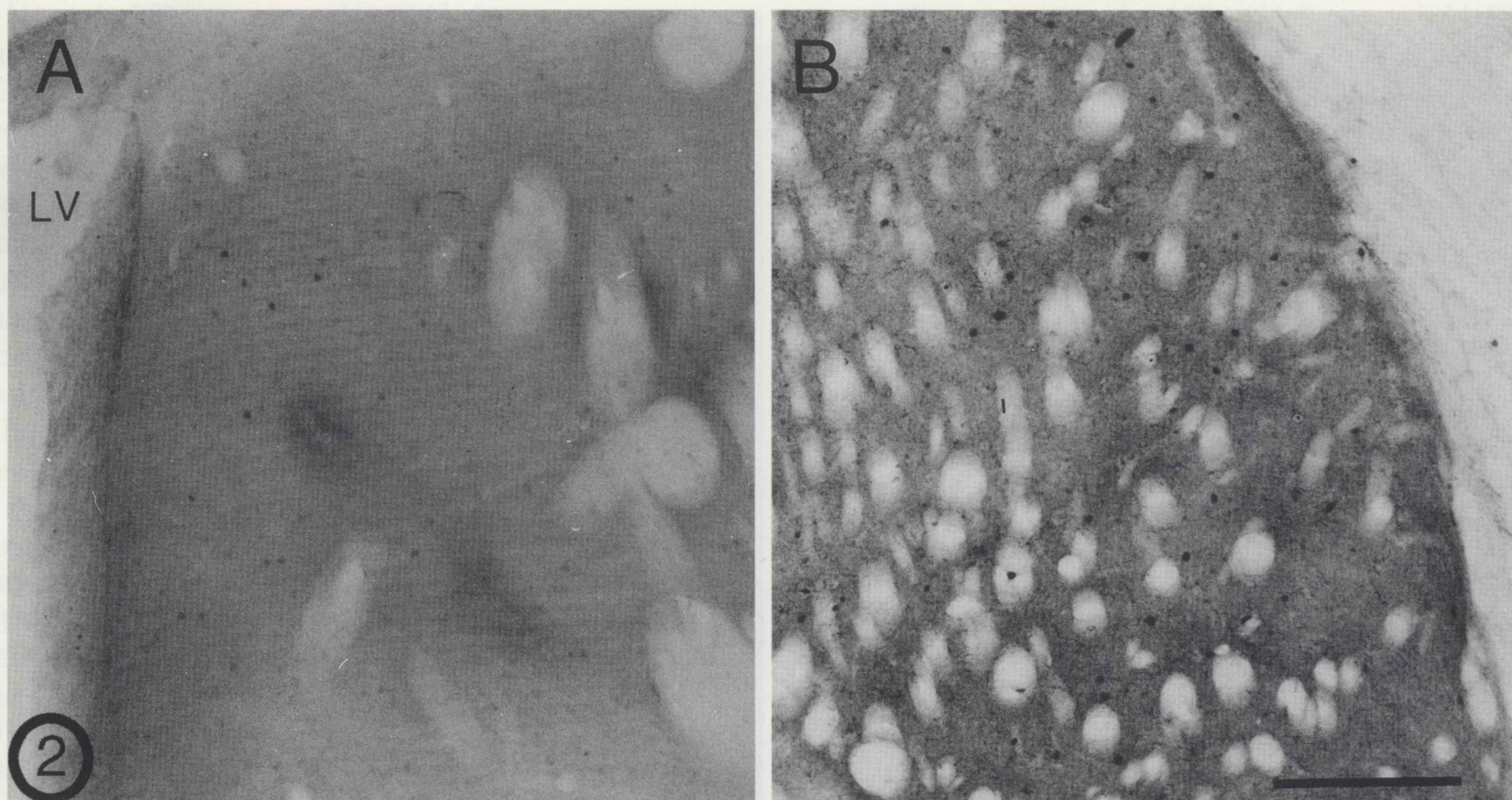


Fig. 2. Photomicrographs taken at the same level of the CPu. (A) Distribution of Fos-IR cells in the dorsomedial quadrant after saline injection (SAL-SAL). (B) Fos-IR cells in the dorsolateral quadrant in a rat treated with MK801 (MK5-SAL). LV = lateral ventricle. Scale bar in B applies to A as well, and = 200 μ m.

acetate sheet. The length of the horizontal line was calculated to be one-third the distance along a straight line between the lateral edge of the corpus callosum to the most dorsal point of the lateral ventricle; the vertical line was drawn at a right angle to the horizontal line and was calculated to be one-fourth the distance from the corpus callosum to the base of the brain. To control for plane of section and shrinkage, a set of lines delineating the quadrant was fitted separately for each brain section analyzed.

Acetate sheets delineating territorial divisions in the striatum were placed over the line drawings of the plotted sections, using blood vessels, prominent fiber bundles, and/or the lateral ventricle as alignment guides. The distribution of cells in each territory was digitized using a tablet attached to a computer. A software program (Macstereology, Ranfurly Microsystems, Renfrewshire, UK) was used to estimate the cross-sectional areas of the entire striatum and its restricted regions, i.e., the dorsolateral quadrant of the CPu and the shell, core, and rostral pole of nucleus accumbens (Figs. 1A,B, 2), and to analyze the density of Fos-IR cells. The Fos-IR cells along the entire rostro-caudal axis of nucleus accumbens and CPu (bregma +2.7 mm to +0.2 mm; Paxinos and Watson, 1986) were also reconstructed from a minimum of 12 sections. To achieve this, sections were aligned and rotated to produce a three-dimensional image of Fos-IR cells throughout the striatum.

Counts of Fos-IR cells, which were recorded at rostral and caudal levels of both dorsal and ventral parts of the

striatum (Fig. 1A,B), were expressed as cells/500 μ m² for each region (dorsolateral CPu, remainder CPu, shell and core of nucleus accumbens). A single central level at the rostral pole was also selected for analysis. For statistical tests, data were transformed by taking the log of the values before analysis (Matthews and Farewell, 1996). This resulted in a good correlation with the expected normal distribution ($r = 0.983$). To distinguish effects due to drug treatment (SAL-HAL vs. MK5-HAL), region and perfusion/immunohistochemical batches (cohorts), the data were analyzed using a general linear model design with repeated measures. In this way, values from individual animals were nested in treatment by cohort and region was entered as a random factor.

RESULTS

In the present study we examined the regional distribution of Fos-IR cells in the CPu (dorsolateral quadrant and remainder) and nucleus accumbens (rostral pole, shell, and core). We used sections immunoreacted for CBP that were adjacent to Fos-IR sections to demarcate nucleus accumbens' territories (Fig. 1A,B). In control animals treated with saline (SAL-SAL), a few Fos-IR cells were found in the dorsomedial part of the CPu (Fig. 2A) and scattered in nucleus accumbens, especially at the rostral pole (Fig. 3A). In rats treated with MK801 (MK5-SAL), cells appeared denser in dorsolateral (Fig. 2B) than in dorsomedial or ventral parts of the CPu, and were sparse in the core and shell

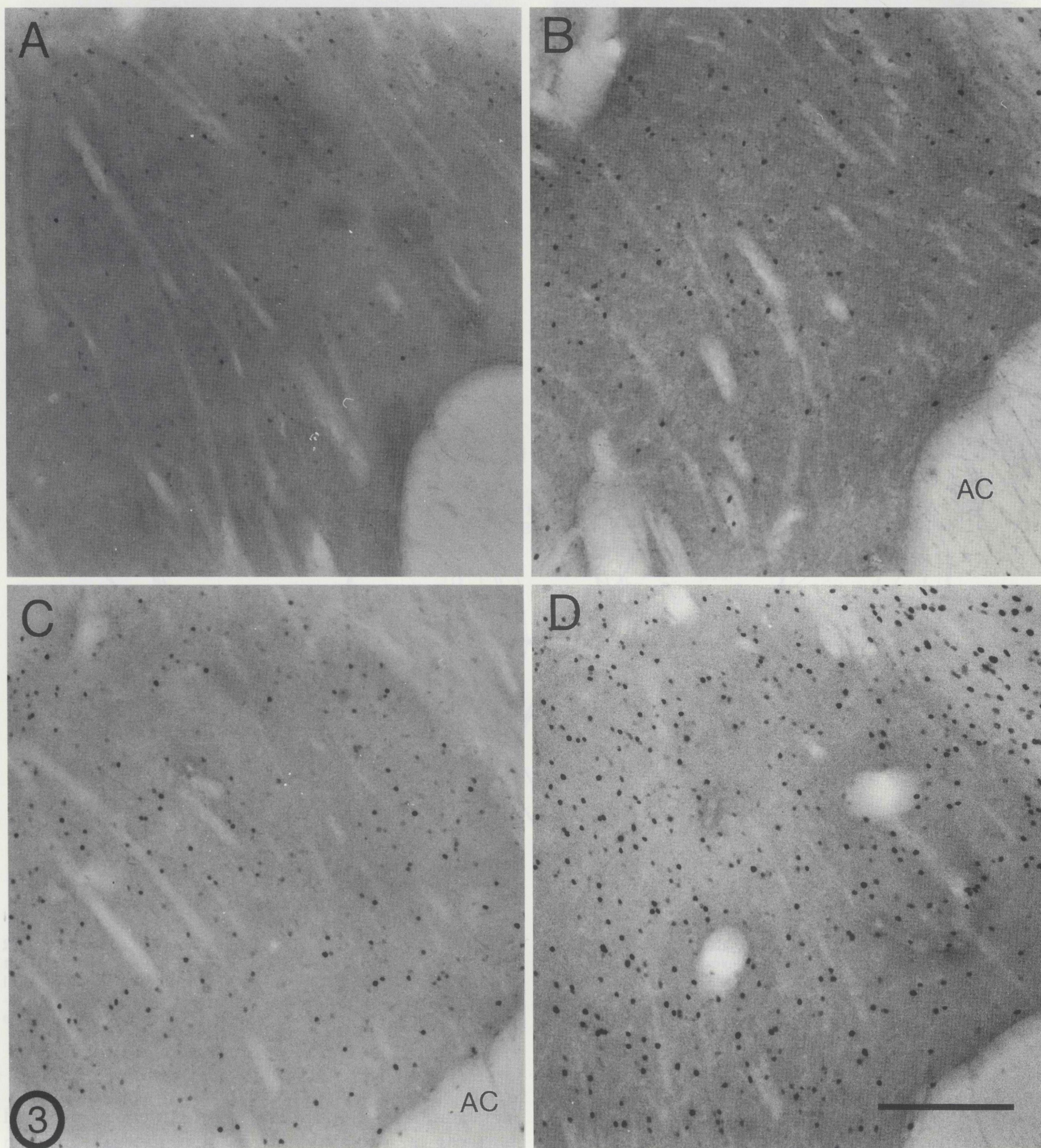


Fig. 3. Photomicrographs of Fos-IR cells in the rostral pole of the nucleus accumbens in rats treated with (A) saline (SAL-SAL), (B) MK801 (MK5-SAL), (C) haloperidol (SAL-HAL), and (D) MK801 followed by haloperidol (MK5-HAL). A comparison of C with D illustrates that Fos-IR cells are increased in number in rats pretreated with MK801. AC = anterior commissure. Scale bar in D applies to all panels, and = 200 μ m.

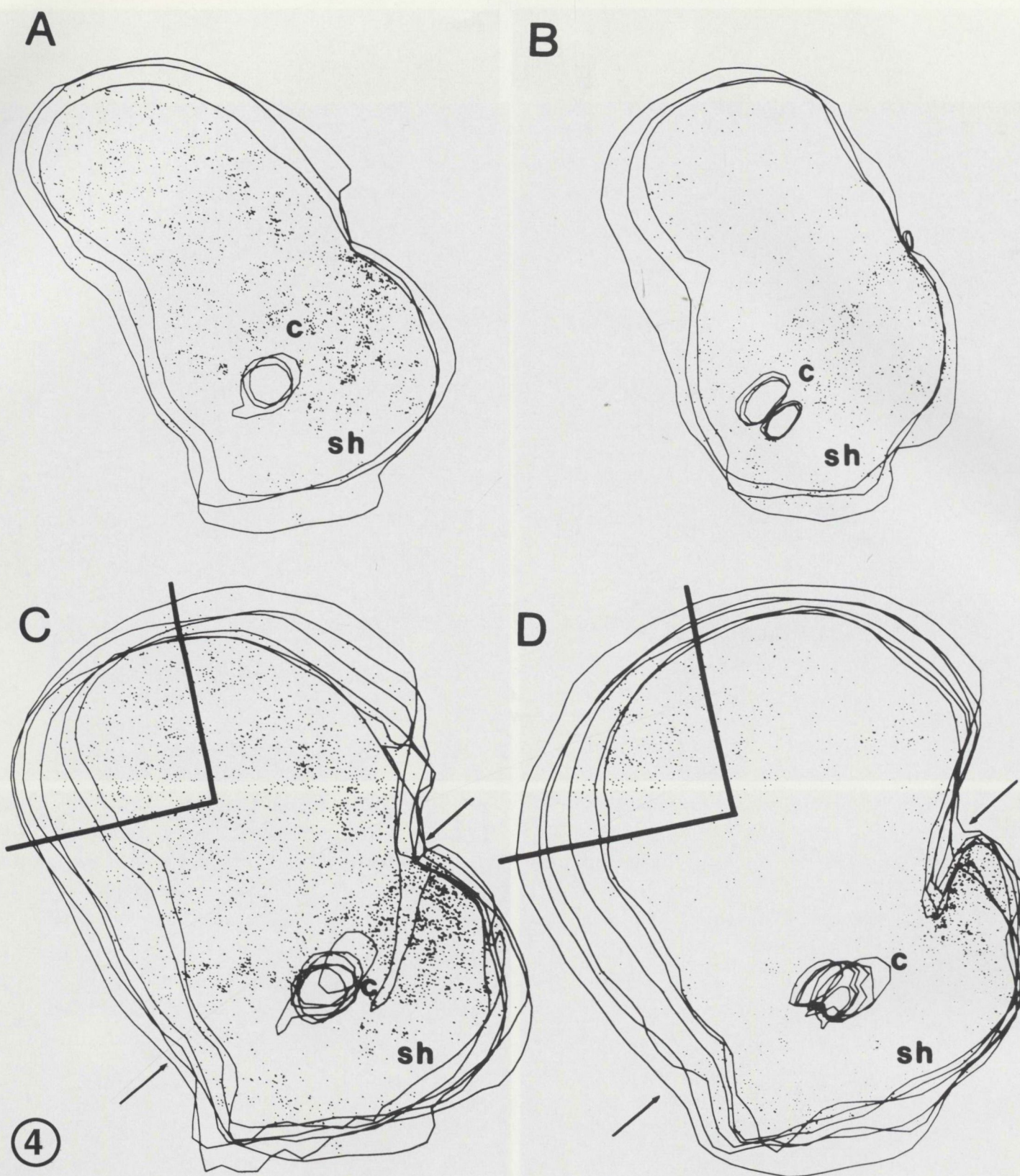


Fig. 4. Four digitized reconstructions of alternate sections, 70 μ m apart, showing rostral and caudal striatal levels (half brains, midline on the right) with Fos-IR cells (dots) plotted onto superimposed sections. (A,B) Three superimposed, rostral sections each. (C,D) Six serial, caudal sections each. The cell plots in A and C are in a haloperidol-treated rat and in B and D in a rat treated with MK5-HAL. Note the sparser distribution of Fos-IR cells in the MK5-HAL

treatment (compare B to A); note that at more caudal levels, Fos-IR cells are reduced in number throughout the CPu (compare D to C) but not in nucleus accumbens. C = core of nucleus accumbens; SH = shell of nucleus accumbens. Long arrows delineate the approximate boundary between nucleus accumbens and CPu. Thick black lines outline the borders of the dorsolateral quadrant of the CPu.

of nucleus accumbens. Fos-IR cells appeared to be densest at the rostral pole (Fig. 3B).

Effects of MK801 on haloperidol-induced changes in Fos immunoreactivity

In rats treated with haloperidol (SAL-HAL) or with MK801 followed by haloperidol administration (MK5-HAL), Fos-IR cells were distributed densely but heterogeneously throughout dorsal and ventral parts of the

striatum (Fig. 4A–D). The reconstructions revealed patterns of cell distributions that differed between treatments but not between animals receiving the same treatment. The Fos-IR cells in the CPu appeared less dense in MK5-HAL animals (Fig. 4B,D) than in SAL-HAL brains (Fig. 4A,C); this difference was particularly evident in the dorsomedial part of the CPu (Fig. 5A,B). In nucleus accumbens, however, changes in density were less evident, except in the caudal core where cells

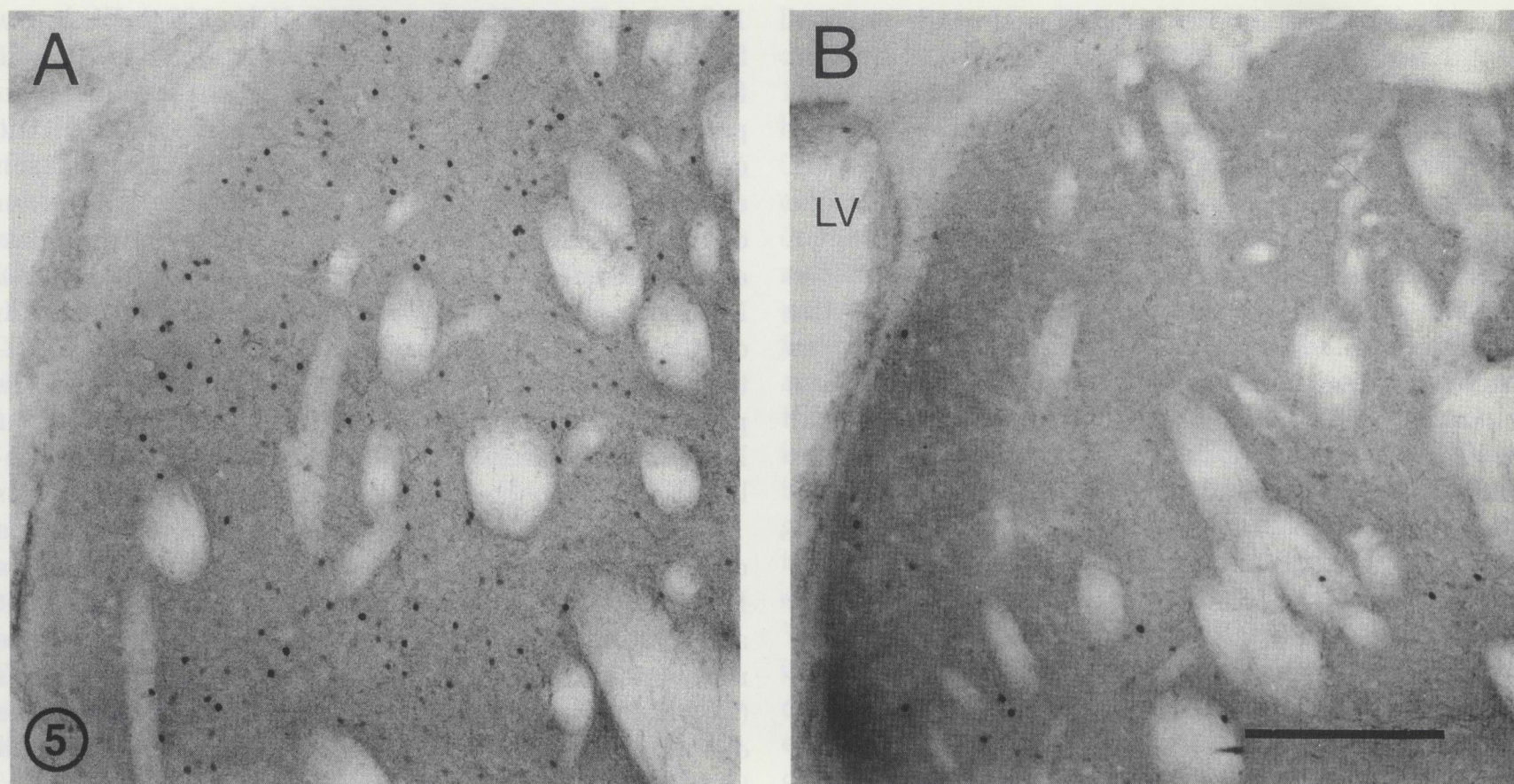


Fig. 5. Photomicrographs taken at the same level of the dorsomedial striatum demonstrating Fos-IR cells: (A) after haloperidol treatment (SAL-HAL); (B) pretreatment with MK801 followed by haloperidol treatment (MK5-HAL). A comparison of A with B shows that Fos-IR cells are reduced in density in pretreated rats. LV = lateral ventricle. Scale bar in B applies to A as well, and = 200 μ m.

TABLE I. Effects of MK801 pretreatment on the haloperidol induction of Fos-IR cells in caudate/putamen (mean cell density $\pm$ s.e.m./500 μ m²)

Region	Haloperidol	MK801/ haloperidol
Caudate/putamen	58.68 $\pm$ 8.14	33.05 $\pm$ 4.33*
Rostral dorsolateral quadrant	73.18 $\pm$ 26.37	37.43 $\pm$ 8.56
Caudal dorsolateral quadrant	77.01 $\pm$ 23.5	37.8 $\pm$ 6.73
Rostral CPu (Bregma + 2.2 mm)	61.06 $\pm$ 15.61	46.56 $\pm$ 13.42
Midrostral CPu (Bregma + 1.2 mm)	43.20 $\pm$ 12.43	24.33 $\pm$ 8.31
Midcaudal CPu (Bregma + 0.2 mm)	38.97 $\pm$ 9.25	19.33 $\pm$ 7.14

* $p < 0.005$; significantly different from SAL-HAL treatment.

TABLE II. Effects of MK801 pretreatment on the haloperidol induction of Fos-IR cells in nucleus accumbens (mean cell density $\pm$ s.e.m./500 μ m²)

Region	Haloperidol	MK801/ haloperidol
Nucleus accumbens	49.39 $\pm$ 6.8	55.25 $\pm$ 8.68
Rostral pole (Bregma + 2.7 mm)	60.11 $\pm$ 17.31	74.65 $\pm$ 18.86
Rostral core (Bregma + 1.7 mm)	70.54 $\pm$ 21.62	88.9 $\pm$ 23.63
Caudal core (Bregma + 1.0 mm)	34.9 $\pm$ 9.67	24.33 $\pm$ 10.87
Rostral shell (Bregma + 1.7 mm)	51.35 $\pm$ 12.01	53.49 $\pm$ 11.39
Caudal shell (Bregma + 1.0 mm)	30.06 $\pm$ 6.06	34.86 $\pm$ 5.69

appeared less dense in MK5-HAL (Fig. 4D) than in SAL-HAL (Fig. 4C) treated animals and in the medial shell where immunopositive cells appeared as dense rostrally (Fig. 4A,B) as caudally (Fig. 4C,D).

Densities of Fos-IR cells were calculated for both CPu and nucleus accumbens for all rats treated with SAL-HAL and MK5-HAL. In the CPu, measurements were taken at three rostrocaudal levels; in the dorsolateral quadrant, densities were calculated at two levels (Table I). In nucleus accumbens, density measurements were carried out at the rostral pole and at one rostral and one caudal level of the shell and of the core (Table II). There was no statistically significant inter-animal variation for the CPu ($P = 0.97$) or for nucleus accumbens ($P = 0.4$). However, there was a significant regional effect in the CPu ($F = 16.275$, $df = 4,19$, $P = 0.0001$; for region designations, see Table I). With haloperidol treatment, Fos-IR cell density was found to be greatest at rostral

levels of the CPu, but declined by approximately 35% further caudally (Table I). However, the cell density in the dorsolateral quadrant of SAL-HAL-treated animals did not decrease between rostral and caudal levels (Table I; compare Fig. 4A and C). With MK5-HAL treatment, the density of Fos-IR cells decreased at caudal as compared to rostral levels of all regions in the CPu (Table I).

There was also a marked effect due to region in nucleus accumbens ($F = 21.335$, $df = 4,19$, $P = 0.0001$; for region designations, see Table II). Rostral cell density declines with haloperidol treatment by 50% in the caudal core and by 40% in the caudal shell (Table II). With MK5-HAL treatment, the density of Fos-IR cells also decreased at caudal as compared to rostral levels of nucleus accumbens' territories. The decrease was particularly evident in the core, where the density declined by 73% over the rostro-caudal axis (Table II).

Statistical tests showed that there was a marked effect due to treatment in the CPu (Fig. 5A,B), in that Fos-IR cell density was significantly decreased with MK5-HAL treatment as compared to that with SAL-HAL ($F = 761.31$, $df = 1,19$, $P = 0.0013$; Table I). However, this difference was not evident for any one region, although a trend towards a treatment/region interaction between the three principal levels of the CPu ($P < 0.08$) but not for the two levels of the dorsolateral quadrant ($P = 0.2$), was found. In nucleus accumbens, there was no effect due to treatment; the density of Fos-IR cells was not reduced significantly by MK801 pretreatment for any territory (Table II). There was, however, a trend towards an interaction between region and treatment ($P < 0.07$). Fos-IR cell density at the rostral pole was increased by 24% in MK5-HAL as compared to SAL-HAL-treated rats (Table II; compare Fig. 3C and D). The cell density in the rostral core of pretreated rats was also elevated over that in SAL-HAL rats. No change was evident in the shell. In the caudal core, the mean Fos-IR cell density decreased, but not significantly, in pretreated as compared to SAL-HAL rats (Table II).

In striatal sections taken from animals treated with the higher dose of MK801 (10 mg/kg; MK10-HAL), neither the pattern nor the density of Fos-IR cells differed from those found in animals injected with the low dose (5 mg/kg; MK5-HAL).

DISCUSSION

In the present study, we found that treating animals with haloperidol resulted in large increases in Fos-IR cells in the CPu and in all parts of nucleus accumbens. When we administered the noncompetitive NMDA receptor antagonist MK801 before haloperidol, we found the density of Fos-IR cells to be significantly reduced in the CPu but not in any territory of nucleus accumbens. These findings clarify and extend previous observations for the CPu (Dragunow et al., 1990; Ziolkowska and Höllt, 1993), but more importantly, they illustrate for the first time that the haloperidol induction of Fos is dependent on the activation of NMDA receptors in a regionally specific manner.

By reconstructing Fos-IR cell distributions along the rostrocaudal axis of the striatum, we consistently found the same pattern of cell distributions across experiments. When we compared rostral and caudal levels for each treatment, however, a regional pattern emerged. With statistical comparisons, we found a marked effect for SAL-HAL and MK5-HAL groups due to treatment and region, but not to animal. The latter presumably means that with the cohort design (Mijnster et al., 1996) we controlled adequately for individual animal differences. The significant regional effects are not surprising in light of heterogenous density of D2 and NMDA receptors in dorsal and ventral striata (Dubois and Scatton, 1985; Boyson et al., 1986; Maragos et al.,

1988; Bardo and Hammer, 1991; Rêlo, 1994). We know, for example, that the higher numbers of Fos-IR cells following haloperidol administration at rostral as compared to caudal levels of the CPu correlate well with the density of D2 receptors (Joyce et al., 1985; Robertson and Fibiger, 1992), as presumably does the rostrocaudal decline in cell density that we found in nucleus accumbens (Bardo and Hammer, 1991; Rêlo, 1994). Moreover, the decline in Fos-IR cells along the rostrocaudal axis of both dorsal and ventral striata in MK5-HAL-treated animals could also reflect the decrease in Fos protein evident with D2 blockade but may be related, at least in part, to NMDA receptor distribution (see below).

In relation to SAL-HAL treatment, there is some question as to whether haloperidol treatment elevates Fos in all territories of nucleus accumbens (Semba et al., 1996). While this drug reportedly increases the numbers of Fos-IR neurons throughout the nucleus (Robertson and Fibiger, 1992, and present results), the *c-fos* gene seems to be upregulated only in the shell (Semba et al., 1996). The reason for this discrepancy is unclear. It could lie in the difference between *in situ* hybridization and immunohistochemical studies, but a more likely explanation relates to how the data were gathered. We calculated cell density for the entire core at rostral and caudal levels, whereas Semba et al. (1996) measured changes in a very restricted ventrolateral part of the core, a region where we, too, found few cells with treatment (unpublished observations). Since the induction of Fos appears to be dependent on the location and density of D2 receptors, it seems important to examine Fos-IR cell densities for entire regions (present results) or to sample areas widely (see Chapman and Zahm, 1996) rather than to select a single, small zone for analysis (Semba et al., 1996).

The present results show that MK801 administration did significantly decrease the density of Fos-IR neurons induced by haloperidol treatment in the CPu. These data contrast with those of Dragunow et al. (1990), who showed that Fos-IR cell density in the CPu could not be attenuated with MK801 unless a very high dose (10 mg/kg) of MK801 was administered. Such a dose may have been toxic, since maximal effects are reached at a much lower dosage (Ziolkowska and Höllt, 1993). The reason for the discrepancy between our results and those of Dragunow et al. (1990) is unclear, but could be reflected in the regional, rather than restricted, manner, respectively, in which these analyses were conducted. Although insignificant in tests, the present work showed an interaction between treatment and region suggesting that the selection of rostro-caudal level to be investigated may be important. Furthermore, the inhomogeneous distribution of NMDA receptors in the striatum (Maragos et al., 1988) could be directly related to the sites at which these receptors can modulate Fos expression. The fact that MK801 is able

to attenuate the haloperidol induction of Fos in the CPu, however, suggests that the actions of haloperidol are dependent, at least in part, on glutamate acting at the NMDA receptor (Tarazi et al., 1996). The blockade of D2 receptors presynaptically on corticostriatal terminals is known to stimulate glutamate efflux (Mitchell and Doggett, 1980; Garside et al., 1996), and chronic haloperidol treatment raises levels of extracellular glutamate (Yamamoto and Cooperman, 1994; See and Chapman, 1994). Therefore, early antagonism of the NMDA receptor may be important for preventing such increases, at least in the CPu.

In the nucleus accumbens, MK801 failed to block the haloperidol-induced increase in Fos. Indeed, here NMDA antagonism preceding haloperidol administration brought about an increase, although insignificant in tests, in the mean number of Fos-IR cells at the rostral pole and rostral core but little change in other parts. This may mean that elevations in glutamate with neuroleptic blockade do not involve the NMDA receptor in the ventral striatum or these receptors are located on different cells from those with D2 receptors. Nevertheless, NMDA receptors are thought to be involved in dopaminergic neurotoxicity brought about by methamphetamine (Ohmori et al., 1993). Nucleus accumbens is innervated by glutamatergic fibers originating in the hippocampus, amygdala, prefrontal cortex, and thalamus (Groenewegen et al., 1987; Berendse et al., 1992; Berendse and Groenewegen, 1990; Zahm and Brog, 1992; Brog et al., 1993; Wright and Groenewegen, 1996) but glutamate neurotransmission is mediated primarily by AMPA/kainate receptors (Pennartz, 1990; Meredith et al., 1993). Under conditions of decreased inhibition, however, NMDA receptors are activated (Pennartz, 1990). Classical neuroleptic drugs seem to increase rather than decrease inhibition in nucleus accumbens by elevating GABA transmission (Johnson et al., 1994), which may explain why NMDA antagonism here has little effect on the haloperidol-induction of Fos.

There is a greater density of NMDA receptors in nucleus accumbens as compared to the CPu (Maragos et al., 1988). This may explain why pretreatment with MK801 elevates rather than decreases the number of Fos-IR cells over haloperidol treatment at the rostral pole and in the rostral core. The fact that MK801 pretreatment does not reduce Fos anywhere in nucleus accumbens could reflect NMDA receptor localization, i.e., receptors are not located where they can be influenced by D2 blockade. In the CPu, the glutamatergic input to striatal principal neurons is primarily onto the heads of spines and the dopaminergic input onto their necks (Freund et al., 1984). This arrangement is a potential site for balanced interactions between the two inputs and for enhanced glutamate release if presynaptic D2 receptors on nigrostriatal terminals are blocked. In nucleus accumbens, however, other relationships

between these two inputs have been described, especially in the shell (Zahm, 1992; Totterdell and Smith, 1989; Meredith et al., 1993). The rostral pole has not been examined. Recent work by Gracy and Pickel (1996) found the R1 subunit of the NMDA receptor to be colocalized with tyrosine hydroxylase — presumably dopaminergic — axons in the shell. Although there is no anatomical evidence for presynaptic contacts in any part of the striatum, there is pharmacological evidence for such action. If NMDA receptors are located presynaptically on dopaminergic terminals, as Gracy and Pickel (1996) find, they will not be in a position to influence the effects of D2 blockade. The arrangement in the core or at the rostral pole has yet to be investigated. Certainly, additional studies are needed to ascertain where D2 and NMDA receptors interact within cells in the ventral striatum.

Differences in the dependence of the NMDA receptor in the haloperidol activation of Fos may have functional consequences for both the desirable and undesirable effects of antipsychotic drug treatment. Extrapyramidal syndromes develop with chronic administration of classical neuroleptics (Jaber et al., 1996), and Meshul et al. (1996a) and Mijster et al. (1996) hypothesized that morphological change may underlie these abnormal movements. If these syndromes are brought about by the deleterious effects of glutamate on striatal neurons (Meshul et al., 1996b), then it is important to locate the site(s) where these effects may occur. The dorsolateral CPu, important for motor control, has been suggested as a likely place for the development of these syndromes (Roberts et al., 1995; Meshul et al., 1996a), but others (Prinssen et al., 1994; Egan et al., 1994) have suggested that nucleus accumbens is important. The present results point to the CPu as a likely site for glutamatergic stimulation of NMDA receptors, data that are in agreement with earlier work by Meshul et al. (1994), who showed that NMDA antagonists can attenuate haloperidol-induced synaptic changes in the dorsolateral striatum. Although NMDA channels permit rapid rises in calcium which can alter striatal neurons (Rothman et al., 1987; Choi, 1988), calcium-permeable AMPA receptors have an even greater potential to be neurotoxic if they are activated for prolonged periods (Choi, 1992; Lu et al., 1996). The latter receptors are readily activated in nucleus accumbens (Meredith et al., 1993) and it seems important, therefore, to assess their role in haloperidol's action in ventral striatal regions where antipsychotic action appears to be NMDA-independent.

ACKNOWLEDGMENTS

We thank Mr. Peter Kelleghan for technical assistance, Dr. Ronan Conroy for advice and help with the statistical measures, RCSI Media Services for photographic assistance, and Prof. B.L. Roberts for comments on the manuscript.

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