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ELECTROPHYSIOLOGICAL CHARACTERISATION OF EMBRYONIC STRIATAL GRAFTS

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THESIS SUBMITTED FOR EXAMINATION FOR THE HIGHER DEGREE DOCTOR OF PHILOSOPHY AT CARDIFF UNIVERSITY

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ACKNOWLEDGEMENTS

"No; we have been as usual asking the wrong question. It does not matter a hoot what the mockingbird on the chimney is singing. The real and proper question is: Why is it beautiful?"

Bertrand Russell

I would like to begin this list of thanks by first of all mentioning the large impact my parents have had on enabling me to complete this thesis. Mandy, my mother for always being supportive, driving me on, and picking me up when times were low. Peter, my father, for aways pushing my intellectual thought, for challenging my ideas, and making me the academic I am. In a major way, the eventual completion and successful defence of this thesis is as much a reflection upon their own efforts as my own.

I owe a great debt of gratitude to my supervisor Steve, for supporting me through the research program and writing stage of this thesis, for enabling me to pursue my own direction, and for refining my theories, ideas, and experiments into the thesis you will undoubtably now be reading. A special thanks goes to Máté who has painstakingly pushed me through the bad times, and the good times of thesis, all with small bouts of his trademark refreshing sarcasm; megköszön Máté. Without question I would like to acknowledge those within the university who have helped me over the last four years. Paul Chapman, for setting up this PhD project. Mark Ramsay and Simon Knevitt, for teaching me the intricacies of *in vitro* electrophysiology. Phil 'The Silver Fox' Blanning and Nikki Walker, for aid with animal work. Ali Baird, for the constant support in setting up transgenic time mates, and for letting buy stuff on occasion. Jane Heath and Jeff Williams, for teaching me the fine art of histology. I would like to thank the rest of the Brain Repair Group for being a great bunch of people to work with. Finally, I would like to thank Riccardo for supporting me through the writing up stage of this thesis, and letting me play with slices as a way of a healthy distraction.

Without doubt I need to acknowledge those outside of work who have put up with me over the last four stressful years. Gareth, Owen, Sam, Helen, Gemma, Simon, Julia and Debs for

living with me and keeping me sane. Berry and Jam, for ensuring that I still enjoied a good night out. The Irish, German, and Portugese, for making me go to the pub when I should have been writing. To Gwyn and Lucy, for always being there and being understanding. To Yorkie, for being the best friend I have ever known. To the Jackal faction, for letting me shout at them, and forget all about work. Last, and by far not least, I thank Llinos, for her love and support over the final stages of this work. I would never have got this far if it wasn't for you.

This thesis is dedicated to the memory of Kenneth 'Bryn' Jones, a father, and moral foundation, whom is sorely missed.

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III. ABBREVIATIONS

3-NP	3-Nitroproprionic Acid
4-AP	4-Aminopyridine
6-OHDA	6-hydroxydopamine
α -CamKII	α- Calmodulin Kinase II
A1	Adenosine Receptor 1
A2A	Adenosine Receptor 2A
AC	Adenyl Cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
aCSF	Artificial Cerebro-spinal Fluid
AMP	Adenosine monophosphate
AMPA	α-Amino-5-hydroxy-3-Methyl-4-isoxazole Propionic Acid
APV	2-amino-5-phosphonopentanoic acid
ATP	Adenosine 5'-triphosphate
BDNF	Brain Derived Neurotrophic Factor
BIC	Bicuculline
CA1	Cornu Ammonis 1
CaM	Calmodulin
CaMKII	Calmodulin Kinase II
cAMP	3'-5'-cyclic adenosine monophosphate
CB1	Canabinoid Receptor 1
CB2	Canabinoid Receptor 2
cGMP	Cyclic guanosine monophosphate
ChAT	Choline acetyltransferase
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPEB	Cytoplasmic Polyadenylation Element Binding Protein
CREB	cAMP response element-binding Protein
CRE	cAMP response element
D1	Dopamine Receptor 1
D2	Dopamine Receptor 2
DA	Dopamine

DAB	Diaminobenzidine
DARPP-32	dopamine- and cyclic AMP-regulated phosphoprotein with molecular weight 32 kDa
DMEM	Dulbecco's Modified Eagle Medium
EPAC	Exchange protein directly activated by cAMP
EPSC	Excitatory Post-Synaptic Current
EPSP	Excitatory Post-Syanptic Potential
ERK	Extracellular signal-regulated kinase
fEPSP	Field Excitatory Post Synaptic Potential
GABA	Gamma-aminobutyric acid
GAD	Glutamic Acid Decarboxylase
GC	Guanylate Cyclase
GE	Ganglionic Eminence
GFP	Green Flourescent Protein
Girk	G protein-regulated inwardly rectifying K^+ Channel
GP	Globus Pallidus
GPe	Globus Pallidus (External Segment)
GPi	Globus Pallidus (Internal Segment)
HD	Huntington's Disease
HFS	High-Frequency Stimualtion
IA	Ibotinic Acid
I_{as}	Inwardly Slow Activating Current
Ia_f	Inwardly Fast Activating Current
I_{Na}	Inwardly Hyperpolarising Sodium Current
IPSP	Inhibitory Post-Synaptic Potential
l-DOPA	levodopa
LGE	Lateral Ganglionic Eminence
LTD	Long-Term Depression
LTP	Long-Term Potentiation
M1	Muscarinic Receptor 1
M2	Muscarinic Receptor 2
MAPK	Mitogen-activated protein kinase
MGE	Medial Ganglionic Eminence
mGluR	Metabotrophic Glutamate Receptor
mRNA	Messenger Ribonucleic Acid

MSN	Medium Spiny Neurone
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PET	Positron Emission Topography
PI	Phosphatidylinositol
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PP1	Protein Phosphatase 1
PPE	Proenkephalin A
PPF	Paired Pulse Facilitation
PPT	Proenkephalin T
QA	Quinolinic Acid
RAP	Ras-Related Protein
RT-PCR	Real Time Polymerase chain reaction
SC	Superior Colliculus
SEM	Standard Error of the Mean
SN	Substantia Nigra
SNpc	Substantia Nigra (Pars Compacta)
SNpr	Substantia Nigra (Pars Reticulata)
SOM	Somatostatin
S-R	Stimulus-Response
STD	Short-Term Depression
STN	Sub-Thalamic Nucleus
STP	Short-Term Potentiation
SULP	Sulpiride
SVZ	Sub-Ventricular Zone
t-ACPD	(1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid
TBS	Tis Buffered Saline
TTX	Tetrodotoxin

TXTBS	Triton and Tris Buffered Saline
WGE	Whole Ganglionic Eminence

1.0 INTRODUCTION

The striatum, a region of the rodent forebrain, is part of an interconnected set of structures known as the basal ganglia and has long been implicated in the initiation and control of motor behaviour (Graybiel et al., 1994; Groenewegen, 2003). However, emerging evidence suggests that the basal ganglia also plays a role in a variety of other cognitive functions such as motor learning, habit formation, and goal rewarded behaviour (Graybiel, 2000; Oberg and Divac, 1975).

1.1 ANATOMY AND PHYSIOLOGY OF THE STRIATUM

The primate basal ganglia consists of the caudate nucleus, putamen, globus pallidus (GP), subthalamic nucleus (STN), and substantia nigra (SN). Each of these structures originates from different regions of the embryonic brain. The GP is of diencephalic origin and comprises of both an internal (GPi) and external segment (GPe). The SN is of midbrain origin, and can be divided into the pars compacta (SNc) and pars reticulata (SNr). The caudate nucleus and putamen are of telencephalic origin, and termed the neostriatum. Within rodents the caudate nucleus and putamen are indistinguishable into discrete parts.

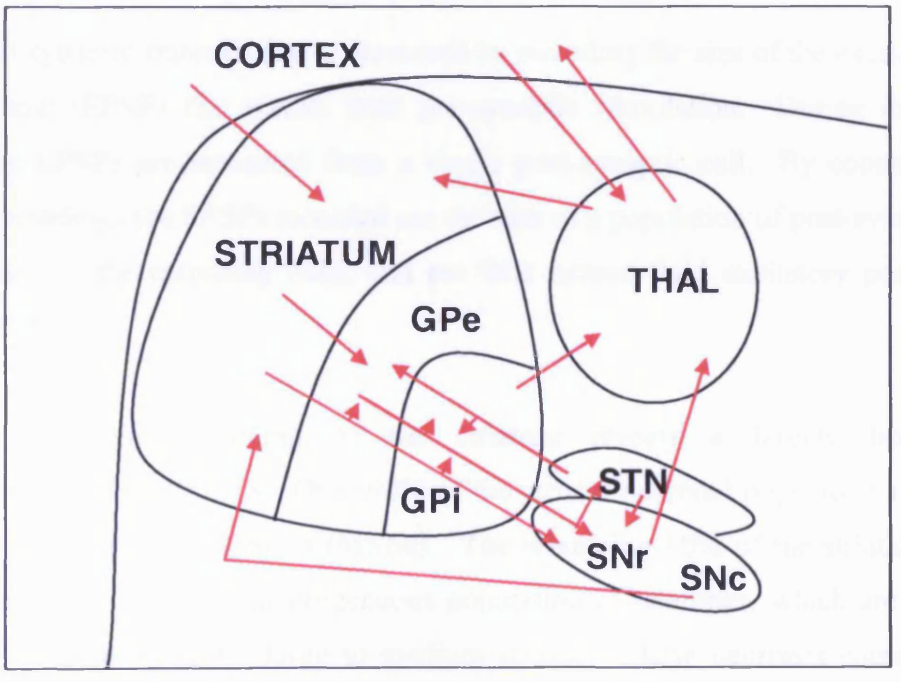

Figure 1.1 Schematic of the structural components of the Basal Ganglia. Arrows indicate neuronal pathways between structures. Adapted from Wilson (2004).

1.1.2 CYTOARCHITECTURE AND PHYSIOLOGY

The last two decades has seen huge advances in the understanding of the physiology of the striatum. In particular, refinement of the *in vitro* slice preparation has enabled more precise electrophysiological recordings from the striatum, permitting a more detailed analysis of the subsequent function.

In 1977, the advent of *in vitro* slice recordings allowed physiological investigations of function to advance to another level. Technological improvements made it possible to take thin slices of whole brains, keep them alive in artificial cerebrospinal fluid (aCSF), record responses and induce plasticity (Andersen et al., 1977). Due to further refinements over the decades, the *in vitro* slice preparation has become one of the more popular methods for studying synaptic physiology. The advantages of this method are that it permits study of brain regions in isolation, it easily permits the use of known concentrations of pharmacological agents, and it enables application of electrodes under visual guidance. Unfortunately, the major disadvantage of this technique is the detrimental effect of trauma caused by dissection and slicing of the brain.

The strength of synaptic transmission is measured by recording the size of the excitatory post-synaptic potential (EPSP) that results from pre-synaptic stimulation. During intracellular recording these EPSPs are measured from a single post-synaptic cell. By contrast, during extracellular recordings the EPSPs recorded are the sum of a population of post-synaptic cells, within the range of the recording field, and are thus termed field excitatory post-synaptic potentials (fEPSP).

Conventional histological staining of the striatum reveals a largely homogenous morphological population of cells. Over 90% of the striatal neuronal population is made up of medium spiny projection neurones (MSNs). The remaining 10% of the striatal neuronal population consists of a largely heterogeneous population of neurones, which are aspiny or sparsely spiny neurones that are large to medium in size. These neurones contain axonal branches which form extensive collateral aborisations with the local cellular network, and refrain from exiting the striatum, indicating that these neurones represent an interneuronal network within the striatum. Of the numerous subtypes of striatal interneurones, two major groups can be classified via their morphological and physiological characteristics.

1.1.2.1 MEDIUM SPINY NEURONES

These neurones are characterised by a medium soma (12-20μm). A small number of dendritic trunks (diameter 2-3μm), which are devoid of spines, give rise to an extensive dendritic tree (radius 300-500μm), which is densely studded with spines. The axonal output from these neurones is seen to emit a number of collaterals prior to exiting the striatum, and these neurones serve as the major output relays from the striatum towards the GP and SN.

Intracellular recordings of MSNs either *in vitro* or *in vivo* have enabled detailed analysis of the normal physiological mechanisms of function. Rodent medium spiny neurones have a highly negative resting potential of approximately -90mV, and an input resistance of 20-60 MΩ (Kita et al., 1984). Though MSN's recorded *in vitro* display an approximately consistent highly negative resting membrane potential, MSNs neurones recorded *in vivo* exhibit spontaneous fluctuations in membrane potentials, which govern the transition between two preferred potentials (Wilson and Groves, 1981). This fluctuation between the polarised "Down" state, and depolarised "Up" state is thought to be governed by both synaptic input and intrinsic voltage-dependant currents (Wilson, 1986). When observed experimentally MSNs lie in a predominantly silent state ("Down"), occasionally firing to produce a burst of several action potentials ("Up"), lasting 0.1-2.0 seconds (Wilson and Groves, 1981).

MSNs, like most other neuronal cell types, display a non-linear relationship between membrane potential and intracellularly injected current. This phenomenon is mediated by the presence of a fast anomalous rectification, unique to this cell type. At membrane potentials near resting this anomalous rectification is mediated via the action of a depolarising potassium conductance (G_{irk}) (Wilson, 1986). This conductance increases during hyperpolarisation to be replaced during depolarisation by a hyperpolarising conductance. Under conditions where the membrane becomes depolarised, injection of current produces a ramp like increase in membrane potential. This ramp-like response is eliminated via the application of a combination of both the potassium channel blocker 4-amino-pyridine (4-AP), and the sodium channel blocker TTX, suggesting that it is mediated via both sodium and potassium currents. Further analysis enables the identification of three sub-types of current involved MSN membrane conductance. The main depolarisation of the ramp-like response is mediated via both a slow transiently activating potassium current (I_{as}), and a fast inactivating potassium current (I_{af}). The rectification of this response is via the hyperpolarising sodium

current (I_{Na}), which becomes activated at membrane potentials close to spike threshold (Nisenbaum and Wilson, 1995).

1.1.2.2 CHOLINERGIC INTERNEURONES

These neurones represent the largest sized cells within the striatum, yet constitute less than 2% of the total neuronal population. The neurones possess an elongated cell body (50-60μm in length, 15-25μm diameter), with a high level of local dendritic branching (100um radius), though some dendrites branch to a distance of 500-750um). Axonal outputs arborise to form local collateral connections with medium spiny neurones predominantly within the region of the dendritic field, although occasionally beyond this. These neurones express acetylcholine (ACh) as their sole neurotransmitter, and stain positively for AChE and ChAT.

The large cholinergic neurones of the striatum display a number of unique electrophysiological properties (Kawaguchi, 1993). They have a more depolarised membrane resting potential -55 to -60 mV, compared to other neuronal types in the striatum. Small injections of depolarising current produces their characteristic firing of long duration action potentials (Kawaguchi, 1993). The most interesting electrophysiological characteristic of these neurones is the observation that they display a degree of spontaneous firing of action potentials, which occurs even in the absence of any synaptic input (Bennett and Wilson, 1999). Due to this unique characteristic amongst cells of the striatum, the large cholinergic neurones are regarded as pacemaker-like neurones.

The ionic mechanisms underlying the pacemaker activity of cholinergic interneurones has been extensively studied. Intracellular recordings from cholinergic interneurones indicates that these cells demonstrate an abnormal voltage-current relationship, in that they maintain a negative conductance below threshold membrane potential for action potential firing (Bennett et al., 2000). The results of this negative conductance is that these cells do not display a stable resting membrane potential, and instead shift towards the threshold membrane potential for action potential firing. This pacemaking activity is made possible through a number of unique ion currents within cholinergic interneurones. During action potential firing, high voltage calcium currents are activated, resulting in an influx of calcium into the cell and a prolongation of the action potential duration. As these currents are slow inactivating, and continue post-action potential firing, calcium builds up within the cell, resulting in the

activation of calcium dependant potassium currents (I_{ahp}). The activation of I_{ahp} causes a long lasting hyperporalization immediately after spiking of the action potential, which in turn activates the hyperpolarisation-activated cation current (I_h). Activation of the I_h current results in cell depolarisation which is continued by activation of the persistent sodium current (I_{Nap}). It is the activation of these two currents that causes the negative conductance below the action potential firing threshold, as depolarisation of the cell, via I_h, results in continued depolarisation of the cell via activation of I_{Nap}. This results in a negative deflection of the voltage-current curve, with cells under positive depolarising feedback, resulting in a slow shift towards threshold membrane potential and action potential firing.

1.1.2.3 GABAergic INTERNEURONES

The GABAergic interneuones of the striatum can be sub-divided into three groups, dependant on their morphological, physiological and biochemical properties.

The first group of GABAergic interneuones stain positive for both GABA (Bolam et al., 1983) and parvalbumin (Cowan et al., 1990; Kita et al., 1990). These neurones represent 3-5% of the striatal neuronal population. Similar in size to MSN's, though with a smoother more rounded soma, these neurones are characterised by their intensely branching local axonal arborisations which often from baskets around the soma of medium spiny neurones. Interestingly, these neurones constitute a small proportion of the total striatal neuronal population yet they are evenly dispersed around the striatum, and remain connected to one another via gap junctions at the point of overlap in dendritic fields (Kita et al., 1990). It has therefore been speculated that these neurones display an electrically coupled network that extends beyond a single neurone's dendritic tree (Koos and Tepper, 1999). These neurones can be divided into two separate subgroups dependant on whether their axons and dendrites form a local (100-150um radius) or extended (300um radius) relationship (Kawaguchi, 1993).

Intracellular recordings from these neurones display characteristic short duration action potentials, short spike after-hyperpolarisations and the ability to fire at high rates (up to 200 spikes/sec with no significant adaptation (Kawaguchi, 1993; Koos and Tepper, 1999). Due to these characteristics this subgroup of GABAergic interneurones are termed fast spiking GABAergic basket neurones.

A second group of GABAergic interneurones are similar in size and morphology to the basket interneurones, though lacking basket axonal arborisations. These neurones are characterised by their positive staining for nitric oxide synthetase (NOS) and somatostatin (SOM) (Vincent et al., 1983), and constitute 1-2% of the striatal neuronal population. These neurones are GABAergic, staining positive for both GAD and GABA within their axonal terminals (Kubota and Kawaguchi, 2000). Due to the scarcity of the GABAergic NOS positive interneurones, and the lack of any possible way to visually identify them *in vitro* these cells have not been extensively studied via electrophysiological recordings.

Finally, a third group of GABAergic interneuones have been identified due to their positive staining for calretinin (Bennett and Bolam, 1993). The cells represent an exceptionally small proportion of the striatal cellular population and have yet to be studied in detail morphologically or physiologically.

1.1.3 NEUROCHEMICAL ORGANISATION

The striatum exhibits an extraordinary level of neurochemical differentiation creating compartments of contiguous groups of cells sharing a common phenotype. These two compartments, termed the 'striosomes' and 'matrix' (Graybiel et al., 1981), are arranged in a mosaic like fashion. Initially these compartments were distinguished by their heterogeneous staining produced by μ-opiate receptor binding and AChE histochemistry (Graybiel et al., 1981). However, further analysis demonstrates that these compartments develop at different stages in embryonic development (Goldman-Rakic, 1981; Lanca et al., 1986; Van der and Fishell, 1987), whilst also displaying differing efferent and afferent connections (Gerfen, 1984; Gerfen, 1985; Gerfen, 1989; Goldman-Rakic, 1981).

During development of the rodent striatum the striosomal compartment is the first to develop, beginning around embryonic day 13 (E13), and forming the striatal primordium. The matrix compartment does not begin to form until embryonic day 16 (E16), when matrix cells from the subventricular zone (SVZ) migrate into the striatal primordium. This large influx of cells causes division of striosomal cells into densely packed clusters of cells, with the less dense matrix regions occupying the area surrounding. This migration and compartmentalisation creates the mosaic appearance seen within the adult striatum (Johnston et al., 1990; Van der and Fishell, 1987).

1.1.4.1 GLUTAMETERGIC AFFERENTS

The corticostriatal and thalamostriatal projections represent the major excitatory input to the striatum. It has been estimated that the rodent striatum contains roughly 900 million excitatory cortical and thalamic synapses per cubic millimetre (Wilson and Groves, 1980). Taking into account the approximate medium spiny cell density within the rodent striatum (84000 cells per cubic millimetre), it can be concluded that each medium spiny neurone receives approximately 11,000 excitatory synaptic contacts from afferent cortical and thalamic inputs (Wilson and Groves, 1980).

Unlike other targets of cortical and thalamic projection, the relationship between post-synaptic medium spiny neurones and pre-synaptic cortical and thalamic projection neurones does not appear to be 'one-to-one'. Quantative analysis of the potential maximal number of synapses formed by corticostriatal afferent fibres on each individual medium spiny neurone, suggests that each cortical projection neurone could at most form roughly 40 synapses within the striatum (Kincaid et al., 1998). Should these 40 synapses form on a single medium spiny neurone, this would equate to 0.4% of the total innervation of the cell. Likewise, should these cortical projection neurones make synapses non-preferentially with all medium spiny neurones within a dendritic field, a single cortical neurone would still only innervate 1.4% of the total medium spiny neuronal population of that field. Further to this, it has been demonstrated that each cortical projection neurone innervates 1-15% of the total striatal volume (Zheng and Wilson, 2002). This would suggest that each medium spiny neurone receives inputs from a relatively large number of cortical and thalamic projection neurones.

Activation of the corticostriatal system causes the release of glutamate from post-synaptic terminals. Glutamate diffuses into the synaptic cleft where it binds to post-synaptic glutametergic receptors resulting in receptor activation. Activation of receptors causes an influx of positive ions into the post-synaptic bouton, resulting in depolarisation of the cell. The subsequent EPSP's can be detected via electrophysiological recordings, and corresponds to the efficacy of synaptic transmission. The latency of the corticostriatal EPSP's does not vary with increased stimulus intensity or frequency, suggesting that the corticostriatal pathway is monosynaptic.

Corticostriatal EPSP's are blocked by the application of kynurenic acid, a broad-spectrum glutamate receptor antagonist, but not by the application of selective N-methyl-D-aspartate

(NMDA) receptor antagonists (Herrling, 1985). This suggests that corticostriatal EPSP's are mediated by the α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) family of ionotropic glutamate receptors. This concurs with observations from the hippocampus and cortex, where baseline synaptic transmission is mediated predominantly via this form of glutametergic receptor. Selective inhibition of AMPA receptors, with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), inhibits corticostriatal EPSPs (Calabresi et al., 1990), further demonstrating that corticostriatal excitatory transmission is mediated predominantly via AMPA receptors.

Within the striatum it is possible to see the effect of NMDA receptors on baseline transmission. Under 'normal' conditions the NMDA receptor is considered inactive, via a voltage sensitive magnesium block. During depolarisation of the post-synaptic cell the magnesium blockade of the NMDA receptor is removed, enabling the receptor to function. Likewise, in the *in vitro* system, the NMDA receptor can become chronically activated via the removal of magnesium from the perfusate solution. Chronic activation of NMDA receptors permits detection of NMDA-mediated currents in the post-synaptic cell, resulting in a distinct NMDA component of the EPSP, which is blocked via the application of NMDA receptor antagonists (Calabresi et al., 1992e). However, it has been shown *in vivo* that repetitive stimulation of the corticostriatal pathway, results in the expression of long-term potentiation (LTP) within the striatum, which is critically dependant on NMDA receptor activation. However, within the *in vitro* slice preparation, where the NMDA receptor is considered inactive under normal conditions, a large proportion of the corticostriatal innervation is lost. Therefore, it would seem that stimulation of a single group of corticostriatal fibres is not sufficient to activate the NMDA receptor, even under conditions of high frequency stimulation (HFS), though activation of a large number of corticostriatal fibres is capable of causing NMDA receptor activation.

Along with ionotropic glutamate receptors, it has been shown that corticostriatal synapses also contain metabotropic glutamate receptors (mGluR) (Testa et al., 1994; Testa et al., 1995). The predominant form of mGluR found in the striatum is of the group I type, which are coupled to phosphatidylinositol (PI) hydrolysis. However, recent studies using RNA amplification techniques, have shown that group II mGluRs also exist in the striatum (Shave

et al., 2001). These receptors, which are negatively coupled to adenylate cyclase activity, have been shown to occupy a predominantly pre-synaptic location.

Activation of striatal mGluRs has been shown to decrease striatal glutametergic synaptic transmission (Calabresi et al., 1992d; Calabresi et al., 1993b; Lovinger, 1991; Pisani et al., 1997). Application of the mGluR agonist (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid (t-ACPD) produces a dose dependant reduction in the amplitude of EPSP's recorded from MSN, without effecting post-synaptic sensitivity to glutamate, suggesting a pre-synaptic site of action. The proposition of a pre-synaptic site of action for t-ACPD is further corroborated by the observation that putative antagonists of type I mGluRs do not interfere with this effect (Calabresi et al., 1993b). Studies have shown that specific agonists for type II mGluRs depress excitatory synaptic potentials, which are blocked by the application of both type I and type II mGluR antagonists (Lovinger and McCool, 1995).

Activation of mGluRs has also been shown to play a role in modulating striatal GABAergic transmission. Agonists of mGluR receptors have been shown to significantly reduce GABA mediated synaptic potentials, without affecting post-synaptic membrane responses to the application of GABA (Stefani et al., 1994). The mGluR mediated inhibition of GABA mediated synaptic potentials is blocked in the presence of N-type calcium channel antagonists (Stefani et al., 1994). Therefore, it would appear that mGluR activation modulates striatal GABAergic transmission via pre-synaptic activation of N-type calcium channels.

1.1.4.2 DOPAMINERGIC AFFERENTS

Dopamine (DA) has long been linked with an involvement in motor control (Alexander et al., 1990; Boraud et al., 2002; DeLong, 1990). Degeneration of the nigrostriatal pathway is considered responsible for the motor symptoms observed in Parkinson's disease. Furthermore, dopamine has been implicated in goal directed behaviour (Robbins and Everitt, 1996), addiction (Robbins and Everitt, 2002), and reward (Schultz, 2005).

The structure and function of DA receptors has been extensively investigated over the last few decades. To date five individual isoforms of DA receptors have been cloned, and can be divided into two families dependant on their pharmacological and biochemical characteristics. The D1-like family of DA receptors includes both D1 and D5 receptors. These receptors have

been shown to activate subset of G proteins, which includes $G_{s/olf}$, G_o and G_z (Sidhu, 1998). Activation of these G proteins leads to the concurrent activation of adenylyl cyclase (AC), which leads to an elevation of the second messenger, cytosolic cyclic AMP (cAMP). cAMP causes dissociation of the cAMP-dependant protein kinase (PKA) regulatory subunit, which in turn affects cellular function. However, there is a growing body of evidence to support the concept that a number of D1-like receptors exist which are not coupled to AC (Anderson et al., 1992; Arnt et al., 1992; Downes and Waddington, 1993; Friedman et al., 1997; Gnanalingham et al., 1995; Johansen et al., 1991; Mailman et al., 1986; Wang et al., 1995). It has been suggested that this subset of D1-like receptors are linked to the PI pathway (Friedman et al., 1997; Mahan et al., 1990; Undie and Friedman, 1990; Undie and Friedman, 1992; Undie et al., 1994).

The D2-like family of DA receptors includes the D2, D3 and D4 receptors. These receptors activate the $G_{i/o}$ subset of G proteins, which inhibit AC activity.

Historically, the expression of the two families of DA receptors was described to be confined to two subtypes of MSN neurones, which can be separated by their anatomical and biochemical characteristics. One group of MSNs project to the substantia nigra and internal segment of the globus pallidus, and is termed the "direct" pathway (Alexander et al., 1986; Gerfen, 1992). The other group of MSNs project to the external segment of the globus pallidus, and then to the subthalamic nucleus and internal segment of the GP before reaching other output targets, and is known as the "indirect" pathway (Alexander et al., 1986; Gerfen, 1992). MSN's of the "direct" pathway predominantly express high levels of D1-type receptors, as well as substance P. MSN's of the "indirect" pathway predominantly express high levels of D2-type receptors, as well as enkephalin. However, it has been shown that the separation of D1- and D2-like receptors does not conform fully to this rule. Rough 20-25% of the MSN population coexpress both D1- and D2-like receptors, as well as substance P and enkephalin (Surmeier et al., 1996). Furthermore, the implementation of the reverse transcriptase-polymerase chain reaction (RT-PCR) has made identification of the less abundant isoforms of DA receptor (D3, D4, and D5) easier. It has been shown that D4 and D5 receptors are present, although at low levels, within the striatum (Bergson et al., 1995). D3 receptors seem to be expressed at high levels within roughly 40% of MSN of the "direct" pathway, and that following DA depletion and the introduction of l-DOPA there is an up regulation of D3 receptor expression in these neurones (Bordet et al., 1997). This

bservation, coupled with the observation that MSNs of the "direct" pathway can also send xon collaterals to the GPe (Kawaguchi et al., 1990), suggests that the two pathways are not ruly segregated.

Though MSN's represent the main target of DA innervation with the striatum, the various nterneurons have also been shown to receive functional DA inputs. However, due to the carcity of the neurones it has proved difficult to characterise the subtype of DA receptors resent on these neurones in any but the cholinergic interneurones. Although initially these eurones where thought to express only D2-like receptors (Le Moine et al., 1990), RT-PCR tudies have revealed that these neurones also coexpress high levels of D5 receptors (Bergson t al., 1995; Yan and Surmeier, 1997).

Though striatal DA has been linked with a number of behavioural responses and pathological conditions, the mechanisms of DA on striatal neurones is still a matter for debate within basal ganglia research. Early studies using DA iontophoresis on anaesthetised animals demonstrated that DA depressed spontaneous firing of striatal neurones (Bloom et al., 1978; Brown and Arbuthnott, 1983; Chiodo and Berger, 1986; Hu and Wang, 1988; Johnson et al., 983; Nisenbaum et al., 1988). Further, studies have looked at the effect of DA on single-unit ctivity, either evoked by cortical stimulation or via direct application of glutamate. Although heses studies reported a DA-mediated decrease in evoked spiking (Brown and Arbuthnott, 983; Johnson et al., 1983; Wachtel et al., 1989), this effect has been described as dose-dependant, with lower doses of DA facilitating glutamate-evoked spiking (Chiodo and Berger, 1986; Hu and Wang, 1988; Hu and White, 1997; Nisenbaum et al., 1988; Shen et al., 992). Furthermore, it has been shown that following stimulation of the medial forebrain undle, which increases DA release in the striatum, there is an enhancement of spontaneous iring in a subset of MSNs (Gonon, 1997). This effect is blocked by application of D1-type eceptors antagonists, suggesting a D1-like receptor mediated response (Gonon, 1997).

The cellular mechanism of striatal DA receptor activation has been studied extensively with he in vitro slice preparation. Activation of D1-like receptors has been shown to reduce spike ctivity induced by current injection (Akaike et al., 1987; Calabresi et al., 1987; Uchimura et l., 1986). The mechanism underlying this inhibition of spike firing has been shown to be aused by D1-like receptor activation of the cAMP pathway, leading to protein kinase (PKA) nediated phosphorylation of type II sodium channels, causing a reduction in sodium peak

current (Schiffmann et al., 1995; Surmeier et al., 1992a; Zhang et al., 1998). Furthermore, activation of D1-like receptors has been shown to increase anomalous potassium rectifier currents in MSNs (Pacheco-Cano et al., 1996), which regulate the resting membrane potential of MSNs. This effect is mediated predominantly by Kir2 channels, which show a highly significant degree of colocalisation with MSNs expressing D1-like receptors (Mermelstein et al., 1998).

D1-like receptors have also been shown to act upon voltage-dependant calcium channels. Activation of D1 receptors has been shown to decrease N- and P/Q-type calcium currents, whilst increasing L-type calcium currents, within MSNs (Surmeier et al., 1995). D1-like receptor suppression of N- and P/Q-type calcium channels, has been shown to be mediated by the PI pathway (Fienberg et al., 1998; Hubbard and Trugman, 1993). D1-like receptor up regulation of L-type channel efficacy relies on the cAMP pathway (Surmeier et al., 1996) and is synonymous with modulation of calcium channels within cardiac cells (Yue et al., 1990).

The functional consequence of D2-like receptor activation on MSNs is less well understood than that of D1-like receptors. It has been shown that activation of D2-like receptors can either facilitate or inhibit voltage-dependant sodium currents (Surmeier et al., 1992a). Facilitation of sodium currents is thought to involve a reversal of the D1-receptor-AC-PKA-mediated inhibition (Stoof and Kebabian, 1984). Inhibition of sodium currents has been shown to involve a negative shift in the voltage dependence of inactivation, whilst peak conductance remains unchanged (Ma et al., 1994).

Activation of D2-like receptors on MSNs has been shown to activate weak inwardly rectifying potassium channels (Freedman and Weight, 1988; Freedman and Weight, 1989), whilst also suppressing currents produced by Kir2 channels (Uchimura and North, 1990). Furthermore, it has been shown that in a certain subset of MSNs D2-like receptor activation enhances depolarization-activated potassium currents (Kita, 1996).

A number of studies have looked at the effect of DA on cholinergic interneurons. Activation of D2-like receptors has been shown to decrease release of ACh (Lehmann and Langer, 1983; Stoof et al., 1992). As D2-like receptors negatively couple to N-type calcium channels (Yan et al., 1997), activation of this type of receptor will inhibit calcium influx, therefore inhibiting

ACh release. Activation of D1-like receptors has been shown to enhance ACh release (Consolo et al., 1992; Damsma et al., 1990).

DA has been shown to affect basal striatal synaptic transmission. Activation of D2-like receptors reduces AMPA-mediated EPSPs when evoked via cortical stimulation, application of glutamate, or application of AMPA (Cepeda et al., 1993; Hsu et al., 1995; Levine et al., 1996b). D1-like receptor activation enhances the NMDA-mediated synaptic response (Cepeda et al., 1993; Cepeda et al., 1998; Levine et al., 1996b; Levine et al., 1996a). Furthermore, some studies have shown that D1-like receptor activation can also enhance AMPA-mediated excitatory post-synaptic currents (EPSCs) and EPSPs (Galarraga et al., 1997; Umemiya and Raymond, 1997), which is thought to involve PKA phosphorylation of DARPP-32 (Yan et al., 1999). It must be noted however that a number of studies have demonstrated that DA has no effect on either AMPA or NMDA mediated responses within the normal striatum (Malenka and Kocsis, 1988; Nicola and Malenka, 1998; Stefani et al., 1995). Within conditions where there is up-regulation of DA receptors, such as following 6-OHDA lesions, application of DA has an inhibitory effect on EPSPs recorded from MSNs (Calabresi et al., 1988; Calabresi et al., 1992a; Calabresi et al., 1993a).

1.1.4.3 STRIATAL INTERNEURONES

Cholinergic Inteneurones

Cholinergic interneurones represent the main source of ACh within the striatum (Woolf and Butcher, 1981; Woolf, 1991), with an addional minor cholinergic projection from the peduncolopontine tegmental nucleus (Woolf et al., 1986; Woolf and Butcher, 1986). Although these neurones represent a rather small proportion of the total striatal neuronal population, their extensive dendritic network indicates that they can form a large number of synaptic contacts with MSN's over an extensive area (Kawaguchi et al., 1995). Cholinergic interneurones receive both glutametergic innervation from the corticostriatal and thalamic projections (Lapper and Bolam, 1992), whilst also receiving dopaminergic inputs from nigral projections (Chang, 1988; Kubota et al., 1987). The extensive dendritic network of the cholinergic interneurones forms predominantly symmetrical synapses to the perikarya, dendritic shafts and spines of MSNs.

Activation of cholinergic interneurones leads to the release of ACh, which can act on striatal neurones via M1 like muscarinic receptors located on the post-synaptic MSNs. Activation of M1 receptors, via the direct application of M1 receptor agonists, leads to depolarisation of MSNs via an inward current (Calabresi et al., 1998b; Hsu et al., 1996). Both the inward current and the depolarising effect are blocked by application of barium, a potassium channel blocker, and by pirenzepine, a M1 receptor antagonist (Calabresi et al., 1998b; Hsu et al., 1996). Furthermore, this current is reversed at the same potential as that seen for normal potassium channels, indicating that the observed M1-activated inward depolarising current is mediated via potassium channels.

Muscarinic receptor activation has also been shown to act on calcium currents within MSNs. Activation of muscarinic receptors leads to a reduction in the duration of calcium dependant plateau potentials (Misgeld et al., 1986). This reduction in calcium plateau potentials has been shown to be mediated via two distinct signalling pathways, both regulated by G proteins, which are separated by their sensitivity to pertussis toxin and the subtypes of calcium channel they act on (Howe and Surmeier, 1995). The first pathway is sensitive to pertussis-toxin and inhibits both N- and P-type calcium channels (Howe and Surmeier, 1995). The second pathway is insensitive to pertussis-toxin and inhibits L-type calcium channels (Howe and Surmeier, 1995). However, the exact subtype of muscarinic receptor involved in the modulation of calcium channels is presently unknown.

The effect of muscarinic receptor activation on ionotropic glutametergic receptors has been investigated. It has been shown that application of low concentrations of muscarine increase the excitability of MSNs by enhancing the membrane depolarisation mediated by NMDA-modulated inward currents, whilst AMPA-modulated inward currents are unaffected (Calabresi et al., 1998b). This effect is antagonised by M1 receptor antagonists, and mimicked via application of M1 receptor agonists, suggesting that it is mediated by post-synaptic M1 receptors (Calabresi et al., 1998a). Interestingly, the facilitatory action of M1 receptor activation is blocked by either calcium chelating agents, or PKC inhibitors (Calabresi et al., 1998a). Therefore, modulation of NMDA receptor currents via M1 receptor activation is likely to rely on direct phosphorylation of the NMDA receptor by protein kinase C (PKC), as a consequence of an increase in internal calcium concentrations.

The location of muscarinic receptors within the striatum is not confined to MSNs. It has been shown that ACh can also exert a functional effect on corticostriatal afferent fibres, GABAergic interneurones, and interestingly upon itself. Application of muscarinic agonists has been shown to reduce ACh release within the striatum (James and Cubeddu, 1987). This autoregulation of neurotransmitter release has been proposed to be mediated by activation of pre-synaptic M2 receptors leading to the inhibition of both N- and P-type calcium currents within cholinergic interneurones, and a consequent reduction in ACh release (Yan and Surmeier, 1996).

Along with autoregulation of ACh release, striatal cholinergic interneurones also play a role in regulating the release of glutamate and GABA from pre-synaptic corticostriatal afferents and GABAergic interneurones. Application of ACh has been shown to increase the spontaneous action potential firing rate in MSNs, whilst also reducing EPSPs, when conducted *in vivo* (Bernardi et al., 1976). Furthermore, application of ACh *in vitro* has an inhibitory effect on action potentials evoked by local striatal stimulation but has no effect on action potential firing evoked by local application of glutamate (Akaike et al., 1988; Dodt and Misgeld, 1986; Takagi and Yamamoto, 1978). This would suggest that ACh-mediated inhibitory effects on intra-striatal EPSPs are mediated via presynaptic mechanisms. It has been suggested that the focus of this pre-synaptic effect on synaptic transmission is mediated via M2 receptor inhibition of both GABA and glutamate. It has been shown that GABA mediated post-synaptic synaptic potentials are reduced by the application of muscarine, or neostigmine (Sugita et al., 1991). Likewise, both neostigmine and muscarine inhibit glutamate mediated post-synaptic potentials, which is reversed by M2 receptor antagonists (Calabresi et al., 1998a).

Nitric Oxide Interneurones

NOS-positive neurones represent a rather small proportion of the total striatal population (Kawaguchi, 1993). They receive glutamatergic excitatory inputs from the corticostriatal projection, which forms glutamatergic synapses on the cell body and proximal dendrites (Salin et al., 1990; Vuillet et al., 1989). Furthermore, it has been shown that NOS-positive interneurones contain mRNA encoding for iontropic glutamate receptors (East et al., 1996). Application of either kainate (Kendrick et al., 1996; Marin et al., 1993) or NMDA (East et al., 1996; Iravani et al., 1998; Kendrick et al., 1996; Marin et al., 1993) results in an elevation of NO levels in the striatum. It has also been demonstrated that NOS-positive neurones form

synapses with nigral DAergic inputs (Fujiyama and Masuko, 1996; Gomez-Urquijo et al., 1999; Vuillet et al., 1989). Furthermore, 6-OHDA lesions of the DA system results in a reduction of NOS activity (de Vente et al., 2000; Sahach et al., 2000). It is theorised that the action of DA on NOS-positive interneurones is mediated by pre-synaptic D1-like and D2-like receptors. Application of D1-like receptor antagonists has been shown to decrease the intensity of NADPH-diaphorase staining, a measure of NOS activity, where it is upregulated following application of D2-like receptor antagonists (Morris et al., 1997).

NOS-positive interneurones form synaptic contacts with the dendrites of MSN's (Morello et al., 1997). The role of NO in the striatum is not clearly defined. Although it appears that NO is capable of modulating MSN synaptic transmission, no clearly defined mechanism of NO action exists. A number of studies have been conducted linking NO with guanylyl cyclase (GC). Activation of GC, has been shown to stimulate cGMP, which in turn activates PKC, a powerful phosphorylator of neuronal receptors (Garthwaite et al., 1995). Furthermore, cGMP can directly influence ion channel function via PKG posphorylation (Fagni and Bockaert, 1996; Robello et al., 1996). NO has been shown to influence NMDA receptor function directly, inhibiting calcium currents (Fagni and Bockaert, 1996; Hoyt et al., 1992; Manzoni et al., 1992). This effect is also induced via application of multiple NO donors (Fagni and Bockaert, 1996). Likewise, NO can directly effect $GABA_A$ receptor function, inhibiting chloride influx, which is believed to be dependant on the cGMP/PKG-mediated pathway (Robello et al., 1996).

The action of NO in the striatum is not confined to glutamatergic or GABAergic transmission. It has been shown that infusions of NOS increase striatal DA concentrations (Hanbauer et al., 1992; Stewart et al., 1996; Zhu and Luo, 1992). Furthermore, infusions of NOS-inhibitors reduce basal DA concentrations (Black et al., 1994), DA release following intra-striatal stimulation (Sandor et al., 1995), and DA release following application of NMDA (Hanbauer et al., 1992; Leslie et al., 1994). Conversely, some studies have shown that application of NO increases DA release to a greater degree following administration in the dorsolateral striatum than in the dorsolmedial striatum (Iravani et al., 1998).

The mechanism underlying NO-facilitated DA release is not clearly understood. It has been shown that NO-facilitated DA release is both calcium dependant (Buyukuysal, 1997; Lonart

et al., 1993; Trabace and Kendrick, 2000; West and Galloway, 1996; West and Galloway, 1998), and calcium independent (Black et al., 1994; Stewart et al., 1996).

It has been postulated that NO also acts on gap junction transmission between MSNs. NO has been shown to increase the incidence of dye coupling between MSNs, which is thought to be mediated by increases in gap junction permeability. Furthermore, this effect is blocked via application of NOS inhibitors, and mimicked by the application of NO donors. These observations suggest that NO may play a critical role in priming neuronal clusters to fire synchronised bursts of action potentials.

GABAergic Interneurones

GABA mediated synaptic transmission within the striatum is facilitated by the two subtypes of GABAergic interneurones (Kawaguchi et al., 1995). However, it is important to note that the MSNs receive GABAergic synaptic contacts from both the pallidal and nigral afferents (Kita, 1993), and axonal collaterals from local MSN's (Kita, 1993; Park et al., 1980; Wilson and Groves, 1980).

GABA released into the synaptic cleft binds with two types of GABA receptors on the post-synaptic MSNs. These two types of GABA receptors are separated according to their sensitivity to the GABA antagonist bicucullune, with $GABA_A$ receptors being insensitive, and $GABA_B$ receptors sensitive to the drug (Calabresi et al., 1991; Nisenbaum et al., 1993; Seabrook et al., 1990).

Activation of $GABA_A$ receptors within MSNs results in a fast membrane depolarisation (Calabresi et al., 1991). This fast membrane depolarisation is thought to be due to the activation of chloride currents, which at the highly negative resting potential of MSNs (-80mV) would cause membrane depolarisation (Calabresi et al., 1991; Mercuri et al., 1991). It has therefore been speculated that activation of $GABA_A$ receptors on MSN's may play a role in the generation of 'up-states' in these neurones. This theory is further corroborated by the observation that $GABA_A$ receptor mediated responses reverse at around -60mV (Kita, 1996; Mercuri et al., 1991), which is within the membrane potential range of resting states (Plenz and Kitai, 1998).

It has been theorised that $GABA_B$ receptors occupy a predominantly pre-synaptic location. Activation of $GABA_B$ receptors has been shown to reduce glutamate mediated EPSPs, whilst also reducing GABA mediated depolarising potentials (Calabresi et al., 1991). Furthermore, as the effect of $GABA_B$ receptor stimulation is not coupled with post-synaptic sensitivity to glutamate or GABA, this effect is considered to be predominantly pre-synaptic. Therefore, $GABA_B$ receptors may play a pre-synaptic role in regulating release of glutamate and GABA.

Adenosine

Adenosine is an active metabolite produced during the breakdown of various intracellular compounds, such as ATP, to produce energy. Extracellular concentrations of adenosine are elevated when the demand on cellular energy exceeds that normally produced by the cell.

Adenosine acts upon four subtypes of adenosine receptors to produce a potent depression like effect. Of the receptor subtypes, A1 adenosine receptors are widely distributed around the mammalian brain, including the striatum, whereas the A2A subtype is expressed almost exclusively with the striatum.

Activation of A1 receptors has been postulated to have a pre-synaptic effect on the suppression of transmitter release (Greene and Haas, 1991). The suppression of transmitter release is often coupled with post-synaptic changes in membrane hyperpolarisation and increased membrane conductance (Uchimura and North, 1991). Within the striatum, activation of adenosine receptors results in a reduction of MSNs' EPSP amplitude, with no discernable effect on the MSN membrane response or the response following application of glutamate (Calabresi et al., 1997b). Furthermore, activation of adensosine receptors is associated with an increase in MSN paired pulse facilitation (PPF), which would further indicate a pre-synaptic site of action (Calabresi et al., 1997b).

Activation of A2A receptors have been shown to have two main physiological consequences. Application of A2A receptor agonists has been shown to suppress $GABA_A$-mediated synaptic currents (Mori et al., 1996). The suppression of $GABA_A$-mediated synaptic currents, via A2A receptor activation, has been attributed to a pre-synaptic site of action (Mori et al., 1996). Therefore, MSN output activity may be regulated by A2A receptors, by reducing $GABA_A$ mediated inhibition of spiny neurones. Activation of A2A receptors has also been shown to inhibit the conductance of NMDA receptors, though only in a subset of MSNs (Norenberg et

al., 1997). However, the mechanisms for this effect, or the consequences, are not fully understood.

1.2 SYNAPTIC PLASTICITY WITHIN THE STRIATUM

Synaptic plasticity can be defined as the ability of synaptic contacts between neurones to change their strengths, according to their patterns of activation, over time. Plastic synapses are able to respond to changes in input by modifying the strength of their output. Synaptic plasticity is widely considered to underlie many cognitive functions involving learning and memory (Martin et al., 2000), and addiction (Gerdeman et al., 2003).

The concept that synapses are able to respond to changes in their environment was first suggested by Donald Hebb in 1949. Hebb, suggested a co-incidence detection rule, whereby the synapse that links two cells would become strengthened if both cells fired at the same time (Hebb , 1949). This theory was first observed *in vivo* at the excitatory synapses between the perforant path and granule cells of the hippocampus (Bliss and Lomo, 1973). A control level of stimulus was given, and the response from a population of cells measured. The cells were subjected to a high frequency tetanus pulse that activated both pre- and post-synaptic cells simultaneously. It was observed that size of the response gained had become larger when the control stimulus was applied. This increase in the response is known as long-term potentiation (LTP), and was shown to last many hours. Today, the phenomenon know as LTP is the focus of many investigations, and has been proposed as the cellular correlate underlying many if not all aspects of learning and memory (Martin et al., 2000).

Synapses can be described as bi-directionally modifiable as they display the ability to decrease their synaptic strength as well as to increase it. This decrease in synaptic efficacy has been shown to follow a similar time course of development and maintenance as LTP, and is termed long–term depression (LTD). LTD can be divided into two types; heterosynaptic LTD which occurs at inactive synapses during high frequency stimulation of a converging input, and the more common homosynaptic LTD which occurs at active synapses (Bear and Malenka, 1994; Bear and Abraham, 1996; Dudek and Bear, 1992).

There appear to be three basic principles that define synaptic plasticity (Bliss and Collingridge, 1993); they were first used to describe LTP, but are readily transferable to LTD. Firstly, a specific intensity threshold exists for the induction of plasticity, a weak tetanus (which only activates a small number of afferent fibres) will not be sufficient to induce plasticity, whereas a strong tetanus will. Also, a low frequency tetanus will not induce plasticity. However increasing the stimulus frequency can render it effective. There remains a balance between tetanus intensity and frequency, where an increase in one can compensate for a decrease in the other. The underlying mechanism regulating induction threshold as a product of both intensity and frequency is termed 'co-operativity'. Secondly, plasticity is considered to be associative. Weak inputs can induce plasticity if they are active at the same time as a stronger, yet convergent input. Thirdly, plasticity can be considered as input specific, as only inputs that are active at the time of tetanus display plasticity.

Since the first observation of synaptic plasticity in the hippocampus, both LTP and LTD have been observed in other brain regions including the amygdala (Heinbockel and Pape, 2000; Racine et al., 1983; Racine and Milgram, 1983), cerebellum (Ito and Kano, 1982; Racine et al., 1986), neocortex (Baranyi and Feher, 1981) and the striatum (Boeijinga et al., 1993; Calabresi et al., 1992c; Calabresi et al., 1992e).

The observation of synaptic plasticity in the striatum proves important, as PET studies in humans have shown that the basal ganglia are heavily involved in the storage of motor memories (Seitz et al., 1990). The striatum, and more particularly the corticostriatal projection, is considered to play a major role in motor learning (Kimura, 1995). Interestingly, recent studies have shown that in conditions where corticostriatal synaptic plasticity is impaired, learning of striatal specific behavioural tasks is also impaired (Pittenger et al., 2006).

Synaptic plasticity within the striatum can be induced via a number of methods including HFS of the cortex or thalamus, focal application of glutamate (Calabresi et al., 1999a), or stimulation of the NO/cGMP pathway (Calabresi et al., 1999b). HFS stimulation of the corticostriatal input fibres remains the favoured method of inducing striatal synaptic plasticity.

1.2.1 CORTICOSTRIATAL LTD

The preferential form of synaptic plasticity expressed at corticostriatal synapses *in vitro* is a matter of debate. Early studies demonstrated that HFS stimulation of the corticostriatal pathway *in vitro*, resulted in the expression of LTD (Calabresi et al., 1992c). Paradoxically, under conditions where magnesium is omitted from the aCSF perfusate solution, HFS results in the expression of LTP (Calabresi et al., 1992e). This interesting phenomenon is considered to be due to the high level magnesium blockade of NMDA receptors observed within the striatum. Within the *in vitro* slice HFS of the corticostriatal fibres is not considered to be sufficient to provide suitable membrane depolarisation to remove the magnesium block. Interestingly, studies *in vivo* have shown that cortical stimulation can induce striatal LTP (Charpier et al., 1999; Reynolds and Wickens, 2000). However, within the *in vivo* preparation, where the whole corticostriatal project is kept intact, HFS probably provides suitable depolarisation to remove the magnesium block of NMDA receptors. Furthermore, recent studies have demonstrated that it is possible to obtain corticostriatal LTP *in vitro* in the presence of magnesium (Mahon et al., 2004; Spencer and Murphy, 2000), where it is suggested that stimulation intensity and slicing plane may play a role in determining the preferential form of synaptic plasticity expressed.

Striatal LTD is dependant on a number of crucial pharmacological interactions. First of all the AMPA family of glutametergic ionotropic receptors plays a vital role, as application of AMPA antagonists blocks the expression of LTD. It has been postulated that AMPA receptors are not essential for the induction of LTD, and merely provide a mechanisms for the more critical membrane depolarisation. Studies have shown that LTD can be induced in situations where a mild stimulation of the corticostriatal pathway is coupled with enforced depolarisation of the post-synaptic neurone (Choi and Lovinger, 1997). Interestingly, intracellular application of voltage-dependant sodium channel antangonsists blocks the expression of LTD during HFS of the corticostriatal fibres, although expression of LTD is restored when the neurone is depolarised via current injection (Calabresi et al., 1994). It is suggested that membrane depolarisation via voltage-dependant sodium currents activates various voltage-gated calcium currents, since application of L-type calcium channel antagonists blocks the expression of LTD (Calabresi et al., 1994; Choi and Lovinger, 1997).

The post-synaptic increases in calcium concentration represent a critical step in the generation of LTD. Intracellular application of calcium chelating agents prior to administration of HFS prevents the expression of LTD (Calabresi et al., 1994). The intracellular mechanisms linked to calcium dependant regulation of striatal LTD share a high degree of similarity with those observed in other neuronal systems. Selective blockers of PKC activity also block LTD expression in a dose dependant manner (Calabresi et al., 1994).

Though a number of sub-types of the mGluR receptor exist within the striatum, type mGluR I receptors are the most predominant sub-type present. Type I mGluRs have been implicated in the formation of LTD within the hippocampus (Bashir, 2003), cortex (Shave et al., 2001) and cerebellum (Shave et al., 2001). Application of mGluR I antagonists has no effect on corticostriatal baseline transmission, but does inhibit LTD in a dose dependant manner (Calabresi et al., 1992d). Further evidence for the important role of mGluR I receptors in LTD expression is that following chronic lithium treatment, corticostriatal LTD is blocked (Calabresi et al., 1993b). Lithium treatment is known to reduce the supply of inositol, which is the substrate of PI metabolism. mGluR I receptors are coupled to PI and rely on PI metabolism to affect protein kinases. Therefore, it would seem that the activation of mGluR receptors is essential for corticostriatal LTD expression (Sung et al., 2001).

Corticostriatal LTD is also critically dependant on the activation of the nigrostriatal projection system, release of DA and activation of DA receptors on the post-synaptic MSN. Antagonists for either D1-like or D2-like DA receptors prevent the expression of LTD (Calabresi et al., 1992c; Calabresi et al., 1992a; Calabresi et al., 1992b; Choi and Lovinger, 1997), demonstrating that activation of both receptor sub-types is critical for LTD expression. Furthermore, LTD induced by the focal application of glutamate is blocked by D2-like receptor antagonists (Calabresi et al., 1999a). Interestingly, unilateral 6-OHDA lesions of the striatum result in a loss of LTD in the ipsilateral but not contralateral striatum (Calabresi et al., 1992e; Calabresi et al., 1992a; Calabresi et al., 1992b). LTD is restored following administration of DA, or coadministration of D1-like and D2-like receptor agonists, but not when either agonist is applied alone (Calabresi et al., 1992e; Calabresi et al., 1992a; Calabresi et al., 1992b). Finally, studies in D2-like receptor knockout mice have demonstrated a lack of corticostriatal LTD, though they do demonstrate a NMDA-dependant LTP following HFS, even in the presence of magnesium (Calabresi et al., 1997c).

There is a large body of evidence to demonstrate that DAergic nigrostriatal afferents display ionotropic glutamate receptors on their pre-synaptic terminals, which are capable of modulating DA release (Carrozza et al., 1992; Carter et al., 1988; Cheramy et al., 1986a; Cheramy et al., 1986b; Clow and Jhamandas, 1989; Imperato et al., 1990; Moghaddam et al., 1990; Morari et al., 1993). Furthermore, it has been shown that glutamate can modulate nigrostriatal DA release via pre-synaptic mGluR receptors (Verma and Moghaddam, 1998). Application of mGluR agonists has been shown to facilitate DA release under baseline conditions, whilst inhibiting DA release during hyperstimulation, such as that seen during HFS (Verma and Moghaddam, 1998).

Within MSNs, the main substrate for protein kinase activity is the dopamine- and cAMP-regulated protein with molecular weight of 32 kDa (DARPP-32). This protein is expressed in high concentrations within dopaminoceptive MSNs (Brene et al., 1995). In its phosphorylated state DARPP-32 inhibits protein phosphatase-1 (PP-1), which in turn regulates the phosphorylation and activity of NMDA and AMPA channels (Greengard et al., 1999). Mice lacking DARPP-32 show normal baseline transmission, but fail to express LTP or LTD (Calabresi et al., 2000b). Interestingly, pharmacological inhibition of PP-1 in these mice, restores both LTP and LTD (Calabresi et al., 2000b).

The role of NO/cGMP activation in striatal LTD has recently been investigated. Pharmacological inhibition of either NO, or guanylyl cyclase (GC) prevents the expression of corticostriatal LTD (Calabresi et al., 1999b). Application of NO donor agents has been shown to induce LTD (Calabresi et al., 2000b). However, within DARPP-32 knockout mice, application of NO donors fails to induce LTD (Calabresi et al., 2000b). These findings suggest that the expression of corticostriatal LTD requires activation, in this case via PKG mediated signalling cascades.

Studies over the last decade have discovered a role for endocannabinoids in striatal LTD. Two isoforms of cannabinoid receptor exist (CB1 and CB2), which are almost exclusively expressed within the basal ganglia (Herkenham et al., 1991). Whilst activation of CB1 receptors is essential for the induction of corticostriatal LTD (Gerdeman et al., 2002), little is known regarding the mechanism of endocannabinoid mediated synaptic plasticity. Activation of CB1 receptors has been shown to inhibit release of glutamate and GABA (Gerdeman and Lovinger, 2001; Szabo et al., 1998). Furthermore, endogenous production of

endocannabinoids has been shown to occur following D2-like receptor activation in the post-synaptic neurone (Giuffrida et al., 1999), with it's release facilitated via MSN membrane depolarisation (Di Marzo and Deutsch, 1998). Therefore, it is possible that endocannabinoids could act as a retrograde messenger.

1.2.2 COTRICOSTRIATAL LTP

As previously described, under normal physiological conditions NMDA receptors located post-synaptically on MSNs are considered inactive, even following HFS. Removal of magnesium from the aCSF perfusate solution results in the chronic disinhibtion of the NMDA receptor and the expression of corticostriatal LTP following HFS (Calabresi et al., 1992e). It must be noted however that HFS of the corticostriatal fibres can also lead to the expression of LTP, even in the presence of physiologically normal levels of magnesium (Akopian et al., 2000; Garcia-Munoz et al., 1996; Lovinger et al., 1993; Partridge et al., 2000; Smith et al., 2001; Spencer and Murphy, 2000; Walsh and Dunia, 1993; Wickens et al., 1996). The intensity of stimulation used during HFS to induce synaptic plasticity could be critical to determining whether striatal LTP or LTD is expressed. Intense HFS, reliably induces a preferential expression of striatal LTD, which is dependant on contiguous activation of DA synapses (Calabresi et al., 1996). Less intense HFS, which is likely to only weakly activate DA synapses, produces a mix of short-term depression (STD), LTD, short-term potentiation (STP) and LTP (Akopian et al., 2000; Lovinger et al., 1993). This suggests that activation of the dopaminergic system may play a critical modulatory role between the expression of striatal LTP and LTD. Furthermore, it has been shown that regional differences in the recording location within the striatum dictate a preferential expression of either LTP or LTD (Smith et al., 2001). It has been shown that the striatum receives topographic projections from the cortex, with visual and limbic structures projecting more medially, and motor structures projecting to a more dorsal and lateral location (Deniau et al., 1996; Donoghue and Herkenham, 1986; Gerfen, 1989; McGeorge and Faull, 1989). Similarly there are reported regional differences in the synaptic structures formed by the nigrostriatal system (Joyce and Marshall, 1987; Szele et al., 1991). Therefore, whilst irregularities occur in the expression of LTP and LTD, the predisposed state of the NMDA receptor and the modulatory action of DA provide a compelling explanation for the mechanism of selection between these two forms of plasticity.

The expression of corticostriatal LTP is critically dependant on the activation of post-synaptic NMDA receptors. Application of NMDA receptor antagonists blocks the expression of corticostriatal LTP, whilst application of AMPA antagonists has no effect on the expression of LTP (Calabresi et al., 1992c).

As with corticostriatal LTD, LTP is dependant on activation of the DAergic system. Whilst application of D1-like receptor antagonists blocks expression of corticostriatal LTP (Kerr and Wickens, 2001), application of D2-like receptor antagonists greatly enhances LTP (Boeijinga et al., 1993; Calabresi et al., 1997a; Calabresi et al., 1999a; Centonze et al., 1999). Furthermore, corticostriatal LTP is enhanced in D2-like receptor knock out mice (Calabresi et al., 1997c). This would suggest that activation of D1-like receptors is critical for the expression of corticostriatal LTP, where activation of D2-like receptors negatively controls the expression of LTP.

As previously described for corticostriatal LTD, the main target for protein kinase activity within MSNs is DARPP-32, which is capable of modifying both AMPA and NMDA receptor activity (Greengard et al., 1999). Interestingly, application of PKA inhibitors has been shown to inhibit corticostriatal LTP but not LTD (Calabresi et al., 2000b). Suggesting that corticostriatal LTP is mediated by posphorylation of DARPP-32 by the PKA signalling cascade.

The involvement of ACh in corticostriatal LTP has been studied. Corticostriatal LTP is blocked by M1 receptor antagonists (Calabresi et al., 1999a), whilst it is enhanced by application of M2 receptor antagonists (Calabresi et al., 1998a).

1.2.3 MOLECULAR MECHANISMS OF SYNAPTIC PLASTICITY

Synaptic plasticity has been shown to last for a number of hours *in vitro* (Bliss and Lomo, 1973; Bliss and Collingridge, 1993), and up to several weeks *in vivo* (Bliss and Collingridge, 1993). It has therefore been postulated that in order for long lasting changes to occur in synaptic efficacy some form of molecular change must take place. These changes are dependant on activity-mediated modification of protein expression. Both LTP and LTD result

in an inevitable increase in intracellular calcium concentration. Over the past few decades it has become clear that this rise in intracellular calcium can act as a signalling mechanism, not just by influencing protein kinase molecules to modify channel efficacy, but also by influencing a variety of transcriptional factors that modify gene expression.

Since the first conception of intracellular calcium as a signalling molecule for the molecular mechanisms of synaptic plasticity, the role of the calcium binding protein calmodulin (CaM) has been strongly implicated. CaM is expressed at exceptionally high levels within neurones (Cimler et al., 1985), and has been shown to influence a number of CaM-regulated molecules, that have been implicated in synaptic plasticity. Binding of calcium to CaM has been shown to increase its affinity for its target proteins (LaPorte et al., 1980). These changes in target affinity are mediated by a calcium-induced conformational change, resulting in the exposure of a hydrophobic domain, which is the main site of intereaction between CaM and its target proteins (Babu et al., 1988; Chapman et al., 1992; LaPorte et al., 1980).

During basal resting state, CaM is considered inactive, where it is bound to neuromodulin and neurogranin (Alexander et al., 1988; Andreasen et al., 1983). During this state CaM is not considered to play a role in signal transduction. However, a number of studies have shown that both neuromodulin and neurogranin may play a role in PKC-regulated phosporylation of ion channels (Alexander et al., 1988; Apel et al., 1990; Chao et al., 1996). Studies within the hippocampus demonstrate that during LTP neuromodulin is phosphorylated. Furthermore, transgenic mice with mutated neuromodulin that mimics the phosphoryalated isoform display enhanced hippocampal plasticity (Hulo et al., 2002). Neurograninn has been shown to act on PKC action much like that seen by neuromodulin (Baudier et al., 1991). However, localisation studies have demonstrated that whilst neuromodulin occupies a predominantly pre-synaptic location, neurogranin is located almost exclusively post-synaptically (Represa et al., 1990).

During conditions where intracellular calcium concentrations are increased, such as during LTP and LTD, CaM becomes unbound from neuromodulin and neurogranin, due to its higher affinity for binding to free calcium (Alexander et al., 1988; Andreasen et al., 1983). Under these conditions CaM is able to bind to a number of other intracellular signalling molecules.

The calcium/calmodulin-dependant protein kinase II (CaMKII) is a calcium stimulated enzyme that has been extensively studied for its involvement in synaptic plasticity. Binding of calcium to CaM enables the calcium/CaM complex to subsequently bind to CaMKII causing activation of the CaMKII enzyme. Activation of CaMKII enables it to perform a number of functions on other intracellular molecules. Interestingly, activated CaMKII can also autoposhporylate itself (De Koninck and Schulman, 1998). Autophosphorylation of CaMKII enables it to disassociate from membrane bound actin where it can diffuse into the synapse and act upon the NR2B subunit of NMDA receptors (Bayer et al., 2001; Shen and Meyer, 1999; Torok et al., 2001).

The role of CaMKII in LTP was initially discovered within the CA1 synapse (Malenka et al., 1989b; Malenka et al., 1989a). It was found that inhibition of CaMKII blocks the induction of LTP (Malenka et al., 1989b; Malenka et al., 1989a), whilst application of CaMKII to the post-synaptic neurone produces LTP (Lledo et al., 1995; McGlade-McCulloh et al., 1993). Furthermore, α-CaMKII knockout mice demonstrate deficits in both LTP and spatial memory (Silva et al., 1992b; Silva et al., 1992a). Due to the high co-localisation of α-CaMKII with the post-syantpic densitiy, it has been postulated that α-CaMKII has a predominantly post-synaptic method of action. Within most forms of synaptic plasticity, the AMPA family of glutametergic receptors plays a critical role in the induction, and maintenance of synaptic plasticity (Hayashi et al., 2000; Kauer et al., 1988; Muller and Lynch, 1988; Nusser et al., 1998). α-CaMKII has been shown to modulate AMPA receptor conductance by directly phosphorylating the GluR1 subunit (Barria et al., 1997; Mammen et al., 1997). Furthermore, α-CaMKII has been shown to play a role in the insertion of new AMPA receptors into the syanspe (Lisman and Zhabotinsky, 2001; Wu et al., 1996). Transgenic mice which lack both α-CaMKII and PKA phosphorylation sites display impaired LTP and LTD, and spatial memory (Lee et al., 2003).

Whilst α-CaMKII plays an important role in the induction and maintenance of synaptic plasticity, it has also been thought to play a role in long-lasting synaptic plasticity. It has been postulated that enhanced protein synthesis within dendrites (Steward and Worley, 2001; Tiedge and Brosius, 1996) may play a role in the concept of 'synaptic tagging', where activated neurones are marked to distinguish the non-active synapses (Frey and Morris, 1997). mRNA for α-CaMKII has been shown to contain a region which targets it to dendrites (Mayford et al., 1996) where it is able to accumulate (Thomas et al., 1994) with levels of

expression elevated during synaptic plasticity (Ouyang et al., 1999). Interestingly, transgenic mice in which the region of mRNA encoding for dendritic targeting is inhibited demonstrate impaired late-phase LTP and deficits in spatial memory (Miller et al., 2002). Furthermore, it has recently been demonstrated that α-CaMKII can phosphorylate, and in doing so stimulate the activity of the cytoplasmic polyadenylation element binding protein (CPEB) (Atkins et al., 2004). CPEB is able to influence mRNA transcription and protein synthesis, and therefore may provide a second mechanism for α-CaMKII modification of long-term synaptic plasticity (Atkins et al., 2004).

Calcium bound CaM can also influence cAMP signalling, which regulates a number of other internal molecules. Calcium-CaM is able to stimulate increased levels of cAMP via interaction with adenylyl cyclases. Although a number of AC isoforms exist, only AC1 and AC8 are stimulated by CaM. AC1 is neurospecific and expressed predominantly in the hippocampus, neocortex, and the olfactory system, whilst AC8 is expressed throughout the brain and is not neuro-specific (Wu et al., 1995; Xia et al., 1991). Both AC1 and AC8 knockout mice display impaired synaptic plasticity in a number of brain regions (Otmakhova et al., 2000; Villacres et al., 1998; Wu et al., 1995). Knockouts for either AC1 or AC8 do display long-lasting synaptic plasticity, although it is impaired slightly in AC1 knockouts (Wu et al., 1995). However, transgenic knockouts for both AC1 and AC8 do not display long lasting synaptic plasticity (Wong et al., 1999). Furthermore, transgenic animals with enhanced AC1 expression also demonstrate enhanced synaptic plasticity and recognition memory (Wang et al., 2004).

cAMP activation has been shown to activate EPACs, which act as exchange factors for the Ras-related proteins 1 and 2 (RAP1 and 2) (Bos 2003). Activation of either RAP1 or 2 results in the consequent activation of both the extracellular signal-related kinase (ERK) and the mitogen-activated protein kinase (MAPK). Both ERK and MAPK signalling has been shown to be critical for synaptic plasticity (English and Sweatt, 1996; Impey et al., 1998; Mazzucchelli et al., 2002).

Both cAMP and ERK/MAPK signalling cascades terminate in the activation of CREB, which in turn activates CRE mediated transcription. CREB has long been implicated in both memory and synaptic plasticity in *Aplysia* (Bartsch et al., 1998; Dash et al., 1990; Martin et al., 1997), *Drosophila* (Yin et al., 1994; Yin et al., 1995), mammalian hippocampus (Pittenger

et al., 2002), amygdala (Josselyn et al., 2001), cerebellum (Ahn et al., 1999) and more recently the striatum (Pittenger et al., 2006). HFS of the CA1 region of the hippocampus has been shown to lead to phospohrylation of CREB and activation of CRE-mediated transcription (Abel et al., 1997). Interestingly, the expression of constitutively active CREB has been shown to enhance the late phase of synaptic plasticity (Barco et al., 2002). Furthermore, both striatal LTP and LTD are abolished in animals expressing a striatal specific dominant negative mutation of CREB (Pittenger et al., 2006).

1.3 HUNTINGTON'S DISEASE AND CELL REPLACEMENT THERAPY

The extensive neuronal loss brought on by neurodegenerative disorders results in distinct and adverse symptoms. Grafting of embryonic tissue into sites of degeneration proves exciting as such methods aim to treat degeneration both at the source and at a symptomatic level.

1.3.1 AEITIOLOGY AND GENETIC BACKGROUND

Huntington's disease (HD), was first described by George Huntington in 1872 in his landmark article 'On Chorea', published in The Medical and Surgical Reporter (Huntington, 1872). In this paper, Huntington described a form of hereditary chorea where '...one or more of the offspring...' of an effected parent '...almost invariably suffer from the disease...'. Along with a fairly detailed description of the hereditary nature of the disease, Huntington also adequately described the chorea itself, the psychiatric symptoms such as onset of dementia, and the select window within a suffers lifespan for expression of symptoms.

Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder caused by a CAG trinucleotide repeat expansion in the HD gene (The Huntington's Disease Collaborative Research Group, 1993). The gene, and subsequent mutation, responsible for HD was discovered by the Huntington's Disease Collaborative Research Group in 1993.

The HD gene was found to express a protein, now known as huntingtin, with a molecular mass of roughly 350kDa. It is within the N terminal end of this protein that the poly-glutamine tract lies. Normal huntingtin contains between 8 and 36 glutamines within this region, whilst HD suffers contain in excess of 38 glutamines in this region. The poly-glutamine tract of huntingtin is encoded within exon 1 of the Huntingtin gene. The number of poly-glutamine repeats is directly proportional to the number of CAG repeats within the exon. Therefore, HD suffers contain an expanded CAG repeat in excess of 38 repeats. However, there is a degree of instability in the expression of CAG repeats, where repeat lengths are not maintained either between generations ('paternal anticipation' effect (Abe, 1997)) or between cells of the body ('somatic mosaicism').

The huntingtin protein is ubiquitously expressed in all cells of the human body, though highest levels of expression are found within the neurones of the brain (Strong et al., 1993). The protein itself is expressed from early stages of development, where it appears to play a critical role in functional development. Animals that do not express huntingtin die at embryonic day 6-10 (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). By contrast lack of huntingtin in cells grown in culture is non-lethal (Metzler et al., 1999). It has been shown that reduction of normal huntingtin can result in abnormal brain development (O'Kusky et al., 1999; White et al., 1997), though the profile of changes does not replicate that seen in HD.

It is apparent that normal huntingtin plays an essential role in neurogenesis, and a possible protective effect in later life. One possible mechanism for such function is the observation that up regulation of brain-derived neurotrophic factor (BDNF) is associated with normal huntingtin (Zuccato et al., 2001). BDNF is an important neuronal survival factor generated by cortical neurones and released within the striatum (Canals et al., 2001). Normal huntingtin has also been implicated to play a role in a number of cellular and synaptic processes.

1.3.2 PATHOLOGY

Macroscopic investigation of HD brains reveals a characteristic reduction in brain size, corresponding to an approximate 10-20% reduction in brain weight. Neuronal atrophy occurs throughout the cerebral hemispheres, the diencephalon, cerebellum, brainstem and spinal cord. The most apparent pathological feature of the disease is the shrinkage and the marked gross pathology of the caudate nucleus and putamen, with later cortical atrophy. Typically around 57% of the cross sectional area of the caudate nucleus and 65% within the putamen, is observed in post-mortem samples (Rubinsztein, 2002).

Microscopically, HD pathology in the striatum shows a marked neuronal loss and increased gliosis. Interestingly, the MSNs of the striatum are the predominant cell type lost in HD, with the striatal interneurones relatively spared (Bruyn, 1979; Roos et al., 1985; Vonsattel et al., 1985). It is suggested that this selective neuronal loss is a causative factor of the motor and cognitive symptoms observed in HD patients (Albin et al., 1989; Crossman, 1987; DeLong, 1990), and the majority of the experimental models of HD are based on this pathology and

target the MSNs using excitotoxins (DiFiglia, 1990). More recently, transgenic models of HD with expanded poly-glutamine repeats have been introduced as an alternative experimental model (Rubinsztein, 2002; Mangiarini et al., 1996).

One of the main cellular consequences of HD, is the aggregation of mutant huntingtin into intranulcear and extranuclear inclusions (Davies et al., 1997; Davies et al., 1998; DiFiglia et al., 1997). These inclusions indicate that mutant huntingtin is processed via different methods to normal huntingtin. Although no conclusive answer as to how mutant huntintin can cause cell death has yet been given, there is a suggestion that mutant huntingtin can cause proteasome dysfunction (Bence et al., 2001; Jana et al., 2001) and altered gene expression via transcriptional changes (Boutell et al., 1999; Nucifora, Jr. et al., 2001; Steffan et al., 2000). However it is important to note that how inclusions may cause, or be linked with, HD pathology is a matter of debate. Emerging evidence suggests that inclusions may provide a neuroprotective role (Zhai et al., 2005; Gauthier et al., 2004), possibly by 'moping ' up the toxic mutant huntingtin.

1.3.3 MOTOR SYMPTOMS

The most prevalent of symptoms observed in HD is the appearance of involuntary choreiform movements, which provide the original namesake of the disease. Chorea is defined as:

'A state of excessive, spontaneous movements, irregularly timed, randomly distributed and abrupt. Severity may vary from restlessness with mild, intermittent exaggeration of gesture and expression, fidgeting movements of the hands, unstable, dance-like gait to a continuous flow of disabling, violent movements'
(Barbeau et al., 1981)

Chorea is observed in the majority of HD patients, and typically increases in severity during the initial period of disease progression. Interestingly, during the later stages of the disease there appears to be a reversal of motor symptoms towards bradykinesia and rigidity, though this reversal often occurs so late on in the disease progression that it becomes masked by the final stages of life.

The chorea observed in HD patients is present at all times when the patient is awake, and is seen to worsen with increased stress. The movements are totally involuntary, regardless of any conscious effort the patient may make. Although chorea differs between patients, there is a degree of stereotypical behaviour associated with this condition, such that most patients show involuntary facial, neck, thoracic, lumbar and digit movement, all of which contribute to produce the characteristic Huntington's chorea.

1.3.4 COGNITIVE SYMPTOMS

Typically the cognitive symptoms observed in HD patients are attributed to the broad title of "Subcortical dementia". However, the many facets of cognitive symptoms observed in HD patients are well documented. Such symptoms occur early on in the time course of the disease (Butters et al., 1978) and increase in severity with disease progression. These cognitive symptoms are of the frontal-type and were originally attributed to the later cortical pathology observed in HD. However, more recent refinement of cognitive and behavioural tests has led to the acceptance that striatal degeneration and disruption of corticostriatal circuits plays a role in the observed cognitive symptoms.

One of the most marked cognitive symptoms of HD is a deficit in executive function. Executive function is a generic term applied to cognitive functions underlying selection, sequencing and control of plans for action. Within the scope of HD, patients seem to demonstrate a poor ability to plan and judge complex situations, often making judgements based on short term rather than long term goals. Likewise patients demonstrate an impaired ability to self-monitor mistakes, even when such errors are relatively apparent to others. Patients are often noted as being 'rigid' of thought, preferring routine to adaptation of thought.

Studies conducted by (Backman et al., 1997; Harris et al., 1992), have looked at correlating executive function deficits and striatal degeneration. Their findings show a correlation of impaired executive function with striatal atrophy and D1/D2 receptor binding.

1.3.5 LESIONING OF THE RODENT STRIATUM

Early methods of lesioning the rodent striatum relied on ablative, electrolytic or cryogenic techniques. Such lesions presented the undesirable condition where phenotypic effect of the

lesion could not separate destruction of the striatum or fibres that transversed the structure (Laursen 1963).

The introduction of a number of excitotoxins revolutionised the used of striatal lesions to mimic HD pathology. The first descriptive use of such excitotoxins was by Coyle and Schwarcz (1976), who used the glutamate receptor agonist kainic acid (KA) to make selective lesions of the rodent striatum (Coyle and Schwarcz, 1976). They observed a marked atrophy of the striatum with a 90% loss of MSNs and large cholinergic neurones, whilst the myelinated axons of corticothalamic fibres of passage were spared (Coyle and Schwarcz, 1976). Importantly, the close correlation between the pathology observed in lesioned rodents and that of HD patients suggested the KA rodent lesion as a good model of HD. This observation was later ratified by Mason and Fibiger (1979), who demonstrated that bilateral striatal KA lesions in rodents produced locomotor hyperactivity, which they considered as a homologue to human chorea (Mason and Fibiger, 1979).

Since the initial observation that excitotoxins produce HD like pathology, a number of other more selective compounds of the same class have been developed. In the early 80's ibotenic acid (IA) replaced KA as the excitotoxin of choice, due to its lower epileptogenic effects. Subsequently, the toxin quinolinic acid (QA) has become more popular. As compared to previous agents, QA – at appropriate doses - demonstrates a selective degeneration of MSNs, whilst sparing other striatal neuronal types, which better replicates the profile of striatal cellular pathology observed in HD patients.

In addition to the excitatory amino acids, a second group of lesion toxins has become popular for producing rodent models of HD. 'Metabolic' toxins such as 3-nitroproprionic (3-NP) acid reproduce mitochondrial dysfunction, producing pathology by a similar process of cell death to that seen in HD patients (Beal et al., 1993). This compound's popularity is increased by its ability to target the striatum even after peripheral application, preventing the need for complex stereotaxic surgery. Although peripheral application results in reduction of neuronal metabolism throughout the whole brain, there is a selective degeneration of striatal neurones (Beal et al., 1993; Palfi et al., 1998). With the current understanding of the causes of human HD pathology, the 3-NP model of HD represents a favourable replication of the pathology of the disease, though variability in the lesion size whatever the dosing regime make this model

of HD less reliable to assess deficits and recovery whether using behavioural, biochemical or anatomical techniques.

Functional descriptions of excitotoxic striatal lesions have been performed using an array of techniques. The most notable characteristic of QA striatal lesions is the expression of a behavioural phenotype similar to HD, which contains a number of distinct behavioural components.

The most striking behavioural phenotype of excitotoxic lesions is the advent of locomotor hyperactivity. Rodents who have received a striatal lesion demonstrate a higher level of locomotor hyperactivity (Deckel et al., 1983; Isacson et al., 1984), which is observed to peak at night time (Isacson et al., 1986).

Unilateral lesions of the striatum produced motor asymmetries that can be facilitated following injection of apomorphine or amphetamine. Although the rotation phenomenon was has been described in the context of unilateral nigral lesions (Ungerstedt et al 1970), a similar, though less reliable response can be seen following striatal lesions (Schwartz 1979, Dunnett 1982, Dunnett 1988, Klug 1993). It must be noted however that rotational turning behaviour following striatal lesions is not consistent from experiment to experiment and does not correlate well to cell loss (Fricker et al., 1996), in contrasts to that seen following nigral lesions (Ungerstedt and Arbuthnott, 1970; Ungerstedt, 1971a; Ungerstedt, 1971b).

More recently the use of more complex behavioural tests has enabled further characterisation of striatal lesions. Early studies within the nigral lesion model reported a defecit in paw reaching (Hamilton et al., 1985), which was later also reported within the striatal lesion model (Dunnett et al., 1988). Subsequently, the paw reaching test developed by Montoya et al (1990), requires animals to reach for food pellets, with each paw being confined to one side of a staircase apparatus. Unilateral lesions of the striatum disrupt the animals ability to reach for food pellets with the contralateral paw (Montoya et al., 1991).

More advanced behavioural testing has permitted the identification of a number of cognitive deficits following striatal lesions. It was first demonstrated by Divac et al (1967), that selective striatal lesions produced delayed response and alternation deficits in monkeys. Further studies have demonstrated this on a number of different behavioural tasks in rats

(Divac et al., 1978; Dunnett and Iversen, 1981; Dunnett et al., 1999). More complex behavioural testing, using the operant conditioning technique, has further demonstrated that lesioning specific sub-sections of the striatum have differing effects on behavioural performance (Brasted et al., 1999a; Fricker et al., 1996). Whilst striatal lesions do not interfere with an animals ability to perform the basic operant response of lever pressing (Sanberg et al., 1979), animals do display impaired performance on various operant tasks (Brasted et al., 1998; Brasted et al., 1999a; Dobrossy et al., 1995; Dobrossy et al., 1996; Dobrossy and Dunnett, 1997). Furthermore, such deficits are more marked following ventral striatal lesions, than in medial striatal lesions (Brasted et al., 1999a).

1.3.6 GRAFTING IN RODENT MODELS OF HUNTINGTON'S DISEASE

The concept of transplanting neural tissue from one subject to another as a strategy for repair is not a modern one. The first report of neural transplantation was published by Gilson Thompson in 1890, where cortical tissue from adult cats was transplanted into the brain of adult dogs. Though Thompson reported living cells a number of weeks post transplantation, with hindsight it is likely that such cells were most likely inflammatory or glial cells, which may have been of host origin. Nevertheless, the first reports of clearly surviving neurons transplanted into the brains of experimental animals is most likely that of Elizabeth Dunn, reporting on 10 years of studies of grafting cortical tissues between neonatal rats.

Modern graft techniques utilise the dissociated cell suspension method (Bjorklund et al., 1983a; Bjorklund et al., 1983b; Dunnett et al., 1983; Schmidt et al., 1983). Although this method has been refined over the last two decades, the basic protocol has remained constant throughout. For striatal transplants, cells are prepared from the ganglionic eminence (GE) of rodent embryos from gestational age E13-15. This range of embryonic ages are selected as E14 represents the peak period of striatal neurogenesis within the ganglionic eminence (Marchand and Lajoie, 1986). Following removal, the WGE is subjected to enzymatic digestion, and mechanical dissociation to produce a cell suspension that can be injected into the host brain.

The advantages of the cell suspension technique are that these injections rely on a thin injection needle which results in very little surgical damage to the host brain (Bjorklund et al., 1983a; Nikkhah et al., 1994). Grafts can be placed in multiple sites, using multiple deposits of either the same or different tissue. Furthermore, the use of a cellular suspension means that the grafted cells require no special manipulation to ensure vascularisation. Therefore, it is possible to implant cell suspensions into deep regions of the host brain, with little surgical damage and no need for extensive vascular support.

1.3.7 ANATOMICAL CHARACTERISTICS OF STRIATAL GRAFTS

One of the most striking characteristics of striatal grafts is that they form a mosaic patch like appearance which was initially considered to reflect the patches and striosomes of the normal striatum (Graybiel et al., 1989; Isacson et al., 1985). However, it turns out that the patch zones ('P-Zones'), which comprise roughly 30-50% of the total graft volume, contain cell types with a morphology resembling all adult striatal neurones (Clarke et al., 1988a; Helm et al., 1990; McAllister et al., 1995). Furthermore, neurones within the P zones stain positive for DARPP-32 (Wictorin, 1992) and AChE (Graybiel et al., 1989) and a variety of other markers of striatal specific neurones (Graybiel et al., 1990).

The remaining 50-70% of the graft volume are termed non-patch zones ('NP-Zones'). NP zones are characterised by not staining positive for either DARPP-32 (Wictorin, 1992) or AChE (Graybiel et al., 1989). Cells within NP zones display morphology consistent with the adult pallidum, cortex and other non-striatal cell types (DiFiglia et al., 1988; Isacson et al., 1985). Studies have shown that the cells within this region come from the transplanted embryonic cellular suspension (Graybiel et al., 1989; Wictorin, 1992). Therefore the existence of cells of a non-striatal phenotype in the NP zones has been attributed to the fact that both striatal and non-striatal cells originate from the common germinal zone primordium, and cannot be differentially dissected based on regional cues alone. Moreover, it has not proved possible to sort cells of different fates so early on in their development, so that inclusion of cells of a non-striatal phenotype is inevitable within the transplanted cellular suspension.

It has been suggested that graft suspensions prepared from lateral ganglionic eminence (LGE), yeild a much higher proportion of striatal tissue within the graft (Pakzaban et al., 1993). In such cases, it has been reported that up to 90% of the graft volume is occupied by P-zones (Pakzaban et al., 1993). However, whilst it is true that the LGE grafts yield a higher proportion of medium spiny neurons in the graft than the whole ganglionic eminence (WGE), this dissection excludes many of the precursors of interneurons, which originate in the medial ganglionic eminence (MGE) (Olsson et al., 1998).

Embryonic striatal grafts have been shown to undergo a certain amount of anatomically assessed reconnection with the host circuitry. A number of studies have demonstrated that for a certain period of time post-transplantation, afferent fibres begin to innervate the graft region (Pritzel et al., 1986; Wictorin, 1992). Although such transplants receive afferent innervation from nearly all of the input phenotypes, there is a notable variation in the type and density of innervation. Cortical and thalamic inputs appear initially to innervate the outer edge of the graft densely, with a significantly lower innervation of the core regions of the graft Wictorin (1998). Although over time the fibre ingrowth by such efferents increases, it fails to reach the level observed in the intact striatum (Xu et al., 1989). The dopaminergic (DA) afferents from the substantia nigra (SN) appear to form patchy innervation of the transplant (Pritzel et al., 1986), innervating the striatal-like "P-Zones" compartment selectively (Graybiel et al., 1989), with similar density of innervation to that seen in the normal striatum as the transplant matures (Wictorin et al., 1989). Finally, the serotonergic innervation from the mesencephalic raphe appears to innervate the whole transplant, and like DA input from the SN, eventually reaches similar densities to that observed in the intact striatum (Wictorin, 1992).

Ultrastructural studies have demonstrated that afferent projections form synaptic contacts with transplanted neurones, within striatal grafts (Clarke and Dunnett, 1993; Wictorin et al., 1989; Xu et al., 1989; Xu et al., 1991a). Whilst it has been shown that the various afferent fibres form synaptic contacts with transplanted MSNs, not all of these synapses are located on the correct region of the post-synaptic neurones. In a study conducted by Xu et al. (1992) labelled cortical inputs only form synapses with the spines of MSNs roughly 50% of the time, whereas in the normal striatum over 90% of these contacts are found on the spines. A similar disparity was found with inputs from the thalamus (Xu et al., 1991a). It must be noted however that in a similar study, Wictorin et al (1989), found that roughly 87% of corticostriatal fibres form synaptic contacts on the heads of spines.

The concept of efferent projections from striatal grafts to the host brain was first postulated based on evidence gained from biochemical studies. Following excitotoxic lesions, the levels of glutamate decarboxylase (GAD) in the SN and GP are greatly reduced (Isacson et al., 1985). Following transplantation, the levels of GAD in both the SN and GP increase to 90% of that seen in the normal adult brain (Isacson et al., 1985). A later study demonstrated a similar result in both the SN and GP, though this time using in vivo push-pull perfusion (Sirinathsinghji et al., 1988). Efferent fibres projecting from the graft to the GP were first demonstrated by Pritzel et al (1986), although the frequency of this observation was reported as being quite rare. Later anatomical studies, using longer survival times and more sensitive pathway tracing methods, have demonstrated that striatal grafts send out projections to the adjacent GP and to the SN, and form antomically correct synapses (Wictorin et al., 1989; Wictorin and Bjorklund, 1989; Wictorin, 1992).

1.3.8 FUNCTIONAL ASSESSMENT OF STRIATAL GRAFTS

Studies by Deckel et al (1983), and Isacson et al (1984), were the first to report a positive functional effect of striatal grafts. These studies demonstrated the motor hyperactivity seen post lesion, is significantly reduced following transplantation. Further studies have corroborated this observation (Deckel et al., 1986a; Deckel et al., 1986b; Deckel et al., 1988a; Deckel et al., 1988b; Isacson et al., 1985; Isacson et al., 1986; Sanberg et al., 1986), whilst also demonstrating that transplantation of striatal tissues into the GP (Isacson et al., 1986) or using control tissues implanted into the striatum (Sanberg et al., 1986) has no positive effect. Moreover, lesioning of the graft reverses the motor improvement (Sanberg et al., 1986).

As previously described, unilateral lesions of the striatum produce a rotational like behaviour when stimulated by either apomorphine or amphetamine. Following transplantation the degree of both spontaneous rotation (Deckel et al., 1988a; Deckel et al., 1988b) and stimulated rotation (Dunnett et al., 1988; Fricker et al., 1996; Isacson et al., 1984; Labandeira-Garcia et al., 1995; Norman et al., 1989) is reduced. Again, rotational behaviour is not seen to be alleviated following transplantation of cortical tissue (Labandeira-Garcia et al., 1995) astrocytes, or P zone poor MGE tissue (Nakao et al., 1998).

As previously described, unilateral lesions of the striatum disrupt the animals ability to perform in the paw reaching tests, and grafts are seen also to alleviate performance of more complex motor tasks (Dunnett et al., 1988; Fricker et al., 1997; Montoya et al., 1990). Graft induced recovery is only seen following transplantation of WGE cellular suspensions, and not following transplantation of nigral cellular suspensions, or cellular suspensions prepared from the MGE (Montoya et al., 1990). Furthermore, the degree of recovery has been shown to correlate with DARPP-32 P zone volume (Nakao et al., 1996). Therefore, it would seem that recovery of skilled motor performance requires functional reconstruction of striatal circuitry.

Graft induced functional recovery of behavioural performance is not confined to the recovery of motor deficits. Studies involving a delayed alternation task in the T-maze have demonstrated that grafts improve acquisition and retention of the task (Deckel et al., 1986b; Deckel et al., 1988b; Isacson et al., 1986). More precise behavioural analysis using operant testing demonstrate a similar recovery on various operant tasks involving delayed response and stimulus-response (S-R) associative learning (Brasted et al., 1999b; Dobrossy and Dunnett, 1998; Mayer et al., 1992). Therefore, transplanted tissue not only restores the ability to perform motor tasks, but also provides a substrate for the re-learning of motor skills and habits, lost post-lesion.

The concept of "learning to use the graft" was first described by Coffey, et al (1989), within the context of retinotectal transplantation. Within the striatal graft environment, "learning to use the graft" was first investigated by Mayer et al (1992), and later expanded on by Brasted et al (1999). Both studies utilised the Carli S-R task (Carli et al., 1985), to explore the learning and retention of motor skills and habits. Unilateral excitotoxic lesions of the striatum disrupt the initiation of responses on the contralateral side, without affecting the ability to respond to the eliciting stimulus (Brasted et al., 1997; Carli et al., 1985). Interestingly, following transplantation, S-R performance remains similarly impaired, with no improvement compared to that seen post-lesion, when tested 4 (Brasted et al., 1999b) or 6 (Mayer et al., 1992) months post-surgery. However, further training on the task, whilst lesioned animals display no significant improvement over time, grafted animals can now relearn to perform the S-R task (Brasted et al., 1999b; Mayer et al., 1992). Therefore, it seems essential that grafts not only integrate with the host circuitry to form anatomically appropriate connections, but that the transplant itself is retrained on the task. Interestingly, the ability to relearn skills and habits lost post-lesion occurs over a similar time period to that required by normal animals to

learn the task initially. It has therefore been theorised that the transplant itself contains the substrates for learning of motor memory, seen within the normal organism.

1.4 PLAN OF THESIS

Embryonic striatal grafts connect with host striatal circuitry, receiving afferent projection from the host cortex, thalamus and SN, and projecting anatomically correct efferent connections to host GP. Previous neurochemical analysis has demonstrated that grafts not only connect appropriately with the host, but are able to influence host neuronal function, restoring the neurochemical balance in target structures. Restoration of host cortico-striatal-pallidal circuits is believed to provide a mechanism for the restoration of motor performance observed post-transplantation.

Interestingly, the phenomenon 'learning to use the graft' demonstrates that striatal grafts are not only capable of restoring motor performance, but that the graft itself possibly provides the substrate for the ability to re-learn motor skills and habits lost post-lesion. The specific question this thesis aims to answer is; does this form of 'new learning' use similar physiological mechanisms to that seen in the normal striatum?

Learning and memory within the mammalian nervous system has been extensively studied for many years, with many theories emerging regarding the cellular mechanisms underlying it. To date, synaptic plasticity is considered as the most appropriate model of learning and memory within the mammalian nervous system. Therefore, the major theme to be investigated within the context of this thesis is: Do striatal grafts display synaptic plasticity, and does such plasticity mimic that seen in the normal striatum?

Firstly, transplanted striatal cells have been show to be physiologically active to host stimulation, suggesting that transplanted cells reconnect with the host circuitry to form functional host-graft synapses, capable of transducing afferent stimulation into suitable post-synaptic responses. However, whether this activity includes restoration of tonic baseline transmission to the lesioned striatum is, as of yet, not known. Furthermore, whilst it is known that striatal grafts express a variety of neurotransmitters seen within the normal striatum, limited evidence exists to demonstrate that striatal grafts display pharmacological

mechanisms of transmission in keeping with the normal striatum. Experiments presented within this thesis will aim to demonstrate that striatal grafts restore baseline transmission, and display pharmacological mechanisms of neurotransmission in keeping with the normal striatum.

Secondly, the phenomenon of 'learning to use the graft' suggested that striatal grafts display cellular mechanisms of learning and memory, consistent with that seen in the normal striatum. Therefore, it is hypothesised that striatal grafts contain the ability to express synaptic plasticity, which is considered the most appropriate model of learning and memory in the nervous system. Experiments presented within this thesis will aim to demonstrate that striatal grafts express synaptic plasticity in keeping with the normal striatum.

Thirdly, synaptic plasticity is critically dependant on the interplay of a number of neurotransmitter systems. Within the mammalian nervous system the variety of neurotransmitter expression across a number of brain structures provides distinct pharmacological mechanisms of synaptic plasticity to specific structures. Therefore, experiments presented in this thesis will attempt to investigate the pharmacological mechanisms of graft synaptic plasticity.

Finally, the mammalian brain is at its most plastic during development and following injury. Previous experiments have demonstrated that subtle changes in the environment can have a functional effect on the adult brain, which is exacerbated following injury. Furthermore, changes in environment have been shown to have a significant effect on graft tissue, improving motor recovery, biochemical, and morphological characteristics associated with graft function. Experiments presented within this thesis will aim to investigate the effect of environmental enrichment on graft synaptic plasticity.

2. GENERAL METHODS

2.1 ANIMALS

Male C57/BL6 mice (Harlan, U.K.), were used as control animals and hosts for transplantation. All animals were housed 6 per cage in a temperature (21°C) and humidity (50%) controlled room, with a 12h:12h light cycle (lights on during the day). All animals were give free access to food *Ad libitum*. All experiments were undertaken in accordance with personal and project licenses issued under the UK Animals (Scientific Procedures) Act 1986.

For graft experiments all donor tissue was collected from the PrP-L-EGFP-L transgenic mouse (Feil et al., 1996). This transgenic mouse exhibits endogenous expression of green fluorescent protein (GFP) in all cells (Feil et al., 1996).

2.2 SURGERY

For all surgical techniques mice were anaesthetised in an induction chamber by inhalation anaesthetic (Isoflurane, Abbott, UK), using O_2 and NO as carrier gases, and placed in a stereotaxic frame (Kopf Instruments). The animals were kept anaesthetised throughout the entire surgical procedure. Prior to regaining consciousness, animals received 5 ml glucose saline s.c. in the neck and 0.15 ml diazepam i.m. in the hind leg. A 500 mg paracetamol tablet was dissolved in 1 litre of the drinking water for 48 hours post surgery.

2.2.1 LESIONING OF THE HOST STRIATUM

Unilateral dorsal striatum lesions were made by injecting 2 x 0.24ml of 0.12M of the excitotoxin quinolinic acid (Cambridge Research Biochemicals), dissolved in 0.1M phosphate buffered saline (pH = 7.4), at two depths within the same needle track in the left neostriatum. Each injection was administered via a 30 gauge stainless steel cannula connected to a microdrive pump, permitting a further 2 min for diffusion. The injection co-ordinates were: A= +1.0, L = +1.8, V = 3.1 and 2.5 with measurements in mm anterior (A) in front of bregma, lateral (L) to the midline, vertical (V) below dura, and the nose bar set at the same horizontal plane as the interaural line. Please note the two measurements given for vertical positioning refer to the dorsal movement of the cannula for the second injection site.

2.2.2 HARVESTING OF EMBRYOS AND GRAFT TISSUE

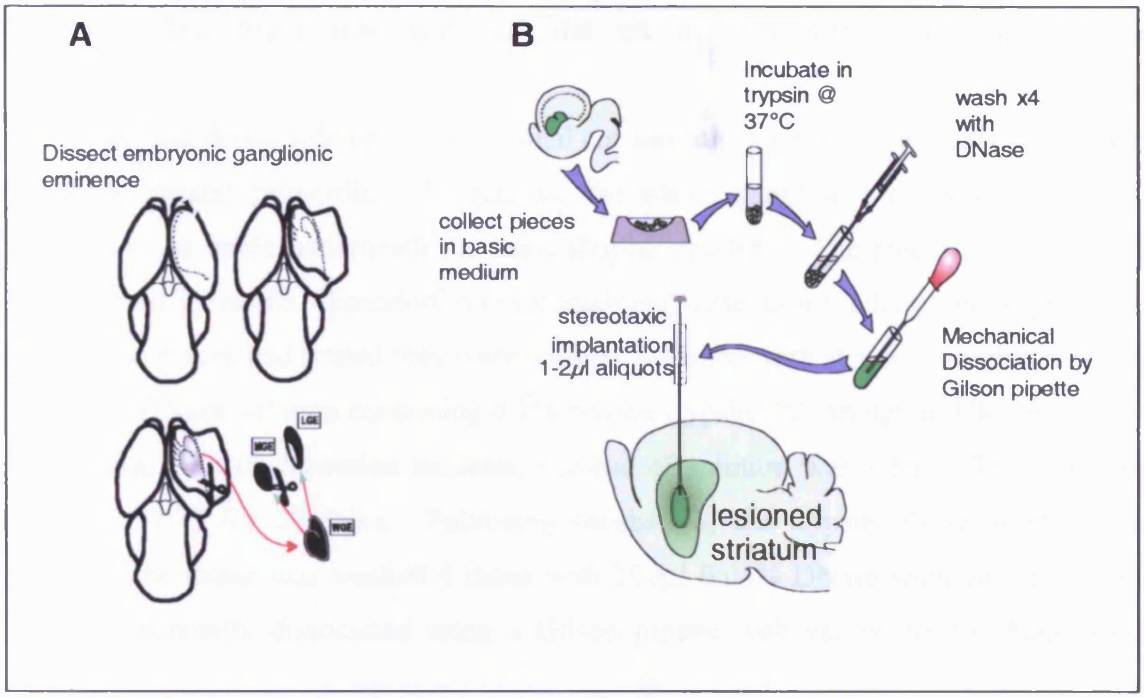

Figure 2.1 Collection and Preparation of Graft Suspension. (A) Diagrammatic representation of WGE dissection from the developing embryonic brain. (B) Schematic of the steps taken to produce a cellular suspension from the dissected WGE tissue. Schematic and diagram modified from (Dunnett et al., 2000). WGE=Whole Ganglionic Emminence, MGE=Medial Ganglionic Emminence, LGE=Lateral Ganglionic Emminence.

Time mated female mice, with embryos at E13 on the day of grafting were killed via terminal anaesthesia and decapitation. The abdomen was shaved, then sterilised with betadine followed by 70% alcohol. The peritoneum was opened using a vertical midline incision of first the skin then the fascia of the abdomen. Each uterine horn was removed in turn, being careful not to touch the uterine horn against the mothers skin. These horns were then placed in 45ml dissection medium containing 95.5% 1x Dulbecco's modified Eagle medium (DMEM), 2.2% Glucose (30%), 1.6% $NaHCO_3$ (7.5%), and 0.5% HEPES (1M).

The uterine horns were transferred to a sterile Petri dish under sterile conditions (dissection hood). Embryos were removed one at a time from the uterine horn and placed on their side in a second sterile Petri dish containing dissection medium. Measurements of crown-rump length were taken, as a method of confirming gestational age of the embryo. The embryos

were then killed via decapitation. The brains were dissected out under a dissecting microscope. The brain was laid out flat on a Petri dish surrounded by ice.

With the brain laid dorsal side up, a longitudinal cut was made across the medial dorsal cortex to reveal the striatal primordia. To remove the whole ganglionic eminence (WGE), a horizontal cut was made underneath the heart shaped structure. The pieces of WGE tissue were transferred to sterile Eppendorf tubes containing dissection medium and kept on ice. Once the tissue pieces had settled they were washed 3-4 times with sterile dissection medium, then a trypsin/DNase soltuion containing 0.1% bovine trypsin (Worthington, UK) and 0.05% DNase (Sigma, UK) in dissection medium, volume of solution was 1.5ml. This was then incubated at 37^{O}C for 20 mins. Following incubation, the trypsin/DNase solution was removed and the tissue was washed 4 times with 200µl 0.05% DNase solution. The tissue was then mechanically dissociated using a Gilson pipette with yellow tip to obtain a cell suspension.

Cell numbers and viability were calculated by the trypan blue dye exclusion method using a haemocytometer. Cell suspensions with 95% or over viability were resuspended in DNase solution and used for the transplant procedure.

2.2.3 TRANSPLANT PROCEDURE

Seven days post-lesion the mice were brought back to surgery for transplantation of embryonic striatal cells. The cells were harvested and prepared on the day of transplantation.

Mice were anaesthetised with inhalation anaesthetic, and placed in a stereotaxic frame (same methods, compounds, and equipment as the lesion surgery). Each animal received 2 x 1µl aliquots of graft suspension (approximately 200,000 cells per µl, 400,000 cells per graft), at the two lesion injection sites A= +1, L= +1.8, V= 3.1/2.5 and I.O = 0.0.

2.3 ELECTROPHYSIOLOGY

2.3.1 SLICE PREPARATION

Mice were killed via cervical dislocation and decapitated. The brain was quickly removed and placed into ice-cold artificial cerebrospinal fluid (aCSF – composition 126 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1mM $NaHPO_4$, 26 mM $NaHCO_3$ and 11 mM D-glucose) which was constantly bubbled with 95%O_2/5%CO_2 to maintain the pH at 7.4. During dissection and subsequent slicing this solution also contained 1mM Kynurenic acid (Sigma Aldrige, UK), a broad spectrum glutamate antagonist, to protect against excitotoxicity during slicing. Sagital sections of 300μm thickness were cut on a vibratome (Leica., GM). They were then transferred to a holding chamber containing room temperature aCSF (Same composition as above), and incubated for at least 1 hour prior to recording. To perform extracellular recording, slices were transferred to a submersion recording chamber. During the equilibration period and remainder of the experiment slices were perfused with aCSF at $4mlmin^{-1}$ at a temperature of $31 \pm 0.5^{O}C$. Slices were left in the chamber for 5-10 minutes to equilibrate before electrode placement.

2.3.2 ELECTRODE PLACEMENT

For all experiments slices were viewed under 50x magnification by a microscope (Olympus, UK). Grafts neurones from GFP embryonic tissue were visualised using UV light, under the same magnification (see Fig 2.2B).

For all experiments stainless steel monopolar stimulating electrodes, with a tip resistance of 0.60 MΩ (Intracel, UK), were placed in the host corpus callosum 1-2 mm away from the graft site (see Fig 2.2A). Recording electrodes were produced from boroscilicate glass capillaries (Harvard Apparatus, UK), with an external diameter of 1.5mm and an internal diameter of 0.86mm. Glass recording electrodes were pulled using a motorised micropipette puller (Sutter Instrument Co., USA), to produce finely pulled glass electrodes, and filled with 1M KCl. The glass pulled recording electrode was placed within the core of the graft region (region of dense GFP possitve cells) in a ventral location to the stimulating electrode. For control experiment where graft tissue was not present, the recording electrode was placed in a ventral position to the stimulating electrode, in the same commonly observed region for graft sites.

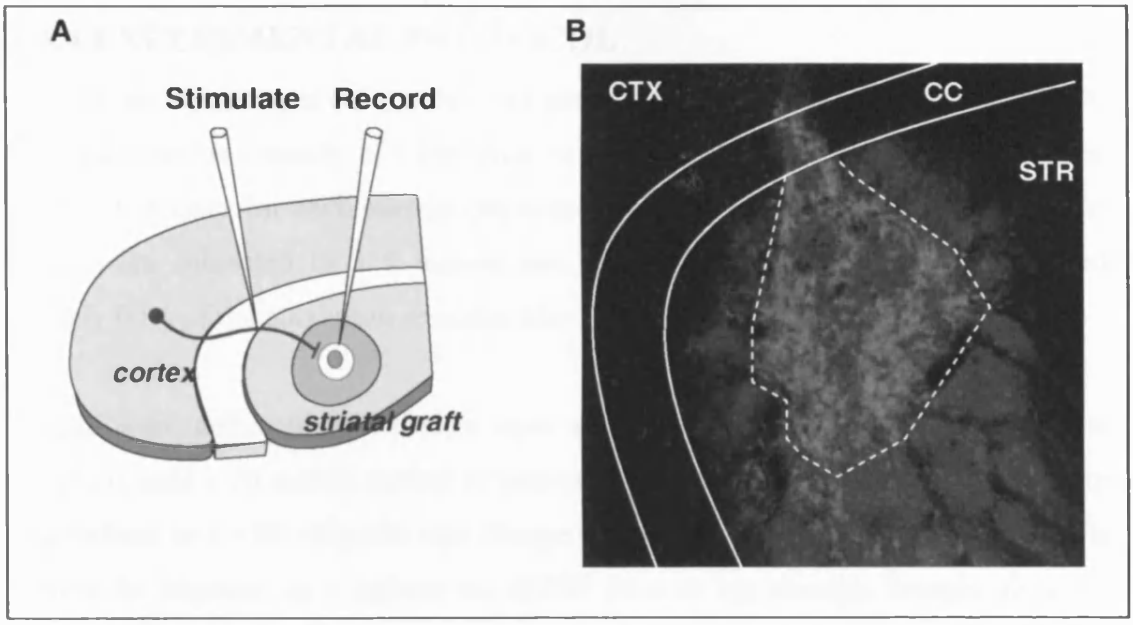

Figure 2.2 The Corticostriatal *'in vitro'* graft preparation. (A) Schematical representation of electrode positions within the striatal graft *in vitro* slice preparation. (B) Photomicrograph demonstrating the endogenous GFP fluorescence of the transplanted tissue within the striatum. CTX = Cortex, CC = Corpus Callosum, STR = Striatum.

2.3.3 STIMULUS AND RECORDING

In all case, stimuli patterns were generated using a Master 8 pulse generator (Intracel, UK) and a Neurolog stimulus isolator (Digitimer, UK). Responses were amplified using a differential amplifier (Warner Instrument Corp., USA), and Axoclamp (Axon Instruments, UK). Responses were monitored and stored for off-line analysis using custom software written in LabView (National Instruments, USA) running on a Macintosh G4 computer.

In all experiments, the initial slope of the fEPSP was used a measure of synaptic strength (Anderson et al, 1977). Once a response had been gained, the slice was submitted to a 30 min rest period, where the slice was stimulated every 5-10 minutes, until the fEPSP slope became stabilised.

2.3.4 EXPERIMENTAL PROTOCOL

Initially, an input/output (I/O) curve was performed. The fEPSP slope was measured across a stimulus intensity of 10-100mA, in steps of 10mA. Four stimuli were given every 10 seconds for each current intensity. Following I/O curve measurement, the slices were subjected to a 5 minute rest period, before an intensity that evoked roughly 60% of the maximum response size was selected.

Stimuli were delivered to the slice once every 20sec (square wave pulses, 0.2ms duration) until a 20 minute period of response stability was seen. Response stability was defined as a >5% response size change over the 20 minute period. This period is termed the baseline; as it reflects the fEPSP prior to any changes brought about by tetanus or drug application.

Attempts to produce plasticity within both grafted and control tissue were made via high frequency stimulation, termed tetanus. Theta-burst stimulation (TBS) comprising of 6 trains, each train consisting of 10 bursts at 5Hz and each burst consisting of 4 pulses at 100Hz, with a pulse width of 0.4ms and an inter-train interval of 20s. Following tetanus, slices were stimulated at baseline levels (0.05Hz), and recording continued for a further 65 minutes.

2.3.5 DATA ANALYSIS AND STATISTICS

Data was analysed off-line. All data were normalised to a baseline level, which was defined as the average response size over the 20 minutes of baseline stimulation. These normalised values were expressed as a percentage of the baseline response (Baseline = 100%) and averaged into 2 minute bins. Results are presented as the mean result gained ± the standard error of the mean (SEM).

Multi-factorial analyses of variance were conducted on the data as appropriate, using the STATISCTICA statistical package (StatSoft, USA).

2.4 HISTOLOGY

2.4.1 PERFUSION AND PREPARATION

Following terminal anaesthesia with sodium pentobarbatone (1ml per Kg), each mouse was perfused transcardially with phosphate-buffered saline (PBS; pH 7.4) for 1 min, followed by 4% paraformaldehyde in 0.1M PBS over 2 minutes.

Brains were removed, post-fixed in 4% paraformaldehyde for a further 3 hours and then immersed in 30% sucrose in PBS, until they sank (~24 hours).

2.4.2 SECTIONING

The tissue was serially sectioned at 50μm on a freezing microtome. The sections were stained either in a free floating or in a mounted state. (See Appendix 1 for detailed protocols)

2.4.3 STAINING

2.4.3.1 Immunohistochemistry

For immunohistological staining all sections were stained free floating in groups of roughly 10 sections per 'Grenier pot' and kept on an orbital shaker at all times.

Sections were initially subjected to a 5 minute quench period (See appendix 1 for solution details), before being washed in TBS three times for ten minutes each period. An initial blocking step, whereby all sections were incubated at room temperature in a 3% concentration of 'normal' serum made up in TXTBS. 'Normal' serum corresponds to the species of animal the secondary antibody was raised in. Following the blocking step all section were transferred, without washing, to a solution containing the primary antibody (See Table 2.1 for concentrations and suppliers), 1% 'normal' serum, with the final concentration made up in TXTBS. Sections were incubated for a period of 48 hours at 4^0C.

Once primary antibody incubation had been completed, sections were submitted to a second 3x10 minute TBS wash, before being subjected to a two hour incubation with

a IgG conjugated secondary antibody (See Table 1 for concentrations and suppliers). Once secondary antibody incubation had been completed, sections were submitted to a third 3x10 minute TBS wash, before being treated with a DAKO streptavidin kit for two hours (DAKO, Denmark). Sections were then subjected to a further two separate washes, first with TBS (3x10 minutes), then with TNS (2x5 minutes). Following this all sections were incubated in DAB at room temperature for revelation of antibody reaction.

Stained free floating sections were serially mounted on gelatinised slides, air dried overnight, and dehydrated via an ascending series of alcohol concentrations, finishing in a 5 minute xylene incubation. Mounted dehydrated sections were rapidly coverslipped with DPX.

Primary Antibody	Raised In	Dilution	Block Serum	Secondary Antibody	Diluation	Supplier
Anti-TH	Mouse	1:1000	Horse	Horse Anti-Mouse	1:200	Chemicon UK
Anti-GFP	Rabbit	1:1000	Goat	Goat Anti-rabbit	1:200	Chemicon UK
Anti-D32	Mouse	1:30000	Horse	Horse Anti-Mouse	1:200	Cornell Uni USA

Table 2.1 Primary and Secondary antibody Details. The above table demonstrates the dilutions and suppliers for all antibodies used during immunhistochemical experiments.

2.5 Common Post-Mortem Histological Analysis of Graft Survival Within the *In Vitro* Slice

For all the experimental groups presented within this thesis, a sample of the experimental population was processed for post-mortem histological analysis. Routing histological staining for GFP, and DARPP-32 was performed in order to clearly demonstrate that transplanted embryonic GFP positive WGE tissue survives transplantation, and demonstrates similar histological properties to that seen with embryonic WGE tissue from the normal striatum.

The following histological images provide examples of the results from the routine histological analysis.

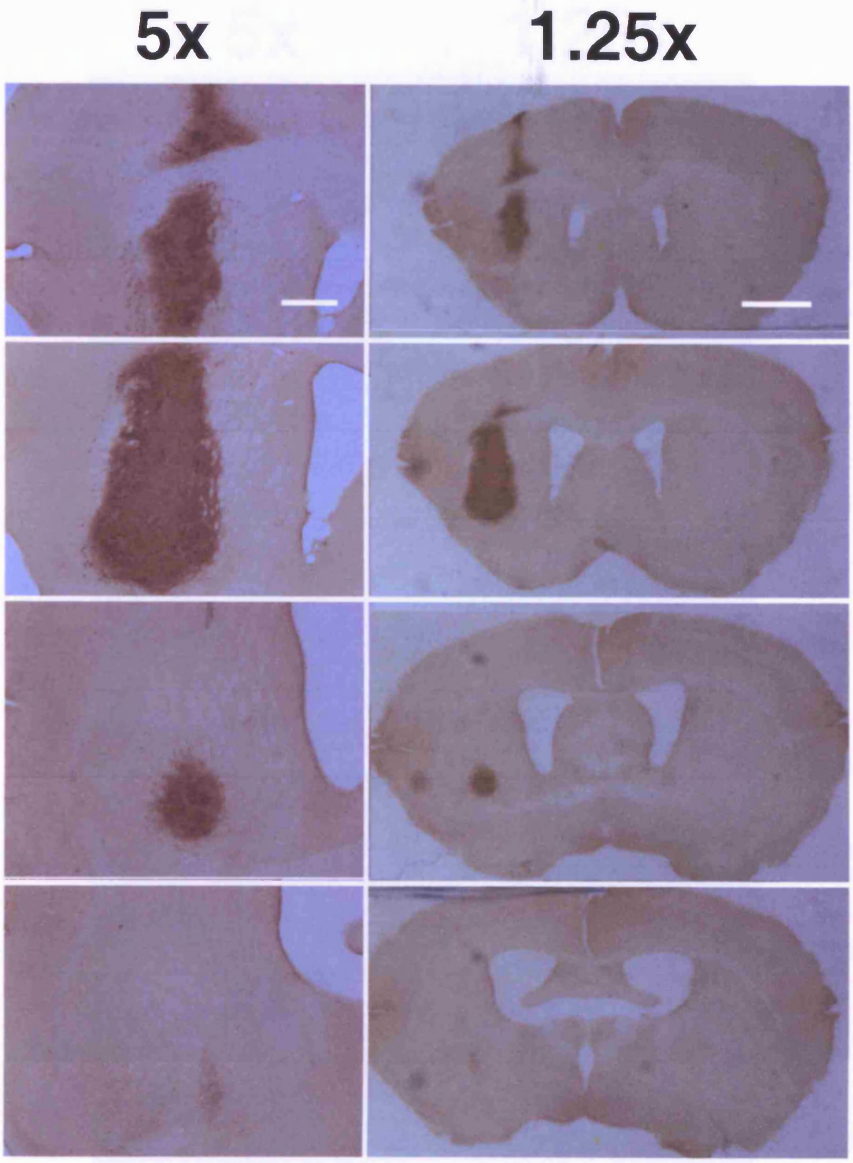

Fig 2.3 Immunohistochemical Staining For GFP. The above photomicrographs demonstrate immunohistochemical staining for GFP in post-mortem sections from E13 GFP WGE grafted animals. Scale bar = 4mm (5x) =1mm (1.25x)

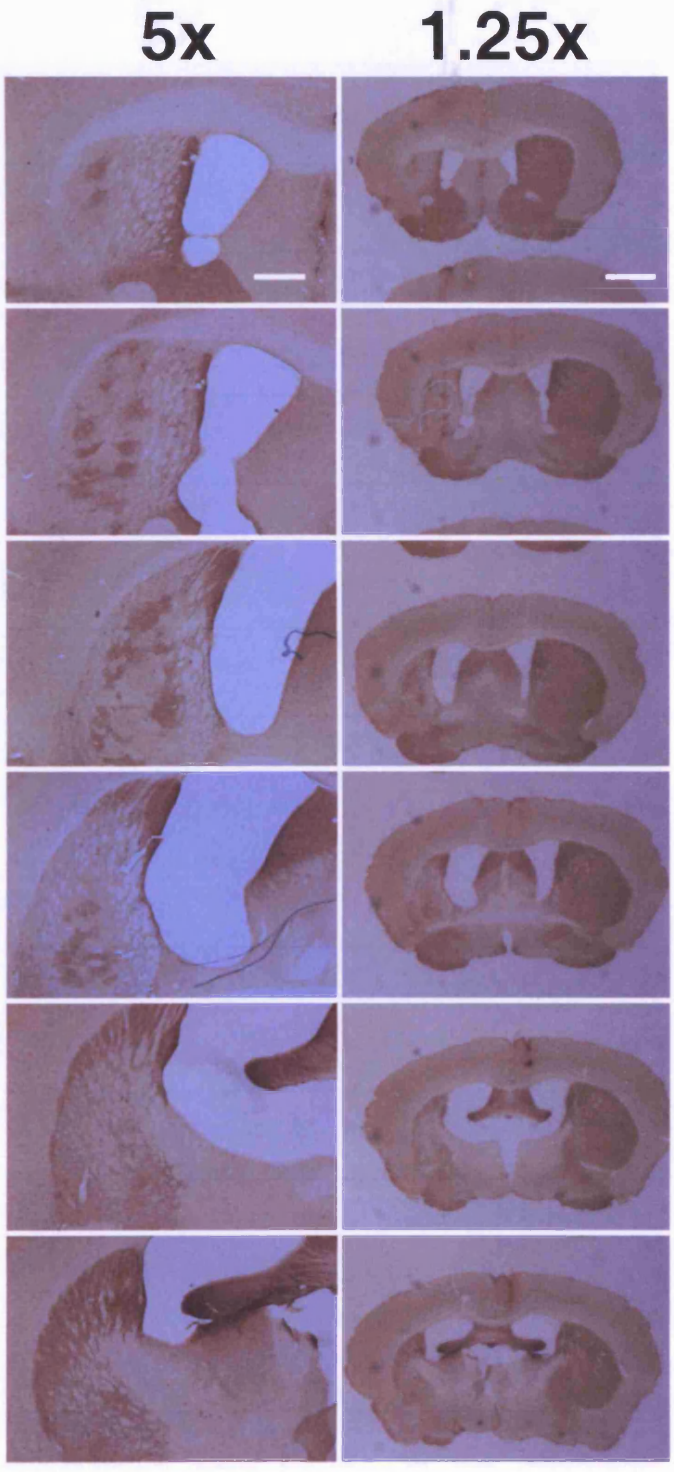

Fig 2.4 Immunohistochemical Staining For DARPP-32. The above photomicrographs demonstrate immunohistochemical staining for DARPP-32 in post-mortem sections from E13 GFP WGE grafted animals. Scale bar = 4mm (5x) = 1mm (1.25x)

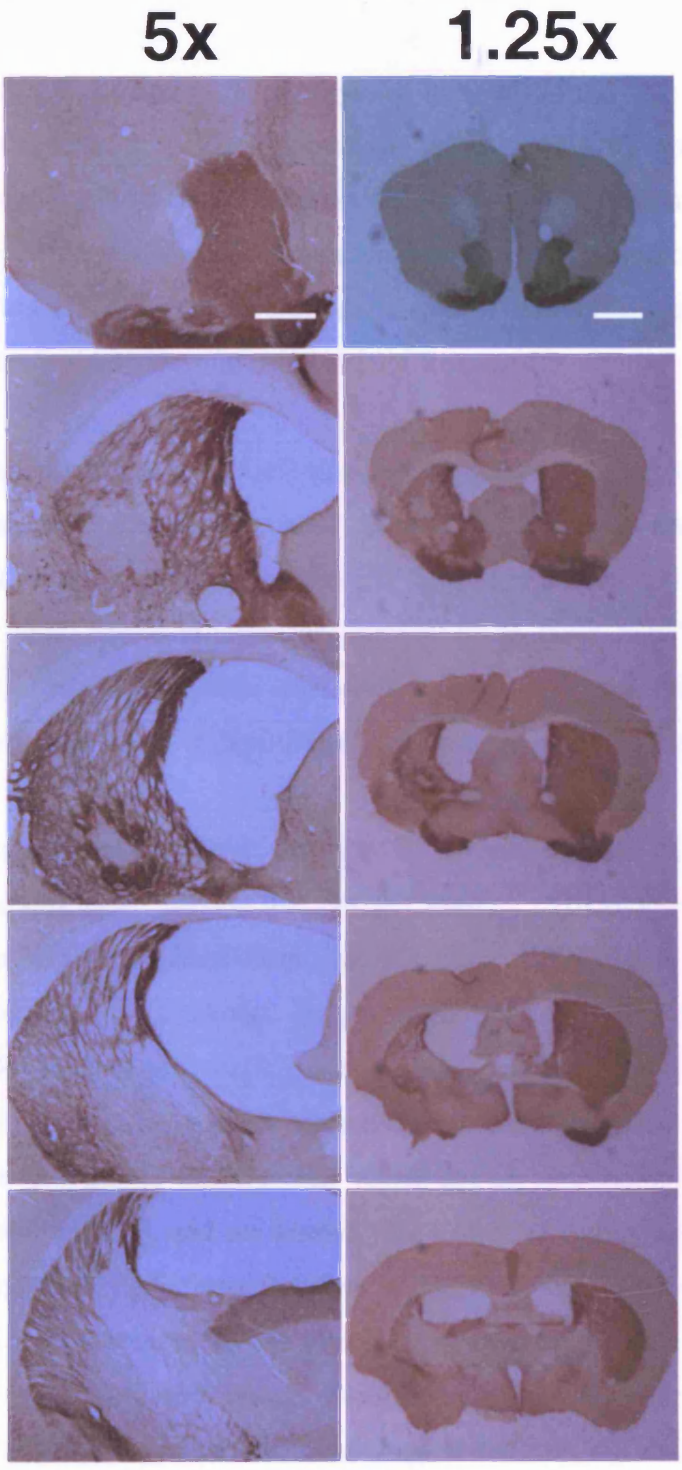

Fig 2.5 Immunohistochemical Staining For TH. The above photomicrographs demonstrate immunohistochemical staining for TH in post-mortem sections from E13 GFP WGE grafted animals. Scale bar = 4mm (5x) =1mm (1.25x)

2.5.1 Discussion of Post-Mortem Anatomical Studies

Previous histological analysis of embryonic striatal grafts has revealed that transplanted striatal tissue not only survives and develops post transplantation, but is also capable of forming functional synapses with the host striatal circuitry. Within the context of this thesis, it is essential to demonstrate that transplantation of embryonic striatal tissue, from GFP transgenic animals, displays anatomically similar grafts to those seen from transplantation of normal striatal tissue.

Immunohistochemical staining for GFP demonstrates that all transplanted cells stain possitive, whilst cells from the host striatum show no immunoreactivity. Furthermore, within there are regions of dense GFP immunoreactivity, which when examined under high power microscopy correspond to large bundles of dendritic fibres. Therefore, transplanted embryonic GFP positive cells not only survive post-transplantation but also display a large degree of dendritic branching and arborisation.

Immunohistochemical staining for DARPP-32 reveals regions of positive and negative immunoreactivity with GFP WGE grafts. Histological analysis of embryonic striatal grafts prepared from normal striatal WGE tissue display similar DARPP-32 immunoreactive patterns. As previously described, patches which stain positive for DARPP-32 display cells which appear morphologically similar to the MSNs of the normal striatum, and are termed 'P-zones'. Patches of the graft which stain negative for DARPP-32 contain cells which appear morphologically similar to host cortical or pallidal cells, and are termed 'NP-Zones'. Within the context of this thesis, it was impossible to distinguish between 'P-zones' and 'NP-zones' within the in vitro graft slice preparation, as all transplanted cells, regardless of phenotype, display endogenous GFP fluorescence. Though steps were taken to recover slices post-recording, and perform post-mortem histological analysis, such attempts failed to yield any suitable histological findings. Therefore, all electrophysiological recordings performed within this thesis are undertaken without the ability to distinguish between 'P-zones' and 'NP-zones', though it must be noted that all recordings were performed within the GFP positive graft region.

Finally immunohistochemical staining for TH demonstrates that whilst the lesioned striatum demonstrates positive immunoreactivity for TH, the graft region is mainly devoid of any dopaminergic input from the SN. Interestingly there are dense patches of TH immunoreactivity surrounding the graft region and the presence of a small number of TH immunoreactive fibres within the graft.

3. 'FUNCTIONAL INTEGRATION' OF EMBRYONIC STRIATAL GRAFTS

3.1 INTRODUCTION

Embryonic striatal grafts have been shown to receive efferent projections from the host prefrontal cortex, thalamus, SN, and RN, forming anatomically correct synapses (Clarke et al., 1988b; Clarke et al., 1988a; Clarke and Dunnett, 1993; Wictorin et al., 1988b; Wictorin et al., 1989; Wictorin, 1992). Furthermore, embryonic striatal grafts project fibres to the host GP and SN (Wictorin et al., 1988a; Wictorin et al., 1988b; Wictorin et al., 1989; Wictorin and Bjorklund, 1989; Wictorin, 1992), where they form anatomically correct synapses (Wictorin et al., 1988a; Wictorin and Bjorklund, 1989; Wictorin, 1992), capable of influencing neurotransmission (Sirinathsinghji et al., 1988). Therefore, it would seem that embryonic striatal grafts are capable of integrating with the host striatal circuitry (Dunnett, 1994). Furthermore, such integration has been shown to include a number of correlates of neuronal function (Dunnett, 1994), including restoration of neurotransmission (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b; Wilson et al., 1990; Xu et al., 1991b).

Only a handful of studies exist in which electrophysiological recordings have been made from embryonic striatal grafts. Rutherford et al (1987) were the first to report that transplanted embryonic striatal neurons (TSNs) are physiologically active post-transplantation. Using the *in vitro* slice preparation, they reported that following stimulation of the host cortex, it was possible to detect EPSPs from TSNs (Rutherford et al., 1987). Although EPSPs from TSNs resembled those from MSNs of the normal striatum, the authors noted that on average EPSPs from TSNs appeared smaller than those from MSNs. Later studies conducted *in vivo* demonstrate that TSNs are also responsive to stimulation of the host thalamus (Wilson et al., 1990; Xu et al., 1991b). However, the authors report that following stimulation of either the host cortex or thalamus does not result in the exclusive expression of EPSPs, and that TSNs are also capable of expressing IPSPs (Wilson et al., 1990; Xu et al., 1991b). There appears to be no apparent discrimination between expression of EPSP or IPSP, save that immediately following impalation of a cell for intracellular recordings there is an

immediate predominate expression of IPSPs (Wilson et al., 1990; Xu et al., 1991b). The expression of these immediate IPSPs only lasts for a few minutes, disappearing gradually to reveal the predominant expression of EPSPs. Due to the higher level of IPSP expression in neonatal MSNs, it has been speculated that the indiscriminate expression of IPSPs in TSNs may reflect certain mechanisms of their immaturity. Interestingly, it has been shown that certain voltage sensitive potassium currents, which are regulated over development, are similar to those seen in normal MSNs (Surmeier et al., 1992b). However, studies by Siviy et al (1993), demonstrate that TSNs display enhanced NMDA mediated transmission (Siviy et al., 1993). Such enhanced NMDA mediated transmission has been shown to exist in neonatal MSNs (Siviy et al., 1991), which would argue for a more neonatal phenotype.

The most striking difference between MSNs and TSNs is that TSNs appear to lack the rhythmic depolarizing cycles between "Up" and "Down" states (Xu et al., 1991b) which in the intact striatum is thought to play a critical role in priming subsections of the striatum for burst firing of action potentials (Wilson and Groves, 1981). Though TSNs display a lack of this rhythmic depolarisation, burst firing of action potentials has been observed following artificial depolarisation (Xu et al., 1991b). This would suggest a pre-synaptic mechanism for this observed phenotype.

Whilst a number of studies have demonstrated that TSNs are responsive to host stimulation (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b; Wilson et al., 1990; Xu et al., 1991b), little is known regarding the 'functional integration' of TSNs into the host striatal circuitry. Specifically, it is unclear if striatal grafts 'functionally integrate' to a degree where they are able to express baseline transmission. Furthermore, though anatomical and molecular studies have demonstrated that grafts express receptors and proteins associated with normal striatal function, it is unknown whether such connection with the host projection systems recapitulates functionally what is seen in the normal striatum.

The studies presented in this chapter aim to investigate the physiological and pharmacological mechanisms underlying the restoration of cortico-striatal baseline transmission, in striatal grafts.

3.2 METHODS

Experiments were performed as described in the general methods chapter, with additional details here:

All control animals in all five experiments were 10-14 weeks of age at time of testing. All grafted animals were 4-6 weeks of age at point of transplantation, 10-14 weeks of age at time of experimentation, with a graft age of 6-8 weeks.

3.2.1 Experiment 1: TSNs are Physiologically Active To Host Stimulation

Animals were divided into two groups: Control (n=12) and Graft (n=10). For sharp electrode recordings TSNs were impaled under visual guidance following positive identification for GFP fluorescence.

3.2.2 Experiment 2: Restoration of Baseline Transmission

Animals were divided into two groups: Control (n=28) and Graft (n=33). Baseline recordings were performed as previously detailed in the general methods.

3.2.3 Experiment 3: Glutamatergic Characterisation

Animals were divided into six groups: Control CNQX (n=6), Graft CNQX (n=5), Control APV (n=6), Graft APV (n=6), Control APV Zero Mg2+ (n=7), and Graft APV Zero Mg2+ (n=8). For The CNQX experiments a normal aCSF perfusate solution was used. Following an initial 20 minute baseline 10μM CNQX was applied to the perfusate solution and the response to the drug was measured over a 60 minute period. For the APV experiments a normal aCSF perfusate solution was used. Following an initial 20 minute baseline 30μM APV was applied to the perfusate solution and the response to the drug was measured over a 60 minute period. For the zero magnesium APV experiments a zero magnesium aCSF perfusate solution was used, to ensure activation of NMDA receptors. Following an initial 20 minute

baseline 30μM APV was applied to the perfusate solution and the response to the drug was measured over a 60 minute period.

3.2.4 Experiment 4: GABAergic Characterisation

Animals were divided into two groups: Control BIC (n=6), and Graft BIC (n=5). Throughout all experiments normal aCSF perfusate was used. Following an initial 20 minute baseline 30μM Bicuculline was applied to the perfusate solution and the response to the drug was measured over a 60 minute period.

3.2.5 Experiment 5: Monoamergic Characterisation

Animals were divided into four groups: Control SCH 23390 (n=8), Control SULP (n=9), Graft SCH 23390 (n=8), and Graft SULP (n=9). Throughout all experiments normal aCSF perfusate was used. For the SCH 23390 experiments an initial 20 minute baseline 10μM SCH 23390 was applied to the perfusate solution and the response to the drug was measured over a 60 minute period. For the SULP experiments an initial 20 minute baseline 3μM Sulpiride was applied to the perfusate solution and the response to the drug was measured over a 60 minute period.

3.3 RESULTS

3.3.1 Experiment 1: TSNs are Physiologically Active To Host Stimulation

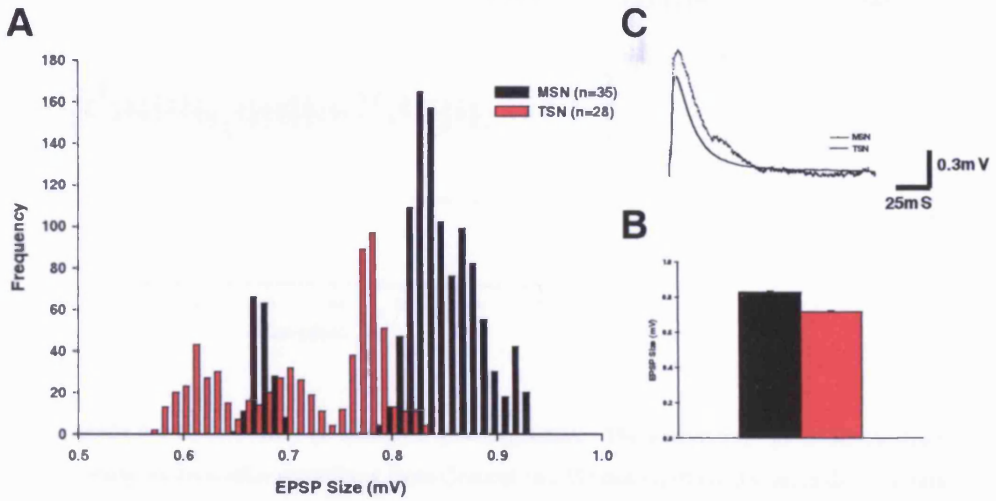

Figure 3.1 TSNs Respond to Host Stimulation. (A) Histogram representation of the frequency of distribution of the absolute size of EPSPs measured from TSNs and MSNs . (B) Mean histogram plot of the average EPSP size recorded from MSNs and TSNs. (C) Example EPSP trace recorded from TSNs and MSNs following 50mA stimulation of the host corticostriatal fibres, during sharp electrode recordings.

Figure 3.1C demonstrates an example EPSP trace from a MSN of the normal striatum, and a TSN, following stimulation of the host corticostriatal fibres, during sharp electrode recordings. EPSPs evoked from both MSNs and TSNs appear visually similar, although it must be noted that TSN evoked EPSPs are on average smaller than those evoked from MSNs. Figure 3.2A demonstrates a frequency distribution plot of the size of the EPSP gained during intracellular sharp electrode baseline recordings (50mA stimulation) from either MSNs or TSNs, and is summarized in the averaged histogram (Fig 3.1B). In total 35 MSNs from 12 control animals, and 28 TSNs from 10 grafted animals expressed EPSPs. There is a higher frequency of TSNs expressing basal EPSPs of lower than 0.8mV of magnitude. Basal EPSPs evoked from MSNs show an average EPSP size of 0.8369mV $\pm$0.007mV, which is significantly larger than the average EPSP size for TSNs which is 0.7154mV $\pm$0.008mV (Groups, $t_{(1945)}$=32.81, p=0.00).

3.3.2 Experiment 2: Restoration of Baseline Transmission

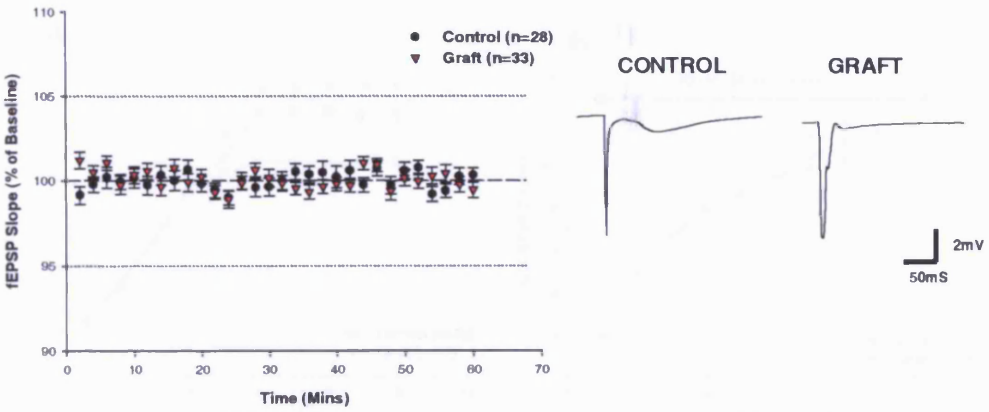

Figure 3.2 Restoration of Baseline Transmission. The above time plots demonstrate baseline extracellular recordings from Control (n=28) and Graft (n=33) animals. All data are normalised to their respective averaged fEPSP size across the whole 60 minute recording period. Insert traces display examples of fEPSPs taken from graft and control recordings.

Following basal stimulation of the host corticostriatal fibres it is possible to detect fEPSPs from the graft region (Fig. 3.2). Example traces (Insert) taken from control and graft based recordings demonstrate that graft fEPSPs appear visually similar to those recorded from the control striatum, albeit consistently smaller than that seen in control recordings. Both control and graft basal recordings demonstrate that following consistent basal stimulation there is no significant difference in size of the normalised fEPSP between graft and control recordings over time (Groups, $F_{(1,59)}=0.79$, $p=0.71$, n.s.).

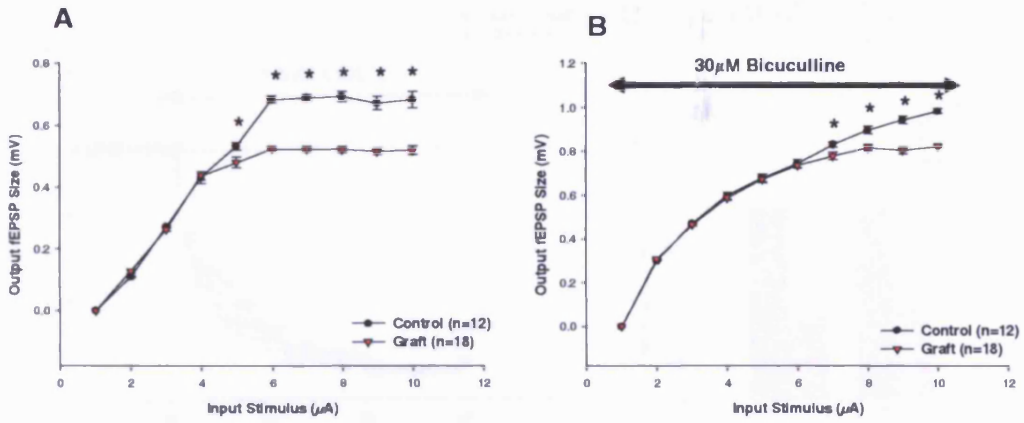

Figure 3.3 Input/Output Curves. Stepped increases in input current (Input) are plotted against the size of the fEPSP (Output), under both normal conditions (A), and in the presence of 30μM Bicuculline (B).

Input/Ouput curves generated from control and graft recordings demonstrate that under normal conditions, and below 4mA stimulation, both control and graft fEPSPs show no significant difference in the size of the fEPSP (Fig. 3.3A) (Groups, $t_{(28)}$=0.03, p=0.97 n.s.). However, over stimulation intensities greater than 5mA, there is highly significant difference between the size of the fEPSP evoked from control and graft recordings (Groups, $T_{(28)}$=5.48, p=0.00), with control fEPSPs being of a greater size than those evoked from the grafts.

Following application of 30μM bicuculline there is no significant difference in the size of the fEPSP between graft and control recordings, below 7mA stimulation (Fig. 4.3B) (Groups, $T_{(28)}$=0.024, p=0.98 n.s.). However, at stimulation intensities of 7mA and above a significant difference occurs in the size of the fEPSP between graft and control recordings (Groups, $T_{(28)}$=3.17, p=0.01), with control fEPSPs being of a greater size than their graft counterparts.

3.3.3 Experiment 3: Glutamatergic Characterisation

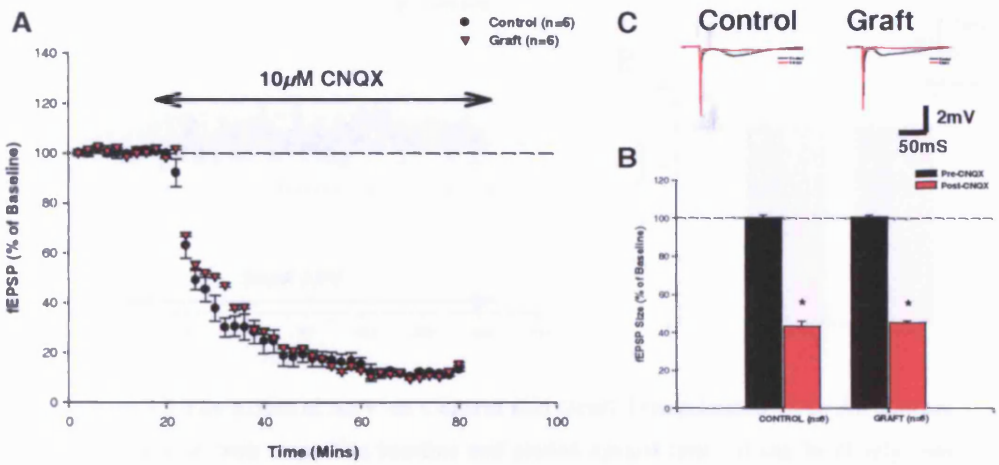

Figure 3.4 Effect of CNQX on Baseline Transmission. (A) All data are normalized to their respective baseline and plotted against time. It can be clearly seen that application of 10μM CNQX results in a reduction in the size of both graft and control fEPSPs. (B) Mean reduction in fEPSP size following application of 10μM CNQX. (C) Example fEPSP traces for control and graft recording prior to, and during, application of 10μM CNQX.

Figure 3.4 demonstrates the effect of CNQX on graft and control transmission. Example fEPSP traces (Fig. 3.4C), demonstrate that application of CNQX causes a reduction in the size of both control and graft evoked fEPSPs. Prior to administration of CNQX a twenty minute baseline is recorded, for the remainder of the experiment all data points are normalized to their averaged baseline, respectively. There is no significant difference in the normalized responses during the baseline period (Groups, $F_{(1,11)}$=2.13, p=0.17, n.s.). Application of 10μM CNQX to the aCSF perfusion medium produces a significant decrease in the size of the fEPSP; Graft CNQX (n=6) 45.51% $\pm$ 0.78% of baseline, Control CNQX (n=6) 43.47% $\pm$ 2.62% of baseline, with no significant difference in the size of the response during application of 10μM CNQX in both graft and control recordings (Groups, $F_{(1,11)}$=0.89, p=0.36, n.s.). Figure 3.4B demonstrates a histogram plot of the mean size of the fEPSP evoked from both control and graft recordings during baseline conditions and during application of 10μM CNQX.

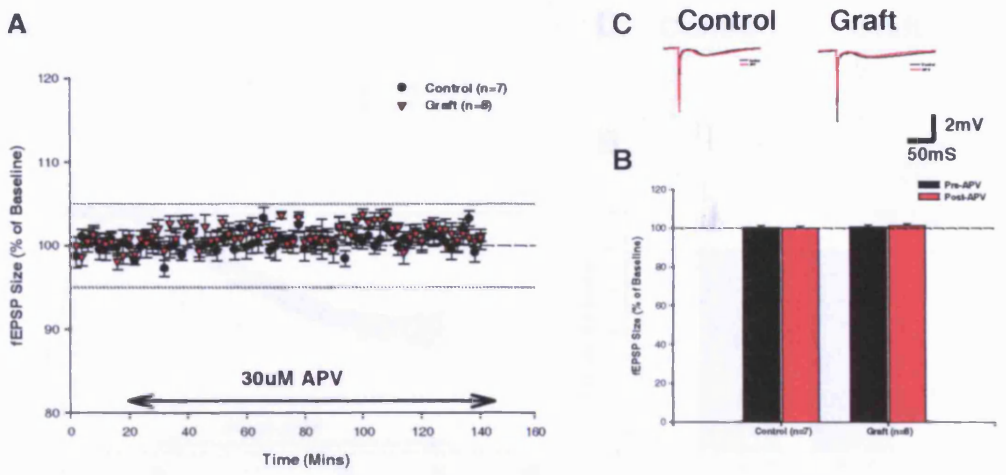

Figure 3.5 The Effect of APV on Control and Graft Transmission. (A) All data are normalized to their respective baseline and plotted against time. It can be clearly seen that application of 30μM APV results in no reduction in the size of both graft and control fEPSPs. (B) Mean reduction in fEPSP size following application of 30μM APV. (C) Example fEPSP traces for control and graft recording prior to, and during, application of 30μM APV.

Figure 3.5 demonstrates the effect of APV on graft and control transmission. Example fEPSP traces (Fig. 3.5C), demonstrate that application of APV causes no visible effect on both control and graft evoked fEPSPs. Prior to administration of APV a twenty minute baseline is recorded, for the remainder of the experiment all data points are normalized to their averaged baseline, respectively. There is no significant difference in the normalized responses during the baseline period (Groups, $F_{(1,11)}$=0.21, p=0.65, n.s.). Application of 30μM APV to the aCSF perfusion medium has no significant effect on the size of the fEPSP; Graft APV (n=8) 99.94% $\pm$ 0.994% of baseline, Control APV (n=7) 100.14% $\pm$ 1.16% of baseline, with no significant difference in the size of the response during application of 30μM APV in both graft and control recordings (Groups, $F_{(1,13)}$=4.02, p=0.065, n.s.). Figure 3.5B demonstrates a histogram plot of the mean size of the fEPSP evoked from both control and graft recordings during baseline conditions and during application of 30μM APV.

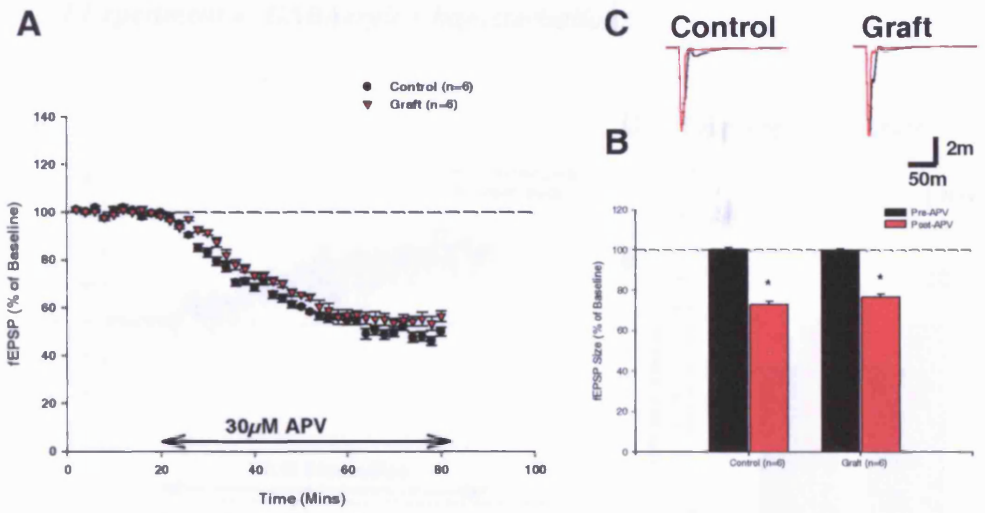

Figure 3.6 The Effect of APV on Control and Graft Transmission. (A) All data are normalized to their respective baseline and plotted against time. (B) Mean reduction in fEPSP size following application of 30μM APV. (C) Example fEPSP traces for control and graft recording prior to, and during, application of 30μM APV.

Figure 3.6 demonstrates the effect of 30μM APV on graft (n=6) and control (n=6) transmission, under conditions where magnesium is omitted from the perfusate solution. Example fEPSP traces (Fig. 3.6C), demonstrate that application of 30μM APV causes a visible reduction in the size of both graft (n=6) and control (n=6) fEPSPs. Prior to administration of 30μM APV a twenty minute baseline is recorded, for the remainder of the experiment all data points are normalized to their averaged baseline, respectively. There is no significant difference in the normalized responses during the baseline period (Groups, $F_{(1,10)}$=1.00, p=0.33, n.s.). Application of 30μM APV to the aCSF perfusion medium results in a significant reduction in the size of the fEPSP; Graft APV (n=6) 76.60% $\pm$ 1.57% of baseline, Control APV (n=6) 73.04% $\pm$ 1.43% of baseline, with no significant difference in the level of depression observed in both graft (n=6) and control (n=6) recordings following application of 30μM APV (Groups, $F_{(1,10)}$=16.06, p=0.002.). Figure 3.6B demonstrates a histogram plot of the mean size of the fEPSP evoked from both control and graft recordings during baseline conditions and during application of 30μM APV.

3.3.4 Experiment 4: GABAergic Characterisation

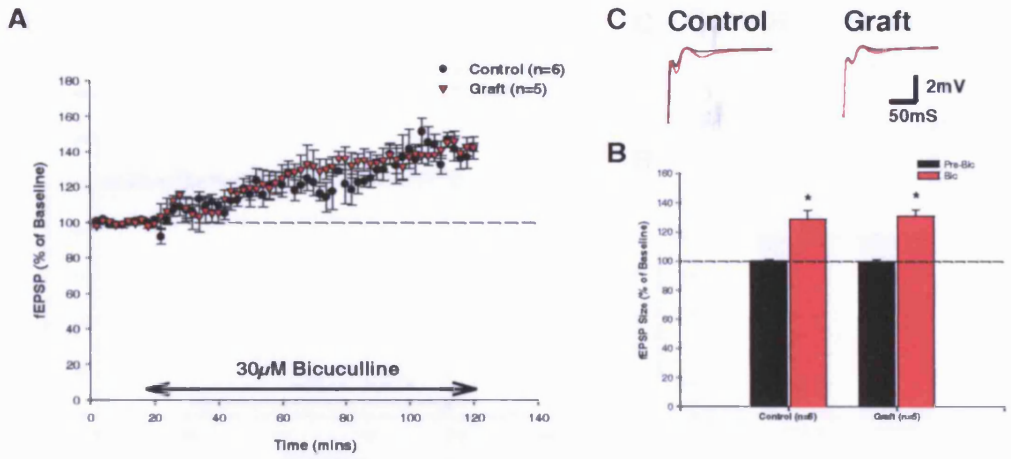

Figure 3.7 The Effect of 30 μM Bicuculline on Control and Graft Transmission. (A) All data are normalized to their respective baseline and plotted against time. Dashed line represents the baseline value. (B) Mean reduction in fEPSP size following application of 30μM Bicuculline. (C) Example fEPSP traces for control and graft recording prior to, and during, application of 30μM Bicuculline.

Figure 3.7 demonstrates the effect of 30μM bicuculline on graft (n=5) and control (n=6) transmission, under normal physiological conditions. Example fEPSP traces (Fig. 3.7C), demonstrate that application of 30μM bicuculline causes a visible increase in the size of both control (n=6) and graft (n=5) fEPSPs. Prior to administration of 30μM bicuculline a twenty minute baseline is recorded, for the remainder of the experiment all data points are normalized to their averaged baseline, respectively. There is no significant difference in the normalized responses during the baseline period (Groups, $F_{(1,9)}$=0.58, p=0.46, n.s.). Application of 30μM bicuculline to the aCSF perfusion medium causes a gradual significant increase in the size of the fEPSP; Graft BIC (n=5) 131.12% ± 4.10% of baseline, Control BIC (n=6) 129.07% ± 5.94% of baseline, with no significant difference in the level of potentiation observed in both graft (n=5) and control (n=6) recordings following application of 30μM bicuculline (Groups, $F_{(1,9)}$=0.73, p=0.412, n.s.). Figure 3.7B demonstrates a histogram plot of the mean size of the fEPSP evoked from both control and graft recordings during baseline conditions and during application of 30μM bicuculline.

3.3.5 Experiment 5: Monoamergic Characterisation

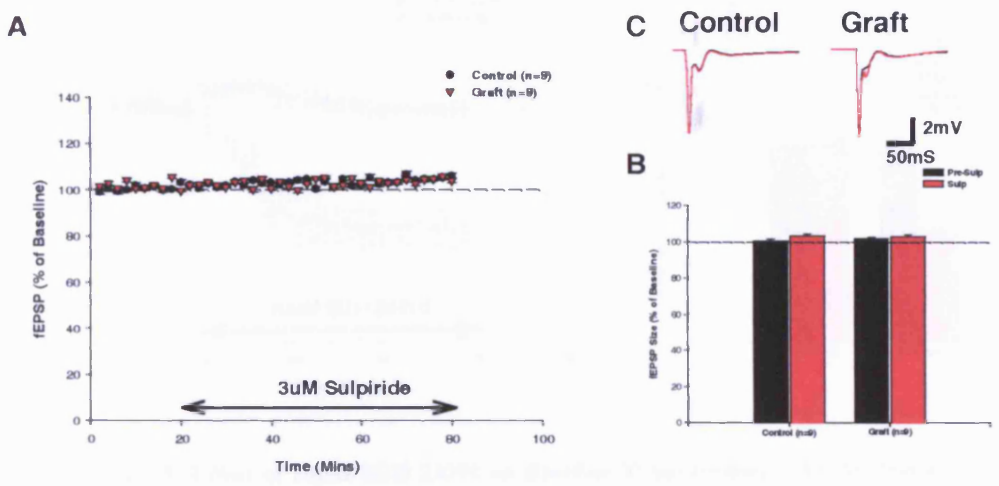

Figure 3.8 The Effect of 3μM Sulpiride on Control and Graft Transmission. (A) All data are normalized to their respective baseline and plotted against time. Dashed line represents baseline. (B) Histogram plot of the mean size of the fEPSP pre- & during application of 3μM sulpiride. (C) Example fEPSP traces for control and graft recording prior to, and during, application of 3μM sulpiride.

Figure 3.8 demonstrates the effect of 3μM sulpiride, a D2 antagonist, on graft (n=9) and control (n=9) transmission, under normal physiological conditions. Example fEPSP traces (Fig. 3.8C), demonstrate that application of 3μM sulpiride causes no visible effect on both control (n=9) and graft (n=9) fEPSPs. Prior to administration of 3μM sulpiride a twenty minute baseline is recorded, for the remainder of the experiment all data points are normalized to their averaged baseline, respectively. There is no significant difference in the normalized responses during the baseline period (Groups, $F_{(1,16)}=0.06$, p=0.79, n.s.). Application of 3μM sulpiride to the aCSF perfusion medium has no significant effect on the size of the fEPSP; Graft SULP (n=9) 101.10% ± 3.40% of baseline, Control SULP (n=9) 99.40% ± 0.54% of baseline, with no significant difference in the size of the response during application of 3μM sulpiride between graft (n=9) and control (n=9) recordings (Groups, $F_{(1,16)}=0.22$, p=0.64, n.s.). Figure 3.8B demonstrates a histogram plot of the mean size of the fEPSP evoked from both control (n=9) and graft (n=9) recordings during baseline conditions and during application of 3μM sulpiride.

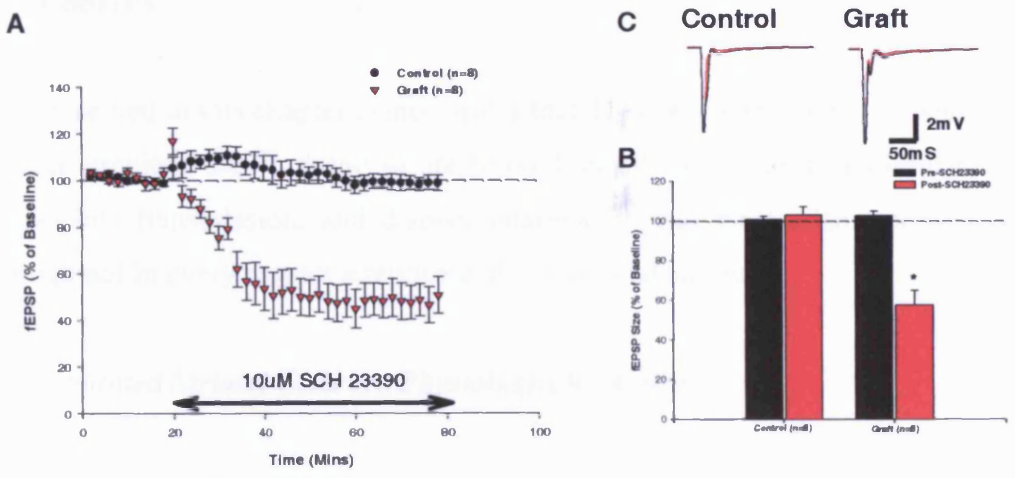

Figure 3.9 Effect of 10μM SCH 23390 on Baseline Transmission. (A) All data are normalized to their respective baseline and plotted against time. (B) Mean reduction in fEPSP size following application of 10μM SCH 23390. (C) Example fEPSP traces for control and graft recording prior to, and during, application of 10μM SCH 23390.

Figure 3.9 demonstrates the effect of 10μM SCH 23390, a D1 antagonist, on graft (n=8) and control (n=8) transmission. Example fEPSP traces (Fig. 3.9C), demonstrate that application of 10μM SCH 23390 causes no visible reduction in the size of control (n=8) fEPSPs, whilst showing a visible reduction in the size of graft (n=8) fEPSPs. Prior to administration of 10μM SCH 23390 a twenty minute baseline is recorded, for the remainder of the experiment all data points are normalized to their averaged baseline, respectively. There is no significant difference in the normalized responses during the baseline period (Groups, $F_{(1,14)}$=2.54, p=0.132, n.s.). Application of 10μM SCH 23390 to the aCSF perfusion medium has no significant effect on the size of control (n=8) fEPSPs, but does cause a significant reduction in the size of graft (n=8) fEPSPs; Graft SCH 23390 (n=8) 57.57% ± 6.81% of baseline, Control SCH 23390 (n=8) 103.16% ± 3.60% of baseline (Groups, $F_{(1,14)}$=35.12, p=<0.005.). Figure 3.9B demonstrates a histogram plot of the mean size of the fEPSP evoked from both control (n=8) and graft (n=8) recordings during baseline conditions and during application of 10μM SCH 23390.

3.4 DISCUSSION

The data presented in this chapter demonstrates that TSNs become integrated with the host striatal circuitry, and in doing so are physiologically active to host stimulation, restore baseline transmission, and display pharmacological mechanisms consistent with, though not in every respect a replicate of, the normal striatum.

3.4.1 Transplanted Striatal Cells are Physiologically Active

A number of previous studies have shown that TSNs are physiologically active (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b; Wilson et al., 1990; Xu et al., 1991b), and responsive to stimulation of the host cortex (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b; Wilson et al., 1990; Xu et al., 1991b), or thalamus (Xu et al., 1991b). The data presented in this chapter further corroborates these findings.

At four weeks post-transplantation it is possible to detect EPSPs from TSNs following stimulation of the host cortex. However, at this point in the post-transplantation period only a proportion of the cells tested exhibited EPSPs following stimulation of the host cortex, suggesting that only a proportion of TSNs receive functional innervation from the host corticostriatal projection. Interestingly, visual observations as to the location of TSNs responsive to host stimulation suggests that such 'connected' cells seem to cluster within the 'periphery' of the graft, whilst the 'core' regions display a lower probability of obtaining 'connected' TSNs. Interestingly, as the graft develops over the post-transplantation period (4-8 weeks post-transplantation) the probability of obtaining 'connected' TSNs from the 'core' region increases. This apparent gradient in the functional innervation of the graft has been shown previously via immunohistochemical tracing of host afferent fibres innervating the graft (Clarke and Dunnett, 1990; Clarke and Dunnett, 1993; Wictorin et al., 1988b; Wictorin et al., 1989; Wictorin, 1992). It has been shown following transplantation that host afferent fibres first innervate the 'periphery', before innervating 'core' regions later on in post-transplantation development. Beginning at roughly 6 weeks post-transplantation nearly all TSNs are responsive to localised stimulation of the graft tissue, with a high proportion also responsive to host

corticostriatal stimulation. Interestingly, studies conducted *in vivo* report that at 6 months post-transplantation nearly all TSNs are responsive to stimulation of either the host cortex or thalamus (Xu et al., 1991b). Therefore, it appears that 'functional connection' between the graft and host circuitry begins at 4 weeks post-transplantation, increasing to innervate most of the graft region over 4-24 weeks post-transplantation.

TSNs display similar current-voltage relationships to MSNs, baring a high input resistance seen in TSNs, suggesting that differences in the ionic mechanisms of TSN excitability, may contribute to, but do not fully account for the differences in the average EPSP size. The most logical explanation for the decreased EPSP size observed from TSNs is the decreased 'functional innervation' of the graft, when compared to the normal striatum. As previously mentioned, innervation of the graft develops over the post-transplantation period, though never recapitulates that seen in the normal striatum. Specifically, a decreased level of corticostriatal innervation of the graft would result in a lower level of excitation delivered to the TSN, which in turn may result in a lower level of excitation and the expression of smaller EPSPs. Furthermore, anatomical studies have shown that striatal grafts demonstrate a decreased level of "Point-to-Point" innervation (Wictorin et al., 1989; Wictorin, 1992; Wilson et al., 1990; Xu et al., 1989; Xu et al., 1991a), where between 20-50% of excitatory synapses from the corticostriatal projection synapse with the shaft of TSN dendritic spines, rather than the head (Wictorin et al., 1989; Wictorin, 1992; Wilson et al., 1990; Xu et al., 1989; Xu et al., 1991a). The functional consequences of such incorrectly targeted synaptic contacts on TSNs in unknown. However, a decreased level of excitatory innervation of the graft, coupled with a reduction in "Point-to-Point" connection could explain why TSNs express smaller EPSPs than MSNs. Interestingly, direct stimulation of the graft region surrounding a TSN increase the size of the EPSP, though such EPSPs never reach the same magnitude as those recorded from MSNs. Therefore, although decreased excitatory drive and "Point-to-Point" connection could be responsible for the observed differences, other irregularities within TSNs (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b; Wilson et al., 1990; Xu et al., 1991b) must contribute. A number of studies have reported that TSNs display ionic characteristics attributed to immature striatal neurones (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b; Wilson et

al., 1990; Xu et al., 1991b). However, other studies have demonstrated that TSNs display ionic mechanisms consistent with a more adult phenotype (Surmeier et al., 1992b). Nevertheless, it remains clear that although TSNs integrate with the host circuitry, the host-graft corticostriatal synapses are not as functionally strong as those seen in the normal striatum.

Extracellular field potential recordings enable a greater understanding of the physiological characteristics of networks of TSNs. Field potential recordings demonstrate similar findings, regarding the size of the fEPSP, as that seen during intracellular recordings. fEPSPs elicited from striatal grafts are on average much smaller in size than those recorded from the normal striatum. Furthermore, when probing graft tissue for extracellular recordings there are distinct regions of the graft that do not show any form of excitatory response. However, unlike the electrophysiological 'dead zones' seen during intracellular recordings, these 'dead zones' are not uniformly divided into 'periphery' and 'core' regions of the graft, and are randomly located within the graft boundary. Furthermore, though these regions of the graft are non-responsive to host stimulation, fEPSPs can be detected following local stimulation of the graft tissue. However, such fEPSPs are exceptionally small (0.25-0.34mV), and display no stable response over time.

There are a number of possible explanations for the electrophysiological 'dead zones' seen in striatal grafts. Firstly, corticostriatal innervation of the graft is not uniform over the whole graft area (Wictorin et al., 1988b; Wictorin et al., 1989; Wictorin, 1992). It is plausible that the regions of the graft which are non-responsive may correspond to regions of low corticostriatal innervation. Therefore, stimulation of the host corpus callosum many not activate sufficient fibres to result in the expression of fEPSPs from the 'dead zones', whilst localised stimulation may activate enough afferent fibres to result in the expression of fEPSPs. A second important factor to consider is the mosaic localisation of cells of striatal phenotype within the graft region. It has been shown that following transplantation cells or a striatal phenotype cluster into zones ('P-zones'), whilst cells of a non-striatal phenotype cluster into other zones ('NP-zones'). Within the eGFP transplant model used during the experiments presented here GFP is expressed in neuronal cells (Feil et al., 1996), and therefore it is impossible to distinguish between 'P-zones' and 'NP-zones', at the time

of placing electrodes. Therefore, it is possible that the electrophysiological 'dead zones' may correspond to 'NP-zones' where the cells are of a cortical and pallidal phenotype (Graybiel et al., 1989). It has been shown in anatomical studies that innervation of the graft region favours the 'P-zones' over the 'NP-zones' (Wictorin et al., 1988b; Wictorin et al., 1989; Wictorin, 1992). Furthermore, expression of receptors and neuro-peptides found within the normal striatum is much denser within the 'P-zones' than the 'NP-zones' (Campbell et al., 1995b). Therefore, it is plausible that 'NP-zones' would display little, if any, response to host stimulation. Further evidence for this theory was sought by attempting to recover *in vitro* slice post recording, and to perform immunohistochemistry to identify the phenotype of cells within the recording site. Unfortunately, it was not possible to obtain suitable resectioned *in vitro* slices within the time frame of thesis, rendering immunohistochemical identification of the recordings site impossible. Immunohistochemical identification of the recording site would provide a conclusive example of the phenotype of the cells recorded from during intracellular sharp electrode recordings. However, extracellular recordings are the result of the sum of synaptic responses from a neuronal field, and therefore immunohistological identification of the cellular phenotype within the localised region of the electrode placement, may not fully reflect the neuronal field recorded from. Therefore, to make accurate recordings from specified regions of the graft, a higher degree of accuracy would be achieved using donor tissue from transgenic animals which express GFP exclusively within the target cell type (see General Discussion).

3.4.2 Restoration of Baseline Transmission

Excitotoxic lesions of the host striatum, via the application of excitotoxins, results in a pronounced loss of MSNs (Coyle and Schwarcz, 1976), which represent the major post-synaptic cellular population of the striatum. It was impossible to detect any form of excitatory response from the lesion site when tested 2-4 weeks post-lesion (n=15). Recordings from the contralateral, lesion spared, striatum demonstrate visually normal fEPSPs to that seen in control non-lesioned animals. The functional significance of excitotoxic lesions has been described both behaviourally (Dunnett and Iversen, 1980; Dunnett and Iversen, 1981; Mason and Fibiger, 1979), and anatomically, but the electrophysiological consequences are far less clear. However,

the loss of baseline transmission within the lesioned striatum must contribute significantly to the behavioural phenotype seen post-lesion, and consequently is investigated in later chapters.

Basal stimulation of the host corticostriatal fibres results in the expression of fEPSPs within the graft. These fEPSPs display stability, with a constant level and rate of stimulation, over a 60 minute period. Though previous studies have shown that TSNs are physiologically active to host stimulation (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b; Wilson et al., 1990; Xu et al., 1991b), the data presented within this chapter builds on previous findings, demonstrating that TSNs are not only physiologically active, but also restore baseline transmission to lesioned striatum. This observation is important as restoration of baseline transmission to the lesioned striatum may provide a functional mechanism for the observed beneficial effects of striatal grafts. Striatal grafts have been shown to dramatically reduce the adverse motor symptoms of HD in experimental models (Deckel et al., 1983; Deckel et al., 1986a; Deckel et al., 1986b; Deckel et al., 1988a; Deckel et al., 1988b; Isacson et al., 1985; Isacson et al., 1986; Sanberg et al., 1986) and clinical trials (Bachoud-Levi et al., 2000a; Bachoud-Levi et al., 2000b). It would be logical to assume that restoration of baseline transmission, much like that seen in the normal striatum, would facilitate recovery of motor abilities. More specifically, the striatum has been heavily implicated with, amongst other things, a strong neurological control over the initiation and control of movement (Graybiel, 2000). Futhermore, it has been shown within experimental models of PD that baseline transmission, and more specifically synaptic plasticity, plays a role in movement disorders such as l-DOPA induced dyskinesias (Picconi et al., 2003). Therefore, it seems logical that restoration of baseline transmission, would play a major role in the recovery of normal behavioural function post-transplantation.

As previously stated, fEPSPs recorded from the graft region are on average much smaller than those recorded from the normal striatum. Normalising fEPSPs to the averaged response size over the baseline period show that recordings from grafts show no significant difference to baseline recordings in the normal striatum. To investigate the efficacy of host-graft baseline transmission input-output curves were generated, using uniform increases in stimulation current, in the absence of any

changes in stimulation rate. Under physiologically normal conditions, and below 4mA stimulation, there is no significant difference in the absolute size of the fEPSP between graft and control recordings. Above 4mA stimulation there is an apparent discrepancy between graft and control responses. Whilst control recordings display an increased response size with increased stimulation, graft recordings reach a asymptote of response by 4mA. Interestingly, following application of GABA receptor antagonists it is possible to see an increased response size from graft based recordings. Although there is a detectable difference between graft and control recordings, this is present at much higher stimulus levels, 7mA as opposed to 4mA.

The data from the input-output experiments suggests a number of interesting facets regarding host-graft transmission. Under normal conditions striatal grafts display a much lower level of maximal stimulation (5mA), to the normal striatum (8mA). This maximal level of stimulation is increased in conditions where the GABA inhibitory effect is blocked (8mA), but is still lower than that seen in the normal striatum (10mA). These findings suggest that the difference in output voltage to input stimulus is mediated to a certain degree by a higher level of GABA inhibition within striatal grafts than the normal striatum. Two possible explanations could account for this interesting phenomenon.

Firstly, it is possible that regions where successful extracellular recordings are obtained correspond to the 'P-zones', as previously described. It has been shown that within the "P zones" of striatal grafts there is higher proportion of GABAergic interneurones (Liste et al., 1997; Zhou et al., 1989), than that seen within the normal striatum. Therefore, it is plausible that the higher proportion of GABAergic interneuones would correspond functionally to a higher GABAergic inhibition of graft transmission.

A second possible hypothesis for the observed higher level of GABAergic inhibition within striatal grafts is the observed higher level of axonal arborisation. MSNs of the normal striatum display a certain degree of axonal arborisation, producing inhibitory GABAergic synaptic contacts with other MSNs (Wilson and Groves, 1980). Within striatal grafts, the level of axonal arborisation by TSNs has been shown to be much higher than that observed in the normal striatum (Clarke et al., 1988b; Clarke et al.,

1988a; Clarke and Dunnett, 1990; Clarke and Dunnett, 1993; Wictorin et al., 1988b; Wictorin et al., 1989; Wictorin and Bjorklund, 1989; Wictorin, 1992; Wilson et al., 1990; Xu et al., 1989; Xu et al., 1991a). Therefore, it is plausible that the higher level of TSN axonal arborisation could contribute to this higher level of GABAergic inhibition.

Interestingly, when GABAergic inhibition is removed there still remains a disparity in the input-output relationship between striatal grafts and the normal striatum, albeit at a higher stimulus intensity that that seen under normal physiological conditions. Therefore even though the higher level of GABAergic inhibition in striatal grafts may govern the differences in response size, this is only the case up to a specific stimulation intensity. Above this stimulus intensity, it is plausible that other mechanisms, which have been previously described, such as decreased corticostriatal innervation and "point-to-point" connection governs the observed differences between the grafted and normal striatum.

3.4.3 Glutamatergic Mechanisms of Host-Graft Transmission

The corticostriatal projection represents the major glutamatergic input to the striatum. Within the normal striatum glutamatergic transmission provides the driving force for excitatory synaptic transmission, and therefore is crucial to the restoration of baseline transmission.

Pharmacological inhibition of AMPA receptors via the application of CNQX has been shown to cause a dramatic reduction in the EPSPs recorded from MSNs (Calabresi et al., 1992c; Calabresi et al., 1992e). Recordings from the control striatum demonstrate that application of CNQX causes an immediate reduction in the size of the fEPSP (Fig 3.4), clearly demonstrating that, under normal conditions, the AMPA receptor contributes a significant proportion of the fEPSP response. Recordings from striatal grafts demonstrate that application of CNQX causes a similar reduction in the response size to that seen in control recordings, with no significant difference between the size of control and graft fEPSPs following CNQX application. Furthermore, the time course of CNQX mediated reduction of the fEPSP is similar in both grafts and

control recordings. These findings would suggest that AMPA mediated synaptic transmission is comparable in control and graft baseline transmission.

Studies conducted within the normal striatum, *in vitro*, show that under normal physiological conditions, application of the selective NMDA receptor antagonist APV has no effect on the size of the fEPSP (Calabresi et al., 1992c; Calabresi et al., 1992e). Under normal conditions the NMDA receptor is considered to be inactive due to the high affinity magnesium ion block of the receptor (Calabresi et al., 1992e). The experiments presented in this chapter demonstrate that application of APV has no effect on control or graft baseline transmission (Fig 3.6). However, following removal of magnesium from the aCSF perfusate solution it is possible to detect a NMDA mediated component of excitatory synaptic transmission, which is sensitive to APV (Calabresi et al., 1992e). The experiments presented here demonstrate that application of APV in magnesium free conditions results in a reduction in the size of the fEPSP in both control and graft recordings, with no significant difference in the size of the fEPSP in the presence of APV between graft and control recordings. These findings would suggest that NMDA mediated transmission is similar in grafts, as to that seen in the normal striatum.

3.4.4 GABAergic Mechanisms of Host-Graft Transmission

Data presented earlier on in this chapter has suggested that striatal grafts display a higher level of GABAergic inhibition, than that seen in the normal striatum. Application of bicuculline within the normal striatum has been shown to increase the size of the fEPSP (Calabresi et al., 2000a). The data presented here demonstrates that application of bicuculline results in an increase in the size of the fEPSP in both control and graft recordings, with no significant difference in the size of the fEPSP between the two. Whilst slightly unexpected this observation can be easily explained. For the majority of experiments a stimulus intensity of 5mA is used, at this intensity the size of response gained is similar in both control and graft recordings, when in the presence of bicuculline (Fig 3.4). Therefore, the effect of GABA receptor blockade is similar in both graft and control preparations. Observations from the input-output studies in the presence of bicuculline indicated that differences in response size between control and graft recordings is only present at stimulus intensities above

8mA. Therefore it would be interesting to see if bicuculline has a similar effect on transmission at stimulus intensities above 8mA. Unfortunately, at these stimulus intensities it is almost impossible to gain a stable response over a 20 minute baseline period, and therefore impossible to establish an accurate comparison between the effect of bicuculline application and basal effects.

3.4.5 Monoamergic Mechanisms of Host-Graft Transmission

Within the normal striatum, projections from the SN form DAergic synapses with MSNs, as well as with other neurons within the striatum (Wilson and Groves, 1980). Anatomical studies have shown that striatal grafts receive projections from the SN, which like the corticostriatal projection, innervate the "P zones" more densely than the remainder of the graft (Clarke et al., 1988b; Clarke et al., 1988a; Clarke and Dunnett, 1990; Clarke and Dunnett, 1993; Wictorin et al., 1988b; Wictorin et al., 1989; Wictorin and Bjorklund, 1989; Wictorin, 1992; Wilson et al., 1990). The data presented here demonstrates that DAergic innervation of striatal grafts form functional synapses capable of affecting baseline transmission.

Application of sulpiride, a selective D2-like receptor antagonist, has been shown to have no effect on baseline transmission within the normal striatum (Calabresi et al., 2000a). Similarly, the results presented here demonstrate that sulpiride has no effect on either control or graft baseline transmission, with no additional effect on the size of the fEPSP following increasing concentrations, indicating that the D1-like mediated component of graft excitatory transmission is similar to that seen in the normal striatum.

Application of SCH 23390, a selective D1-like receptor antagonist has an interesting effect on control and graft baseline transmission. Within control recordings, application of SCH 23390 causes a slight increase in the fEPSP size, which, following a 20 minute period returns to baseline levels (Fig 3.10). Within graft based recordings application of SCH 23390 causes a gradual reduction in the size of the fEPSP stabilizing to roughly 50% of baseline following 20 minute period of application. This observation is interesting as it indicates a significant difference between normal and graft baseline transmission. It would therefore seem that the D2-like mediated

component of excitatory synaptic transmission is different in grafts to that seen in the normal striatum. A number of anatomical studies have shown that within the "P-zones" of striatal grafts, the level of D2-like receptor expression is lower than that seen within the normal striatum (Campbell et al., 1995b; Sirinathsinghji et al., 1994). Furthermore, studies utilising PET analysis have shown an increased D2-like receptor binding in striatal grafts (Torres et al., 1995), though it must be noted that other studies has reported no increase in D2-like receptor binding (Pundt et al., 1997). Therefore, it is plausible that the abnormal differences in D2-like mediated transmission seen between graft and control recordings could be attributed to a lower level of D2 receptors seen within striatal grafts.

Finally, it is important to consider the time frame of post-transplantation recovery with respect to reconstruction of the normal striatal-nigral circuitry. It has previously been shown that innervation of the graft by host nigral fibres occurs at approximately 3 months post-transplantation, with more extensive innervation of the graft region by 6 months post-transplantation (Pritzel et al., 1986; Wictorin et al., 1988a; Wictorin et al., 1988b). Therefore, it is plausible that differences observed in the D2-like mediated component of graft excitatory transmission may simply reflect that within the time frame of post-transplantation recovery, within these experiments, functional reconnection between the graft and host nigral fibres is impaired.

3.5 CONCLUSION

TSN's are physiologically active to host stimulation, given suitable post-transplantation development to form functional synapses with the host corticostriatal projection system. Embryonic striatal grafts 'functionally integrate' with the host striatal circuitry restoring baseline transmission to the lesioned striatum, and restoring a number of functional connections with host striatal networks. Within striatal grafts blockade of D1-like receptors produces a different effect on baseline transmission than that seen in the normal striatum.

4. EMBRYONIC STRIATAL GRAFTS DISPLAY BIDIRECTIONAL SYNAPTIC PLASTICITY

4.1 INTRODUCTION

"Learning to use the graft" describes a mechanism whereby functions that had been established in the intrinsic circuits of the brain through a lifetimes' training and experience prior to lesion need to be re-established - through training leading to relearning - using the graft circuitry. The concept that transplanted tissue could provide recovery of learned skills and habits was first observed in the context of functional recovery following enucleation and retinal transplantation (Coffey et al 1989). These early experiments showed that following transplantation the recovery of visual pathways was not sufficient to permit the rat to immediately "see" and respond accordingly. In order to regain behavioural performance transplanted animals needed to be trained to interpret the meaning of the visual stimuli and to respond accordingly, therefore permitting the relearning of the specific task.

The concept of "learning to use the graft" has been more extensively investigated within the striatal graft environment. First by Mayer and later expanded on by Brasted et al, they assessed functional recovery using the '9 hole' box apparatus, which utilises an array of tests specifically aimed at measuring deficits in cognitive mechanisms intrinsic to the striatum. Unilateral excitotoxic lesions of the striatum impair the initiation of responses on the contralateral side without affecting the animals' ability to detect or respond to the eliciting stimulus (Brasted et al., 1997; Carli et al., 1985). Interestingly, animals which have received a striatal graft displayed a similar level of profound behavioural deficit to their lesion only counterparts when returned to the test 4 (Brasted et al., 1999b) or 6 (Mayer et al., 1992) months post-surgery. However, with repeated testing, whilst lesioned animals showed no significant improvement over time, grafted animals improved with training (Brasted et al., 1999b; Mayer et al., 1992). It would therefore seem essential that grafts not only form anatomically appropriate connections, but also that the animal is re-trained on the task itself with the transplant serving as the substrate for the re-acquisition of the skill. Such relearning takes place over a similar time period to that

required for naïve animals to learn the task. It has recently been shown that relearning can occur in low repetition non-operant tasks, such as paw reaching, with much less exposure and practice (Dobrossy and Dunnett, 2005). Furthermore, such relearning is critically dependant on training to be specific in targeting the limb controlled by the damaged striatum with the striatal implant (Dobrossy and Dunnett, 2003).

Whilst it has been clearly demonstrated that striatal grafts display the ability to relearn previously trained tasks, it still remains unknown whether such grafts similarly display cellular correlates of learning and memory. Synaptic plasticity is widely regarded as the most appropriate model of cellular learning (Bliss and Collingridge, 1993). It has been shown that selective blockade of NMDA receptors in the hippocampus, blocks both hippocampal synaptic plasticity, and impairs behavioural performance on tasks relying heavily on appropriate hippocampal memory function (Morris et al., 1986). Within the striatum, genetic manipulation of CREB, a key protein involved in the maintenance of synaptic plasticity, both eliminates bidirectional striatal synaptic plasticity, and causes deficits in behavioural tasks requiring striatal specific mechanisms of memory (Pittenger et al., 2006). Therefore, it appears clear that learning and memory, and synaptic plasticity are critically linked.

As the previous chapter reports, a small number of studies have shown that embryonic striatal grafts are capable of forming anatomically correct synaptic contacts with the host circuitry (Clarke et al., 1988b; Clarke et al., 1988a; Clarke and Dunnett, 1990; Clarke and Dunnett, 1993; Wictorin et al., 1989). Gene expression studies have shown that grafted cells express neurotransmitters appropriate to that seen in normal striatal neurones (Campbell et al., 1995b). Furthermore, experiments using "push-pull" perfusion demonstrate that striatal grafts restore only 34% of baseline levels of GABA in the host globus pallidus and substantia nigra (Sirinathsinghji et al., 1988).

Host-graft synapses are physiologically active to stimulation of host afferent fibres, displaying mechanisms of neuronal excitability (Rutherford et al., 1987; Siviy et al., 1993; Xu et al., 1991b; Wilson et al., 1990). Grafted cells also express immediate early gene responses, which are in part mediated by neuronal excitability, and display

increase expression during mechanisms of plasticity (Mandel et al., 1992). However, it is still unknown whether grafted embryonic striatal neurones are capable of expressing synaptic plasticity, and how this relates to the normal striatal neurones.

The studies presented in this chapter aim to demonstrate that embryonic striatal grafts display synaptic plasticity, in keeping with that observed in the normal striatum.

4.2 METHODS

Experiments were performed as described in the General Methods chapter, with additional details here:

All control animals in all five experiments were 10-14 weeks of age at time of testing. All grafted animals were and 4-6 weeks of age at point of transplantation, 10-14 weeks of age at time of experimentation, with a graft age of 6-8 weeks.

4.2.1 Experiment 1: Long-Term Depression in Embryonic Striatal Grafts.

For extracellular recordings animals were divided into two groups: Control (n=11), and Graft (n=12). Extracellular recordings were performed as indicated in the general methods section. For intracellular recordings animals were divided into two groups: Control (n=15), and Graft (n=14). Intracellular sharp electrode recordings were performed as indicated in the previous chapter. During both extracellular and intracellular recordings LTD was induced via HFS in the presence of normal aCSF perfusate.

4.2.2 Experiment 2: Long-Term Potentiation in Embryonic Striatal Grafts.

For extracellular recordings animals were divided into two groups: Control (n=23), and Graft (n=25). Extracellular recordings were performed as indicated in the general methods section For intracellular recordings animals were divided into two groups: Control (n=18), and Graft (n=18). Intracellular sharp electrode recordings were performed as indicated in the previous chapter. During both extracellular and

intracellular recordings LTP was induced via HFS in the presence of magnesium free aCSF perfusate.

4.3 RESULTS

4.3.1 Experiment 1: Long-Term Depression in Embryonic Striatal Grafts

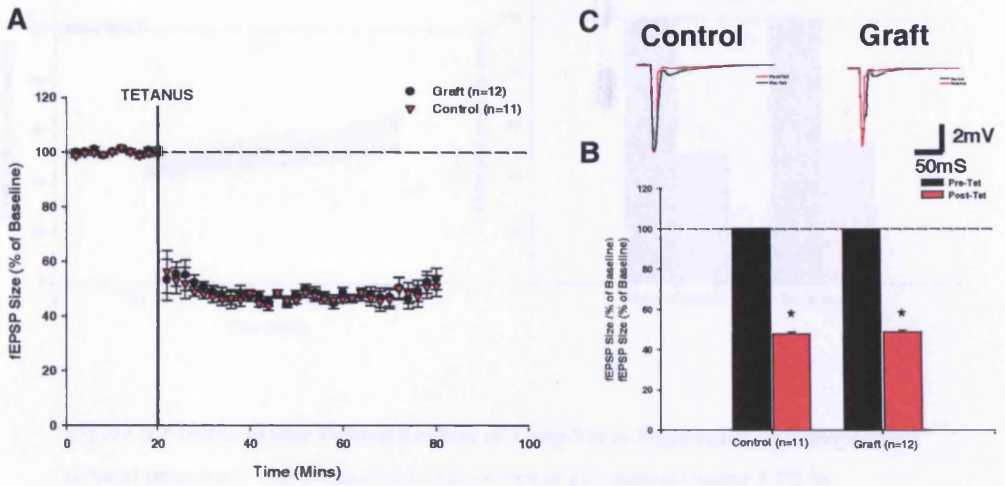

Figure 4.1. Extracellular Demonstration of Long-Term Depression in Embryonic Striatal Grafts. (A) Normalised time-response plot demonstrating LTD in embryonic striatal grafts. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 4.1 demonstrates the effect of HFS on graft and control recordings, under physiologically normal conditions. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between graft and control recordings (Groups, $F_{(1,21)}=2.24$, p=0.14, n.s.). Tetanic stimulation of the host corticostriatal fibres results in an immediate depression in the size of the fEPSP within both control (n=11) 47.90% $\pm$0.77%, and graft (n=12) 48.80% $\pm$0.78% recordings (Fig 4.1a+b). Both control (n=11) and graft (n=12) (Groups vs Time, $F_{(29,609)}=1.50$, p=0.04.) recordings display a significant reduction in the size of the fEPSP when compared to their respective baselines, with no significant difference in the level of depression seen between graft and control recordings (Groups, $F_{(1,21)}=0.68$, p=0.41, n.s.).

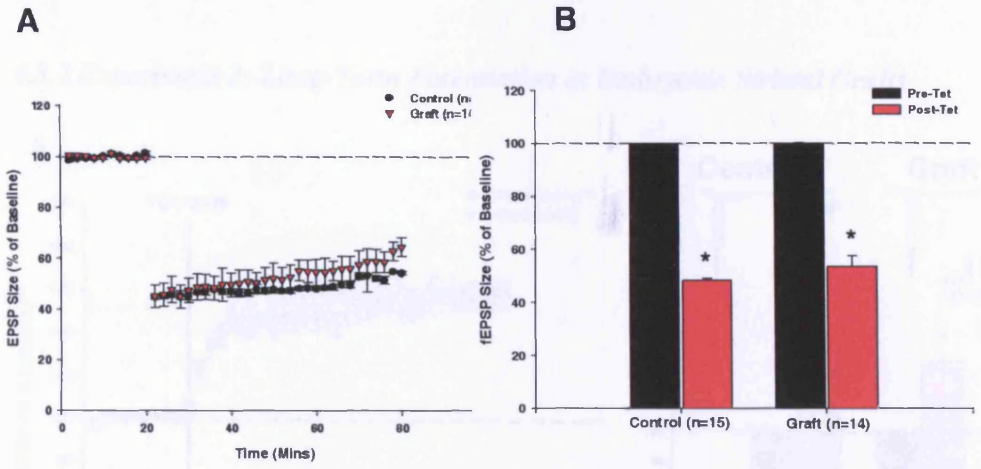

Figure 4.2 Intracellular Demonstration of Long-Term Depression in transplanted striatal neurones. (A) Normalised time-response plot demonstrating LTD in transplanted striatal neurones. Dashed line represents baseline, bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response seen pre- & post-tetanus.

Figure 4.2 demonstrates LTD in TSNs and MSNs during intracellular sharp electrode recordings, under physiologically normal conditions. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between graft and control recordings (Groups, $F_{(1,27)}$=0.014, p=0.90, n.s.). Tetanic stimulation of the host corticostriatal fibres results in an immediate depression in the size of the EPSP within both control (n=15) 48.40% $\pm$0.57%, and graft (n=14) 53.50% $\pm$4.03% recordings (Fig 4.1a+b). Both control (n=15) and graft (n=14) (Groups vs Time, $F_{(29,783)}$=5.03, p=<0.005.) recordings display a significant reduction in the size of the EPSP when compared to their respective baselines, with no significant difference in the level of depression seen between graft and control recordings (Groups, $F_{(1,27)}$=1.19, p=0.28, n.s.).

4.3.2 Experiment 2: Long-Term Potentiation in Embryonic Striatal Grafts

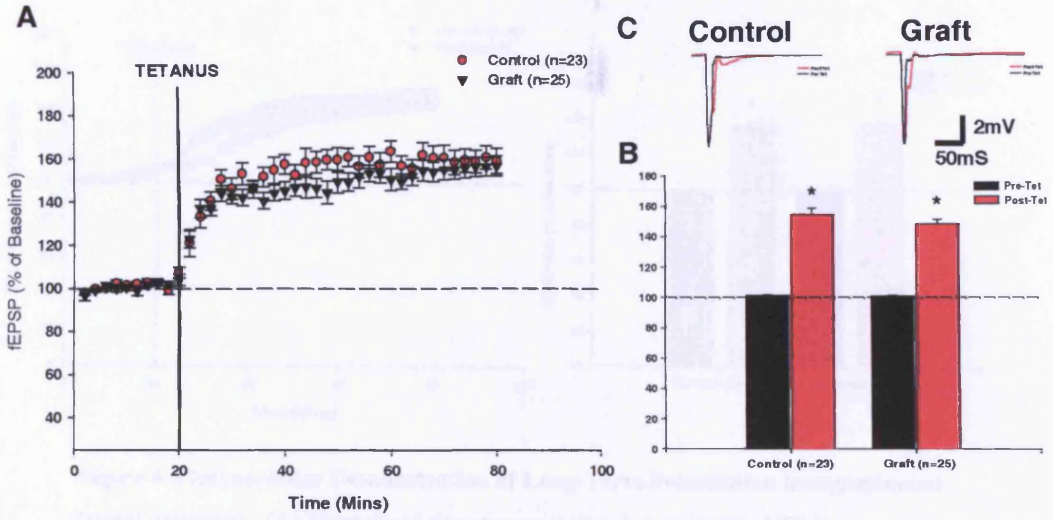

Figure 4.3 Extracellular Demonstration of Long-Term Potentiation in Embryonic Striatal Grafts. (A) Normalised time-response plot demonstrating LTP in embryonic striatal grafts, note that magnesium is omitted from the aCSF perfusate throughout the entire experiment. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 4.3 demonstrates the effect of HFS on graft and control recordings, under conditions where magnesium is omitted from the perfusate solution. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between graft and control recordings (Groups, $F_{(1,44)}$=1.60, p=0.21, n.s.). Tetanic stimulation of the host corticostriatal fibres results in an immediate potentiation in the size of the fEPSP within both control (n=23) 154.69% $\pm$3.78%, and graft (n=25) 148.06% $\pm$3.29% recordings (Fig 4.1a-b). Both control (n=11) and graft (n=12) (Groups vs Time, $F_{(29,1276)}$=12.15, p=<0.005.) recordings display a significant increase in the size of the fEPSP when compared to their respective baselines, with no significant difference in the level of potentiation seen between graft and control recordings (Groups, $F_{(1,44)}$=1.52, p=0.22, n.s.).

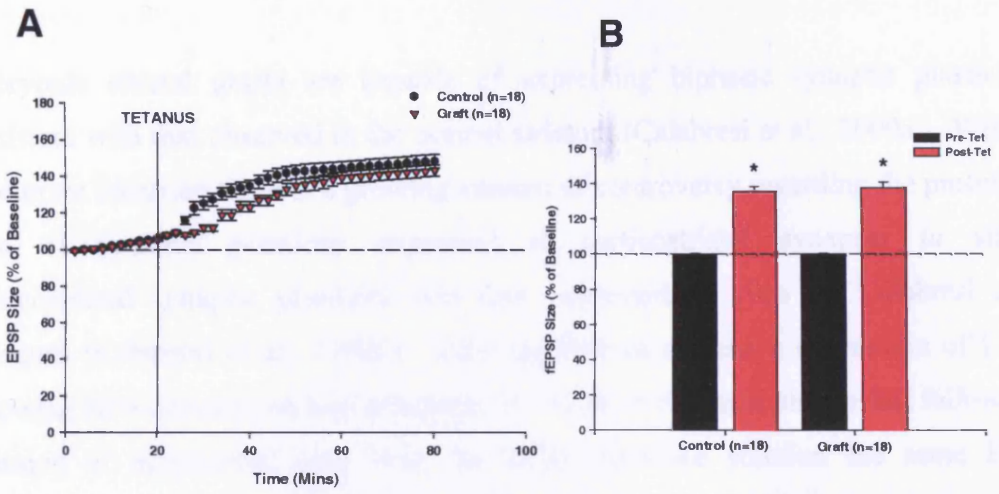

Figure 4.4 Intracellular Demonstration of Long-Term Potentiation in transplanted striatal neurones. (A) Normalised time-response plot demonstrating LTP in transplanted striatal neurones. Dashed line represents baseline, bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response seen pre- & post-tetanus.

Figure 4.4 demonstrates LTP in TSNs and MSNs during intracellular sharp electrode recordings, under conditions where magnesium is omitted from the perfusate solution. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between graft and control recordings (Groups, $F_{(1,34)}$=0.098, p=0.76, n.s.). Tetanic stimulation of the host corticostriatal fibres results in an immediate potentiation in the size of the EPSP within both control (n=18) 135.50% $\pm$2.70%, and graft (n=18) 137.50% $\pm$3.30% recordings (Fig 4.4a+b). Both control (n=15) and graft (n=14) recordings display a significant increase in the size of the EPSP when compared to their respective baselines (Groups vs Time, $F_{(29,986)}$=9.60, p=<0.005.), with no significant difference in the level of potentiation seen between graft and control recordings (Groups, $F_{(1,34)}$=0.21, p=0.64, n.s.).

4.4 DISCUSSION

Embryonic striatal grafts are capable of expressing biphasic synaptic plasticity, consistent with that observed in the normal striatum (Calabresi et al., 2000a). Within the current literature there is a growing amount of controversy regarding the preferred form of synaptic plasticity expressed at corticostriatal synapses *in vitro*. Corticostriatal synaptic plasticity was first expressed in vitro by Calabresi and collegues (Calabresi et al., 1992c). They reported an exclusive expression of LTD following HFS of corticostriatal afferents. However, it was later shown that following omission of magnesium ions from the aCSF perfusate solution the same HFS produced an exclusive expression of LTP (Calabresi et al., 1992e). More recent studies have shown that it is possible to express LTP in vitro following HFS of corticostriatal afferents, under normal physiological conditions (Partridge et al., 2000; Smith et al., 2001; Spencer and Murphy, 2000; Wickens et al., 1996) which is critically dependant on dopaminergic action and independent of NMDA receptor activation (Reynolds and Wickens, 2002).

Within the context of the experiments presented in this chapter, under normal physiological conditions HFS of corticostriatal afferents results in the expression of LTD. Within control recordings, HFS results in a predominant expression of LTD (27 out of 28), with a single case expressing LTP (1 out of 28). Whilst in the absence of magnesium HFS resulted in an exclusive expression of LTP (41 out of 41). Recordings from striatal grafts always express LTD in the presence of magnesium (26 out of 26), and LTP in its absence (43 out of 43). Studies have demonstrated that it is possible to obtain LTP within *in vitro* slices, under normal physiological conditions (Partridge et al., 2000; Smith et al., 2001; Spencer and Murphy, 2000; Wickens et al., 1996). Smith et al, attempt to explain the mechanistic underpinning of this disparity by demonstrating that within the striatum *in vitro* there is a regional specificity in the predominant expression of LTP/LTD (Smith et al., 2001). Using coronally prepared striatal *in vitro* slices, Smith et al demonstrated that recordings taken from the lateral region of the striatum demonstrate a preferential expression of LTD (Smith et al., 2001). Conversely, recordings from the medial region of the striatum demonstrate a preferential expression of the LTP. The most favourable explanation for the observed

regional specificity in the expression of LTP/LTD, is the observation that projections to the striatum organise themselves in a topographic manner. Specifically, it appears that the topographical separation within the cortex is conserved in the corticostriatal projections. Visual and limbic regions of the cortex, have been shown to project to medial regions of the striatum, whilst motor regions project to lateral regions of the striatum (Deniau et al., 1996; Donoghue and Herkenham, 1986; Gerfen, 1989; McGeorge and Faull, 1989). Interestingly, there is a predominant expression of LTP from the striatum *in vivo*, which is attributed to the conservation of dense corticostriatal projections, and intrinsic interneuronal networks. Traditionally, the predominant expression of LTD *in vitro* has been attributed to the loss of the dense corticostriatal projection following tissue sectioning. It has been shown that the expression of LTP *in vitro* is critically dependant on removal of the NMDA receptor magnesium block (Calabresi et al., 1992e). Removal of the magnesium blockade of striatal NMDA receptors requires a large membrane depolarisation, which is provided by the corticostriatal projection. Though HFS of corticostriatal projections may be sufficient to activate NMDA receptors *in vivo*, it may not be sufficient to do the same *in vitro*. As previously stated, different regions of the striatum receive differing topographical origins of corticostriatal innervation (Smith et al., 2001). Within a specific *in vitro* slice the level of cortical innervation of the striatum may be sufficient, under normal physiological conditions, to express LTP in the medial region, though insufficient to do so in the lateral regions. Within the literature, recording sites are rarely defined into medial or lateral regions, thus it is highly plausible that some of the disparities regarding the preferred form of synaptic plasticity observed from corticostriatal synapses *in vitro* could in fact be due to these reported topographical differences. All of the recordings presented in this chapter were obtained from the dorsal lateral striatum, with within the site of transplantation (A= +1.0, L = +1.8, V = 2.5), or within the same location in the normal striatum. This region of the striatum has been shown to receive projections, predominantly, from the motor cortex (Deniau et al., 1996; Donoghue and Herkenham, 1986; Gerfen, 1989; McGeorge and Faull, 1989), and preferentially to express LTD (Smith et al., 2001). However, the experiments presented in this chapter do not provide any further clarification of the mechanisms underlying the regional specificity in striatal LTP/LTD *in vitro*. It can be clearly seen that within control recordings, under physiological conditions, there is a preferential expression of LTD (27 out of 28);

though under conditions where magnesium is removed from the aCSF perfusate solution this switches to a total expression of LTP.

Though the level of cortical innervation preserved within the in vitro slice provides a compelling argument for the preferential expression of LTP/LTD, a number of other factors must be considered. Firstly, due to the proximity of the stimulating electrode to the striatum it is more than likely that current spread across the slice will activate other projection systems, such as the DAergic projection from the SN. Interestingly, differing strengths and intensities of HFS have been shown to produce a mix of plasticities. Intense HFS stimulation of the corpus callosum results in the predominant expression of LTD (Calabresi et al., 1992c). Weaker, less intense HFS of the corpus callosum results in the expression of a mixture of STD, STP, LTD and LTP (Akopian et al., 2000; Calabresi et al., 1992c; Calabresi et al., 1992e; Lovinger et al., 1993). Therefore, the differing intensities of HFS used, and the location of the stimulating electrode may in some way contribute to the differing forms of plasticity observed. Within the experiments presented here, a theta burst stimulation protocol was used to induce synaptic plasticity. Though intense forms of HFS used in other studies produces stable reliable synaptic plasticity within the normal striatum (Calabresi et al., 1992c), attempts to induce plasticity from striatal grafts using this protocol yielded limited results, with very few recordings displaying stable synaptic plasticity. This observation is hardly surprising given the observation that striatal grafts receive a less dense corticostriatal innervation than that seen in the normal striatum (Wictorin, 1992). Therefore, it is highly likely that intense HFS of the limited corticostriatal projection will results in over-stimulation of the pathway. Interestingly, in experiments where a strong HFS protocol is used, a large STD is observed from striatal grafts. In hindsight, this observation fits with the theory that strong HFS over-stimulates the limited corticostriatal inputs to striatal grafts, preventing the expression of LTD.

4.4.1 Striatal Grafts Display Bidirectional Synaptic Plasticity

Beginning at four weeks post-transplantation it is readily possible to readily induce both LTD and LTP within embryonic striatal grafts. Within the normal striatum HFS results in a periodic expression of LTD. Within the experiments presented in this

chapter, HFS induced LTD in 27 out of 40 recordings. Failure to induce LTD was not confined to either particular slices, or particular animals. In many cases recordings from one site within the striatum failed to yield LTD, whilst recordings form other sites with the slice displayed LTD following HFS. Therefore, it would appear that induction of LTD within the normal striatum is critically dependant on the environment in which HFS occurs, and the location of recording. Within embryonic striatal grafts the failure to express LTD following HFS was much higher (26 out of 65) than that seen in control recordings from the normal striatum. Unlike control recordings, moving the location of the recording and stimulating electrode does not always yield LTD. However, this observation is not quite as clear as that seen in the normal striatum and the ability of striatal grafts to express LTD is more random than that seen in control recordings. Therefore, whilst it is clear that obtaining LTD from striatal grafts is much more difficult than within the normal striatum, evidence for the mechanisms underlying this is limited only to electrophysiological observations. Yet, two important factors regarding the integration of grafts into the host striatum provide compelling arguments for these observations.

Following transplantation, functional innervation of the graft is time dependant and varied across the numerous striatal afferents (Wictorin et al., 1988b; Wictorin, 1992). Furthermore, innervation of the graft is not a total process, with differing regions of the graft receiving differing levels of afferent innervation across the post-transplantation period. Anatomical studies have clearly shown that the outer regions of the graft are most densely innervated around four weeks post transplantation, following this period innervation of the graft begins to reach the innermost regions (Wictorin et al., 1988b; Wictorin, 1992). In the context of the experiments presented in this chapter, LTD is first sought from the inner-most regions of the graft, before moving to the other regions should LTD fail to be expressed. Though success is not guaranteed from the outer regions of the graft the probability of gaining LTD is increased. Additionally, it has been shown that within striatal grafts only 50-80% of the corticostriatal afferents form anatomically correct synapses with transplanted striatal neurones (Wictorin et al., 1989; Xu et al., 1989). Though it is not yet known whether the incorrect synapses affect TSN neuronal function, a reduction in the number of functionally normal synapses is likely to affect the ability to induce LTD.

When ascertaining the possible mechanisms for the increased failure to express LTD observed within striatal grafts, it is important to consider the cellular mosaic unique to the graft environment (Graybiel et al., 1989). Many studies have demonstrated, anatomically, that the expression of receptors and proteins indicative of a striatal phenotype, particularly DARPP-32, are exclusively located within the patches or 'P-zones', as opposed to the non-patch or "NP"-zones, of the graft. Electrophysiological studies, conducted *in vivo*, have also demonstrated on a single cell level that almost all neurones within the graft are responsive to host stimulation (Wilson et al., 1990; Xu et al., 1991b; Xu et al., 1991a). Therefore, what is unknown is whether TSNs from the 'NP-zones' respond in a similar electrophysiological fashion to those from the 'P-zones'. One possible way TSNs from 'P-zones' and 'NP-zones' could differ would be in the expression of synaptic plasticity. It is highly plausible that the inability to express LTD in some of the recordings could reflect the proximity of the recording site to 'NP-zones' within the graft. Transplanted cells within the 'NP-zones' have been shown to display a cortical or pallidal phenotype (Wictorin, 1992). Furthermore, afferent connections from the host brain, such as dopaminergic inputs from the substantia nigra, have been shown to preferentially innervate the 'P-zones' (Wictorin, 1992). Therefore, it is logical to assume that although such transplanted cells may express mechanisms of excitatory transmission, they do not display any mechanisms of synaptic plasticity.

Although recordings from striatal grafts display a lower probability of expressing LTD than that seen in control recordings there is no significant difference in the level of depression observed in recordings from striatal grafts when compared to those from the normal striatum. It is important to note however, that on average the size of the fEPSP recorded from striatal grafts is lower than that recorded from the control striatum, and thus although the size of the fEPSP recorded from striatal grafts, post-tetanus, is smaller than that recorded post-tetanus in controls both responses decrease in size by the same proportion following HFS. Although striatal grafts display a lower probability of expressing LTD than the normal striatum, successful expression of LTD occurs to similar levels to that seen within control recordings. Therefore, it would appear that within striatal grafts the host-graft corticostriatal synapse is not only functional to host stimulation, but is also plastic to changes to host stimulation.

As with LTD, it is possible to express LTP from striatal grafts from 4 weeks post-transplantation. One interesting observation is that following removal of magnesium the probability of obtaining synaptic plasticity, in this case LTP, is much higher (43 out of 50) than the probability of obtaining synaptic plasticity, in this case LTD, under normal physiological conditions (26 Out of 65). This proves interesting as it would be expected that the suggested causative factors for the lower probability of expressing synaptic plasticity from striatal grafts under normal physiological conditions would also affect the probability of expressing synaptic plasticity under conditions where magnesium is removed. Assuming such factors do contribute to the ability to express LTP in the absence of magnesium, there are a number of possible reasons for the higher probability of obtaining synaptic plasticity.

Under magnesium free conditions the NMDA receptor is chronically active, and therefore able to contribute to baseline transmission and LTP induction. LTP induction is critically dependant on internal calcium concentrations, which subsequently is critically dependant on calcium flow into the cell. Chronic activation of NMDA receptors results in a large increase in calcium currents into the cell, and therefore increases the cells ability to potentiate following HFS. Furthermore, increased calcium entry into the post-synaptic cell would make the cell more responsive to weaker inputs. Therefore, weak stimulation intensity, may not be sufficient to induce LTD, yet is sufficient to induce LTP, due to the higher potential of the post-synaptic cells to be potentiated following removal of magnesium. This increased potentiation effect can be seen as a positive drift in the baseline values during low frequency baseline recordings, prior to LTP induction. Additionally, it is important to note that a number of studies looking at the physiological properties of TSN, have shown that TSNs display a higher degree of excitability than the MSN of the normal striatum (Siviy et al., 1993). Studies by Sivy et al have demonstrated that one component of higher excitability is attributed to NMDA mediated excitatory responses, which are more excitable in TSNs than MSNs. Therefore, it is possible that an increased NMDA excitability may be contributing to the increased probability of expressing synaptic plasticity in magnesium free conditions.

4.4.2 Synaptic Plasticity and "Learning to use the graft"

A number of studies have demonstrated that the ability to learn motor skills and habits, lost post-lesion, can be restored by transplantation of embryonic striatal grafts (Brasted et al., 1999b; Mayer et al., 1992; Wictorin et al., 1989). Furthermore, this 'new learning', facilitated by embryonic striatal grafts, can be disrupted by delaying transplantation, thereby impairing formation of host-graft corticostriatal synapses (Brasted et al., 2000). Therefore, "learning to use the graft" appears to be critically dependant on functional reconnection between the host cortical afferents and TSNs.

The data presented in this chapter demonstrates that the host-graft corticostriatal synapse is capable of expressing bidirectional synaptic plasticity consistent with that observed in the normal striatum. Within the normal striatum it has been shown that conditions which impair striatal synaptic plasticity also impair behavioural tests of learning and memory, which are heavily dependant on corticostriatal mechanisms of memory (Pittenger et al., 2006). It would appear logical that 'new learning', facilitated by embryonic striatal grafts, would likewise be critically dependant on the expression of host-graft corticostriatal synaptic plasticity.

4.5 CONCLUSION

The findings reported in this chapter propose not only that embryonic striatal grafts can restore cortico-striatal connectivity lost due to the lesion, but that striatal grafts are also capable of expressing bidirectional synaptic plasticity. Both LTP and LTD expressed at host-graft corticostriatal synapses resembles that seen in the normal striatum. However, the probability of expressing synaptic plasticity is lower in striatal grafts, suggesting that the density of host cortical innervation may be lower than that seen in the normal striatum. Nevertheless, the expression of synaptic plasticity from embryonic striatal grafts could provide a cellular mechanism for the behaviourally observed "learning to use the graft" phenomenon, and more generally the substrate for new S-R or habit learning in striatally grafted animals.

5. PHARMACOLOGICAL INVESTIGATION OF HOST-GRAFT SYNAPTIC PLASTICITY

5.1 INTRODUCTION

The previous chapter demonstrates that both embryonic striatal grafts, and TSNs display bidirectional synaptic plasticity in keeping with the normal striatum. Though synaptic plasticity expressed from striatal grafts is similar to that seen in the normal striatum, little is known about the pharmacological mechanisms governing the synaptic plasticity of grafts. The corticostriatal synapse receives a varied heterological range of neurotransmitter inputs from striatal interneurons, cholinergic, for example, and from extrinsic projections from other brain structures, such as dopaminergic afferents from the substantia nigra. Therefore, to demonstrate that striatal grafts functionally integrate into the host striatal circuitry it is vital that graft synaptic plasticity shares similar pharmacological mechanisms as that seen in the ungrafted host.

Conventional staining of striatal grafts with either DARPP-32 or AChE reveals a cellular mosaic, representing two distinct heterogeneous compartments of cells. Regions, or patches, of the graft that stain positive for DARPP-32 or AChE are termed 'P-zones', and refer to cells with morphological and biochemical characteristics of normal striatal cells (Graybiel et al., 1989). Regions of the graft that do not stain positive for DARPP-32 or AChE are termed 'NP-zones' and stain positive for neuronal markers of cortical or pallidal cell types (See the General Introduction for a more detailed description of "P-" and "NP-Zones").

Within the 'P-zones' of striatal grafts it is possible to detect a number of neurotransmitters and neuropeptides found within the normal striatum. Levels of precursor peptides for enkephalin (PPE) and substance P (PPT), are similar to that seen in the normal striatum, where roughly 50% of all neurones express either PPE or PPT (Campbell et al., 1995a). Levels of ChAT, an enzyme involved in the synthesis of acetylcholine, is roughly 40% of that seen in the normal striatum, when quantified across the whole graft volume (Campbell et al., 1995a). However, cells which mark

positive for ChAT are predominantly found within the 'P-zones' where they are densely located (Campbell et al., 1995a). Therefore, it is likely that the levels of ChAT within the 'P-zones' of striatal grafts is similar to that seen in the normal striatum. Levels of GAD67, an enzyme involved in the synthesis of GABA, displays regions of high and low levels of expression within both the normal striatum, and striatal grafts. Correlation of cells which mark positive for GAD67 with those that mark positive for DARPP-32 demonstrate that only 50% of GAD67 positive neurones are also positive for DARPP-32 (Campbell et al., 1995a). Actual levels of GAD67 expressed within striatal grafts shows that regions of low GAD67 expression correlate with regions of DARPP-32 expression, within the 'P-zones'. Within 'NP-zones' levels of GAD67 expression are 2.2-2.6 times higher than that seen in the normal striatum (Campbell et al., 1995a). These cells display morphological characteristics similar to GABAergic cells of the cortex and globus pallidus (Campbell et al., 1995a).

The expression of receptors found within the normal striatum has been quantified in striatal grafts. Expression of D1 and D2 receptors has been well documented (Campbell and Bjorklund, 1995; Campbell et al., 1995a; Pundt et al., 1997; Sirinathsinghji et al., 1994; Torres et al., 1995). In situ hybridization for D1 and D2 receptors demonstrates that both receptor sub-types are present in striatal grafts, though largely confined to the 'P-zone, the area receiving the dopaminergic innervation. Within the normal striatum, almost all of the MSNs express either D1 or D2 receptors. Within the 'P-zones' of striatal grafts, whilst expression of D1 is similar to that seen in the normal striatum, expression of D2 receptors is approximately 13% lower (Campbell et al., 1995a). Furthermore, receptor binding studies have demonstrated increased levels of D1 receptor binding in striatal grafts, whilst there is no change in D2 receptor binding between grafts and lesioned controls (Pundt et al., 1997). Though it must be noted that some studies using PET to monitor D2 receptor binding report a moderate increase in this measure (Torres et al., 1995).

This chapter details experiments conducted to provide pharmacological classification of synaptic plasticity expressed from striatal grafts.

5.2 METHODS

Experiments were performed as described in the general methods chapter, with additional details here:

All control animals in all five experiments were 10-14 weeks of age at time of testing. All grafted animals were 4-6 weeks of age at point of transplantation, 10-14 weeks of age at time of experimentation, with graft a graft age of 6-8 weeks.

5.2.1 Experiment 1: Glutamatergic Characterisation

Animals were divided into two groups: Control APV (n=7), and Graft APV (n=11). For all experiments a zero magnesium aCSF perfusate solution was used, to ensure activation of NMDA receptors. Following an initial 20 minute baseline 30μM APV was applied to the perfusate solution and a second 20 minute baseline was recorded prior to the administration of tetanus.

5.2.2 Experiment 2: GABAergic Characterisation

Animals were divided into two groups: Control BIC (n=9), and Graft BIC (n=8). Throughout all experiments normal aCSF perfusate was used. Following an initial 20 minute baseline 30μM Bicuculline was applied to the perfusate solution and a second 20 minute baseline was recorded prior to the administration of a tetanus.

5.2.3 Experiment 3: Monoamergic Characterisation

Animals were divided into four groups: Control SCH 23390 (n=8), Control Sulpiride (n=8), Graft SCH 23390 (n=7) and Graft SULP (n=7). Throughout all experiments normal aCSF perfusate was used. For the SCH 23390 experiments an initial 20 minute baseline 10μM SCH 23390 was applied to the perfusate solution and a second 20 minute baseline was recorded prior to the administration of a tetanus. For the SULP experiments an initial 20 minute baseline 10μM Sulpiride was applied to the

perfusate solution and a second 20 minute baseline was recorded prior to the administration of a tetanus.

5.3 RESULTS

5.3.1 Experiment 1: Glutametergic Characterisation

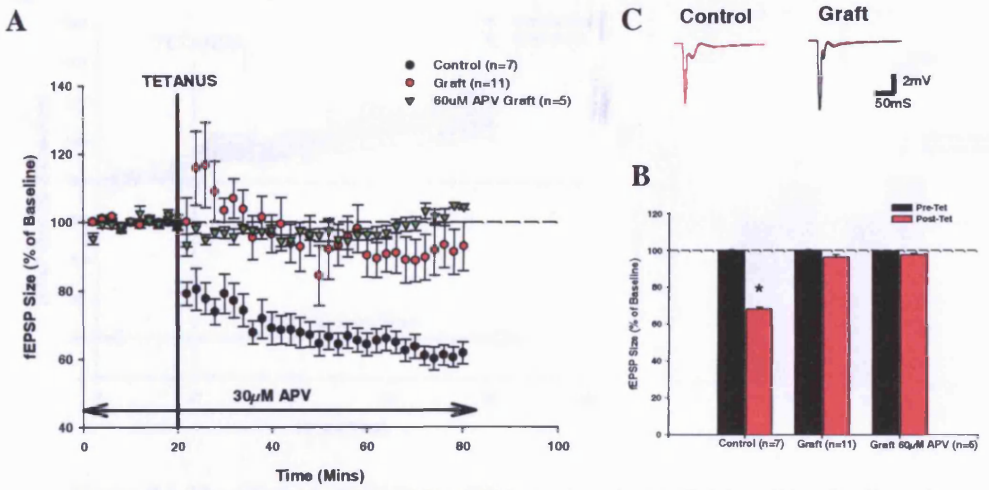

Figure 5.1 The effect of 30μM APV on synaptic plasticity within Embryonic Striatal Grafts. (A) Normalised time-response plot demonstrating the effect of both 30μM APV and tetanic stimulation in embryonic striatal grafts. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 5.1 demonstrates the effect of 30μM APV on graft and control synaptic plasticity. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between graft and control recordings (Groups, $F_{(1,17)}$=0.73, p=0.40, n.s.). Tetanic stimulation of the host corticostriatal fibres within control (n=7) recordings results in an immediate moderate depression of the fEPSP 67.97% ±3.30%. Whilst tetanic stimulation of host corticostriatal fibres within graft (n=11) recordings results in a short lasting (~10 mins) potentiation followed by a gradual depression in the size of the fEPSP within graft recordings (n=11) 96.41% ±4.95%, of baseline (Fig 5.1a+b). Graft recordings (n=11) display no decrease in the size of the fEPSP. Control recordings (n=7) display a reduction in the size of the fEPSP when compared to their respective baselines, with a significant difference in the level of depression seen between graft and control recordings (Groups, $F_{(1,17)}$=19.23, p=<0.005, n.s.). Within graft recordings at 60 μm concentration of APV (n=5), tetanic stimulation of the host corticostriatal fibres results in no change in the size of the fEPSP 97.76% ±0.71%.

5.3.2 Experiment 2: GABAergic Characterisation

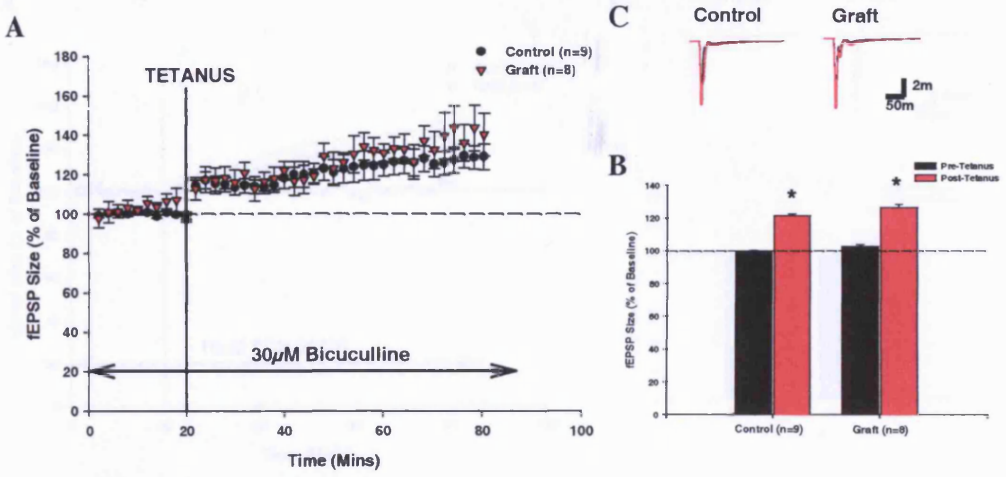

Figure 5.2 The effect of 30μM Bicuculline on synaptic plasticity within Embryonic Striatal Grafts. (A) Normalised time-response plot demonstrating the effect of both 30μM Bicuculline and tetanic stimulation in embryonic striatal grafts. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 5.2 demonstrates the effect of 30μM Bicuculline on graft and control synaptic plasticity. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between graft and control recordings (Groups, $F_{(1,21)}=1.18$, $p=0.28$, n.s.). Tetanic stimulation of the host corticostriatal fibres results in a gradual potentiation of the fEPSP, which increases over time to reach an average of control (n=9) 121.51%±0.98% and graft (n=8) 126.48%±1.73% of baseline over the 60 min post-tetanus period, with no significant difference in the level of potentiation seen between graft and control recordings (Groups, $F_{(1,21)}=1.35$, $p=0.25$, n.s.).

5.3.3 Experiment 3: Monoamergic Characterisation

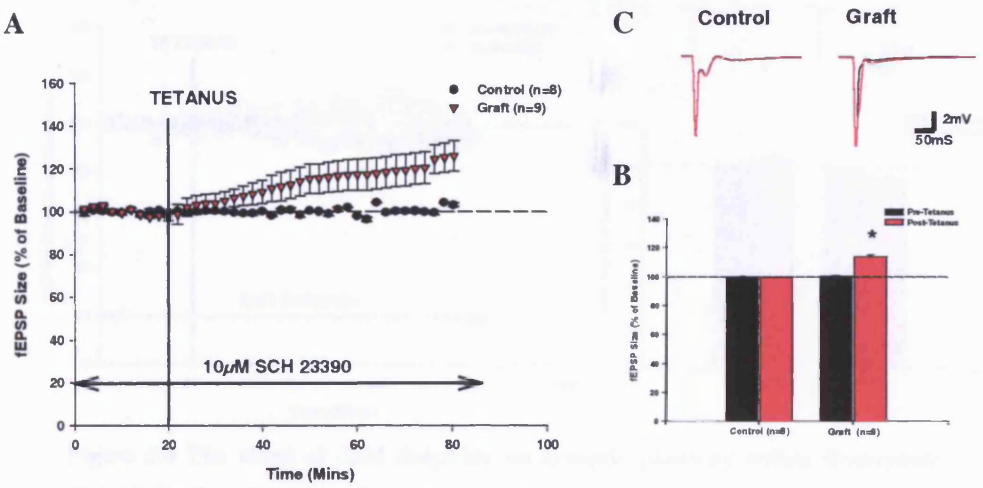

Figure 5.3 The effect of 10μM SCH 23390 on synaptic plasticity within Embryonic Striatal Grafts. (A) Normalised time-response plot demonstrating the effect of both 30μM Bicuculline and tetanic stimulation in embryonic striatal grafts. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 5.3 demonstrates the effect of 10μM SCH 23390 on graft (n=9) and control (n=8) synaptic plasticity. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between graft and control recordings (Groups, $F_{(1,15)}$=2.29, p=0.15, n.s.). Tetanic stimulation of the host corticostriatal fibres results in a gradual potentiation of the fEPSP, which increases over time to reach an average of control (n=8) 99.28%$\pm$0.69% and graft (n=9) 113.47%$\pm$6.09% of baseline over the 60 min post-tetanus period, with a significant difference in the level of potentiation seen between graft and control recordings (Groups, $F_{(1,15)}$=4.68, p=0.046).

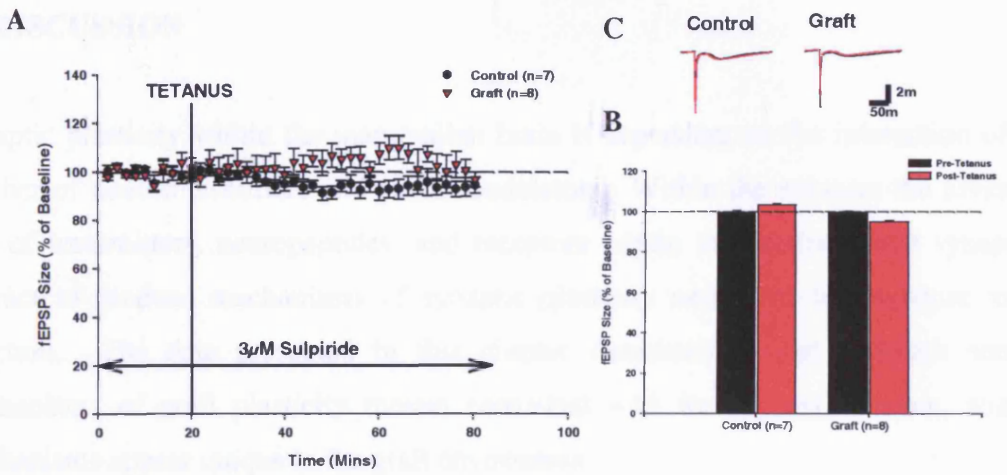

Figure 5.4 The effect of 3μM Sulpiride on synaptic plasticity within Embryonic Striatal Grafts. (A) Normalised time-response plot demonstrating the effect of both 30μM Bicuculline and tetanic stimulation in embryonic striatal grafts. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 5.4 demonstrates the effect of 3μM Sulpiride on graft (n=8) and control (n=7) synaptic plasticity. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between graft and control recordings (Groups, $F_{(1,13)}$=0.01, p=0.91, n.s.). Tetanic stimulation of the host corticostriatal fibres results in no real change in the size of the fEPSP, control (n=7) 96.19%±2.94% and graft (n=8) 105.13%±4.071% of baseline over the 60 min post-tetanus period, with no significant difference in the level of potentiation seen between graft and control recordings (Groups, $F_{(1,13)}$=3.00, p=0.10, n.s.).

5.4 DISCUSSION

Synaptic plasticity within the mammalian brain is dependant on the interaction of a number of neurotransmitters and neuro-modulators. Within the striatum the diverse mix of transmitters, neuropeptides, and receptors within the corticostriatal synapse interact to produce mechanisms of synaptic plasticity unique to this synapse and structure. The data presented in this chapter demonstrates that although some mechanisms of graft plasticity remain consistent with the normal striatum, some mechanisms appear unique to the graft environment.

5.4.1 Glutamatergic Mechanisms of Graft Plasticity

As with baseline transmission, glutamate mediated excitatory transmission represents a critical component of striatal synaptic plasticity. Within the normal striatum the form of synaptic plasticity expressed at the corticostriatal synapse is critically dependant on the combination of ionotropic glutamate receptors activated during HFS of the corticostriatal efferents. Activation of AMPA receptors, without NMDA receptor activation, results in the expression of LTD (Calabresi et al., 1992c). Paradoxically, expression of LTP is dependant on activation of both AMPA and NMDA receptors (Calabresi et al., 1992e). Embryonic striatal grafts have been shown to express similar pharmacological mechanisms of baseline transmission as that seen in the normal striatum (See chapter 3), including similar responses to application of AMPA and NMDA receptor antagonists. Furthermore, embryonic striatal grafts are capable of expressing bidirectional synaptic plasticity, in keeping with the normal striatum (See chapter 4). However, studies have indicated that embryonic striatal grafts display mechanisms of increased excitability, which maybe attributed to the NMDA receptor (Siviy et al., 1993). Yet, though blockade of either NMDA or AMPA receptors produces similar effects in both control and graft recordings, under baseline conditions, the same is not true following HFS of the corticostriatal afferents.

Application of APV in the absence of magnesium, has an interesting effect on synaptic plasticity from embryonic striatal grafts. Under such conditions HFS of the host corticostriatal afferents results in an immediate, short duration potentiation of

graft recordings, which return to baseline conditions within ten minutes. Whilst the mechanism for this apparent disparity is unknown, a number of possible explanations exist. Previous analysis of the AMPA receptor, within embryonic striatal grafts, has shown it to function in a similar manner to that seen in the normal striatum. Therefore, it is likely that the observed effects may be facilitated by the NMDA receptor. Interestingly, APV concentrations of 60μM and above results in a loss of the immediate STP, followed by a further reduction in the size of the fEPSP. This would suggest that within embryonic striatal grafts the NMDA receptor may contribute to the expression of LTD. It has been previously reported that TSNs display a higher level of excitability than MSNs (Siviy et al., 1993). This observation has been attributed to an early developmental age of TSNs, as it has been shown that developing striatal neurones display similar characteristics of increased excitability (Siviy et al., 1991). This increased excitability has been attributed to the NMDA receptor, which has been shown to be regulated throughout development (van Zundert et al., 2004). Within the NMDA receptor complex, whilst the NR1 sub-units remain constant, the NR2 subunits, of which four subtypes exist, are regulated over development and activity (Cull-Candy et al., 2001). It is the NR2 subunits which regulate receptor calcium ion permeability, magnesium ion affinity, and selectivity of antagonists (Anson et al., 2000; Tovar et al., 2000). It has been shown that the NR2 subunit is regulated over development. Studies have shown a higher incidence of NMDA receptors containing the NR2B subunit within the embryonic brain, which gradually decreases post-natal, switching to a higher incidence of NMDA receptors expressing the NR2A subunit that is more common in adults (Monyer et al., 1994). The data presented within this chapter demonstrates that within embryonic striatal grafts twice the concentration of APV is required to antagonise the NMDA receptors to produce a switch in expression of LTP to LTD, as seen in the normal striatum under conditions where magnesium is not present. It is important to note that the antagonist selectivity is regulated by the NR2 sub-unit (Laube et al., 1997). Furthermore, it has been shown that APV displays a higher selectivity for NR2A subunits over NR2B (Laube et al., 1997). Therefore, it is plausible that the higher concentrations of APV needed to produce similar electrophysiological effects as that seen in the normal striatum could indicate that NMDA receptors from embryonic striatal grafts show a higher incidence of the NR2B subunit over the NR2A. Also, it has been shown that the NR2B subunit has a higher calcium permeability than the

NR2A (Monyer et al., 1994), which could account for the reported higher excitability observed from striatal graft recordings (Siviy et al., 1993). Regardless, the findings reported here would suggest that embryonic striatal grafts demonstrate high levels of NR2B subunits at four weeks post-transplantation, which would suggest that TSNs retain some electrophysiological characteristics of an immature neuronal phenotype.

5.4.2 GABAergic Mechanisms of Host-Graft Synaptic Plasticity

Application of the GABA receptor antagonist bicuculline, in the presence of magnesium, results in the expression of LTP from both graft and control recordings following HFS. Under normal conditions, HFS in the presence of magnesium results in the expression of LTD. The mechanisms underlying this switch in expression of LTP to LTD are not clearly understood. It is plausible that blockade of GABAergic inhibition enables the post-synaptic MSNs and TSNs to be sufficiently depolarised so that pre-synaptic stimulation is sufficient to activate NMDA receptors resulting in the expression of LTP. Interestingly, the level of potentiation observed following GABAergic blockade is less than that seen under conditions where LTP is induced in the absence of magnesium. If inhibition of GABAergic transmission enables the expression of LTP, it would be expected that similar levels of LTP would be expressed as that seen in zero magnesium conditions. However, it is worth noting that bicuculline is selective to $GABA_A$ receptors over $GABA_B$, leaving $GABA_B$ receptors active (Bowery et al., 2002). Therefore, it could be essential that for total expression of LTP both $GABA_A$ and $GABA_B$ receptors must be antagonised. It is important to note that following application of bicuculline the mean size of the fEPSP increases to roughly 130% of baseline. Thus it is plausible that under such conditions HFS increases the size of the fEPSP to maximal levels prior to reaching the level of potentiation seen under zero magnesium conditions.

Interestingly, the data demonstrate that, under conditions where $GABA_A$ receptors are inhibited by bicuculline, HFS produces similar levels of LTP in both control and graft recordings. This would suggest that GABAergic mechanisms of synaptic plasticity, modulated by $GABA_A$ receptors, is similar in graft and control recordings.

5.4.3 Monoamergic Mechanisms of Host-Graft Synaptic Plasticity

The data presented within this chapter demonstrates that monoamergic mechanisms of host-graft synaptic plasticity are different to that observed within the normal striatum. Application of sulpiride, a D2 receptor antagonist has been shown to have no effect on either graft or control baseline transmission (see chapter 3, Fig 3.9). However, following HFS, whilst control recordings display no change in fEPSP size, recordings from striatal grafts demonstrate a gradual potentiation. Within the normal striatum it has been shown that application of sulpiride, followed by HFS, has no effect on fEPSP size, thus eliminating the expression of either LTP or LTD (Calabresi et al., 1992b). The results presented here confirm the observation that within the normal striatum application of sulpiride blocks the expression of both LTP and LTD.

Interestingly application of sulpiride, followed by HFS, to striatal grafts results in the expression of LTP, although it must be noted that the level of LTP observed is lower than that seen in either control or graft recordings under conditions where magnesium is omitted from the perfusate solution. Interestingly, similar levels of LTP have been observed in recordings from animals which have received unilateral 6-OHDA lesions (Calabresi et al., 1992b). Such lesions ablate the dopaminergic inputs to the striatum, resulting in a pronounced loss of DA within the striatum (Calabresi et al., 1992b). Recordings from DA depleted slices demonstrate a complete lack of striatal LTP or LTD following HFS of the corticostriatal afferents (Calabresi et al., 1992b). However, in the presence of sulpiride and DA, HFS results in the expression of LTP (Calabresi et al., 1992b). Therefore it seems essential that expression of LTP, following blockade of D2 receptors, requires activation of other DA receptors. Further studies conducted within D2 receptor knock out mice demonstrate that following HFS LTP is expressed from the corticostriatal synapse (Calabresi et al., 1997c). As would be expected application of sulpiride has no effect on the expression of LTP from D2 knockout mice (Calabresi et al., 1997c). Furthermore application of the D1 receptor antagonist SCH 23390 has no effect on the expression, or level of, LTP observed following HFS (Calabresi et al., 1997c). Therefore it would seem that within the normal striatum activation of both D1 and D2 receptors is essential for the expression of LTD, whilst inhibition of D2 receptors is sufficient for the expression of LTP, which is independent of D1 receptor activation.

The data presented within this chapter demonstrates that in the presence of sulpiride HFS results in the expression of LTP from graft recordings, whilst control recordings display neither LTP nor LTD. Interestingly, the level of LTP observed from striatal grafts closely resembles those reported by Calabresi et al in the DA depleted and D2 knockout striatal recordings (Calabresi et al., 1992b; Calabresi et al., 1997c). The similarity in these observations suggest several features of graft function and connection. Firstly, the similarity in expression of LTP between graft recordings and those from the DA depleted striatum proves interesting. In the dopamine depleted studies conducted by Calabresi et al, a unilateral 6-OHDA lesion of the SN, resulted in a near total loss of DAergic cells from the SN, with a similar total loss of DAergic terminals from the striatum (Calabresi et al., 1992b). Within the lesion model of HD used throughout the experiments presented within this thesis, it has been shown that post-lesion there is a moderate degeneration of the DAergic fibres from the host SN (Wictorin et al., 1988b; Wictorin, 1992). Yet, it has been shown that embryonic striatal grafts contain fibres from the host SN that stain positive for TH, and which correspond with the areas containing the striatal-like "P-zones" within the grafts (Wictorin et al., 1988a). Furthermore, it has been shown that TSNs form anatomically appropriate synaptic contacts with the host DAergic fibres from the SN (Clarke et al., 1988b; Wictorin, 1992). The data presented within this chapter demonstrates that pharmacological blockade of D2 receptors, and administration of HFS, results in the expression of LTP from striatal graft recordings. This clearly demonstrates that following transplantation, DAergic fibres from the SN make synaptic contacts with TSNs to form functional connections between the host SN and the graft. Within the DA depleted striatum, the sulpiride induced expression of LTP requires the co-application of DA (Calabresi et al., 1992b), which is not required for the sulpiride induced expression of LTP from striatal grafts. This would be expected as application of DA to slices from the 6-OHDA lesioned striatum is presumably required to activate other DA receptors, where the functional connection observed within striatal grafts provides intrinsic DA sufficient to activate receptors.

The sulpiride dependant expression of LTP within striatal grafts poses an interesting question regarding functional connections between striatal grafts and the host dopaminergic network. Functional connection is clearly plausible since the DAergic

projections are able to release DA, and DA receptors located on TSNs are responsive to application of antagonists. However, the sulpiride dependant expression of LTP within striatal grafts would suggest that receptor mechanisms observed within striatal grafts may be somewhat similar to that seen within the DA depleted striatum. A possible explanation for this observed similarity would be dopamine receptor supersensitivity following lesion. Firstly, it is know that following 6-OHDA lesions the dopamine receptors located on MSNs become sensitised to the application of DA (Arnt, 1985). This supersensitivity has been suggested to be regulated via increased cAMP-dependant signalling (Gnanalingham et al., 1995; Missale et al., 1989). Furthermore, it has been shown that cAMP dependant signalling, involving CAMKII, mediates serine phosphorylation of NMDA NR2 subunits (Kotter, 1994; Omkumar et al., 1996), augmenting NMDA receptor currents (Rostas et al., 1996). Interestingly, it has been shown that DA mediated phosporylation of NMDA receptors is increased in 6-OHDA lesioned animals (Oh et al., 1999). This, in turn, suggests that dopamine receptor supersensitivity results in enhanced glutamatergic excitatory transmission.

Within the context of the experiments presented within this chapter, it is plausible that the sulpiride induced expression of LTP observed within grafts could be due to D1 receptor supersensitivity. The observation that NMDA receptor efficacy plays a major role in determining the direction of synaptic plasticity induced at the corticostriatal synapse, via HFS, is pertinent in this observation, as enhanced NMDA receptor efficacy as a result of receptor supersensitivity could result in a preferential switch from LTD to LTP, as seen in figure 5.3.

One would expect that dopamine receptor supersensitivity would result in the supersensitisation of both D1 and D2 receptors. The data presented in this chapter demonstrates HFS in the presence of SCH 23390, a D1 receptor antagonist, results in a lack of LTP/LTD expression (Fig 5.4). Interestingly, other studies have demonstrated that behavioural expression of dopamine receptor supersensitivity is confined to D1 receptor activation (Cai et al., 2002). Furthermore, D2 receptors have been shown to negatively couple to cAMP dependant signalling (McAllister et al., 1995; Onali et al., 1985; Potenza et al., 1994; Tang et al., 1994b; Tang et al., 1994a). It is more than likely that D2 receptor supersensitivity would negatively affect serine phosphorylation of NMDA receptors, resulting in decreased NMDA receptor efficacy.

Though it would be expected that in the absence of LTP, LTD would be expressed, it has been shown that expression of LTD requires co-activation of both D1 and D2 receptors (Calabresi et al., 1992b). Therefore, one would expect the total lack of expression of synaptic plasticity observed.

The monoaminergic influences on host-graft plasticity would suggest an interesting mechanism underlying connection of monoamergic circuitry in the grafted striatum. The observation that dopaminergic regulated plasticity is similar to that seen in 6-OHDA lesion models, following application of DA, would suggest a similarity in the receptor mechanisms observed between the two lesion models. Whilst dopamine receptor sensitivity provides a logical hypothesis for the observed phenomenon within the striatal lesion and graft model, it is hard to ignore the relevance of the developmental age of the tissue transplanted into the lesioned striatum. It has been theorised that enhanced glutamatergic transmission within striatal grafts is a result of increased glutamatergic receptor efficacy. Whilst it has not been demonstrated it is possible that dopaminergic receptors may display similar mechanisms of increased efficacy due to developmental age. However, it has been demonstrated that D1 receptors within striatal grafts display increased levels of receptor binding (Pundt et al., 1997), therefore suggesting that D1 receptor sensitivity within striatal grafts is responsible for sulpiride dependant switch in expression of LTP over LTD.

Finally, it is important to consider a potential time course effect. It has been shown that DA fibres from the nigra can take up to 6 months to fully innervate the graft region. Within the post-transplantation periods studied in the experiments presented in this chapter, it is likely that dopaminergic innervation of the graft is at an early stage. Therefore, supersensitive TSNs are only just receiving a new DA input with insufficient time to compensate back to normal following the lesion induced denervation.

5.5 CONCLUSION

The findings presented in this chapter propose that although embryonic striatal grafts restore synaptic plasticity to the lesioned striatum, the pharmacological mechanisms of host/graft synaptic plasticity are different to that seen in the normal striatum. Specifically, NMDA mediated and D2 mediated mechanisms of host/graft plasticity differ significantly from that seen in the normal striatum. The differences in NMDA mediated mechanisms of synaptic plasticity between striatal grafts and the normal striatum are hypothesised to be due to a higher expression of NR2B subunits with embryonic striatal grafts. Therefore, it is plausible that during development post-transplantation, TSNs retain some phenotypes of an embryonic development age. The differences in D2 mediated mechanisms of host/graft plasticity are suggested to be due to functional connection between striatal grafts, and the host DAergic projection from the SN, resulting in supersensitisation of D2 receptors.

6. ENVIRONMENTAL ENRICHMENT AND GRAFT PHYSIOLOGICAL AND ANATOMICAL PLASTICITY

6.1 INTRODUCTION

Environmental enrichment refers to measures taken to enhance the sensory and motor experiences of experimental animals. Though studies involving enrichment do not conform to any form of standardised protocol, nearly all studies utilise manipulations of the animals' environment in order to increase social interaction, exploratory behaviour, and exercise levels. Common environmental manipulations taken in order to enrich the environment in which animals inhabit include increasing the number of animals housed in a single cage, increasing the cage size, and introduction of novel items. What is remarkable is that such environmental manipulations can impinge on neuronal development and function.

Hebb first introduced the concept that the behavioural experience, acquired throughout ones life, can modify or promote activity dependant changes in neuronal function (Hebb , 1949). Pioneering experiments by Rosenweig demonstrated that environmental enrichment produces both structural changes in the brain, such as increased cortical thickness, and morphological changes, including increased soma size, dendritic branching and density of dendritic spines (Rosenzweig et al., 1967). Later experiments have demonstrated that environmental enrichment can also impact positively upon a number of cognitive and motor tasks (Fernandez-Teruel et al., 1997; Tees, 1999; Xerri et al., 1996). The observed enhancement of behavioural performance is considered to be dependant on morphological, physiological, and biochemical changes, induced by the enriched environment.

Environmental enrichment has been shown to induce increased expression of a number of genes encoding for proteins which play a key role in neuronal development and function (Rampon and Tsien, 2000; Rampon et al., 2000). There is an up regulation in expression of key genes involved in synaptic transmission and plasticity (Rampon et al., 2000), and expression of trophic factors (Pham et al., 1999).

Furthermore, it has been shown that environmental enrichment can impact upon neuronal transmission (Foster et al., 1996; Irvine and Abraham, 2005). Studies conducted within the hippocampus have further demonstrated that environmental enrichment can enhance synaptic plasticity expressed from the CA1 region (Artola et al., 2006; Duffy et al., 2001), whilst also positively effecting LTP induction (Artola et al., 2006).

The adult brain is at its most plastic during development and following injury, and these initial observations lead to studies looking at the effect of environmental enrichment in animal models of HD and cell transplantation. Environmental enrichment has been shown to increase behavioural performance on tasks which rely on the striatal hemisphere controlled by the graft (Dobrossy et al., 2000; Dobrossy and Dunnett, 2001; Dobrossy and Dunnett, 2003; Dobrossy and Dunnett, 2004; Dobrossy and Dunnett, 2005). Furthermore, it has been demonstrated that grafts in animals housed in enriched environments display bigger DARPP-32 positive cell soma, and receive denser innervation from the host DAergic projection system (Dobrossy and Dunnett, 2004). Studies have also shown that grafts within animals housed in enriched environments demonstrate an increase in brain-derived neurotrophic factor (BDNF) (Dobrossy and Dunnett, 2004). Similar increases in BDNF levels have been seen in both control animals (Neeper et al., 1996), and HD transgenic mice (Hockly et al., 2002; Spires et al., 2004), where it has been shown to slow disease progression (Hockly et al., 2002; Spires et al., 2004).

Therefore, it would seem that environmental enrichment can enhance behavioural performance through anatomical, biochemical and physiological mechanisms. Though it has been clearly demonstrated that environmental enrichment can influence graft mediated behavioural performance, anatomical characteristics, and biochemical properties it is still unclear whether enrichment can influence physiological properties such as synaptic plasticity expressed from striatal grafts.

Therefore, the aim of this chapter is to identify the effect of environmental enrichment on synaptic plasticity within the normal striatum and striatal grafts.

6.2 METHODS

Experiments were performed as described in the general methods chapter, with additional details here:

Enrichment Protocol

Upon arrival, animals were divided into enriched and non-enriched housing. Animals housed within enriched environments were housed in groups of six, within a 40 x 25 x 12cm sized cage. The cage was equipped with a number of novel toys, and environmental stimuli designed to induce increased social interaction and exploratory behaviour (Bio-Serv, NJ, USA). Additionally all enriched environment cages contained a horizontal mouse running wheel, designed to provide the animals with free access to exercise (Bio-Serv, NJ, USA).

Animals housed within non-enriched environments were housed in groups of two, within a 30 x12 x 12cm sized cage. The cage was devoid of any environmental stimuli bar the standard mouse housing medium (a single cardboard tube) required under Home Office guidelines. Both enriched and non-enriched animals were given free access to the standard laboratory animal food and water, *ad libitum*, and housed within the same room with the standard light:dark cycle, temperature, and humidity settings (as per General Methods).

Animals remained housed within their respective environments, during all surgery procedures, and until the point of sacrifice for experimentation.

6.2.1 Experiment 1: The Effect of Enrichment on LTP/LTD from the Normal Striatum.

Animals were divided into two groups. Standard Control (n=13), animals were 10-14 weeks of age at the time of experiment, and housed in a standard environment from 4 weeks of age. Enriched Control (n=15), animals were 10-14 weeks of age at the time of experiment, and house in an enriched environment from 4 weeks of age. For LTD

experiments a normal aCSF perfusate was used. For LTP experiments magnesium was omitted from the aCSF perfusate.

6.2.2 Experiment 2: The Effect of Enrichment on LTP/LTD from Striatal Grafts.

Animals were divided into two groups. Standard Graft (n=14), host animals were 6-8 weeks of age at the point of transplantation, 10-14 weeks of age at the time of experiment, and housed in a standard environment from 4 weeks of age. Enriched Graft (n=20), host animals were 6-8 weeks of age at the point of transplantation, 10-14 weeks of age at the time of experiment, and housed in an enriched environment from 4 weeks of age. For LTD experiments a normal aCSF perfusate was used. For LTP experiments magnesium was omitted from the aCSF perfusate.

6.2.3 Experiment 3: The Effect of Enrichment on Synaptic Plasticity from the Normal Hippocampus.

Animals were divided into two groups. Standard Control (n=9), animals were 10-14 weeks of age at the time of experiment, and housed in a standard environment from 4 weeks of age. Enriched Control (n=10), animals were 10-14 weeks of age at the time of experiment, and house in an enriched environment from 4 weeks of age. For all experiments a normal aCSF perfusate was used. For all hippocampal experiments a HFS protocol consisting of 3 trains, each train consisting of 10 bursts at 5Hz and each burst consisting of 4 pulses at 100Hz, within an inter-train interval of 10s.

6.2.4 Experiment 4: Assessing the Levels of BDNF Following Enrichment.

Three animals from each group (Standard Control, Enriched Control, Standard Graft, Enriched Graft) were sacrificed via cervical dislocation, decapitated, and their brains quickly removed and submersed in ice cold aCSF, containing 1mM Kynurenic acid. Brain were hemisected, and the left hemisphere, containing the striatal graft, was stored for preparation of *in vitro* slices for electrophysiology (as per General Methods). The right hemisphere was quickly dissected, and samples of the Cortex, Striatum, Hippocampus, and Cerebellum were taken, and stored at -80°C in

preweighed Eppendorf tubes. The tubes containing the tissue samples were weighed to determine the weight of the sample. The levels of BDNF present in the tissue were ascertained by following the protocol for the enzyme-linked immunosorbent assay (ELISA) method using E_{max} ImmunoAssay System (Promega, Madison WI, USA). The tissue was homogenized and centrifuged and the supernatant fluid, containing the extracted BDNF, was aliquoted in duplicate into 96-well plate precoated with antiBDNF monoclonal antibody. Following blocking to reduce non-specific reactions, the capture BDNF was incubated with 1:500 antihuman BDNF polyclonal antibody (Promega, Madison WI, USA). The specifically bound polyclonal antibody was detected using anti IgY conjugated with horseradish peroxidase antibody which, when incubated with a chromogenic substrate, resulted in a colour change proportional to the amount of BDNF present. The absorbance of the colour development was measured in a microplate reader at 450nm wavelength to allow quantification of the BDNF concentrations present in the test samples based on the absorbance from the standardised samples of known concentrations. The concentrations in each sample were determined in duplicate and the mean value was used for analysis.

6.3 RESULTS

6.3.1 Experiment 1. The Effect of Enrichment on LTP/LTD from the Normal Striatum.

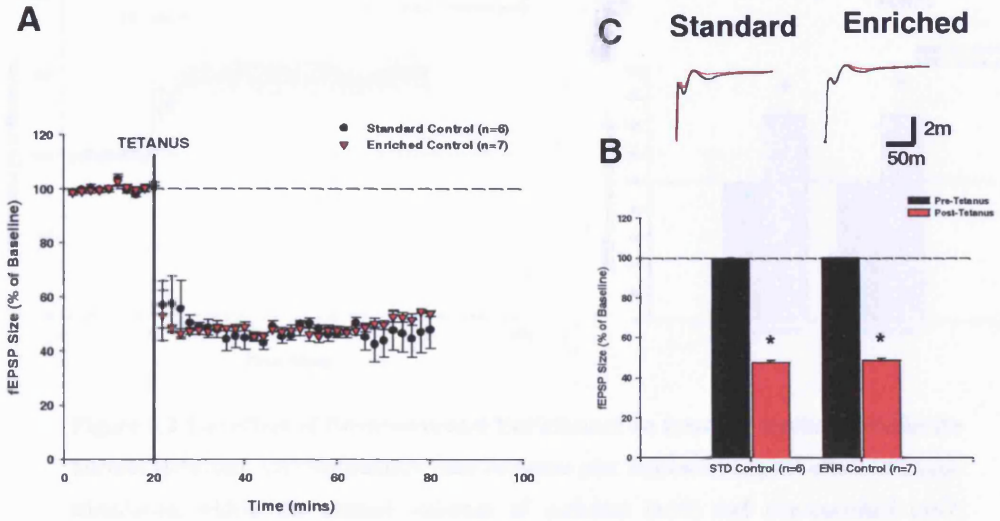

Figure 6.1 The effect of Environmental Enrichment on synaptic plasticity within the normal striatum. (A) Normalised time-response plot demonstrating the effect of tetanic stimulation within the normal striatum of enriched (n=7) and non-enriched (n=6) animals. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 6.1 demonstrates the effect of tetanic stimulation on synaptic plasticity from the enriched (n=7) and non-enriched (n=6) normal striatum, under physiologically normal conditions. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between enriched (n=7) and non-enriched (n=6) recordings (Groups, $F_{(1,11)}=0.24$, $p=0.62$, n.s.). Tetanic stimulation of the host corticostriatal fibres results in an immediate depression in the size of the fEPSP, enriched (n=7) 52.39%$\pm$7.13% and non-enriched (n=6) 51.35%$\pm$4.67% of baseline over the 60 min post-tetanus period, with no significant difference in the level of depression seen between graft and control recordings (Groups, $F_{(1,11)}=0.39$, $p=0.54$, n.s.).

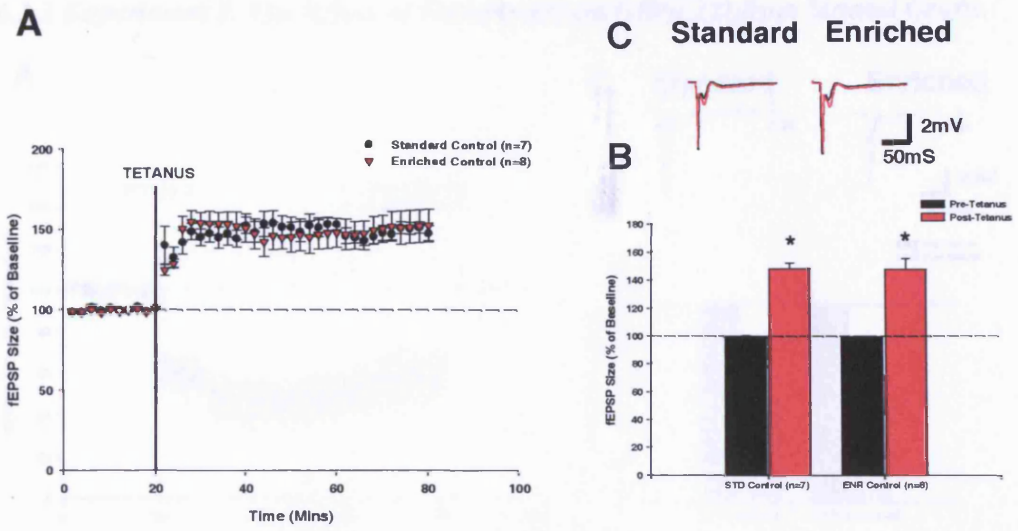

Figure 6.2 The effect of Environmental Enrichment on synaptic plasticity within the normal striatum. (A) Normalised time-response plot demonstrating the effect of tetanic stimulation within the normal striatum of enriched (n=8) and non-enriched (n=7) animals, under conditions where magnesium is omitted from the perfusate solution. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 6.2 demonstrates the effect of tetanic stimulation on synaptic plasticity from the enriched (n=8) and non-enriched (n=7) normal striatum, under conditions where magnesium is omitted from the perfusate solution. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between enriched (n=8) and non-enriched (n=7) recordings (Groups, $F_{(1,11)}=0.10$, p=0.75, n.s.). Tetanic stimulation of the host corticostriatal fibres results in an immediate potentiation in the size of the fEPSP, enriched (n=8) 51.35%$\pm$4.67% and non-enriched (n=7) 52.39%$\pm$7.13% of baseline over the 60 min post-tetanus period, with no significant difference in the level of potentiation seen between graft and control recordings (Groups, $F_{(1,13)}=3.00$, p=0.10, n.s.).

6.3.2 Experiment 2. The Effect of Enrichment on LTP/LTD from Striatal Grafts.

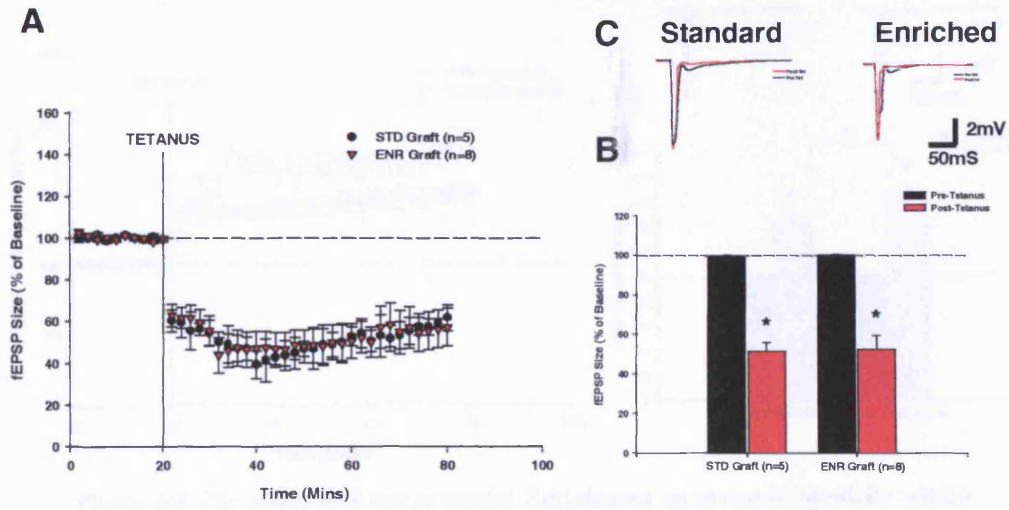

Figure 6.3 The effect of Environmental Enrichment on synaptic plasticity within embryonic striatal grafts (with Mg^{2+}). (A) Normalised time-response plot demonstrating the effect of tetanic stimulation within the embryonic striatal grafts of enriched (n=8) and non-enriched (n=5) animals. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 6.3 demonstrates the effect of tetanic stimulation on synaptic plasticity from the enriched (n=8) and non-enriched (n=5) embryonic striatal grafts, under physiologically normal (with magnesium) conditions. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between enriched (n=8) and non-enriched (n=5) recordings (Groups, $F_{(1,11)}=0.01$, p=0.91, n.s.). Tetanic stimulation of the host corticostriatal fibres results in an immediate depression in the size of the fEPSP, enriched (n=8) 52.39%$\pm$7.13% and non-enriched (n=5) 51.35%$\pm$4.67% of baseline over the 60 min post-tetanus period, with no significant difference in the level of potentiation seen between graft and control recordings (Groups, $F_{(1,11)}=0.10$, p=0.91, n.s.).

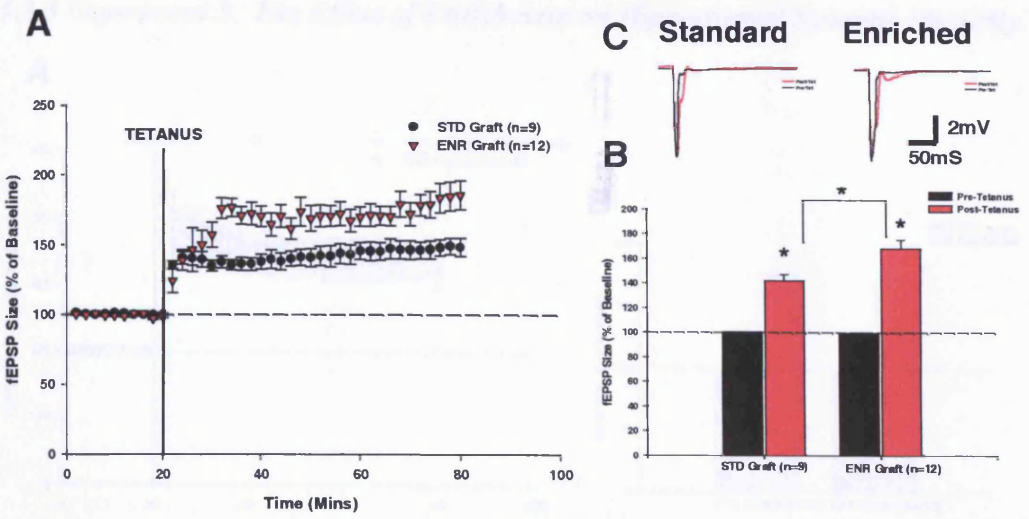

Figure 6.4 The effect of Environmental Enrichment on synaptic plasticity within embryonic striatal grafts (without Mg^{2+}). (A) Normalised time-response plot demonstrating the effect of tetanic stimulation within the normal striatum of enriched (n=12) and non-enriched (n=9) animals, under conditions where magnesium is omitted from the perfusate solution. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 6.4 demonstrates the effect of tetanic stimulation on synaptic plasticity from the enriched (n=12) and non-enriched (n=9) embryonic striatal grafts, under conditions where magnesium is omitted from the perfusate solution. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between enriched (n=12) and non-enriched (n=9) recordings (Groups, $F_{(1,19)}=2.97$, $p=0.10$, n.s.). Tetanic stimulation of the host corticostriatal fibres produces an immediate potentiation of the fEPSP in both enriched (n=12) 168.52%$\pm$6.79% and non-enriched (n=9) 142.19%$\pm$5.21% recordings, with enriched (n=12) recordings potentiating to a significantly higher degree than non-enriched (n=9) recordings (Groups, $F_{(1,19)}=8.41$, $p=0.009$, n.s.).

6.3.3 Experiment 3. The Effect of Enrichment on Hippocampal Synaptic Plasticity.

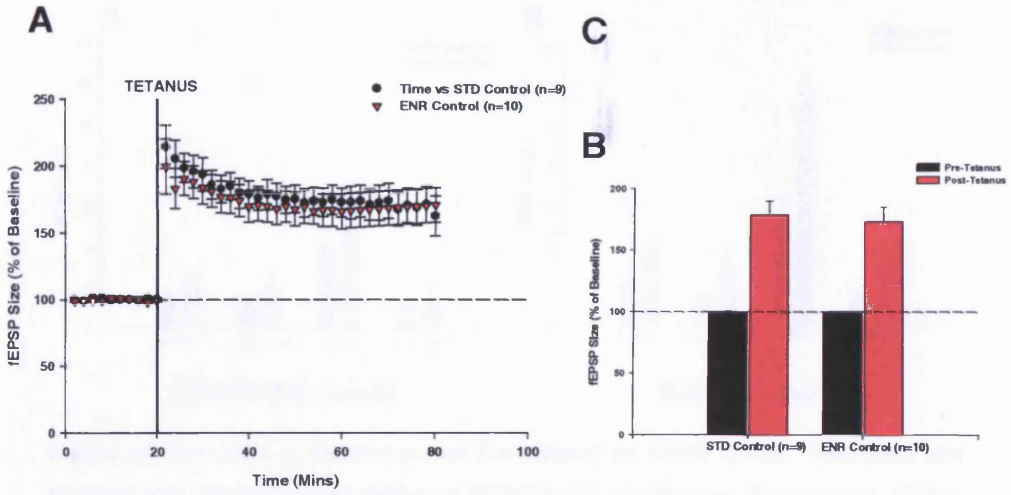

Figure 6.5 The effect of Environmental Enrichment on synaptic plasticity within the CA1. (A) Normalised time-response plot demonstrating the effect of tetanic stimulation within the CA1 of enriched (n=10) and non-enriched (n=9) animals, under conditions where magnesium is omitted from the perfusate solution. Dashed line represents baseline. Bold vertical line represents point of tetanus (See General Methods). (B) Histogram plot of the mean normalised response size seen pre- & post-tetanus. (C) Example fEPSP traces of both groups pre- & post-tetanus.

Figure 6.5 demonstrates the effect of tetanic stimulation on synaptic plasticity from the enriched (n=10) and non-enriched (n=9) CA1 region of the hippocampus, under physiologically normal conditions. All data points are normalised to their respective baseline periods. There is no significant difference in the normalised responses during the baseline period, between enriched (n=10) and non-enriched (n=9) recordings (Groups, $F_{(1,17)}=1.56$, p=0.22, n.s.). Tetanic stimulation of the CA1 produces an immediate potentiation of the fEPSP in both enriched (n=10) 172.89%$\pm$11.94% and non-enriched (n=9) 178.88%$\pm$10.64% recordings, with no significant difference in the level of potentiation seen between enriched (n=10) and non-enriched (n=9) recordings (Groups, $F_{(1,17)}=0.137$, p=0.71, n.s.).

6.3.4 Experiment 4. Assessing the Levels of BDNF Following Enrichment.

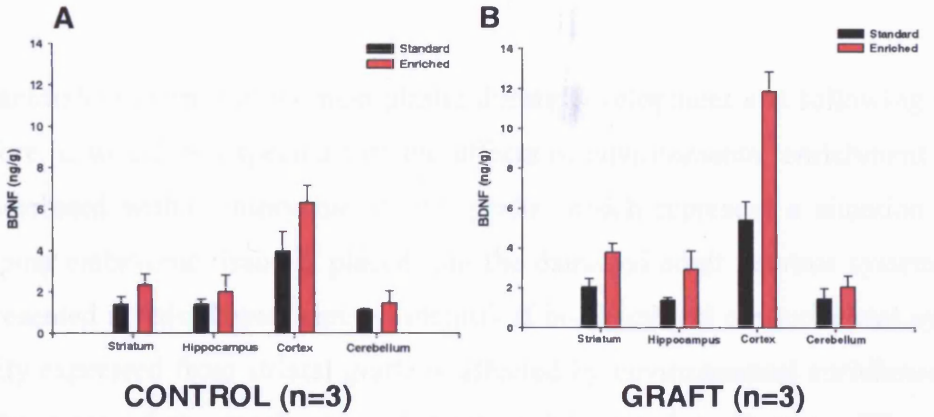

Figure 6.6 The effect of Environmental Enrichment on BDNF levels. Histogram plot demonstrating the mean concentration of BDNF (ng/g) in Striatum, Hippocampus, Cortex and Cerebellum samples collected from (B) grafted and (A) control animals housed in standard and enriched environments.

Figure 6.6 demonstrates the effect of environmental enrichment on BDNF levels within control and grafted animals. Enriched Control (n=3), Standard Control (n=3), Enriched Graft (n=3) and Standard Graft (n=3) animals were dissected for tissue samples of the Striatum, Hippocampus, Cortex and Cerebellum. Each tissue sample was tested via ELISA in duplicate, with the mean value used as a representative figure for BDNF concentration within the sample. Samples were then averaged across the three animals to produce an overall mean for each structure, and housing condition, and plotted as a histogram (Fig 6.6). Due to large differences in the variance between regions, the data was log transformed in order to homogenise the variance. The log transformed data was subjected to a 2 way ANOVA to assess significance. There is a significant increase in BDNF levels across all brain regions (Groups, $F_{(1,23)}$=47.43, p=<0.001) following environmental enrichment, with no significant interaction between regions and groups (Groups, $F_{(3,21)}$=0.20, p=0.895, n.s).

6.4 DISCUSSION

The mammalian brain is at its most plastic during development and following injury. Therefore, it would be expected that the effects of environmental enrichment would be exacerbated within embryonic striatal grafts, which represent a situation where developing embryonic tissue is placed into the damaged adult nervous system. The data presented in this chapter aims to identify if bi-directional corticostriatal synaptic plasticity expressed from striatal grafts is affected by environmental enrichment, and to probe some of the mechanisms behind enrichment dependant modification of neuronal function.

6.4.1 The Effect of Enrichment on LTP/LTD from the Normal Striatum

Studies have demonstrated that environmental enrichment can affect hippocampal synaptic transmission (Duffy et al., 2001; Irvine and Abraham, 2005) and increase both LTP and LTD expressed from the CA1 region (Artola et al., 2006). However, the effect of environmental enrichment on bi-directional corticostriatal synaptic plasticity had yet to be investigated.

The data presented in this chapter demonstrates that environmental enrichment (under the parameters employed) had no effect on the level of corticostriatal LTD expressed from the normal striatum. Whilst it would be logical that environmental enrichment would increase LTD within the normal striatum, the findings presented here are not totally unexpected. Previous studies demonstrate that LTD is increased, following enrichment, have concentrated on LTD expressed from the CA1 region of the hippocampus (Artola et al., 2006; Duffy et al., 2001). Within the hippocampus expression of LTD is induced by repetitive low frequency stimulation (LFS) and is dependant on NMDA receptor activation. LTD expressed from the striatum is independent of NMDA receptor activation (Calabresi et al., 1992c; Calabresi et al., 1992e). This proves important, as the effects of enrichment on synaptic plasticity have been suggested to be heavily dependant on modification of NMDA receptor (Artola et al., 2006; Duffy et al., 2001). Therefore it is plausible that corticostriatal LTD is not overtly effected by enrichment.

Also, it is important to note, that previous studies on the effect of enrichment on CA1 synaptic plasticity, have used differing enrichment protocols to the one employed in the studies presented here. Duffy et al report that CA1 LTP is only enhanced following 8-week exposure to an enriched environment (Duffy et al., 2001), whilst animals exposed to an enriched environment display no change in CA1 LTP, after shorter exposure periods (Duffy et al., 2001). Furthermore, Artola et al report that both CA1 LTP and LTD are enhanced following a 5-week exposure to an enriched environment (Artola et al., 2006), which is not reversed following a further 3-5 week exposure to a standard environment (Artola et al., 2006). In this present study mice were exposed to the enriched environment for 8-12 weeks prior to experimentation. Whilst, this time period may be sufficient to induce changes in CA1 LTD, the same time period may not be sufficient to induced changes in corticostriatal LTD. Interestingly, gene expression studies have demonstrated that the effect of enrichment on promoting up regulation of gene expression is time dependant (Rampon et al., 2000). It is plausible that 8-weeks exposure to an enriched environment provides an early stage of the enrichment effects within the striatum. Therefore, it is impossible to rule out that corticostriatal LTD may be affected following longer periods of enrichment.

It is also important to note that previous studies demonstrating a change in CA1 LTD have used animals at a much later stage in adult development 7-9 months of age (Artola et al., 2006). Furthermore, it has been shown that CA1 synaptic plasticity decreases with increased age (Foster et al., 1996). It is plausible that the increase in CA1 LTD following enrichment could be facilitated by a decline in LTD with age, resulting in an increased enrichment effect. Therefore, it would be interesting to analyse the effect of environmental enrichment on corticostriatal LTD and LTP from aged normal mice.

Interestingly, the data presented in this chapter clearly demonstrates that environmental enrichment causes an increased expression of LTP. In the presence of magnesium, HFS resulted in the expression of synaptic plasticity from enriched animals in 10 out of 15 recordings, with 7 out of 15 being LTD, and 3 out of 15 being LTP. Under similar conditions, animals housed in standard environments expressed

synaptic plasticity in 6 out of 15 recordings, with a total expression of LTD (6 out of 15). Furthermore, in conditions where magnesium is omitted from the perfusate solution, animals from standard housing demonstrated LTP in 7 out of 10 recordings, whilst those from enriched housing demonstrated LTP in 10 out of 10 recordings (Two recordings clearly displayed LTP, but were omitted from the final analysis due to data not being collected for a full 60 minute post-tetanus period). These observations prove interesting for a number of reasons. It can clearly be seen that environmental enrichment improves the success rate of expressing corticostriatal synaptic plasticity from the normal striatum. Interestingly, similar findings have been found, with regard to CA1 LTP (Artola et al., 2006; Duffy et al., 2001). It has been suggested that increased expression of synaptic plasticity may provide a physiological mechanism for the observed increases in behavioural performance following enrichment (Artola et al., 2006). Therefore, though the enrichment paradigm utilized in the experiments presented here does not affect bidirectional corticostriatal synaptic plasticity size, it does result in increased expression of corticostriatal synaptic plasticity, which may have an effect on behavioural performance.

Additionally, environmental enrichment appears to increase the expression of LTP under normal physiological conditions. As previously described, LTP within the normal striatum appears to be critically dependant on the activation of NMDA receptors, which under *in vitro* conditions requires the omission of magnesium from the perfusate solution (Calabresi et al., 1992c; Calabresi et al., 1992e). Striatal NMDA receptors are distinct from other NMDA receptors in that they have a high strength magnesium block, requiring strong depolarisation from corticostriatal afferents in order to be activated (Calabresi et al., 1992c). It is theorised that the lack of NMDA receptor activation under normal conditions in the striatal *in vitro* slice is the result of poor preservation of corticostriatal afferents within the slice (See Chapter 4). Furthermore, it has been shown that enrichment can influence morphological changes (Rosenzweig et al., 1967) which may include changes in the density of the corticostriatal input. However, it would seem that environmental enrichment can offset some of the difficulties in activating the NMDA receptor, resulting in the increased expression of LTP under normal conditions. Yet, how could enrichment result in increased ease of NMDA receptor activation?

There are two possible ways environmental enrichment could affect striatal NMDA receptor activation within the normal striatum. Firstly, enrichment could directly affect pre-synaptic mechanisms of LTP induction. A number of studies have demonstrated that enrichment causes a reduction in the threshold for LTP induction (Artola et al., 2006; Duffy et al., 2001). Under such conditions a lower number of tetanic bursts are required for induction of LTP. Though the mechanisms for this reduction in threshold are unknown, it has been suggested that it may involve enhanced neurotransmitter release from the pre-synaptic terminals, therefore reducing the pre-synaptic activation requirement (Artola et al., 2006). It has also been shown that environmental enrichment causes morphological changes, such as increased dendritic branching and spine density, which could increase the pre-synaptic activation of striatal MSNs. Furthermore, gene expression studies have demonstrated that enrichment causes an increase in expression of genes involved with formation of new synapses (Rampon et al., 2000). Therefore, it is highly plausible that increasing and strengthening the corticostriatal synapses may be a causative factor in enrichment facilitated expression of LTP. It is also important to note that enrichment could affect post-synaptic mechanisms of LTP induction and expression, such as internal signalling cascades. Studies have shown that the enrichment induced facilitation of CA1 LTP is blocked by PKA inhibitors, whilst this is not the case in standard animals (Duffy et al., 2001). Furthermore, enrichment has been shown to cause an increase in expression of gene encoding for key proteins involved in signal transduction (Duffy et al., 2001). Therefore, enrichment may modify post-synaptic signalling cascades involved in the transduction of synaptic signalling.

It would therefore seem that the environmental enrichment paradigm presented within this chapter is capable of altering the induction of LTP at corticostriatal synapses, resulting in an increased expression of LTP.

6.4.2 The Effect of Enrichment on LTP/LTD from Striatal Grafts.

Previous studies have demonstrated that environmental enrichment can enhance behavioural performance on motor learning tasks under the control of the grafted striatum (Dobrossy and Dunnett, 2001; Dobrossy and Dunnett, 2004). Though the

mechanisms of this increased behavioural performance are not clearly understood, it has been shown that enrichment can induce morphological changes in the graft tissue, such as increased cell size, dopaminergic innervation, and BDNF expression (Dobrossy and Dunnett, 2001; Dobrossy and Dunnett, 2004). The data presented in this chapter demonstrates that enrichment can also induce changes in host-graft synaptic plasticity.

Within striatal grafts whilst expression of LTD is similar in animals housed in both standard and enriched environments, LTP expressed from enriched animals is facilitated with respect to those housed in standard environments. Therefore, though LTP is not facilitated within the normal striatum, the grafted striatum clearly demonstrates an increase in LTP. This observation proves interesting as it would suggest that the grafted striatum, in some way, facilitates the effects of environmental enrichment.

As previously mentioned, enrichment induced facilitation of LTP is considered to be dependant on both pre- and post-synaptic modifications of the transplanted tissue. It has been shown that enrichment causes activity dependant changes in the morphology of transplanted cells (Dobrossy and Dunnett, 2001; Dobrossy and Dunnett, 2004). Such morphological changes would be consistent with increased activation of TSNs, which is more than likely facilitated by increased synapse formation. These morphological changes could account for the observed increase in host-graft corticostriatal LTP, seen within enriched animals. Furthermore, it has been shown that dopaminergic innervation of the graft is increased in enriched animals (Dobrossy and Dunnett, 2001; Dobrossy and Dunnett, 2004)). Though there is no direct evidence, it would be plausible that cortical innervation of the graft would also be increased by environmental enrichment. Therefore, enrichment induced changes in host-graft corticostriatal LTP could be facilitated by increased cortical innervation of the graft region.

Reported experience dependant changes induced by enrichment have also included changes in post-synaptic signalling (Duffy et al., 2001), and changes in gene expression (Rampon et al., 2000). Furthermore, it has been suggested that striatal grafts display a higher expression of neonatal NMDA receptors, which display

increased calcium permeability (Siviy et al., 1993) (Also see Chapter 5). It is possible that enrichment induced changes in intracellular signalling may be amplified by the higher presence of neonatal NMDA receptors present in TSNs. Therefore, the enrichment protocol employed in the studies presented here may not be sufficient to induce changes in LTP expressed from the normal striatum, due to the predominant expression of the less conductive adult NMDA receptors.

Finally, it is important to note that enrichment results in up regulation of growth factors such as BDNF (Dobrossy and Dunnett, 2001; Dobrossy and Dunnett, 2004; Hockly et al., 2002; Spires et al., 2004). Inhibition of BDNF has been shown to impair hippocampal LTP (Ma et al., 1998). Therefore up regulation of BDNF may contribute to enrichment induced facilitation of LTP.

6.4.3 The Effect of Enrichment on Hippocampal Synaptic Plasticity.

The data presented in this chapter did not replicate the obervation that environmental enrichment (under the parameters employed) can affect the level or expression of LTP from the CA1 region of the hippocampus. Previous studies have demonstrated that enrichment causes a facilitation of LTP (Artola et al., 2006; Duffy et al., 2001) and LTD (Artola et al., 2006). However, both Artola et al, and Duffy et al, used the 100hz stimulation paradigm in order to express LTP from the CA1 region. In the studies presented here a standard hippocampal theta burst was used to express CA1 LTP. Thus, the differences in HFS protocols could account for the lack of enrichment induced facilitation in the studies presented here, with respect to those of Artola et al, and Duffy et al. It has been shown that the mechanisms of enrichment induced changes of CA1 LTP/LTD do not involve modulation of AMPA and NMDA currents (Artola et al., 2006; Duffy et al., 2001). Rather, enrichment reduces the threshold for LTP induction, which is critically dependant on the HFS protocol employed (Artola et al., 2006; Duffy et al., 2001). Therefore, it is likely that the changes induced by 100hz HFS, are not sufficiently induced by theta burst HFS.

6.4.4 Assessing the Levels of BDNF Following Enrichment.

Previous studies have demonstrated that BDNF levels are increased in animals receiving striatal grafts following enrichment (Dobrossy and Dunnett, 2001; Dobrossy and Dunnett, 2004). Interestingly, levels of BDNF are increased in transgenic models of HD (Hockly et al., 2002; Spires et al., 2004), and are considered to play a neuroprotective role in slowing down the disease progression (Hockly et al., 2002; Spires et al., 2004). BDNF has also been shown to promote cell survival, growth, and enhance development and plasticity of MSNs (Ivkovic et al., 1997; Mizuno et al., 1994; Ventimiglia et al., 1995).

The data presented in this chapter demonstrates that under normal conditions, animals housed in enriched environments display an increase in neuronal BDNF levels across all four brain regions as a consequence of their housing. Within the context of the studies present here, enrichment resulted in no change in the level, or expression of either corticostriatal, or hippocampal synaptic plasticity, from control animals. Furthermore, it has been shown that behavioural performance, relying on the corticostriatal synapse, is not overtly affected by environmental enrichment (Dobrossy and Dunnett, 2001; Dobrossy and Dunnett, 2004). Therefore, though enrichment causes an increase in BDNF levels, within control animals enrichment causes no major affect on striatal and hippocampal physiology, and function. This adds further evidence to the argument that the enrichment paradigm employed during the experiments presented here is either not rigorous enough, or extensive enough to induce global changes in neuronal function. Furthermore, studies have shown that enrichment induced increases in gene expression is time dependant (Rampon et al., 2000). Therefore, the observation that BDNF levels are increased would suggest that the enrichment paradigm used represents an early stage in the enrichment induced changes on function.

Enrichment induced changes in the levels of BDNF is dramatically different following lesion and cell transplantation. Grafted animals display a higher level of BDNF expression than control animals. This observation proves interesting as it would suggest that lesion and transplantation increases the effects of enrichment on BDNF levels. More specifically, it would seem that unilateral lesions, and grafts, are

capable of effecting enrichment induced BDNF expression in the contralateral striatum cortex and even hippocampus. As previously stated, the enrichment paradigm employed in the studies presented here caused no change in neuronal function, but a significant change in BDNF levels. However, it would seem that the lesion and graft is capable of inducing enrichment effect, suggesting that such injury and repair may 'kick-start' the effects of environmental enrichment, so that a paradigm which previously had little effect is now exacerbated.

Interestingly, grafted animals display an increase in both BDNF levels and corticostriatal LTP following enrichment. Previous studies have shown that BNDF is implicated in synaptic plasticity. BDNF knockout mice demonstrate impaired LTP (Korte et al., 1995; Patterson et al., 1996), which is reversed by transfection with lentiviruses expressing BDNF (Korte et al., 1995). Furthermore, spatial learning has been shown to activate BDNF expression (Gomez-Pinilla et al., 2001), whilst inactivation of BDNF impairs spatial learning (Ma et al., 1998), Also mice lacking the BDNF receptor show impaired learning (Minichiello et al., 1999), whilst BDNF receptor over expresser mice display improved cognitive performance (Koponen et al., 2004). It would therefore seem clear that BDNF plays a significant role in synaptic plasticity, learning and memory. However, most such experiments have been undertaken using hippocampal-dependant tests of spatial memory rather than using tests of motor associative and habit learning more associated with striatal plasticity. Increased levels of BDNF expression is quite likely to only play a contributing role in the functional effects observed following enrichment. It is important to consider that BDNF can influence other factors such as morphological changes, increased neuronal survival, and increased innervation of the graft may contribute to the enrichment effects observed. Furthermore, enrichment induced up regulation of other growth factors, and intracellular signalling compounds more than likely contribute to the observed effects.

6.5 CONCLUSIONS

The studies presented in this chapter demonstrate that subtle manipulations of the environment in which an animal is housed can influence neuronal correlates of function, such as expression and levels of synaptic plasticity, and expression of BDNF. Interestingly, it would seem that both the level, and the time course of enrichment, plays a role in the degree of positive effects on neuronal function. The enrichment paradigm employed within this chapter results in minor effects on striatal synaptic plasticity, and BDNF expression, which would indicate an early stage in the enrichment effects. However, the same paradigm results in enhanced LTP, and BDNF expression within animals which have received unilateral lesions and striatal grafts. Therefore, it would seem that neuronal injury and repair provides a 'kick-start' for the observed enrichment effects.

7. GENERAL DISCUSSION

The aims of this thesis were to evaluate the extent to which embryonic striatal grafts integrate functionally with the host tissue, and display cellular correlates of learning and memory. Previous functional assessment of striatal grafts has relied upon behavioural (Dunnett, 1995; Dunnett et al., 2000), anatomical (Wictorin, 1992), and biochemical techniques (Sirinathsinghji et al., 1994). Physiological analysis of graft function has demonstrated that TSNs are active to stimulation of the host cortex (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b; Wilson et al., 1990; Xu et al., 1991b), and thalamus (Xu et al., 1991b), both *in vivo* (Xu et al., 1991b) and *in vitro* (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b). Furthermore, emerging evidence has suggested that TSNs retain some immature physiological characteristics (Siviy et al., 1993; Xu et al., 1991b).

This studies presented in this thesis build on previous physiological analysis of graft function by providing evidence for the expression of LTP and LTD, pharmacological analysis of both baseline transmission and host-graft bidirectional synaptic plasticity, and the effect of environmental enrichment on graft physiological function. This chapter will detail how these factors interact to influence graft function.

7.1 Limitations of the current in vitro graft slice model.

All of the experiments presented in this thesis have been conducted within the same *in vitro* graft model. Over the course of this thesis a number limitations have arisen which though not addressed within the available time frame, would need to be considered before performing further analysis.

The primary limitation of the current model is the lack of distinction between recordings from "P-zones" and "NP-zones". During this thesis work extensive attempts were made to recover slices post-recording, resection, and stain using markers positive for "P-zones" locality (DARPP-32, AChE). Successful recovery and histological processing of slices would have enabled me to match the recording site with immunohistological evidence of "P-zone" locality. Whilst it is fustrating that such analysis was not successful within the time frame of this thesis, I believe the

results obtained have not overtly suffered from the lack of histological confirmation. Furthermore, though such analysis would add further depth to the results obtained, positive identification of "P-zone" locality would be confined to post-recording analysis.

One method of addressing this issue, whilst also enabling identification of "P-zone" locality prior to recording would be to utalise a transgenic tissue donor with GFP linked to a "P-zone" positive marker. In the last year a transgenic mouse with GFP driven under the DARPP-32 promotor has become available. Using the D32-GFP transgenic as a tissue donor would enable positive identification of "P-zones" within the graft, prior to electrode placement. Unfortunately, the availiability of these transgenic mice fell outside of the time frame for this thesis. However, the use of this transgenic in future studies will enable direct electrophysiological investigation of "P-zone" function.

A second limitation of the experimental model presented in this thesis is the inability to study graft function on a systems level. Whilst *in vitro* analysis enables a more direct assessment of 'functional integration' at a monosynaptic level, one must also consider 'functional integration' at a systems level. *In vivo* analysis would permit the study of 'functional integration', not just in the context of multiple brain regions constituting a functional circuit, but also in the context of the 'normal' system in which the graft would integrate functionally. Therefore, whilst the experiments presented in this thesis build on, and develop, our understanding of host-graft synaptic transmission, one would seek to perform comparative studies in a suitable *in vivo* model. Therefore, whilst my findings provide interesting revelations regarding graft function and integration, one would hope that further *in vitro* and *in vivo* investigation takes place.

7.2 Do Embryonic Striatal Grafts 'Functionally Integrate' With the Host Striatal Circuitry?

It has been clearly demonstrated that striatal grafts receive projections from the host cortex, thalamus, and SN, and that these projections form anatomically correct synapses with TSNs (Clarke et al., 1988b; Clarke et al., 1988a; Clarke and Dunnett,

1990; Clarke and Dunnett, 1993; Wictorin et al., 1988a; Wictorin et al., 1988b; Wictorin et al., 1989; Wictorin and Bjorklund, 1989; Wictorin, 1992; Wilson et al., 1990; Xu et al., 1989; Xu et al., 1991a). Furthermore, efferent projections from the graft project to the host GP, and SN (Wictorin, 1992) where they are capable of influencing neuronal transmission (Sirinathsinghji et al., 1994).

Behavioural analysis post-transplantation has shown that cognitive performance on a number of behavioural techniques, relying on intact cortico-striatal-pallidal circuits is improved (Dunnett, 1995). Additionally, 'push-pull' perfusion techniques have demonstrated that innervation of the host GP by the striatal graft influences pallidal levels of GABA (Sirinathsinghji et al., 1994). Finally, electrophysiological experiments have demonstrated that TSNs are responsive to stimulation of the host cortex and thalamus (Rutherford et al., 1987; Siviy et al., 1993; Surmeier et al., 1992b; Wilson et al., 1990; Xu et al., 1991b). Therefore, prior to the work carried out in this thesis there existed a growing body of indirect evidence to support the concept that embryonic striatal grafts do indeed 'functionally integrate' with host circuitry.

The experimental findings of this thesis provide further evidence to support the concept that embryonic striatal grafts can 'functionally integrate' with the host. Experiments conducted within this thesis have focused on the host-graft corticostriatal projection, as this represents the major afferent host input to striatal grafts, and has been shown to be critical to the mediation of plasticity and restoration of cognitive function, involved in learning and memory (Brasted et al., 1999b; Brasted et al., 1999a). however, one would hope that future experiments would seek to investigate other host inputs to the graft, and inputs to the host of graft origin.

It can be clearly seen that given sufficient time post-transplantation the host cortex is capable of forming functional corticostriatal synapses with embryonic striatal grafts. Furthermore, such 'functional integration' of the graft with the host circuitry includes restoration of baseline transmission, which could be critical to the observed improvement in motor performance post-transplantation. It can be clearly seen that the host-graft corticostriatal synapses are functional. Data presented in this thesis demonstrates that striatal grafts display tonic baseline transmission that is stable over continuous recording periods (60 mins). However, though baseline transmission is

restored, does the efficacy of such baseline transmission replicate that seen in the normal striatum? On average extracellular responses from the graft are consistently smaller than those expressed from the normal striatum. Therefore, though baseline transmission is restored, the level of transmission is lower within the grafts. It is difficult to draw a functional significance of this impaired transmission, because it may simply reflect the smaller net number of connections formed. A reduction in the size of extracellular responses would suggest that although striatal grafts reconnect with the host neuronal system, such reconnection is not as dense, nor as robust as that seen in the normal striatum.

A second factor in assessing the efficacy of host-graft baseline transmission is its ability to respond to changes in input stimulation. The data presented in this thesis demonstrates that increases in input stimulation reaches an output saturation point at a much lower input current in grafts, than that seen in the normal striatum. Furthermore, this saturation point can be increased by application of GABA antagonists. Therefore, it would appear that although host-graft corticostriatal synapses are robust enough to respond to increased stimulation, their saturation point is much lower than that seen in normal striatum, and is more heavily modulated by GABAergic inhibition. This increased GABAergic inhibition is considered to be facilitated by increased synapse formation with collateral TSNs. Whilst collateral inhibition between MSNs is present in the normal striatum, there appears to be a higher prevalence of its occurrence in the grafted striatum. The functional significant of this increased collateral inhibition is unknown, and is more likely a consequence of the highly plastic environment in which graft connections develop. Regardless, it would appear that host-graft cortical connections do indeed functionally connect, in a manner much like that seen in the normal striatum.

This thesis presents a large body of evidence to demonstrate that the various neurotransmitter systems seen within the normal striatum are functionally replicated within striatal grafts. Both glutamatergic and GABAergic mechanisms of baseline transmission appear consistent with that seen in the normal striatum. However, there is a striking difference in the DAergic mechanisms seen within striatal grafts. Specifically, D2 mediated transmission appears different to that seen in the normal striatum, both under baseline conditions and during synaptic plasticity. Data

presented in this thesis demonstrates that D2 mediated mechanisms of synaptic plasticity mimic that seen in 6-OHDA lesioned striatum following DA application (Calabresi et al., 1992c). This has led to the speculation that within the striatal lesion and graft model similar mechanisms of DAergic reconnection and D2 receptor supersensitivity occur. Therefore, it would seem that in order to improve the 'functional integration' of embryonic striatal grafts due attention must be paid to the formation of functionally normal DAergic synapses. One hypothesis to account for this pattern is that it reflects the relatively short time frame of the experiments whereas a longer survival period will allow restored DA transmission to stabilise and receptor sensitivity to normalise. Clarification of this issue will involve further experiments on the time course that have fallen outside of the time frame available for this thesis work.

It must be noted that the experiments presented within this thesis provide a broad overview of the various neurotransmitter and receptor relationships seen in the normal striatum. Therefore, it would be interesting to see if 'functional integration' of embryonic striatal grafts includes restoration of other systems such as functional connections between TSNs and the large cholinergic interneurones.

7.3 Do TSNs Retain Physiological Characteristics of A Neonatal or Embryonic Development Age?

Previous analysis of TSN physiological function has shown that they display a number of characteristics consistent with an immature phenotype, such as increased expression of IPSPs (Xu et al., 1991b), and increased NMDA receptor efficacy (Siviy et al., 1993). This thesis provides further evidence for the theory that NMDA receptors located on TSNs display an immature phenotype consistent with early neonatal MSNs.

The NMDA receptor proves to a key site is probing the developmental characteristics of TSNs for a number of reasons. Firstly, the NMDA receptor contributes a large proportion of the excitatory current during enhanced synaptic transmission, and plays a key role in governing the preferential expression of LTP or LTD within *in vitro* striatal slices. Secondly, the NR2 sub-unit of the NMDA receptor has been shown to

be regulated over development so that NR2B sub-units are predominantly expressed during embryonic and neonatal neurones, whilst the NR2A sub-unit is predominantly expressed in adult neurones. Thirdly, the NR2B displays a higher calcium conductance, lower magnesium ion affinity, and decreased selectivity to antagonist such as APV, when compared to the NR2A sub-unit.

This thesis demonstrates quite clearly that NMDA receptor mediated mechanisms of synaptic plasticity are different within embryonic striatal grafts, when compared to the normal striatum. Specifically a number of characteristics such as an increased concentration of APV required to fully antagonise NMDA receptors, and lower strength of magnesium ion blockade would suggest that within TSNs there is a preferential expression of NR2B NMDA receptors over NR2A. However, it is important to note that further experimentation, using voltage clamp recordings and sub-unit selective NMDA receptor antagonists and agonist would be prudent.

Though not conclusive proof, the evidence presented within this thesis does provide a compelling argument that TSNs do retain some physiological characteristics of a embryonic or neonatal development age, at least up to 8 weeks post transplantation.

7.4 *Learning to Use the Graft and Corticostriatal Synaptic Plasticity, does Corticostriatal Synaptic Plasticity Influence Learning and Memory?*

One of the aims of this thesis has been to demonstrate that embryonic striatal grafts display biphasic synaptic plasticity. Though I have clearly demonstrated that striatal grafts can express synaptic plasticity, this observation provokes more questions regarding how behavioural and cellular correlates of learning and memory interact.

Studies by Rosvold in the early 1960s demonstrated that lesions of the striatum can produce deficits in a number of cognitive tasks, relying on learning and memory. This observation was further refined by studies conducted by Divac which demonstrate that the corticostriatal projection plays an essential role in some forms of learning and memory (Divac et al., 1967).

Synaptic plasticity was first demonstrated by Bliss, et al in the anaesthetised rabbit hippocampus (Bliss and Lomo, 1973), and was subsequently theorised to provide a cellular model for learning and memory (Bliss and Collingridge, 1993). Later studies have demonstrated that the measures taken to inhibit synaptic plasticity also impair performance in behavioural tasks relying on learning and memory (Morris et al., 1986). It would therefore seem apparent that both learning and memory, and synaptic plasticity are critically linked.

Studies conducted in parallel to this thesis have further demonstrated that both corticostriatal synaptic plasticity and behavioural tasks involving striatum dependant forms of learning and memory are inhibited in a transgenic animal expressing dominant negative forms of CREB (Pittenger et al., 2006), thereby, demonstrating that corticostriatal synaptic plasticity is essential for striatal forms of learning and memory.

In my studies excitotoxic lesions of the striatum eliminate all corticostriatal transmission, not just the lack of expression of synaptic plasticity. Yet, given suitable amounts of time post-transplantation embryonic striatal grafts expresses bidirectional synaptic plasticity in keeping with the normal striatum. Studies conducted by Brasted et al, demonstrate that unilateral excitotoxic lesions disrupt the ability to learn new motor skills or habits (Brasted et al., 1999b). However, following transplantation such animals relearn tasks in a time frame similar to that taken by naïve animals to learn the task (Brasted et al., 1999b). Furthermore, such relearning appears to rely on the formation of an intact corticostriatal projection (Brasted et al., 2000). Therefore, the evidence presented in thesis, combined with the behavioural data presented by Brasted et al, adds further weight to the argument that corticostriatal synaptic plasticity is essential for, and a potential substrate of, the learning of new motor skills and habits.

Additionally, I have demonstrated that grafted animals which have been housed in enriched environments display increased levels of LTP. Studies conducted by Dobrossy et al, have shown that in similar enrichment paradigms behavioural performance is improved in animals which have received striatal grafts (Dobrossy and

Dunnett, 2005). Therefore, it would seem that conditions which improve corticostriatal synaptic plasticity, also improve behavioural performance.

7.5 FINAL SUMMARY AND CONCLUSIONS

In this thesis, I have sought to address some of the basic scientific questions regarding graft integration and function. Physiologically, it would appear that embryonic striatal grafts reconnect functionally with the host striatal circuitry in a manner that replicates many of the physiological properties of the normal striatum. The observation that striatal grafts restore synaptic plasticity to the lesioned striatum adds further weight to the working hypothesis that striatal grafts facilitate true repair of the lesioned striatum.

The driving force behind research into cell transplantation is the potential for its application as a therapy for neurodegeneration. Clinical trials have demonstrated that cell transplantation can produce beneficial effects in patients with HD (Bachoud-Levi et al., 2000a; Bachoud-Levi et al., 2000b; Bachoud-Levi et al., 2006). Embryonic striatal grafts are required to reconstruct the damaged circuitry to represent true repair. This thesis demonstrates that embryonic striatal grafts are capable of integrating with the host striatum, restoring functional circuits which are present in the normal striatum. It is believed that this functional integration facilitates improvement in behavioural performance. Therefore, probing the physiological characteristics of striatal grafts is essential if we are to improve on the technique, and provide greater functional benefits.

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9. APPENDIX

IMMUMOHISTOCHEMISTRY ON RAT AND MOUSE SECTIONS.

Sections.

Suitable for 30-60um free-floating sections. Cut from tissue which has been perfused with phosphate buffer, fixed in 4% buffered paraformaldehyde, and then equilibrated with 25% buffered Sucrose. 10-20um slide mounted paraffin or cryostat sections may also be used for this method.

Free floating sections are processed in "Greiner pots" on a rotating mixer. The lids of the pots are cut away partly and a gauze square is fitted between the lid and the pot in a way such as to retain the sections but allow the liquid to be tipped away and more added.

Method:

1	Quench (Solution 1) below	5mins
2	Wash in TBS(solution 2 below)	3x10 mins
3	Block in 3% Serum block in TXTBS (see solution 3 below)	1hr
4	Transfer without washing to primary antibody in TXTBS with 1% serum.	Overnight at room temp. or 2-3 days at 4 degrees On a rotating mixer.
5	Wash in TBS	3x10 mins
6	Biotinylated Secondary antibody in TBS with 1% serum.	2 hours
7	Wash in TBS	3x10mins
8	ABC Kit DAKO (solution 4 below) in TBS with 1% serum	2 Hours
9	Wash in TBS	3x10 mins
10	Wash in TNS (solution 5 below)	2x5mins May be left overnight at 4 degrees overnight.
11	DAB Solution (solution 6 below)	Until light background But dark specific stain Check microscopically
12	Wash in TNS	2x5mins

13 Wash in TBS Store at 4 degrees until

14 Mount on gelatinised slides, air dry, dehydrate in Ascending series of alchohols. Clear in xylene. Coverslip with DPX

Solutions.

Solution 1 Quench.

	Methanol (98%)	5ml
	Hydrogen peroxide (30%)	5ml
	Distilled Water	40 ml
Solution 2 TBS	Trizma Base	12g
	Sodium Chloride	9g
	Distilled water	Make up to 1L
	Adjust to pH 7.4 with conc HCL	
Solution 3 TXTBS	TBS	250ml
	Triton X-100	500ul
	Adjust to ph 7.4 with conc HCL	
Solution 4 ABC	DAKO Streptavidin Kit	
	Per ml of solution	
	Solution A	5ul
	Solution B	5ul
	TBS with 1% serum.	
Solution 5 TNS	Prepare fresh prior to use	
	Trizma base 6g	
	Distilled water	Make up to 1L

Solution 6 DAB

A stock solution of DAB is prepared where 1g DAB (Sigma D5637 3,3'-Diaminobenzidene tetra hydrochloride) is dissolved in a fresh TNS solution.
The resulting solution is then aliquoted into2ml amounts in bijous and stored at -20 C for future use . This solution contains 20 mg of DAB per aliquot.

DAB Soln.	2ml
TNS(fresh)	40ml
Hydrogen peroxide soln	12ul.

This solution may be diluted to 1in 5 with TNS if the reaction proceeds too quickly.

This method for Tyrosine hydroxylase staining is used as follows.

Using Horse serum block , Chemicon MAB 318 Anti Tyrosine Hydroxylase (mouse monoclonal at a primary dilution of 1in 1000 . Secondary Horse anti Mouse (rat adsorbed) Vector BA2001 at a dilution of 1in 200