

DOPAMINE-GLUTAMATE INTERACTIONS
IN THE STRIATUM

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DOPAMINE-GLUTAMATE INTERACTIONS
IN THE STRIATUM

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ABSTRACT

The striatum is part of a neural feedback network that modifies the functioning of the cerebral cortex. The importance of the striatum is underlined by the clinical consequences of striatal dysfunction: disordered signaling in the striatum gives rise to the clinical syndrome of Parkinson's disease, while degeneration of striatal output neurons produces the clinical manifestations of Huntington's disease.

The striatum is a complex structure comprised of two major populations of neurons: the spiny projection neurons that carry the striatal output to other nuclei in the basal ganglia; and several subtypes of aspiny cells that project locally within the striatum to modify striatal output. The two major inputs to the striatum are the glutamatergic pathway from the cerebral cortex and the dopaminergic pathway from the substantia nigra. The goal of my research is to explore the nature and functional significance of dopamine-glutamate signaling and its role in the striatum and basal ganglia.

My first series of studies *in vivo* demonstrated that altering dopaminergic tone in the striatum by D2-dopamine receptor blockade or by 6-hydroxy-dopamine lesion of the nigrostriatal dopamine projection in the rat could modify the pathological, neurochemical and behavioural consequences of glutamate-receptor-mediated-stimulation. In order to investigate the details of this interaction, I developed an *in vitro* tissue culture system. I showed initially that the growth of striatal neurons in serum free culture parallels their *in vivo* development. I then went on to use this *in vitro* system to demonstrate the differential effects of selective glutamate receptor agonists on transmitter release from subpopulations

of the two major classes of striatal neurons: i) those in which somatostatin and neuropeptide Y are colocalised with nitric oxide and ii) the substance P-containing spiny projection neurons. This series of studies demonstrated that substance P release was selectively stimulated through the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor while somatostatin and neuropeptide Y release were selectively provoked by stimulation of the kainate receptor. Stimulation of the metabotropic glutamate receptor had little effect on the release of any of the three peptides.

My final series of experiments examined the differential effects of selective dopamine receptor stimulation on glutamate-receptor-induced release of substance P, somatostatin and neuropeptide Y. The D1 agonist SKF 38393, and to a significantly lesser extent the D2 agonist quinpirole, attenuated glutamatergic release of substance P from the spiny neurons. In contrast, the D2 agonist quinpirole potentiated the release of neuropeptide Y and somatostatin from aspiny neurons. The D1 agonist SKF 38393 attenuated glutamate receptor stimulated release of neuropeptide Y, without significantly affecting the release of somatostatin from the same cultures. This latter result indicates that dopamine can differentially regulate transmitter release not only from separate populations of striatal neurons but also differentially control release of transmitter that are colocalised within a single population of neurons.

To my knowledge these studies are the first to demonstrate this differential regulation in the striatum, and implies that the delicate balance required for both normal cognition and movement may be intimately related to the balance of signaling between the intrinsic (somatostatin-neuropeptide Y-containing) and extrinsic (substance P-containing) neuronal populations in the striatum.

ACKNOWLEDGEMENTS

There is a tide in the affairs of man,
Which taken at the flood, leads on to fortune;
Omitted, all the voyage of their life
Is bound in shallows and in miseries.

Shakespeare, Julius Caesar

The writing of these acknowledgements is a task that I have often thought would never come to pass. And it would never have been possible without the input from many people, a few I would like to mention especially.

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TABLE OF CONTENTS

PRELIMINARIES.....	I
DESCRIPTIVE NOTEII
ABSTRACT.....	..III
ACKNOWLEDGEMENTS.....	..V
TABLE OF CONTENTS.....	..VI
LIST OF ILLUSTRATIONS.....	..XII
CHAPTER 1: SUMMARY OF AIMS AND RATIONALE.....	1
CHAPTER 2: BACKGROUND LITERATURE.....	3
2.1 THE STRIATUM AND BASAL GANGLIA: ANATOMICAL CONNECTIONS	3
2.2 AFFERENT PROJECTIONS TO THE STRIATUM	5
<i>A. Glutamatergic Afferents.....</i>	<i>5</i>
i) Topography.....	5
ii) Glutamate receptors	8
iii) Glutamate-Mediated Excitotoxicity.....	12
<i>B. Dopaminergic Afferents.....</i>	<i>16</i>
i) Topography.....	16
ii) DA receptors.....	19
2.3 INTRINSIC ANATOMY OF THE STRIATUM	21
<i>A. Medium-sized Spiny Neurons.....</i>	<i>21</i>
i) Extrinsic afferents.....	23
ii) Axon collaterals of medium spiny neurons.....	25
<i>B. Aspiny Neurons.....</i>	<i>26</i>
i) Large aspiny cholinergic interneurons.....	26
ii) Medium-sized aspiny SS/NPY/NOS containing interneurons.....	28
iii) GABA interneurons.....	29

2.4 THE FUNCTIONS OF THE STRIATUM: LESSONS FROM THE CLINIC	30
A. <i>Consequences of Striatal Dysfunction for Movement</i>	30
B. <i>Consequences of Striatal Dysfunction on Cognition</i>	35

**CHAPTER 3: DOPAMINE-GLUTAMATE INTERACTIONS IN THE STRIATUM:
BEHAVIOURALLY RELEVANT MODIFICATION OF EXCITOTOXICITY BY
DOPAMINE-RECEPTOR-MEDIATED MECHANISMS..... 38**

3.1 ABSTRACT	39
3.2 INTRODUCTION.....	41
3.3 EXPERIMENTAL PROCEDURES.....	42
A. <i>Unilateral Striatal Lesions</i>	42
B. <i>Bilateral Striatal Lesions</i>	45
3.4 RESULTS	47
A. <i>Unilateral Lesions</i>	47
B. <i>Bilateral Striatal Lesions</i>	49
3.5 DISCUSSION	51
<i>Dopamine-Glutamate Interactions in the Striatum: Anatomical Considerations</i>	52
<i>Functional Interactions Between Dopamine and Glutamate in the Striatum:</i>	53
<i>Significance</i>	56
3.6 REFERENCES	58
3.7 FIGURES.....	69
<i>Table 1</i>	75
<i>Table 2</i>	76

CHAPTER 4: THE ONTOGENY OF NADPH-DIAPHORASE NEURONS IN SERUM-FREE STRIATAL CULTURES PARALLELS IN VIVO DEVELOPMENT	77
4.1 ABSTRACT	78
4.2 INTRODUCTION.....	79
4.3 METHODS.....	80
<i>A. In Vitro Studies</i>	80
<i>B. In Vivo Preparation</i>	83
4.4 RESULTS	84
4.5 DISCUSSION	87
<i>Conclusions</i>	90
4.6 REFERENCES	91
4.7 FIGURES.....	96
4.8 PHOTOGRAPH LEGEND.....	100
<i>Table 1</i>	105
<i>Table 2</i>	106

CHAPTER 5: THE ROLE OF GLUTAMATE RECEPTOR SUBTYPES IN THE DIFFERENTIAL RELEASE OF SOMATOSTATIN, NEUROPEPTIDE Y AND SUBSTANCE P IN PRIMARY SERUM-FREE CULTURES OF STRIATAL NEURONS	107
5.1 ABSTRACT.....	108
5.2 INTRODUCTION.....	109
5.3 METHODS.....	110
<i>A. Striatal culture method</i>	110
<i>B. Immunohistochemistry</i>	110
<i>C. Protocol for the peptide release experiments</i>	111
<i>D. Radioimmunoassay</i>	112
<i>E. Data analysis</i>	113
5.3 RESULTS	113
<i>A. Immunohistochemistry</i>	113

<i>B. Evoked release of SS, NPY and SP</i>	114
5.4 DISCUSSION	115
5.6 BIBLIOGRAPHY	118
5.7 FIGURES.....	121
CHAPTER 6. DOPAMINE-GLUTAMATE INTERACTIONS IN PRIMARY SERUM-FREE CULTURES OF STRIATAL NEURONS	126
6.1 ABSTRACT	127
6.2 INTRODUCTION.....	128
6.3 METHODS.....	129
<i>A. Striatal culture method</i>	129
<i>B. Release experiments</i>	130
<i>C. Radioimmunoassay</i>	130
<i>D. Data analysis</i>	131
6.4 RESULTS	131
6.5 DISCUSSION	133
6.6 REFERENCES	138
6.7 FIGURES.....	142
CHAPTER 7. DISCUSSION.....	147
BIBLIOGRAPHY.....	151

LIST OF ILLUSTRATIONS

CHAPTER 2.

<i>Figure 1. Circuit diagram of the normal basal ganglia.</i>	4
<i>Figure 2. Normal cortex: laminar distribution of neurotransmitters and neuropeptides.</i>	6
<i>Figure 3. Topography of cortico-striatal projection. (Adapted from McGeorge and Faull, 1989.)</i>	7
<i>Figure 4. Diagrammatic representation of the NMDA receptor (top) and different classes of metabotropic receptor (bottom).</i>	10
<i>Figure 5. Possible mechanisms involved in excitotoxic cell death.</i>	14
<i>Figure 6. Topography of the nigrostriatal pathway in the rat. (Adapted from Moore and Fallon, 1978.)</i>	18
<i>Figure 7. Synaptic organization of medium spiny neuron.</i>	23
<i>Figure 8. Basal ganglia circuit diagram depicting changes associated with Parkinson's disease.</i>	32
<i>Figure 9. Basal ganglia circuit diagram depicting changes associated with Huntington's disease.</i>	35

CHAPTER 3.

<i>Figure 1. Range of unilateral striatal lesions in the different treatment groups.</i>	69
<i>Figure 2. Unilateral striatal lesion: morphometric analyses.</i>	70
<i>Figure 3. Unilateral striatal lesion: neurochemical analyses.</i>	71
<i>Figure 4. Range of bilateral striatal QUIN lesions in the different treatment groups.</i>	72
<i>Figure 5. Bilateral striatal lesions: behavioural testing.</i>	73
<i>Figure 6. Bilateral striatal lesions: neurochemical analyses.</i>	74

CHAPTER 4.

<i>Figure 1. Time course of in vivo and in vitro expression of NADPH-d positive striatal neurons.....</i>	<i>96</i>
<i>Figure 2. Total number of neurons per coverslip in primary striatal cultures from days-in-vitro 1-16.....</i>	<i>97</i>
<i>Figure 3. Camera lucida drawings of the three morphological subtypes of in vivo and in vitro NADPH-d positive neurons.....</i>	<i>98</i>
<i>Figure 4. Typical NADPH-d positive neurons from DIV 5 to 7.....</i>	<i>101</i>
<i>Figure 5. Typical NADPH-d positive neurons from DIV 11-21.....</i>	<i>102</i>
<i>Figure 6. Development and topography of NADPH-d neurons in vivo from E18 to day 14 (equivalent to DIV 18).....</i>	<i>99</i>
<i>Figure 7. Typical NADPH-d positive neurons in vivo</i>	<i>103</i>
<i>Figure 8. Single cultured striatal neuron co-labelled for somatostatin and neuropeptide Y</i>	<i>104</i>

CHAPTER 5.

<i>Figure 1. Single cultured striatal neuron co-labelled for somatostatin and neuropeptide Y</i>	<i>121</i>
<i>Figure 2. Single cultured striatal neuron expressing immunoreactivity for substance P</i>	<i>122</i>
<i>Figure 3. Typical photo of primary neuronal striatal culture</i>	<i>123</i>
<i>Figure 4. Comparative release of SS, NPY, and SP after incubation with PBS, KCL, or various glutamatergic agonists.....</i>	<i>124</i>
<i>Figure 5. Total immunoreactive SS (top), NPY (middle) and SP (bottom) from each of the experimental groups.....</i>	<i>125</i>

CHAPTER 6.

<i>Figure 1. Dose response of quinpirole and SKF 38393 induced release of NPY and SS</i>	<i>142</i>
<i>Figure 2. Effect of D1 and D2 receptor agonists on kainate-induced release of SS, NPY, SP</i>	<i>143</i>
<i>Figure 3. Effect of D1 and D2 receptor agonists on NMDA-induced release of SS, NPY, SP</i>	<i>144</i>
<i>Figure 4. Effect of D1 and D2 receptor agonists on QUIS-induced release of SS, NPY and SP</i>	<i>145</i>

CHAPTER 1: SUMMARY OF AIMS AND RATIONALE

The striatum is the largest and most important structure in the basal ganglia, the function of which is to act as a feedback circuit for the cerebral cortex. Dysfunction of the striatum, such as occurs in Huntington's disease and Parkinson's disease, results in characteristic impairments of movement and cognition. The major inputs to the striatum are the glutamatergic pathway from the cortex and the dopaminergic pathway from the substantia nigra compacta. There are two major subtypes of neurons in the striatum: the spiny projection neurons and the aspiny interneurons. A considerable amount of research has been devoted to the study of the spiny projection neurons, however, there is little understanding of the aspiny interneurons, and in particular the subpopulation of cells containing somatostatin and neuropeptide Y.

The **goal** of this thesis is to study the actions and interactions of glutamate and dopamine in the modulation of striatal function with particular emphasis on the aspiny somatostatin/neuropeptide Y-containing neurons.

The **specific aims** are :

1. To study the ability of a dopamine receptor antagonist to modify glutamate-receptor mediated excitotoxicity in the striatum *in vivo*.
2. To develop an *in vitro* system to permit the study of neurotransmitter release from serum-free striatal cultures.

3. To characterize the ontogeny of the somatostatin/neuropeptide Y/nitric oxide synthase-containing subpopulation of striatal neurons in the culture system, and to compare this with the ontogeny of this same cell population *in vivo*.

4. To characterize the differential effects of selective glutamate receptor agonists on neurotransmitter release from two subpopulations of cultured striatal neurons: the somatostatin/neuropeptide Y-containing aspiny interneurons and the substance P-containing spiny projection neurons.

5. To study the differential effects of selective DA receptor agonists on somatostatin, neuropeptide Y and substance P release *in vitro*; and to analyze the ability of selective DA receptor agonists to modify glutamate-receptor-mediated release of somatostatin, neuropeptide Y and substance P.

CHAPTER 2: BACKGROUND LITERATURE

2.1 THE STRIATUM AND BASAL GANGLIA: ANATOMICAL CONNECTIONS

The striatum plays a role in both movement and cognition, and abnormalities in the striatum have been implicated in a number of pathological conditions such as Huntington's disease, Parkinson's disease and stroke. It has been recognized in the past 15 years that the basal ganglia acts as a 'cortical appendage', forming a feedback circuit to modify the functioning of the cerebral cortex. A simplified model of the two main pathways of the basal ganglia circuitry is shown in figure 1. The direct pathway is a feedback loop which runs from cortex—striatum—internal pallidum (GPi) (entopeduncular nucleus or EP in the rat)—ventral lateral thalamus—cortex. Glutamatergic pyramidal neurons originating mostly in layer 5 of the cerebral cortex project to the striatum and are excitatory in nature. The dorsal striatum sends an inhibitory gamma-aminobutyric acid (GABA) projection to the GPi and to the reticular part of the substantia nigra (SNr). The GPi and SNr in turn send inhibitory GABAergic projections to the ventral–anterior and ventral–lateral nuclei of the thalamus. The thalamo–cortical pathway, which is thought to be excitatory, then completes the circuit. The activity of this main feedback loop is modified by several subsidiary circuits. (1) The substantia nigra, which receives input from the cerebral cortex (pathway not shown) as well as from the striatum and subthalamic nucleus (STN), sends a dopaminergic projection from its compact part (SNc) to the striatum. (2) The striatal output to GPi and SNr is complemented by an inhibitory, GABAergic pathway to external pallidum (GPe). The GPe sends inhibitory GABAergic fibres to the STN, which in turn acts to stimulate the GPi via a glutamatergic projection. This is known as the indirect pathway. (3) A third modulatory pathway is from the intralaminar nuclei of the thalamus to the striatum (pathway not shown in figure 1).

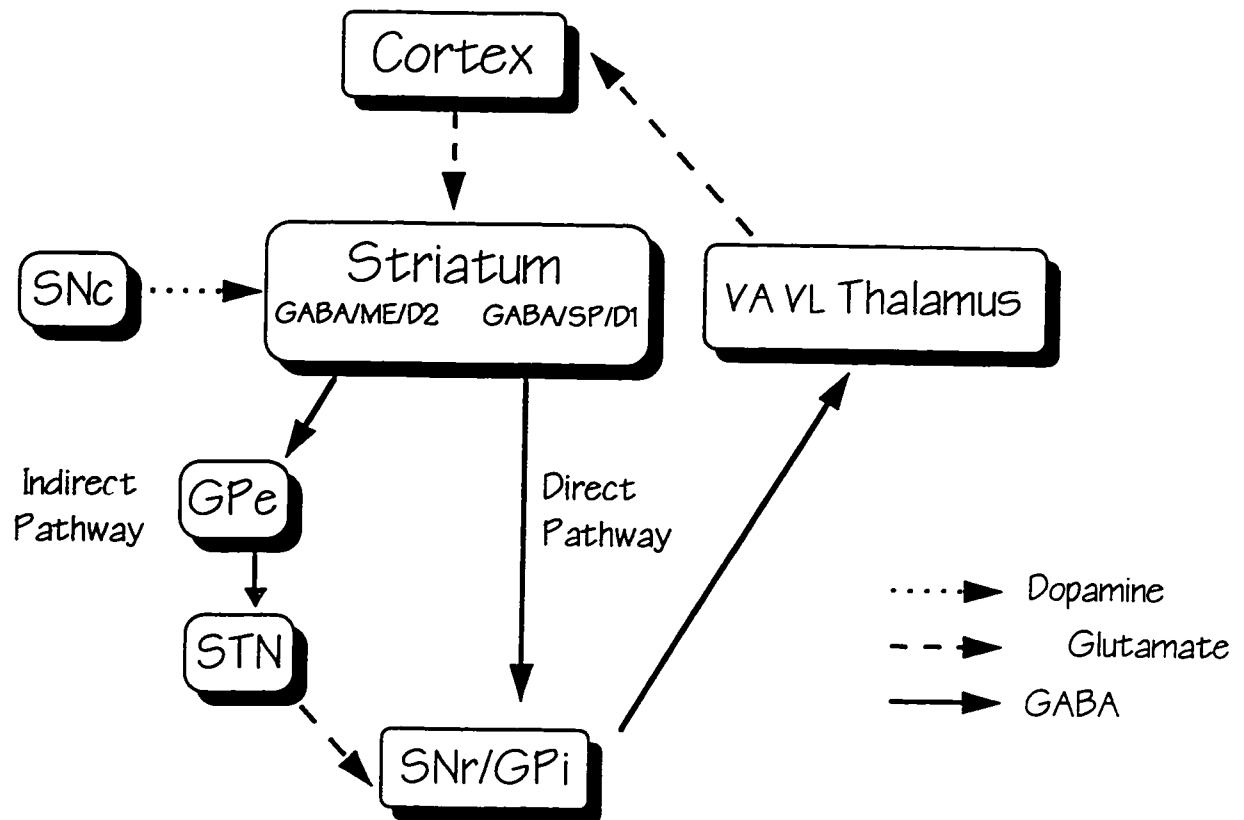


Figure 1. Circuit diagram of the normal basal ganglia.

2.2 AFFERENT PROJECTIONS TO THE STRIATUM

A. Glutamatergic Afferents

i) Topography

Glutamate is the major excitatory neurotransmitter in the central nervous system and the principal neurotransmitter of the corticostriatal pathway which forms one of two major afferents to the striatum. It is well-established that all major regions of the cerebral cortex project onto the striatum. Many components of this pathway have bilateral projections and thus provide a mechanism for intrahemispheric co-ordination and integration. In a detailed study of the corticostriatal projection in the rat by McGeorge and Faull (1989) using retrograde transport of horseradish peroxidase conjugated to lectin, it was found that all major regions of the cortex project to the striatum on both sides of the brain with an ipsilateral predominance (McGeorge and Faull, 1989). The cells of origin are mainly in layer V of the cortex (especially lamina Va) with very small numbers in lamina III (see figure 2).

Furthermore, each striatal locus receives inputs from several different areas of the cortex with each cortical region projecting onto a longitudinally oriented region of the striatum in an antero-posterior orientation. In addition, specific areas of cortex primarily project to a given area of the striatum (with some overlap), such that: i) the neocortex projects mainly to the caudate-putamen; ii) the mesocortex projects predominantly to the medial and ventral regions of the caudate-putamen and iii) the allocortex projects mainly to the nucleus accumbens but also to the medial and ventral parts of the caudate-putamen. Within each of these major projection systems there is a further sub-organization such that the sensory and motor areas project topographically onto the dorsolateral striatum; and the

rostral sensorimotor cortex projects to dorsal regions of the dorsolateral striatum; the visual area projects to dorsomedial striatum; and the auditory area projects to medial striatum (figure 3).

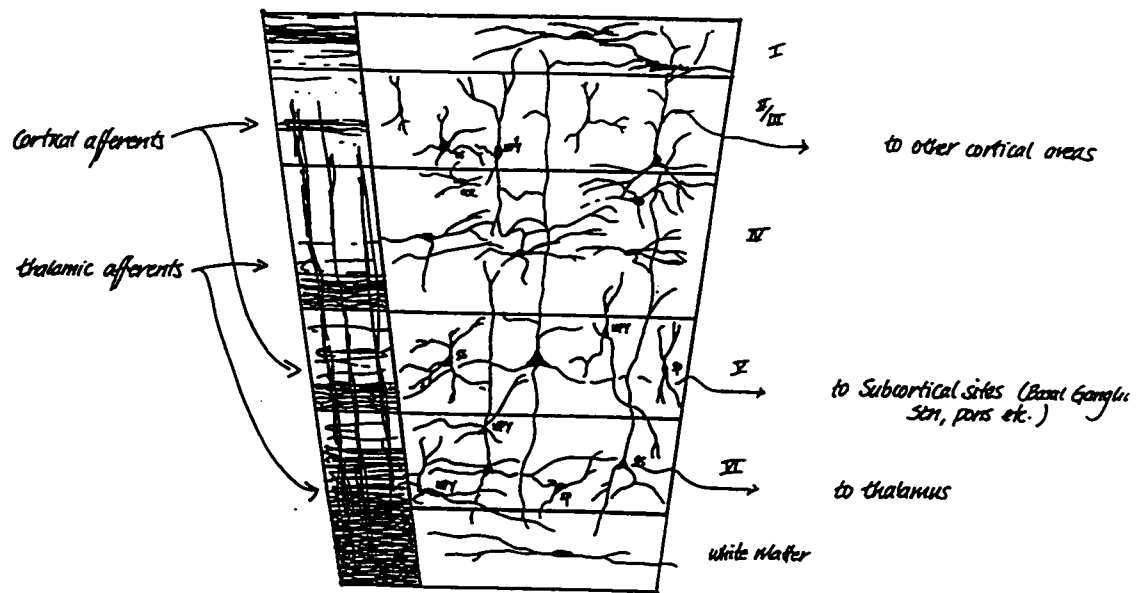


Figure 2. Normal cortex: laminar distribution of neurotransmitters and neuropeptides.

Peptide abbreviations: SS=somatostatin; NPY=neuropeptide Y; SP=substance P; CCK=cholecystokinin.



neocortex
 ■ sensorimotor □ entorhinal/periform
 ■ visual ■ mesocortex
 ■ auditory

figure 3. Topography of cortico-striatal projection (Adapted from McGeorge and Faull, 1989)

ii) Glutamate receptors

Two principal types of glutamatergic receptors have been identified: 1) Ligand-gated channels, each named after the analog that selectively excites it: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA) and kainate (KA) (Watkins and Olverman, 1987). The KA and AMPA receptors are associated with ionophores that permit sodium (Na^+) and potassium (K^+) conductance, while NMDA receptors gate calcium (Ca^{2+}) channels as well as Na^+ . 2) The second type of glutamate receptor is a G-protein coupled metabotropic quisqualate (QUIS) receptor.

NMDA receptor:

The NMDA receptor (see figure 4A, *top*) is both voltage- and chemical-dependent. In the resting state the NMDA receptor channel is blocked by a magnesium (Mg^{2+}) ion, but with sufficient depolarization the Mg^{2+} ion is dislodged allowing the influx of Ca^{2+} and Na^+ ions (Nowak et al, 1984). The NMDA receptor can be selectively activated by several endogenous compounds, including L-aspartate, homocysteate and quinolinate (QUIN) (Do et al, 1986; Stone and Connick, 1985). Activation of this receptor is dependent on the availability of glycine, which acts at a strychnine-insensitive allosteric binding site within the NMDA receptor complex (Johnson and Ascher, 1987). The action of glutamate at the NMDA receptor can also be selectively antagonized: competitively by 2-amino-5-phosphonovalerate (2-APV) and 2-amino-5-phosphonoheptanoate (2-APH) (Watkins and Olverman, 1987), or non-competitively by zinc (Zn^{2+}) or drugs that bind to the phencyclidine site within the open channel, such as phencyclidine (PCP), MK-801, or ketamine (Kemp and et al, 1987; Lodge and et al, 1987; Peters and et al, 1987; Westbrook and Mayer, 1987).

There are five NMDA receptor channel subunits, all of which share significant amino acid sequence identity with the AMPA and kainate receptor channel subunits (NR1, NR2A-D) (Seeburg, 1993; Lynch et al., 1994; Greenamyre and Porter, 1994). Each NMDA receptor is composed of two different protein subunits: NR1 and one of the NR2A-D subunits (Gozlan and Yehezkel, 1995; Lipton, 1993).

There are at least six pharmacologically relevant sites for modification of NMDA receptor function and they are summarized in the diagram on the following page (figure 4A, top).

Non-NMDA ionotropic receptors

The non-NMDA receptors fall into two main classes: AMPA and KA. GluR1-4 (or A-D) are AMPA subunits. GluR5-7, KA1 and KA2 assemble into KA receptors. The GluR5 gene is expressed mainly in the Purkinje cells of the cerebellum while GluR6 mRNA is present in most regions of the hippocampus and also in the granule cells of the cerebellum. Messenger RNA for GluR7 is highest in the neocortex and the dentate gyrus of the hippocampus. Phosphorylation of ionotropic glutamate receptors, especially KA receptors and KA receptor binding proteins by enzymes such as Ca²⁺-dependent protein kinase, protein kinase C, and cAMP-dependent protein kinase has been suggested to regulate their function (Wang et al., 1993). AMPA activates channels with fast kinetics and very low Ca²⁺ permeability indeed AMPA receptors appear to mediate the majority of all fast excitatory transmission. KA acts at AMPA receptors to generate a current that persists in its continued presence, and at KA receptors (or high-affinity KA receptors) to activate fast desensitizing currents.

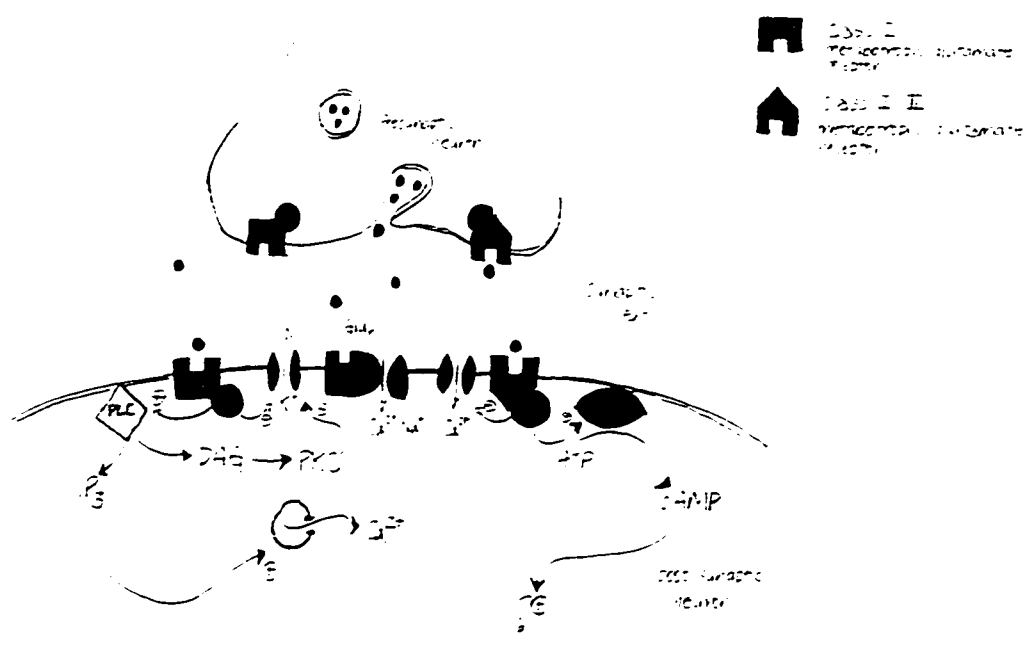
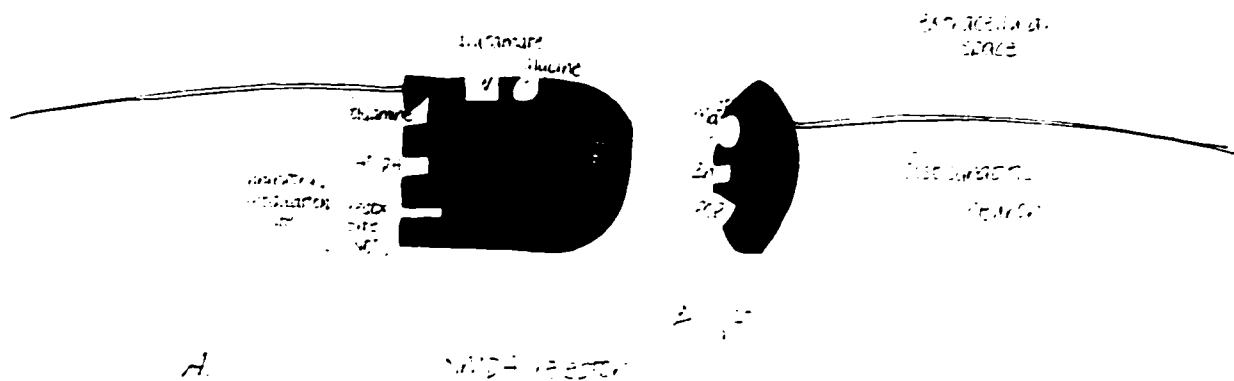


Figure 4 Diagrammatic representation of the NMDA receptor (top) and the different classes of metabotropic receptor (bottom).

Metabotropic Receptors

Eight metabotropic glutamate receptor subtypes have been cloned (mGlu1–mGlu8) that fall into 3 general categories termed classes I, II and III (see figure 4B, *bottom*). Sequence homology within classes is approximately 70% and between classes is approximately 40% (Nakanishi, 1992). Class I comprises mGlu1 and mGlu5; class II mGlu2 and mGlu3; and class III mGlu4 and mGlu6–8. Activation of class I mGlu receptors produces a second messenger response mediated by receptor interaction with a GTP binding protein that stimulates phospholipase C (PLC) and initiates phosphoinositide (PI) hydrolysis. The resultant second messengers, inositol 1,4,5–triphosphate (IP_3) and diacylglycerol, mobilize intracellular Ca^{2+} and activate protein kinase C (Sladeczek and et al, 1985; Nicoletti et al., 1996; Sugiyama and et al, 1987; Houamed et al., 1991; Masu et al., 1991). This class of metabotropic receptor is located at the periphery of the postsynaptic membrane whereas the ionotropic receptors occupy the core of the synapse (Nusser et al., 1994). In addition there are presynaptic class I mGlu receptors whose activation has been shown to enhance glutamate release in the presence of arachidonic acid (Herrero et al., 1992).

In contrast to the class I mGlu receptors, activation of classes II and III mGlu receptors inhibits adenylate cyclase and thus reduces cAMP; it is also negatively coupled to voltage-gated calcium channels. While the precise localization and function of these different subtypes have not yet been determined, in general class I metabotropic glutamate receptors increase neuronal excitation and excitability, whereas classes II and III metabotropic glutamate receptors reduce excitation.

iii) Glutamate-Mediated Excitotoxicity

As mentioned above, glutamate is the principal mediator of excitatory synaptic transmission in the central nervous system. Normally, extracellular levels of glutamate only rise to high levels in the synaptic cleft during signalling between neurons; however, when extracellular levels are sustained, as a result of disease or injury, glutamate becomes an instrument of neuronal death. In 1957, Lucas and Newhouse demonstrated that prolonged exposure to glutamate could destroy retinal neurons (Lucas and Newhouse, 1957). Subsequently, Olney showed that this neurotoxic potential was not limited to retinal neurons, but a feature common to the actions of all excitatory amino acids (EAA) on central neurons (Olney and Sharpe, 1969; Olney, 1971). He further postulated that EAAs might be responsible for the neuronal death observed in some neurological diseases. In recent years this hypothesis, known as 'excitotoxicity', has been fueled by new insights into glutamate receptor function and experiments revealing a role for EAAs in the pathophysiology of Huntington's disease, ischemia, hypoglycemia and stroke.

Mechanism of Toxicity

Excitotoxicity is most easily discussed in terms of three stages: induction, amplification, and expression.

Induction: In response to glutamate, NMDA receptors open calcium channels and allow large quantities of Ca^{2+} into the cell. At the same time, Na^+ ions rush in both through channels gated by the NMDA-receptor and through the quisqualate and AMPA/kainate receptor channels. Once the Na^+ ions begin to flow into the cell, Cl^- ions follow and the cell begins to swell; this is the acute, reversible Na^+ -dependent component of excitotoxicity. This influx of Na^+ and Ca^{2+} reversibly disrupts the ability of neurons to

respond normally to signals from other nerve cells. Activation of the quisqualate metabotropic receptor triggers an increase in the production of two intracellular messengers, diacyl glycerol (DAG) and inositol tri-phosphate (IP₃), which may also play a role in the induction of excitotoxicity.

Amplification: In this phase, Ca²⁺ levels rise to very high levels — partially due to Ca²⁺ influx through the NMDA-receptor channels, partially as a result of IP₃ mobilization of intracellular Ca²⁺ stores, and additionally by a carrier molecule that exchanges sodium for calcium. Voltage-gated calcium channels may also increase intracellular calcium levels.

The resulting excess of Ca²⁺, coupled with the increase in DAG, alters the activity of several families of enzymes that are known to modify membrane proteins (most likely including glutamate receptors) and increase the sensitivity of the neurons to excitatory signals. This serves as a positive feedback loop that increases the amount of damage and decreases the chances of cell survival. The increase in Ca²⁺ leads to the release of glutamate and the amplification of the excitotoxic process to other cells.

Expression: Early in this phase the damage produced becomes irreversible. The excess Ca²⁺ activates enzymes that degrade DNA, proteins and phospholipids. An increase in intracellular calcium activates the protease calpain, which catalyses the reaction of xanthine dehydrogenase to xanthine oxidase, which in turn results in the formation of O₂⁻ from the oxidation of xanthine (McCord, 1993). An increase in intracellular calcium can also activate phospholipase A₂, with subsequent phospholipid breakdown and metabolism of arachidonic acid leading to the formation of the free radicals such as O₂⁻ and the hydroxyl radical (Olanow, 1993). The hydroxyl radical initiates the process of lipid peroxidation by removing a hydrogen atom from a polyunsaturated fatty-acid side chain

(written lipid-H in figure 5) of the phospholipid cell membrane (McCord, 1993; Cheeseman and Slater, 1993). This starts a chain reaction that results in the breakdown of many phospholipids to lipid-O₂H (lipid hydroperoxides), which severely disrupts membrane integrity and function, allowing the influx of calcium, eventual destruction of the cell membrane, and subsequent cell death (McCord, 1993; Halliwell and Gutteridge, 1985; Cheeseman and Slater, 1993).

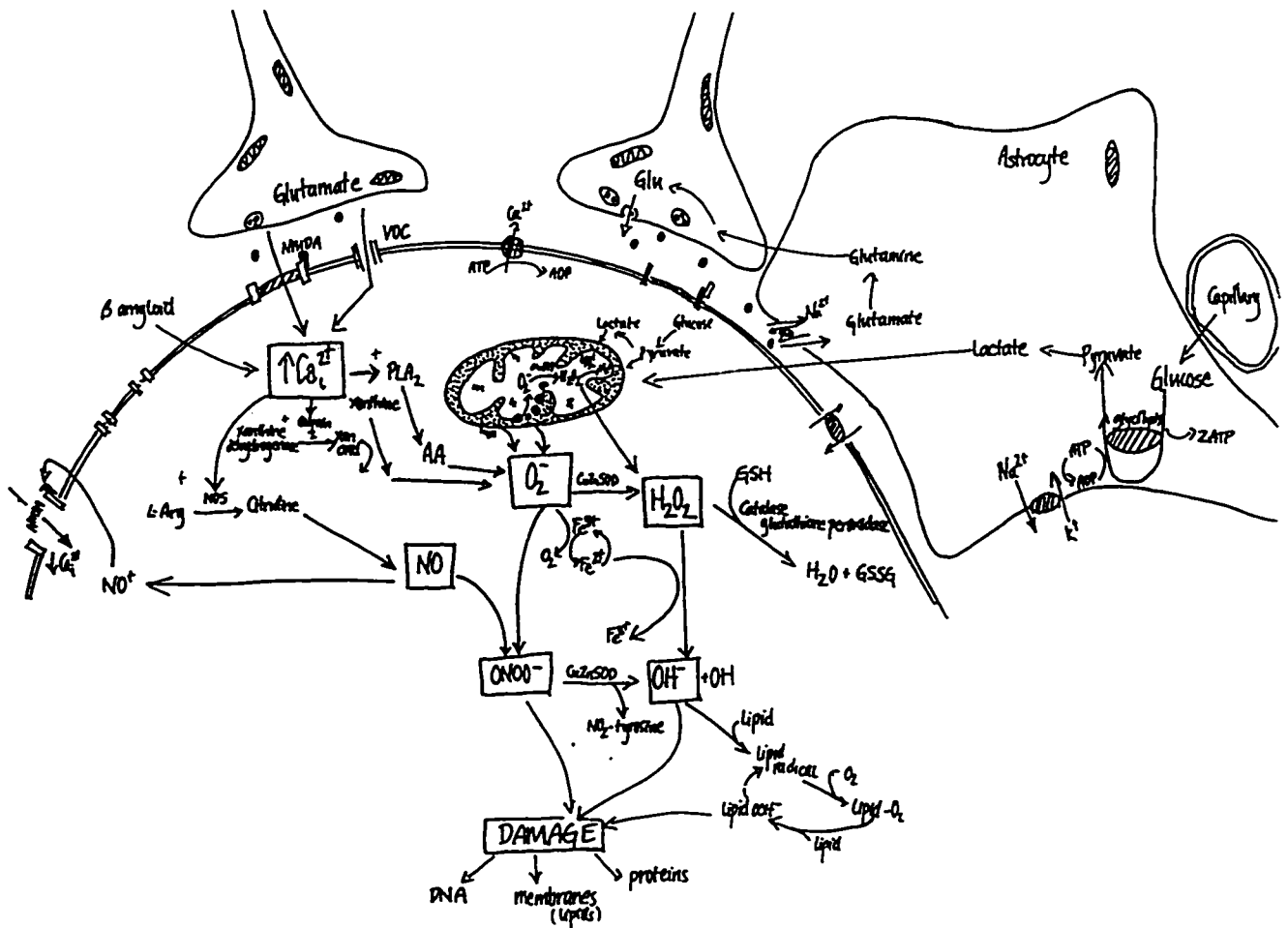


Figure 5. Possible mechanisms involved in excitotoxic cell death.

Kainate Toxicity

Kainic acid (KA), an analogue of glutamic acid has proven to be an extremely potent excitatory and neurotoxic agent. The neurotoxicity of KA is thought to be due to the combined effects of direct neuronal depolarization via kainate receptors coupled with an increased release of glutamate which also acts to depolarize the susceptible neurons (Ferkany et al., 1982; Coyle, 1983). It is also possible that KA inhibits the uptake of glutamate (Johnston et al., 1979). In terms of mechanisms of excitotoxicity, KA produces both acute neuronal swelling and, at least in some systems, Ca^{2+} -mediated degeneration (Garthwaite and Garthwaite, 1986; Rothman et al., 1987; Berdichevsky et al., 1983).

Kainate receptors are distributed throughout the brain but are most concentrated in the cortex, hippocampus and striatum. Injection of KA into any of these areas produces degeneration of cell bodies while sparing terminals and axons of passage (Coyle and Schwarcz, 1976). It has been noted that the axon-sparing quality of these lesions is similar to that observed in Huntington's disease (HD) and ischemic brain injury.

Quinolinic Acid

In 1983, Schwarcz et al. published data demonstrating that quinolinic acid (QUIN) could produce an axon-sparing lesion (Schwarcz et al., 1983). Furthermore, the neurochemical profile proved to be strikingly similar to HD, with the selective destruction of medium spiny neurons and the relative sparing of the aspiny neurons (Beal et al., 1986). Beal et al. showed that an intrastriatal injection of 240 nmols of QUIN resulted in marked depletions of GABA and substance P, and a relative sparing of somatostatin/neuropeptide Y (SS/NPY) and dopamine, mimicking the pattern seen in HD. Since that time, it has been reported by several others (Davies and Roberts, 1987; Boegman and Parent, 1988; Roberts and DiFiglia, 1988) that the large cholinergic aspiny neurons of the striatum, which are spared in HD, are also spared following QUIN lesions. As neither ibotenic acid nor KA

spared the SS/NPY or acetylcholine neurons, QUIN has become the toxin of choice in animal models of HD. Receptor studies have also supported the QUIN model, with QUIN-lesions resulting in preferential depletion of NMDA receptors, similar to observations in HD (Young et al., 1988; Greenamyre and Young, 1989).

Despite the volume of data supporting an excitotoxic mechanism in HD, there remains an atmosphere of controversy about the sparing of SS/NPY neurons. Although the sparing has been demonstrated numerous times neurochemically, it has been harder to demonstrate immunocytochemically. Some investigators have found that while the ACh neurons are spared, the SS/NPY neurons seem to disappear (Davies and Roberts, 1987; Boegman et al., 1987; Boegman and Parent, 1988). Others have found sparing of the SS/NPY neurons (Beal et al., 1986; Beal et al., 1989; Koh et al., 1986; Koh and Choi, 1988; Koh and Choi, 1988). The controversy may prove to be an artifact of methodology. One of the downfalls of the QUIN model is that, due to the nature of intrastriatal injections, there is necessarily a lesion core where everything is destroyed, a penumbra where there is selective sparing, and an area outside of the lesion which remains undamaged. The key issue is whether there is *relative* sparing of SS/NPY neurons. This could only be found in the penumbra of the lesion; and, indeed, a recent study (Beal et al., 1989) suggests that there is in fact relative sparing of SS/NPY in the penumbra of QUIN lesions but not in lesions using either ibotenic acid or KA.

B. Dopaminergic Afferents

i) Topography

Dopamine (DA) afferents to the striatum originate in the mesencephalic cell bodies of the substantia nigra (SN) and the ventral tegmental area (VTA) (areas A9 and A10, respectively, according to the classification of Dahlström and Fuxe (Dahlström and Fuxe,

1964)). For the most part, dopaminergic neurons originating in the pars compacta of the substantia nigra (SNc) project to the caudate and putamen as the nigrostriatal pathway (Anden et al., 1964; Björklund and Lindvall, 1984). The A10 cell body group of the VTA projects predominantly to the nucleus accumbens, known as the mesolimbic pathway (Simon et al., 1979; Oades and Halliday, 1987; Björklund and Lindvall, 1984). The VTA also sends DA efferents to other areas of the ventral striatum such as the olfactory tubercle and bed nucleus of the stria terminalis, as well as other limbic structures such as the amygdala, lateral septum, lateral habenula and ventral hippocampus. There is some overlap between the A9 and A10 projection areas, with VTA innervating ventromedial caudate-putamen and cell bodies of the ventro-medial SNc projecting to the nucleus accumbens (Björklund and Lindvall, 1984). Tyrosine hydroxylase (TH) immunohistochemistry has demonstrated the highest density DAergic terminals in the caudate and putamen, with less staining evident in the nucleus accumbens and olfactory tubercle (Björklund and Lindvall, 1984). Although projections are predominantly ipsilateral, like the corticostriatal projections the DA afferents have been shown to innervate contralateral striatum as well (Altar et al., 1983).

Tracing studies in the rodent demonstrate that the ascending midbrain projections to basal forebrain and caudate-putamen are topographically organised (Lynd-Balta and Haber, 1994; Beckstead et al., 1979; Moore and Fallon, 1978; Nauta et al., 1978). The mesostriatal projection is organised in three planes: dorsal-ventral, medial-lateral and anterior-posterior; with an inverse dorsoventral topography and direct mediolateral and anteroposterior projections. Ventral cells of the SN and VTA have dendrites that are oriented ventrally, and project primarily to the striatum but also to the septum and nucleus accumbens (Gerfen et al., 1987). Dorsal midbrain DA neurons of the SNc, the VTA and retrorubral group (areas A9, A10 and A8, respectively), whose dendrites are oriented

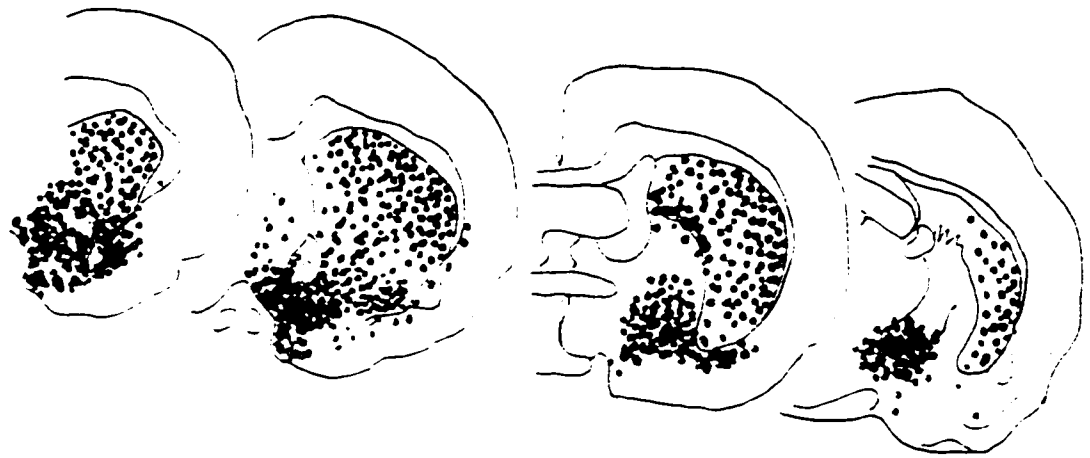


Figure 6. Topography of the nigrostriatal pathway in the rat. (Adapted from Moore and Fallon, 1978)

mediolaterally, project to the ventral forebrain as the mesolimbic pathway. The lateral and caudal SN projects to the caudal putamen while dorsal cells of the more medial areas of the SN and VTA project to the amygdala. A summary of the topography of the nigrostriatal projection is shown in figure 6 (adapted from Moore and Fallon, 1978; see also Haber and Groenewegen, 1989; Lavoie and Parent, 1991).

ii) DA receptors

There are two main subtypes of DA receptors, referred to as D1 and D2. In recent years, several new DA receptors have been found that share characteristics and conserved amino acid sequences with one or the other of these two DA receptor subtypes. The current nomenclature classifies the D1 and D5 DA receptors as belonging to the D1 subtype, while the D2 subtype consists of D2 long and short, D3 and D4 DA receptors (Seeman and Van Tol, 1994).

The distinction between D1 and D2 receptor subtypes is based largely on their differential effects on second messenger signal transduction. Both receptor classes belong to the category of G-protein coupled receptors with seven transmembrane-spanning domains. However, D1 receptors are coupled to a stimulatory G-protein (Gs), causing an increase in adenylyl cyclase activity and subsequent increase in cyclic AMP (cAMP) levels; while D2 receptors are coupled to an inhibitory G-protein (Gi), causing a decrease or no change in adenylyl cyclase activity and cAMP levels (Onali et al., 1985; Stoof and Keibadian, 1981).

Both receptor subtypes may also be involved in non-adenylyl cyclase coupled signal transduction. The D1 receptor has been shown to be coupled to phospholipase C in a stimulatory way (Mahan et al., 1990). In addition, the phosphorylation of the protein DARPP-32 — contained by most DA-receptive cells — is enhanced by the increased levels of cAMP following D1 receptor stimulation. This in turn may regulate a phosphatase that

would alter the phosphorylation states of other effector proteins (Walaas et al., 1983; Halpain et al., 1990). Non- adenylyl cyclase transduction mechanisms that have been proposed for the D2 receptor include coupling to phosphoinositide metabolism (Pizzi et al., 1988) and direct effects on ion (K^+ and Ca^{2+}) channel activity (Lacey et al., 1987; Drukarch et al., 1989).

Although D1 and D2 receptors can have opposite effects on adenylyl cyclase activity, it is apparent that the physiological significance of their interaction is more complex. Despite their apparently antagonistic functions, many studies have shown a synergistic interaction between them. Several investigators have proposed that the D1 receptor may in fact have a permissive effect on D2 receptor-mediated activity (Andersen et al., 1990; Robertson, 1992).

Precise localisation of DA receptors in the striatum has not been completely clarified. Most of the evidence points toward a segregation of D1 and D2 receptor subtypes, confining them largely to different striatal efferent projections (Gerfen et al., 1990; Gerfen, 1992; Le Moine et al., 1990); although some investigators argue for colocalisation of D1 and D2 receptors in the striatum (Surmeier et al., 1993). A detailed immunohistochemical study of DA receptor localisation in the rat by Yung et al (1995) found that D1 and D2 receptor immunoreactivity was most abundant in the striatum, where it was mainly on the medium spiny neurons, although some of the immunopositive perikarya resembled interneurons (Yung et al., 1995).

An interesting relationship between DA receptor subtype, neuropeptide colocalisation and striatal efferent projections has emerged in the literature. Striatal output to the GPi and SNr (known as the direct pathway) appears to have a large density of D1 receptors in the striatum and in the terminal fields, uses GABA as a neurotransmitter, with colocalisation of the neuropeptides substance P (SP) and dynorphin (DYN). Striatal output

to the GPe (the indirect pathway) has a preponderance of D2 receptors and has met-Enk colocalised with GABA.

The nigrostriatal DAergic system has also been shown to possess autoreceptors that may modulate DA transmitter synthesis and release, as well as firing rate (Chiodo et al., 1995). These autoreceptors are of the D2 subtype and are located somatodendritically in the SN, as well as in the terminal fields in the striatum. Many have postulated the presence of DA receptors on the ACh interneurons (Chang, 1988; Le Moine et al., 1990), and indeed the neurochemical data suggesting that stimulation of the DA D2 receptor inhibits acetylcholine release has been replicated repeatedly (Chang, 1988; Sethy, 1979). However, the anatomical evidence for a direct synaptic contact between DAergic nigrostriatal fibers and intrinsic cholinergic neurons has been difficult to find (Stoof et al., 1992; Pickel and Chan, 1990).

2.3 INTRINSIC ANATOMY OF THE STRIATUM

Neurons with cell bodies intrinsic to the striatum fall into two morphologically distinct groups: medium spiny GABAergic neurons and aspiny neurons.

A. Medium-sized Spiny Neurons

The medium spiny neurons account for as many as 95% of the neurons in the striatum (Kemp and Powell, 1971). The majority of these neurons are generally Type 1 neurons (10–20 μ m diameter cell bodies) and can be identified on the basis of differences in their axonal projections and cytochemical contents into two main types (Kawaguchi et al., 1990): i) those that contain GABA and met-enkephalin, and project to the GPe; and ii) those that contain GABA, substance P (SP) and dynorphin (DYN) and project to the SNr

and GPi (or EP in non-primates). These neurons radiate dendrites that extend up to 200–300 μ m across. The first 30 μ m of dendrite tends to be mostly smooth, the remaining portion branches extensively and is densely laden with spinous processes. These neurons often have a local axon collateral distributed within the same domain as the dendritic arbour, as well as sending long axon collaterals to the GP and SN.

These medium spiny neurons are the synaptic targets for the vast majority of striatal afferents, including those from the cerebral cortex, intralaminar thalamic nuclei, dopaminergic neurons of the SN and the serotonergic fibres from the dorsal raphe nucleus (Smith and Bolam, 1990; Wilson, 1990).

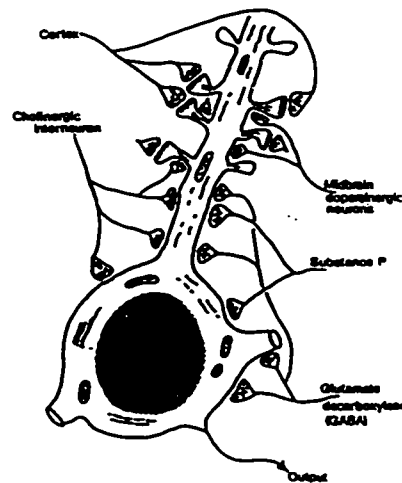
The distribution of inputs can be separated into 3 major categories (figure 7):

1) **soma and smooth dendrites.** Although synapses on this area are relatively sparse, there are at least two types of symmetrical synapses found: those from medium spiny neurons and those from interneurons and TH-positive neurons.

2) **proximal dendrites.** This portion of the neuron contains both symmetric and asymmetric synapses. The symmetric synapses are identical to those present on the soma and smooth dendrites. The asymmetric synapses contain glutamatergic vesicles.

3) **distal dendrites.** This area of the neuron receives similar inputs as the proximal dendrites, with the exception that no interneurons have been found. The dendritic spine can be divided into two portions — the neck region which receives primarily symmetrical synapses from TH-positive cells and the head region which receives primarily asymmetric synapses from the cortex.

Inputs to medium spiny neurons



distal dendrites

CTX>sn(TH)>m. spiny

proximal dendrites

CTX>m.spiny>SN(TH)>interneuron

smooth dendrites and soma

m.spiny>SN(TH)>interneuron

Figure 7. Synaptic organization of medium spiny neuron. (Adapted from Bolam, 1990.)

i) Extrinsic afferents

Extrinsic afferents to the striatum arise from the cerebral cortex, the substantia nigra, the thalamus, dorsal raphe, amygdala and globus pallidus. Of these, the morphology of synapses from the cortex and the substantia nigra are best understood.

Corticostriatal inputs

These inputs are almost always asymmetric and contain small round vesicles. Pasik et al. (1976) estimates that approximately 80% of striatal synapses are corticostriatal (Pasik et al., 1976). However, the actual input to any single cortical area to an individual medium spiny neuron is relatively sparse (Somogyi et al., 1981). Therefore any single

striatal area receives convergent inputs from multiple cortical areas such that corticostriatal afferents are rather dense on individual medium spiny neurons.

Nigrostriatal inputs

Although a consensus for the synaptic organization of dopamine afferents is emerging, there has been a history of controversy regarding these inputs; especially regarding whether classical synaptic contacts are made or whether the synapses are symmetrical or asymmetrical. Pickel et al. (1981) provided the first clear morphological evidence for symmetrical synapses at TH-positive terminals in the striatum (Pickel et al., 1981). They showed that TH containing axon terminals constituted approximately 21% of the total number of terminals into the striatum and consisted of three types: the most prevalent type 1 (82% of the total), is small and forms symmetric junctions with dendrites and dendritic spines. The other two types, Types II and III, have medium to large diameters and show either no membrane specialization or are asymmetric with dendrites. Freund et al. (1984) further characterised the distribution of inputs of the symmetric TH-positive synapses demonstrating that roughly 59% of TH-positive synapses are on the neck of dendritic spines, 35% are on the interspine shafts of distal dendrites and 6% are on the cell body and proximal dendrites (Freund et al., 1984). These fibres make multiple contacts with interspine dendritic shafts.

Furthermore, the dendritic spines of striatonigral neurons that received TH-positive input to the neck also invariably received input, usually more distally, from asymmetric — and therefore probably corticostriatal glutamate — fibres. This organisation was confirmed by Bouyer et al. (1984) who demonstrated that following decortication degenerating corticostriatal afferents make synaptic contact with dendritic spines that also receive TH-positive synapses (Bouyer et al., 1984). Their results also suggested that the TH-labelled neurons and cortical projection neurons may be in direct contact with one another.

Approximately 30% of the degenerating neurons that they studied were in direct contact with the TH-positive synapses. If this were not an artifact of the technique, this could partially explain the controversy over the release of dopamine by glutamate and *vice versa* (Schwarcz et al., 1978).

The location and function of Types II and III TH-positive neurons has not been well characterised .

ii) Axon collaterals of medium spiny neurons

In addition to their projection to the SN or GP (Chang et al., 1981; Grofova, 1975; Somogyi and Smith, 1979; Somogyi et al., 1981), the medium spiny neurons have local axon collaterals that are distributed within their dendritic arbour (Bishop et al., 1982; Somogyi et al., 1981; Wilson and Groves, 1980). These collaterals make symmetric contact with other medium spiny neurons and other cell types in the striatum: 12% communicate with the smooth proximal dendrite or cell body; 48% are located on the distal interspine dendritic shafts; and 40% are on the synaptic spines (Wilson and Groves, 1980). Synapses of local axon collaterals onto dendritic spines are generally located on the necks of spines that also receive an asymmetric synapse, most likely from a corticostriatal neuron.

Bolam et al reported in 1986 that local axon collaterals expressing substance P target the more proximal parts of medium spiny neurons, in contrast to the extrinsic neurons that target the more distal portions of the medium spiny neuron (Bolam et al., 1986). The medium spiny neurons also synapse on other cell types in the striatum such as the large cholinergic interneurons (Bolam and Izzo, 1986).

B. Aspiny Neurons

There are at least 4 major classes of interneurons: 1) large Type II (20–30 μ m diameter cell bodies) neurons containing acetylcholine (Bolam et al., 1984; Levy et al., 1983; Phelps et al., 1985) which are identifiable by the presence of choline acetyltransferase (ChAT); 2) medium-sized Type 1 neurons, the best characterized of which contain both somatostatin and neuropeptide Y, as well as the histochemical marker for nitric oxide synthase (NOS), NADPH–diaphorase (DiFiglia and Aronin, 1982; Takagi et al., 1983); 3) GABAergic interneurons that contain parvalbumin, one of the calcium binding proteins (Gerfen et al., 1985); and 4) GABAergic interneurons that contain calretinin (Jacobowitz and Winsky, 1991; Résibois and Robers, 1992; Bennett and Bolam, 1993). Double-labelling studies have shown that these classes of cells are non-overlapping. Smaller numbers of cholecystinin and vasoactive intestinal peptide (VIP-positive) aspiny neurons have been described but it is not yet clear whether these represent additional classes of cells or subclasses of the four well-defined interneuronal types (Takagi et al., 1984; Theriault and Landis, 1987).

i) Large aspiny cholinergic interneurons

The large aspiny cholinergic neurons account for approximately 1.7% of all striatal neurons and have a large somatic size (20–50 μ m). The key to their identification was the discovery that they are the source of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT). Initially, AChE was used as a marker for these cells, but it is now known that AChE is expressed in both the cholinergic and somatostatin-containing interneurons and is therefore a non-specific marker. ChAT is a reliable marker for these cells.

These giant neurons have long dendrites and extensive axon collaterals distributed within the striatum (Phelps et al., 1985). They make both symmetric and asymmetric

synapses, with the greatest proportion on distal dendrites (Izzo and Bolam, 1988; Bolam and Izzo, 1986; Phelps et al., 1985). The asymmetrical synapses are similar to those of the corticostriatal afferents and probably contain glutamate. They are most abundant on secondary and tertiary distal dendrites. The symmetric synapses are most common on the cell soma and proximal dendrites with at least a portion of them containing substance P, and are therefore thought to be from medium spiny axon collaterals (Bolam et al., 1986). The remainder may be dopaminergic-- although as mentioned earlier, direct anatomical evidence for this has been difficult to obtain. A smaller portion may represent input from SS/NPY interneurons.

The cholinergic interneurons have widespread dendritic trees, subtending a region much larger than that of the spiny projection neurons (Wilson et al., 1990), and are thus capable of integrating synaptic inputs over relatively large regions.

Cholinergic neurons have been shown to be monosynaptically excited by stimulation of the cerebral cortex *in vivo*, but anatomical studies have shown that they are predominantly innervated by fibres from the thalamus (Lapper and Bolam, 1992). While the functional importance of this is not known, this suggests that interneuronal types may specialize in the processing of cortical or thalamic inputs; in contrast to the projection neurons, which receive input from both of the main afferent inputs.

Cholinergic interneurons express the NK-1 (SP) receptor, which indicates that the cholinergic cells are likely to be a direct target for SP released by recurrent SP containing GABAergic collaterals. SP-positive axonal terminals, which probably arise from spiny projection neurons, have been observed to make synaptic contact with dendrites of cholinergic neurons; and SP has been shown to release endogenous ACh *in vivo* and *in vitro* from the rat striatum (Bolam et al., 1986; Bolam et al., 1983; Guevara Guzman et al., 1993). Spiny projection neurons of both major types (GABA-SP/DYN and GABA-

MetEnk) are relatively enriched in the m1 and m4 muscarinic ACh receptors (Izzo and Bolam, 1988).

ii) Medium-sized aspiny SS/NPY/NOS containing interneurons

The medium aspiny SS/NPY/NOS containing interneurons are aspiny cells 12-25 μ m in diameter and constitute 1-2% of striatal neurons. In comparison with cholinergic- and parvalbumin-positive cells, neurons of this class have fewer dendritic branches and their axonal arbourizations are less dense but more extensive than the medium spiny neurons within the striatum (DiFiglia and Aronin, 1982; Takagi et al., 1983). Afferent synapses are relatively sparse with proximal portions receiving both symmetric and asymmetric synapses and distal dendrites receiving primarily asymmetric synapses similar to the corticostriatal synapses.

These cells were first visualized histochemically by staining for NADPH-diaphorase using NADPH and nitroblue-tetrazolium (NBT). NADPH-diaphorase staining is a reliable marker for NOS-containing cells, due to the ability of the carboxy-terminus portion of the NOS enzyme to transfer electrons from NADPH to NBT. This reaction results in the characteristic diaphorase-formazan staining, and is independent of NOS activity levels. In addition to NOS, these cells contain somatostatin and neuropeptide Y and receive direct cortical inputs which are probably glutamatergic (Vuillet et al., 1989). Most of these cells do not possess high levels of NR1 mRNA (NMDA) or transcripts for Glu1-Glu4 receptors (AMPA), unlike the majority of other striatal neurons. Therefore, cortical glutamatergic transmission to SS-containing cells may be mediated through the metabotropic receptors. SS/NPY/NOS cells also receive cholinergic (symmetrical synapses) (Vuillet et al., 1992) and dopaminergic (probably only D1) (Vuillet et al., 1989; Aoki and Pickel, 1988) innervation and express the NK-1 (SP) receptor (Kaneko et al., 1993).

SS/NPY/NOS-containing neurons are the only striatal neurons that are capable of producing nitric oxide (NO). NO is likely to have at least two functions in the striatum: i) as an endothelium-derived relaxing factor (EDRF) (Snyder, 1991; Bredt and Snyder, 1992) that acts directly on guanylate cyclase in vascular smooth muscle cells and causes vasodilation; ii) as a neurotransmitter, either directly through interactions with channels (Manzoni et al., 1992) or indirectly through effects on surrounding neurons. As the majority of spiny projection neurons are enriched in soluble guanylate cyclase and express mRNAs that encode soluble guanylate cyclase, these cells may be among the potential targets for NO produced by the SS interneurons. There is some evidence that NO may influence the functioning of the other striatal neurons as the *in vivo* infusion of NO to the striatum enhances the release of ACh, GABA and glutamate; whereas the use of NO inhibitors, such as N-methyl nitroarginine, results in the gradual reduction in the release of all striatal neurotransmitters (DA, GLU, GABA, ACh) (Ariano and Matus, 1981; Ariano et al., 1982; Ariano, 1983).

It is thought that these neurons make symmetric synapses, however their targets are as yet not clarified. A large number of SS-immunoreactive varicosities have been found in direct apposition to striatal cell bodies and dendrites (Takagi et al., 1983). The majority of synapses are statistically most likely to be located on spiny striatal neurons, however, symmetrical synapses have also been seen on the large cholinergic interneurons (Vuillet et al., 1992).

iii) GABA interneurons

Initial studies that stained striatal sections for glutamic acid decarboxylase (GAD) – a marker for GABA-containing neurons – revealed that only a small number of neurons were strongly stained, while many were weakly stained (Ribak et al., 1979; Bolam et al., 1985). Bolam and colleagues showed that the strongly stained neurons had the

morphological properties of interneurons (Bolam et al., 1985; Bolam et al., 1983). Subsequent histochemical studies revealed that these strongly GAD-containing interneurons did not contain detectable amounts of neuropeptides, while the more common but less densely GAD-positive striatal neurons co-expressed SP or met-Enk (Reiner and Anderson, 1990; Penny et al., 1986; Kita and Kitai, 1988). Furthermore, staining with two different isoforms of GAD – GAD65 and GAD67 – differentially labelled the interneurons and the spiny projection neurons. Specifically, labelling with GAD65 preferentially labelled the projection neurons while GAD67 labelled the interneurons (Gonzales et al., 1991; Kaufman et al., 1991; Chesselet et al., 1993). Very little is known about these interneurons.

2.4 THE FUNCTIONS OF THE STRIATUM: LESSONS FROM THE CLINIC

A. Consequences of Striatal Dysfunction for Movement

Although the caudate nucleus and the putamen are cytologically uniform structures and for many years were thought to have similar functions, it now appears as if they play somewhat different roles. The putamen receives most of its cortical inputs from motor and somatosensory areas and projects by way of the globus pallidum and thalamus to the premotor and supplementary motor areas. In accordance with this, neurons in the putamen fire in conjunction with particular movements or positions, and stimulation of small areas of the putamen causes discrete movements. Thus the putamen is most likely involved in the motor functions of the basal ganglia. In contrast, the caudate nucleus receives most of its inputs from association areas of cortex and projects by way of the globus pallidus and

thalamus mostly to prefrontal cortical areas. Few caudate neurons respond to movements or positions, suggesting that this pathway may play a more important role in cognition.

Evidence for involvement of the basal ganglia in movement has come primarily from clinico-pathological evidence. The elucidation of the neuropathology of Parkinson's disease (PD) in the 1960's provided a model for the development of bradykinesia. PD is a slowly progressive neurodegenerative disorder of the basal ganglia characterised by tremor, muscular rigidity, difficulty in initiating motor activity, and loss of postural reflexes. It afflicts approximately 1% of the population over age 55. Initial complaints may be of slowness of movements and heaviness, stiffness or aching in the limbs, a loss of affect, and malaise (Adams and Victor, 1993). As the disease progresses difficulties in the initiation of movement and postural disturbances, including a loss of arm swing while walking and difficulties in maintaining balance, appear. The handwriting becomes small and shaky, tending to get smaller as the sentence continues (micrographia). Rigidity is found in most muscles, although it may be confined to one limb initially; and eventually develops into severe 'cogwheel' rigidity. A 'pill-rolling', low hertz tremor often develops characterised by a four-to-six-per-second alternating flexion-extension movement that is most noticeable in the thumb when the hand is at rest. At first, most people with PD are able to return to a reasonable level of function with the addition of L-dopa (a precursor in the biosynthesis of dopamine), but as the disease progresses the response to the drug is less predictable and may include periods of freezing alternating with severe chorea ('on/off' phenomena) (Marsden and Parkes, 1976). Approximately 2/3 of patients are disabled within 5 years and 80% within 10 years (Adams and Victor, 1993).

In PD there is a primary loss of dopamine neurons from the SNc and subsequent diminution of its dopaminergic influence on the striatum (figure 8). Even in patients with mild symptoms, a striatal dopamine loss of 70%-80% is observed, while severely impaired

subjects have striatal dopamine depletions in excess of 90%. As described above, DA has an inhibitory role on the GPe (indirect) pathway and an excitatory role on the SNr/GPi (direct) pathway. Loss of DA, as in the case of PD, leads to loss of facilitation of movement by both direct and indirect pathways (Hallett, 1993). The symptoms of rigidity and difficulty initiating movement are the consequences of an overactive indirect pathway, which results in an increase in GPi inhibition of thalamic output to the cortex. Bradykinesia is the consequence of an underactive direct pathway, with the disinhibited GPi again suppressing the thalamo-cortical output. These same effects on movement are also seen in neuroleptic-induced parkinsonism, where blockade of the DA D2 receptor is thought to produce the imbalance between the direct and indirect pathways required to produce bradykinesia and rigidity.

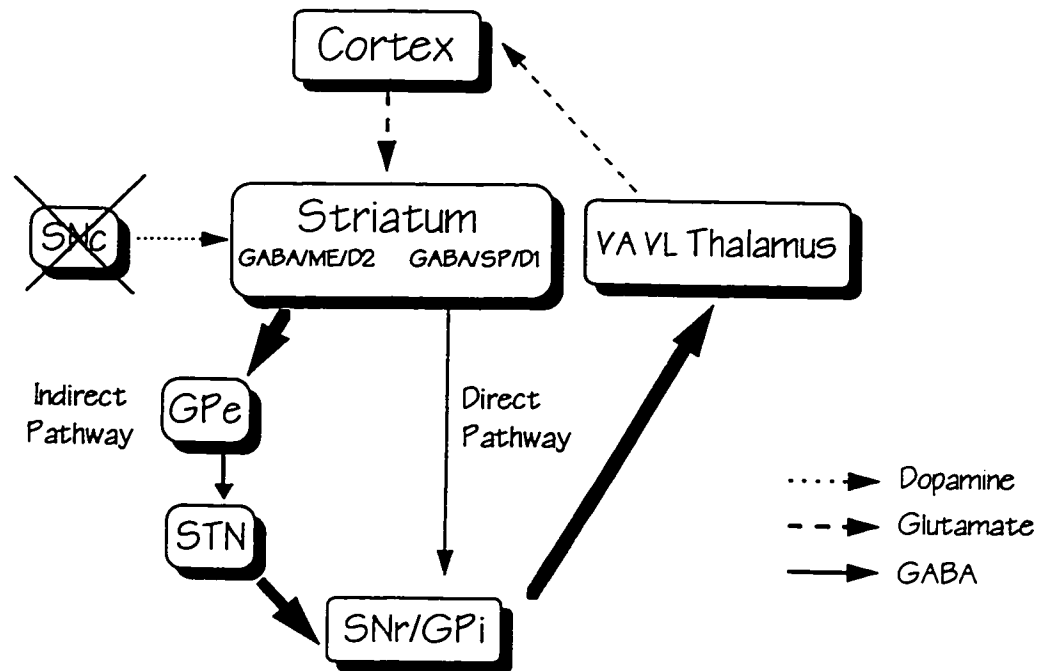


Figure 8. Basal ganglia circuit diagram depicting changes associated with Parkinson's disease.

Huntington's disease (HD) is another progressive neurodegenerative disease of the basal ganglia with profound motor disturbances (Adams and Victor, 1993; Folstein et al., 1986). The motor symptoms of HD develop gradually, initially characterised by involuntary movements. In the early years of the disease, an affected person may be unable to remain still or he/she may have tic-like movements of an isolated muscle group. This is followed by increased restlessness and clumsiness, as well difficulties with speech articulation. As the illness develops, obvious involuntary movements occur — usually jerky and unpredictable, with a characteristic pattern for each patient. Simultaneously, voluntary movements also become clumsy and slow. Both voluntary and involuntary movements deteriorate over many years, eventually affecting the ability to speak, walk, and eat. Balance is also severely affected as a result of choreic movements and falls are common. Eating is compromised by both the chorea and a decreased ability to chew and swallow. At this point, 13–15 years after onset, the patient cannot be left unattended and total care is required.

A variety of involuntary movements occur in HD. Chorea is the most common, especially in the early stages of HD, but motor restlessness, dystonia, tremor, and myoclonus may also be present (Adams and Victor, 1993; Folstein et al., 1986). Bradykinesia develops in the later stages of HD. The chorea, meaning 'dance', is characterised by sudden, involuntary movements of almost any part of the body and it frequently has a writhing quality (choreoathetosis). It is not stereotyped or repetitive like a tic, and is almost always aggravated by stress and decreased by intense concentration. Toward the end of life the involuntary movements usually decrease and the patient suffers from bradykinesia, often appearing to be 'locked-in' to their bodies. The impairments in both voluntary and involuntary movements are likely to be related to different subsystems

in the basal ganglia, as they respond differently to pharmacological manipulations (Hallett, 1993).

The pathophysiology of HD is characterised by an initial selective loss of the striatal GABA neurons of the indirect pathway which contain the neuropeptide met-enkephalin (figure 9) (De la Monte et al., 1988; Vonsattel et al., 1985; Vonsattel et al., 1987). These neurons normally inhibit the GPe, and the resulting disinhibition of the GPe leads to suppression of the subthalamic nucleus (STN). Both clinically and experimentally, the destruction of the STN produces severe hemiballismus (Dubinsky et al., 1989; Mitchell et al., 1989). In HD, the incomplete inhibition of the STN and loss of indirect pathway tone give rise to a lesser form of hyperkinesia known as choreoathetosis. (A similar mechanism may underlie the pathophysiology of tardive dyskinesia.) Later in the course of HD, corresponding to the clinical development of bradykinesia, there is further degeneration encompassing the GABA neurons of the direct pathway, which contain the neuropeptides substance P and dynorphin. This gives rise to an underactive direct pathway, similar to what is seen with PD (Hallett, 1993). Indeed, most end-stage HD patients are profoundly parkinsonian.

Chorea or dyskinesia can also be induced by dopaminergic agents. In particular, L-dopa-induced dyskinesias seen in PD are very similar in appearance to the chorea seen in HD. The pathophysiology is assumed to be an excessive stimulation of a dopamine system chronically understimulated due to the disease-related loss of SNc dopamine neurons (Marsden and Parkes, 1976).

In summary, dysfunction of the striatum and basal ganglia circuitry in disease states causes a variety of movement disorders. The pathophysiology of these diseases has contributed to the understanding of the role of the striatum for normal motor functions. The execution of voluntary movements — as well as the avoidance of involuntary movements

— occurs by activating and inhibiting selective motor pathways. The direct pathway appears to selectively activate, while the indirect pathway serves to inhibit, specific motor programs in order to perform specific motor tasks.

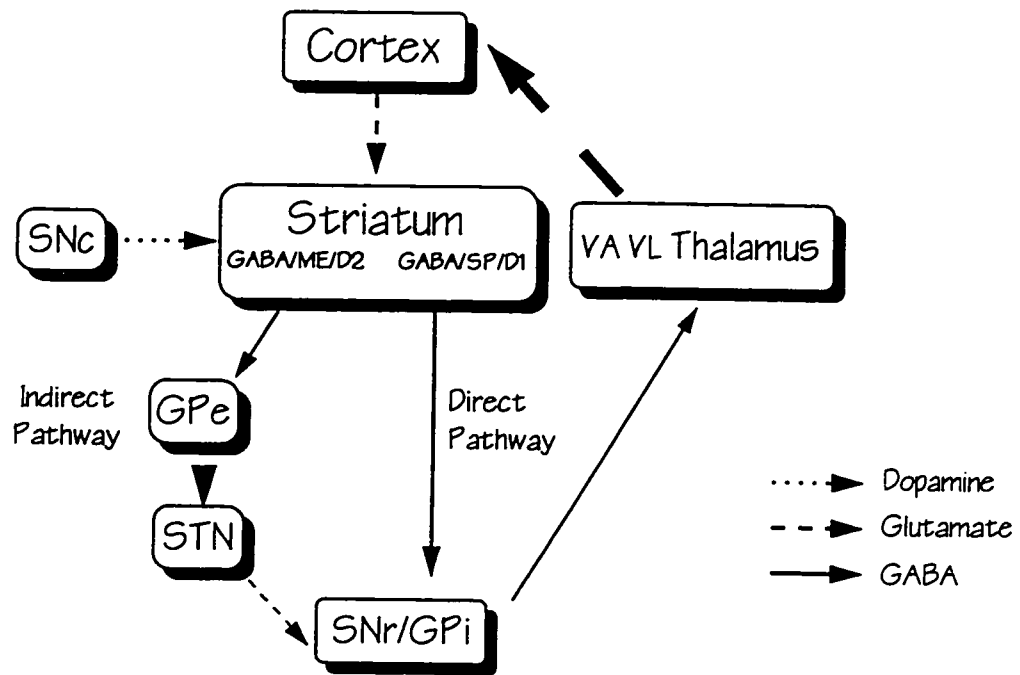


Figure 9. Basal ganglia circuit diagram depicted changes associated with Huntington's disease.

B. Consequences of Striatal Dysfunction on Cognition

While pathways incorporating the putamen and somatosensory portions of the cortex are associated with movement, pathways involving the caudate and prefrontal cortex are associated with cognition. This is also illustrated by the clinical-pathological data demonstrating impaired cognition in disorders of the basal ganglia such as HD and PD (Saint-Cyr et al., 1995).

Although there is some variability in the initial symptoms of HD, the cognitive disorder tends to begin early in the course of the illness, often years prior to the onset of any motor dysfunction. Common presenting emotional symptoms are depression, irritability, apathy, decreased concentration and loss of energy; although occasionally the initial manifestations of a thought disorder may be psychosis (Cummings, 1995; Shoulson, 1990). As the illness progresses, most psychiatric symptoms persist and worsen, until the inevitable onset of dementia (Morris, 1995).

The most striking neuropathologic change in HD is atrophy and neuronal loss in the striatum (Lange et al., 1976; Vonsattel et al., 1985; Vonsattel et al., 1987). In a detailed and comprehensive study, Vonsattel et al documented the most profound loss of neurons in the caudate; in particular the anteromedial aspects of this nucleus. These neuropathological changes occurring primarily in the caudate correspond to the early stages of the disease and the clinical findings of mood and personality changes. Taken as a whole, the neuropsychological pathology of HD strongly suggests a role for the caudate in cognition.

As in HD, patients with PD frequently present with depression and often progress to dementia (Saint-Cyr et al., 1995). Depression is one of the earliest presenting symptoms in patients with PD and is often associated with bradyphrenia. The mood disorder can be severe enough to provoke suicide, and may be accompanied by psychosis. Interestingly, in the early stages of the disease these psychiatric symptoms may be relieved by the administration of low dose L-dopa, suggesting that the nigrostriatal dopaminergic tone between direct and indirect pathways of the striatum may play a key role in normal cognition and emotion.

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CHAPTER 3: DOPAMINE-GLUTAMATE INTERACTIONS IN THE
STRIATUM: BEHAVIOURALLY RELEVANT MODIFICATION OF
EXCITOTOXICITY BY DOPAMINE-RECEPTOR-MEDIATED
MECHANISMS

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3.1 ABSTRACT

The two most important afferent projections to the striatum contain glutamate and dopamine, respectively. Excitotoxic damage resulting from excessive stimulation of the NMDA subtype of glutamate receptor has been implicated in pathophysiology of ischaemic stroke, hypoglycaemic brain damage and Huntington's disease. We studied the ability of the dopamine system to modify the anatomical, neurochemical and behavioural consequences of glutamatergic toxicity in the striatum.

In a first set of experiments the specific NMDA-receptor agonist quinolinate (QUIN) was injected unilaterally into the striatum of rats pretreated with one of (i) intraperitoneal (i.p.) saline (controls); (ii) i.p. haloperidol (HAL), a D2 dopamine receptor agonist; or (iii) 6-hydroxydopamine (6-OHDA) lesion of the ipsilateral nigrostriatal tract. QUIN-induced striatal damage, as assessed by morphometric and neurochemical criteria, was significantly attenuated in the animals with 6-OHDA lesions and in those pretreated with HAL, compared with saline-pretreated controls. There were no significant differences between the 6-OHDA and HAL groups. In a second set of experiments, animals received (a) bilateral intrastriatal QUIN plus perioperative i.p. saline (BILAT QUIN); (b) bilateral intrastriatal QUIN plus i.p. haloperidol (BILAT QUIN+HAL); or (c) bilateral intrastriatal saline (CONT). Again, the QUIN-lesioned animals treated with perioperative HAL had significantly less striatal damage than the BILAT QUIN rats. Behavioural assessment in the Morris Water Maze showed the BILAT QUIN+HAL group to be significantly less impaired on a spatial acquisition task than the BILAT QUIN animals. Measures of spontaneous daytime motor activity showed significant differences in average speed and rest time between the BILAT QUIN+HAL rats and the BILAT QUIN group. The performance of the BILAT QUIN+HAL group was not significantly different from that of controls on any of the behavioural tasks.

These results indicate an important role for D2 dopamine receptor-mediated mechanisms in striatal excitotoxicity. Since the excitotoxic process involves the same fundamental signaling mechanism that is involved in normal glutamatergic transmission, these findings imply an ability of D2 receptor blockade to modify glutamate signaling in the striatum. These results may have implications for treatment strategies in ischaemic stroke, hypoglycemic brain damage and schizophrenia.

Key words: haloperidol, Huntington's disease, schizophrenia, quinolinic acid, stroke.

3.2 INTRODUCTION

The striatum plays a central role in the striatal-pallidal-thalamic feedback circuit that modulates function of the cerebral cortex. Striatal dysfunction can produce clinically important abnormalities of motor and cognitive abilities. Deficiency of striatal dopamine gives rise to the bradykinesia and akinesia that characterize Parkinson's disease, while striatal degeneration is associated with the choreoathetotic movement disorder and neuropsychiatric impairments found in Huntington's disease.

The major input to the striatum is a glutamatergic projection from cerebral cortex. Excessive stimulation of glutamate receptors can produce neuronal damage through an excitotoxic mechanism.² This excitotoxic process may play an important role in the pathogenesis of acute stroke, hypoglycemic brain damage and Huntington's disease.^{2,3,58,65} Experimental studies have demonstrated the importance of transmitter-receptor interactions in the development of this damage. Glutamate receptor antagonists have been shown to protect the striatum and hippocampus from hypoglycemia and focal ischemia,^{3,58,64,65} and removal of the corticostriatal glutamatergic system can completely block excitotoxin-induced damage in the striatum.^{39,66}

The second important input to the striatum is the dopaminergic fibre system that originates in the substantia nigra compacta. Selective lesioning of this pathway can attenuate ischemia- and hypoglycemia-induced neuronal damage.^{6,16,29,40} The mechanism for this neuroprotective effect has not been elucidated. Golgi studies have shown that 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal tract result in a reduced density of dendritic spines in the striatum.³³ Since glutamatergic receptors are localized to the dendritic spines, it is possible that the dopaminergic lesions may exert their neuroprotective effect by simply decreasing the number of glutamate receptors available for excitotoxic stimulation. Alternatively, the protective effect observed with 6-OHDA

lesions may reflect a direct interference with dopamine-glutamate interactions at the receptor level. The present study was undertaken to clarify whether dopamine is capable of modifying glutamatergic toxicity in the striatum by a receptor-mediated mechanism.

3.3 EXPERIMENTAL PROCEDURES

A. Unilateral Striatal Lesions

Surgical Procedures

Forty-eight male Sprague-Dawley rats (Charles-River Quebec) were housed in group cages, with free access to food and water, on a 12h:12h day:night light cycle.

Surgery was performed under pentobarbital anesthesia in 2 stages:

i) Unilateral injections into the substantia nigra were carried out when the animals weighed 200-250 grams. Each anaesthetized rat was placed in a stereotaxic apparatus (David Kopf, Tujunga, California) with the toothbar 3.0 mm below the interaural line. A 10 μ l Hamilton syringe with a 30-gauge blunt-tipped needle (Hamilton Company, Reno, Nevada) mounted on an electrode carrier was used to deliver the solutions. The stereotaxic coordinates were: A/P +4.5 mm; M/L +0.9 mm. In 39 animals, the cannula was lowered 7 mm below the dura (to avoid damaging the ascending DA fibre bundle) and saline 4 μ l was injected over the course of four minutes. In the remaining 9 rats, the needle was lowered to 8.0 mm below the dura, and 8 μ g 6-OHDA (Sigma Company, St Louis, MO) was administered into the rostromedial aspect of the substantia nigra on the right side. The 6-OHDA was delivered in a volume of 4 μ l saline at a rate of 1 μ l/minute. The adequacy of the 6-OHDA lesions was assessed by measuring rotations following an

apomorphine challenge (0.25mg/kg subcutaneously). All 9 6-OHDA -lesioned rats showed > 10 rotations per minute to the left, reflecting >90% dopamine depletion in the right (ipsilateral) striatum. ii) The second surgical procedure was carried out 3 weeks after the first. On this occasion, 20 of the animals with sham lesions of the DA pathway were pretreated with 5 mg/kg haloperidol, a dopamine D2 receptor antagonist (McNeil Pharmaceuticals, Stouffville, ON) by intraperitoneal (i.p.) injection, while all other animals received an i.p. injection of saline. Twelve hours after the i.p. injections, all animals received a unilateral injection of 240 nanomoles of quinolinate (QUIN; Sigma Co. St Louis, MO) into the right striatum at the following coordinates: A/P +0.7 mm; M/L +2.5 mm; D/V 4.5 mm (below the dura). QUIN, a selective agonist at the NMDA subtype of glutamate receptor, produces an excitotoxic lesion when injected into the striatum.² The QUIN was delivered in a volume of 1 μ l of saline at the rate of 1 μ l/minute. After the injection was complete, the needle remained in place for an additional two minutes prior to retraction and the closing of the wound. All animals received a second injection of either vehicle or haloperidol 5-6 hours post-surgery.

This 2-stage process produced three experimental groups: (1) unilateral striatal lesion with pre-operative 6-OHDA lesion of the ipsilateral nigrostriatal dopaminergic pathway (QUIN+6-OHDA; N=9); (2) unilateral striatal lesion with perioperative pharmacological blockade of the D2 dopamine receptor by haloperidol (QUIN+HAL; N=20); (3) unilateral striatal lesion with perioperative injection of i.p. saline (QUIN+Saline; N= 19).

Morphometric Studies

One week post-surgery, the animals were killed by decapitation. The brains were removed, placed in ice-cold 0.9% saline and cut in the coronal plane through the site of the needle track (A/P +0.7 mm). Coronal sections 2 mm thick anterior and posterior to the injection site were placed on an iced Petri dish and photographed using an Olympus OM-2 camera equipped with bellows and macro lens. Contact sheets were then developed from the photographs and coded, so that the treatment status of each animal was not apparent from the contact sheets. The lesions appeared white on all slices in contrast to normal striatum, which was a shade of gray. The lesions were rank ordered according to size by two independent observers blind to the treatment status of the animals. The area of the lesion was then calculated using bioquant IV software (R and M Biometrics, Nashville Tennessee). The individual tracings were placed on a Hipad Digitizer software tablet (Houston Instruments, Austin Texas) connected to an IBM personal computer. Area was measured using a cursor (Houston Instruments) attached to the software tablet. In each instance two measurements were taken and the mean of the two was calculated and expressed as a percentage of total striatal area. Statistical analysis of the data was performed using analysis of variance or Kruskal-Wallis H test followed by Mann Whitney U tests or Kruskal-Wallis tests.

Neurochemical Studies

The striatum was dissected from both the control and the QUIN-lesioned sides and placed in individual test tubes containing 1 ml 0.1 N HCl. Peptides and amino acids were extracted by boiling the tissue for 10 minutes. Three 250 μ l aliquots of supernatant were lyophilized and stored at -20°C. Radioimmunoassays for somatostatin, neuropeptide

Y, substance P and cholecystokinin were carried out as previously described.⁴⁶ Amino acids were measured by high-performance liquid chromatography (HPLC) with fluorescence detection. Neurotransmitter levels on the lesioned side were expressed as percentage of values on the unlesioned side. Statistical analysis was carried out by 1-way analysis of variance, followed by post hoc Mann-Whitney U tests or T-tests, where appropriate.

B. Bilateral Striatal Lesions

Surgical Procedures

Thirty male Sprague-Dawley rats, each weighing 225-250 gms, were randomized (ten in each group) to one of three treatment protocols: (1) bilateral intrastriatal injection of QUIN (120 nmols/hemisphere), preceded by haloperidol 5 mg/kg i.p. twelve hours prior to surgery and followed by a second injection of haloperidol 5-6 hours post-surgery (BILAT QUIN+HAL); (2) bilateral intrastriatal injection of QUIN (120 nmols/hemisphere), preceded by injection of vehicle i.p. twelve hours prior to surgery, followed by a second injection of vehicle 5-6 hours post-surgery (BILAT QUIN); (3) bilateral intrastriatal injection of saline, preceded and followed by i.p. saline as for group two (CONT). The protocols and stereotaxic coordinates for intrastriatal injections were identical to those described for the unilateral lesions.

Behavioural Testing

After a recovery period of two weeks the animals were tested in the Morris Water Maze (MWM). The testing was conducted in a circular tank, 1.83 metres in diameter.

One of the four quadrants contained a clear plexiglass platform, 30 cm high. The maze was filled with water (18-19°C) such that the top of the platform was 1-1.5 cm below the surface of the water and not visible to the swimming rat. The location of the platform was marked on the bottom of the pool to ensure consistency of placement.

The animals were tested using a protocol described by Whishaw et al.⁶⁷ Each trial consisted of placing the rat into the pool at one of four equidistant points. Each rat was given four trials per day, the order of which was chosen randomly such that no location recurred for a given day. If the animal did not find the platform within 60 seconds, it was removed from the pool and placed on the platform for an additional 20 seconds. If the animal found the platform, it was allowed to remain on the platform for 20 seconds. After six consecutive days of training with the transparent submerged platform, the animals were tested on day seven with a visible platform constructed of black plastic and projecting 1-2 cm above the surface of the water. Latency to find the platform was recorded for each trial. Each testing session was recorded by a camera suspended above the pool and connected to a VHS video recorder. The data were analysed by ANOVA.

After completion of the MWM testing, the animals were assessed for spontaneous activity during their subjective day. The rats were placed in Digiscan Animal Activity Monitors (Omnitech, Columbus, Ohio) which are programmed to gather data for a number of variables, and spontaneous activity was recorded from 10:00 to 12:00. The variables of average speed, rest time and total distance travelled were analysed. The data were analysed by ANOVA followed by Mann-Whitney U-tests.

Morphometric and Neurochemical Studies

All animals were killed one week after the conclusion of testing in the MWM, and the striatal lesions were rank-ordered according to size, as described earlier. The tissue was then processed and striatal neuropeptides were measured as described for unilateral lesions. In these studies the rats treated with i.p. injections of saline served as controls.

3.4 RESULTS

A. Unilateral Lesions

Morphometric Analyses (Figures 1,2, Table 1)

The lesions produced by unilateral intrastriatal injection of QUIN are depicted in Figure 1. Total striatal area was unchanged on the lesioned side as compared with the unlesioned side in all 3 groups (Table 1), indicating that there had been no significant atrophy of striatal tissue in the week between the time of lesioning and sacrifice of the animals.

When the striatal lesions were rank-ordered according to lesion size (Figure 2a), there was a highly significant effect for group ($H= 16.23$; $p<0.00001$). The QUIN+HAL and QUIN+6-OHDA groups were each found to have significantly smaller lesions than did saline-pretreated controls (QUIN+HAL vs QUIN+Saline, $p<0.01$; QUIN+6-OHDA vs QUIN+Saline $p<0.01$; QUIN+HAL vs QUIN+6-OHDA, not significant).

Quantitative analysis of lesion size (Figure 2b) likewise showed a significant effect for group ($F=6.39$, $p<0.01$). Striatal lesions in the QUIN+HAL group were 42% smaller than in saline pretreated controls ($p<0.005$). Animals with 6-OHDA lesions of

the ipsilateral nigrostriatal tract had striatal lesions 48% smaller than control lesions ($p < 0.01$). There was no significant difference in lesion size between the QUIN+HAL vs QUIN+6-OHDA groups.

Neurochemical Analyses (Figure 3)

Levels of Substance P, which is localized to a subpopulation of medium-sized spiny neurons in the striatum, were reduced by 56% in the QUIN-lesioned striatum of animals pretreated with saline vehicle, but by only 21% in rats pretreated with haloperidol and by 29% in rats with pre-existing 6-OHDA lesions (Figure 3a). This represented a significant sparing of substance P immunoreactivity in the striatum of the QUIN+HAL and the QUIN+6-OHDA groups, as compared with QUIN + Saline rats ($F = 4.43$, $p = 0.026$; QUIN+Saline vs QUIN+HAL, $p < 0.05$; QUIN+Saline vs. QUIN+6-OHDA, $p < 0.05$; QUIN+HAL vs QUIN+6-OHDA, not significant).

Levels of somatostatin and neuropeptide Y (NPY), which are contained in a subpopulation of locally projecting medium aspiny neurons in the striatum, were reduced by 26% and 24%, respectively, in the QUIN-lesioned striatum of rats pretreated with saline vehicle (Figure 3a). Pretreatment with haloperidol or 6-OHDA did not provide significant sparing of somatostatin or NPY levels.

Concentrations of cholecystinin (CCK), which is believed to be localized to afferent terminals in the striatum, were not significantly changed in the QUIN-lesioned striatum of any of the 3 groups.

Amino acid analysis (Figure 3b) showed a 46% depletion of GABA levels in the QUIN-lesioned striatum of saline-pretreated animals, compared with 23% reduction in

the QUIN+HAL group and 24% decrease in QUIN + 6-OHDA rats ($F= 4.43$, $p=0.026$). This represented a significant attenuation of striatal GABA loss in the group treated with haloperidol ($p<0.05$) and in the group with pre-existing 6-OHDA lesions ($p<0.05$). Taurine showed similar changes, with 43% reduction of taurine concentrations in the QUIN-lesioned striatum of the QUIN+Saline group, but only 17% and 21% in the QUIN+HAL and QUIN+6-OHDA groups respectively ($F=5.3$, $p=0.015$); QUIN+Saline vs QUIN+HAL, $p<0.01$; QUIN+Saline vs QUIN+6-OHDA, $p<0.02$).

Glutamate, the transmitter pool of which is localized mostly to the terminals of the corticostriatal tract, was reduced 16-17% in the lesioned striatum in all 3 groups, with no significant differences between the groups.

B. Bilateral Striatal Lesions

Morphometry (Figure 4)

The range of lesions produced by the bilateral intrastriatal injection of QUIN is shown in figure 4. As was the case with unilateral injections, animals treated perioperatively with haloperidol had significantly smaller lesions than did QUIN-lesioned rats receiving perioperative saline ($p<0.02$).

Behavioural Studies (Figure 5, Table 2)

Animals with bilateral QUIN lesions of the striatum (BILAT QUIN) were significantly slower than controls in locating the hidden platform of the Morris Water Maze ($F=10.06$, $p<0.001$) (Figure 5). Animals treated pre- and post-lesioning with haloperidol (BILAT QUIN+HAL) showed significantly better performance in the MWM

than did BILAT QUIN rats ($F=4.48$, $p<0.05$). Indeed, the performance of the BILAT QUIN+HAL group was not significantly different from that of controls. On day 7 of the MWM, when the platform was visible to the rats, all three groups showed comparable performance in swimming to the platform, suggesting comparable levels of motivation and motor function.

Measures of spontaneous daytime motor activity (Table 2) showed a significant effect for average speed ($F=3.97$, $p=0.031$) and total rest time ($F=4.96$, $p<0.015$). In each of these measures the BILAT QUIN group was significantly different from both controls and BILAT QUIN+HAL animals, while the BILAT QUIN+HAL rats did not differ significantly from controls. A similar effect was observed for total distance travelled ($F=3.57$, $p<0.043$) as the BILAT QUIN rats were significantly different from controls ($p<0.03$) while the BILAT QUIN+HAL group did not differ significantly from controls.

Neurochemical Analyses (Figure 6)

Animals pretreated with haloperidol (BILAT QUIN+HAL) had significant protection from the substance P-depleting effect of QUIN, as compared with BILAT QUIN rats pretreated with saline ($p<0.01$). Levels of somatostatin were likewise significantly spared in the striatum of haloperidol-pretreated rats compared with saline-pretreated animals (BILAT QUIN+HAL vs BILAT QUIN: $p<0.001$). Haloperidol did not provide a comparable sparing of neuropeptide Y or CCK levels.

3.5 DISCUSSION

These results show that excitotoxicity in the striatum can be modified in a behaviourally relevant manner by pharmacological blockade of the D2 dopamine receptor. Short-term treatment with the D2 receptor antagonist haloperidol resulted in significant attenuation of the striatal lesion produced by quinolinic acid. The striatal sparing in haloperidol-treated animals, as assessed by morphometric and neurochemical measures, was comparable to that observed in rats with 6-OHDA lesions, suggesting an important role for D2 dopamine receptor-mediated mechanisms in striatal excitotoxicity.

Neurochemical analysis confirmed previous observations that levels of substance P and GABA are depleted to a greater extent than somatostatin or NPY in quinolinate-lesioned striatum.² Treatment with haloperidol or 6-OHDA significantly reduced the extent of substance P and GABA depletion, but failed to attenuate the NPY changes in either unilaterally- or bilaterally-lesioned rats and did not protect against the decreases of somatostatin immunoreactivity in animals with unilateral lesions. This suggests that dopamine may selectively influence NMDA receptor-mediated toxicity on the medium spiny neurons of the striatum, without a comparable effect on the medium aspiny populations.

Taurine levels were decreased by 43% in QUIN-lesioned striatum. As with GABA and substance P, there was significant sparing in animals pretreated with haloperidol or 6-OHDA. Taurine is believed to be localized to spiny neurons in striatum.^{9,15} The ability of dopamine receptor blockade or 6-OHDA lesions to attenuate the taurine depletion is again consistent with a role for dopamine in excitotoxic damage to spiny striatal neurons.

When tested in the Morris Water Maze, animals with bilateral excitotoxic lesions of the striatum (BILAT QUIN) were impaired in the acquisition of the hidden platform

task, confirming the observations of Whishaw et al.⁶⁷ and ourselves.²⁶ The lesioned rats showed no impairment in their ability to swim to a visible platform, suggesting adequate levels of motivation and motor function for performance of the MWM task. Treatment with haloperidol at the time of lesioning (BILAT QUIN+HAL) protected the rats from this behavioral impairment, resulting in a level of performance that was not significantly different from that of sham-lesioned controls.

Dopamine-Glutamate Interactions in the Striatum: Anatomical Considerations

The main target of glutamatergic excitotoxicity in the striatum is the medium spiny neuron. Glutamatergic input to the medium spiny population occurs primarily at the level of the distal dendrite.³⁶ Two types of distal dendrites have been identified on medium-spiny neurons. The first, comprising approximately 53% of the total spine population, consists of a single asymmetric (glutamatergic) synaptic bouton, allowing for unimpeded glutamate transmission.²³ The second type of spine receives dual input, with dopaminergic and glutamatergic projections converging on the same dendritic spine. Roughly 47% of corticostriatal glutamate fibres, and almost 60% of the nigrostriatal dopamine input, terminate on this latter type of spine.^{23,36,59} These dual-input dendritic spines provide a major locus for direct interaction between the dopaminergic and glutamatergic projections to the striatum.

There are several other potential sites at which dopamine might modulate glutamate toxicity in the striatum. Approximately 35% of the dopaminergic synapses on the medium spiny neuron are located on the distal dendritic shaft, where they are in a position to modify electrical changes flowing from the spines.^{31,61} A further 5-6% of dopamine synapses are found on the proximal dendritic shafts and cell body, providing

another site for dopamine to influence signaling in the medium spiny neuron.^{21,59} In addition to these possible direct interactions with glutamatergic transmission, dopamine might also indirectly modify glutamatergic neurotransmission by influencing somatostatin/NPY and cholinergic interneurons which synapse on the medium spiny neurons of the striatum.

Functional Interactions Between Dopamine and Glutamate in the Striatum:

i) The Role of DARPP-32

Details of the functional interaction between glutamate and dopamine in the striatum have begun to be clarified at the molecular level. Specifically, it has recently become clear that there is a direct interaction at the level of the dendritic spine between D1 receptors and NMDA receptors via the intracellular protein called DARPP-32.³² The active form of DARPP-32 inhibits protein phosphatase-1 and the subsequent dephosphorylation of intracellular proteins. Activation of the NMDA receptor by glutamate, on the other hand, leads to the dephosphorylation of DARPP-32 and the activation of protein phosphatase-1.

These opposing effects of dopamine and glutamate on the phosphorylation state of DARPP-32 could potentially explain the haloperidol-induced effects obtained in this experiment. Haloperidol, in the dose used in these experiments, is an antagonist at the D2 and D3 subtypes of dopamine receptor, but not at the D1 receptor. Since D2 receptors on presynaptic terminals of the nigrostriatal pathway are known to inhibit dopamine release, short-term administration of haloperidol might be expected to increase dopamine release in the striatum. This in turn would lead to increased stimulation of postsynaptic D1

receptors, without concomitant stimulation of the D2 receptors (which would be competitively blocked by the haloperidol). The resulting increase in D1 receptor stimulation would antagonize the effect of glutamate on the phosphorylation state of DARPP-32, thereby potentially resulting in a reduction of excitotoxicity.

While this might account for the neuroprotective effect of haloperidol, the protective effect of 6-OHDA cannot readily be understood in terms of this model. If the major determinant of NMDA receptor-mediated toxicity were indeed related to the phosphorylation state of DARPP-32, then one would have expected the elimination of the nigrostriatal DA tract to have exacerbated, rather than attenuated, the excitotoxicity, as there would now be no D1 receptor stimulation to phosphorylate DARPP-32.

ii) The Role of D2 Receptors

There is evidence of an additional interaction between dopamine and glutamate in the striatum via D2 receptors. Specifically, dopamine and D2 agonists have been shown to inhibit stimulated glutamate efflux in the striatum.^{37,44,45,49,68} It has been hypothesized that dopamine might exert this inhibitory effect on glutamate efflux via presynaptic D2 receptors on corticostriatal nerve terminals,^{22,27,49,56} though there is not yet any direct anatomical evidence for this.

The putative presynaptic inhibition of glutamate release by dopamine would not explain the data from our study, as blocking D2 receptor-mediated transmission through 6-OHDA lesions or the administration of haloperidol would have been expected to disinhibit glutamate release. This in turn would have either enhanced or had no effect on the excitotoxic process, whereas we observed a neuroprotective effect.

iii) Effects on NMDA Receptors

It is recognized that glutamate receptors on the medium spiny neuron are localized to the dendritic spines. Golgi studies have shown that the density of dendritic spines is decreased following 6-OHDA lesions of the ipsilateral nigrostriatal tract.³³ The loss of NMDA receptors that would presumably accompany this reduction of synaptic spines might explain the ability of 6-OHDA lesions to attenuate excitotoxic damage in the striatum. The ability of haloperidol to produce a comparable attenuation of lesion size may reflect a similar effect on NMDA receptors.

iv) The Role of Nerve Growth Factors

Many growth factors including NGF, BDNF, NT-3 and bFGF have been shown to protect against excitotoxic lesions in the striatum, septum, cortex and hippocampus.^{10,13,24,25,43,53,54,57} This neuroprotective effect may be related to the ability of growth factors to stabilize intracellular calcium by increasing the expression of intracellular proteins such as calbindin.^{11-13,42} Alternatively, as in the case of bFGF, the excitoprotective effect may involve downregulation of NMDA receptor proteins, thereby attenuating glutamate-induced increase of intracellular calcium levels.⁴³ A second potential protective mechanism is enhancement of free radical defense systems via increased expression of glutathione peroxidase and superoxide dismutase.^{51,52,63}

Enhanced production of NGF and/or other growth factors could explain the results from both the 6-OHDA lesioned animals and those pretreated with haloperidol. It is well known that NGF can attenuate the toxic effects of QUIN injected in the striatum and a recent report indicated that lesioning nigral dopaminergic neurons with 6-OHDA

increased NGF content in the brain for a period of .5-28 days post-lesion.⁵⁰ It was subsequently shown that striatal growth factors can also be stimulated by haloperidol,^{9b} raising the possibility that this effect might mediate the neuroprotective action of the drug.

v) The Role of Nitric Oxide

The gaseous neurotransmitter nitric oxide (NO) is known to be involved in neural transmission in the CNS and has been reported to play an important role in mediating glutamatergic toxicity.^{14,17,19,20,34,38,41,47,55,62} NO is produced in neurons that contain the enzyme nitric oxide synthase (NOS).^{4,5,18} In the striatum, NOS is found in the subset of locally projecting aspiny neurons that also contain the neuropeptides SS and NPY.¹⁸ Stimulation of these neurons by dopamine through the D2 receptor is known to result in an increase of SS release from cultured striatal neurons and may also produce increased release of NO.²⁸

The neuroprotective effects of 6-OHDA and haloperidol observed in this experiment could be mediated by changes in nitric oxide. Lesioning of the nigrostriatal dopaminergic pathway with 6-OHDA, or receptor blockade with haloperidol, would reduce D2 receptor-mediated stimulation of SS/NPY/NOS-containing neurons in the striatum. A resulting decrease of NO release would be expected to reduce the excitotoxic effect of QUIN.

Significance

The results of these experiments may have clinical implications. The ability of haloperidol to attenuate acute excitotoxic brain damage suggests that D2 receptor

blockers may be useful in the treatment of acute ischaemic stroke and hypoglycaemia. Unlike many of the experimental measures that have been reported to reduce excitotoxic damage, neuroleptic medications are already in clinical use. The role of D2 receptor blockers in disorders that may involve chronic excitotoxic processes, such as Huntington's disease, is more problematic, since long-term administration of haloperidol can itself produce degenerative changes in the basal ganglia.^{7,35,60}

These findings may also have implications for our understanding of schizophrenia. Patients with schizophrenia typically exhibit cognitive abnormalities that are thought to reflect dysfunction of the dorsolateral prefrontal cortex.^{30,48} It has been shown that lesions of the basal ganglia, particularly the dorsal-medial striatum, produce behavioural deficits similar to those associated with prefrontal lesions.^{26,67} The ability of haloperidol to modify excitotoxicity in the dorsal-medial striatum suggests that neuroleptic medications, which are widely used in the treatment of schizophrenia, may exert at least some of their therapeutic effect by modifying glutamatergic signaling in the striatum.

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3.7 FIGURES

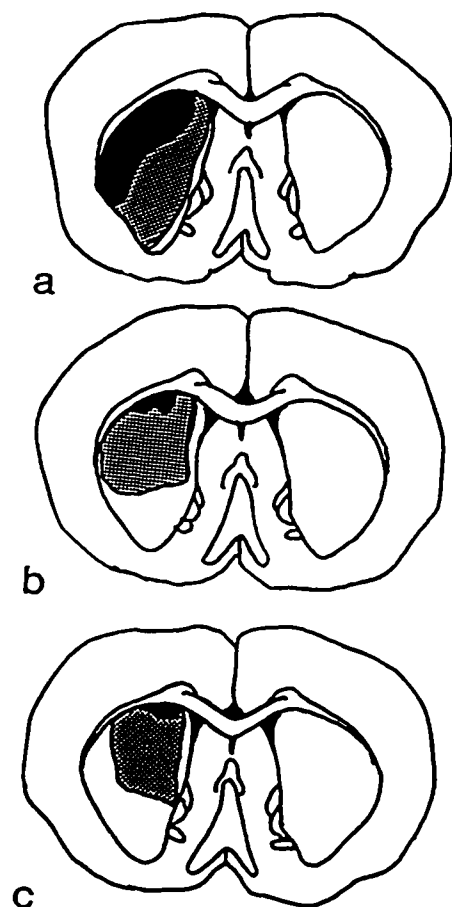


Figure 1. Range of unilateral striatal lesions in the different treatment groups.

Range of unilateral striatal lesions in the different treatment groups. The smallest lesion in each group is depicted in solid black and the largest lesion is represented by the shaded area. a) QUIN + saline; b) QUIN + HAL; c) QUIN + 6-OHDA. The lesions in the QUIN group were significantly larger than those in the other two groups, while there was no statistical difference between the QUIN+HAL and QUIN+6-OHDA groups.

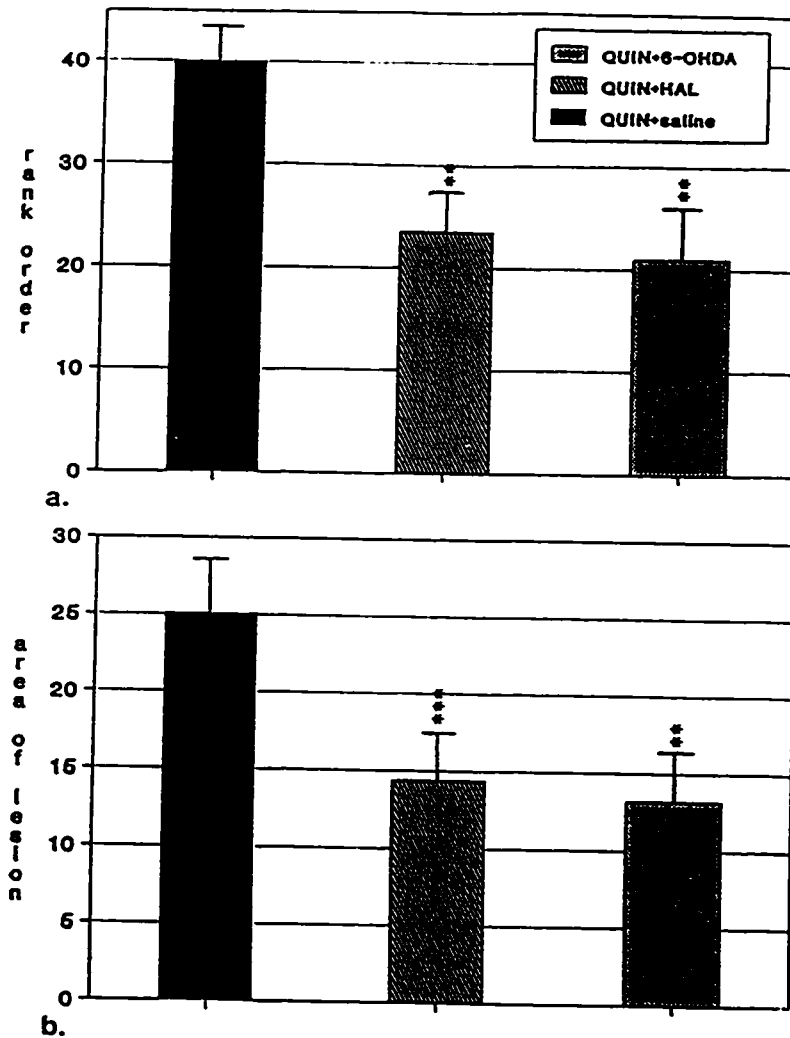


Figure 2. Unilateral striatal lesion: morphometric analyses.

A. (top): Rank ordering according to lesion size; a higher number denotes a larger lesion.
 B. (bottom): Area of lesion (mm²). Values represent mean \pm SEM. By both measures, the QUIN+HAL and QUIN+6-OHDA groups had significantly smaller lesions than QUIN+saline. There was no difference between the two treatment groups. ** $p < .01$; *** $p < .005$.

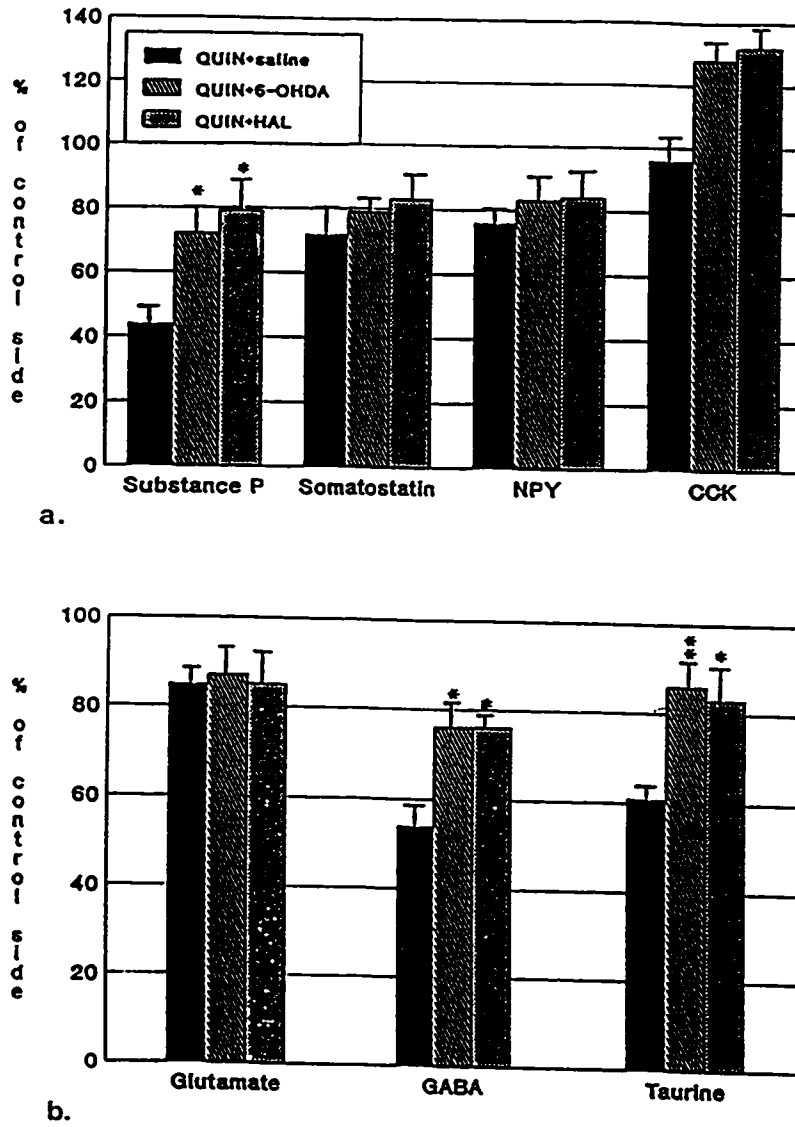


Figure 3. Unilateral striatal lesion: neurochemical analyses.

a. (top): Neuropeptides. b.(bottom): Amino acids. Values represent mean \pm SEM. There was significantly less depletion of substance P, GABA, and taurine (markers for spiny neurons) in the QUIN +HAL and QUIN+6-OHDA groups, as compared with QUIN+saline controls. * $p < 0.05$; ** $p < 0.01$.

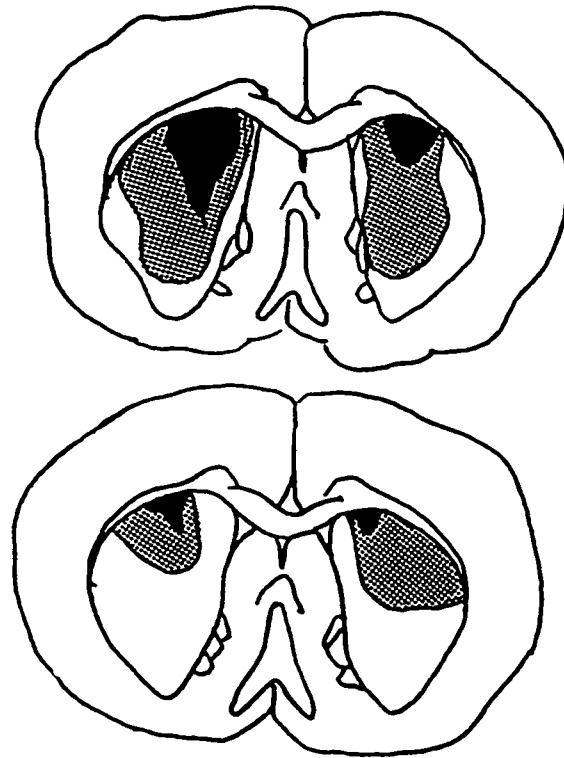


Figure 4. Range of bilateral striatal QUIN lesions in the different treatment groups.

The smallest lesion in each group is depicted in solid black and the largest lesion is represented by the shaded area. (top) BILAT QUIN; (bottom) BILAT QUIN + HAL.

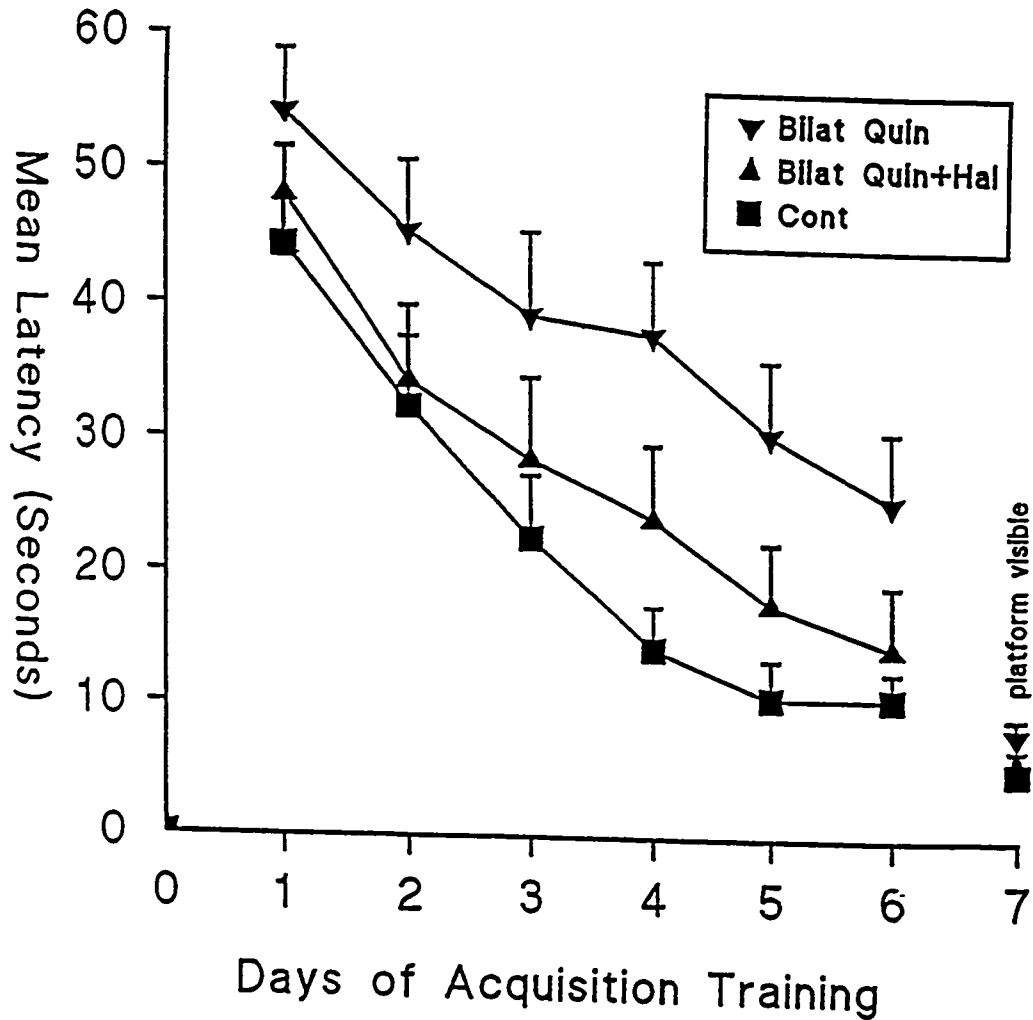


Figure 5. Bilateral striatal lesions: behavioural testing.

Mean latency to find the hidden platform in Morris Water Maze. Each data point represents the mean latency for a block of 4 individual trials. Rats with BILAT QUIN lesions of the striatum showed significant impairment of ability to locate the hidden platform compared to both the controls and the BILAT QUIN+HAL groups. The BILAT QUIN+HAL group was not significantly differently from controls.

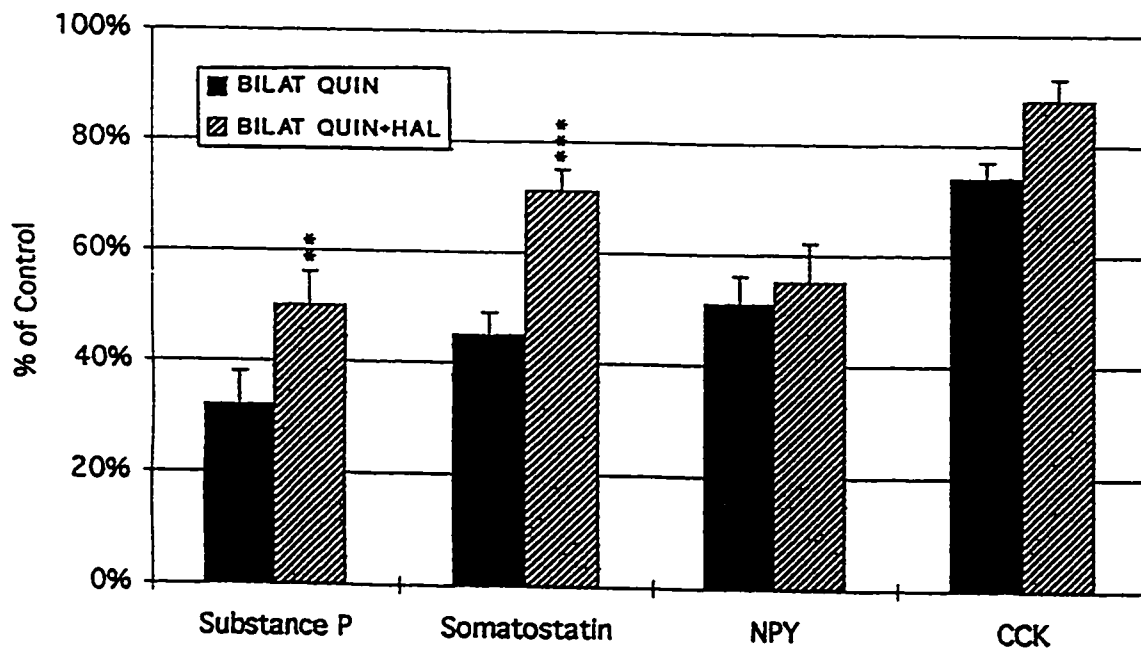


Figure 6. Bilateral striatal lesions: neurochemical analyses.

There was significantly less depletion of substance P and somatostatin immunoreactivities in the striatum of BILAT. QUIN+HAL animals compared with the BILAT. QUIN group. ** $p < 0.01$, *** $p < 0.001$.

Table 1.

Total area of lesioned vs. non-lesioned striatum in rats with unilateral QUIN lesions of the right striatum. There were no significant differences in any of the groups, indicating that there had been no significant atrophy of striatal tissue in the week between lesioning and sacrifice of the animals.

	<i>Non-lesioned side</i>	<i>Lesioned side</i>
Quin + saline	33.76 ± 3.4	34.12 ± 4.2
Quin + HAL	30.96 ± 4.5	32.01 ± 5.1
Quin + 6-OHDA	32.03 ± 3.6	29.75 ± 3.2

Table 2.

Measures of spontaneous daytime motor activity in control rats (CONT), in animals with bilateral quin-induced lesions of the medial striatum plus perioperative i.p. haloperidol (BILAT QUIN+HAL), and in rats with bilateral striatal lesions treated perioperatively with i.p. saline (BILAT QUIN) * $p < 0.05$ compared with CONT; § $p < 0.05$ compared with BILAT QUIN+HAL

	<i>Total rest time (minutes)</i>	<i>Average speed (cm/sec)</i>	<i>Total distance travelled (cm)</i>
CONT	110.5 ± 1.0	9.57 ± 0.39	5540 ± 822
BILAT QUIN+HAL	111.4 ± 1.6	9.96 ± 0.47	4282 ± 795
BILAT QUIN	115.5 ± 0.8 *§	8.47 ± 0.28 *§	2717 ± 383 *

CHAPTER 4: THE ONTOGENY OF NADPH-DIAPHORASE
NEURONS IN SERUM-FREE STRIATAL CULTURES
PARALLELS IN VIVO DEVELOPMENT

Neuroscience (1997) in press.

4.1 ABSTRACT

Nitric oxide synthase is colocalized with somatostatin and neuropeptide Y in a subpopulation of striatal interneurons that stain selectively for NADPH-diaphorase. We studied the ontogeny of diaphorase-positive neurons in striatal serum-free cultures derived from 15-16 day-old CD1 mice. NADPH-diaphorase staining was detected as early as embryological day 18 *in vivo* and day 5 *in vitro*. Over the next seven days the number of neurons staining for NADPH-diaphorase increased rapidly and then levelled off at about 0.5-1% of the total neuronal population both *in vivo* and *in vitro*. The cultured diaphorase neurons were also similar to their *in vivo* counterparts in terms of morphology and dendritic branching.

Striatal neurons expressing NADPH-diaphorase exhibit similar ontogeny, morphology and neurochemical characteristics *in vivo* and in serum-free primary neuronal cultures. The culture system may represent a useful model for studying this important subgroup of striatal neurons.

4.2 INTRODUCTION

Nitric oxide (NO) is a gaseous neurotransmitter which has been implicated in both physiologic and pathologic processes in the brain. It may function physiologically as a retrograde neurotransmitter in the formation of memory^{7,9,28,32}, as well as in development and plasticity.³⁶ It may also play a role in aggression and sexual behaviour.²¹ Pathologically, NO has been connected with the neurotoxicity associated with excessive NMDA-receptor stimulation.^{5,11,12,27}

NADPH-diaphorase (NADPH-d) is a specific marker for cells containing neuronal nitric oxide synthase (nNOS).^{8,10,17,20,38} Somewhat paradoxically, those neurons capable of producing NADPH-d appear to be protected from the neurotoxic effects of NO, as they are selectively spared in such pathological conditions as stroke³³, Huntington's disease¹⁴, and Alzheimer's disease,^{18,25} and in cultured neurons exposed to NMDA-mediated excitotoxic injury.^{22,23} Furthermore, the abnormal migration of NADPH-d neurons in cortex has recently been implicated in the pathogenesis of schizophrenia, as has the alteration of a different subset of NADPH-d neurons in the pedunclopontine nucleus of the brainstem.^{1,2} There is complete co-localisation of NADPH-d and nNOS in striatum²⁰, with NADPH-d selectively localised to a subpopulation of medium-sized aspiny interneurons containing somatostatin (SS) and neuropeptide Y (NPY).^{29,36}

We have investigated the ontogeny of NADPH-d neurons in primary cultures of striatal neurons compared with their *in vivo* development in fetal and neonatal mouse brain. In particular, we looked for the earliest time of expression of NADPH-d; the increase in NADPH-d expression over time; the percentage of striatal neurons expressing NADPH-d; morphological features relating to somata, number and description of neurites, and neuritic branching; and neurochemical characteristics, specifically the colocalization of SS and NPY in cultured neurons.

4.3 METHODS

A. In Vitro Studies

Preparation of neurons in primary culture

The primary neuronal cultures were obtained from 15-16 day old CD1 mouse embryos (E15-16). The striata were dissected from the embryos in 0.1M PBS with 1% penicillin/streptomycin and mechanically dissociated in 1 ml of medium with a fire-narrowed Pasteur pipette. The cell suspension was diluted to a density of 1×10^6 trypan-blue-excluding cells per ml in a serum-free DMEM/F12 media supplemented with a hormone mix containing progesterone, selenium salts, putrecine, transferrin, insulin and sodium bicarbonate. No antibiotics were added to the medium. One ml aliquots of the cell suspension were transferred into 24-well plates (NUNC, Gibco, Burlington, Ontario)

containing 12 mm glass coverslips previously coated with 15 $\mu\text{g/ml}$ poly-l-ornithine (Sigma, St. Louis, Missouri). Cultures were maintained at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂. The medium was replaced after 7 and 14 days. This method produced a virtually glial-free neuronal culture system.

Histochemistry, cell counting and morphometric studies

Cultures were stained each day from day *in vitro* (DIV) 1–21.³¹ On each day 9 coverslips (3 from each of 3 different cultures) were fixed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.3 for 20 minutes. Several post-fixation washes with 1M PBS were followed by 90 minutes incubation at 37°C in a solution of 0.1M PBS, pH 7.4, containing 0.3% triton X-100, 0.1 mg/ml nitroblue tetrazolium and 1.0 mg/ml β -NADPH, which selectively stains neurons containing NOS. The staining reaction was terminated by washing twice in 0.1M PBS. All glass coverslips were mounted on rectangular glass coverslips with gelvisol.

Twenty fields per coverslip (comprising a total of 180 fields/day) were analysed, using light microscopy to count diaphorase neurons and phase contrast to assess background neurons. Time series quantifications were performed to determine earliest expression of NADPH-d, increase of expression over time, absolute number of neurons expressing NADPH-d at various stages of development and percentage of total neurons expressing NADPH-d for each day *in vitro* (DIV). Morphometric studies of at least 40

neurons/day were performed for overall shape, and number of primary, secondary and tertiary dendrites.

Immunohistochemistry

Six coverslips from each of 5 sister co-cultures were taken and immunohistochemically processed for the simultaneous detection of SS and NPY. Briefly, cultured neurons were fixed for 20 minutes in a solution of 0.1M phosphate buffer (PBS) containing 4% paraformaldehyde. The cultured neurons were then incubated for 30 minutes in 5% normal goat serum diluted in 0.1M PBS. Subsequent to further rinsing, the striatal neurons were incubated at 37°C for 2 hours with both a polyclonal antibody raised in rabbit against NPY²⁶ and a monoclonal antibody raised in mouse against SS (MDC, Connecticut). The antisera were diluted to 1:100 for NPY and 1:500 for SS in PBS containing 0.3% triton X-100 and 1% normal goat serum. Following this incubation, the neurons were rinsed again in 0.1M PBS and incubated for 40 minutes in goat anti-rabbit secondary antibody conjugated to rhodamine isothiocyanate (1:100 for NPY) and in goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (1:100 for SS). Following another rinse in 0.1M PBS, the round coverslips were removed from the culture plates, placed on uncoated glass microscope slides and coverslipped with fluorosave (Calbiochem). Immunolabelling was visualised under fluorescence microscopy at the appropriate wavelength of filtered ultraviolet light. A total of 404 neurons from 5 different

cultures were studied using a fluorescent microscope to determine the number of neurons that co-localised SS and NPY.

B. In Vivo Preparation

Five CD1 mouse brains from each of eight time points (E14, E16, E18, postnatal day 1 (equivalent to DIV 5), day 3 (equivalent to DIV 7), day 6 (equivalent to DIV 10), day 10 (equivalent to DIV 14), and day 13 (equivalent to DIV 17)) were removed and placed into 4% paraformaldehyde for 48 hours. All brains were then transferred to 30% sucrose for 24 hours and then flash frozen in isopentane at -50°C . The brains were sliced on a Leitz cryostat into $15\ \mu\text{m}$ sections. Sections were transferred into the NADPH-d staining solution for 90 minutes to stain diaphorase neurons. Half the sections were then counterstained with 1% neutral red for visualisation of background neurons. Stained sections were quickly dehydrated in an ascending graded series of ethanols, delipidated in xylene and mounted with permount. The numbers of NADPH-d neurons from sixteen central striatal sections were counted at each time point. A representative striatum from each of these time points was photographed and copied onto mylar to provide a pictorial representation of the topography of NADPH-d expression.

4.4 RESULTS

Most neuroblasts adhered to the poly-l-ornithine coated slides within 2-5 hours post-plating, with obvious neurite extension by 12 hours. Neurons grown in culture did not display NADPH-d staining until day 5-6 in culture. The absolute numbers of NADPH-d neurons increased dramatically, multiplying more than 50-fold between DIV 4 and DIV 6 (Figure 1), at a time when the total number of non-diaphorase neurons was decreasing (Figure 2). The total number of diaphorase neurons continued to increase rapidly between days 6-13, then stabilized, such that by DIV 16 the NADPH-d neurons accounted for approximately 0.5-1% of the total neuronal population.

The stained neurons in culture displayed three predominant morphologies. These included fusiform neuronal cell bodies with primary neurites extending from each perikaryal pole (accounting for 28% of the neurons examined); multipolar neurons with round somata, many displaying at least one large diameter proximal neurite (70% of total); and triangular somata displaying one primary neurite at each apex (2% of total). These three neuronal morphologies were identical to those found *in vivo* (Figure 3).

The morphological characteristics of the neuronal somata, as well as the relative proportions of different cell morphologies, remained consistent over the time period examined (Table 1). In contrast to the perikarya, the complexity of the branching pattern of NADPH-d labelled neurites changed substantially during the course of *in vitro* development. The number of tertiary neuritic branches displayed by labelled neurons

increased significantly across all morphological types over 21 days *in vitro* (Figures 4, 5). Moreover, increases in the number of secondary neuritic branches were identified only for multipolar neurons with round somata. They also had a comparable increase in tertiary neurites over time. Within fusiform neurons, the greatest change in morphology over time was seen in the number of tertiary neurites, with relatively little change in the number of secondary neurites. Triangular neurons showed the greatest change over time in the number of tertiary neurites, with the number of secondary and tertiary neurites reaching a plateau by DIV 13. At later stages *in vitro* (DIV 7 onwards), NADPH-d labelled neurites could be traced for considerable distances. On high power examination, distal neurites displayed multiple varicosities (Table 2, Figure 5).

The morphology of striatal neurons at different stages of *in vivo* development roughly paralleled that identified *in vitro* with respect to the complexity of dendritic branching. The labelled neurons were of the medium aspiny type of striatal neuron and displayed the three basic perikaryal shapes identified *in vitro* (Figures 3,7). Multipolar neurons were more prominent at later stages of development while the earlier labelled neurons displayed substantially fewer primary neurites. In addition the neuropil of the striatum at later stages of development exhibited a much greater concentration of small diameter varicose fibres, similar to those observed *in vitro* (Figures 3,5,7).

In vivo neurons staining for NADPH-d were identified as early as embryonic day 18 (E18). At this stage labelled neurons were confined to the extreme lateral border of the

caudate-putamen, immediately adjacent to the external capsule. Labelled neurons were densely concentrated in this area as well as more ventrally along the external capsule throughout development. At later stages of development NADPH-d neurons were identified throughout the striatum but remained more concentrated in its lateral half. At rostral levels labelled neurons were observed in the ventral striatum and olfactory tubercle (figure 6). Between E18 and D1 *in vivo* (equivalent to DIV 5 *in vitro*) the number of diaphorase-containing neurons displayed by the striatum increased more than 20 fold. The number of NADPH-d neurons continued to increase, doubling once again before they levelled off at DIV 14 (day 10 *in vivo*)(Figure 1).

Of the 404 double-labelled neurons that were studied, 394 displayed SS/NPY co-localisation (Figure 8). All of the 10 neurons that did not display colocalisation were immunopositive for SS. There were no neurons immunopositive only for NPY.

4.5 DISCUSSION

These results demonstrate that NADPH-d staining can develop normally in serum-free cultures of striatal neurons. As shown in Figure 1, the development of diaphorase-positive neurons *in vitro* was strikingly parallel to what was observed *in vivo*. In both cases, the number of cells expressing NADPH-d increased rapidly over the course of about 7 days, then levelled off at about 0.5-1% of the total neuronal population.

Both *in vivo* and *in vitro* striatal diaphorase neurons expressed NADPH-d before achieving complete morphological maturity (see Figures 4, 7a). NADPH-d was expressed as early as E18 (equivalent to DIV 3) in the mouse striatum *in vivo*, while the *in vitro* expression of NADPH-d occurred at DIV 5 following removal of the fetal striata at E15. Thus, in absolute terms, the expression of NADPH-d occurred approximately 3 days later *in vitro* than *in vivo*. The difference of 3 days in the timing of the detectable expression of NADPH-d may be attributable to the mechanical disruption of the cells prior to plating in the culture dishes. Alternatively, it is conceivable that neurons ready to express NADPH-d at an earlier stage *in vitro* — i.e. those developing adjacent to the external capsule — were not removed during dissection in an attempt to avoid the inclusion of cortical tissue (see Figure 6).

This parallelism between the *in vivo* and *in vitro* development of striatal diaphorase neurons extends to the neurochemical and morphological characteristics of the cells. In rat, monkey and human striatum, NADPH-d-positive cells represent a subpopulation of

medium-sized aspiny neurons in which there is colocalization of the neuropeptides somatostatin and NPY^{15,29,30,37}. We found >97% colocalization of the neuropeptides somatostatin and NPY *in vitro*, indicating expression of the full genetic complement of these cells in the culture system. Both *in vivo* and *in vitro*, the diaphorase/somatostatin/NPY cells conform to one of three morphological subtypes with the multipolar subtype predominating.

In the primary cultures, the number of neurons expressing NADPH-d increased dramatically from DIV6-DIV13, while the absolute number of non-NADPH-d neurons decreased by half over the first 9 days *in vitro*. Both neuronal populations subsequently stabilized, with diaphorase-positive cells representing 0.5-1% of the total population. Since neurons are thought not to reproduce in culture after plating, the steady increase in diaphorase-positive neurons in the first days after plating most likely represents the new expression of NADPH-d in cells that were already present at the time of plating.

To the best of our knowledge, this study represents the first description of the ontogeny of NADPH-d neurons in primary striatal cultures. Uehara-Kunugi and colleagues³⁴ studied diaphorase-staining in cerebral cultures, which would typically have contained, in addition to striatal cells, diaphorase-expressing neuronal populations from neocortex, olfactory bulb, amygdala, hypothalamus, medial septum, nucleus of the diagonal band of Broca, subiculum and ventral endopiriform nucleus.³⁸ Because the cerebral cultures were exposed to cytosine arabinoside, which in our experience is

selectively toxic to striatal neurons, and because the photomicrographs are described as representing “cells from cerebral cortex”,³⁴ the data from the earlier study cannot be directly compared to our results. It is interesting to note, however, that Uehara-Kunugi and co-workers, like ourselves, found delayed diaphorase expression in their cultures compared with *in vivo* expression at a comparable stage of development.

The neurons that express NADPH-d in striatum are relatively resistant to excitotoxic injury, and are selectively spared in Huntington’s disease; indeed, these cells may actually be up regulated in Huntington’s disease, as evidenced by the dramatically increased levels of immunoreactive somatostatin and NPY in the striatum of patients with Huntington’s disease^{5,6,14,15,18,22-24,33}. The mechanism for this relative resistance to neurodegenerative and excitotoxic processes has not been elucidated. Since high concentrations of NO are known to be neurotoxic, it seems somewhat paradoxical that the very cells which produce NO should be protected from its toxic effects^{11,12,22}. One possible explanation is that NO neurons with NADPH-d also contain high concentrations of superoxide dismutase that mop up free superoxide radicals and thereby potentially protect against the toxic metabolites that are activated by the NO^{13,39}. Striatal cells lacking superoxide dismutase, primarily the spiny neurons, are the ones that show particular vulnerability to excitotoxic and neurodegenerative disease processes^{19,39}.

An alternative explanation for the relative resistance of NADPH-d neurons may relate to the NO receptor, soluble guanyl cyclase. In the striatum NADPH-d is localised to

the aspiny interneurons while the cGMP system, including the soluble guanyl cyclase, is predominantly located in the medium spiny neurons of the striatum.^{3,38} Both NOS and the cGMP system appear to be absent from the giant cholinergic interneurons.^{4,35} The release of NO would increase levels of cGMP via guanyl cyclase and secondarily shut-down mitochondrial respiration and impair DNA synthesis and preferentially target the spiny neurons for damage.

Conclusions

The results of this study indicate that NADPH-d expression can develop in cultured striatal neurons even in the absence of extrinsic afferents and glial cells. The similar ontogenic, morphological, and neurochemical characteristics of the NADPH-d neurons *in vitro* and *in vivo* support the potential usefulness of the culture system for studying this important sub-group of striatal interneurons.

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4.7 FIGURES

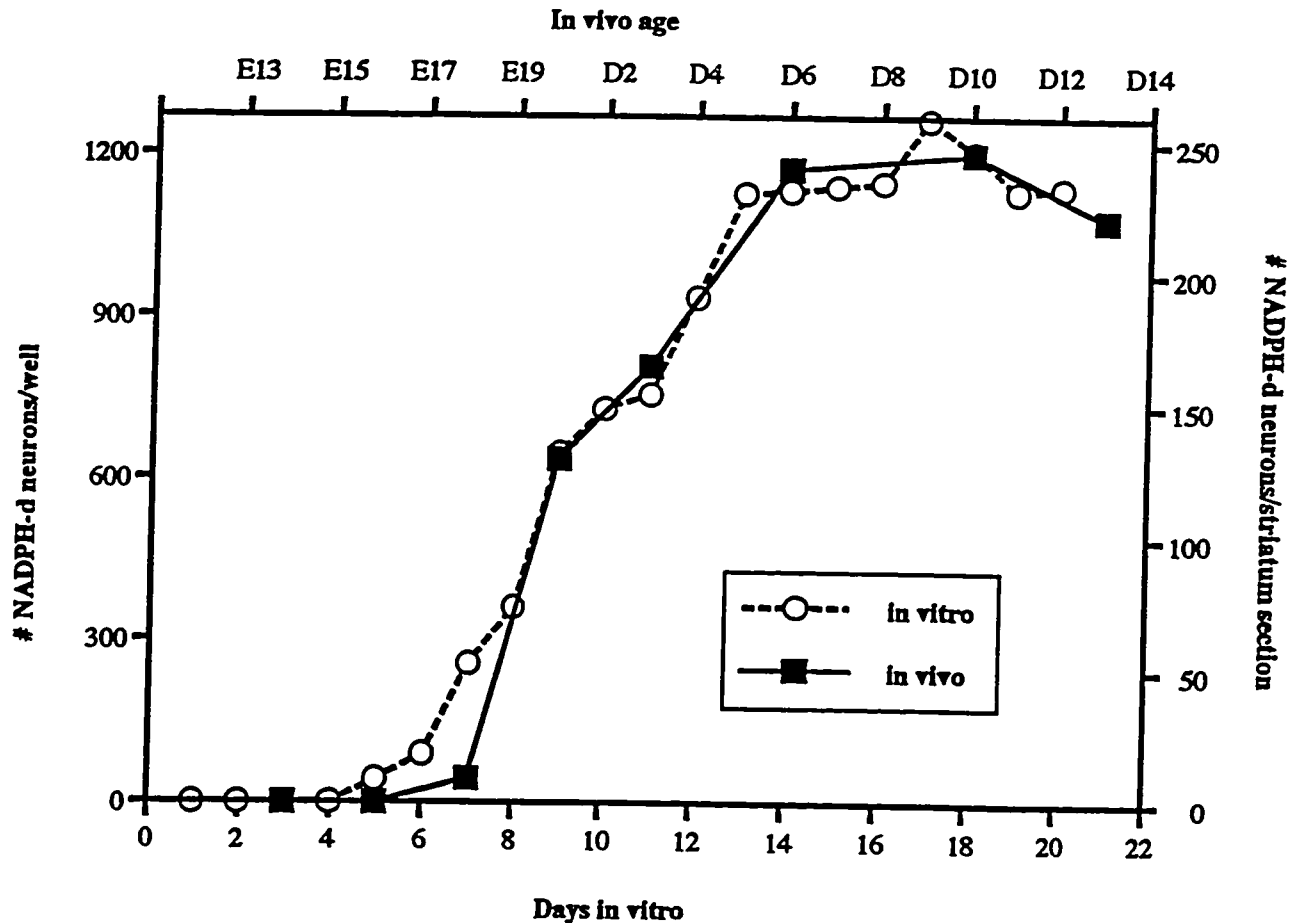


Figure 1. Time course of *in vivo* and *in vitro* expression of NADPH-d positive striatal neurons.

Each *in vitro* data point is derived from cell counts of 180 microscopic field and represents the average number of NADPH-d positive neurons per coverslip for days *in vitro* 1-21. Each *in vivo* data point was obtained by counting sixteen central striatal sections and represents the average number of NADPH-d neurons per 40um coronal hemi-section. The *in vivo* growth curve has been shifted to the right by 3 days for comparison with the *in vitro* data.

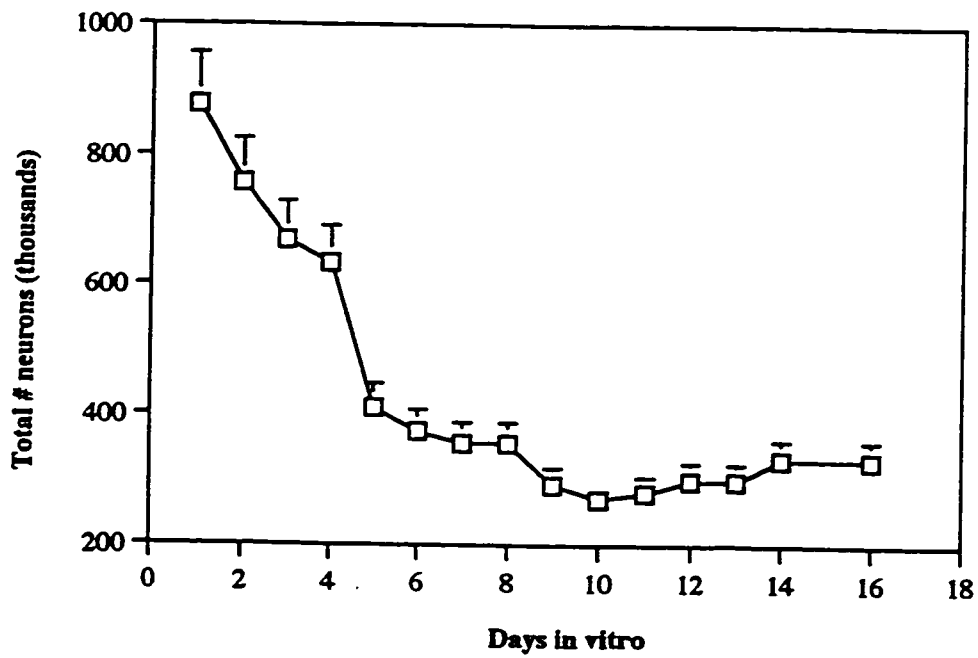


Figure 2. Total number of neurons per coverslip in primary striatal cultures from *days-in-vitro* 1-16.

Each data point represents the average number of neurons per coverslip/day + SEM.

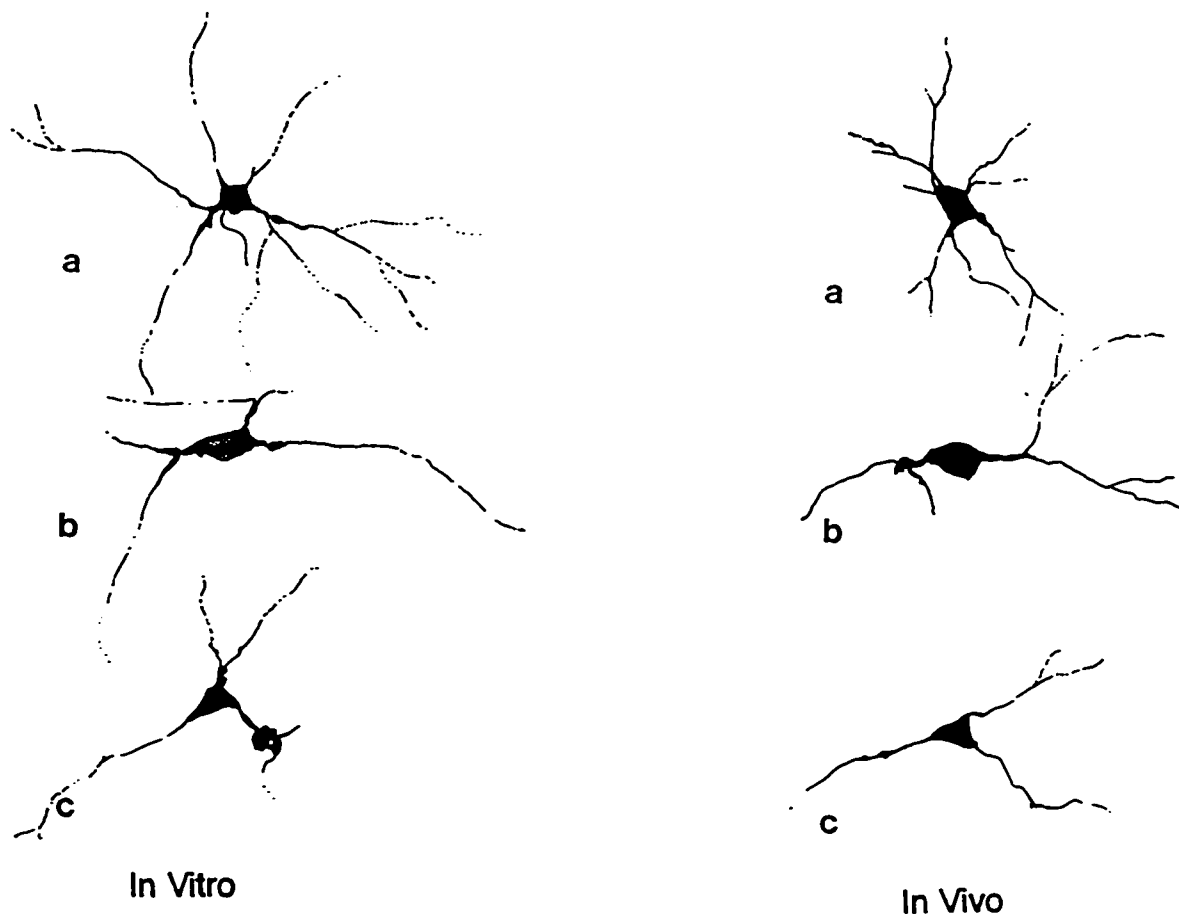


Figure 3. Camera lucida drawings of the three morphological subtypes of *in vivo* and *in vitro* NADPH-d positive neurons.

a=multipolar, b=fusiform, c=triangular.

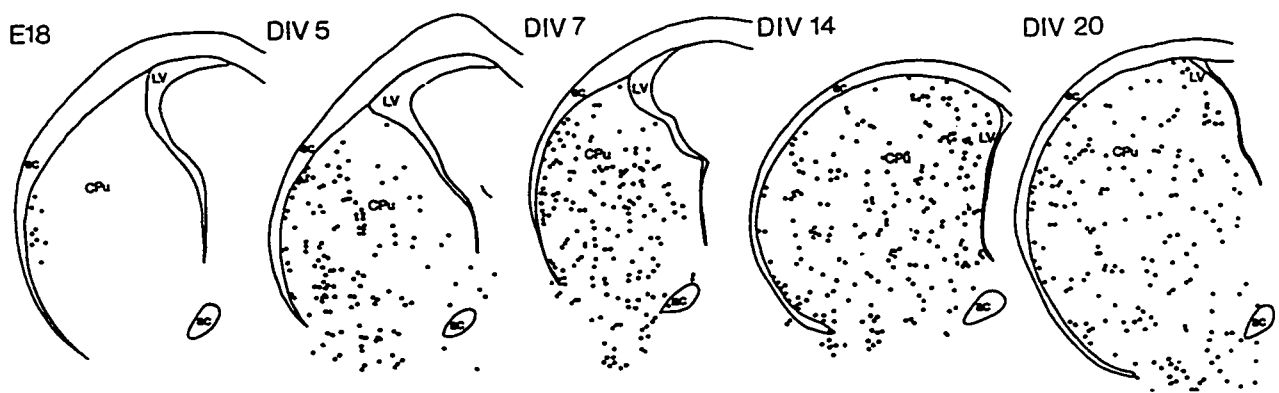


Figure 6. Development and topography of NADPH-d neurons *in vivo* from E18 to day 14 (equivalent to DIV 18).

NADPH-d neurons were identified as early as E18 at the extreme lateral boundary of the striatum. At later stages NADPH-d positive neurons were found throughout the striatum but still remained more concentrated in the lateral areas.

4.8 PHOTOGRAPH LEGEND

Figure 4. Typical NADPH-d positive neurons from DIV 5 to 7. a=DIV 5, b=DIV 6, c=DIV 7.

Figure 5. Typical NADPH-d positive neurons from DIV 11-21. a=DIV 11, b, c=DIV 14, d=DIV 21

Figure 7. Typical NADPH-d positive neurons *in vivo*. a=1 day old mouse striatum; b=14 day old mouse striatum.

Figure 8. Single cultured striatal neuron co-labelled for somatostatin and neuropeptide Y. a=somatostatin, b=neuropeptide Y.

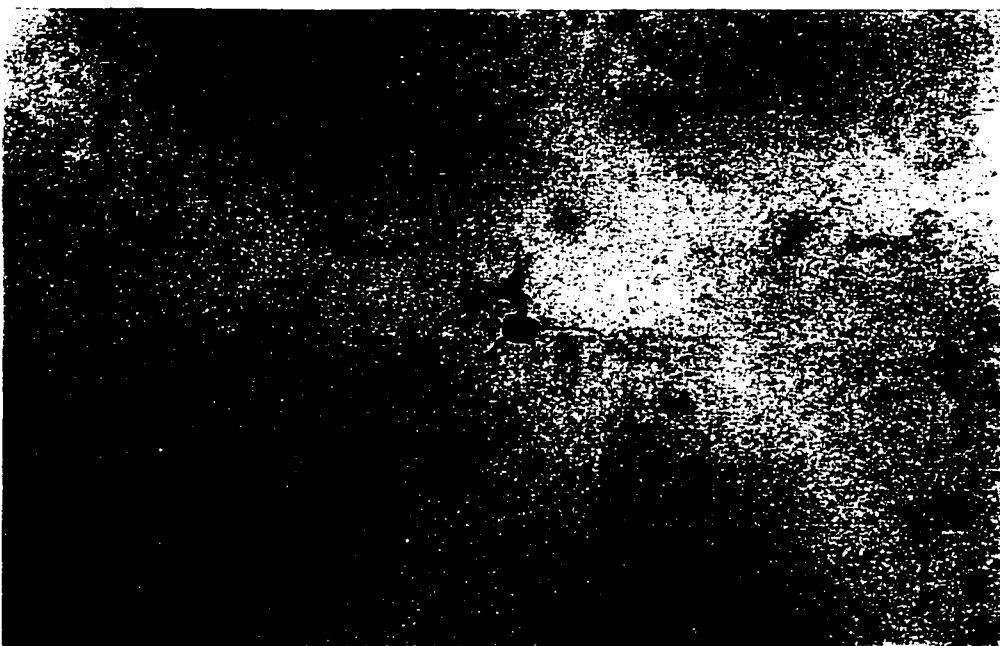
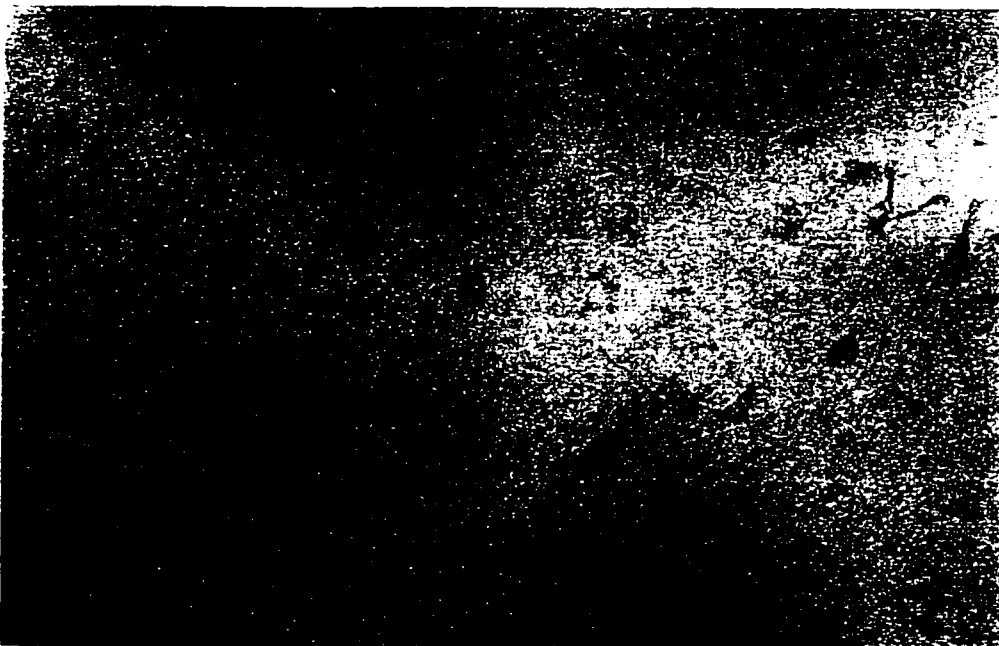
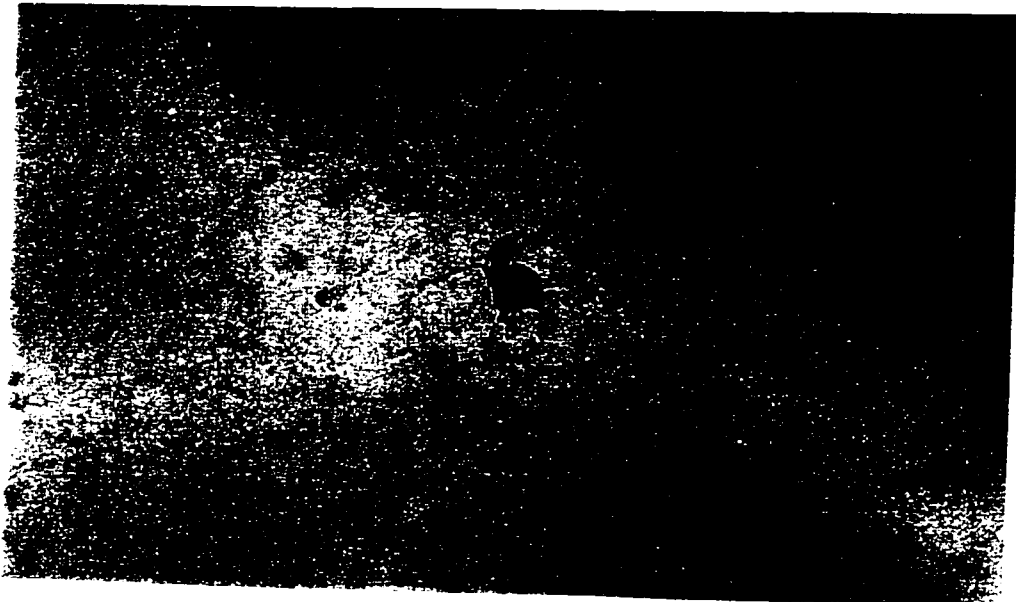
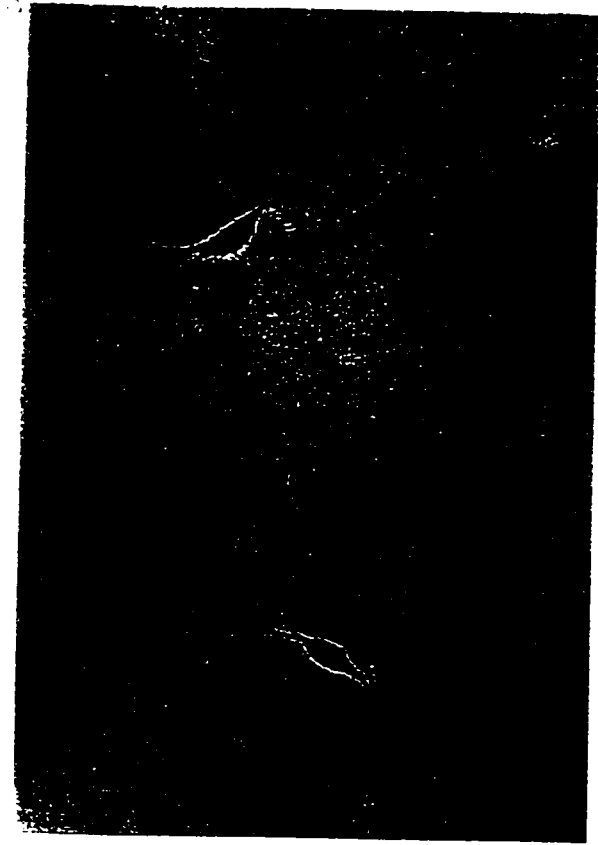
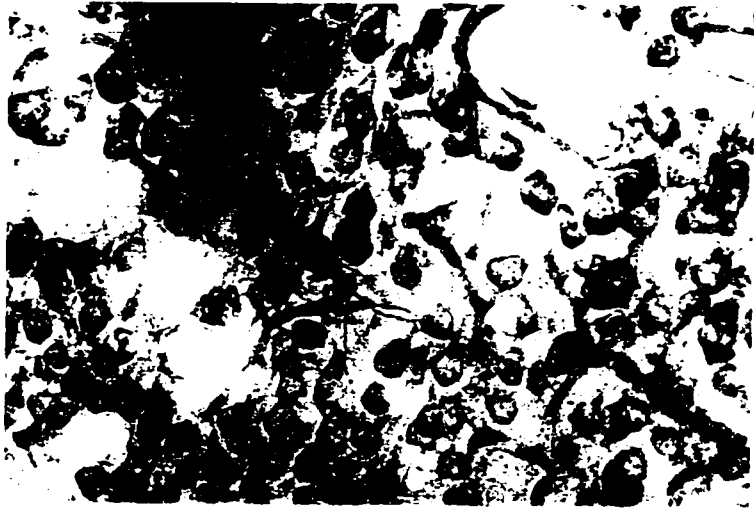
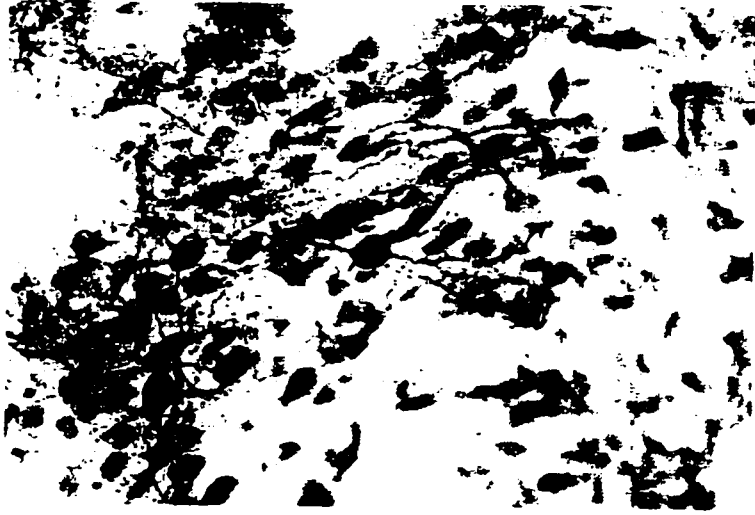
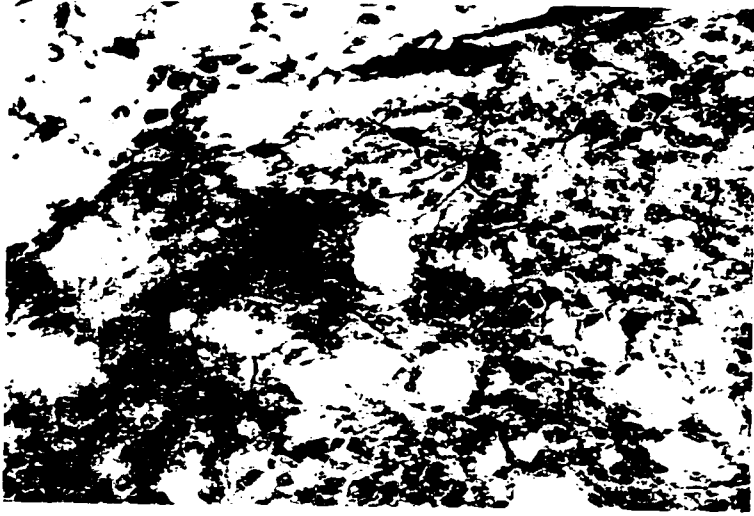
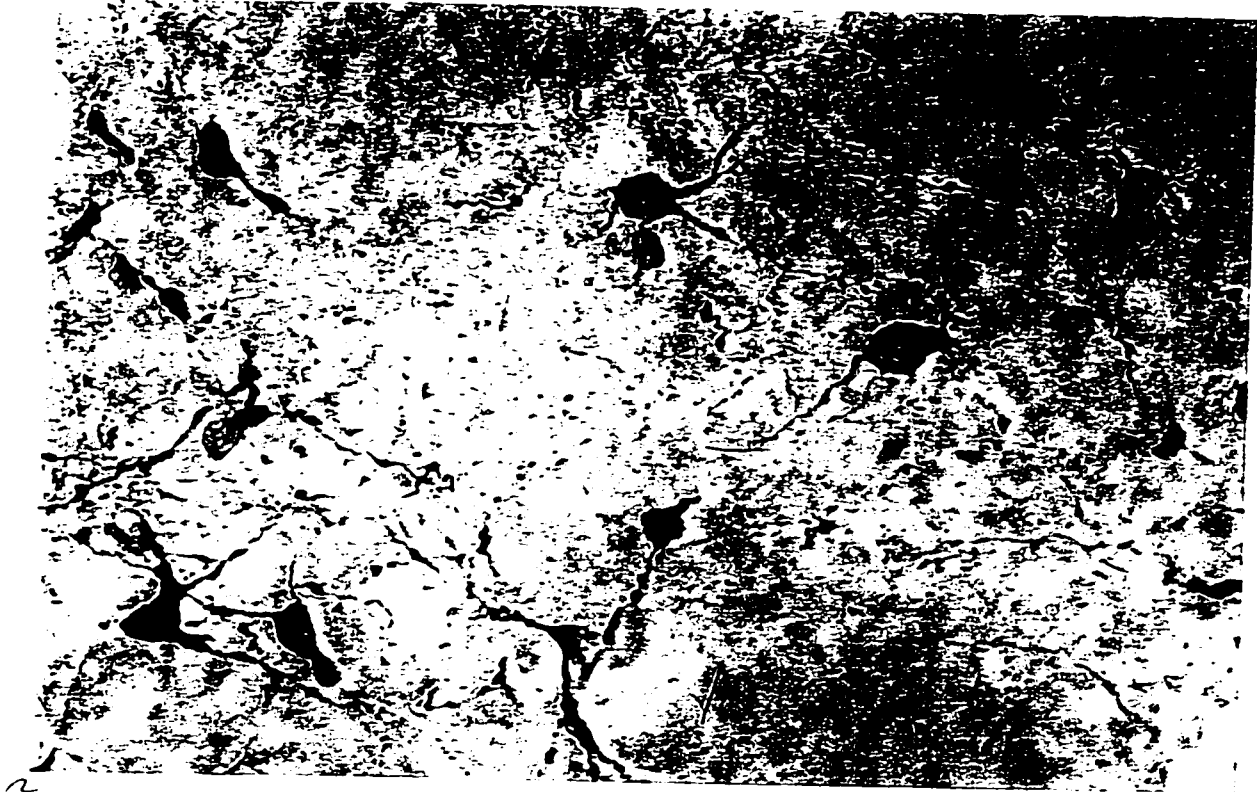


figure 5:





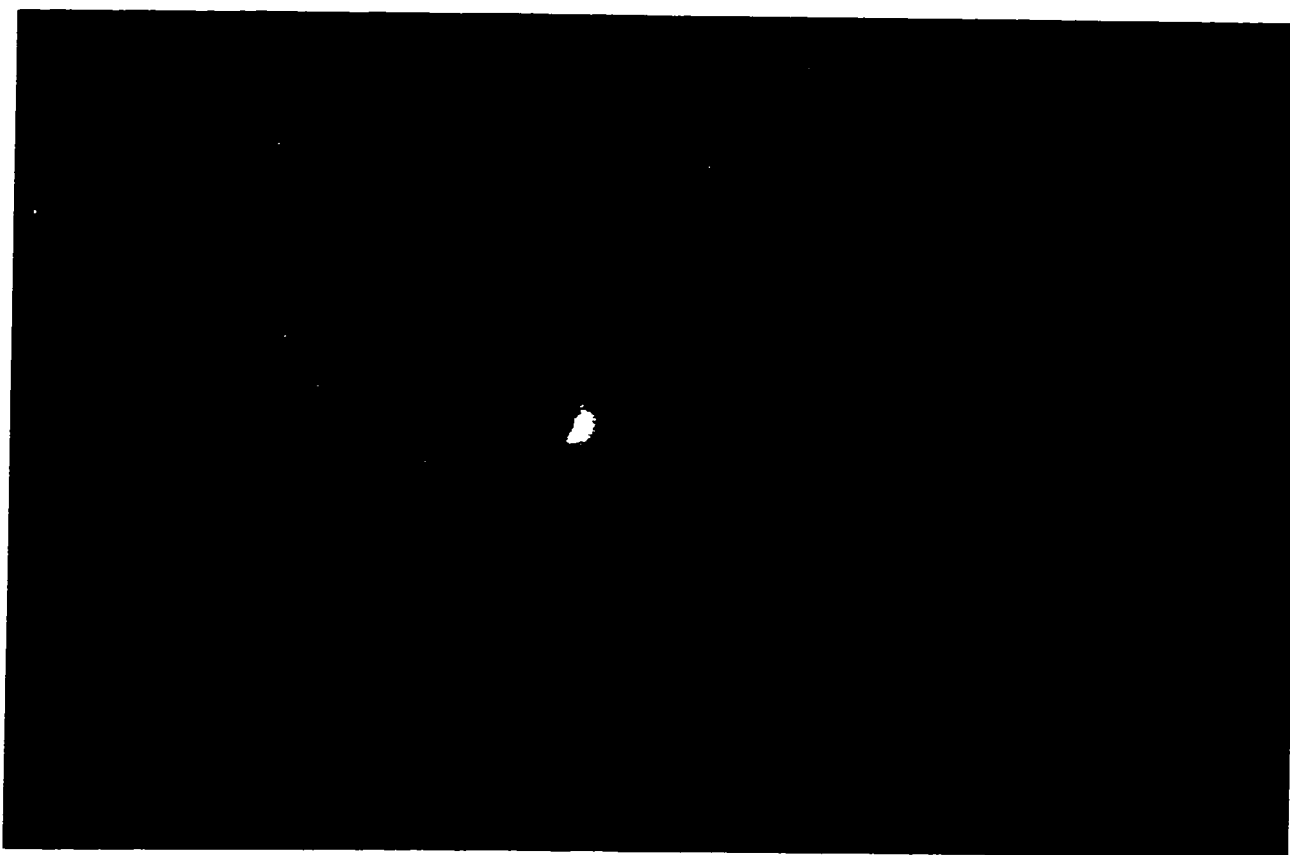


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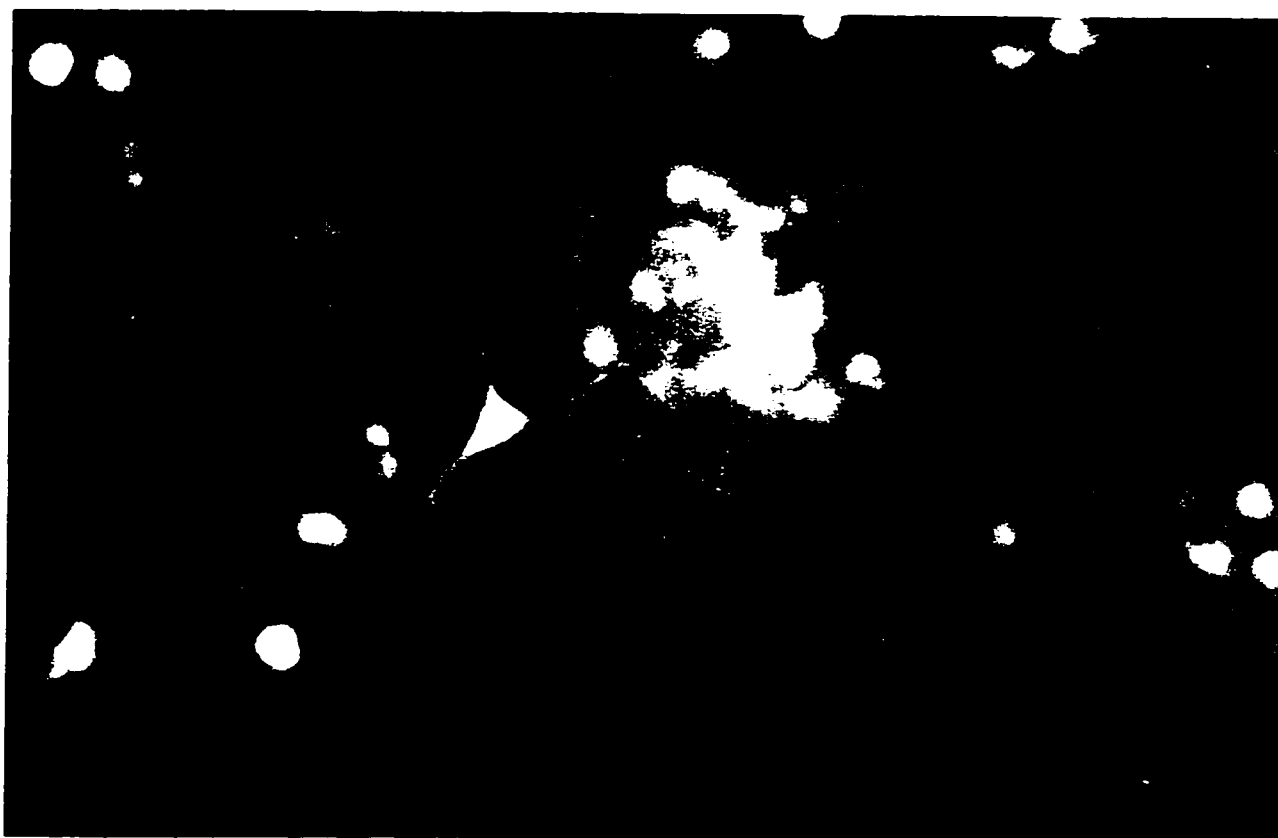


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figure 7



B



B

figure B

Table 1.

Percent of total neurons for each morphological type over time *in vitro*. Relative proportions of each neuronal type remained unchanged over time.

<i>Neuronal Type</i>	<i>DIV 7 n = 68</i>	<i>DIV 9 n = 117</i>	<i>DIV 11 n = 99</i>	<i>DIV 13 n = 98</i>	<i>DIV 17 n = 125</i>
Fusiform	22%	32%	30%	27%	27%
Multipolar	74%	67%	67%	70%	71%
Triangular	4%	1%	3%	3%	2%

Table 2.

Average number of primary, secondary and tertiary neurites for each of the three morphological cell types over time *in vitro*. For fusiform neurons, the greatest change in morphology over time is seen in the number of tertiary neurites, with relatively little change in the number of secondary neurites. In contrast, multipolar neurons demonstrate a small increase in the number of primary neurites, with large increases in both secondary and tertiary neurites over time. Triangular neurons show the greatest change over time in the number of tertiary neurites, with the number of secondary and tertiary neurites reaching a plateau by DIV 13.

<i>Neuritic Type</i>	<i>DIV 7</i> <i>n = 68</i>	<i>DIV 9</i> <i>n = 117</i>	<i>DIV 11</i> <i>n = 99</i>	<i>DIV 13</i> <i>n = 98</i>	<i>DIV 17</i> <i>n = 125</i>
Fusiform					
<i>Primary</i>	2.0	2.0	2.0	2.0	2.0
<i>Secondary</i>	4.4	4.0	4.5	5.1	5.5
<i>Tertiary</i>	1.8	4.0	3.9	4.2	7.5
Multipolar					
<i>Primary</i>	2.7	3.7	3.6	4.1	4.2
<i>Secondary</i>	1.3	4.2	3.9	4.9	6.2
<i>Tertiary</i>	0.1	2.0	1.9	3.3	5.5
Triangular					
<i>Primary</i>	3.0	3.0	3.0	3.0	3.0
<i>Secondary</i>	2.0	4.0	3.7	5.0	5.0
<i>Tertiary</i>	0.0	1.3	1.2	4.0	4.0

CHAPTER 5: THE ROLE OF GLUTAMATE RECEPTOR
SUBTYPES IN THE DIFFERENTIAL RELEASE OF
SOMATOSTATIN, NEUROPEPTIDE Y AND SUBSTANCE P IN
PRIMARY SERUM-FREE CULTURES OF STRIATAL
NEURONS.

Synapse (1997) in press.

5.1 ABSTRACT

The spiny and aspiny neuronal populations of the striatum display differential vulnerability to the toxic effects of glutamatergic agonists. Substance P-containing spiny neurons appear to be more vulnerable to NMDA-receptor-mediated toxicity, and less susceptible to kainate toxicity, than the somatostatin- and neuropeptide Y (NPY)-containing aspiny population. We studied whether selective glutamatergic agonists might have similar differential effects on neuropeptide *release* from the substance P- and somatostatin/NPY-containing neuronal populations. After collection of a baseline sample, striatal neurons in primary culture were treated with one of: phosphate-buffered saline (PBS); 56mM potassium chloride (KCl); 100 μ M N-methyl-D-aspartate (NMDA); 100 μ M quisqualate; 100 μ M kainate; or 100 μ M glutamate. Baseline and treatment samples were measured by radioimmunoassay for somatostatin, NPY and substance P. KCl and kainate provoked a selective release of somatostatin and NPY, while substance P measured in the same samples showed no response. By contrast, NMDA elicited a selective release of substance P, without a similar increase of either somatostatin or NPY. Quisqualate evoked comparable responses in the 3 peptides.

These results indicate that the glutamatergic regulation of somatostatin and NPY release from aspiny striatal neurons in primary culture is preferentially mediated by the kainate receptor, while substance P release is selectively mediated by the NMDA receptor. These findings suggest a preferential expression of functional kainate receptors on the aspiny somatostatin/NPY neurons, and of NMDA receptors on the substance P-containing spiny neurons.

5.2 INTRODUCTION

The neurons of the striatum can be categorized by morphological criteria into two main groups: the spiny efferent neurons and the aspiny interneurons. The medium-sized spiny projection neurons comprise greater than 95% of the neuronal population and contain gamma-aminobutyric acid (GABA). This population can be further subdivided according to target structure and peptide immunoreactivity: striatal neurons projecting to the pallidum contain GABA and met-enkephalin whereas striatonigral neurons contain GABA, substance P and dynorphin. Striatal interneurons are considerably less abundant, accounting for less than 5% of the total population. The population of interneurons containing somatostatin and neuropeptide Y (NPY), colocalized with nitric oxide synthase (Dawson et al., 1991), is selectively spared in Huntington's disease and in some animal models of toxicity (Beal et al., 1986a; Beal et al., 1989a; Ferrante et al., 1987; Garside et al. 1996; Uemara et al, 1990).

Excitotoxicity experiments have shown, both *in vivo* and *in vitro*, that the substance P-containing spiny neurons of the striatum may be more susceptible to NMDA receptor-mediated toxicity than the somatostatin/NPY-containing aspiny neurons (Beal et al., 1986a; Beal et al., 1989a; Koh and Choi, 1988b). Conversely, the somatostatin/NPY cells appear to be more vulnerable than substance P neurons to the effects of non-NMDA receptor-mediated glutamatergic stimulation (Choi and et al., 1987; Koh et al., 1990; Freese et al., 1990).

There is little information available concerning the differential effects of selective glutamate receptor agonists on neuropeptide *release* from the somatostatin/NPY- and substance P-containing neuronal populations. We studied this in primary serum-free cultures of striatal neurons by directly comparing the release of somatostatin, NPY and substance P in response to glutamate and various selective glutamate receptor agonists.

5.3 METHODS

Striatal culture method

Primary neuronal cultures were obtained from 15-16 day old CD1 mouse embryos (E15-16) (Garside et al., 1997). The striata were dissected from the embryos in 0.1M phosphate buffered saline (PBS) with 1% penicillin/streptomycin and mechanically dissociated in 1 ml of medium with a fire-narrowed Pasteur pipette. The cell suspension was diluted to a density of 1×10^6 trypan-blue-excluding cells per ml in a serum-free DMEM/F12 medium supplemented with a hormone mix containing progesterone, selenium salts, putrescine, transferrin, insulin and sodium bicarbonate. No antibiotics were added to the medium. For immunohistochemical studies, one ml aliquots/well of the cell suspension were transferred into a 24-well plates (NUNC, Gibco, Burlington, Ontario) containing 12 mm glass coverslips previously coated with $15 \mu\text{g/ml}$ poly-l-ornithine (Sigma, St. Louis, Missouri) ; two ml aliquots/well of cell suspension were placed in 6-well plates (NUNC, Gibco, Burlington, Ontario) for the release experiments. Cultures were maintained at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2 . The medium was replaced after 7 and 14 days. This method produced a virtually glial-free neuronal culture system (Garside et al., 1997; Weiss et al., 1986).

Immunohistochemistry

Coverslips from several cultures were taken and immunohistochemically processed for the simultaneous detection of somatostatin and NPY ; separate coverslips were stained for substance P. Briefly, cultured neurons were fixed for 20 minutes in a solution of 0.1M PBS containing 4% paraformaldehyde. The cultured neurons were then incubated for 30 minutes in 5% normal goat serum diluted in 0.1M PBS. Following an additional rinse, 3-6 coverslips per culture were incubated at 37°C for 2 hours with both a polyclonal antibody

raised in rabbit against NPY (Beal et al., 1986b) and a monoclonal antibody raised in mouse against somatostatin (MDC, Connecticut). Another 3-6 coverslips per culture were incubated with a polyclonal antibody against substance P (Dimension). The antisera were diluted to 1:100 for NPY, 1:500 for somatostatin and 1:500 for substance P in PBS containing 0.3% Triton X-100 and 1% normal goat serum. Following this incubation, the neurons were again rinsed in 0.1M PBS and incubated for 40 minutes in goat anti-rabbit secondary antibody conjugated to rhodamine isothiocyanate diluted 1:100 (for NPY and substance P) or in goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate diluted to 1:100 (for somatostatin). After another rinse in 0.1M PBS, the round coverslips were removed from the culture plates, placed on uncoated glass microscope slides and coverslipped with fluorosave (Calbiochem). Immunolabelling was visualised under fluorescence microscopy at the appropriate wavelength of filtered ultraviolet light.

Protocol for the peptide release experiments

For the peptide release experiments, the cultures were placed in a water bath maintained at 37°C. Prior to the experiment, the medium was aspirated and discarded. Each of the 6-well plates was then rinsed three times with Earle's balanced salt solution (Gibco, Burlington, Ontario). The third rinse was left for 15 minutes and collected as the *baseline* sample. One ml of one of the following treatment solutions was then added to each culture well: PBS (1M), KCl (56mM), N-methyl-D-aspartate (NMDA; 100µM), quisqualate (QUIS; 100µM), kainate (KA; 100µM) or glutamate (GLU; 100µM). All treatment solutions were made up in PBS; the PBS used for NMDA stimulation did not contain magnesium. After 15 minutes, the *treatment* sample was collected into test tubes containing 100µl of 1N HCl to avoid peptide degradation. Following the treatment incubation, 1ml of 0.1N HCl was added to the 6-well plates and allowed to sit for 30

minutes after which the cells were scraped free of the wells and collected into a third test tube to serve as the *total remaining* peptide pool. All aliquots were lyophilized in a speed vac concentrator overnight and then stored at -20°C until the time of radioimmunoassay.

Each experiment was carried out at least 3 times in each of 3 or more separate cultures (prepared from 3 different sets of animals and set up on 3 different days). This resulted in a minimum of 9 data points (range 9-21) for each of the experimental groups. The data from the different experiments were pooled for purposes of analysis.

Radioimmunoassay

All tissue culture samples were assayed for somatostatin, NPY and substance P with highly sensitive radioimmunoassays (RIA) using antisera raised in rabbit against synthetic peptide (final dilution 1:600,000 for somatostatin; 1:300,000 for NPY; and 1:5 million for substance P)

Assays were initially run in a standard RIA buffer consisting of EDTA (0.01M), sodium chloride (0.5M), bovine serum albumin (RIA grade 0.1%), and sodium azide (0.2%) in 0.1M PBS (pH 7.4). Appropriately diluted antiserum was added to assay tubes containing standards (0.5-64 pg for somatostatin (1-28) and substance P; 2-256 pg for NPY) or samples and then incubated at 4°C for three days. Iodinated peptide was added to all test tubes and the assay was then incubated for a further 3 days. To terminate the assay, secondary antibody was added to all tubes except the totals (goat anti-guinea pig/normal guinea pig serum for substance P and goat anti-rabbit/normal rabbit serum for NPY and somatostatin). Twenty-four hours later the tubes were centrifuged for twenty minutes at 3000g and aspirated. The supernatant containing free radioligand was aspirated and the radioactivity of the pellets was determined using five-minute counting periods on a Micromedic gamma counter. The data were analysed using Micromedic software on an XT computer.

The intra- and inter-assay coefficients of variation for pooled standards were in each case less than 5% and 10%, respectively.

Data analysis

Statistical analysis was performed using a two-factor ANOVA by peptide and treatment followed by *post-hoc* single factor ANOVA and Tukey tests where appropriate.

5.4 RESULTS

Immunohistochemistry

Indirect immunohistochemistry was used to visualize somatostatin/NPY- and substance P-containing neurons (see figures 1 and 2). Approximately 1-2% of the total neuronal population in the cultures was immunoreactive for somatostatin and NPY. The two peptides were co-localized greater than 95% of the time, consistent with previous observations of these interneurons both *in vitro* and *in vivo* (Garside et al. 1997). Substance P-immunoreactive neurons comprised approximately $47 \pm 5\%$ of the total neuronal population and did not appear to contain either somatostatin or NPY. A typical culture is shown in figure 3.

Spontaneous release of somatostatin, NPY and substance P

The spontaneous rate of peptide release in the cultures was assessed by serial measurements of peptide levels in the medium after each of 6 consecutive 15 minute incubation periods. The average *baseline* peptide release per well over the course of a 15 minute incubation period was 7.2 ± 0.4 fmols of somatostatin, 7.0 ± 0.5 fmols of NPY and 4.5 ± 0.2 fmols of substance P. There was a slight decrease in the amount of each

peptide spontaneously released during the second 15 minute period, with stable rates of release over the next four 15-minute blocks. After the final 15 minute incubation period, there were still substantial quantities of each peptide within each culture, as measured by the *total remaining* peptide pool (Table 1).

Evoked release of somatostatin, NPY and substance P

The 2-way ANOVA for the stimulation experiments showed a significant effect for peptide ($F_{(2, 166)} = 4.51$, $p < 0.01$) and for agonist ($F_{(4, 166)} = 6.2$, $p < 0.001$). A summary of these results is represented in figure 4.

The peptide responses to KCl showed a significant effect for group ($F_{(2, 29)} = 4.6$, $p < 0.02$; figure 4). KCl provoked a significant increase over baseline in the release of both somatostatin ($p < 0.01$) and NPY ($p < 0.001$); there was no significant difference between the somatostatin and the NPY responses to KCl. In contrast, substance P showed no significant increase in release following KCl and the substance P response was significantly less than the somatostatin and NPY responses (somatostatin vs substance P, $p < 0.01$; NPY vs substance P, $p < 0.001$).

Kainate treatment also produced a significant effect for peptide ($F_{(2, 29)} = 8.86$, $p < 0.001$). The profile of peptide responses was qualitatively similar to that observed following KCl, with somatostatin levels rising more than 5-fold above baseline ($p < 0.001$), NPY release increasing more than 2-fold ($p < 0.001$), and substance P showing no significant increase over baseline levels. The somatostatin response was significantly greater than that of NPY ($p < 0.01$).

In contrast to kainate and KCl, quisqualate evoked comparable responses in the three peptides, with no significant effect for group ($F_{(2, 32)} = 1.68$, $p = 0.22$). While there was a trend toward increased release of all three peptides, none of the changes was significant from baseline levels.

Unlike the other glutamate agonists, NMDA elicited a greater response in substance P (379% of baseline release) than in somatostatin (133%) or NPY (185%) ($F_{(2,31)} = 21.05$, $p < 0.00001$; substance P *vs* somatostatin, $p < 0.00001$; substance P *vs* NPY, $p < 0.001$; somatostatin *vs* NPY, ns). Indeed, the substance P response to NMDA was significantly greater than it was to any of the other treatments ($F_{(4,52)} = 38.6$, $p < 0.00001$; NMDA *vs* KCl, $p < 0.00001$; NMDA *vs* KA, $p < 0.00001$; NMDA *vs* GLU, $p < 0.00001$; NMDA *vs* QUIS, $p < 0.001$).

Glutamate-induced peptide release showed a significant effect for group ($F_{(2,26)} = 4.34$, $p < 0.02$), with a trend for somatostatin to be increased more than NPY or substance P.

A summary of the total remaining peptide pool per well (i.e. following baseline and treatment) is shown in Table 1. The amount of peptide remaining was inversely proportional to the amount of peptide released following treatment (Figure 5). The total amount of peptide per well (calculated as the sum of basal release plus treatment-induced release plus the amount remaining in each well) did not differ significantly across treatment protocols (Figure 5).

5.5 DISCUSSION

These results show that selective glutamatergic stimuli differentially regulate the release of neuropeptides from spiny and aspiny populations of striatal neurons in primary culture. Levels of somatostatin and NPY, which are markers for a subpopulation of medium-sized aspiny neurons, showed a robust response to stimulation by kainate, but little or no response to NMDA. By contrast, release of substance P, which is localized to a subpopulation of spiny striatal neurons, was unaffected by kainate but was strongly stimulated by NMDA.

To the best of our knowledge, this study is the first to directly compare the differential profiles of peptide release from striatal neurons in response to selective glutamate receptor stimulation. The results suggest that somatostatin/NPY-containing spiny neurons respond preferentially to kainate receptor stimulation, while the glutamatergic regulation of substance P-containing spiny neurons is selectively mediated by the NMDA receptor. These findings are in general agreement with the results of *in vivo* excitotoxicity experiments, which have shown striatal somatostatin/NPY immunoreactive cells to be highly vulnerable to kainate toxicity, with relative sparing of this neuronal population after exposure to doses of NMDA receptor agonists that kill most other striatal neurons.

Baseline peptide release and total peptide content in the cultures were comparable across treatment groups. As has been observed *in vivo* in both humans and animals (Beal et al., 1988a; 1988b; 1989b), absolute quantities of each of the three peptides were similar in the striatal cultures despite the numerical preponderance of substance P-containing cells. Substantial quantities of all 3 peptides remained in the cultures after each of the stimulation paradigms, indicating that not all immunoreactive peptide had been released.

The ability of KCl and kainate to provoke somatostatin release in these experiments is in partial agreement with a previous study, which found kainate to be a potent stimulator of somatostatin release from cultured striatal neurons (Williams et al., 1991). In primary cultures of cortical or diencephalic neurons, KCl elicits a robust somatostatin response similar to what we observed (Tapia-Arancibia and Astier, 1988; Tapia-Arancibia and Astier, 1989). We did not observe the previously-described release of somatostatin in response to NMDA (Williams et al., 1991).

The pattern of NPY release in these experiments generally followed that of somatostatin, with which it is co-localized over 95% of the time (Garside et al., 1997). An exception was the response to kainate, which provoked a significantly greater release of

somatostatin than NPY. The somatostatin/NPY response to glutamate showed a similar trend. A dissociation of somatostatin from NPY has previously been described in cerebral cortex, where the 2 peptides are also co-localized most of the time. In cortical cultures, NMDA receptor agonists increase levels of mRNA for somatostatin but not for NPY (Patel et al., 1995), and in Alzheimer's disease postmortem brain the levels of somatostatin are reduced to a much greater extent than are concentrations of NPY (Allen et al., 1985; Allen et al., 1984; Gabriel et al, 1993}

NMDA proved to be a more potent stimulus to substance P release than any of the other agents used in these experiments. The NMDA effect was selective for substance P, with neither somatostatin nor NPY measured in the same samples showing a significant change from baseline levels. This would suggest a differential distribution of functional NMDA receptors on spiny vs aspiny striatal neurons. Recent direct evidence based on *in situ* hybridization of mRNA for the NMDA receptor subunit NR1 has shown that the NR1 gene is expressed in only 20% of the somatostatin/NPY-containing cells of the striatum, the remaining 80% exhibiting weak or absent signals (Augood et al., 1994).

Acknowledgements.

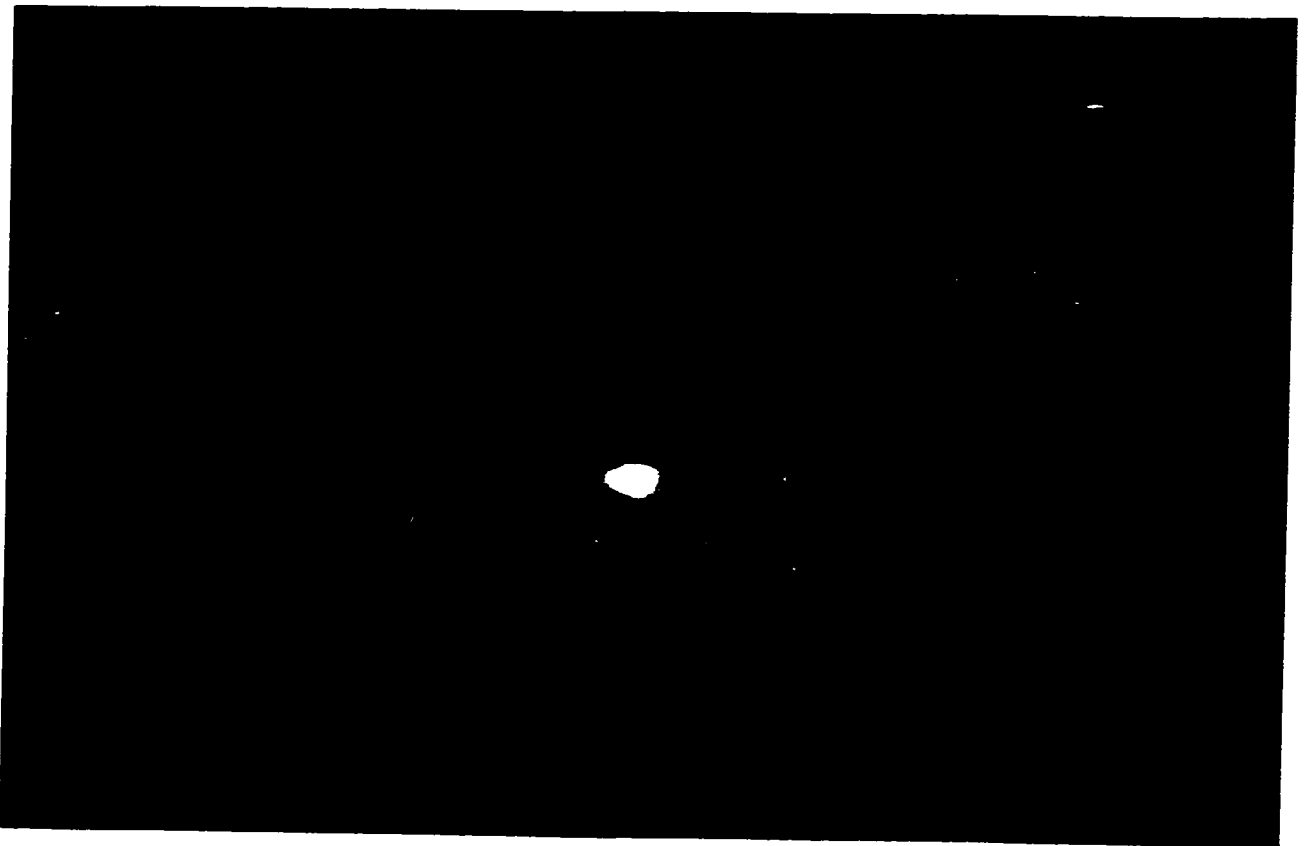
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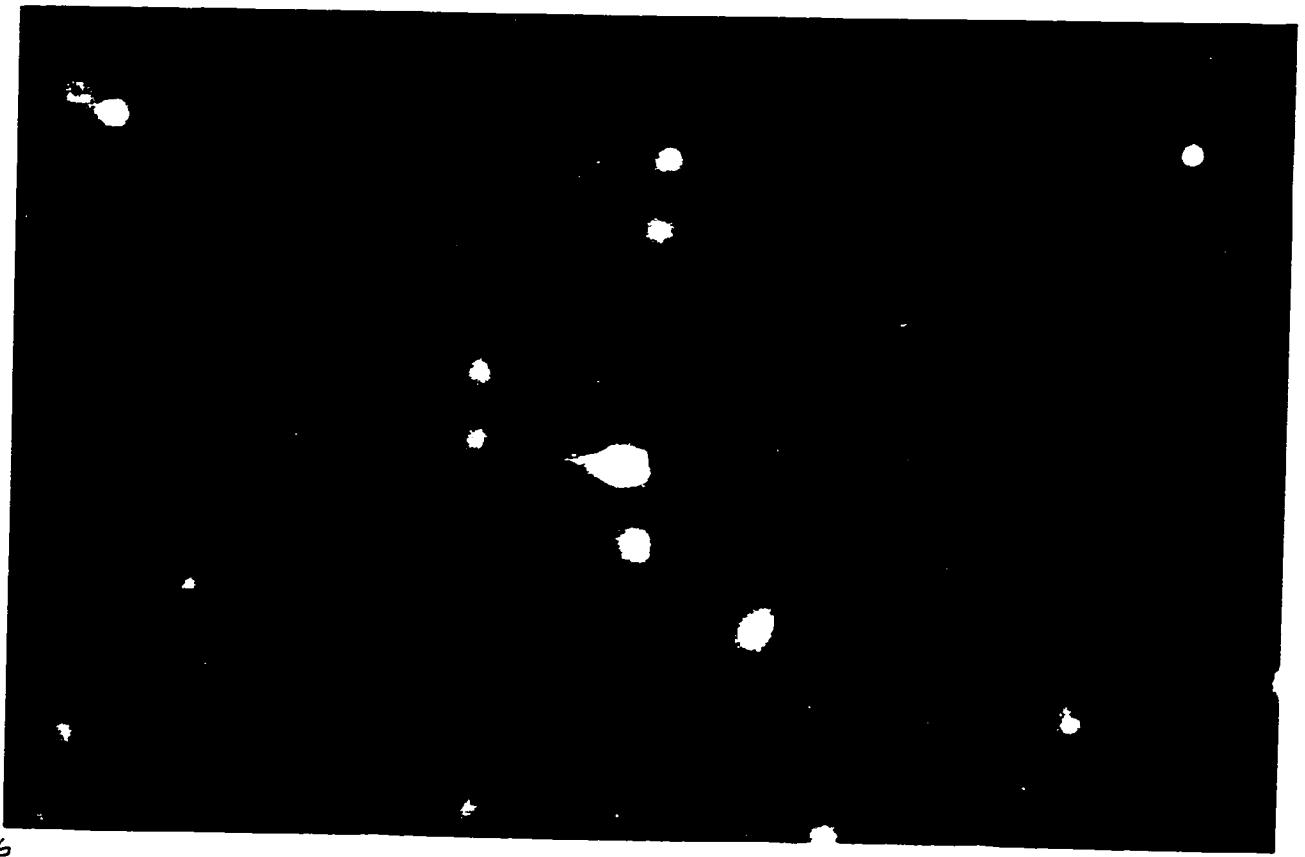
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a



b

figure 1.



figure 2.

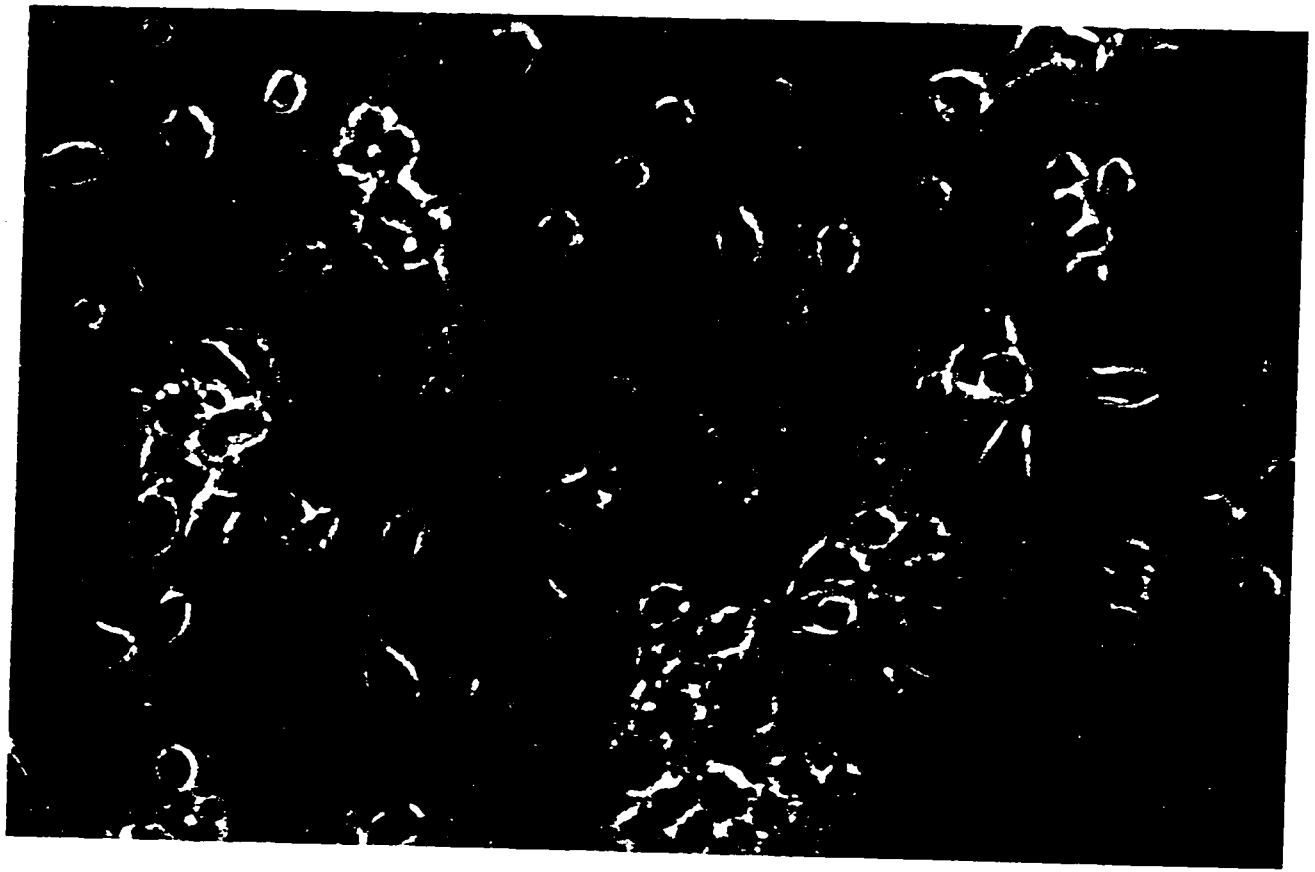


figure 3

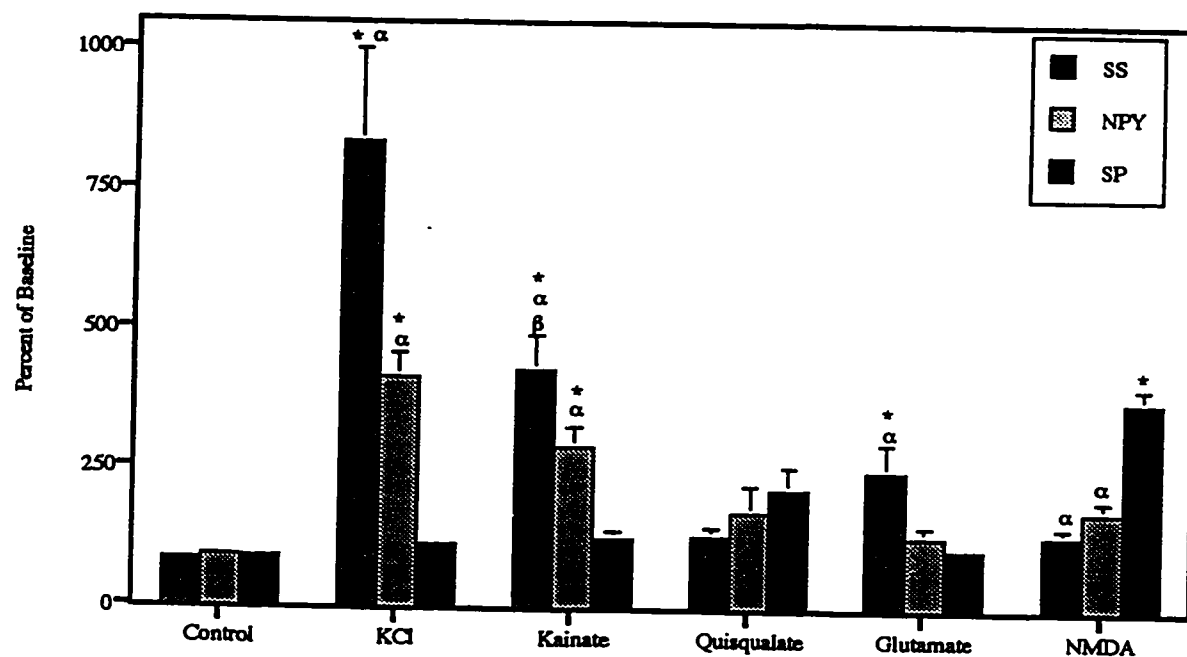


Figure 4: Comparative release of immunoreactive somatostatin (SS), neuropeptide Y (NPY) and substance P (SP) from cultured striatal neurons following incubation with PBS (control), potassium chloride (KCl), or various glutamatergic agonists. KCl and kainate preferentially elicited the release of somatostatin and NPY, while NMDA selectively provoked substance P release. The data were analysed by 2-factor ANOVA followed by post-hoc single-factor ANOVA and Tukey tests (where appropriate) * $p < 0.01$ compared with controls; α $p < 0.01$ compared with substance P response; β $p < 0.01$ compared with NPY response.

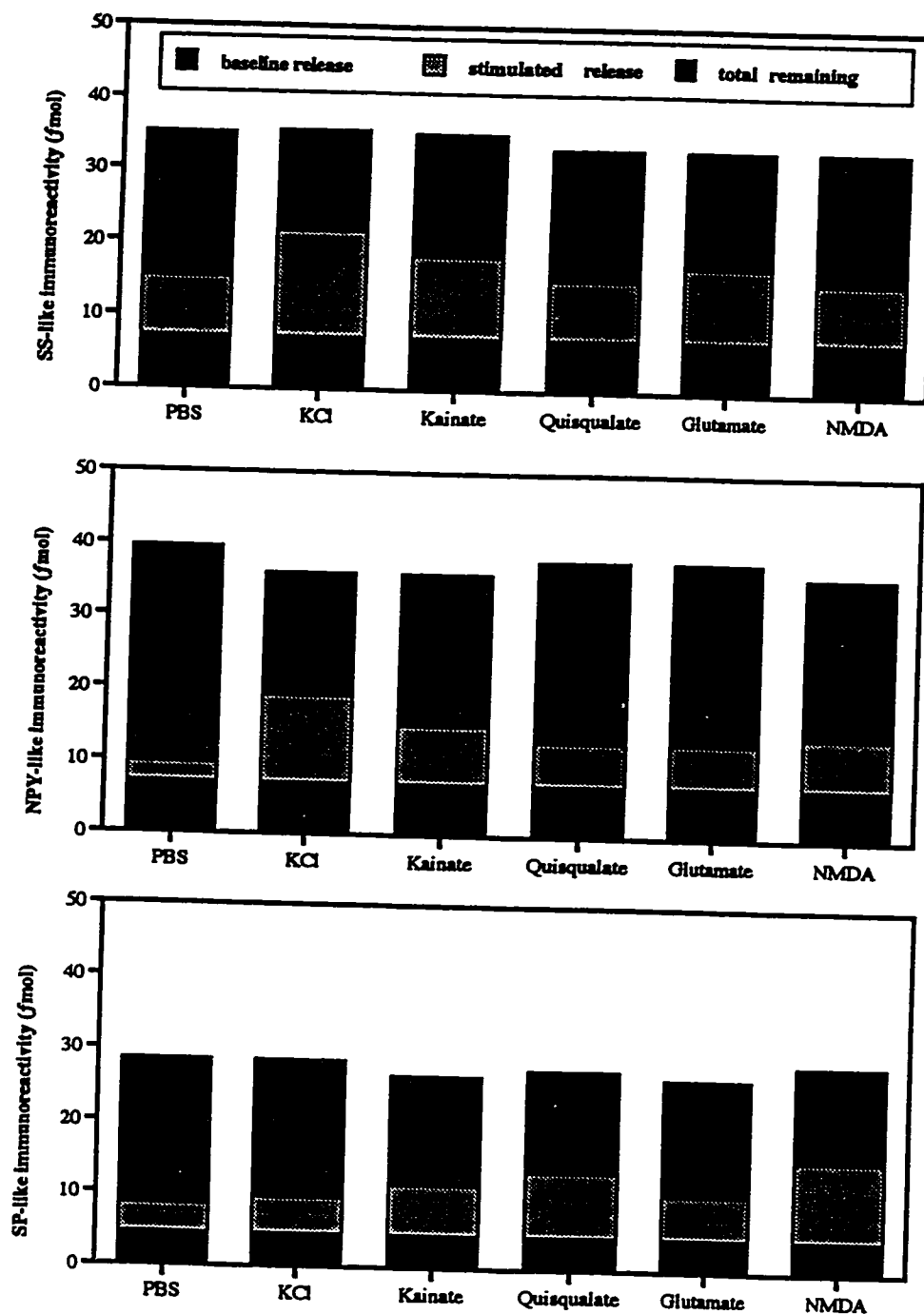


Figure 5: Total immunoreactive somatostatin (top), NPY (middle) and substance P (bottom) from each of the experimental groups, expressed as the sum of *baseline release* plus *stimulated release* plus *residual peptide* remaining after the stimulation. Baseline peptide release and total peptide content in the cultures were comparable across treatment groups. Substantial quantities of all 3 peptides remained in the cultures after each of the stimulation paradigms, indicating that not all immunoreactive peptide had been released.

Table 1. Total amount of peptide remaining in culture wells after completion of the release experiment, expressed in fmols \pm SEM.

	<i>PBS</i>	<i>KCl</i>	<i>Kainate</i>	<i>Quis</i>	<i>Glu</i>	<i>NMDA</i>
<i>SS</i>	20 \pm 1.0	14 \pm 1.2	17 \pm 0.3	18 \pm 0.6	16 \pm 0.6	18 \pm 0.6
<i>NPY</i>	30 \pm 1.4	17 \pm 4.2	21 \pm 1.4	25 \pm 1.7	25 \pm 1.8	22 \pm 2.8
<i>SP</i>	20 \pm 1.0	19 \pm 2.2	15 \pm 1.5	14 \pm 1.0	16 \pm 2.9	13 \pm 1.5

CHAPTER 6. DOPAMINE-GLUTAMATE INTERACTIONS IN
PRIMARY SERUM-FREE CULTURES OF STRIATAL
NEURONS

Submitted to *Neuroscience*.

6.1 ABSTRACT

The two most important afferent projections to the striatum are the glutamatergic pathway from the cerebral cortex and the dopaminergic input from substantia nigra. Little is known about the interaction of glutamate and dopamine in the regulation of neuropeptide release from subpopulations of striatal neurons. We studied the effects of selective dopamine and glutamate receptor agonists, both alone and in combination, on the release of somatostatin, neuropeptide Y and substance P from striatal neurons in primary culture.

The findings were as follows: 1) The D2 dopamine receptor agonist quinpirole produced a robust dose dependent release of somatostatin and NPY, while the D1 agonist SKF 38393 had no significant effect on the release of either peptide. 2) The glutamatergic agonist NMDA selectively evoked the release of substance P, while kainate preferentially stimulated the release of somatostatin and neuropeptide Y. 3) The D1 receptor agonist SKF 38393 consistently inhibited glutamate agonist-induced release of substance P, while generally having little or no effect on the release of somatostatin and neuropeptide Y. 4) The D2 receptor agonist quinpirole potentiated kainate- and quisqualate-induced neuropeptide Y release, while consistently inhibiting glutamatergic substance P release.

These results indicate that selective dopamine receptor agonists differentially modify the receptor-specific glutamatergic regulation of neuropeptide release from spiny and aspiny striatal neurons in primary culture.

6.2 INTRODUCTION

The striatum is comprised of two major populations of neurons: the spiny projection neurons that carry the striatal output to other nuclei in the basal ganglia; and several subtypes of aspiny cells that project locally within the striatum to modify striatal output. The population of medium-sized projection neurons can be further subdivided according to target structure and peptide immunoreactivity: striatal neurons projecting to the pallidum contain GABA and met-enkephalin, while striatonigral neurons contain GABA along with substance P and dynorphin. Striatal interneurons likewise include neurochemically distinct subpopulations, one of which contains somatostatin and neuropeptide Y, colocalized with nitric oxide synthase.

The major excitatory input to the striatum is the glutamatergic projection from the cerebral cortex. The signals from this pathway are transduced by at least three different subtypes of glutamate receptor: a family of metabotropic receptors which are second messenger systems; kainate/AMPA ionotropic receptors which allow selective conductance of sodium ions; and an NMDA ionotropic receptor through which can pass both sodium and calcium ions. The relative distributions of the three glutamate receptor subtypes vary among the different neuronal populations of the striatum.

The other major striatal afferent arises from the substantia nigra pars compacta and utilizes dopamine as its neurotransmitter. Dopamine has differential effects on the two main subpopulations of spiny projection neurons, stimulating gene expression in the substance P-containing cells by an action on D1-like receptors, and inhibiting met-enkephalin immunoreactive neurons through D2-like dopamine receptors. The effects of dopamine on the somatostatin/ neuropeptide Y-containing subpopulation of aspiny neurons are unknown.

While the importance of the dopaminergic and glutamatergic inputs for striatal function are widely recognized, there is relatively little information available regarding the differential effects of selective dopamine and glutamate agonists on neurotransmitter release from the various subpopulations of striatal neurons. There is, in particular, a paucity of information regarding the interaction of the dopaminergic and glutamatergic systems in the regulation of neuropeptide release from striatal neurons.

We investigated the effects of selective dopamine and glutamate receptor agonists, both alone and in combination, on the release of somatostatin, neuropeptide Y and substance P from primary serum-free cultures of striatal neurons.

6.3 METHODS

Striatal culture method

Primary neuronal cultures were obtained from 15-16 day old CD1 mouse embryos (E15-16).^{9, 10} All efforts were made to minimize animal suffering and the number of animals used. The striata were dissected from the embryos in 0.1M phosphate buffered saline (PBS) with 1% penicillin/streptomycin and mechanically dissociated in 1 ml of medium with a fire-narrowed Pasteur pipette. The cell suspension was diluted to a density of 1×10^6 trypan-blue-excluding cells per ml in a serum-free DMEM/F12 medium supplemented with a hormone mix containing progesterone, selenium salts, putrescine, transferrin, insulin and sodium bicarbonate. No antibiotics were added to the medium. Two ml aliquots/well of the cell suspension were then placed in 6-well plates (NUNC, Gibco, Burlington, Ontario) for the release experiments. Cultures were maintained at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂. The medium was replaced after 7 and 14 days. This method produced a virtually glial-free neuronal culture system.^{9,29}

Immunohistochemical staining has shown that approximately 1-2% of the total neuronal population in the cultures are immunoreactive for somatostatin and neuropeptide Y (NPY) and that the two peptides co-localize greater than 98% of the time. Substance P-immunoreactive neurons composed approximately 47+ 5% of the total neuronal population and do not contain either somatostatin or NPY. A typical culture is shown in figure 1.

Release experiments

For the peptide release experiments, the cultures were placed in a water bath maintained at 37°C. Prior to the experiment, the medium was aspirated and discarded. Each of the 6-well plates was then rinsed three times with Earle's balanced salt solution (Gibco, Burlington, Ontario). The third rinse was left for 15 minutes and collected as the *baseline* sample. One ml of one of the following treatment solutions was then added to each culture well: Phosphate buffered saline (PBS) (1M), N-methyl-D-aspartate (NMDA) (100 μ M), quisqualate (100 μ M), kainate (100 μ M), quinpirole (1 μ M), SKF 38393 (1 μ M), NMDA + quinpirole, NMDA + SKF 38393, kainate + quinpirole, kainate + SKF 38393, quisqualate + quinpirole, or quisqualate + SKF 38393. All treatment solutions were made up in PBS; the PBS used for NMDA stimulation did not contain magnesium. After 15 minutes, the *treatment* sample was collected into test tubes containing 100 μ l of 1N HCl to avoid peptide degradation. For the dopamine agonist dose response curves the protocol was the same as described above except the following concentrations were used for both quinpirole and SKF 38393: 0.01, 0.1, 1.0, 10, and 100 μ M. All aliquots were lyophilized in a speed vac concentrator overnight and then stored at -20°C until the time of radioimmunoassay.

Each experiment was carried out at least 3 times in each of 3 or more separate cultures (prepared from 3 different sets of animals and set up on 3 different days). This

resulted in a minimum of 9 data points (range 9-21) for each of the experimental groups. The data from the different experiments were pooled for purposes of analysis.

Radioimmunoassay

All tissue culture samples were assayed for somatostatin, NPY and substance P with highly sensitive radioimmunoassays (RIA) using antisera raised in rabbit or guinea pig against synthetic peptide (final dilution 1:600,000 for somatostatin; 1:300,000 for NPY; and 1:5 million for substance P).²³

Assays were initially run in a standard RIA buffer consisting of EDTA (0.01M), sodium chloride (0.5M), bovine serum albumin (RIA grade 0.1%), and sodium azide (0.2%) in 0.1M PBS (pH 7.4). Appropriately diluted antiserum was added to assay tubes containing standards (0.5-64 pg for somatostatin (1-28) and substance P; 2-256 pg for NPY) or samples and then incubated at 4°C for three days. Iodinated peptide was added to all test tubes and the assay was then incubated for a further 3 days. To terminate the assay, secondary antibody was added to all tubes except the totals (goat anti-guinea pig/normal guinea pig serum for substance P and goat anti-rabbit/normal rabbit serum for NPY and somatostatin). Twenty-four hours later the tubes were centrifuged for twenty minutes at 3000g. The supernatant containing free radioligand was aspirated and the radioactivity of the pellets was determined using five-minute counting periods on a Micromedic gamma counter. The data were analysed using Micromedic software on an XT computer.

The intra- and inter-assay coefficients of variation for pooled standards were in each case less than 5% and 10%, respectively.

Data analysis

Statistical analysis was performed using a two-factor ANOVA by peptide and treatment followed by *post-hoc* single factor ANOVA and Tukey tests where appropriate.

6.4 RESULTS

The dose-response curve for the D2 agonist quinpirole showed a significant dose-dependent stimulation of both somatostatin ($F=8.86$; $p<0.005$) and NPY ($F=18.23$; $p<0.001$) with 3-8 fold increases of both peptides at concentrations of $10\mu\text{M}$ and $100\mu\text{M}$ (Fig. 2). The D1 agonist SKF 38393, on the other hand, had a tendency to inhibit somatostatin release ($F=3.35$; $p=0.06$) without producing any change of NPY levels measured in the same samples ($F=0.38$, $p=0.82$). When used in concentrations of $1\mu\text{M}$ or less (the doses used in the dopamine-glutamate interaction experiments), neither the D1 agonist SKF 38393 nor the D2 agonist quinpirole had a significant effect on somatostatin or NPY release from the cultures.

The two-way ANOVA for the dopamine glutamate experiments showed a significant effect for peptide ($F=9.24$, $p<0.001$) and for agonist ($F= 2.47$, $p<0.02$) and peptide by agonist interaction ($F=5.47$, $p<0.001$).

Treatment with kainate stimulated the release of somatostatin and NPY by 437% and 316% respectively but had little effect on substance P ($F=8.86$, $p<0.001$; somatostatin vs control, $p<0.001$; NPY vs control, $p<0.001$; substance P vs control, ns; Fig. 3). The addition of quinpirole *potentiated* the kainate-induced release of NPY ($p<0.05$), and to a lesser extent somatostatin, but *inhibited* the kainate-evoked release of substance P ($p<0.01$). The addition of SKF 38393 had an inhibitory effect on the kainate-stimulated release of both NPY ($p<0.05$) and substance P ($p<0.0001$); the D1 agonist SKF 38393

had a significantly more potent inhibitory effect than did the D2 agonist quinpirole on substance P release ($p < 0.05$).

In contrast to what was seen with kainate stimulation, NMDA provoked a greater release of substance P than of somatostatin or NPY ($F = 21.05$, $p < 0.0001$; substance P *vs* control, $p < 0.00001$; somatostatin *vs* control, ns; NPY *vs* control, ns; *Fig. 4*). Both the D1 agonist SKF 38393 and the D2 agonist quinpirole significantly attenuated the NMDA-stimulated release of substance P ($p < 0.0001$ in both cases), with the D1 agonist having a significantly stronger inhibitory effect (NMDA + quinpirole *vs* NMDA + SKF 38393, $p < 0.01$). Neither of the dopamine agonists had any effect on NMDA-induced release of somatostatin or NPY.

The presence of quisqualate had no significant effect on the release of somatostatin, NPY or NMDA. The addition of quinpirole significantly potentiated NPY release ($p < 0.05$) and markedly decreased substance P release ($p < 0.05$) (*Fig. 5*). The addition of SKF 38393 had no effect on somatostatin or NPY release but significantly attenuated the quisqualate-induced substance P response ($p < 0.05$). Once again the inhibitory effect of SKF 38393 was significantly more potent than that of quinpirole on substance P release ($p < 0.05$).

6.5 DISCUSSION

These results show the ability of selective dopamine receptor agonists to differentially modify the glutamatergic release of somatostatin, NPY and substance P from cultured striatal neurons.

A. Dopaminergic Control of Somatostatin and NPY Release from Striatal Neurons

When administered alone, the D1 dopamine receptor agonist SKF 38393 had little effect on the release of somatostatin or NPY, while the D2 agonist potentiated the release of both peptides in a dose-dependent manner. These findings are in general agreement with those of Engber *et al.* (1992)⁶ who found that regular administration of a selective D2 agonist led to elevated levels of somatostatin and NPY in striatum, while a seven day course of a D1 agonist resulted in decreased levels of the two peptides. Thermos *et al.* (1996),²⁶ using an *in vivo* microdialysis system, were not able to detect significant changes in somatostatin release after infusions of the nonspecific dopamine agonist apomorphine or selective dopamine receptor antagonists.

We considered whether these observations might reflect a direct action of dopamine on the somatostatin/NPY containing cells or whether, alternatively, the dopaminergic effects might be indirectly mediated by other neuronal populations. Arguing against a direct site of action are *in situ* hybridization studies which have shown that somatostatin-immunoreactive striatal neurons contain little or no mRNA for either the D1 or the D2 receptor,^{21, 22} while ultrastructural investigations have yielded conflicting reports regarding the existence of dopaminergic synapses onto the somatostatin/NPY-staining neurons of the striatum.^{1, 18, 27} This contrasts with the substantial dopaminergic input to cholinergic and substance P-containing striatal neurons, either of which could in turn influence the regulation of the somatostatin/NPY-releasing cells.^{14, 28} Since cholinergic striatal neurons preferentially express mRNA for D2 receptors, while substance P-positive cells have a predominance of D1 receptor mRNA,^{11, 17, 21, 22, 31} our finding that quinpirole but not SKF 38393 can stimulate somatostatin and NPY release might reflect an indirect action mediated by cholinergic interneurons. In this model, D2 receptor stimulation would result

in decreased release of acetylcholine in the striatum, thereby reducing the cholinergic inhibition of the somatostatin/NPY cells.

B. Glutamatergic Regulation of Somatostatin, NPY and Substance P Release from Striatal Neurons

The selective glutamatergic agonists used in these experiments differentially affected peptide release from aspiny and spiny populations of striatal neurons. Levels of somatostatin and NPY showed a robust response to stimulation by kainate, but little or no response to NMDA. The release of substance P, by contrast, was unaffected by kainate but was strongly stimulated by NMDA.

This pattern of differential peptide release is similar to the pattern of glutamatergic toxicity observed both *in vivo* and *in vitro*. Excitotoxicity experiments have shown that the substance P-containing spiny neurons of the striatum may be more susceptible to NMDA receptor-mediated toxicity than the somatostatin/NPY-containing aspiny neurons.^{2, 3, 8, 15} Conversely, the somatostatin/NPY cells appear to be more vulnerable than substance P neurons to the effects of non-NMDA receptor-mediated glutamatergic stimulation.^{5, 7, 16} These observations, taken together with the findings of the present study, suggest a differential distribution of functional NMDA and kainate receptors on substance P-containing spiny neurons *vs* somatostatin/NPY immunoreactive aspiny striatal neurons.

C. Dopamine-Glutamate Interactions in the Regulation of Substance P Release

In the experiments summarized in figures 2-4, the D1 receptor agonist SKF 38393 consistently attenuated the glutamate agonist-induced release of substance P. It has been shown that there is a direct interaction between D1 receptors and NMDA receptors in regulating the phosphorylation state of the intracellular protein DARPP-32 in spiny striatal neurons: D1-receptor stimulation activates DARPP-32 and thereby inhibits protein phosphatase-1, while NMDA-receptor stimulation produces the opposite effect.¹³ The present results indicate that D1 receptor stimulation and NMDA receptor activation may likewise have opposite effects on substance P release from these neurons.

Like SKF 38393, the D2 receptor agonist quinpirole consistently inhibited glutamatergic release of substance P. With all three glutamate agonists, the inhibitory effect of quinpirole was significantly less potent than that of SKF 38393. This may reflect the reported predominance of the D1 receptor subtype on substance P-containing striatal neurons^{11, 21} (but see also Surmeier *et al.*, 1993²⁵). Alternatively, or additionally, the actions of the D2 agonist may be mediated by another subtype of striatal cell. A likely candidate in this regard might be the cholinergic interneurons, which express high levels of inhibitory D2 receptors, and which appear to promote the release of substance P in the striatum.^{4, 12, 17, 20, 22, 24} Stimulation of D2 dopamine receptors on the cholinergic neurons would be expected to inhibit acetylcholine release and thereby decrease the amount of substance P released into the culture medium. The GABA/met-enkephalin-containing neurons of the striatum also express D2 receptors, but these cells form symmetric, presumably inhibitory, contacts on substance P-containing neurons.^{11, 19, 30} Suppression of GABA/met-enkephalin release by a D2 agonist would be expected to result in potentiation of substance P release, which is not what we observed in the present experiments.

D. Dopamine-Glutamate Interactions in Somatostatin and NPY Release

At concentrations which had no effect on the spontaneous release of somatostatin or NPY, the D2 receptor agonist quinpirole significantly potentiated both kainate- and quisqualate-induced NPY release. A similar, though not statistically significant, pattern was observed with somatostatin. Quinpirole had no effect on NMDA-stimulated release of either peptide. As noted earlier, it is not clear whether these findings reflect a direct action of the D2 agonist on the somatostatin/NPY cells, as opposed to an indirect effect mediated by cholinergic interneurons. The potentiating effect of the D2 agonist on glutamatergic NPY release contrasts with its inhibitory effect on substance P release.

Whereas the D2 agonist potentiated kainate-evoked NPY release, the D1 agonist SKF 38393 produced the opposite effect. SKF 38393 had no other significant effects on somatostatin or NPY release, suggesting a minor role for the D1 dopamine receptor subtype in regulating peptide release from the somatostatin/NPY cells of the striatum.

Summary

These results indicate that: 1) selective glutamate receptor agonists differentially stimulate peptide release from subpopulations of striatal neurons. Substance P release was selectively evoked by NMDA, while somatostatin and NPY preferentially responded to kainate. 2) Selective dopamine receptor agonists differentially modify glutamate receptor-mediated peptide release from striatal neurons. The D1 receptor agonist SKF 38393 consistently inhibited glutamate agonist-induced release of substance P, while generally having little or no effect on somatostatin or NPY release. D2 receptor stimulation, on the other hand, potentiated kainate and quisqualate-induced NPY release, while consistently inhibiting glutamatergic substance P release.

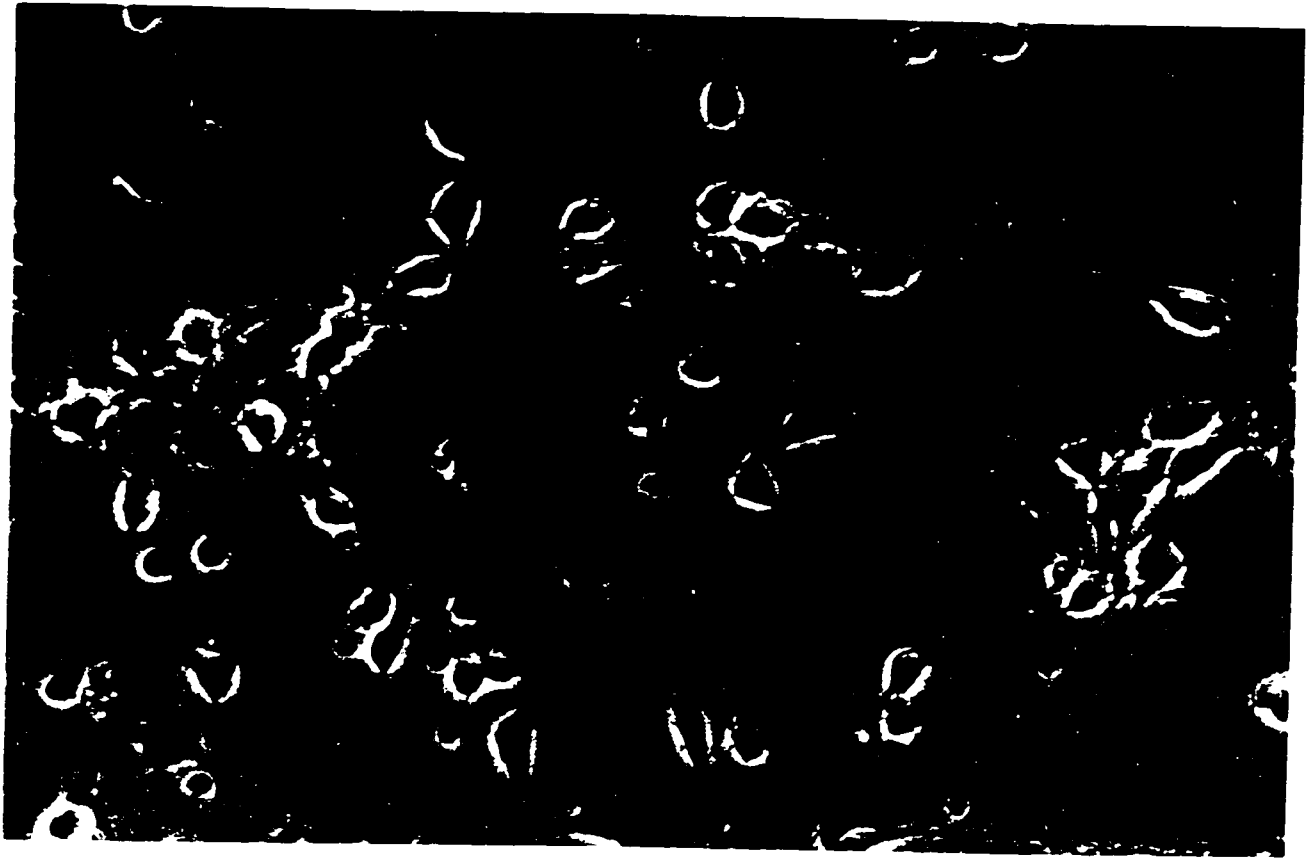
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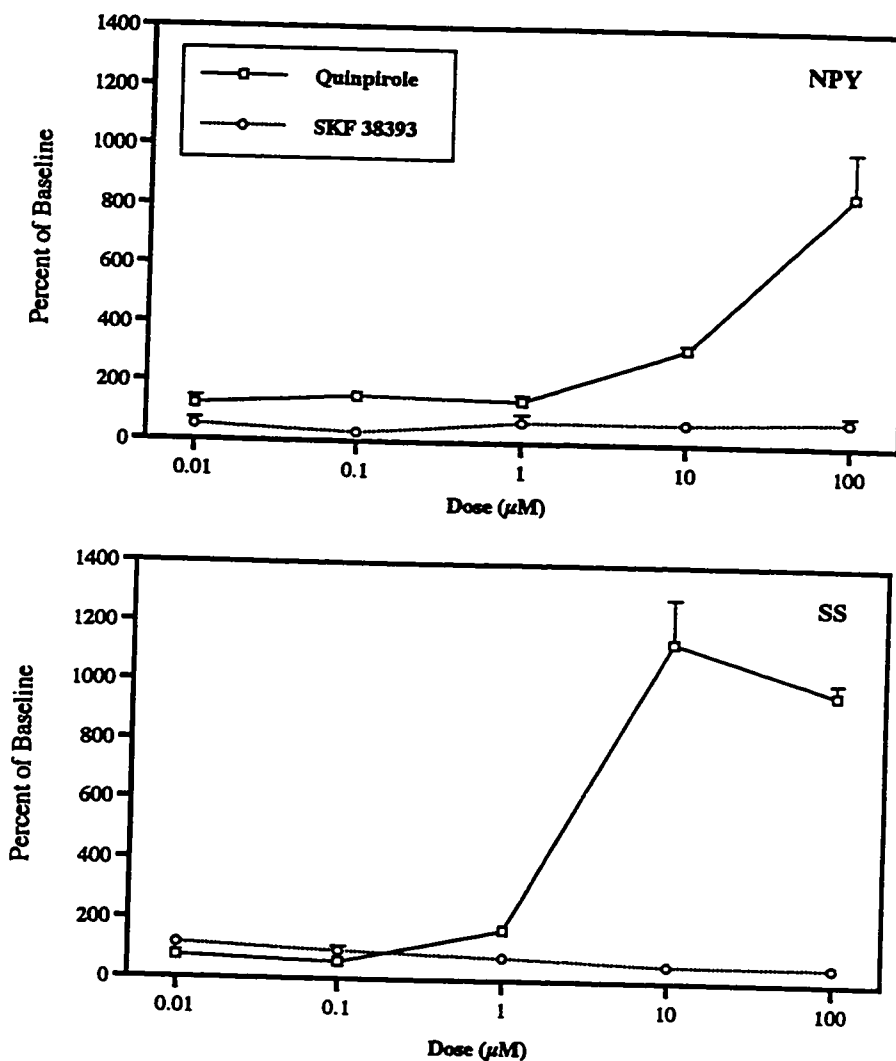


Figure 2. Dose response of quinpirole- and SKF 38393-induced release of NPY (top) and somatostatin (bottom) from cultured striatal neurons. The D1 agonist SKF 38393 had no effect on the release of either peptide. The D2 agonist quinpirole produced a dose-dependent increase in release of both somatostatin and NPY. Note that the doses used for the experiments shown in figures 3, 4 and 5 had no significant effect on the release of either peptide.

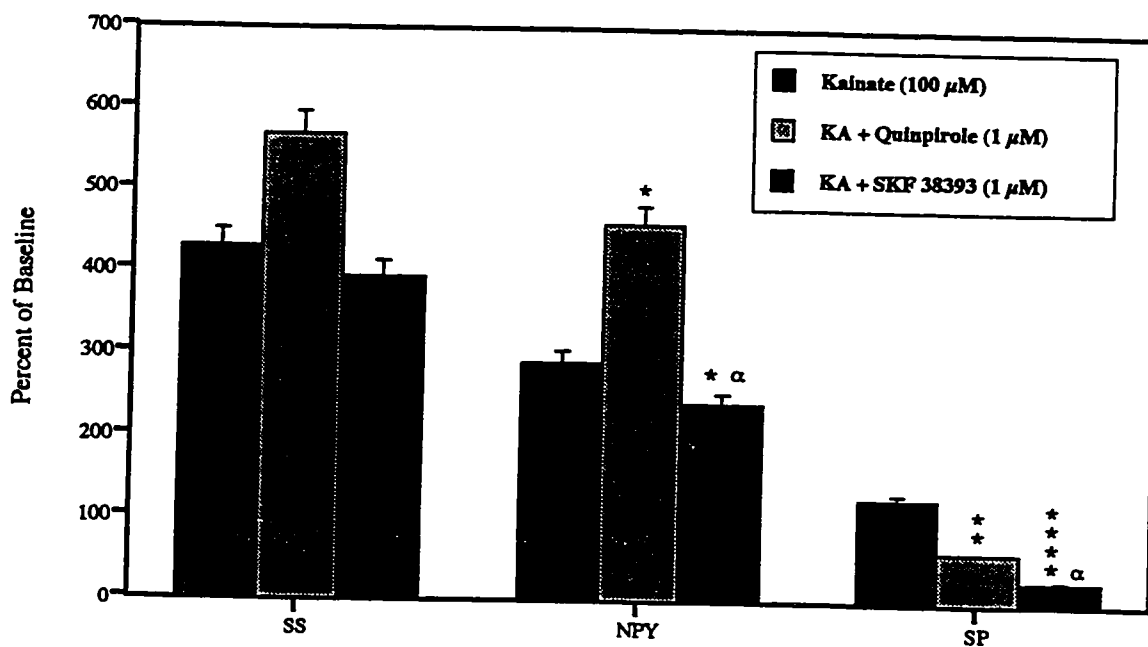


Figure 3. Effect of D1 and D2 dopamine receptor agonists on kainate-induced release of somatostatin, NPY and substance P. Treatment with kainate stimulated the release of somatostatin and NPY by 437% and 316%, respectively, above baseline levels but had little effect on substance P. The addition of quinpirole potentiated the kainate-induced release of NPY but inhibited the kainate-evoked release of substance P. The addition of SKF 38393, in contrast, had an inhibitory effect on the kainate stimulated release of both NPY and Substance P.

- * $p < 0.05$ compared with levels evoked by kainate alone.
- ** $p < 0.01$ compared with levels evoked by kainate alone.
- **** $p < 0.0001$ compared with levels evoked by kainate alone.
- α $p < 0.05$ D1 vs D2 effect on levels evoked by kainate alone.

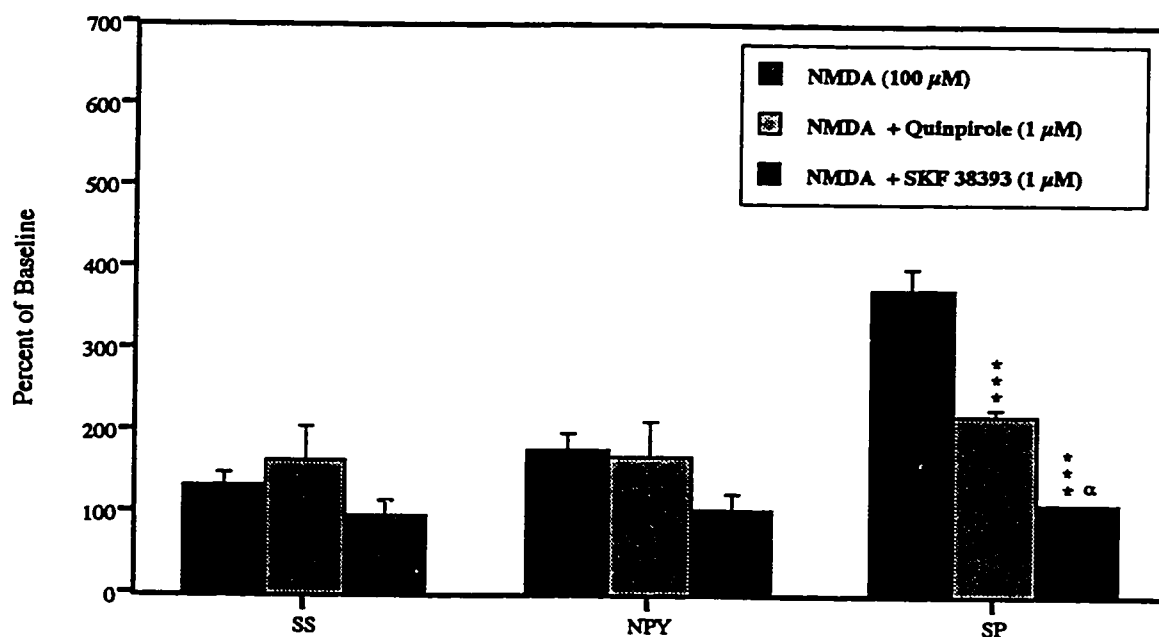


Figure 4. Effect of D1 and D2 dopamine-receptor agonists on NMDA-induced release of somatostatin, NPY and substance P. NMDA provoked a significantly greater release of substance P than of somatostatin or NPY. Both the D1 agonist SKF 38393 and the D2 agonist quinpirole significantly attenuated NMDA-stimulated release of substance P, with the D2 agonist having a significantly stronger inhibitory effect. Neither of the dopamine agonists affected the NMDA-induced release of somatostatin or NPY

*** $p < 0.001$ compared with levels evoked by NMDA alone.

α $p < 0.05$ D1 vs D2 effect on levels evoked by NMDA alone.

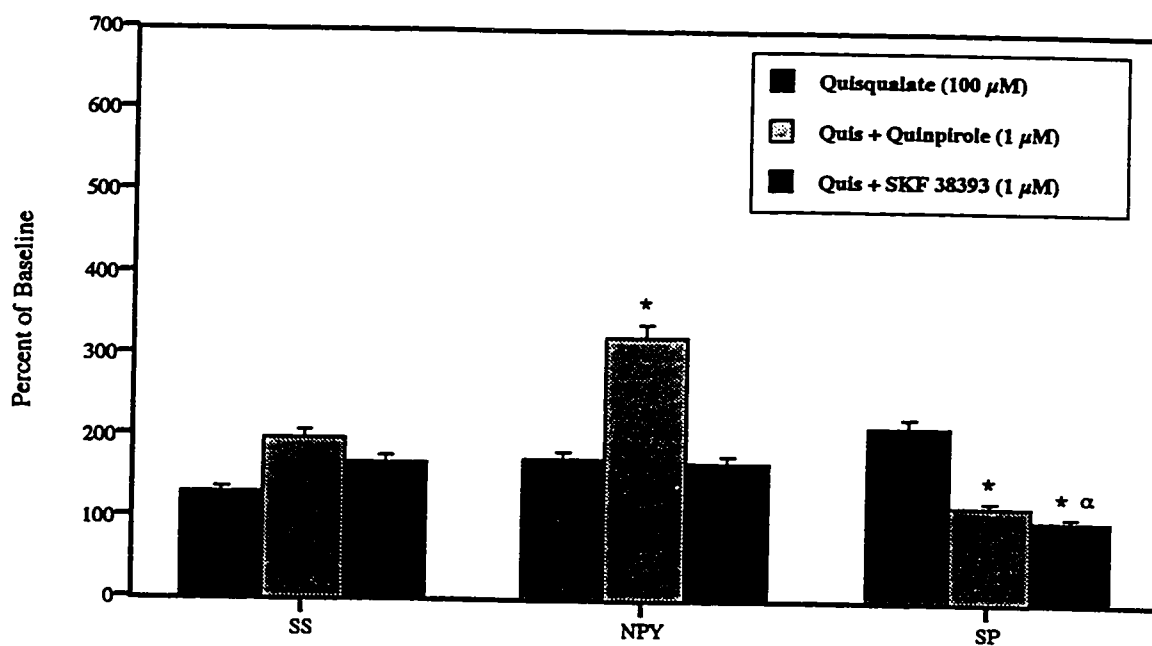


Figure 5. Effect of D1 and D2 dopamine-receptor agonists on quisqualate-induced release of somatostatin, NPY and substance P. Quisqualate on its own had no significant effect on the release of any of the three peptides. The addition of quinpirole significantly potentiated NPY release and decreased substance P release. The addition of SKF 38393 had no effect on somatostatin or NPY release but significantly attenuated the quisqualate-induced substance P response.

* $p < 0.05$ compared with levels evoked by quisqualate alone.

α $p < 0.05$ D1 vs D2 effect on levels evoked by quisqualate alone.

CHAPTER 7: DISCUSSION

The long-term goal of my research is to better understand the physiology and functional significance of dopamine-glutamate signaling in the striatum. The importance of the striatum in normal brain activity is highlighted by the disease states that result from its dysfunction. The loss of striatal dopamine in Parkinson's disease and the resulting changes in striatal signaling produce the bradykinesia, rigidity and mental changes that characterise the disorder. Degeneration of the spiny striatal output neurons in Huntington's disease leads to the striking abnormalities of movement and mental function that are observed in HD patients.

The two most important afferent projections to the striatum contain glutamate and dopamine, respectively. The striatum itself is a heterogeneous structure and many of the details of striatal signaling are still being clarified. Locally projecting intrinsic striatal neurons fall into at least two populations: acetylcholine-containing neurons and somatostatin-neuropeptide Y- nitric oxide synthase-containing neurons. Together, these account for approximately 5% of the total neuronal population of the striatum. The remaining striatal neurons are GABAergic efferents, one population of which co-localises dynorphin and substance P. Despite considerable study of the independent contributions of glutamate and dopamine afferents to the *medium spiny* neurons of the striatum in physiological and pathophysiological processes, little is known about how dopamine and glutamate combine to regulate transmitter release. The effects of dopamine, glutamate on the function of *aspiny* neurons is even less well understood.

In my thesis work I have focused on two subgroups of striatal cells: 1) the somatostatin-neuropeptide Y-nitric oxide -containing subclass of intrinsic neurons; and 2) the substance P containing subpopulation of spiny projection neurons. The *in vivo*

experiments described in chapter three studied the ability of dopamine D2-receptor blockade to modify glutamatergic signaling through the NMDA receptor. I used pathological, neurochemical, behavioural measures of lesion size as an index of NMDA-receptor mediated signaling. I found that systemic administration of the D2-dopamine receptor antagonist haloperidol significantly attenuated the size of a glutamate-receptor mediated lesions in the rat striatum by 42%, compared with a 48% reduction in lesion size after prior lesioning of the afferent dopamine pathway with 6-hydroxydopamine. These results suggested that dopamine alters glutamatergic signaling in the striatum by a receptor-mediated mechanism. I could not determine from the *in vivo* experiments, however, how dopamine and glutamate might interact in the control of *transmitter release* from specific subsets of striatal neurons.

In order to address this issue, I developed a primary neuronal tissue culture system. To confirm the usefulness of the *in vitro* method, I began with a detailed study of the similarities between my *in vitro* system and the *in vivo* animal. Chapter four presents the *in vitro* ontogeny of one of the intrinsic striatal cell types (the somatostatin-neuropeptide Y-nitric oxide containing neurons) compared with its *in vivo* development. This experiment revealed a striking consistency between the *in vivo* and *in vitro* development of the somatostatin- neuropeptide Y- nitric oxide synthase-containing class of interneurons. The proportion and morphology of substance P containing neurons was also very similar to that observed *in vivo* (data not shown).

In the series of *in vitro* experiments described in chapters five and six, I studied the differential effects of various glutamatergic and dopaminergic agonists on the release of transmitters from these two classes of neurons. The experiments presented in chapter five show that selective glutamatergic stimuli differentially regulate the release of neuropeptides from spiny and aspiny populations of striatal neurons. In particular, levels of somatostatin

and neuropeptide Y showed a robust response to stimulation by kainate, but little or no response to NMDA. By contrast, release of substance P was unaffected by kainate but was strongly stimulated by NMDA. Neither of the two groups of neurons showed much response to stimulation by quisqualate.

This pattern of differential peptide release is strikingly similar to the pattern of differential toxicity observed in the *in vivo* studies. The intrastriatal injection of the selective NMDA-receptor agonist quinolinic acid results in marked depletions of substance P and a relative sparing of somatostatin and neuropeptide Y. *In vitro*, exposure to NMDA results in a 3-4 fold increase in the amount of substance P released, with no significant increase in the amount of somatostatin or neuropeptide Y released. Both of these studies suggest a differential distribution of functional NMDA, kainate and quisqualate receptors on spiny vs aspiny striatal neurons.

In the final chapter, I investigated the ability of selective dopamine receptor agonists to modify glutamate-induced signaling. In terms of dopaminergic signaling alone, the D1 agonist SKF 38393 had very little effect on the release of somatostatin or neuropeptide Y *in vitro*, while the D2 agonist quinpirole potentiated the release of both peptides in a dose dependent manner. The interaction between dopamine and glutamate on the functioning of these neurons was more complex but revealed the following general pattern: 1) the D1 agonist SKF 38393 attenuated glutamate-agonist-induced release of substance P, without any effect on somatostatin release. SKF 38393 also significantly decreased the kainate-evoked release of neuropeptide Y. 2) In contrast, the D2 agonist quinpirole, significantly potentiated glutamate-agonist-induced release of neuropeptide Y, with a similar trend in somatostatin, while attenuating the evoked release of substance P by the same glutamate agonists.

Addition of the D1-dopamine agonist to the culture medium attenuated NMDA-induced substance P release, a result that is consistent with the *in vivo* experimental results. *In vivo*, short-term administration of haloperidol increases dopamine transmission via the D1-dopamine receptor as the D2 autoreceptors are blocked. The D1 receptors are preferentially located on the substance P/GABA spiny neurons on the same spines as the NMDA receptors. It has recently been shown that there is a direct interaction between D1 receptors and NMDA receptors via the intracellular protein DARPP-32 (Halpain et al., 1990). The active form of DARPP-32 inhibits protein phosphatase-1 and the subsequent dephosphorylation of intracellular proteins. Activation of the NMDA receptor by glutamate, on the other hand, leads to the dephosphorylation of DARPP-32 and the activation of protein phosphatase-1. Assuming that there is a correlation between the levels of dephosphorylated DARPP-32 and NMDA toxicity, the resulting increase in D1 receptor stimulation would antagonize the effect of glutamate on the phosphorylation state of DARPP-32, and result in a reduction of excitotoxicity. My *in vitro* studies demonstrated that D1-receptor stimulation significantly attenuated NMDA-mediated substance P release, consistent with the *in vivo* data. This confirms the conclusion that D1-dopamine receptor stimulation opposes the actions of NMDA-stimulation on substance P/GABA striatal neurons.

These studies indicate the complexity of the interactions between the different neurotransmitter systems in the striatum. These studies may have implications for the understanding of the pathophysiology of clinical syndromes such as Huntington's disease and Parkinson's disease, in which the primary problem is dysfunction of striatal signaling.

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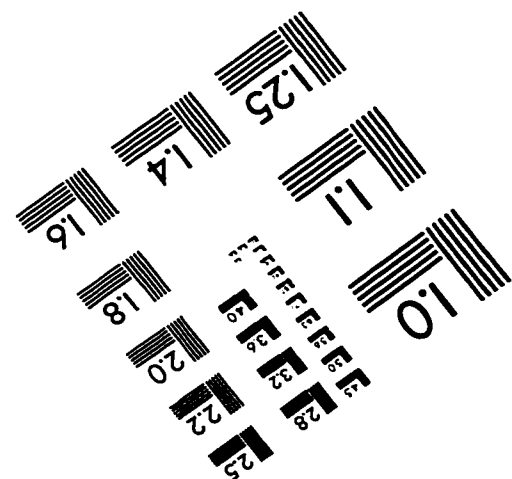
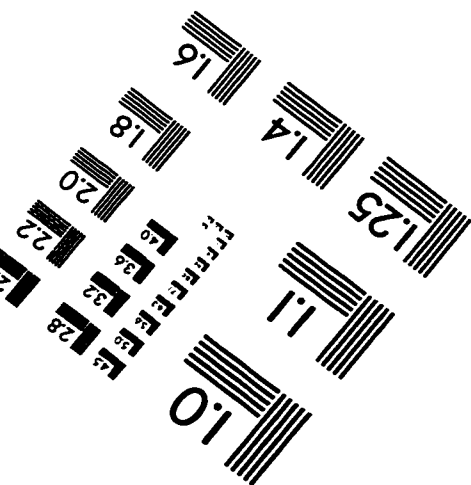
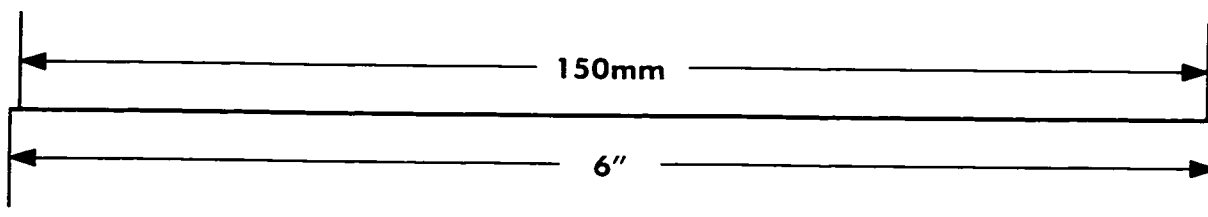
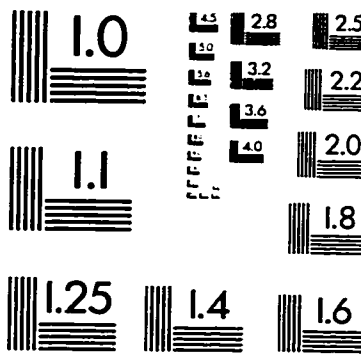
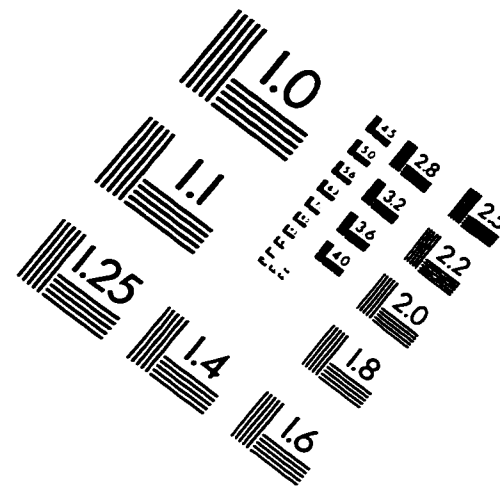
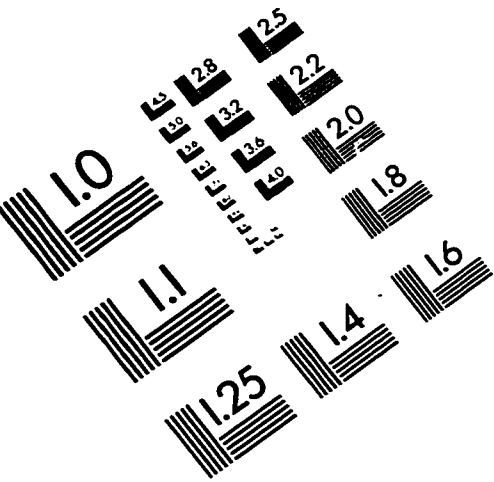
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IMAGE EVALUATION TEST TARGET (QA-3)




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