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Sex-dependent influences of dexamethasone exposure in-utero and stress and stress hormones in adulthood on a rat model of Parkinson's disease.

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ABSTRACT

Stress is increasingly identified as a risk factor for brain disease, but the underlying processes are unkown. This thesis examines the influence of raised levels of the glucocorticoid (GC) stress hormone at different stages of life (during late gestation and/or in adulthood) on pathways known to be altered in parkinson's disease (PD), and compares this with the effects of repeated adult stress. Adult stress or GC treatment produced complex, differential sex and region specific changes in these pathways. Notably, adult stress or GC treatment increased markers of nirosative stress in the male but not female rat substantia nigra (SN). Prenatal GC treatment produced long term effects on both dopamineric (DA) and noradrenergic (NA) nuclei, increasing the number of DA neurons in the SN and ventral tegmental area, and reducing the number of NA neurons in the locus coeruleus of adult rats. Furthermore microglial and astrocyte populations were increased and decreased respectively in these nuclei. Interestingly prenatal GC treatment rendered the male midbrain susceptible to a down regulation of DA measures in response to adult challenge, whereby the number of SN DA neurons and striatal and accumbal DA levels were attenuated. Fascinatingly adult stress or GC treatment, but not prenatal GC treatment exacerbated 6-hydroxydopamine-induced neurotoxicity, that was associated with increased microglial activation and protein nitration in the SN, in males, but afforded neuroprotection in females, neither of which were substantially altered by prenatal treatment. This is the first study to demonstrate that prenatal GC treatment can permanentlty alter the response of the midbrain DA pathways to adult challenge. Furthermore this is one of very few studies looking at male and female responses in parallel and thus highlights some striking sex differences in the response to stress and GCs. Together these novel data strongly support the premise that stress and GCs influence the functional integrity of pathways involved in PD, thereby

potentially contributing to PD pathogenesis. The data also highlight the complex sexually dimorphic nature of their actions. Although further work is required to determine the functional consequences of these stress/GC related changes, these findings may have considerable implications for the use of GCs in clinical practice.

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LIST OF ABBREVIATIONS

Ach	Acetylcholine
ACTH	Adrenocorticotrophic hormone
AD	Alzheimers disease
AIF	Apoptosis inducing factor
ANS	Autonomic nervous system
AP-1	Activating protein 1
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BBB	Blood brain barrier
11-βHSD	11-beta-hydroxysteroid-dehydrogenase
cAMP	Cyclic adenosine monophosphate
CBG	Corticosterone binding globulin
chAT	Choline-acetyl-transferase
CNS	Central nervous system
COMT	Catechol-o-methyl-transferase
CORT	Corticosterone/cortisol/hydrocortisone
COX-2	Cyclo-oxygenase-2
CRH	Corticosterone releasing hormone
CSF	Cerebro spinal fluid
DA	Dopamine
DARP-32	Dopamine and cAMP regulated phosphoprotein
DAT	Dopamine transporter
DβH	Dopamine-beta-hydroxylase
DEX	Dexamethasone
DOPAC	3,4-Dihydroxy-phenylacetic acid
ELISA	Enzyme linked immunosorbent assay
GC	Glucocorticoid
GD	Gestational day
GFAP	Glial fibrillary acidic protein
GPCR	G protein coupled receptor
GPe	Globus Pallidus (external segment)
GPi	Globus Pallidus (internal segment
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HPA	Hypothalamic pituitary adrenal
HPLC	High performance liquid chromatography
HVA	Homanovanillic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HIAA	5-hydoxyindolecetic acid
IgG	ImmunoglobulinG
IFN-γ	Interferon gamma
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-10	Interleukin 10
iNOS	Inducible nitric oxide synthase
LC	Locus coeruleus

L-Dopa	Levodopa
LPS	Lipopolysacharide
LTP	Long term potentiation
MAO	Mono-amine-oxidase
MCDA	Mesocortical dopaminergic system
MDR	Multi-drug resistance
MFB	Medial forebrain bundle
MHC II	Major histocompatability complex
MLDA	Mesolimbic dopaminergic system
MMP-3	Matrix metalloproteinase 3
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MR	Mineralcorticoid receptor
NA	Noradrenaline
NAcc	Nucleus accumbens
NBM	Nucleus basalis of meynert
NeuN	Neuronal Nuclei
NF-κB	Nuclear factor kappa B
NMDA	N-methy-D-aspartic acid
NO	Nitric oxide
NOS	Notric oxide synthase
NSDA	Nigrostriatal dopaminergic system
6-OHDA	6-hydroxydopamine
OX-6	MHC class II antibody
OX-42	CD11b antibody
PD	Parkinosn's disease
PFC	Prefrontal cortex
PND	Post-natal day
POMC	Propiomelanocortin
PPN	Pedunculopontine nucleus
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SERT	Serotonin transporter
SNpc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulate
STN	Sub thalamic nucleus
TGF-β	Transforiming growth factor beta
TH	Tyrosine hydroxylase
UPS	Ubiquitin-proteasome-system
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area

CHAPTER 1

INTRODUCTION

1.1 Parkinson's Disease

Parkinson's disease (PD) is named after James Parkinson who first described the disease in 1817 in his work 'An essay on the shaking palsy'. The disease is principally characterised by a loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) that innervate the striatum and form the nigrostriatal dopaminergic (NSDA) pathway. This loss of neurons and the subsequent loss of dopamine (DA) within the striatum produce the classic symptoms of tremor, rigidity and bradykinesia.

1.1.1. Prevalence

PD is the second most prevalent neurodegenerative disorder after alzheimers disease (Bower et al., 1999) with 1-2% of the population over 65 years suffering from the disease. This increases to 3-5% of people over the age of 85 (Alves et al., 2008) and is more common in developed countries where there is a greater life expectancy. Early onset of sporadic PD is rare with approximately 4% of patients developing symptoms before the age of 50 (Van de beden 2003). Furthermore the incidence of PD is 1.5 times higher in men than women (Wooten et al., 2004).

1.1.2. Clinical Symptoms

Motor Symptoms

The main motor symptoms of PD are a resting tremor, muscle rigidity and bradykinesia which occur early in the disease course. Latter symptoms include a freezing gait, a loss of postural reflexes and a flexed posture of neck, trunk and limbs (Maetzler et al., 2009). When PD patients first present with motor systems they have already suffered considerable NSDA degeneration (Schapira et al., 2006). Indeed it has been suggested

that ~50-60% of SNpc neurons and 60-70% of striatal nerve terminals are lost before the onset of motor symptoms (Foley and Riederer, 1999).

Non-motor symptoms

Most PD patients also exhibit non motor symptoms which are summarised in Table 1.1. These include neuropsychiatric complications such as depression, anxiety and dementia and have been shown to have a significant impact on the quality of life of PD patients (Cubo et al., 2002). As many of these symptoms do not respond to dopaminergic treatment it is thought that they may be a result of degeneration in regions of the brain other than the NSDA (See section 1.1.3). Although some of these symptoms appear late on in the disease course (e.g. dementia and psychosis), some appear early on in the disease course (e.g. depression, anxiety and olfactory disturbances) and have even been shown to precede motor symptoms in some cases (Lim et al., 2009).

Table 1.1. Non-motor symptoms of PD

Neuropsychiatric	Depression, apathy, anxiety. Anhedonia Attention defecit Hallucinations, illusions, delusions Dementia Obsessional behaviour (drug induced), repetitive behaviour Confusion Delirium Panic attacks
Sleep Disorders	Restless leg movement REM disorders Vivid dreams Insomnia Breathing disturbance
Autonomic	Bladder disturbance Sweating Orthostatic hypotension Sexual dysfunction Dry eyes
GI	Dribbling Ageusia Dysphagia Reflux/Vomiting Nausea Constipation Faecal incontinence
Sensory	Pain Paraethesia Olfactory disturbance
Other	Fatigue Diplopia Blurred vision Seborrhoea Weight loss/gain

Taken from (Muzerengi et al., 2007)

1.1.3 Neuropathology

Dopaminergic Pathology

The brains of PD patients show reduced neuromelanin pigmentation in the SN which is correlated with neuronal loss and disease severity (Zecca et al., 2003). Neurons that are prone to degenerate accumulate cytoplasmic inclusions known as Lewy bodies (Mori et al., 2006). Lewy bodies are composed of several proteins including neurofilament, ubiquitin and α-synuclein. They are found in the perikarya of neurons, within neurites, in axons and have also been found in glial cells (Mochizuki et al., 2002)) within the SNpc. It is currently thought that the loss of neurons within the SNpc and the subsequent denervation of dorsal areas of the striatum precipitate many of the motor symptoms of PD.

Non-Dopaminergic Pathology

There is now substantial evidence to suggest that the pathology of PD is not restricted to the NSDA and that there is extensive non-dopaminergic pathology. Neuronal loss and the presence of Lewy bodies in PD patients is evident albeit to a lesser extent in many other brain areas and involves many neuronal types including the ventral tegmental area (dopaminergic; VTA), locus coeruleus (noradrenergic; LC), nucleus basilis of meynert (NBM; cholinergic), dorsal motor nucleus of the vagus (serotonergic) and the pedunculopontine nucleus (PPN; cholinergic) suggesting that many neurotransmitter systems are effected by the pathogenic disease process (Lim et al., 2009). Furthermore, there is increasing evidence to suggest that DA neurons are not the first to degenerate in PD. The work of Braak and colleagues suggests that PD begins in the anterior olfactory nucleus and medulla (Braak stage 1) and ascends through the brain eventually involving

the whole neocortex (Braak stage 5). SNpc degeneration is observed at an intermediate stage in the disease process (Braak stage 3) (Braak et al., 2003).

1.1.4 Dopaminergic neurotransmission

Synthesis

DA is synthesised from tyrosine. The rate limiting step in DA synthesis is the conversion of L-tyrosine to L-Dopa by the enzyme tyrosine hydroxylase. Dopa is then rapidly converted to DA by L-aromatic amino acid decarboxylase.

Uptake

The dopamine transporter (DAT) is present in DA nerve terminal membranes transporting DA in and out of the terminal according to the concentration gradient. DAT is a member of the Na^+/Cl^- dependent plasma membrane transporters and is responsible for the fast uptake of DA into the terminal, thereby regulating DA concentration at the synapse and therefore its interaction with pre and post synaptic receptors.

Metabolism

After reuptake into the nerve terminal DA is converted to dihyroxyphenylacetic acid (DOPAC) by monoamineoxidase (MAO). It is also converted to homovanillic acid (HVA) by the subsequent action of catechol-O-methytransferase (COMT) and MAO.

Receptors

DA receptors are G protein coupled receptors (GPCRs) and post synaptic DA receptors can be classified as either D1-like (D1,D5) or D2-like (D2,D3,D4). Within the striatum post synaptic DA neurons regulate feedback pathways to the SNpc. D2 receptors are also present as autoreceptors. Autoreceptors present on the soma or dendrites act to

slow firing rate whereas autorecerptors present on the nerve terminal inhibit DA synthesis and release. D2 autoreceptors are present within the SNpc and both D1 and D2 receptors can be found within the striatum.

Signal transduction

Activation of D1 receptors results in an increase of adenylate cyclase activity resulting in increased cAMP and the activation of protein kinase A, which in turn phosphorylates DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein). Phosphorylation of DARPP-32 results in potent inhibition of protein phosphatase-1 activity. Conversely D2 receptor activation inhibits adenylate cyclase activity resulting in decreased DARPP-32 phosphorylation. D2 activation also increases calcium influx which in turn also inactivates DARPP-32.

1.1.5 The nigrostriatal pathway and the Basal Ganglia Motor Loop

The NSDA is one of four major dopaminergic pathways within the brain and connects the SNpc to the striatum. The NSDA forms part of a complicated set of connections known as the basal ganglia motor loop. This loop consists of a group of subcortical nuclei that together regulate the initiation and execution of movement. Basal ganglia circuitry is thought to consist of two major projection systems between input structures and output structures; a direct pathway between the striatum and the internal segment of the globus palidus (GPi) and the substantia nigra reticulata (SNr) (GABAergic; inhibitory), and an indirect pathway involving the globus palidus (external segment; GPe) and the subthalamic nucleus (STN). Connections between the GPe and STN are GABAergic (inhibitory), whereas projections from the STN to the GPi/SNr are glutamatergic (excitatory). Activation of the direct pathway results in inhibition of the

GPi/SNr resulting in disinhibition of thalamocortical neurons. Activation of the indirect pathway however, causes net excitation of the GPi/SNr thus causing inhibition of the thalamocortical neurons. The balance between these pathways is important in regulating the output of the basal ganglia and therefore in the control of motor function (Miyachi, 2009); Figure 1.1). These two pathways are differentially affected by dopaminergic projections from the SNpc to the striatum. Most striatal neurons are medium spiny GABAergic neurons that differentially express D1 or D2 dopamine receptors: direct pathway neurons predominately express D1 receptors whilst indirect pathway neurons express D2 receptors. Activation of D1 receptors would activate direct pathway neurons whilst activation of D2 receptors would inhibit activation of the indirect pathway. Thus the effect of dopamine signalling on both pathways would result in a reduction of basal ganglia output to the thalamus. Reduced DA signalling as seen in PD therefore results in increased basal ganglia output producing disinhibition of the thalamus. This classical model of basal ganglia circuitry has greatly improved our understanding of PD pathophysiology and aided the development of treatments for PD. However, more recent evidence suggests that the connections within the basal ganglia are actually much more complex (Obeso et al., 2002).

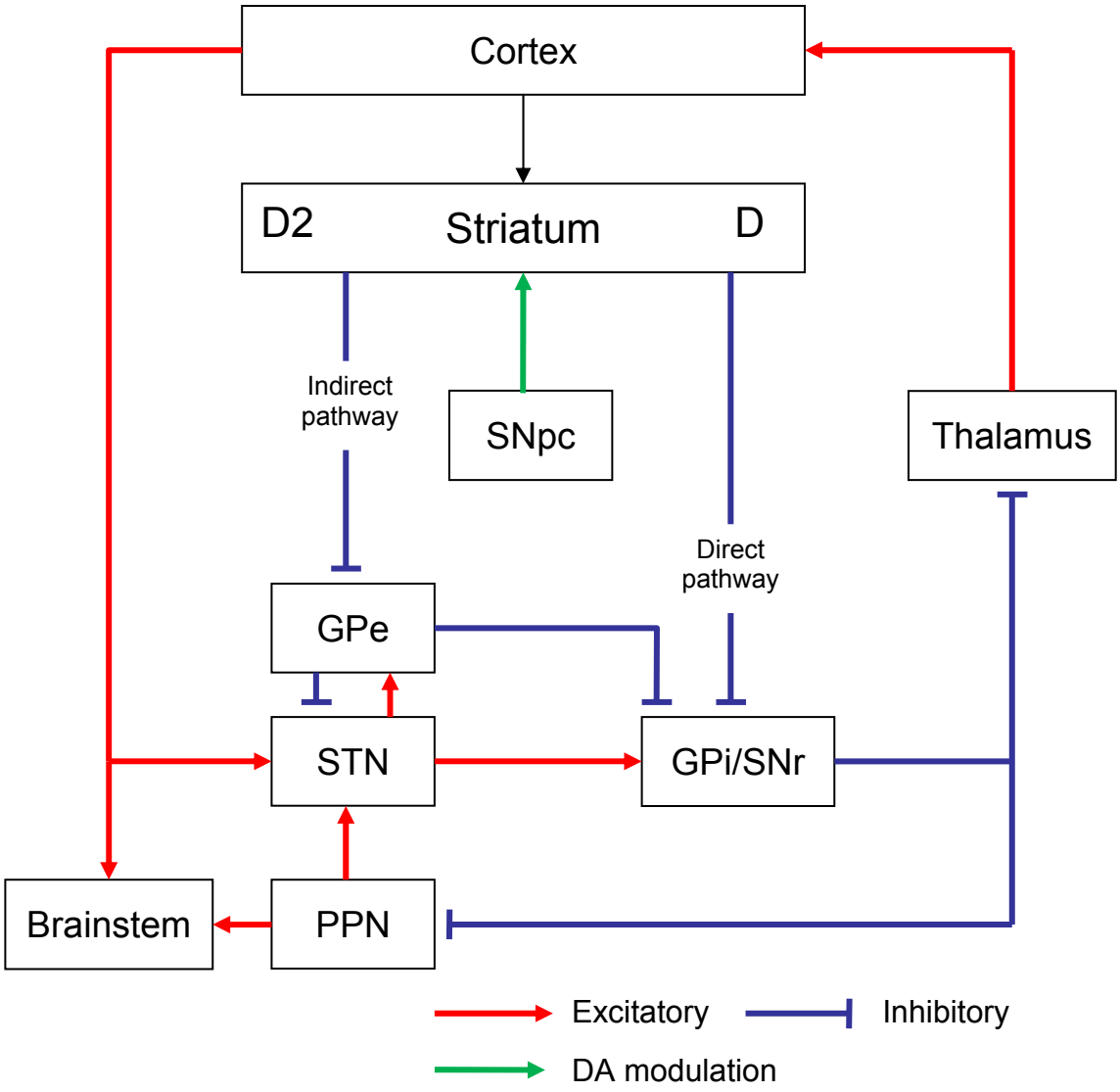


Figure 1.1. Schematic diagram of basal ganglia motor loop

Simplified illustration of basal ganglia anatomy. The direct and indirect pathways from the striatum have net effects of disinhibition and inhibition on the cortex, respectively. STN, subthalamic nucleus; GPe, external globus pallidus; GPi, internal globus pallidus; SNr, substantia nigra pars reticulta; SNc, substantia nigra pars compacta; PPN, pedunculopontine nucleus. Adapted from (Yin and Knowlton, 2006)

1.1.6. Mesolimbic and mesocortical DA system

Although cell loss in PD is most severe in the SNpc there is also significant degeneration of DA neurons, albeit to a lesser extent, within the VTA (German et al.,

1989). These neurons form two major DA projections forming the mesolimbic (MLDA) and mesocortical (MCDA) pathways (Figure 1.2). The MLDA consists of dopaminergic neurons within the VTA that primarily project through the medial forebrain bundle to the nucleus accumbens (NAcc) which is found in the ventral striatum, as well as innervating the amygdala and hippocampus. NAcc DA input from the VTA modulates the activity of GABAergic medium spiny neurons within the NAcc that form part of the cortico-striato-thalamo-cortico-loop. The MLDA forms the brains major reward pathway (Schultz, 2000) and as such has been implicated in depression and addiction. On the other hand the MCDA is composed of DA neurons that primarily innervate the PFC. This pathway is involved in motivational and emotional responses and has been associated with the negative symptoms of schizophrenia.

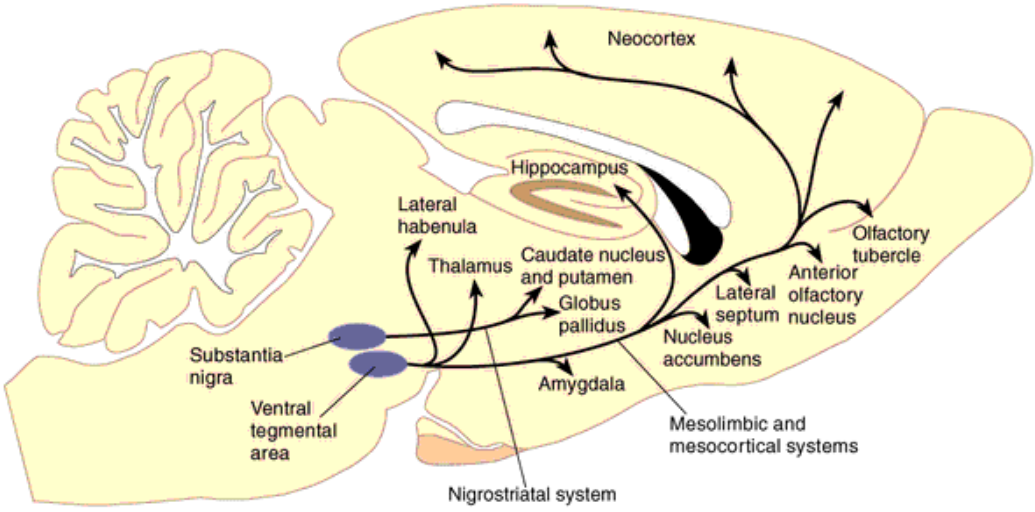


Figure 1.2. Dopaminergic pathways within the rat brain

The nigrostriatal system consists of projections from the SNpc to the striatum. Projections from the VTA primarily innervate the nucleus accumbens and the frontal cortex forming the mesolimbic and mesocortical pathways respectively.

1.1.7 Locus coeruleus

The locus coeruleus (LC) is a nucleus in the rostral pons and is the main source of noradrenergic neurons within the brain. The LC has a mainly excitatory effect on the brain and mediates attention and arousal, playing a major role in the physiological response to stress. The LC innervates many areas of the brain including the spinal cord, brain stem, cerebellum, hypothalamus, thalamus, amygdala and cortex. It receives major inputs from the hypothalamus, cingulated gyrus, amygdala and medial prefrontal cortex.

Noradrenaline (NA) utilises the same biosynthetic pathway as DA. DA is subsequently converted to NA by dopamine-β-hydroxylase (DβH). NA is taken up into synaptic vesicles by VMAT and on its release acts on adrenergic receptors which are broadly classified into α (α1, α2) and β(β1, β2, β3) adrenoceptors. Uptake of NA occurs either presynaptically by uptake 1 or by non neuronal cells in the vicinity (uptake 2). NA is metabolised by COMT and MAO.

1.1.8 Nucleus Basalis Meynert

The nucleus basalis of meynert (NBM) is located in the basal forebrain and is the principal source of cholinergic projections to the cerebral cortex. Central cholinergic activity is thought to be involved in the modulation of behaviour.

Acetylcholine (ACh) is synthesised within cholinergic neurons by choline acetyltransferase from choline and acetyl-co-A. Acetylcholinesterase which is abundant within the synapse converts ACh back to choline and acetyl-co-A and rapidly clears the

synapse of ACh. There are two types of Ach receptors – muscarinic metabotrophic receptors and nicotinic ionotrophic receptors, both of which are found within the CNS.

1.1.9 Current Treatments for Parkinson's disease

Dopaminergic treatments

Dopamine replacement therapy is the mainstay of PD treatment. This works by either replacing the lost DA in the striatum or by mimicking DA action. Three decades after its first use, the DA precursor levodopa (L-Dopa) is still the gold standard for the treatment of Parkinson's disease, primarily due to its high efficacy. However, chronic treatment with L-Dopa results in disabling motor complications due to the pulsatile stimulation of DA receptors such as 'wearing off' effects and dyskinesias (Marsden and Parkes, 1976). Combination therapy with COMT inhibitors such as entacopone reduces L-Dopa metabolism in the periphery, extending the half life of L-dopa thereby prolonging its action. It also results in a more continuous stimulation (rather than pulsatile) of DA receptors which significantly improves motor fluctuations. Alternately, the use of DA agonists provides a more sustained activation of DA receptors due to their longer half life but fail to match the efficacy of L-Dopa. However, they do not produce motor complications to the same degree as with L-Dopa and are therefore often used in younger patients to delay the need for L-Dopa therapy. Side effects of DA agonists include hallucinations, confusion and psychosis. The MAO-B inhibitors selegiline and rasagiline are effective either alone or in combination with L-Dopa, however patients will eventually require L-Dopa treatment. When given in combination MAO-B inhibitors prevent the breakdown of dopamine and hence preserve what dopamine is formed in the brain allowing a marked reduction in the dose of L-Dopa needed to control the motor symptoms.

Non-dopaminergic treatments

A major breakthrough occurred with the development of deep brain stimulation, whereby electrodes are stereotactically implanted to achieve thalamic stimulation. This reduced drug resistant motor fluctuations and dyskinesias and preserves L-dopa responsiveness (Houeto et al., 2002). However, such surgical procedures are not without risks and side effects e.g. depression. Promising potential non-dopaminergic treatments for PD also include α2 receptor agonists (Rascol et al., 2001) and adenosine A_2 antagonists (LeWitt et al., 2008), both of which act on GABAergic striatal neurons, as well as 5-HT agonists (Bara-Jimenez et al., 2005) and glutamate antagonists (Samadi et al., 2008), all with the view of normalising the output pathways from the striatum. (Olanow et al., 1996). The possibility of the future use of stem cells would make this approach more viable. Treatments for the non-dopaminergic symptoms of PD have also been developed. Cholinesterase inhibitors and atypical antipsychotics have been successful in treating the dementia and psychosis associated with PD (Fox et al., 2008). There are currently no reliable biomarkers for preclinical PD. The development of a biomarker for the disease would aid the development of neuroprotective strategies to prevent the degeneration of SNpc neurons.

1.1.10 Epidemiology

The etiology of PD is unknown but both genetic and environmental factors have been implicated in the disease.

1.1.10.1 Environmental risk factors

Several environmental factors such as rural living, drinking well water, pesticide exposure and occupation have been associated with an increased risk of developing PD

(Tanner and Goldman, 1996). Excess weight in midlife has been associated with a greater PD risk whereas a low calorie intake is protective (Love, 2005; Ragonese et al., 2007). This is particularly interesting as DA is involved in the regulation of food intake. Conversely cigarette smoking and coffee/tea consumption have been linked with a decreased risk of PD (Ross and Petrovitch, 2001; Kalda et al., 2006; Quik et al., 2008). Infectious agents such as influenza, nocardia (tuberculosis like germ) and helicobacter have also been connected to PD (Jang et al., 2009). Furthermore, parental PD increases the risk of PD in offspring. This could suggest either a genetic or an environmental influence. However, the fact that the risk increases the younger the child is when the parent develops PD symptoms suggests that this is due to the environment (Chade et al., 2006). Moreover, immigrants from Asia and Africa in the US have an increased risk of PD than they would in their native countries, again supporting a role of the environment (Schoenberg et al., 1988). More recently an interest in the impact of early life events in the development of PD has developed. Indeed, a shorter adult height, that may be associated with early life events affecting growth, is associated with an increased risk of PD in males (Ragonese et al., 2007). Furthermore people born in the year of an influenza pandemic have a higher risk of developing PD (Singer and Weiner, 1989).

1.1.10.2 Familial PD

The causes of almost all cases are unknown but occasionally PD can be inherited as a simple mendelian trait. Although sporadic and familial PD are very similar, inherited forms tend to have an earlier onset and have atypical clinical features. Five genes have been clearly linked to PD and there are a number of other genes or genetic linkages that have been associated with PD (Shown in Table 1.2).

α-synuclein

Mutations in α-synuclein were the first genetic mutations to be linked to PD (Polymeropoulos et al., 1996). As α-synuclein is a major structural component of Lewy bodies found in both sporadic and familial PD it is likely that it plays a role in both forms of PD with high levels or mutated forms of α-synuclein being detrimental (Winkler et al., 2007).

Leucine Rich Repeat Kinase-2 (LRRK2)

LRRK2 is a large protein with multiple functional domains including kinase and GTPase domains (West et al., 2005). There is evidence to suggest that LRRK2 may be involved in vesicular transport within the SNpc (Biskup et al., 2006).

Parkin

Kitada et al., 1998 (Kitada et al., 1998) identified the first autosomal recessive gene to be linked to PD. Parkin is an ubiquitin E3 ligase and mutations in parkin lead to a loss of function resulting in the accumulation of toxic substrates (Zhang et al., 2000). Sporadic PD is also characterised by reduced parkin activity thus accumulation of these substrates may contribute to the pathogenesis of PD.

DJ-1

DJ-1 was identified by Bonifati in 2003 and is thought to function as an antioxidant protein as well as a redox sensitive chaperone (Bonifati et al., 2003). Knockout of DJ-1 led to a deficit in mitochondrial scavenging of hydrogen peroxide (Andres-Mateos et al., 2007).

PINK1

PINK1 was identified in 2004 by Valente et al., (Valente et al., 2004) and is a mitochondrial associated kinase.

These gene defects appear to be linked by a common pathway: the degradation of proteins. Figure 1.3 depicts how these genetic mutations could result in cell death due to impairments in protein handling.

Table 1.2. Genes implicated in familial PD

Locus	Gene	Inheritence	Reference
PARK 1 (+4)	α-synuclein	AD	(Polymeropoulos et al., 1997)
PARK 2	Parkin	AR	(Kitada et al., 1998)
PARK 3	Unknown	AD	(Gasser et al., 1998)
PARK 5	UCH-L1	unclear	(Leroy et al., 1998)
PARK 6	PINK1	AR	(Valente et al., 2004)
PARK 7	DJ-1	AR	(Bonifati et al., 2004)
PARK 8	LRRK2	AD	(Funayama et al., 2002)
PARK 9	ATP13A2	AR	(Ramirez et al., 2006)
PARK 10	Unknown	unclear	(Hicks et al., 2002)
PARK 11	Unknown	unclear	(Pankratz et al., 2003)
PARK 12	Unknown	unclear	(Pankratz et al., 2003)
PARK 13	Omi/HHA2	AD	(Strauss et al., 2003)

Table adapted from (Vila and Przedborski, 2004).

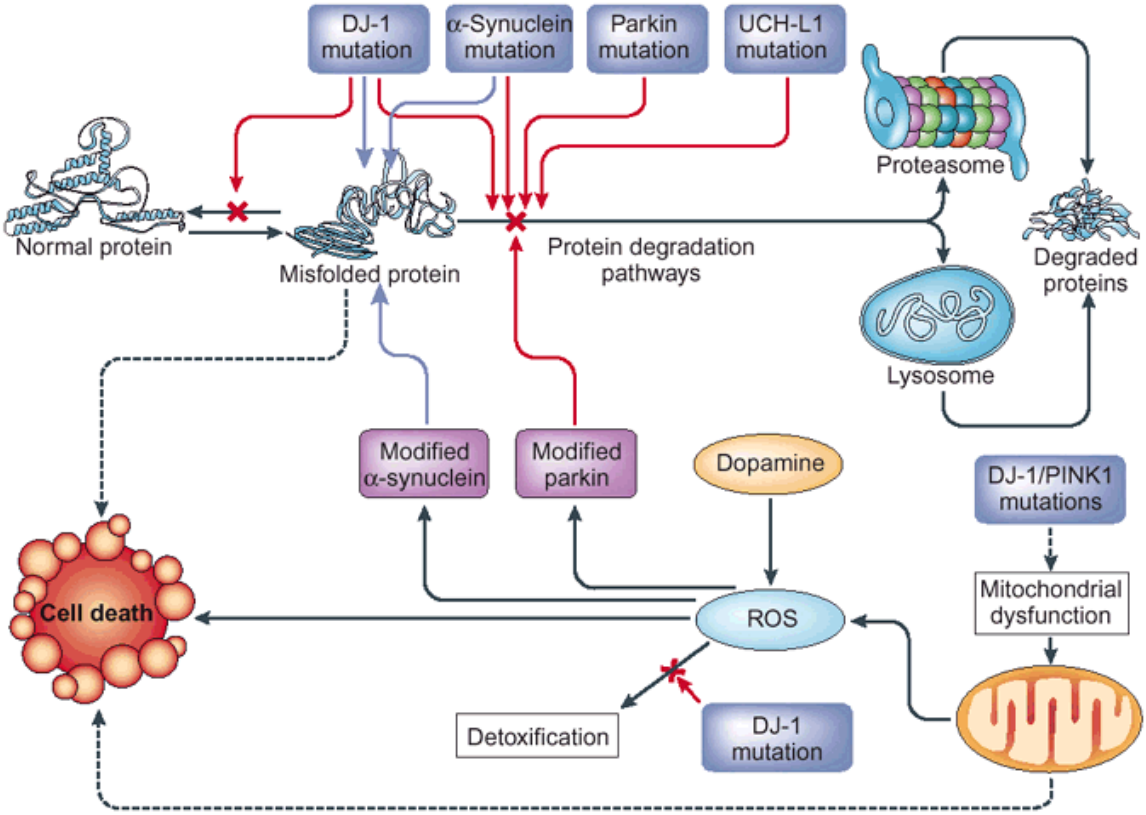


Figure 1.3. Genetic mutations and the pathogenesis of PD.

Genetic mutations that lead to PD suggest that misfolded proteins may contribute to PD neurodegeneration. Mitochondrial dysfunction, oxidative stress and protein mishandling are all tightly interconnected in this hypothesized pathogenic cascade. Adapted from (Vila and Przedborski, 2004).

1.1.11 Pathogenesis of PD

Current thinking suggests that PD is not caused by one single factor but from the convergence of a number of deleterious factors occurring both within DA neurons as well as in the surrounding neurons and glia. Evidence for the involvement of these factors has been obtained mainly through the use of toxin-based models but the recent identification of genes involved in familial PD have increased our understanding of some of these processes. The following sections describe these processes in more detail. These detrimental pathways may interact with each other producing a self perpetuating cascade of detrimental events ultimately resulting in progressive neurodegeneration.

Oxidative stress

Most cellular reactions utilise oxygen for catalysis and energy production. This results in the production of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxy radicals and reactive nitrogen species (RNS) such as peroxynitrite and nitro-tyrosyl radicals. Excessive production of ROS and RNS is detrimental to cell membranes and can ultimately result in cell death. To balance this there are antioxidants such as glutathione and vitamin E and antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase. Disruption of this balance leads to damage of proteins, lipids, DNA and can activate apoptotic pathways. Brain cells are particularly susceptible to oxidative damage due to high levels of polyunsaturated fatty acids in their membranes and the relatively low activity of antioxidant enzymes within the brain (Mariani et al., 2005). Aging is associated with elevated oxidative stress due to the accumulation of oxidative damage and decline in antioxidant status over time (Mariani et al., 2005). This is considered important as aging is a major risk factor for the development of neurodegenerative disease. There is now

extensive evidence for a role of oxidative and nitrosative stress in the pathogenesis of PD. In the SNpc of the PD brain there are reduced levels of glutathione (Pearce et al., 1997); increased lipid peroxidation (Dexter et al., 1989b); increased oxidative protein damage (Good et al., 1998) (Duda et al., 2000) and increased levels of DNA damage in affected neurons (Alam et al., 1997). ROS and RNS are generated by a number of biological processes, for example nitric oxide (NO) can be formed by the enzyme nitric oxide synthase (NOS) and iron can drive the formation of hydroxyl radicals by fenton reactions (Dexter et al., 1989a). DA is highly reactive and is more predisposed to oxidation than other catecholamine (LaVoie et al., 2005). Indeed, the metabolism of DA by MAO-B produces hydrogen peroxide as a by product (Adams et al., 1972) as well as dopamine quinone which directly modifies proteins (Stokes et al., 1999). In addition, several downstream products of DA oxidation result in complex I inhibition (Spencer et al., 1998); (Li et al., 1998). Furthermore, nitration of tyrosine on TH by peroxynitrite has been shown to inhibit TH activity which may contribute to disease progression (Blanchard-Fillion et al., 2001).

Inflammation

In acute conditions the inflammatory response protects tissues against invading agents and promotes healing but when chronically sustained it can cause serious damage to the host's own tissue. The brain was originally thought to be an immune privileged organ but recent evidence demonstrates that it has its own unique defence system. Microglia are the only cells within the CNS capable of producing an immune response (Aloisi, 2001). They are immunocompetent and phagocytic cells that mediate the innate defence system and are therefore critical for normal CNS function (Kim and de Vellis, 2005). They can also act as scavenger cells in the event of a pathologic insult (Beyer et al.,

2000). Microglia actively monitor the CNS parenchymal environment by continual movement of their fine processes (Nimmerjahn et al., 2005). Under normal conditions neurons and astrocytes suppress microglial activation. Microglial activation is inhibited by the engagement of CD200 glycoprotein expressed on the surface of neurons and its receptor on microglia (Hoek et al., 2000). Furthermore electrical activity and soluble factors released from neurons suppress microglial MHC II (major histocompatability complex II) antigen presentation (Wei and Jonakait, 1999; Neuman 2001) and astrocytes suppress microglial activation by releasing TGF-β or IL-10 (Aloisi, 2001). Resting microglia are highly sensitive to changes in their immediate environment and can quickly activate in response to a pathologic event (Wojtera et al., 2005). In their resting state microglia exhibit a ramified morphology and low expression of membrane receptors. On their activation there is an increase in IgG reactivity and up-regulation of cell adhesion molecules (Orr et al., 2002). Microglia then attach to neurons under the direction of chemokines expressed by neurons (Aloisi, 2001). Once activated the microglial cell body becomes enlarged and the number of microglia increases (Raivich et al., 1996). If the pathogenic stimulus is maintained microglia then cluster around the neurons and become phagocytic (McGeer and McGeer, 1998). Activated microglia at the site of injury change their morphology, express increased levels of MHC antigens and become phagocytic (Hayes et al., 1987, 1988). They release inflammatory cytokines that amplify the inflammatory response by activating and recruiting other cells to the lesion. Cytokines are key regulators of innate and adaptive immune responses and microglia are the principal source of cytokines within the brain. Il-1 and TNF-α are the two main proinflammatory cytokines produced by microglia during CNS inflammation but microglia are also capable of producing antinflammatory cytokines such as TGF-β and IL-10. Moreover, microglia can release ROS that mediate neuron damage.

Inflammation was first proposed to be involved in PD by McGeer et al, in 1988 (McGeer and McGeer, 1998). Their studies found that there was upregulation of MHC molecules and an increased number of activated microglia in the brains of PD patients. Further evidence for an inflammatory component for PD was provided by studies showing that there were increased levels of antibodies to proteins modified by DA oxidation (Rowe et al., 1998) and that there was increased levels of the proinflammatory cytokines TNF-α, IL-1β and IL-6 in the CSF (cerebro spinal fluid) and striatum of PD brains (Mogi et al., 1994b; Mogi et al., 1994a; Blum-Degen et al., 1995; Muller et al., 1998). Activated microglia expressing TNF-α, IL-1β, IFN-γ and iNOS have been identified within the SNpc of PD patients (Hunot et al., 1996; Hirsch et al., 1998). Additionally IL-1, IL-6 and TNF-α has been found within the CSF and basal ganglia of PD patients (Nagatsu, 2002). Interestingly post-mortem brains of humans exposed to MPTP (1-methyl--4-phenyl-1,2,3,6-tetrahydropyridine; commonly used to model PD) show activated microglia 16 years after exposure to the toxin suggesting that there is a prolonged inflammatory response.

Kim and Joh (2006) proposed a pathway by which inflammation could result in DA degeneration. They suggested that activated matrix metalloproteinase 3 (MMP-3) released from stressed neurons was responsible for microglial activation and the generation of superoxide as well as NFκB mediated cytokine release. Furthermore, neuromelanin that is found in DA neurons can elicit pro-inflammatory cytokine and nitric oxide release and α-synuclein that is found in Lewy bodies can cause superoxide release from microglia. Activated microglial could then cause DA neuron degeneration either by superoxide, nitric oxide and pro-inflammatory cytokines or by direct

phagocytosis of neurons. This would result in a self propagating cycle that sustains chronic inflammation and induces progressive degeneration.

Mitochondrial dysfunction

The first evidence that mitochondrial dysfunction might be involved in PD came from the observation that MPTP inhibition of complex 1 of the electron transport chain resulted in a PD like syndrome (Langston and Ballard, 1983). Later, decreased activity of complex 1 was found in post-mortem PD brains (Parker et al., 1989). The detrimental effects of complex 1 inhibition are: increased production of reactive oxygen species leading to oxidative damage of lipids proteins and DNA; energy depletion and the activation of apoptotic pathways (Vila et al., 2008). More recently the discovery that mutations in several genes that encode mitochondrial proteins result in PD provides further evidence for a role of mitochondria in the disease process (section 1.1.10.2).

Mitochondria contain molecules that when released can activate caspase dependent or independent cell death. This is regulated by pro (Bax) and anti-apoptotic proteins (Bcl-2 family) that control permeability of mitochondria. Perrier et al., 2007 proposed a pathogenic mechanism by which neuronal death due to complex-1 inhibition results from a self perpetuating cascade of detrimental events. The blockade of complex-1 blocks the electron transport chain resulting in increased ROS production. This in turn increases the availability of cytochrome c within the mitochondria which can be released by the activation and translocation of Bax due to the activation of the transcription factors p53 and JNK resulting from DNA oxidative damage. Several elements of this cascade have now been shown to be present in post-mortem PD brains (Vila et al., 2008).

PD linked genes have the capabilities to either directly or indirectly affect mitochondria. Mitochondrial PINK1 can attenuate cytochrome c release and apoptotic cell death (Plun-Favreau et al., 2007; Pridgeon et al., 2007). This protective effect of PINK1 is mediated by its kinase domain and is abolished in PD mutants (Haque et al., 2008). PINK1 can also interact with parkin (Yang et al., 2006) which is localised in mitochondria and also prevents cytochrome c release and apoptotic cell death (Kuroda et al., 2006). α-synuclein can interact directly with mitochondria to either induce the release of ctochrome c (Parihar et al., 2008) or through the inhibition of complex 1 (Devi et al., 2008). DJ-1 has been identified as an atypical peroxidase able to scavenge mitochondrial hydrogen peroxide (Andres-Mateos et al., 2007). Oxidative stress promotes its localisation to mitochondria (Zhang et al., 2005a). Additionally LRRK2 is partly localised to mitochondria and can induce the release of cytochrome c and therefore apoptotic cell death via caspase cascade (West et al., 2005; Iaccarino et al., 2007).

Mitochondria also have a role in aging and as aging is the biggest risk factor for PD this is of significant importance. Mitochondria are a major source of ROS as they are a by product of respiratory chain function. Wallace (2005) postulated that due to their close proximity to mitochondria, ROS could damage mitochondrial components during aging resulting in mitochondrial dysfunction and further ROS production.

Ubiquitin-proteasome-system (UPS) dysfunction

The UPS is an intracellular protein degradation system responsible for protein turnover within the cell (Hershko and Ciechanover, 1998). Proteins for degradation are covalently tagged with ubiquitin protein. This involves an elaborate ligation reaction requiring the sequential activation of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligating enzyme (E3). This ubiquitination process is repeated several times and the polyubiquinated protein is then targeted for degeneration by the 20S proteasome.

Direct evidence for a role of the UPS in the pathogenesis of PD came from the identification of mutations in the parkin gene that resulted in PD (Kitada et al., 1998). Parkin encodes a ubiquitin ligase (Zhang et al., 2000) thus loss of function of this gene impaired UPS functioning. In addition, missense mutations in UCHL1 that are linked to PD result in decreased ubiquitin hydroxylase activity (Leroy et al., 1998). Although α-synuclein does not form part of the UPS machinery its over expression can inhibit proteasome function (Tanaka et al., 2001; Chen et al., 2006). Further evidence for an involvement of the UPS in PD came from studies showing that there was reduced expression of subunits of the proteasome in sporadic PD patients and that in SN extracts isolated from PD brains there was decreased activity of the 20S proteasome (McNaught et al., 2003; Tofaris et al., 2003). Rotenone and MPTP treated mice have also been shown to exhibit reduced proteasome function (Betarbet et al., 2006) (Fornai et al., 2005). Aberrant protein homeostasis in PD patients could result in toxic accumulation of intracellular proteins. Indeed, misfolded and aggregated α-synuclein is thought to be a primary event in PD caused by mutations in α-synuclein. Additionally, aggregated α-

synuclein can inhibit proteasome function (Snyder et al., 2003) resulting in a vicious cycle of UPS impairment.

Glutamate excitotoxicity

Glutamate is the primary excitatory neurotransmitter within the mammalian CNS. When neurons are injured they release higher concentrations of glutamate than usual that leads to a prolonged post synaptic response. This can ultimately result in cellular dysfunction and the initiation of apoptosis, known as excitotoxicity (Olney and de Gubareff, 1978). Increased levels of extracellular glutamate result in prolonged postsynaptic activation of NMDA receptors causing a greater influx of Ca^{2+} into neurons (Meldrum, 1990). Fast excitotoxicity is mediated by NMDA activation and the activation of downstream signalling pathways where as slow excitotoxicity is caused by mitochondrial dysfunction. Mitochondria act to sequester excess intracellular Ca^{2+} (Peng and Greenamyre, 1998). If sustained this excess Ca^{2+} leads to uncoupling of the electron transport chain resulting in decreased ATP synthesis producing an energy crisis as well as increasing the generation of ROS (Dykens, 1994). Furthermore Ca^{2+} overload results in mitochondrial depolarisation thereby increasing mitochondrial permeability and inducing the release of apoptosis initiating factor (AIF) and cytochrome c leading to the activation of apoptotic pathways.

There are excitatory glutamate connections between the STN, PPN and SN. These pathways have been found to be hyperactive in both PD patients (Benazzouz et al., 2002) and in animal models of PD (Orieux et al., 2000; Oueslati et al., 2005)). Moreover DA neurons express NMDA receptors (Mereu et al., 1991) and pharmacological blockade of these receptors is neuroprotective (Turski et al., 1991).

Further evidence for a role of glutamate in PD comes from studies in which the pathways from the STN and PPN are lesioned, reducing excitatory drive to the SN and resulting in neuroprotection.

1.1.12 Animal models of PD

Toxin based models aim to reproduce the pathological and behavioural features of the human disease in rodents and primates. Although genetic models of PD are now available, mimicking familial gene defects, such models have been disappointing failing to mimic the pathological features of PD. Consequently, toxin based models are still widely used due to their ability to induce the loss of SNpc neurons. The advantages and disadvantages of the most commonly used models are displayed in table 1.3 and are discussed further below.

6-OHDA

6-hydroxydopamine (6-OHDA) is a noradrenaline analogue that was introduced as a catecholaminergic neurotoxin over 40 years ago and was used to produce the first animal model of PD (Ungerstedt, 1968) and is still widely used today. Due to its structural similarities to DA and noradrenaline, 6-OHDA is selectively taken up by DA and noradrenaline transporters (Luthman et al., 1989). The subsequent accumulation of 6-OHDA within the cytosol promotes a high rate of free radical and quinone formation (Figure 1.5) due to the rapid autoxidation of 6-OHDA. Additionally, 6-OHDA can accumulate in mitochondria where it inhibits the activity of the electron transport chain by inhibiting complex I. 6-OHDA does not cross the blood brain barrier so has to be administered stereotactically. 6-OHDA is injected into either the SNpc, the medial

forebrain bundle (MFB; contains ascending DA and 5-HT projections to the forebrain) or the striatum. Administration to the SNpc results in DA neuron degeneration 24 hours after injection that occurs without apoptotic morphology (Jeon et al., 1995) whereas injections to the MFB result in apoptotic DA neuron degeneration (He et al., 2000; Zuch et al., 2000). On the other hand intrastriatal injections of 6-OHDA result in a prompt degeneration of striatal DA terminals but a delayed, retrograde loss of SNpc neurons occurring over several weeks. None of these models result in the formation of Lewy bodies. Generally unilateral models have been used where the contralateral hemisphere can serve as an internal control. In addition, unilaterally lesioned rats exhibit characteristic circling behaviour in response to DA releasing drugs that is correlated with the degree of neuronal loss. However, this is only observed in animals that have an extensive nigrostriatal lesion. Bilateral injections are rare due to high mortality rates due to aphagia and adipsia (Ungerstedt, 1971). There is also a marked inflammatory response to 6-OHDA characterised by the activation of microglia – the brain's macrophages. This correlates well with the human disease in which inflammation has been identified as a key mechanism involved in the degenerative process. The 6-OHDA model is also associated with an increase in iron and oxidative stress in the SNc. The 6-OHDA model is still the most widely used PD model today, mainly due to its versatility; the fact that lesions are highly reproducible and the relatively low cost of the procedure.

MPTP

The neurotoxic properties of MPTP were accidently discovered when drug users who had injected MPTP developed symptoms remarkably similar to that of PD. MPTP is able to cross the blood brain barrier where it is metabolised by MAO-B in astrocytes to

the active metabolite MPP+ (1-methyl-4-phenylpyridinium). MPP+ is selectively taken up into the cytosol by catecholaminergic transporters (Javitch et al., 1985) (Bezard et al., 1999). Within neurons MPP+ can be taken up by the vesicular monoamine transporter-2 (VMAT2) into synaptic vesicles which appears to prevent toxicity (Takahashi et al., 1997) or it can react with cytosolic enzymes (Klaidman et al., 1993). The toxic effects of MPP+ however are due to it's accumulation in mitochondria where it inhibits complex I of the electron transport chain (Nicklas et al., 1985; Mizuno et al., 1987) resulting in reduced levels of ATP in the striatum and ventral midbrain as well as increased ROS formation (Hasegawa et al., 1997; Figure 1.4). Lewy bodies are not formed in the MPTP model (Forno et al., 1993). Susceptibility to MPTP neurotoxicity is species dependent: rats are relatively resistant to the toxin whereas mice and non human primates are more susceptible to its neurotoxic effects. Mice treated with MPTP do not develop motor symptoms (Przedborski et al., 2001) so have been used to study molecular mechanisms underling degeneration, whereas MPTP non-human primates have largely been used to study behavioural and physiological consequences of DA neuron degeneration. In the acute model of MPTP administration there is neurodegeneration without apoptosis (Jackson-Lewis et al., 1995), as well as an inflammatory response (Kurkowska-Jastrzebska et al., 1999). In the chronic model of PD however, MPTP administration results in cell death by apoptosis (Tatton and Kish, 1997). As in PD DA neurons of the SNpc are more susceptible to MPTP neurotoxicity than VTA neurons. Additionally neurons that contain neuromelanin are more susceptible to MPTP induced degeneration (Herrero et al., 1993).

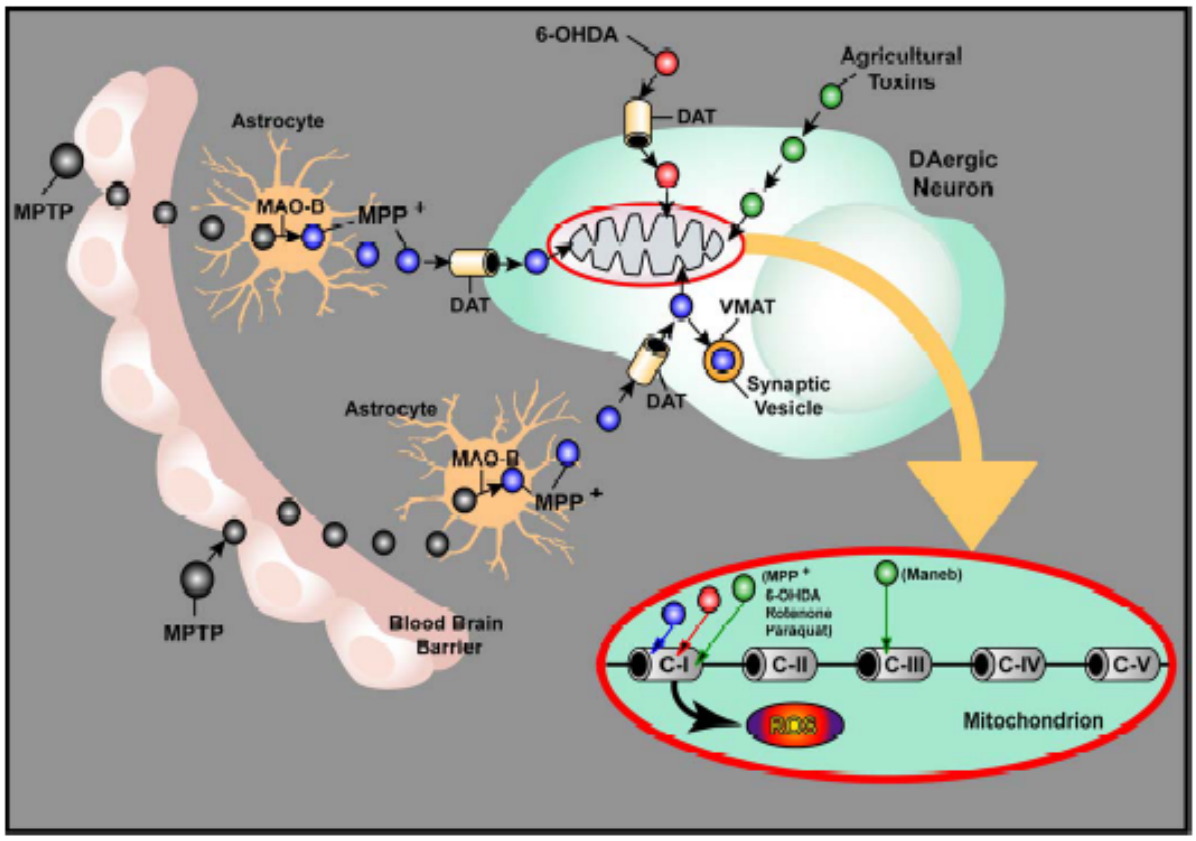


Figure 1.4. Schematic overview of molecular and intracellular pathways of dopaminergic neurotoxins applied in animal models of Parkinson's disease.

MPTP (black vesicles) enters the brain by crossing the blood–brain barrier after systemic injection. Thereafter, MPTP (black vesicles) is taken up by astrocytes and converted in its active form: MPP+ (blue vesicles, catalyzed by MAO-B). Then, MPP+ (blue vesicles) is released from astrocytes into the extracellular space and can be specifically transported into dopaminergic neurons via DAT. Inside the dopaminergic neuron MPP+ (blue vesicle) can be (i) concentrated in mitochondria or can (ii) be sequestrated into synaptic vesicles (yellow-blue vesicle) via VMAT. 6-OHDA (red vesicles) has to be stereotactically targeted into the substantia nigra, MFB or the striatum. 6-OHDA (red vesicles) is selectively taken up via DAT by dopaminergic neurons. 6-OHDA (red vesicle) can also be accumulated by mitochondria. Agricultural toxins (green vesicles), penetrate into the dopaminergic neuron unspecifically and accumulate inside the mitochondria. MPP+ (blue vesicle), rotenone (red vesicle) and paraquat (green vesicle) affect complex 1 (C1) directly, leading to C-1 inhibition and the generation of reactive oxygen species.

Rotenone

Rotenone is widely used as an insecticide and fish poison and has only relatively recently been developed as a rodent model of PD (Betarbet et al., 2000). It is highly lipophillic and can therefore easily cross biological membranes. It binds to the same site as MPP+ on complex 1 thus impairing oxidative phosphorylation within mitochrondria (Schuler and Casida, 2001). It has been reported that systemic administration of rotenone results in selective degeneration of DA SNpc neurons however other studies suggest that this degeneration is more widespread (Hoglinger et al., 2003); (Lapointe et al., 2004). In the rotenone model α-synuclein positive inclusions have been identified within DA neurons of the SNpc (Betarbet et al., 2000). However, in this model the extent of the lesion is highly variable and the mortality rate is high.

Paraquat

Paraquat (N,N'-dimethyl-4-4'-bipiridinium) is a herbicide that has a similar structure to MPP+. Epidemiologic studies suggest that environmental exposure to paraquat increases the risk of developing PD (Liou et al., 1997). Its toxicity is likely to be due to the formation of superoxide free radicals (Day et al., 1999) (Przedborski and Ischiropoulos, 2005). Systemic administration of paraquat results in degeneration of SNpc neurons and their projections, which is accompanied by the presence of α-synuclein inclusions (McCormack et al., 2002). There are also increases in levels of α-synuclein in the frontal cortex and ventral midbrain (Manning-Bog et al., 2002). However, in this model the extent of the lesion is highly variable and the mortality rate is high.

Proteosome Inhibitors

In 2004, McNaught (McNaught et al., 2004) described a new model of PD. This model was based on findings that there were defects in the ubiquitin-proteasome-system in PD patients (Furukawa et al., 2002; McNaught et al., 2003) and that mutations in Parkin, an ubiquitin E3 ligase causes early onset PD (Kitada et al., 1998). In the McNaught study, animals were treated with either a natural (epoxomicin) or synthetic proteasome inhibitor (PSI). These rats developed Parkinson like symptoms within 1-2 weeks that were alleviated by L-Dopa or apomorphine administration. These animals also had reduced levels of DAT, reduced striatal DA levels and DA neuron death with apoptosis. In addition, neuronal degeneration was present within the locus coeruleus, dorsal motor nucleus of the vagus and the nucleus basalis of meynert. Furthermore degenerating neurons contained cytoplasmic inclusions that resembled Lewy bodies. Unfortunately several groups, including this group, have failed to reproduce the findings of this study and thus the model is surrounded by considerable controversy (Bove et al., 2006; Kordower et al., 2006; Manning-Bog et al., 2006). Although highly attractive due to its potential to recapitulate many aspects of the disease, the proteasome inhibitor model will be unable to provide a suitable for PD until these discrepancies are resolved.

Gene based models

Although inherited PD accounts for less than 5% of all cases it is likely that they share similar pathogenic mechanisms with sporadic PD cases, thus gene based models of PD have been generated. For instance a variety of transgenic mice models have been developed that over express α-synuclein. Although they are a useful tool to investigate the pathogenesis of PD they often exhibit very little SNpc pathology.

Table 1.3. Advantages and disadvantages of animal models of PD

	ADVANTAGES	DISADVANTAGES
6-OHDA	-Specific loss of DA neurons -Uniform lesions -Low inter-animal variability -Quantifiable motor deficits -Unilateral lesions possible	-No Lewy body formation -Only one type of cell loss -Rapid cell death -Possible non specific damage
MPTP	-Known to produce PD like symptoms in humans	-No Lewy body formation -No stable motor defecit -Inter-species variability
ROTENONE	-Selective NSDA degeneration -Lewy body formation -Complex-1 Inhibition -Microglial activation -Oxidative stress -Proteosomal dysfunction -Extra nigral pathology	-Considerable variability -High mortality
PARAQUAT	-Lewy body formation -Only modest neurochemical modifications	-Poor reproducibility -High mortality
PROTEOSOME INHIBITORS	-Extra-nigral pathology -Lewy bodies	-Lack of reproducibility
GENE BASED MODELS	-Recapitulate many disease aspects -Extra nigral pathology	-Absence of NSDA degeneration

1.2 Stress

Biological stress was defined by Hans Seyle as 'the non specific response of the body to a demand placed upon it'. Later in 1988 Sterling and Eyer coined the termed 'allostasis' to encompass the physiological response of the body to daily events that enables the maintenance of homeostasis, literally meaning 'achieving stability through change'. Modern convention thus defines stress as anything that can disrupt homeostasis, with the resultant stress response acting to restore the delicate homeostatic balance via an adaptive process enabling the organism to cope both physiologically and behaviourally. The physiological response to stress is characterised by activation of the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis.

1.2.1 Autonomic response to stress

In response to stress there is immediate activation of the sympathetic nervous system with the consequent release of NA from nerve terminals and adrenaline from the adrenal medulla. This mediates the 'flight or fight response' whereby mental and physical activity is enhanced. There is also a concomitant decrease in parasympathetic drive.

1.2.2 HPA axis response to stress

The HPA axis (Figure 1.6) mediates the release of glucocorticoids (GCs) in response to diurnal cues and stress. In response to stress catecholaminergic, serotonergic and cholinergic inputs from limbic and brain stem centres that encode a stress stimulus converge on the hypothalamus. Stimulation of the parvocellular neurons of the paraventricular nucleus (PVN) results in secretion of corticotrophin releasing hormone

(CRH) and arginine vasopressin (AVP) into the hypophyseal portal circulation. CRH and AVP act via GPCRs at the anterior pituitary to synergistically stimulate the synthesis and cleavage of propiomelanocortin (POMC) into corticotrophin (ACTH). ACTH is released into the systemic circulation and transported to the zona fasiculata of the adrenal cortex where through action on membrane bound receptors it influences the activity of the rate limiting enzyme in the conversion of cholesterol to pregnalone to cortisol (humans and primates) and corticosterone (rodents), the endogenous GC hormones. GCs are steroid hormones that when released into the systemic circulation, exert diverse actions on the body including effects on glucose/lipid metabolism, immune function, inflammatory processes, host defence, fluid/electrolyte balance, vascular tone and bone metabolism. Within the CNS GCs can act on both neurons and glia where they exert organisational effects during development and influence neuronal plasticity in adults. GCs are implicated in the regulation of mood, behaviour, food intake and body temperature. GCs also regulate the circadian rhythm with GC secretion peaking during the morning in man (diurnal) and in the evening in rodents (nocturnal).

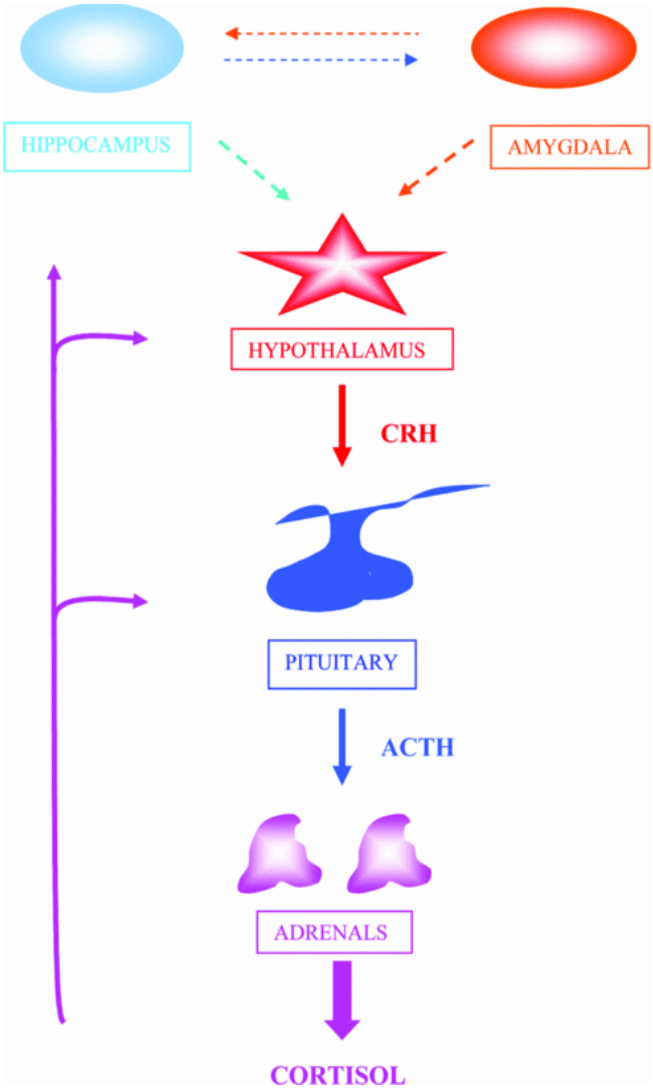


Figure 1.5 Schematic diagram of the HPA axis

CRH is synthesized in neurons of the paraventricular nucleus of the hypothalamus and released into the hypophysial portal circulation. CRH stimulates ACTH synthesis and release from corticotrophs in the anterior pituitary gland. ACTH is released into the systemic circulation where it then stimulates production of glucocorticoids from the adrenal cortex. GCs exert numerous functions on physiological processes and exert negative feedback on several levels of the HPA axis by inhibiting CRH and ACTH. The HPA axis is also modulated by higher centres, notably the hippocampus and amygdala. Adapted from (Drake et al., 2007).

1.2.3 Mechanisms of GC action

Receptors

The effects of GCs are mediated by the high affinity (Kd 0.5-2nM) mineralcorticoid receptor (MR) and the low affinity (Kd 10-20nM) glucocorticoid receptor (GR), both are members of the nuclear hormone receptor family. Within the CNS, GCs act on both neurons and glia. Basal levels of CORT occupy MR receptors, the highest CNS levels of which are found within limbic regions. They exert tonic inhibition on the HPA axis and regulate the normal circadian release of ACTH and CORT. GR receptors are more ubiquitously expressed throughout the brain and are progressively activated during stress. The density of these receptors varies in relation to circadian rhythm, age and stressful life experiences (Meyer et al., 2001).

Regulation of GC signalling

I) **Corticosteroid binding globulin (CBG):** Only ~ 4% of circulating GCs are unbound, the rest is bound to CBG which determines the availability of free GC to its receptors (Siiteri and Stites, 1982).

II) **Multidrug resistance (MDR) P glycoprotein:** The presence of MDRP on endothelial cells of the blood brain barrier (BBB)(de kloet 1998) prevents the penetration of synthetic GCs and cortisol but not corticosterone into the rodent brain (Meijer et al., 1998).

III) **11-β-hydroxysteroid-dehydrogenase (11βHSD):** The pre-receptor metabolism of GCs by 11βHSD allows for tissue specific delivery of GCs to target cells. There are two isoforms of this enzyme: 11βHSD-1 works as a reductase converting the inactive cortisone into cortisol and 11βHSD-2 works as a dehydrogenase converting CORT into inert cortisone.

Genomic action of GCs

Transactivation: GCs are lipophillic, thus easily pass through plasma membranes where they can bind to the ligand binding domain of cytosolic GRs. Association of GCs with their receptor results in the activation of GR and its dissociation from its multiprotein complex. The receptor can then translocate to the nucleus where GR homodimers bind to DNA binding sites (GC response element; GRE) in the promoter region of target genes, thus activating gene transcription of GC sensitive genes (Almawi and Tamim, 2001). This is known as transactivation.

Transrepression: The GC-GR complex can also repress transcription of genes by binding to negative GRE's (Sakai et al., 1988) known as transrepression. Moreover, the GC-GR complex can inhibit the transactivation function of transcription factors such as AP-1 and NFκB through direct or indirect interactions. The GC-GR complex can either bind to transcription factor subunits to prevent their association with DNA or can compete with transcription factors for nuclear co-activators (Almawi and Tamim, 2001).

GCs regulate growth, development, metabolism, behaviour and apoptosis. It is thought that this diversity in function arises from isoforms of the GR that are a result of alternative RNA splicing and post-translational modifications of GR mRNA that may modulate the function and stability of the receptor. This would allow for cell type specific regulation of gene expression by a single transcription factor (Zhou and Cidlowski, 2005).

Non-genomic action of GCs

Some effects of GCs occur too fast for them to be genomic actions. GCs can interact with cellular membranes influencing membrane associated proteins, thus membrane permeability and lipid peroxidation (Buttergeit 2002). GCs can also diminish ATP production by inhibiting oxidative phosphorylation. Furthermore the dissociation of proteins (e.g. heat shock proteins) from the GR-multiprotein complex on ligand binding, provides an indirect action of GCs (Croxtall et al., 2000). Membrane bound GR's have also been reported (Orchinik et al., 1991). Although their function is currently unknown they have been implicated in rheumatoid arthritis. (Bartholome et al., 2004).

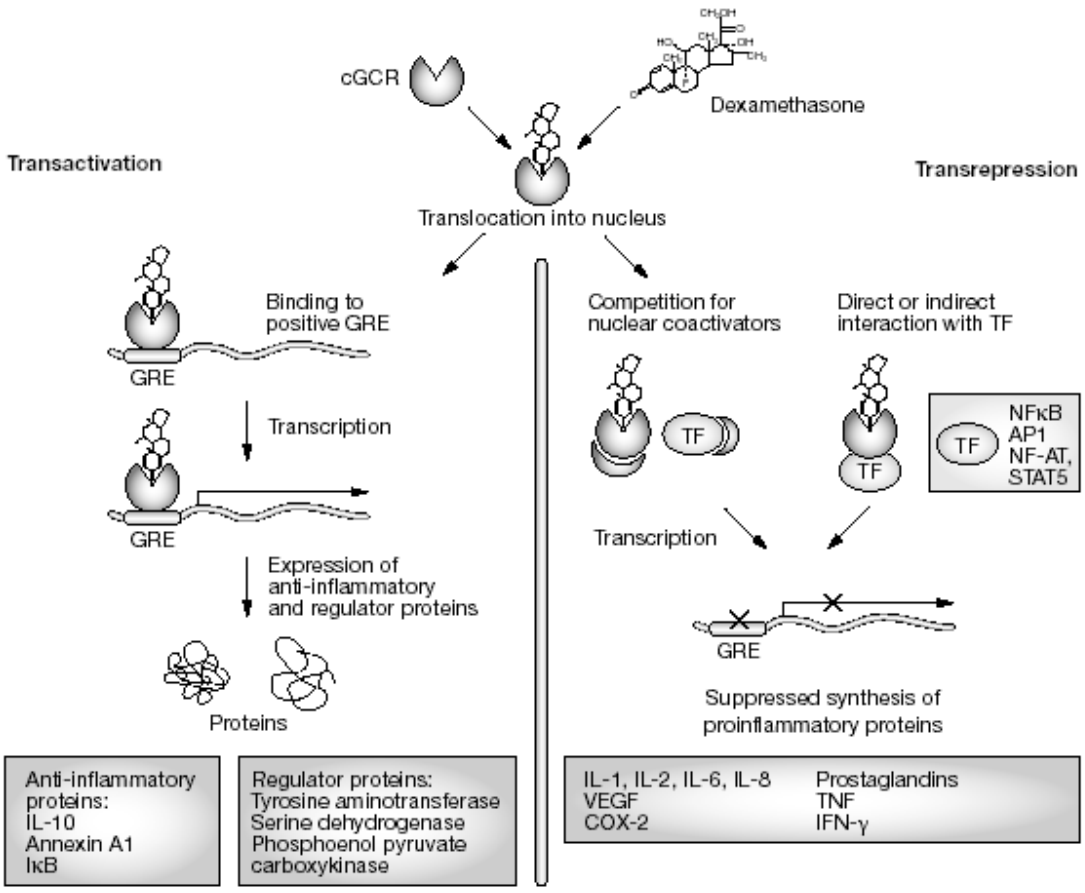


Figure 1.6 Genomic actions of GCs

Genomic effects of glucocorticoids include transactivation and transrepression. Binding of the GC-GR complex to GREs leads to expression of GC sensitive genes (transactivation). Transrepression is caused by competition for nuclear coactivators, or direct or indirect interaction with transcription factors.

Taken from (Stahn and Buttgereit, 2008).

1.2.4 Negative feedback within the HPA axis

GC's exert negative feedback actions on the brain via MR and GR receptors. MRs are localised in the hippocampus and other regions of the limbic brain and maintain basal HPA activity. GRs are more abundant throughout the brain with the highest expression found in the hypothalamus and pituitary corticotrophs and are involved with the feedback of both basal and stress induced levels of GCs, enabling termination of the stress response. Alterations in the effectiveness of GC negative feedback has profound effects on HPA activity and the regulation of the stress response and can increase vulnerability to disease (De Kloet et al., 1998; Gomez et al., 1998).

1.2.5 Animal models of stress

Animal models of stress have been widely used to aid our understanding of the basic mechanisms of stress on physiology. These include a wide variety of escapable and unescapable stressors which can be physical, phsychological, acute or chronic. Table 1 shows the most commonly used models of stress in rodents. The most extensively studied is an unavoidable physical stress such as immobilisation, extreme temperature, fasting, immersion, or electric shocks. Emotional stressors such as crowding or social isolation are useful for the study of psychiatric disorders but it has been suggested that immobilisation/restraint stress is most useful in the study of stress on degeneration, post traumatic stress disorder (PTSD) and the effects of stress on the immune system (Southwick et al., 1994; Garcia-Bueno et al., 2008). The most commonly used stressors in human studies are stressful interviews, free speech, mental arithmetic tasks and exercise, however these can only be used to study the effects of acute stress.

Table 1.4 Rodent models of stress

Immobilisation/Restraint Unpredictable Stress	Physical/Psychological	Acute / Chronic
Sleep deprivation Intruder Predator Maternal separation Crowding Social isolation Noise	Psychological	Acute/ Chronic
Swim stress Exercise Electric shocks Thermal Injury Extreme temperatures	Physical	Acute/Chronic

1.2.6 Habituation / adaptation to stress

There is a decrement in HPA response with repeated exposure to the same (homotypic) stressor (Jaferi and Bhatnagar, 2006) known as habituation. However, exposure to a repeated homotoypic stressor results in an exaggerated response to a novel stressor. This is known as facilitation (Bhatnagar and Dallman, 1998). This is thought to be regulated by GC mediated negative feedback

1.2.7 Clinical uses of GCs

GCs were first used clinically for the treatment of rheumatoid arthritis. Manipulations of cortisol structure in the 1950's led to the development of synthetic GCs that vary in their potencies, duration of effect and receptor affinities (Table 1.5; Figure 1.8). GCs have a wide range of therapeutic applications. They are used in the treatment of adrenal insufficiency (addisons disease), hypoglycaemia, hypercalcaemia, and can be used in the treatment of tumours (due to their effects on cell maturation, cell differentiation and apoptosis). However their major clinical use is in the treatment of inflammatory and immune diseases (Longui, 2007) such as asthma, allergy, inflammatory bowel disease and rheumatoid arthritis.

Table 1.5 Comparison of the main corticosteroid agents

Compound	GR affinity	Relative potency (anti-inflammatory)	Duration of action
Hydrocortisone (cortisol)	1	1	Short
Cortisone	0.01	0.8	Short
Corticosterone	0.85	0.3	Short
Prednisolone	2.2	4	Intermediate
Prednisone	0.05	4	Intermediate
Methylprednisolone	11.9	5	Intermediate
Triamcinolone	1.9	5	Intermediate
Dexamethsone	7.1	30	Long
Betamethasone	5.4	30	Long

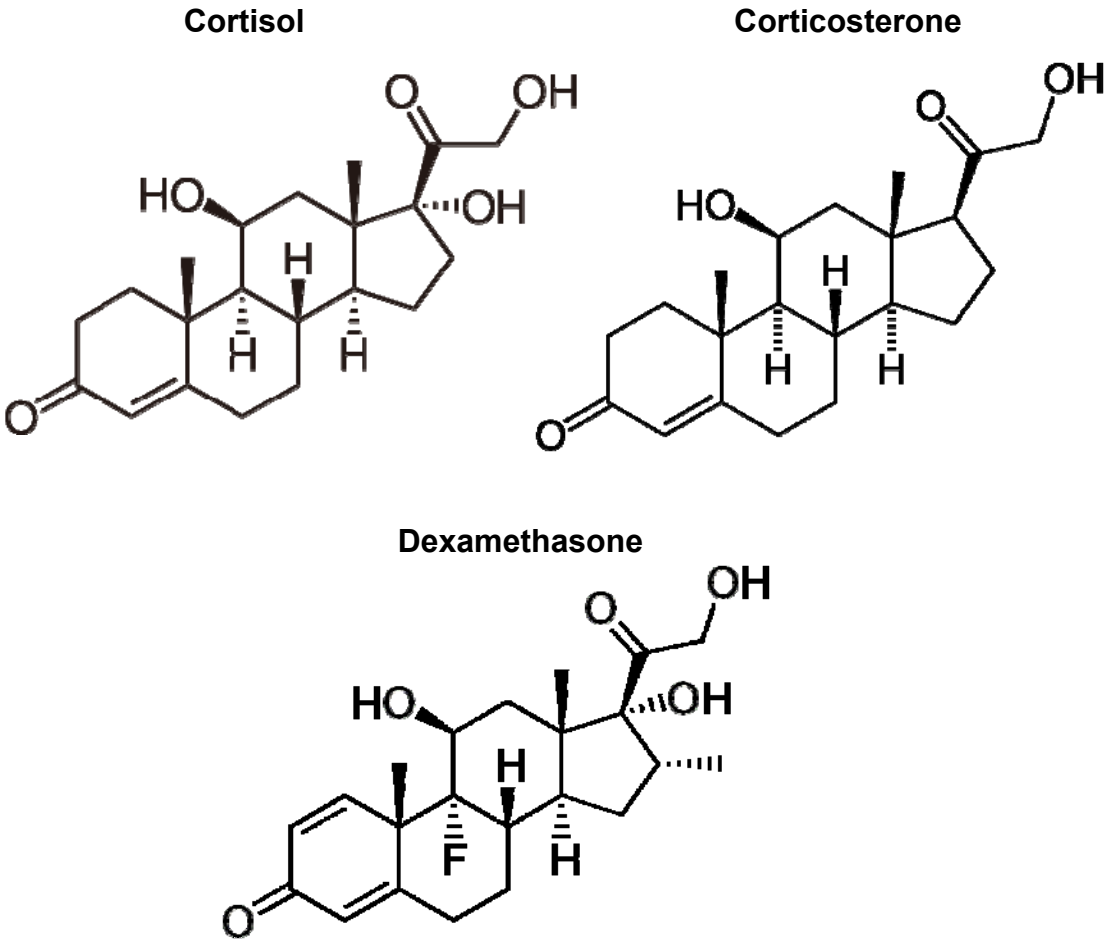


Figure 1.7 Structure of cortisol, corticosterone and the synthetic GC dexamethasone

1.2.8 Detrimental effects of stress and GCs.

Although activation of the HPA axis enables adaptive changes that enable physiological and behavioural coping, prolonged activation of the HPA axis presents a health risk. As GCs antagonise the effects of insulin, persistent elevation of GCs can increase blood pressure and the risk of diabetes. GCs also suppress the immune system thus may lead to an increased risk of infection. Furthermore elevated GC levels pose an increased risk for obesity, osteoporosis and cardiovascular disease (McEwen, 2000).

Detrimental effects of GCs in the CNS

Increased HPA activity has been implicated in major depression; anorexia nervosa, obsessive compulsive disorder/ panic disorder and decreased HPA activity implicated in PTSD and seasonal affective disorder (Schuppenies et al., 2006; Handwerger, 2009). Depression is often precipitated by stressful life events. HPA dysfunction has been established in depression and elevated cortisol levels often precede the onset of depression (Posener et al., 2000). Depressed patients exhibit greater cortisol levels, an increased response to ACTH and elevated CRH levels along with failure of GC negative feedback (Pariante, 2004). A decreased hippocampal volume has been reported in both depressed and Cushing's patients that correlates with disease progression (Sheline et al., 1999) (Starkman et al., 1992; Lupien et al., 2007b). Moreover, the depressive symptoms of Cushing's disease can be relieved by the correction of hypercortisolaemia (Murphy 1991). Salivary and cerebral spinal fluid (CSF) cortisol levels have been shown to correlate with the severity of Alzheimer's disease (AD) (Giubilei et al., 2001; Hoogendijk et al., 2006). There is a high prevalence of depression in AD (Greenwood et al., 2003) suggesting that they may share a common pathogenesis (Meynen et al.,

2007). Furthermore, GCs increased pathology in a mouse model of AD (Green et al., 2006).

Hippocampus

Much of the work on the detrimental effects of stress and GCs has focused on the hippoacampus as it has a high density of GC receptors and is involved in the regulation of HPA activity. The studies of Landfield at al., 1981 (Landfield et al., 1981) demonstrated that chronic exposure to GCs could influence hippocampal neuronal viability and function, typified by the degenerative loss of pyramidal neurons and subsequent deficits in memory (Sapolsky, 1986). It is now known that within the hippocampus chronic GC exposure produces glial cell proliferation, reduced dendritic branching, decreased volume, a reduction in CA1 and CA3 cell number and impaired long term potentiation (LTP) (Joels et al., 2004; Swaab et al., 2005). Chronic stress studies using daily immobilisation for several weeks; a selection of random stressors twice daily or repetitive social stressors have been shown to produce similar effects to that of chronic GCs with deficits in hippocampus-related learning, decreased branching and length of CA3 dendrites, decreased synaptic contacts, reduced proliferation and increased apoptosis (Woolley et al., 1990; Magarinos et al., 1996; Kim and Diamond, 2002) (Heine et al., 2004b). There is evidence to suggest that these effects are mediated by elevations in glutamate and subsequent NMDA receptor activation (McEwen and Magarinos, 1997). Chronic CORT treatment in humans results in smaller hippocampal volumes and declarative memory deficits when compared to controls (Brown et al., 2004).

Amygdala

Opposite effects of stress are seen on the amygdala where repeated stress produces hypertrophy, dendritic growth in neurons (Vyas et al., 2002) and enhances amygdala dependent unlearned fear and fear conditioning (Conrad et al., 1999).

Medial prefrontal cortex

Within the mPFC repeated stress leads to dendritic shortening (Sousa et al., 2000) (Wellman, 2001; Vyas et al., 2002) and attention impairment (Liston et al., 2006) that may reflect structural remodelling in the mPFC.

Pro-inflammatory effects of GCs

GCs are considered anti-inflammatory, immunosuppressive and immunomodulatory. However in recent years this view has been challenged in light of findings that suggest GCs can also exert pro-inflammatory effects within the CNS (Sorrells and Sapolsky, 2007). This is exemplified by studies showing that chronic stress in rats fails to suppress increases in inflammatory cytokines following LPS infusion (de Pablos et al., 2006). Moreover stress through GR activation increases TNF-α, IL-1β and iNOS within the hippocampus and cortex when LPS is administered peripherally (Munhoz et al., 2006). Stress and GCs can also increase microglial proliferation through a glutamate/NMDA mechanism (Nair and Bonneau, 2006).

Table 1.6 Evidence for proinflammatory actions of stress in the rodent CNS

Pro-inflammatory mediator	Stressor	Effect in CNS	Reference
Glucocorticoids	Immobilisation	Increased IL-1β, TNF-α, IL-6, NFκB	Minami 1991, yamasu 1992, nukina 1998
Catecholamines	Inescapable tail shock Immobilisation Footshock	Increased IL-1β, IL-6 Increased NFκB Increased NFκB	Johnson 2005 Madrigal 2001 Black 2006
Glutamate	Immobilisation	Increased COX-2, iNOS	Madrigal 2003
Cytokines	Immobilisation, tailshock, footshock swim stress Immbobilisation Immobilisation, footshock	Increased IL-1β Decreased IL-1β, TNF-α Increased TNF-α, NFκB, iNOS Increased IL-6	Deak 2005 Connor 2005 Madrigal 2001, 2002 Kronfol 2000
Pro-inflammatory enzymes	Swim stress, heat exposure, immobilisation	Increased COX-2, iNOS	Katafuchi 2003, Madrigal 2003

Abbreviations COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; IL, interleukin; NFκB, nuclear factor kappa B; TNF-α, tumor necrosis factor-alpha. Adapted from (Garcia-Bueno et al., 2008)

1.2.9 Effect of sex and age on the HPA axis

There is generally a greater stress response in adult (pre-menopausal) females than males (Kajantie and Phillips, 2006). During aging the concentration of cortisol in CSF (cerebrospinal fluid) increases progressively (Laughlin and Barrett-Connor, 2000) and there is an age related decline in hippocampal volume in men but not women (Pruessner et al., 2001). There is also an age related increase in CRH neurons in men but not women (Bao and Swaab, 2007). In addition men have greater AVP levels than females (van Londen et al., 1997). Basal CORT levels in rodents are not altered in aging. However, stress responses are greater and recovery takes longer in older animals (Lucassen et al., 2001; Heine et al., 2004a). Hippocampal damage resulting from age related degeneration results in the disinhibition of GC negative feedback promoting further activation of the HPA axis, increasing GC levels thus causing accumulating damage. This is known as the GC cascade hypothesis (Sapolsky et al., 1986). Further evidence for dissociative effects of stress and GCs in males in females is provided by experiments showing that chronic foot shock stress decreases proliferation in the dentate gyrus of males but increases proliferation in females (Westenbroek et al., 2004). Chronic restraint stress induces the retraction of dendrites in the CA3 hippocampal neurons in males but not females (unless they are ovariectomised) (Galea et al., 1997) and enhances or has no effect on spatial learning in females but is detrimental in males (Bowman et al., 2003; Conrad et al., 2003).

1.2.10 HPA axis interaction with the hypothalamic-pituitary-gonadal (HPG) axis

Both CRH and GCs can prevent GnRH (gonadotrophin releasing hormone) release. Moreover GCs can directly influence the testes and ovaries to reduce the production of

sex hormones (Swaab et al., 2003; Bao and Swaab, 2007). CRH fibres from the PVN also innervate GnRH neurons in the infundibular nucleus (Dudas and Merchenthaler, 2003). Conversely sex hormones control CRH gene expression and estrogen and androgen receptors are co-localised with CRH neurons (Bao et al., 2005; Bao et al., 2006). Furthermore, ovarian steroids increase HPA activity and enhance the HPA response to psychological stress (Kirschbaum et al., 1996).

1.2.11 Early life stress

Early life manipulations have been carried out in primates, guinea pigs, sheep, cattle, pigs, rats and mice. Manipulations can be split into prenatal manipulations and postnatal manipulations that impact upon different stages of development. Table 1.7 shows the main manipulations used in rodents.

Table 1.7 Early life manipulations in rodents

Manipulation	Nature of treatment	Timing of treatment	Effect later in life	Reference
Prenatal stress	Noise / Restraint	Last week of gestation	Increased HPA reactivity, emotionality and exploratory behaviour	Macari 2001
Postnatal handling	Brief separation from mother	PND 1-14	Decreased HPA reactivity, emotionality and exploaratory behaviour	Levine 1967
Maternal care	Licking and grooming of pups	PND 1-14	Decreased HPA reactivity, emotionality and exploaratory behaviour	Meaney 1994/ francis 2002
Maternal separation	Prolonged separation from mother	PND1-14	Increased HPA reactivity, emotionality and exploratory behaviour	Plotsky 2005
Novelty exposure	Exposure to novelty	PND1-21	Enhanced spatial memory, social competion and larger HPA response to unexpected stressor	Tang 2007
Aversive conditioning	Odor-shock conditioning	PND8 PND23 PND12-15 * PND12-15 #	Odor preference Odor avoidance Odor preference Odor avoidance	Moriceau 2006

PND: post natal day. *With mother present. #without mother present. Table adapted from (McEwen, 2007)

It is now widely accepted that early life events impact upon adult physiology and behaviour. The idea of 'programming' or 'imprinting' can be described as the association between an environmental factor and its action during a critical window on the development and organisation of tissues that persist throughout life. Different cells/tissues can be sensitive at different times and to different challenges. The purpose of this programming that occurs early in life is thought to be to allow adaptation for its future environment. Two hypotheses have formed that are thought to underpin this programming effect. The first is maternal malnutrition, the second being exposure to glucocorticoids or stress. Interestingly, these are interrelated whereby GCs can modulate food intake and malnutrition can alter GC exposure.

1.2.11.1 Prenatal Stress

The offspring of prenatally stressed rats exhibit increased basal corticosterone and ACTH levels and an increased HPA response to stress (Review (Matthews, 2002)), sensitises to postnatal stressors such as handling (Smythe et al., 1996) and decreased habituation to stress (Fride et al., 1986). Prenatally stressed rats also have decreased expression of GR and MR (Henry et al., 1994; Koehl et al., 1997; Szuran et al., 2000). In humans prenatal stress can cause pre-term birth or full term low birth weight (Barker, 1996; Wadhwa et al., 2004). Low birth weight is associated with an increased risk of cardiovascular and metabolic disease later in life (Barker, 1996). Children that grow up in emotionally cold families are at a greater risk of poor mental and physical health in adulthood (Repetti et al., 2002). Furthermore abuse during childhood is a known risk factor for depression, PTSD, substance abuse, obesity, diabetes and cardiovascular disease (Heim and Nemeroff, 2001; Anda et al., 2006) (Felitti et al., 1998).

1.2.11.2 Prenatal GCs

Within the brain GCs are important for the normal maturation of the CNS where they are involved in initiating terminal maturation, remodelling axons and dendrites, and influencing cell survival (Meyer 1983). Prenatal GCs decrease brain weight at birth (Huang et al., 1999), delay the maturation of neurons and glia (Huang et al., 2001b, 2001a) and influence synapse formation (Antonow-Schlorke et al., 2003) permanently altering brain structure. GR and MR are highly expressed within the developing brain (Kitraki et al., 1997). However until mid-gestation the developing CNS is protected from GCs by 11βHSD2. It is then that 11βHSD2 expression is switched off in the rat brain, coinciding with the terminal stage of neurogenesis, allowing GC access to the fetal brain (Diaz et al., 1998). A similar phenomenon occurs in humans where 11βHSD2 is reduced at weeks 19-26 (Stewart et al., 1994).

Glucocorticoids are the primary candidate for early life programming. A large number of pregnant women are treated with synthetic GCs to prevent respiratory distress syndrome in newborns. GCs are administered to mothers at risk of preterm labour and in antenatal treatment of foetuses at risk of congenital adrenal hyperplasia. The most commonly used GCs are dexamethasone (DEX) and betamethasone due to the fact they are weak MR agonists and have weak immunosuppressant effects (Aghajafari et al., 2001). Dexamethasone and betamethasone are also poor substrates for 11βHSD2 (Albiston et al., 1994) therefore readily cross the placenta and have access to the fetus. No adverse effects of a single dose have been reported but multiple courses may have detrimental effects later in life (Aghajafari et al., 2001). In the rodent brain GR and MR are low throughout gestation but rapidly increase after birth, however there are distinct patterns of GR and MR expression in the foetal rat brain (Diaz et al., 1998). GR's are

present in the hippocampus, hypothalamus and pituitary by GD13 whereas MR are not present in the hippocampus until GD16/17.

The HPA axis is particularly sensitive to programming where it produces an increase in basal CORT levels in adult rats and decreases GR and MR expression within the hippocampus, thus attenuating feedback sensitivity (Levitt et al., 1996; Welberg et al., 2001). Primates also exhibit elevated basal and stress levels of cortisol (Uno et al., 1994). GCs also program behaviour where they seem to impair coping mechanisms later in life (Welberg et al., 2001), program anxiety related behaviours (Dunn and Berridge, 1990; Welberg et al., 2000) and affect the development of dopamine systems with implications for the development of schizophrenia, addiction and extrapyramidal disorders (Diaz et al., 1997).

In humans antenatal GC treatment results in lower birth weights (French et al., 1999; Bloom et al., 2001). However there is a lack of long term follow up studies to ascertain their long term implications on adult physiology, pathology and behaviour. Currently there is evidence that adolescents that were exposed to synthetic GCs in utero have higher blood pressure (Doyle et al., 2000) and that multiple antenatal doses of DEX result in a reduced head circumference, a lower IQ and behavioural problems (French et al., 1998). Furthermore treatment with DEX of foetuses at risk of cogenital adrenal hyperplasia has been associated with increased emotionality and behavioural problems (Trautman et al., 1995). Children of low birth weight have higher plasma GC levels, have exaggerated adult responses to ACTH, elevated blood pressure, insulin resistance, glucose intolerance and hyperlipidemia (Phillips et al., 2000).

11βHSD2 is also thought to play a role in low birth weight. Indeed, lower 11βHSD2 activity correlates with smaller fetus size in rats (Benediktsson et al., 1993) and low birth weight in humans (Murphy et al., 2002). Babies homozygous for deleterious mutations in 11βHSD2 have low birth weights (Dave-Sharma et al., 1998). Interestingly, dietary protein restriction during pregnancy reduces 11βHSD2 (Bertram et al., 2001).

1.2.12 Potential involvement of stress and GCs in PD

One of the earliest proposed causes of PD was stress (Charcot 1878, Gowers 1888). This idea has started to regain attention and a hypothesis for the involvement of stress and GCs in the pathophysiology of PD is emerging (Kibel and Drenjancevic-Peric, 2008). In PD patients it has been suggested that acute or chronic stress may result in an earlier onset or worsen the symptoms of PD (Macht et al., 2005). Indeed cortisol levels are higher in PD patients and have been associated with gait deficits (Hartmann et al., 1997; Charlett et al., 1998). The presence of GC receptors within the NSDA (Cintra et al., 1994) provides a means by which GCs could exert an effect on the NSDA rendering it vulnerable to excess levels of GCs. Consequently stress and GCs have been shown to negatively affect motor function in both humans (Maki and McIlroy, 1996) and rats (Metz et al., 2001; Metz et al., 2005). Of significant interest is the finding that stress can elevate oxidative damage in the mouse NSDA (Kim et al., 2005) a mechanism that has been heavily implicated in the pathogenesis of PD. Further evidence supporting an effect of stress and GCs on the NSDA is provided by studies demonstrating that chronic corticosterone treatment elevates D1 receptor mRNA in the striatum of mice (Biron et al., 1992); immobilisation stress increases striatal DA levels (Kim et al., 2005) and that repeated restraint stress increases DAT mRNA within the rat striatum (Copeland et al.,

2005). It has also been proposed that the early life environment has a significant impact on disease susceptibility in adulthood and that GCs are an important programming factor (see section 1.2.11). Thus, stress and excess GCs during development may impact upon the developing NSDA. Indeed, prenatal GC treatment permanently alters rat DA system (McArthur et al., 2005; McArthur et al., 2007a)

1.3 Aims

The following chapters aim to determine the effect of GC treatment early in life and the effects of adult GC treatment or stress exposure on pathways implicated in PD. The pathways examined are the dopaminergic NSDA, MLDA and MCDA as well as the noradrenergic LC, cholinergic NBM and serotonergic system which are known to degenerate in PD. The specific aims of the project are outlined below.

1. To determine the effects of prenatal dexamethasone treatment on pathways known to degenerate in PD.

2. To determine the effects of repeated adult stress or adult treatment with GCs on pathways known to degenerate in PD.

3. To determine how prenatal DEX treatment combined with adult stress or GC treatment affects pathways known to degenerate in PD.

4. To examine the influence of prenatal dexamethasone treatment and/or adult repeated stress or chronic adult GC treatment on susceptibility to 6-OHDA neurotoxin.

5. To establish any sex differences in the effects of 1,2,3 and 4.

In order to achieve these aims rats will be bred in-house and treated prenatally with dexamethasone (GD16-19) via the maternal drinking water. Pups will be allowed to mature and adult rats (3.5 months) will receive repeated restraint stress or chronic GC treatment (via drinking water) for a period of 6 weeks. Integrity of the SN, VTA, LC and NBM will be assessed by the use of immunohistochemistry and the stereological quantification of cell numbers within these regions. Levels of microglia, astrocytes, and oxidative damage will also be assessed by immunohistochemistry in these regions. Functionality of neuronal systems will be determined using high performance liquid chromatography whereby the levels of dopamine and its metabolites, serotonin and its metabolite and noradrenaline will be quantified in the striatum, nucleus accumbens and frontal cortex. To determine the effects of stress and GCs on susceptibility to a toxic insult, 6-OHDA will be administered to the MFB, producing a partial lesion of the NSDA. The severity of the lesion and the subsequent DA loss will then be assessed as described above using immunohistochemical markers and HPLC.

CHAPTER TWO - MATERIALS AND METHODS

2.1 – IN VIVO METHODS

2.1.1 - Animals

All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, under project license number PIL 70/6486 with local ethical approval (Ethical Committee of Imperial College, London). Sprague-Dawley rats were purchased from Harlan for timed mating and housed in the Comparative Biology Unit at Charing Cross Hospital. Animals were allowed to acclimatise for one week before undergoing any procedure. Animals were given free access to food and water in a temperature controlled (21 ±2C°) 12 h light-dark cycle room (lights on 07:00am, lights off 19:00pm). All animals were handled regularly and weighed weekly. Male and female animals were housed in separate rooms. Experiments were carried out between 08:30 and 12:00 to prevent complications associated with circadian rhythm.

2.1.2 - Breeding

Two females and one male were housed together overnight. Mating was confirmed by the presence of vaginal plugs the next morning and recorded as gestational day 1 (GD1). Pregnancy was later confirmed by palpation on GD10. Pregnant females were then housed individually for littering and monitored regularly. Offspring were weaned at 3 weeks, when they were randomly assigned to treatment groups with animals from at least three different litters in each group. Males and females were then housed in separate rooms in cages containing two animals.

2.1.3 - Prenatal Dexamethasone Treatment

Rat pups were indirectly exposed to the synthetic glucocorticoid dexamethasone (DEX). 0.5μg/ml dexamethasone sodium phosphate (Rosemont Pharmaceuticals, Leeds, UK) was administered via the maternal drinking water on GD 16 to 19. In this paradigm the developing rats are exposed to DEX via the placenta, a method established by (Theogaraj et al., 2005). Control animals received normal drinking water throughout gestation. Maternal weights and fluid intake were monitored over the period of DEX treatment (see section 2.6.1) Mothers were also closely monitored for any changes in behaviour.

2.1.4 - Adult Restraint Stress

At 3.5 months animals were subjected to physical restraint stress. This was achieved by immobilisation in a plastic restraint tube (Harvard Apparatus, UK) for 1 hour every other day for 6 weeks. Two different sized tubes were used depending on the size of the animal that could be adjusted to achieve a tight fit. The procedure was carried out at the same time each day (8.30-11.30am). This produced a model of repeated, intermittent, psychological stress that replicates the paradigm used by Fontella et al (2005) that reported increased oxidative stress within the hippocampus.

2.1.5 - Adult Hydrocortisone Treatment

3.5 month old rats were treated with the synthetic glucocorticoid hydrocortisone (the synthetic form of cortisol). 400 μg/ml hydrocortisone sodium succinate (Pharmacia Ltd, UK) was added to the drinking water for a period of 6 weeks. This non-invasive glucocorticoid treatment is based on a method used by (Magarinos et al., 1998), which

produced alterations in hippocampal morphology that were comparable to those induced by restraint stress. The paradigm produced stress levels of GCs and gave a circadian profile due to increased feeding/drinking behaviour in the evening (Conrad et al., 2004). Control animals received normal drinking water. Weights and fluid intake were recorded throughout the treatment period (see sections 2.6.2-3).

2.1.6 - Assessment of Estrous Cycle

A subset of female animals from each treatment group were assessed for estrous cycling (see section 2.6.4). At ~5 months of age vaginal lavages were collected on 10 consecutive days and allowed to dry on microscope slides. Slides were then stained with haematoxylin and eosin and examined under a light microscope. Evaluation of estrous stage was achieved by the identification of the cell types present according to published methods (Goldman et al., 2007); (Hubscher et al., 2005); see figure 2.1).

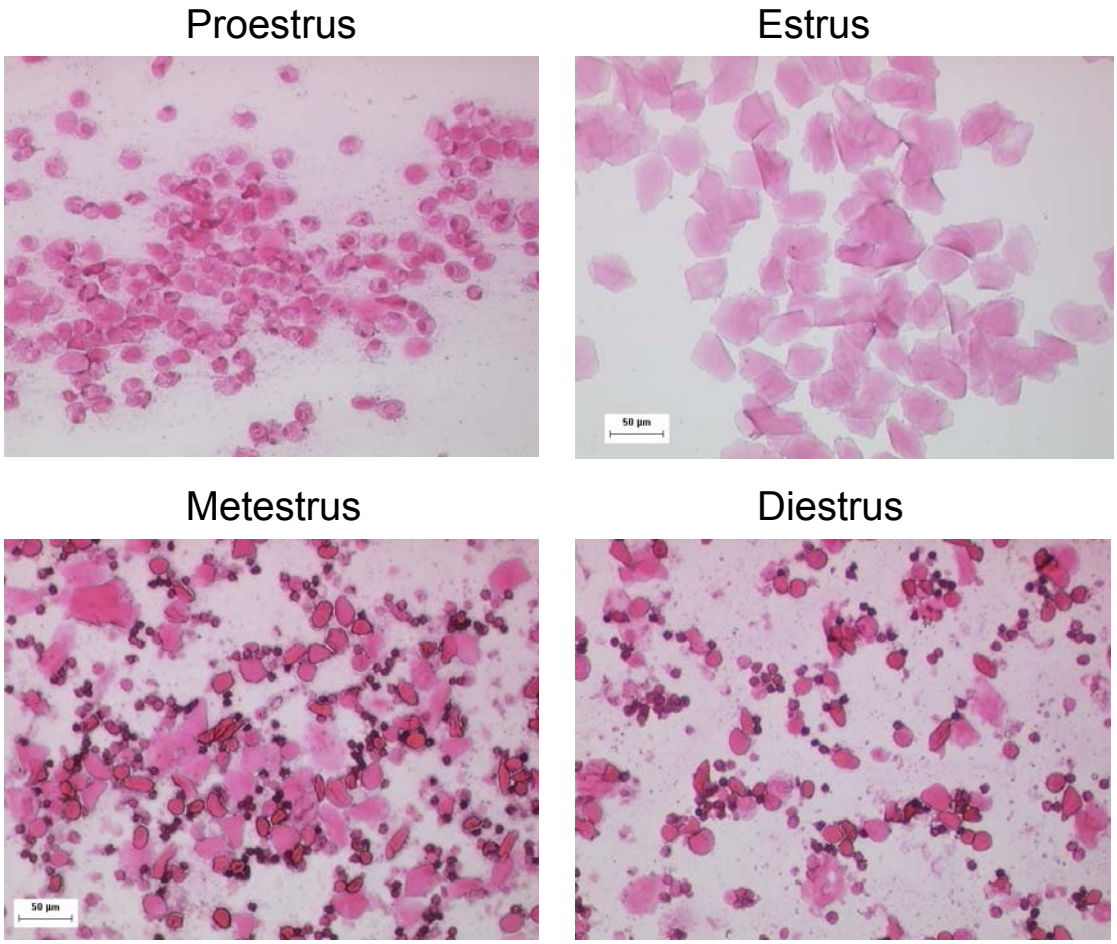


Figure 2.1 – Estrus cycle staging. Haematoxylin and eosin staining of vaginal smears at different stages of the estrous cycle. The proestrus stage lasts 12-14 hours and is characterised by round nucleated cells of uniform size. Estrus lasts 25-27 hours and is distinguished by the appearance of irregularly shaped un-nucleated cornified cells. Metestrus lasts 6-8 hours and is characterised by the appearance of leukocytes. During diestrus, which lasts 55-57 hours, both leukocytes and nucleated cells are present. For the majority of animals the estrus cycle lasts 4-5 days.

2.1.7 - 6-Hydroxydopamine Lesioning of the Nigrostriatal Pathway

To examine the effects of stress or glucococorticoids on the ability of the nigrostriatal system to cope with a toxic insult we employed the neurotoxin 6-OHDA that is used to model PD. 6-OHDA is a hydroxylated analogue of dopamine that is taken up by DAT where its subsequent accumulation in the cytosol produces prolonged oxidative stress, ultimately resulting in neuron death. For more information on the use of 6-OHDA to model PD see section 1.1 12. The magnitude of the lesion produced is dependent on site of injection and the concentration of the 6-OHDA used. For a review of 6-OHDA nigrostriatal lesions see (Deumens et al., 2002). The medial forebrain bundle was chosen as the site of injection as it had previously been used to successfully produce partial lesions of the NSDA (Datla et al., 2004). In the work presented here where older larger animals were used, 5μg of 6-OHDA was injected into the medial forebrain bundle. Preliminary work (see figure 2.3) suggested that this dose produced a 60% striatal dopamine loss in males and 40% loss in females. This unilateral, partial lesion allowed the determination of any positive or negative effects of preceding stress and glucocorticoid treatments on lesion size with the unlesioned hemisphere of the brain serving as an internal control.

Before surgery commenced pre-operative weights were recorded. Anaesthesia was induced using 5% Isoflurane (Abbott, UK) and 2.5% oxygen (BOC, UK) in an induction chamber. Once animals were sufficiently anaesthetised and had lost their righting reflex animals were transferred to a stereotaxic frame (Kopf Instruments, USA) with ear bars 5 mm below the incisor bar to secure the head and placed on a heated mat. Anaesthesia was maintained at 1.5-3% isoflurane and 1 % oxygen and was continually monitored throughout the surgical procedure by monitoring the rate and depth of the

animals breathing and its reflexes**.** Once the animal was fixed in the stereotaxic frame the scalp was shaved and sterilised with iodine. Animals then received 0.1 ml bupivicaine hydrochloride (Marcain, AstraZeneca, UK), a long acting local anaesthetic injected sub cutaneously at 4 different sites on the scalp close to the incision site. 5ml of glucosaline (Baxter, UK) was given by intraperitoneal injection to prevent dehydration. A single incision was made along the scalp from midway between the eyes to midway between the ears to expose the skull. The skull was cleaned by scraping away any membranous tissue to reveal bregma, an anatomical landmark on the skull from which coordinates could be measured. A small hole was made using a dental drill above the proposed site of the medial forebrain bundle, 2.2mm posterior and 1.5mm lateral to bregma (based on coordinates from the standard rat brain atlas of Paxinos and Watson, 1986; see figure 2.2). The meninges were cleared from the surface of the brain and 5µg of 6-OHDA hydrobromide free base (dissolved in 0.1% ascorbic acid in 0.9% sodium chloride solution) was injected at 7.9mm ventral to the dura over a period of 4 minutes (1µl/min) using a 10µl syringe (Hamilton, UK). The needle was then left in place for another 4 minutes to allow the toxin to diffuse and to prevent reflux of the toxin before being removed. Antibiotic powder was applied to the wound to prevent infection and the wound closed with 3 sutures. The animal was then placed in a heated recovery chamber to recover from the anaesthetic were it was closely monitored. Post surgery animals received mashed food, were housed individually and were culled 14 days post-op.

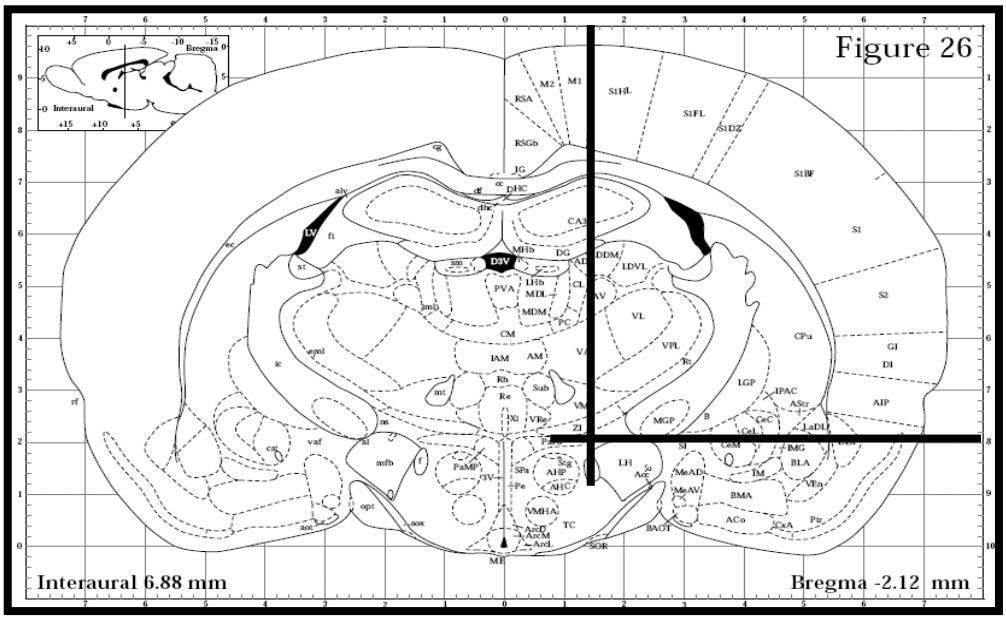


Figure 2.2 Coronal section showing location of the stereotaxic injection of 6-OHDA to the left medial forebrain bundle. 6-OHDA was injected 2.2mm posterior, 1.5mm lateral and 7.9 mm ventral to bregma. Figure adapted from atlas by Paxinos and Watson 1986.

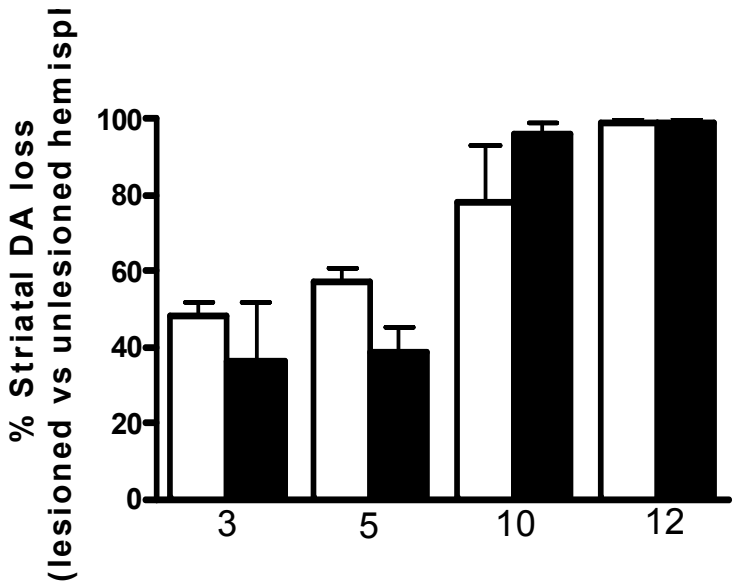


Figure 2.3. Percentage striatal DA loss in response to different doses of 6-OHDA in male (open bars) and female rats (filled bars). 3, 5, 10 and 12μg 6-OHDA were stereotaxically injected into the left medial forebrain bundle. Striatal DA levels were measured 2 weeks post surgery and expressed as a % of the DA concentration in the contralateral striatum. 2 animals of each sex were exposed to each dose.

2.1.8 - Post-Mortem Procedure and Tissue Collection

Animals were culled by decapitation. Trunk blood was collected, allowed to clot on ice and was subsequently centrifuged for 15 minutes at 3000 rpm at 4°C. Serum was collected and stored at -80°C until further analysis. Brains were rapidly removed, the nucleus accumbens, striatum and frontal cortex were dissected out and snap frozen on dry ice. They were then stored at -80°C until further processing. The remaining hind brain blocks were fixed in 4% paraformaldehyde in PBS (phosphate buffered saline) for 7 days and then cryoprotected in 30% sucrose in PBS with 0.1 % sodium azide. Once brains had sunk they were removed from sucrose solution and stored at -80°C.

2.2 - EX VIVO METHODS

2.2.1 - Corticosterone Enzyme Linked Immuno Assay

Analysis of circulating corticosterone levels were assessed using a Corticosterone ELISA kit (IDS, USA). The assay was performed in accordance with kit instructions and all calibrators, controls and solutions were provided within the kit. Each serum sample was diluted 1 in 10 with sample diluent (PBS and horse serum). 100µl of each calibrator, control or sample was then added to the appropriate well of an antibody coated plate (polyclonal rabbit anti-corticosterone) in triplicates. 100µl of enzyme conjugate solution (horseradish peroxidise) was added to all wells and the plates were left to incubate for 18 hours at 2-8°C. After incubation wells were washed 3 times with wash solution followed by the addition of 200µl of the chromagen tetramethylbenzadine (TMB substrate) to each well, to visualise the reaction. Plates were left to incubate for 30 minutes at room temperature after which the reaction was stopped with 100µl of 0.5M hydrochloric acid. The absorbance of each well was read using a microplate reader (Tecan, UK) at 450nm. Calibrator absorbance were used to produce a standard curve (see figure 2.4) from which the concentration of corticosterone in each serum sample could be established.

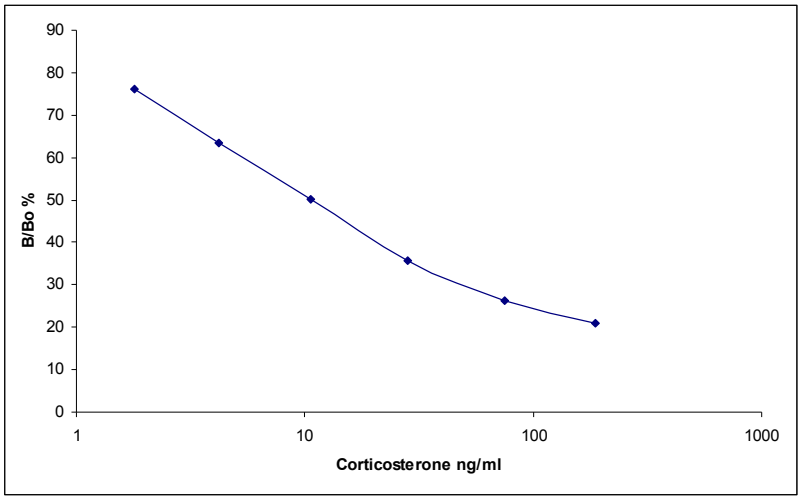


Figure 2.4 Example of a corticosterone standard curve . Corticosterone concentration was plotted against percent binding (B/Bo%).

2.2.2 - Cryostat Sectioning

From fixed and cryoprotected hind brains, 30µm sections were cut using a cryostat (Bright Instruments, UK) maintained at -22ºC. Sections were thaw mounted onto slides coated in poly-l-lysine (0.1%) in series of 6 slides with two sections per slide. This allowed the subsequent staining of adjacent sections. Sections were collected throughout the nucleus basilis of meynert (NBM), substantia nigra (SN) and locus coeruleus (LC). These regions were identified using the standard rat brain atlas (Paxinos and Watson, 1986; see figure 2.5). Slides were stored at -80ºC until further use.

2.2.3 - Immunohistochemistry

All immunohistochemical analysis was performed using an avidin-biotin immunoperoxidase (ABC) method. Optimisation of all antibodies was performed to identify appropriate working dilutions and positive and negative controls were used to ensure the specificity of staining. Positive controls consisted of tissue where the epitope of interest was known to be expressed and negative controls involved the omission of the primary antibody. Working dilutions of all antibodies used can be found in table 2.1.

Sections were circled with a wax blocker and rehydrated with PBS for 15 mins. Sections were covered with 0.3% hydrogen peroxide / methanol solution for 45 minutes to block endogenous peroxidase activity. Sections were then washed 3 x 5 minutes in PBS with 0.1% Triton-X (PBS-T) to enable antibody penetration. Non specific binding was blocked by incubation in normal serum (appropriate to the secondary antibody) in PBS-T for 1 hour. Sections were left to incubate at room temperature overnight in primary antibody made up in normal serum in PBS-T. The following day sections were

washed in PBS-T (3 x 5 mins), then incubated in the appropriate secondary antibody for 1.5 hours. Sections were again washed in PBS-T (3 x 5 mins) and incubated in avidin-biotin complex using the vector elite kit (1:200) (Vector, UK) for 1 hour. Following washing (3 x 5 mins in Tris buffer) the reaction was visualised with 0.05% diaminobenzidene (DAB) dissolved in Tris buffer with 0.01% hydrogen peroxide until desired staining intensity was reached. The reaction was stopped by placing slides in 3 changes of distilled water. Sections were dehydrated in increasing concentrations of alcohol, cleared in xylene and cover slips were attached using DPX mountant. Sections were then stored for analysis by light microscopy.

2.2.4 - Neuronal Immunohistochemistry

To determine the effect of treatments on dopaminergic neurons within the SNpc and VTA, an antibody against tyrosine hydroxylase (TH) was used. TH is the rate limiting enzyme in DA synthesis and can therefore be used as a marker of DA neurons. TH would also be found in noradrenergic neurons but these are not present at the level of the SNpc and VTA. An antibody against dopamine-β-hydroxylase (DβH), the enzyme that converts dopamine to noradrenaline was used as a marker of noradrenergic neurons within the LC. To identify and quantify cholinergic neurons in the NBM, an antibody against choline-acetyltransferase (ChAT) was used. ChAT is an enzyme that combines acetyl coA to choline forming acetylcholine and thus is used as a marker for cholinergic neurons. A general neuronal marker neuronal N (NeuN) was additionally used on adjacent sections in all regions to indicate whether there were changes in neuronal numbers or enzyme expression. When quantifying neurons only cells that displayed robust immunoreactivity with a nucleus, a clear cytoplasm and axonal processes were counted. Cells that appeared severely damaged or deformed were not included.

2.2.5 - Glial Immunohistochemistry

As inflammation is closely linked to HPA axis function and is thought to be involved in the pathogenesis of PD, microglial and astrocyte populations were studied in all brain regions. To study microglial populations an OX-42 antibody was used. This antibody recognises the cell surface antigen CD11b of the type 3 complement receptor found in mononuclear phagocytes. To specifically identify activated microglia an antibody against major histocompatability complex class II antigens, OX6 was used. To study changes in astrocytic morphology and proliferation an antibody against glial fibrillary acidic protein (GFAP), an intermediate filament protein found in astrocytes was used. As well as obtaining total numbers of microglia and astrocytes, their morphology was also assessed. Reactive astrocytes display intense GFAP staining and have enlarged cell bodies with one or two thickened processed whereas resting astrocytes show moderate GFAP staining and have small cell bodies and several processes. Resting microglia display moderate OX-42 reactivity and have small cell bodies with numerous processes with extensive ramifications. Activated microglia however, show intense OX-42 and OX-6 staining, have larger cell bodies and have shorter processes and fewer ramifications (Raivich et al., 1996).

2.2.6 - Oxidative stress Immunohistochemistry

As oxidative stress is heavily implicated in the pathogenesis of PD and stress and GCs have been shown to elevate levels of oxidative stress within the brain, the consequences of oxidative damage within the SNpc, VTA, LC and NBM was determined. An antibody against 3-nitrotyrosine, a marker of protein nitration which is indicative of nitrosative stress (the formation of peroxynitrite and reactive nitrogen species) and

therefore oxidative stress was used. Sections were counterstained with haematoxylin to allow cells positive for protein nitration to be counted.

Table 2.1 Details of all prima ry and se condary a ntibodies used in ABC immunohistochemistry.

	Primary antibody	Source	Secondary antibody	Normal serum
Dopaminergic neurons	Rabbit anti-Tyrosine Hydroxylase (1:1000)	Pel-freez	Biotinylated goat anti-rabbit (1:200)	20% NGS,
Neurons	Mouse anti-Neu-N (1:1000)	Chemicon	Biotinylated horse anti-mouse (1:100)	5%NHS
Activated Microglia	Mouse anti- OX6 (1:750)	Serotech	Biotinylated horse anti-mouse (1:100)	5% NHS
Microglia	Mouse anti- OX-42 (1:500)	Serotech	Biotinylated horse anti-mouse (1:100)	5% NHS
Astrocytes	Mouse anti-GFAP (1:1000)	Sigma	Biotinylated horse anti-mouse (1:100)	5% NHS
Nitrosative stress	Rabbit anti 3-nitrotyrosine (1:1000)	Chemicon	Biotinylated goat anti-rabbit (1:200)	20%NGS
Noradrenergic neurons	Rabbit anti-Dopamine-β-hydroxylase (1:1000)	Chemicon	Biotinylated goat anti-rabbit (1:200)	20% NGS
Cholinergic neurons	Rabbit anti-Choline Acetyltransferase (1:750)	Chemicon	Biotinylated goat anti-rabbit (1:200)	20% NGS

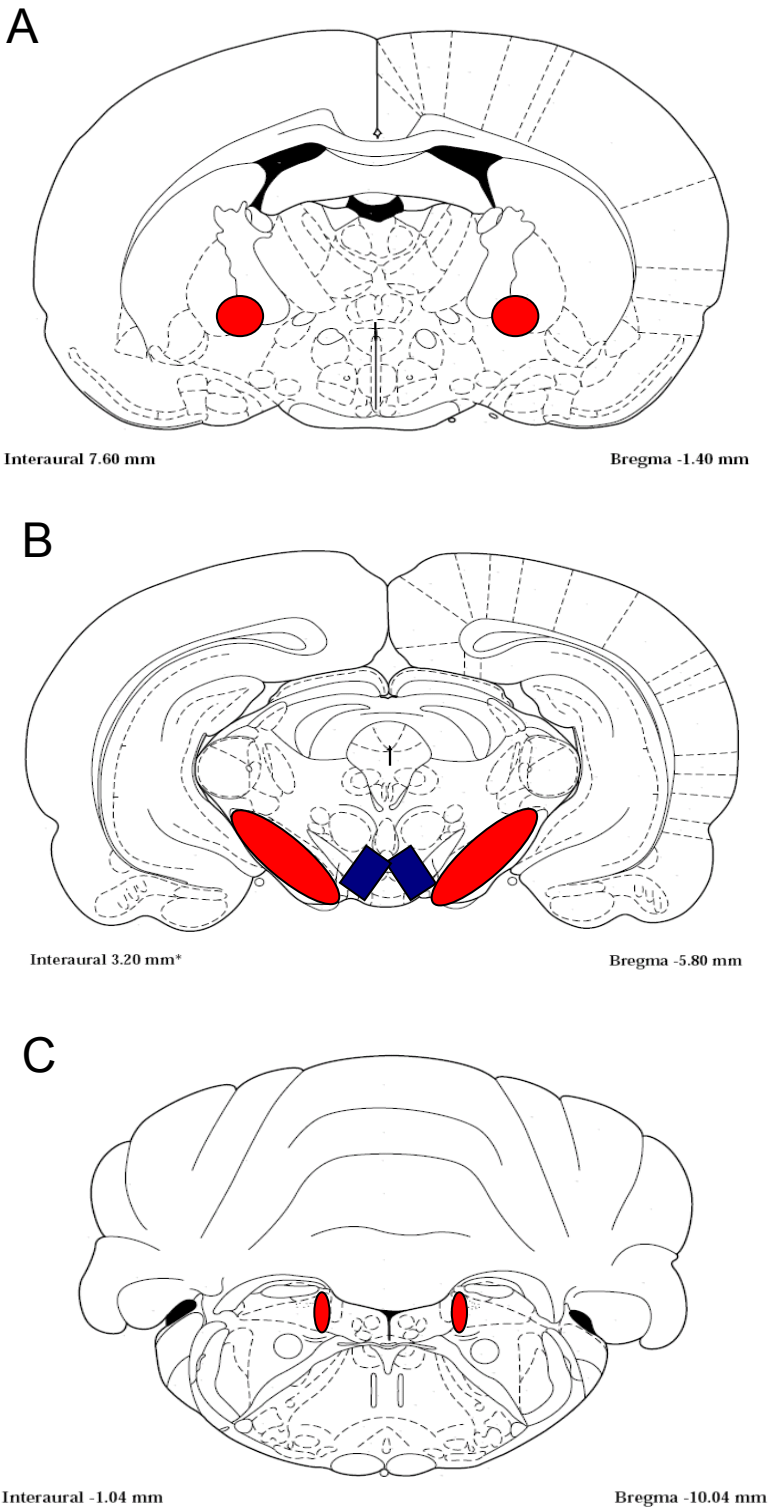


Figure 2.5. Coronal rat brain sections depicting regions of interest. Nucleus basilis of meynert (A), substantia nigra (B in red), ventral tegmental area (B in blue) and locus coeruleus (C). Pictures adapted from atlas by Paxinos and Watson, 1986.

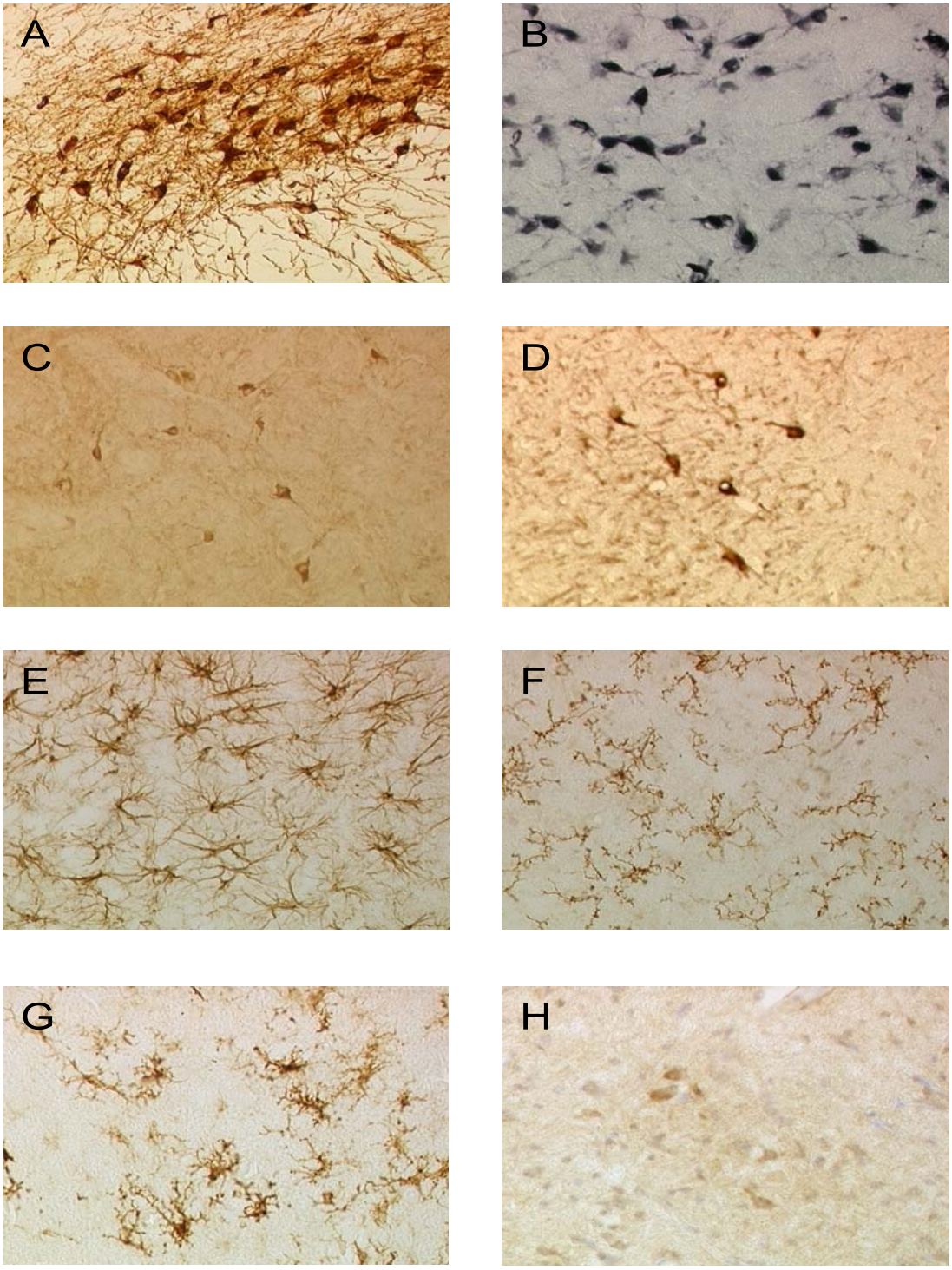


Figure 2.6. Photomicrographs of immunohistochemical staining depiciting tyrosine hydroxylase in SNpc (A), neuronal n in SNpc (B), ChAT in NBM (C), DβH in LC (D),GFAP in SNpc (E), OX-42 in SNpc (F), OX-6 in SNpc (G) and 3-nitrotyrosine positive cells in SNpc (H). Images were taken at 200x magnification.

2.2.7 - Image analysis and stereological quantification

Stereological estimates were made using computer based stereology software (MAG, UK) attached to a Nikon Eclispe E800 microscope and JVC analogue camera. Cells were counted using the optical fractionator method (Schmitz et al., 2005). The area of interest (AOI) was first defined by the user (see Fig 2. A. The software was then used to create counting frames within the AOI using uniform random sampling. The total area of the counting frames relative to the area of the AOI gives the area sampling fraction (asf). The height of the optical dissector which was measured using a heidenhain microcator (Heidenhain, Germany), relative to the actual section thickness gives the height sampling fraction (hsf). The section sampling fraction (ssf) was 1/6 as every 6 section throughout the AOI was analysed. Only cells falling within or touching the counting frame were counted and forbidden lines were used to avoid edge effects (see fig 2.7 B). Total cell estimates (N) were made using the following formula N = n(1/ssf)(1/asf)(1/hsf) where n equals the number of positive cells counted.

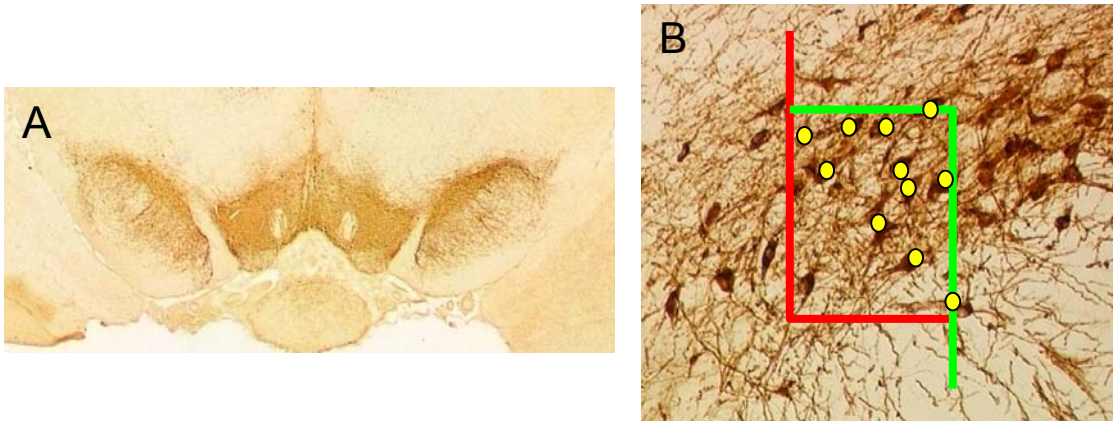


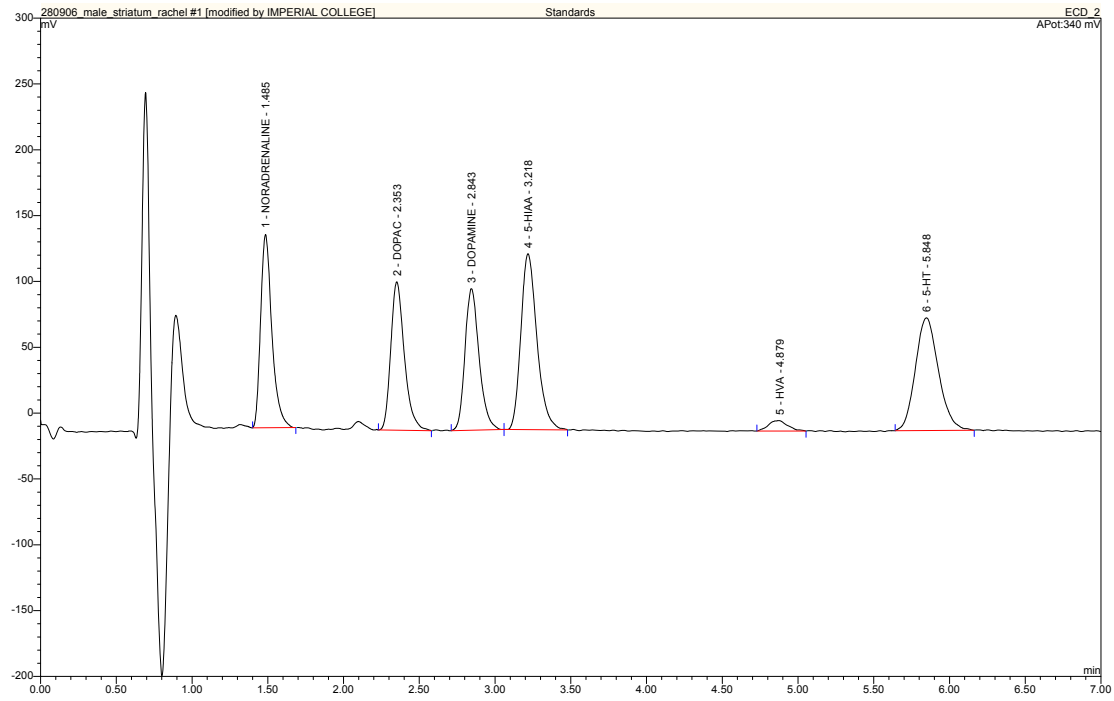
Figure 2.7. A) Selection of AOI. B) Positive cells within a counting frame. Cells falling within the counting frame or touching green lines were counted. If cell was touching a red line it was not counted.

2.2.8 - Monoamine Analysis by HPLC-ECD

High performance liquid chromatography was used to detect the concentrations of monoamines within the striatum, nucleus accumbens and the frontal cortex. The levels of DA and its metabolites dihydroxyphenylaceitic acid (DOPAC) and homovanillic acid (HVA), 5-hydroxytryptamine (5HT), its metabolite 5-hyroxyindoleacetic acid (5HIAA) and noradrenaline (NA) were determined. Individual samples were weighed and then homogenised in 0.5ml of ice cold homogenising medium (50mM trichloroacetic acid, 0.5mM disodium ethylenediaminetetraacetic acid (EDTA) along with 0.5pmol/µl 3,4-dihydroxybenxylamine as an internal standard, using an ultrasonicator (Soniprep, Sanyp UK). Samples were kept on ice for 10 minutes to allow extraction of monoamines and then centrifuged for 10 mins at 13000g at 4ºC. The resulting supernatant was filtered through a 0.45µm pore syringe filter (Whatman, UK) and placed into glass vials (Chromacol UK) on an autosampler (Gina 50 Dionex, UK) maintained at 5ºC. Using Chromeleon software 30µl from each sample was injected and then chromatographically separated on an Altex Ultrasphere 5µm column (Beckman Coulter, High Wycombe, UK) at room temperature using a mobile phase consisting of 0.1 mM KH_2PO_4, 0.1 mM EDTA, 1 mM octyl sodium sulfonate, 12% methanol, adjusted to pH 2.75 with orthophosphoric acid, at a flow rate of 1.2 ml/min. Samples were quantified by an analytical cell (model 5011, ESA Biosciences, UK) attached to a Coulochem II electrochemical detector (ESA Biosciences, UK) with electrode one at -0.20 V and electrode two at +0.34 V with respect to the palladium reference electrode. Data was collected and analysed using chromeleon software (Dionex, UK) which compares sample data to data from external standards for each molecule. External standards of each monoamine (50pmol/ml) were prepared from a stock solution of each monoamine dissolved in 0.1M hydrochloric acid (5µmol/ml) and analysed prior to samples to

determine retention times for each monoamine and to allow the subsequent calculation of the concentration of each monoamine in each sample. Additionally, DA turnover and 5-HT turnover was calculated by measuring the ratio of DA and 5-HT to their respective metabolites (pmol/ml). Control and treated groups were run on the same day with the same external standards. Due to the known variability in baseline across HPLC runs, care should be taken when comparing data from different experiments. Due to the large number of samples it was not possible to run male and female samples together therefore data should not be directly compared. Figure 2.9 shows an example of a trace produced for external standards (A) and a striatal sample (B).

A



B

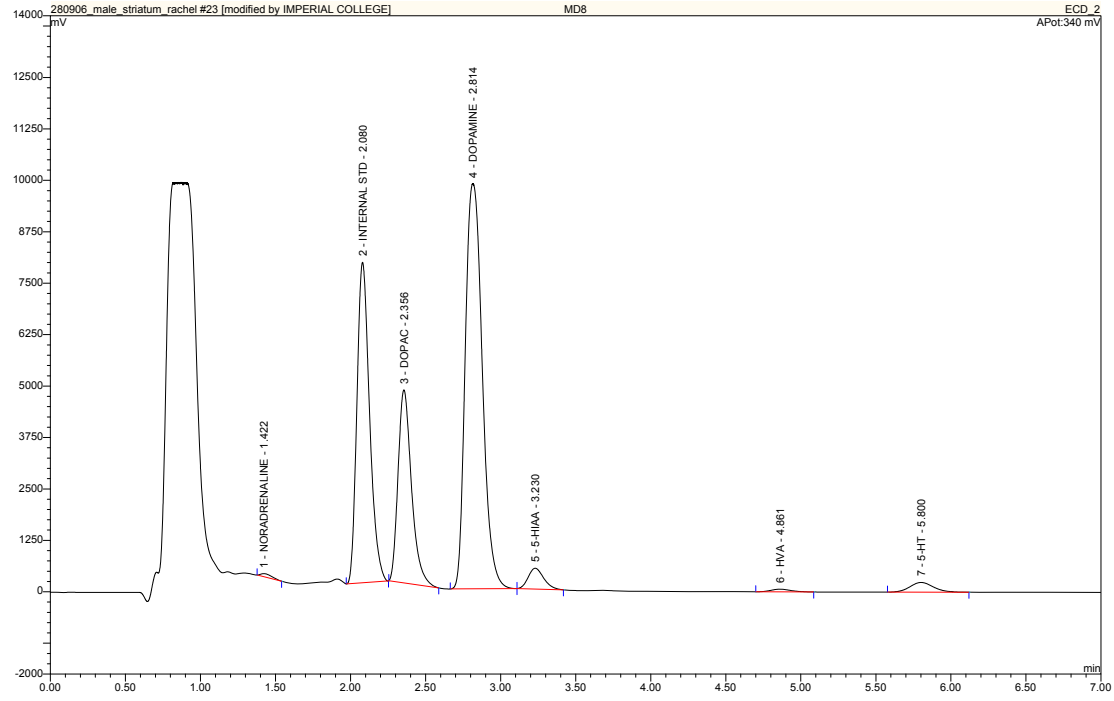


Figure 2.8. Example of an HPLC trace for external standards (A) and a striatal sample (B).

2.3 - STATISTICS

Statistical analysis was carried out using SPSS 16.0 software. Where multiple comparisons were made, a two or three way ANOVA with post hoc multiple comparison tests were performed. Further details of these analyses can be found in appendix 1. Individual comparisons were made using an unpaired, two-tailed student's t-test. All data are expressed as means ± SEM. In all cases differences were considered to be significant if $p<0.05$.

2.4 - MATERIALS AND SOURCES

All materials are from VWR or Sigma unless otherwise stated in the text.

2.5 - EXPERIMENTAL DESIGN

This thesis combines results from 3 large, long term studies on the effects of stress and GC treatments on brain regions affected by PD. The first study examined the effects of prenatal DEX and / or adult repeated restraint stress on brain regions implicated in PD. The second experiment replicated the first set of experiments but also exposed animals to 6-OHDA. This provided information on how stress and GCs affect the response to a toxic insult in the brain regions examined. The third study was identical to study 2 except for a change in the treatment in adulthood where adult repeated restraint was replaced with chronic GC treatment. This allows a comparison of the effects of stress and elevated GCs and may help determine whether effects induced by repeated stress are caused by elevated GC levels. From this point on experiments where the adult treatment was stress is referred to as study 1 and experiments including GC treatment

study 2. A time line for the treatments is shown in figure 2.10 and figure 2.11 shows how animals were split into different treatment groups.

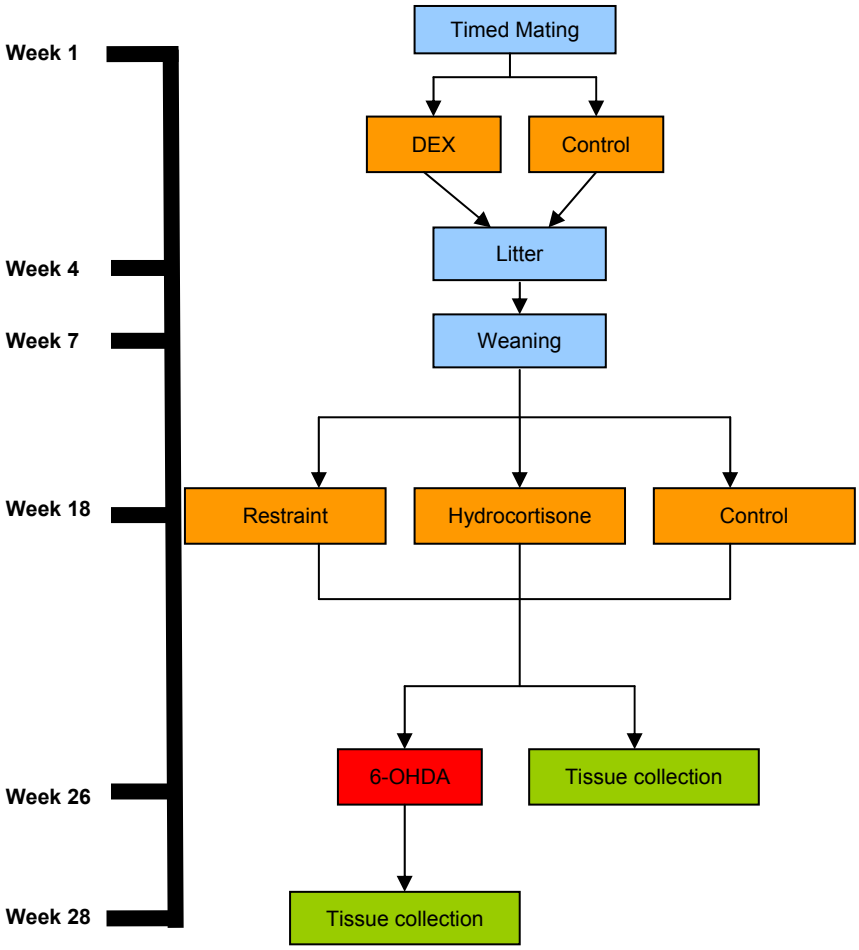


Figure 2.10 Flow chart showing the order and timing of treatment regimes. Time mated pregnant females were treated with DEX on GD 16-19. Offspring were weaned at 3 weeks of age. At 3.5 months of age animals were subjected to repeated restraint or chronic hydrocortisone treatment for a period of 6 weeks. At 5.5 months of age (two weeks after last stress session or termination of GC treatment) animals were either culled for analysis or subjected to a sterotaxic injection of 6-OHDA. 6-OHDA animals were culled 2 weeks after surgery.

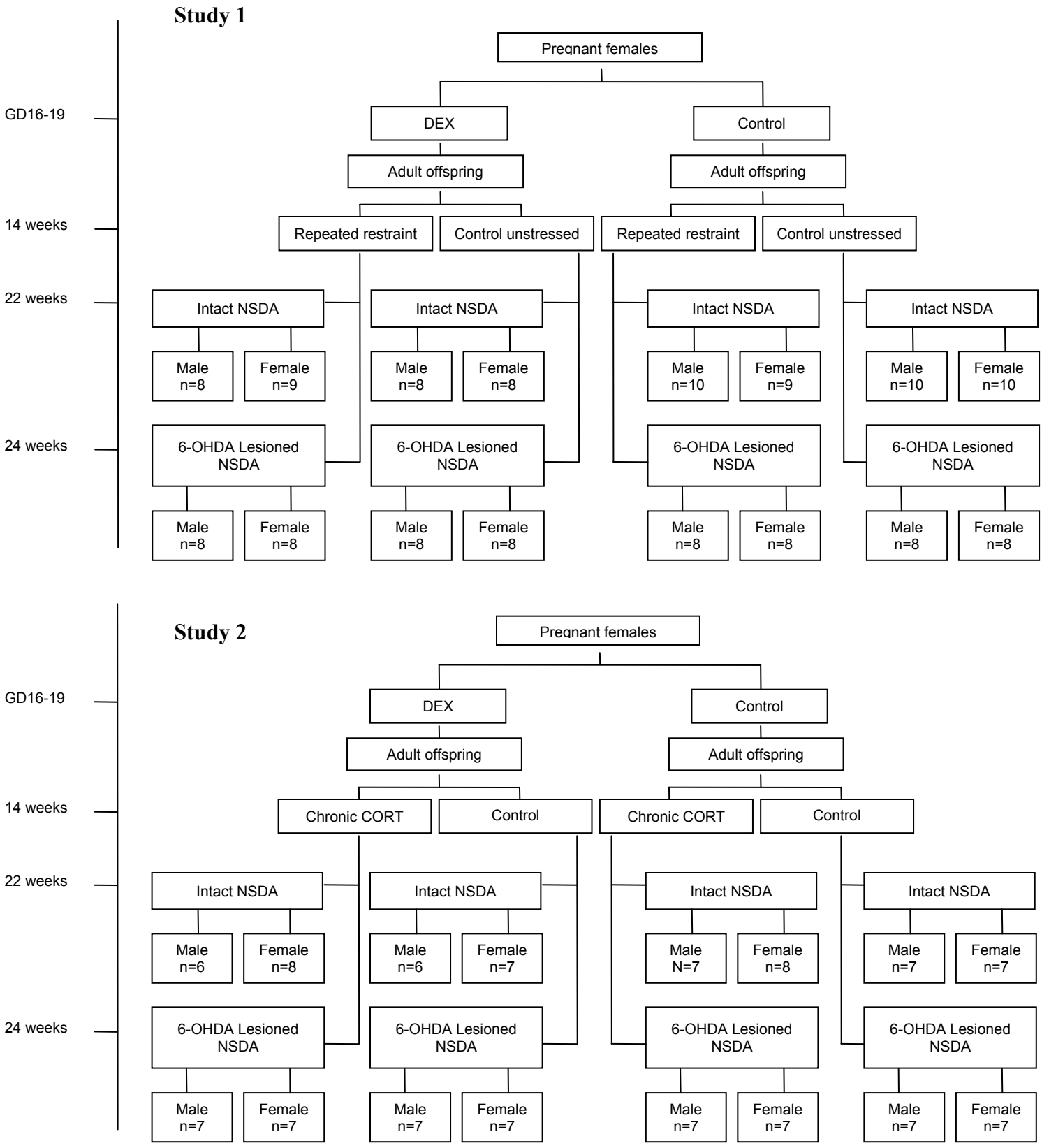


Figure 2.11. Flow chart depiciting the way in which animals were divided into treatment groups. Numbers represent the number of animals per treatment group.

2.6 PHYSIOLOGICAL IMPACT OF TREATMENT REGIMES

2.6.1 Effect of DEX treatment on maternal physiology

Prenatal DEX treatment increased fluid intake ($p<0.05$; Figure 2.12A) and prevented maternal weight gain ($p<0.001$; Figure 2.12B) over GD16-19. DEX exerted no effect on litter size (Figure 2.12C) and had no observable effects on maternal behaviour. Using existing pharmokinetic data (McArthur et al., 2006), average fluid intake and average litter size the concentration of DEX received by the pups was estimated to be ~30ng/ml.

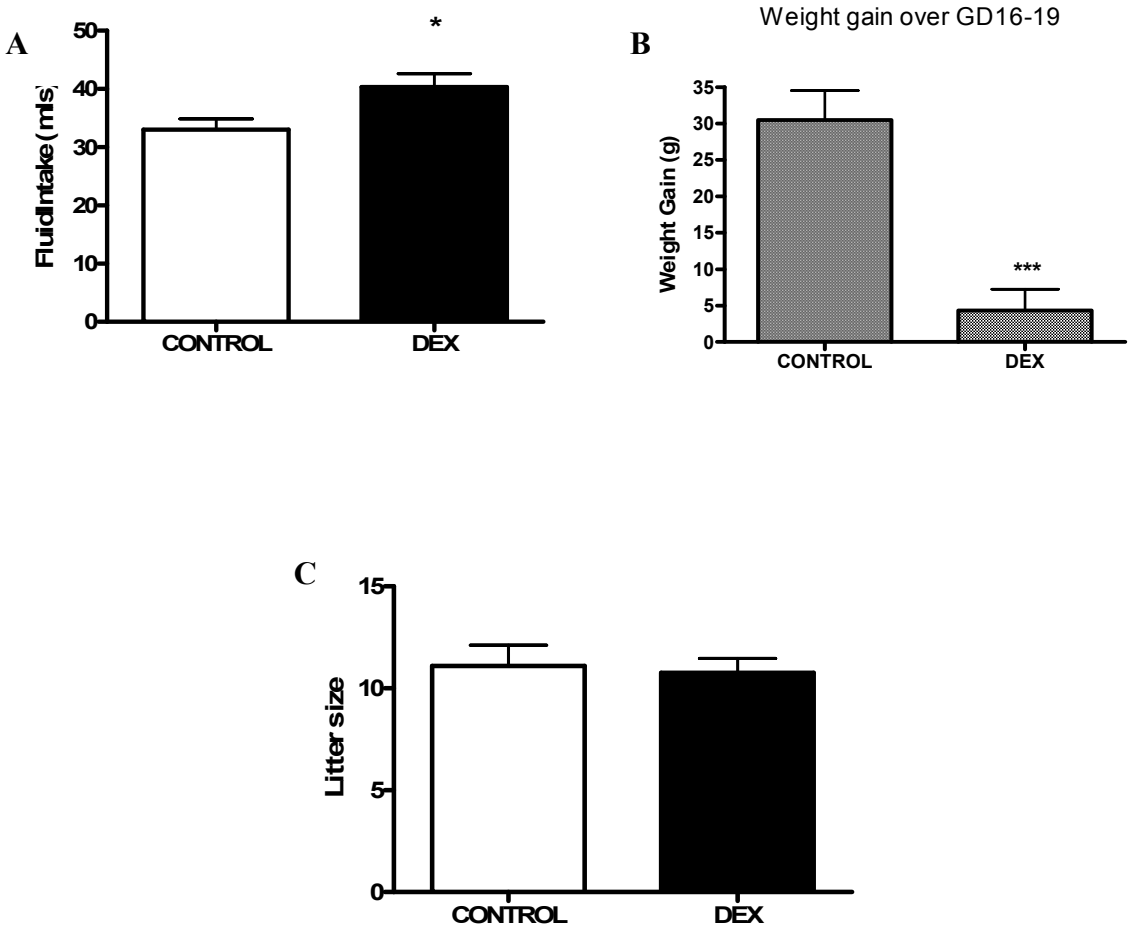


Figure 2.12. Effect of DEX tr eatment (0.5µg/ml via drinking w ater) on GD16-19 o n maternal fluid intake (A) and w eight gain (B) over GD16-19 and litter size (C). *p<0.05, **p<0.01, ***p<0.001 versus controls. N= 11 (control), 13 (DEX).

2.6.2 Effect of treatment regimes on growth

Prenatal DEX treatment or adult repeated restraint stress had no effect on normal growth in either male or female offspring (Figure 2.13A,B). By contrast chronic hydrocortisone treatment prevented weight gain in both male and females over the period of treatment ($p<0.001$; Figures 2.13C,D, 2.14A,B). This was unaffected by prenatal DEX treatment.

A **B**

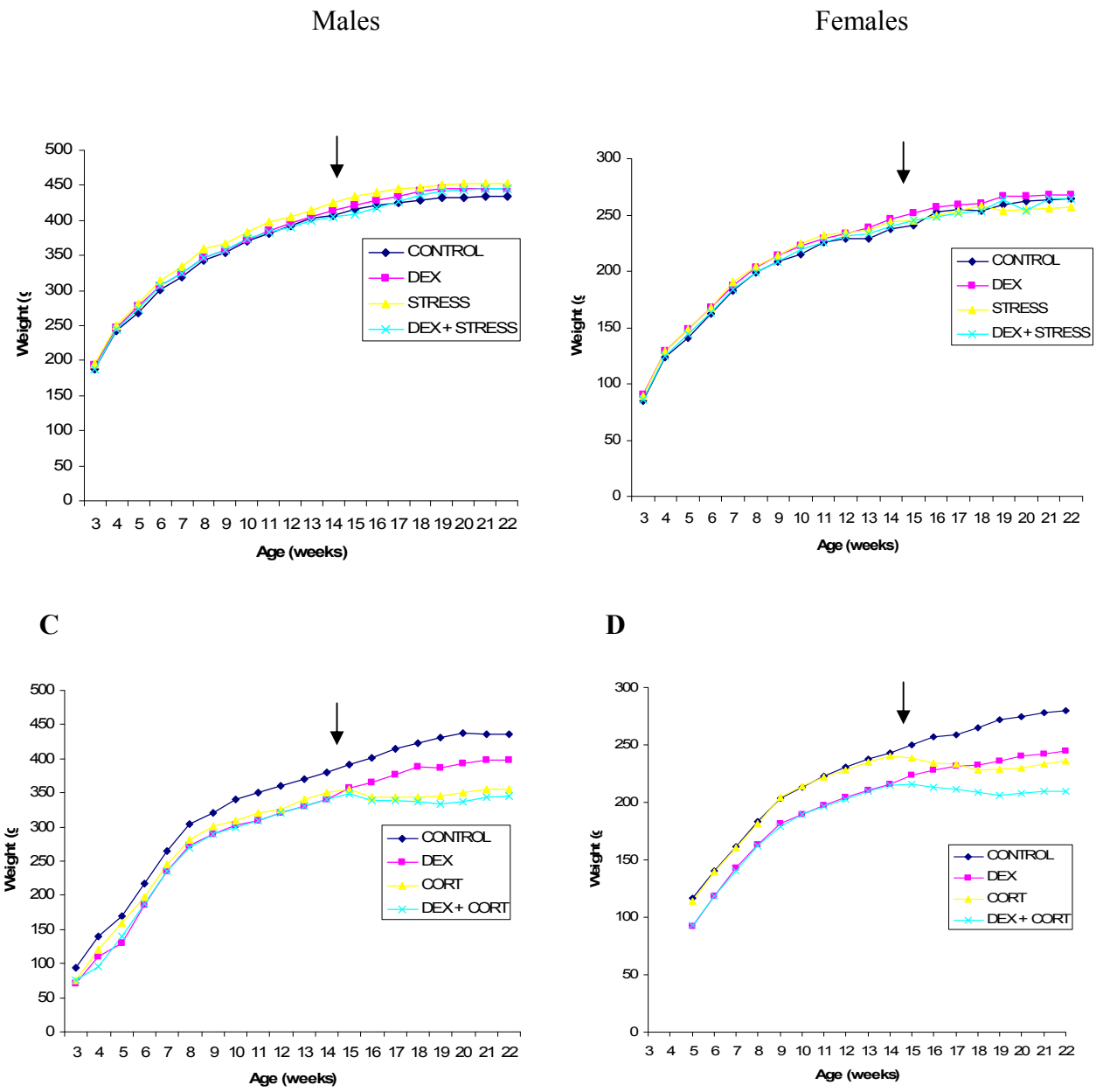


Figure 2.13. Effect of treatment regimes on growth. Weights were recorded weekly following weaning (3 weeks). Effect of DEX (GD16-19) and repeated adult (3.5 months) stress on growth in males and females is shown in A and B respectively. Effect of DEX (GD 16-19) and adult (3.5 month) GC treatment is shown in C (males) and D (females). Data are expressed as means. Initiation of adult treatment shown by arrow. A: n= 16 (control), 17 (DEX), 16 (stress), 16 (stress and DEX). B: n=18 (Control), 16 (DEX), 18 (stress), 18 (DEX and stress). C: n= 14 (control), 13 (DEX), 14 (CORT), 12 (DEX + CORT). D: n= 14 (Control), 16 (DEX), 14 (CORT), 14 (DEX + CORT).

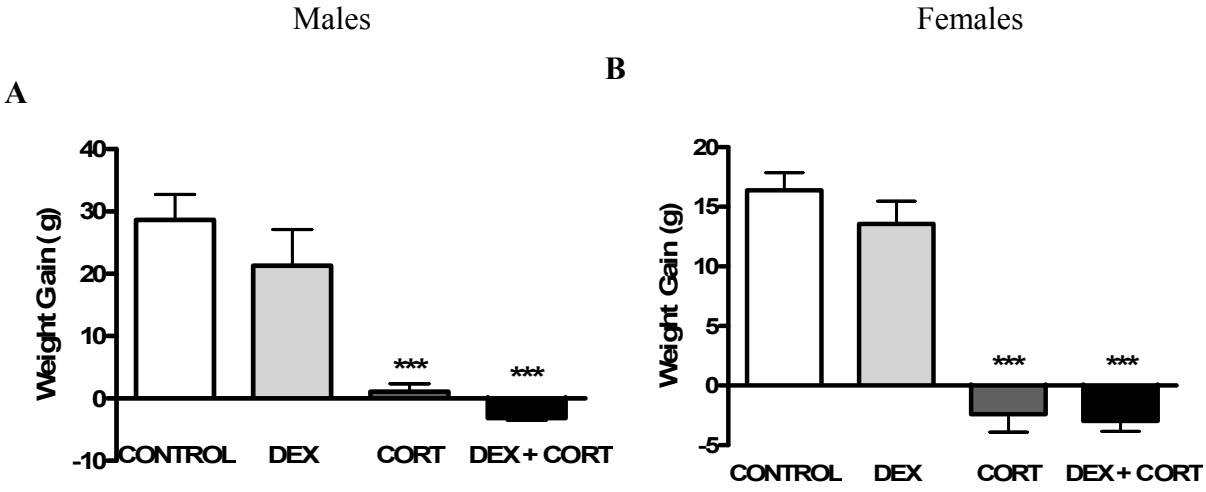


Figure 2.14. Weight gain (g) over adult GC treatment period (14-20 weeks) in males (A) and females (B). Data are expressed as mean ± sem. *** $p<0.001$ versus control. A: n= 14 (control), 13 (DEX), 14 (CORT), 12 (DEX + CORT). B:n= 14 (Control), 16 (DEX), 14 (CORT), 14 (DEX + CORT).

2.6.3 Fluid intake over hydrocortisone treatment period

Males showed no preference for hydrocortisone supplemented water (Figure 2.15A). Females that were prenatally treated with DEX showed a reduced preference for hydrocortisone supplemented water ($p<0.01$; Figure 2.15B). Average daily fluid intake was 30.81 ± 0.91 in males and 30.21 ± 1.39 in females. This equates to a daily dose of ~35mg/kg in males and ~50mg/kg in females.

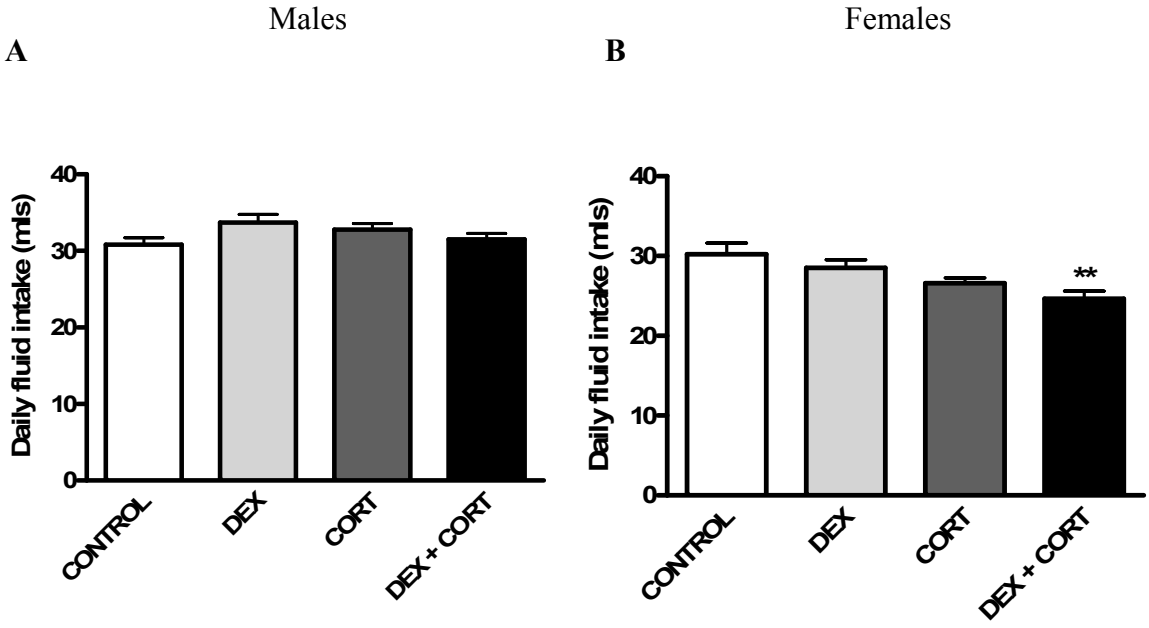


Figure 2.15. Average daily fluid intake (ml) over hydrocortisone treatment period (14-20 weeks) in males (A) and females (B). Data expressed as mean ± sem. **p<0.01 versus control. A: n= 14 (control), 13 (DEX), 14 (CORT), 12 (DEX + CORT). B:n= 14 (Control), 16 (DEX), 14 (CORT), 14 (DEX + CORT).

2.6.4 Effect of treatment regimes on estrus cycling

In the subset of animals examined there was no effect of any treatment on estrus cycling. Stress and HPA activation exerts an inhibitory action on the female reproductive system (Kalantaridou et al., 2004). Repeated stress may not have been a severe enough stress to cause disruption to the cycle. CRH is thought to underlie the negative effects of stress and HPA activation on the female reproductive system. In our GC treatment regime it is likely that CRH release was suppressed due to negative feedback and therefore would not impact on the estrus cycle.

2.6.5 Effect of treatment regimes on serum corticosterone levels at autopsy

There were no significant effects of treatment on serum corticosterone levels in males or females (Figure 2.16). However this does not mean that our treatment regimes did not affect corticosterone levels as serum was collected two weeks after the final session or dose of treatment. Nevertheless it does indicate that any affects on corticosterone levels were not persistent. Serum was collected during the morning (8.30-10.30am) which corresponds with the circadian trough of corticosterone accounting for the low levels of corticosterone reported here. It is also known that our method of serum collection (decapitation) results in lower morning corticosterone levels than other methods (Dunn and Scheving, 1971).

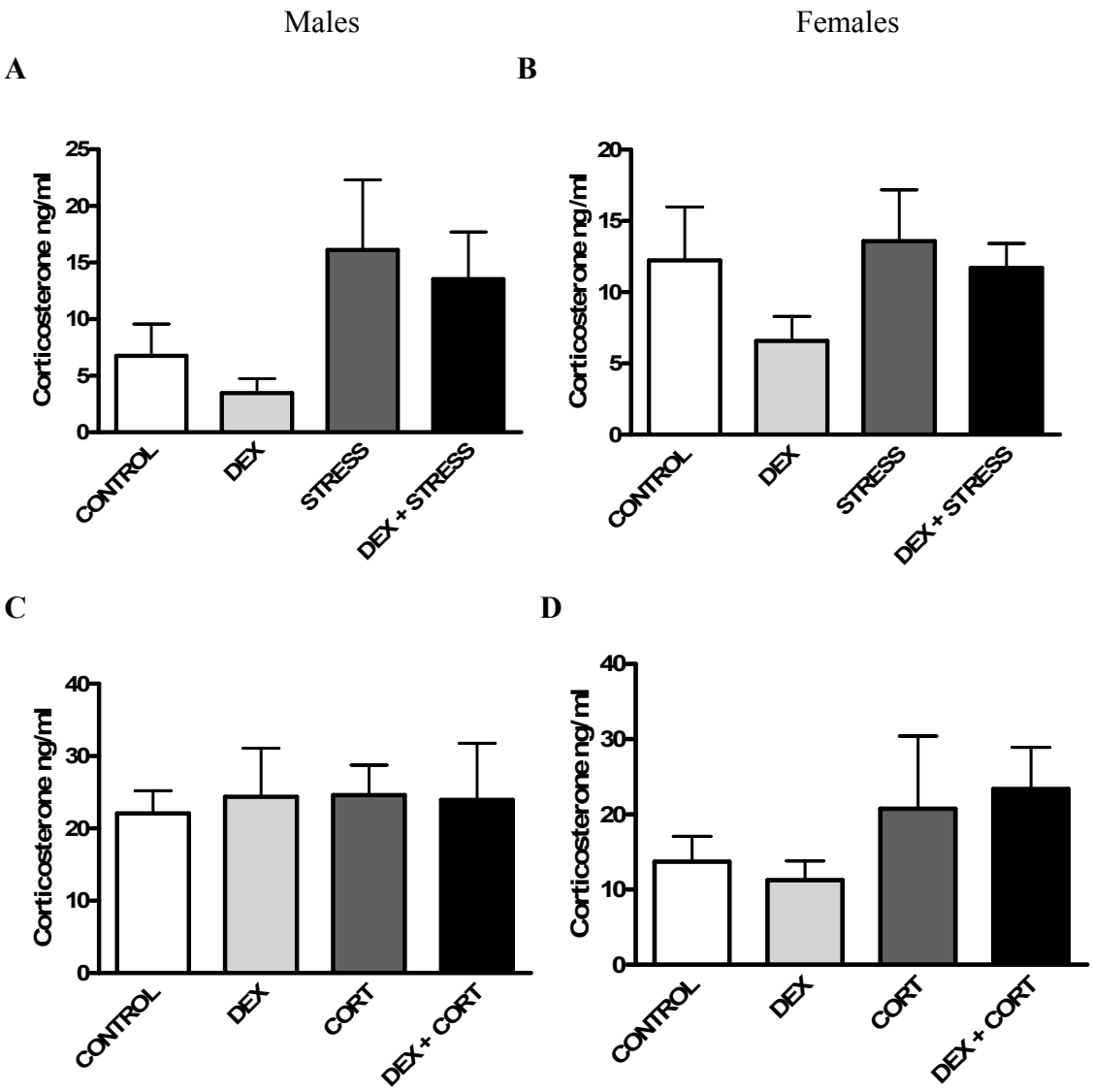


Figure 2.16. Effect of treatment regimes on serum corticosterone levels (ng/ml) at autopsy. In study 1 (A,B) and study 2 (C,D) Serum was collected at 5.5 months and corticosterone levels measured by enzyme immunoassay (females A,C; males B,D). A: n= 8 (control), 9 (DEX), 8 (stress), 8 (stress and DEX). B: n=10 (Control), 8 (DEX), 10 (stress), 10 (DEX and stress). C: n= 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n= 7 (Control), 9 (DEX), 7 (CORT), 7 (DEX + CORT).

CHAPTER 3

The sex-dependent effects of stress and GC treatments on the integrity of the adult NSDA.

3.1 INTRODUCTION

The main pathological feature of PD is the degeneration of DA neurons within the SNpc which results in a loss of DA within the striatum. One of the earliest proposed causes of PD was stress and this idea has started to regain attention and a hypothesis for the involvement of stress and GCs in the pathophysiology of PD is emerging (Kibel and Drenjancevic-Peric, 2008). In PD patients it has been suggested that acute or chronic stress may result in an earlier onset or worsen the symptoms of PD (Macht et al., 2005). Indeed, cortisol levels are higher in PD patients and have been associated with gait deficits (Hartmann et al., 1997; Charlett et al., 1998). The presence of GCs within the NSDA provides a means by which GCs could exert an effect on the NSDA rendering it vulnerable to excess levels of GCs. Consequently stress and GCs have been shown to negatively affect motor function in both humans (Maki and McIlroy, 1996) and rats (Metz et al., 2001; Metz et al., 2005). Of significant interest is the finding that stress can elevate oxidative damage in the NSDA (Kim et al., 2005) a mechanism that has been heavily implicated in the pathogenesis of PD. Further evidence supporting an effect of stress and GCs on the NSDA is provided by studies demonstrating that chronic corticosterone treatment elevates D1 receptor mRNA in the striatum of mice (Biron et al., 1992); immobilisation stress increases striatal DA levels (Kim et al., 2005) and that repeated restraint stress increases DAT mRNA within the striatum (Copeland et al., 2005). It has also been proposed that the early life environment has a significant impact on disease susceptibility in adulthood (see section 1.2.11) and that GCs are an important programming factor. Thus, stress and excess GCs during development may impact upon the developing NSDA. Indeed, prenatal GC treatment has been shown to permanently alter midbrain rat DA systems (McArthur et al., 2005). This chapter aims to address the

hypothesis that stress and GCs at different stages in life may affect the integrity of the NSDA rendering it vulnerable to toxic insults.

The following experiments therefore sought to determine the effects of prenatal treatment with a synthetic glucocorticoid, dexamethasone (DEX), and/or exposure in adulthood to repeated intermittent stress or chronic GC treatment on the morphological and functional integrity of the rat NSDA. As few studies explore responses in males and females in conjunction, the NSDA response to stress and GCs in both male and female animals was examined. This is important as the stress response and the effects of early life programming differ in males and females (Kudielka and Kirschbaum, 2005; Luine et al., 2007; Weinstock, 2007). Moreover, the prevalence of PD is higher in males (Schrag et al., 2000) and previous work has demonstrated that females are less susceptible to the neurotoxin 6-OHDA which specifically targets the NSDA pathway and provides an animal model of PD (Murray et al., 2003).

3.2 EXPERIMENTAL DESIGN AND HYPOTHESES

Two highly novel studies were performed in which the integrity of the NSDA system and its susceptibility to 6-OHDA were explored: the first (study 1) examined the effects of prenatal DEX treatment and/or repeated restraint stress in adulthood and the second (study 2) the effects of prenatal DEX treatment and/or chronic hydrocortisone treatment in adulthood. These protocols enable testing of the hypothesis that GCs are the important mediators of the effects of adult stress within the NSDA system. As stress and GCs have been shown to affect inflammation and oxidative stress within the brain and these processes have been heavily implicated in the pathogenesis of PD (see section 1.1.11) alterations in glial populations and nitrosative protein damage within the SNpc were also examined.

To assess the outcome of treatment regimes, nigrostriatal integrity was assessed using immunohistochemistry. TH immunohistochemistry was used to determine the number of DA neurons within the SNpc and NeuN immunohistochemistry was used to deterimine the total number of neurons within the SNpc. HPLC was then used to assess NSDA functionality whereby the levels of DA, DOPAC and HVA within the striatum were obtained. The effects of these treatment regimes on microglia, astrocytes and protein nitration within the SNpc were assessed using immunohistochemical markers (see section 2.2.5/6).

The consequences of stress and GCs on the outcome of a 6-OHDA unilateral lesion, which is commonly used to model PD (Simola et al., 2007) were also evaluated. Here 5µg of 6-OHDA was injected into the left MFB, which contains the axons of the

neurons within the SNpc, at a dose producing a partial unilateral lesion of the NSDA. This method of administration has previously been found to produce a destruction of the A9 and A10 cell groups of the SNpc, depletion of DA in the ipsilateral striatum, hypersensitivity of postsynaptic receptors in the striatum and characteristic turning behaviour in response to amphetamine or apomorhine produced due to the imbalance in dopamine activity between the two hemispheres (Duemans, 2002). The effect of stress and GC treatment on the lesion was again determined by immunohistochemistry and HPLC. For further details of the experimental design and methodology see chapter 2.

3.3 RESULTS

3.3.1 The intact nigrostriatal system (NSDA) system

***Control values**:* On combination of data from study 1 and 2 the average number of TH-immunoreactive (TH-IR) cells in the normal adult SNpc was estimated to be 8118 ± 0.276 in males and 7185 ± 0.267 in females. These values are in line with other published estimates (Anden et al., 1966; Hedreen and Chalmers, 1972; German and Manaye, 1993; Dewing et al., 2006; McArthur et al., 2007a) and accord with findings that the number of TH-IR cells in the female SNpc is significantly lower than in the male ($p<0.05$) (Dewing et al., 2006; McArthur et al., 2007a) . Although the number of NeuN-IR cells was significantly higher there was also a sex difference in the total number of neurons within the SNpc with 9843 ± 0.26 cells in males and 8869 ± 0.45 cells in adult females ($p<0.05$). The average striatal DA level was 13.06 ± 1.43 ng/mg of tissue in control males and 14.97 ± 0.46 ng/mg of tissue in control females (not significant), thereby agreeing with other reports that there are no sex differences in control levels of striatal DA (Wagner et al., 1993; Murray et al., 2003). However, it is difficult to compare HPLC data across studies as discussed in section 2.2.8.

***Adult treatments in male rats*:** Repeated stress in adulthood had no effect on the male NSDA pathway at SNpc or striatal levels (Fig 3.1, 3.2, Table 1). In contrast to repeated stress, chronic GC treatment in adulthood increased the number of TH-IR cells within the SNpc ($p<0.05$; Fig 2C) but not NeuN-IR cells and raised striatal DA ($p<0.01$) and DOPAC levels ($p<0.001$; Table 2). Repeated stress but not GC treatment increased the number of OX-42-IR cells within the male SNpc ($p<0.05$; Figure 3.4A). No changes

in microglial morphology were observed with the majority of the counted microglia displaying a ramified morphology typical of their resting state. Both adult treatments significantly increased 3-NT-IR cells within the SNpc ($P<0.005$; Figure 3.5A,C) indicating a greater degree of oxidative damage. Neither adult treatment altered GFAP-IR within the SNpc.

Prenatal treatment in male rats: Compared with controls, prenatal DEX treatment increased the number of TH-IR ($p<0.05$) and NeuN-IR ($p<0.05$) cells within the adult male SNpc (Figure 3.1A,C,3.2A,C) without altering DA levels within the striatum (Table 1 and 2). There was a trend for a reduced astrocyte population in DEX treated males but this did not reach significance (Figure 3.3) and there was an increased microglia population within the SNpc of adult rats treated with DEX in study 2 (Figure 3.4C).

Prenatal treatment in combination with adult treatment in male rats: In rats subjected to prenatal DEX, adult stress not only ablated the effects of the prenatal treatment on TH-IR ($p<001$) and NeuN-IR ($p<0.001$), but reduced TH-IR, and striatal DA levels ($p<0.05$) to values significantly lower than those of controls (Figures 3.1,3.2; Table 3.1). Interestingly, the effects of adult GC treatment were also significantly attenuated by prenatal DEX treatment where TH-IR and striatal DA levels in animals treated with GCs both prenatally and in adulthood did not differ from those of the untreated controls ($p<0.001$ and 0.01 respectively; Figure 3.1; Table 3.2) . DA metabolite levels were however elevated ($p<0.05$; Table 3.2) in these animals indicating an increase in the turnover of DA within the striatum ($p<0.05$; Table 1, Table 2).

***Adult treatments in female rats*:** Unlike males, in females repeated adult stress increased the number of TH-IR (but not NeuN-IR) cells in the SNpc ($p<0.001$; Fig 3.1B) and elevated striatal DA ($p<0.001$), DOPAC ($p<0.01$), but not HVA levels (Table 3.1). By comparison GC treatment in adulthood did not affect the female NSDA (Figure 3.1D, Table 3.2), with the exception of elevated HVA levels. Adult treatments exerted no affect on GFAP, OX-42 or 3-NT-IR cell counts in the female SNpc.

Prenatal treatment in female rats: As in males, prenatal DEX treatment increased the number of TH-IR ($p<0.05$) and NeuN-IR ($p<0.05$) cells in the female SNpc (Fig 3.1B,D,3.2B,D) without affecting striatal DA levels. An increase in striatal DOPAC levels, and therefore DA turnover, reached significance ($p<0.05$) in the first, but not second, study (Table 3.1 and 3.2). Prenatal DEX treatment had no effect on OX-42-IR or 3-NT-IR cell numbers but reduced the number of GFAP-IR astrocytes in study 2 ($p<0.05$; Fig 3.3D).

Prenatal treatment in combination with adult treatment in female rats: Stress appeared to enhance the effect of prenatal DEX on TH-IR (Fig 3.1B) without affecting striatal DA. By contrast, DOPAC levels and DA turnover in animals treated with GCs prenatally and then subjected to stress in adulthood did not differ from those of the untreated controls (Table 3.1). Adult GC treatment in DEX treated rats attenuated DOPAC levels ($p<0.001$) but didn't alter turnover (Table 3.2). TH-IR cell counts in animals treated with GCs both prenatally and in adulthood did not differ from those of the untreated controls whilst their HVA levels were similar to those of rats given prenatal DEX only (Table 3.2).

A **B**

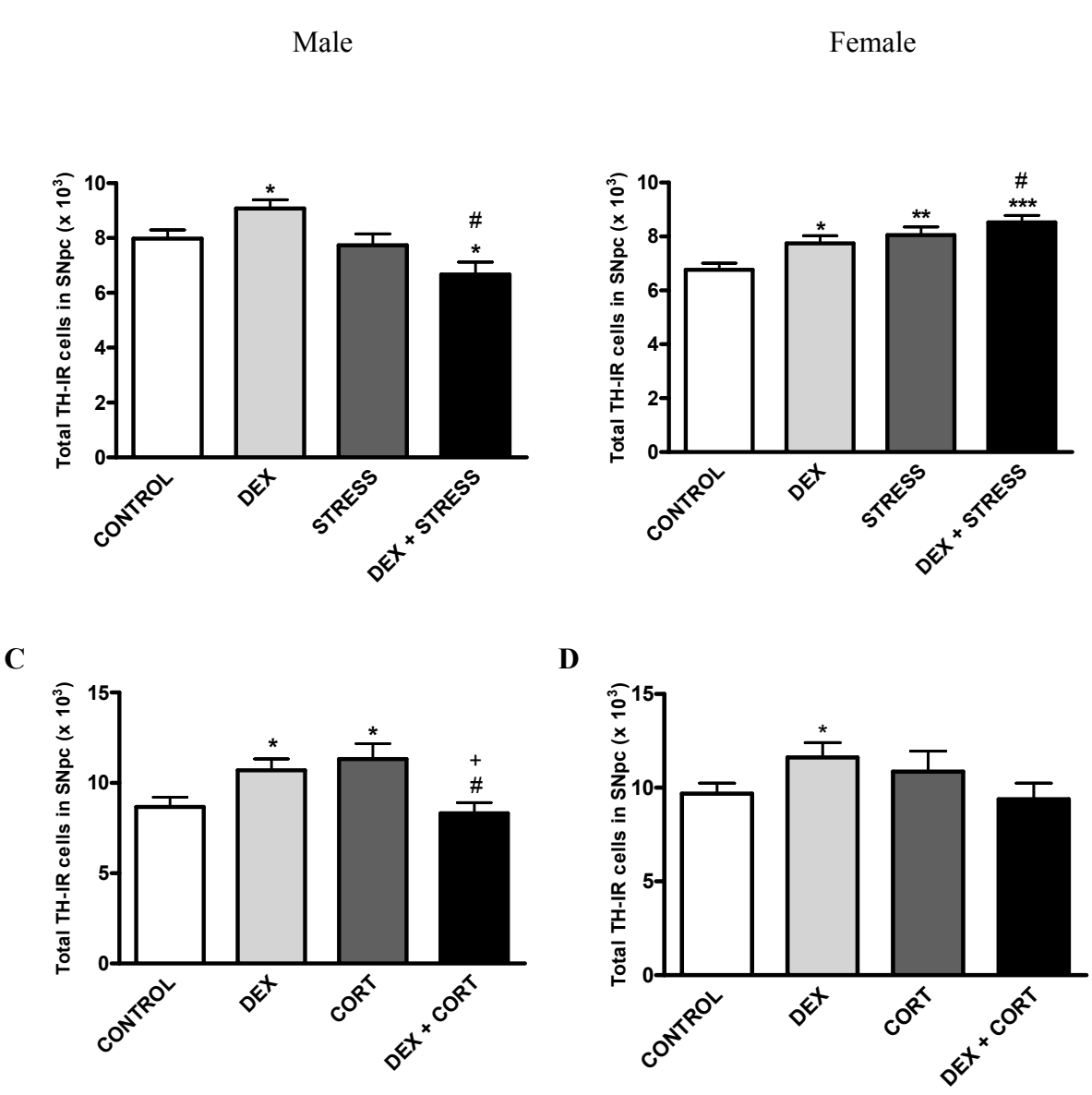


Figure 3.1. Effect of prenatal dexamethasone and/or adult stress (study 1) or GC treatment (study 2) on TH-IR cells within the SNpc. Stereological estimates of total TH-IR cells within the SNpc in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by TH immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

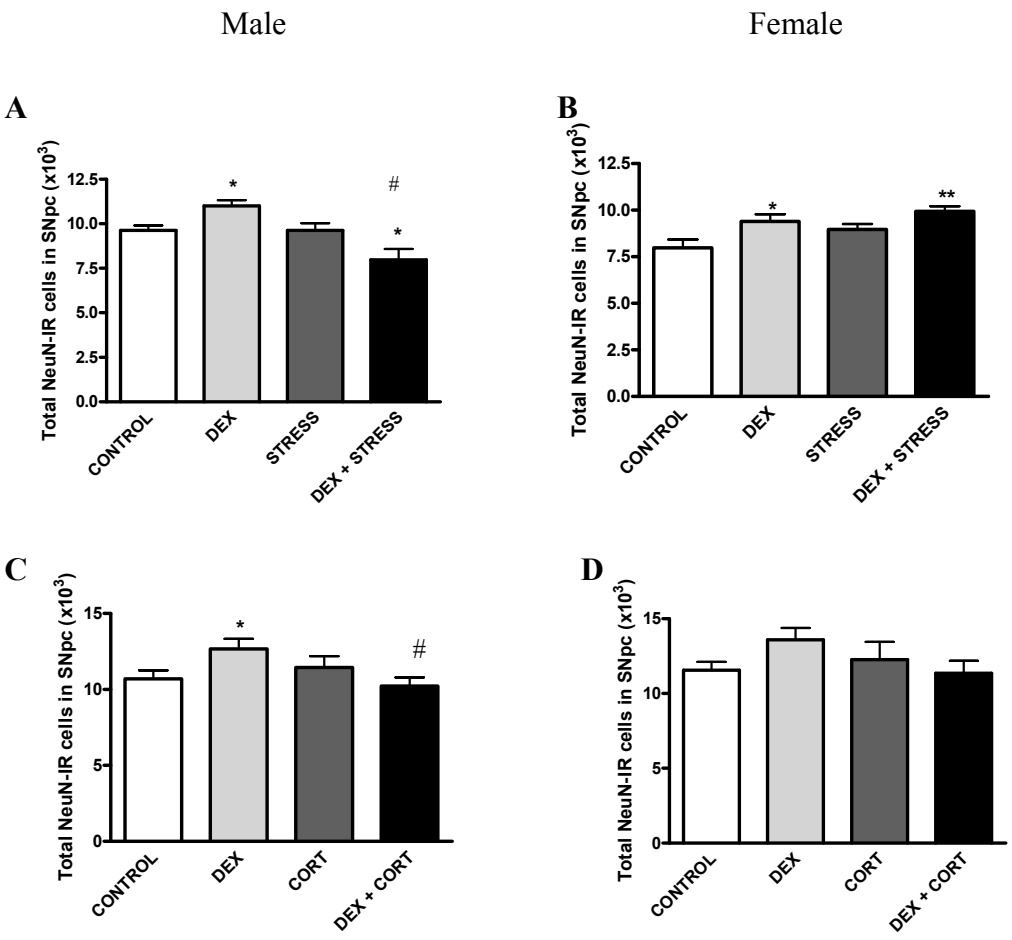


Figure 3.2. Effect of prenatal dexamethasone and/or adult stress (study 1) or GC treatment (study 2) on NeuN-IR cells within the SNpc. Stereological estimates of total NeuN-IR cells within the SNpc in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by NeuN immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls; # vs DEX, + vs adult treatments. A: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

Adult Striatal DA, DOPAC and HVA levels				
	CON	**DEX**	**STRESS**	**DEX + STRESS**
Male				
DA	20.44 ± 1.29	21.23 ± 1.15	22.41 ± 1.09	17.91 ± 0.92 * ## ++
DOPAC	6.71 ± 0.39	8.15 ± 0.57	7.85 ± 0.40	8.04 ± 0.49
HVA	1.36 ± 0.11	1.66 ± 0.09	1.25 ± 0.09	1.88 ± 0.09 ** ++
Turnover	0.44 ± 0.02	0.51 ± 0.03	0.45 ± 0.02	0.62 ± 0.03 *** #
Female				
DA	15.21 ± 0.49	17.24 ± 0.85	21.35 ± 1.1 ***	19.82 ± 0.66 *** #
DOPAC	5.68 ± 0.48	9.82 ± 0.37 **	8.73 ± 1.03 **	8.01 ± 0.58 *
HVA	1.55 ± 0.07	2.01 ± 0.07	1.75 ± 0.13	1.91 ± 0.15
Turnover	0.52 ± 0.03	0.78 ± 0.05 *	0.57 ± 0.09	0.56 ± 0.04

Table 3.1. Effect of prenatal DEX and/or adult stress on striatal DA and metabolite levels. Striatal DA, DA metabolite and DA turnover were assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeated restraint stress. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of DA metabolites (DOPAC and HVA) to DA in pmol/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs controls, # vs DEX, + vs adult treatment. Males: n=8 (control), 8 (DEX), 8(stress), 8 (DEX + stress). Females: n= 9 (control), 8 (DEX), 8 (stress), 8 (DEX + stress).

Adult Striatal DA, DOPAC and HVA levels				
	CON	**DEX**	**CORT**	**DEX + CORT**
Male				
DA	9.13 ± 1.14	12.92 ± 1.23	17.76 ± 2.26 **	10.57 ± 2.51 +
DOPAC	2.43 ± 0.32	4.04 ± 0.35	4.88 ± 0.37 ***	5.62 ± 2.49 ***
HVA	0.63 ± 0.12	0.67 ± 0.07	0.83 ± 0.05	0.98 ± 0.09 *
Turnover	0.41 ± 0.05	0.38 ± 0.04	0.43 ± 0.06	0.77 ± 0.171 * # +
Female				
DA	14.81 ± 0.72	15.99 ± 0.80	17.47 ± 0.84	13.19 ± 0.58 +
DOPAC	2.01 ± 0.17	2.49 ± 0.17	2.73 ± 0.25	1.76 ± 0.25
HVA	1.03 ± 0.08	1.27 ± 0.06 *	1.49 ± 0.06 ***	1.23 ± 0.03
Turnover	0.23 ± 0.01	0.25 ± 0.01 ***	0.27 ± 0.01	0.24 ± 0.002

Table 3.2. Effect of prenatal DEX and/or adult GC treatment on striatal DA and metabolite levels. Striatal DA, DA metabolite and DA turnover were assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) GC treatment. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of DA metabolites (DOPAC and HVA) to DA in pmol/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs controls, # vs DEX, + vs adult treatment. Males: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). Females: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

Male Female

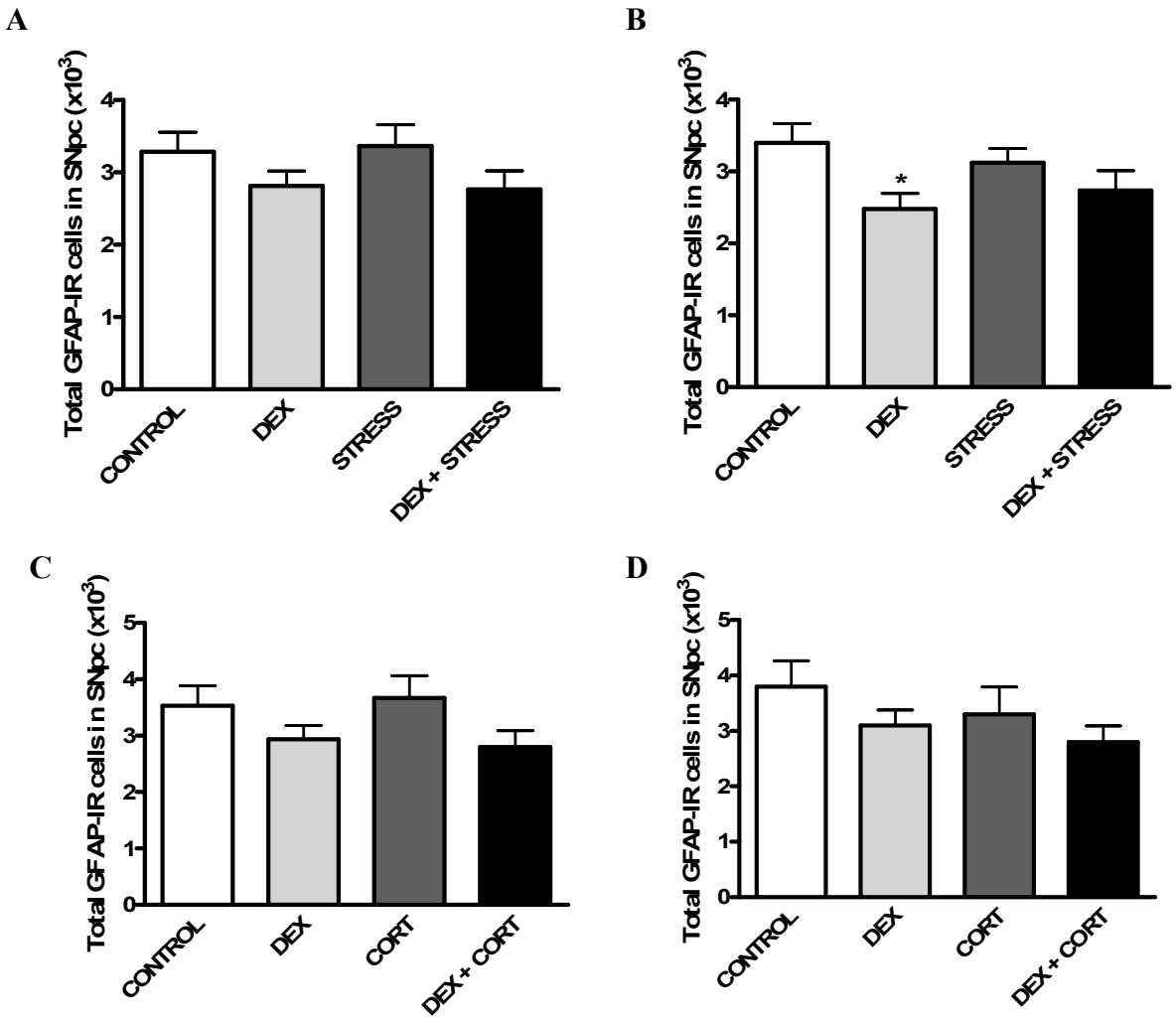


Figure 3.3. Effect of prenat al DEX a nd/or adult stress or GC treatment on GFAP-IR cells within the SNpc. Stereological estimates of total GFAP-IR cells within the SNpc in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by GFAP immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 9 (DEX), 8 (stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

Male Female

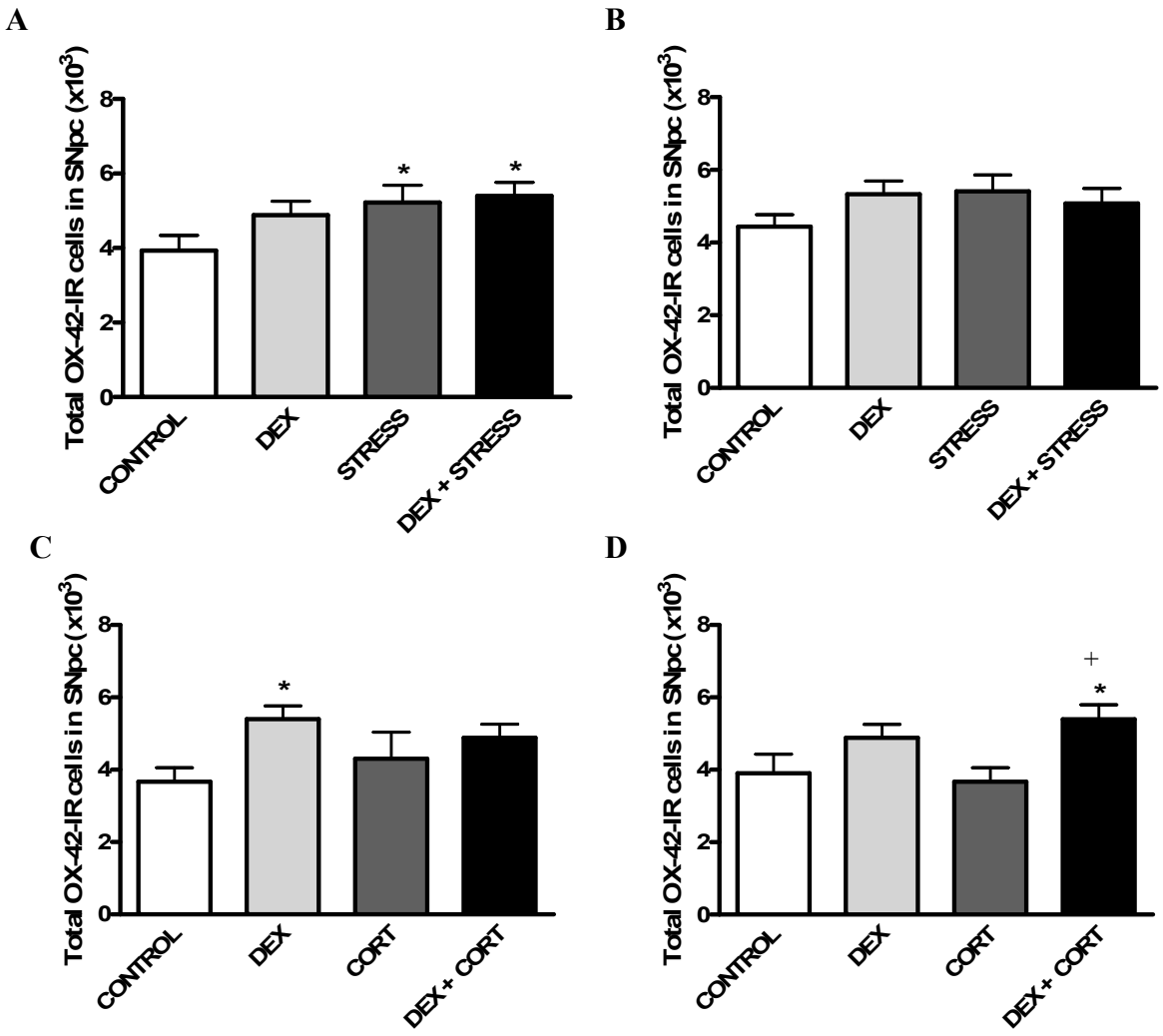


Figure 3.4. Effect of prenatal DEX and/or adult stress or GC treatment on OX-42-IR cells within the SNpc. Stereological estimates of total OX-42-IR cells within the SNpc in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by OX-42 immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. N numbers can be found in figure 2.11. A: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

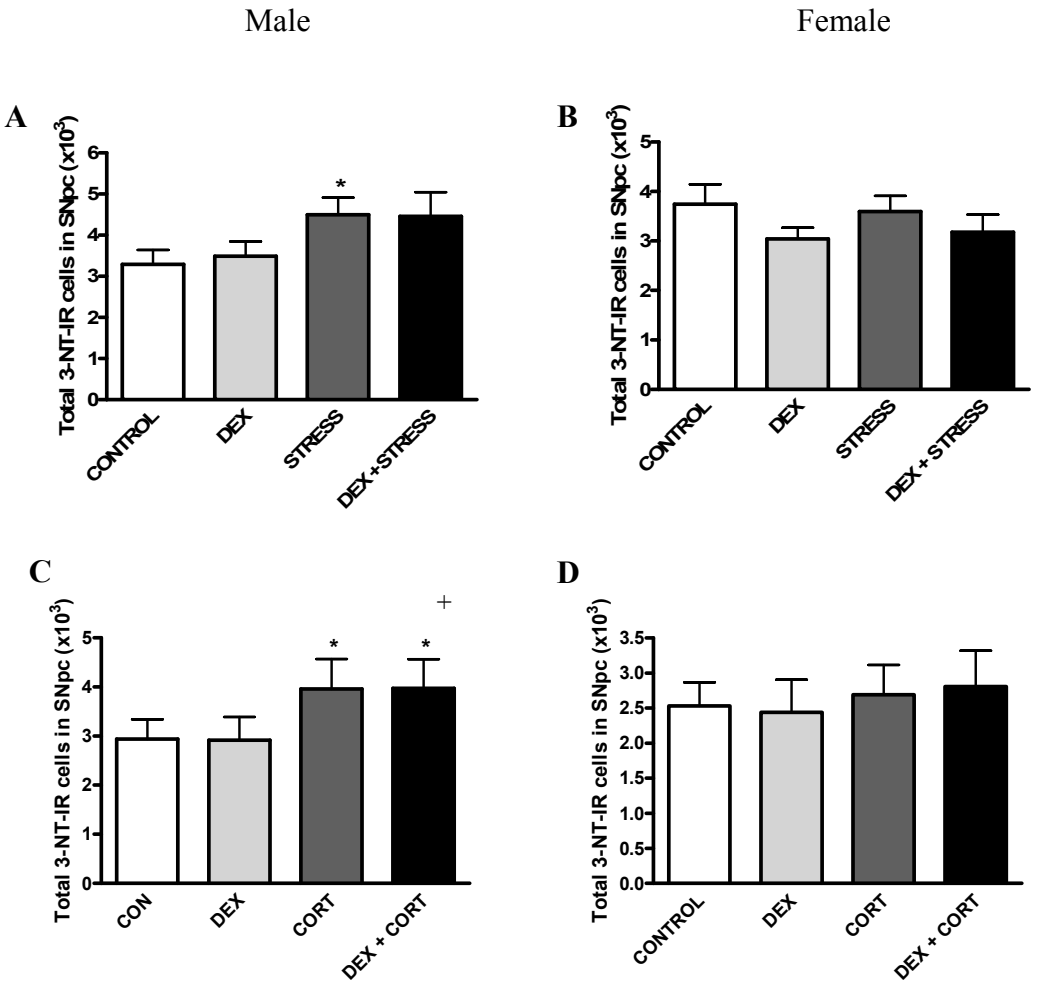


Figure 3.5. Effect of prenatal DEX and/or adult stress or GC treatment on 3-NT-IR cells within the SNpc. Stereological estimates of total 3-NT-IR cells within the SNpc in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by 3-NT immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatments. N numbers can be found in figure 2.11. A: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

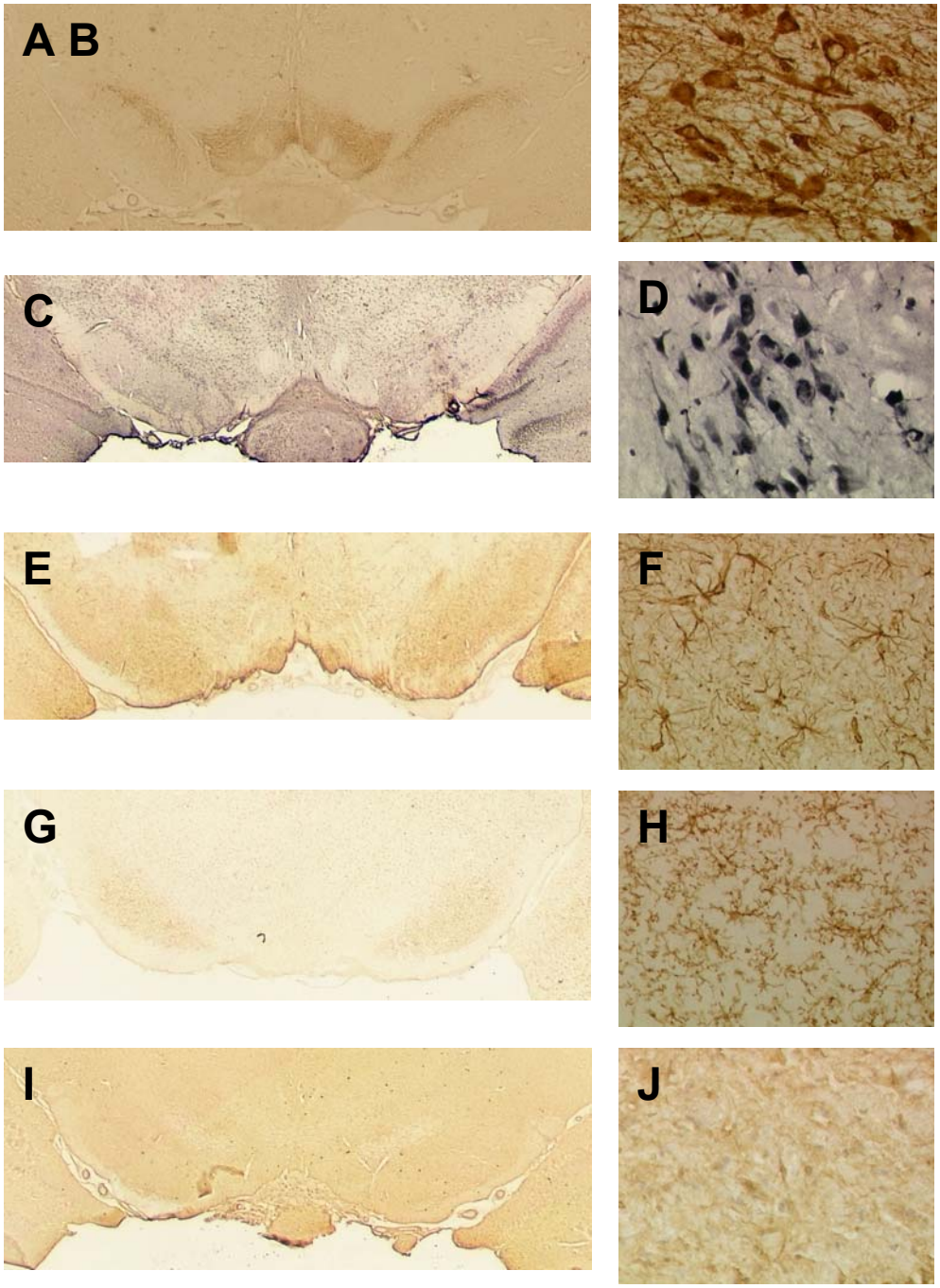


Figure 3.6 Representati ve photomicrographs of immunohistochemistr y within the SNpc. Shown at x10 (ACEGI) and x200 (BDFHI) magnification. TH (AB), NeuN (CD), GFAP (EF), OX-42 (GH), 3-NT (IJ)

3.3.2 The injured NSDA system

The number of TH-IR cells in the SNpc and DA levels within the striatum are expressed both as actual levels (Figure 3.8; Tables 3.4,3.6) in the lesioned and unlesioned hemisphere and as a percentage loss in the lesioned vs unlesioned hemisphere (Figures 3.9,3.12) to aid interpretation of the data.

Controls: In order to examine the implications of our treatment regimes for susceptibility to pathological change, we employed the 6-OHDA model of PD. Here administration of 5µg 6-OHDA to the left medial forebrain bundle consistently produced a significant loss of TH-IR cells within the SNpc and depleted DA levels within the striatum of the ipsilateral hemisphere. On combination of data from study 1 and study 2 in male controls there was a 38.75 ± 6.03 % loss of TH-IR cells in the lesioned SNpc compared to the contralateral SNpc and a 48.9 ± 5.33 % loss of striatal DA. The size of the lesion and subsequent DA loss was not significantly different in females where there was a 45.78 ± 6.2 % loss of TH-IR cells and a 54.14 ± 8.13 % loss of striatal DA. The number of TH-IR cells in the control hemisphere of lesioned animals was greater than in the SNpc of control animals that had not received 6-OHDA (figures 3.8 and 3.1). This was found to be the case in both males and females ($p<0.05$) however, striatal DA levels within the contralateral striatum were lower than in unlesioned controls, although as discussed in section 2.8 it is difficult to compare HPLC data from different runs. It is also interesting to note that the effects previously reported were lost in the contralateral SNpc of lesioned animals.

To dissect further the effects of GCs and stress on the outcome of a 6-OHDA lesion, microglial activation within the lesioned SNpc was assessed using

immunohistochemistry for OX-6 (a MHC class II antigen) which selectively identifies activated microglia. Negligible microglial activation was detected in the contralateral SNpc of lesioned animals but within the ipsilateral SNpc extensive microglial activation was observed therefore the number of OX-6-IR cells was expressed as a percentage change from the number of positive cells in the lesioned SNpc of control rats (Figure 3.14). The average number of OX-6-IR cells within the lesioned SNpc was estimated to be 2332 ± 288 in male controls and 3536 ± 435 in female controls. Administration of 6-OHDA also produced proliferation of GFAP-IR cells within the lesioned SNpc where the number of GFAP-IR+ cells rose by 34.48 ± 4.27% and 28.70 ± 2.91% in male and female controls respectively (Figure 3.13). Additionally there was an increase in the number of GFAP-IR cells displaying an activated reactive morphology (Figure 3.10). Levels of nitrosative stress within the lesioned SNpc were significantly increased when compared to the contralateral SNpc. There were 32.12 ± 2.96% more 3-NT-IR+ cells in the lesioned male SNpc and 31.84 ± 4.22% more 3-NT-IR+ cells in the female lesioned SNpc (Figure 15).

Adult treatments in male 6-OHDA rats: Repeated restraint stress in adult males significantly exacerbated 6-OHDA induced TH-IR cell loss ($p<0.01$; Figure 3.7A), NeuN-IR cell loss ($p<0.05$; Figure 3.9) and striatal DA depletion ($p<0.05$; Table 3). In addition there was an increase in OX6-IR cells ($p<0.001$; Figure 3.12A, 3.13) and 3-NT-IR cells ($p<0.05$; Figure 3.14A,3.15) in the SNpc of repeatedly stressed males above that seen in 6-OHDA control animals. Chronic adult GC treatment also enhanced the loss of TH-IR cells ($p<0.05$; Fig 5E,M), increased the number of OX-6-IR ($p<0.01$; Figure 6C,F) and 3-NT-IR ($p<0.05$; Figure 7C,G) cells, but did not significantly affect DA depletion.

Prenatal treatment in male 6-OHDA rats: Prenatal DEX treatment did not alter the susceptibility of the NSDA to 6-OHDA neurotoxicity at SNpc or striatal levels (Figures 3.7, 3.9, tables 3 and 4).

Prenatal treatment in combination with adult treatments in male 6-OHDA rats: Prenatal DEX treatment did not alter the effect of adult stress on 6-OHDA induced TH-IR or striatal DA depletion. However adult GC treatment in DEX treated males exacerbated 6-OHDA-induced striatal DA loss ($p<0.05$), without producing a further effect on TH-IR cell loss.

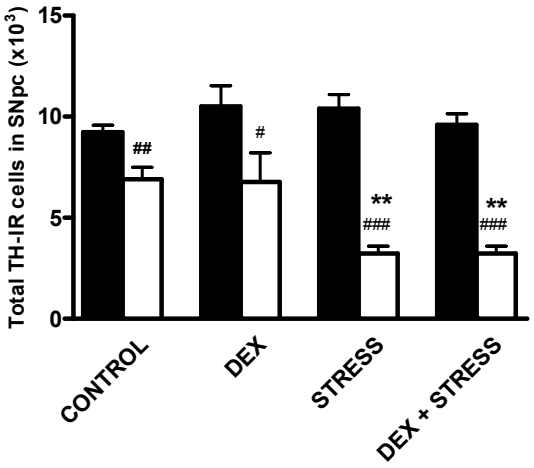
Adult treatments in females: In contrast to its effect in males, repeated stress in adult females attenuated 6-OHDA-induced TH-IR cell loss ($p<0.05$; Figure 3.7B,3.8L), NeuN-IR cell loss ($p<0.05$; Figure 3.8B) and striatal DA loss ($p<0.05$; Table 3). Chronic adult GC treatment similarly reduced TH-IR cell loss ($p<0.05$; Figures 3.7C 3.8F) but did not affect DA depletion (Table 4). Neither treatment altered GFAP-IR, OX6-IR or 3-NT-IR above that observed in 6-OHDA control animals.

***Prenatal treatment in 6-OHDA females*:** As for males, prenatal DEX treatment did not alter the susceptibility of the NSDA to 6-OHDA neurotoxicity at SNpc or striatal levels (Figure 3 B, D, F, H).

Prenatal treatment in combination with adult treatments in 6-OHDA females: In females that received GC treatment striatal DA depletion was unaffected by previous prenatal DEX treatment, whereas the SNpc effects caused by adult restraint

stress were attenuated, such that TH-IR cell loss was no longer significantly reduced after exposure to adult stress. Prenatal DEX treatment didn't alter the response to 6-OHDA in GC treated females.

A **B**



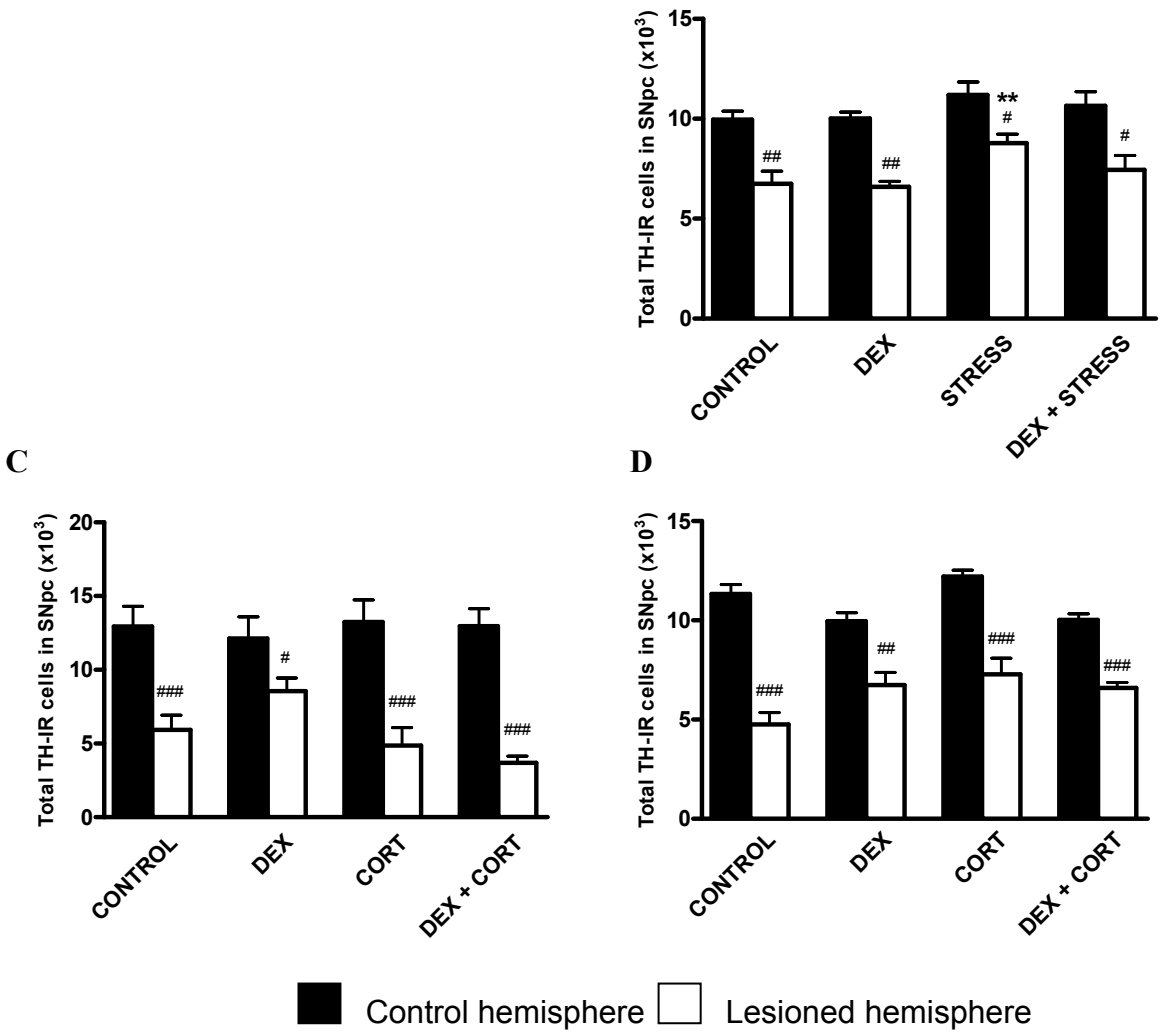


Figure 3.8. Effect of prenatal DEX treatment and/or adult stress or GC treatment on TH-IR cells within the SNpc following 6-OHDA lesioning. Stereological estimates of the number of TH-IR cells within the SNpc of male (A,C) and female (B,D) animals exposed to a 6-OHDA were determined by TH immunohistocemistry. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by three way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs unlesioned hemisphere. A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 6 (DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

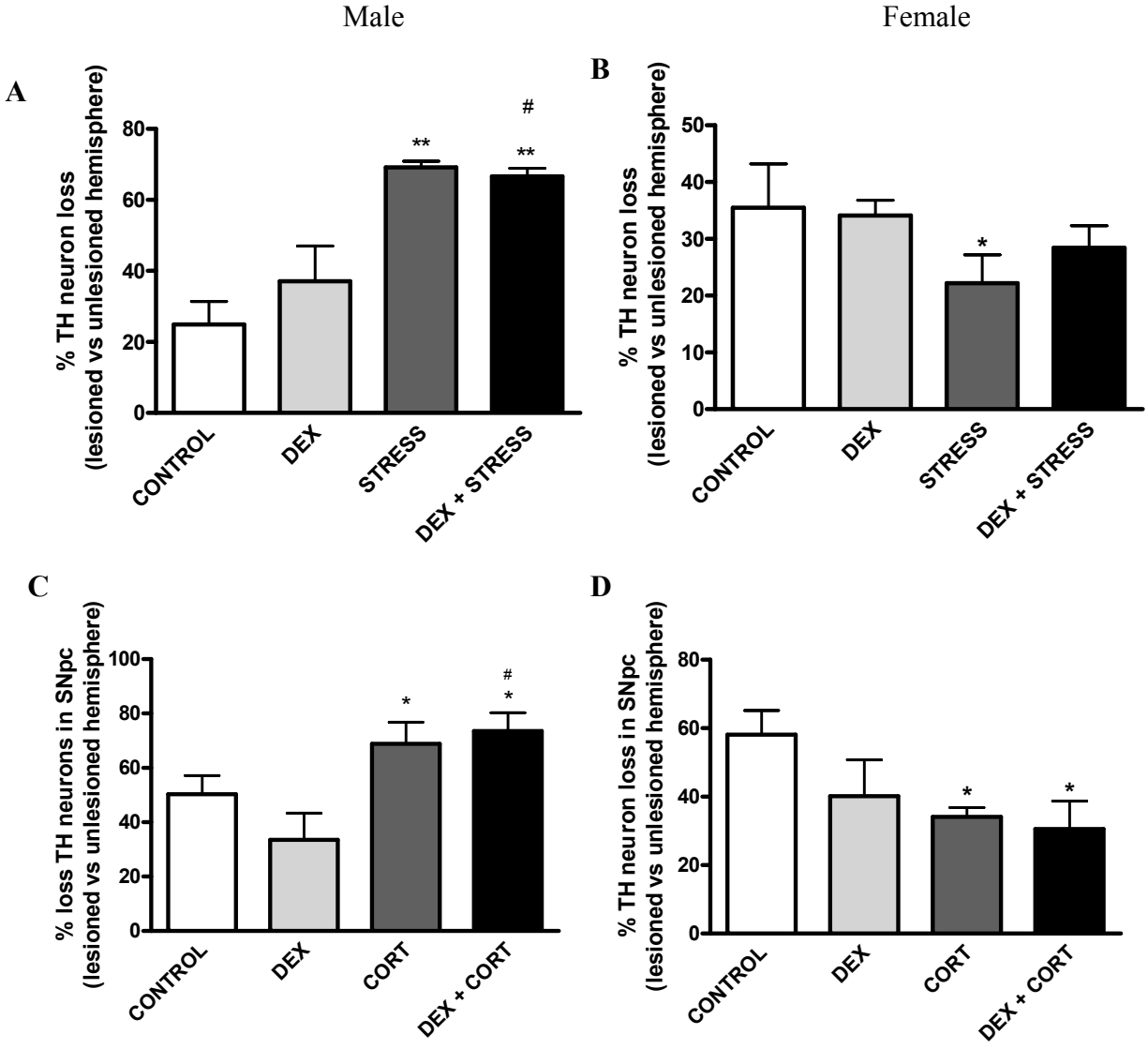


Figure 3.9. Effect of prenatal DEX treat ment and/or adult stress or GC treatment on percentage loss of TH-IR cells in the SNpc follo wing 6-OHDA lesioning. The percentage loss of TH-IR cells in the lesioned SNpc versus the unlesioned SNpc in male (A,C) and female (B,D) animals exposed to a 6-OHDA. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effects of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 6 (DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

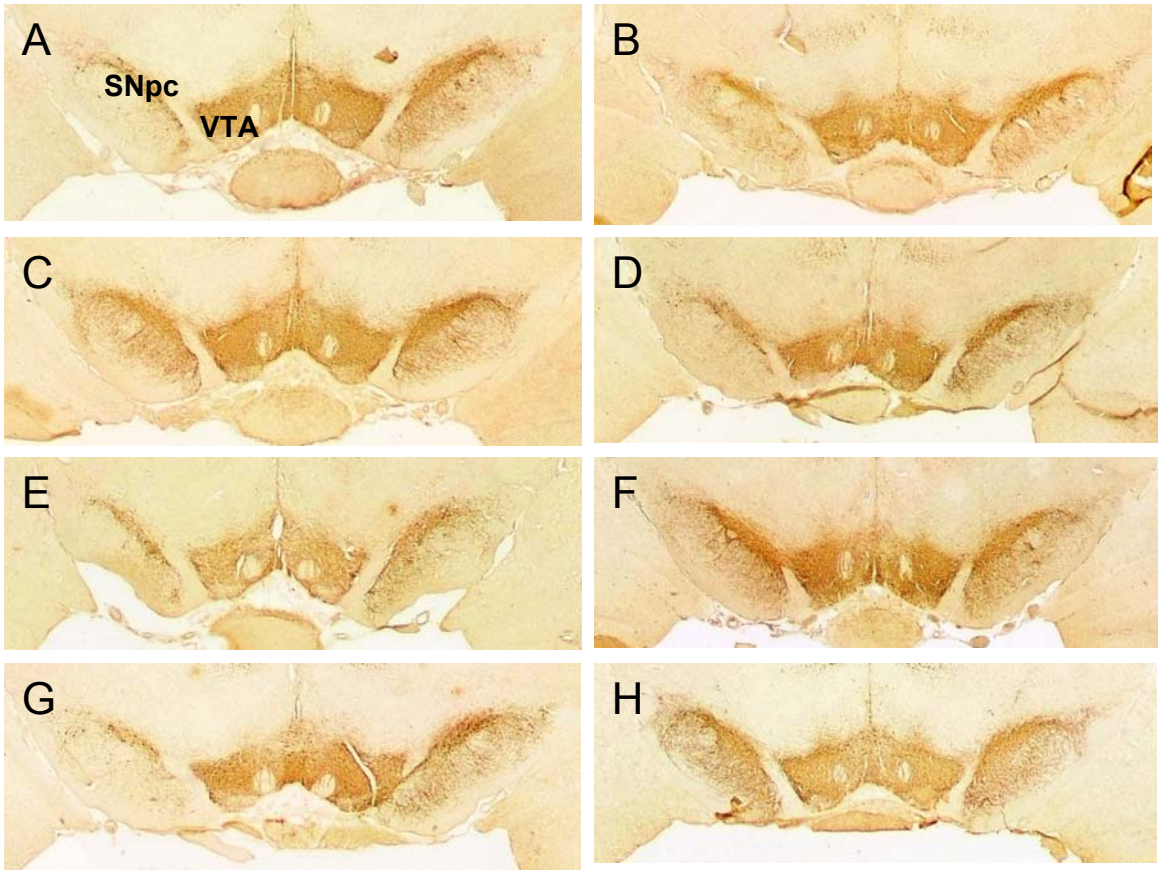


Figure 3.10 Representative photomic rographs of TH-IR staining within the SNpc and VTA of male (A,C,E,G) and female (B,D,F,G) rats. Controls (A,B), DEX treated (C,D), stressed (E,F) and GC treated (G,H). Images were taken at 10x magnification.

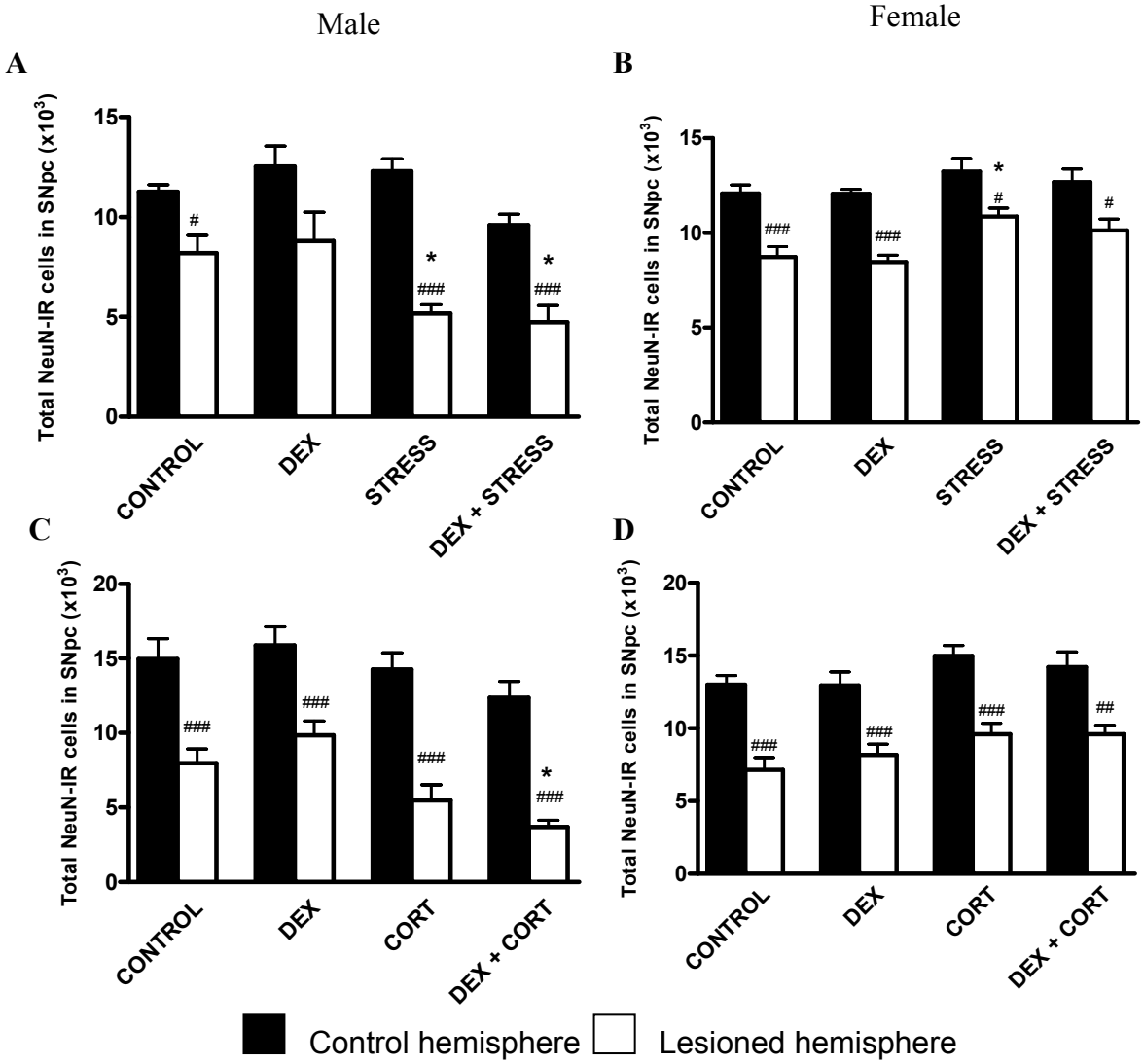


Figure 3.11. Effect of prenatal DEX treatment and/or adult stress or GC treatment on NeuN-IR cells within the SNpc following 6-OHDA lesioning. Stereological estimates of the number of NeuN-IR cells within the SNpc of male (A,C) and female (B,D) animals exposed to a 6-OHDA were determined by immunohistocemistry. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effects of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by three way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls. A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 6 (DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

Striatal DA, DOPAC and HVA in 6-OHDA lesioned animals								
	Control		**DEX**		**STRESS**		**DEX + STRESS**	
	Right	**Left**	**Right**	**Left**	**Right**	**Left**	**Right**	**Left**
Males								
DA	15.05 ± 1.48	8.17 ± 1.65 ##	17.76 ± 1.74	10.74 ± 2.19 #	19.80 ± 1.77	5.03 ± 2.30 ###*	13.71 ± 1.10	3.77 ± 1.26 ###*
DOPAC	2.94 ± 0.31	1.24 ± 0.39	4.29 ± 0.65	2.1 ± 0.47	3.63 ± 0.55	1.09 ± 0.42	2.91 ± 0.47	0.88 ± 0.31
HVA	0.77 ± 0.09	0.41 ± 0.11	1.21 ± 0.15	0.63 ± 0.15	0.91 ± 0.14	0.51 ± 0.14	0.93 ± 0.10	0.32 ± 0.12
Turnover	0.44 ± 0.02	0.91 ± 0.03	0.50 ± 0.03	0.57 ± 0.04	0.78 ± 0.02	1.52 ± 0.05	1.01 ± 0.08	1.41 ± 0.04
Females								
DA	8.01 ± 1.17	4.45 ± 0.63 #	10.50 ± 0.61	5.99 ± 0.98 ##	16.23 ± 0.51	11.78 ± 1.78 # ***	16.28 ± 0.65	12.27 ± 1.62 # ***
DOPAC	6.49 ± 0.81	3.44 ± 0.28	5.61 ± 0.26	2.67 ± 0.40	2.14 ± 0.23	1.28 ± 0.24	2.67 ± 0.20	1.70 ± 0.27
HVA	1.59 ± 0.21	1.23 ± 0.11	1.29 ± 0.08	0.85 ± 0.13	0.83 ± 0.04	0.60 ± 0.09	0.84 ± 0.04	0.63 ± 0.09
Turnover	1.15 ± 0.21	1.13 ± 0.17	0.74 ± 0.07	0.70 ± 0.08	0.34 ± 0.14	0.20 ± 0.02	0.22 ± 0.02	0.22 ± 0.02

Table 3.3. Effect of prenatal DEX treatment and / or adult stress on striatal DA and metabolite levels in response to 6-OHDA lesioning. Striatal DA, DA metabolite and DA turnover were assesed by HPLC at 6 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeated restraint stress. At 5.5 months rats received an injection of 5µg 6-OHDA to the left MFB. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of DA to it's metabolites (DOPAC and HVA) in pmol/mg. Statistical significance was assessed by three way anova with tukeys post hoc multiple comparison tests.*p<0.05, **p<0.01, ***p<0.001 vs controls, # vs unlesioned hemisphere. Males: : n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). Females:: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress).

Striatal DA, DOPAC and HVA in 6-OHDA lesioned animals								
	Control		DEX		CORT		DEX + CORT	
	Right	Left	Right	Left	Right	Left	Right	Left
Males								
DA	7.69 ± 1.04	4.34 ± 1.21 ##	9.58 ± 1.06	6.42 ± 1.61 #	10.04 ± 0.77	3.23 ± 1.05 ###**	8.20 ± 1.93	1.93 ± 1.58 ### *
DOPAC	2.36 ± 0.38	1.27 ± 0.41	3.51 ± 0.17	2.33 ± 0.56	5.86 ± 0.59	3.41 ± 0.90	5.51 ± 0.41	1.83 ± 0.97 *
HVA	0.87 ± 0.13	0.64 ± 0.19	1.11 ± 0.27	1.32 ± 0.10	1.87 ± 0.22	1.41 ± 0.31	1.97 ± 0.15	0.81 ± 0.34
Turnover	0.90 ± 0.21	3.48 ± 1.46	0.58 ± 0.21	2.08 ± 0.86	1.01 ± 0.15	3.81 ± 1.16	1.06 ± 0.13	4.49 ± 1.63
Females								
DA	9.59 ± 0.58	5.57 ± 0.97 ##	9.24 ± 1.78	6.87 ± 1.20	10.77 ± 0.81	5.62 ± 1.39 ##	7.98 ± 0.65	5.19 ± 1.42
DOPAC	3.06 ± 0.14	1.30 ± 0.36	3.08 ± 0.22	1.86 ± 0.45	3.08 ± 0.56	1.34 ± 0.47	2.66 ± 0.22	1.73 ± 0.47
HVA	0.78 ± 0.06	0.46 ± 0.07	0.92 ± 0.09	0.73 ± 0.13	0.76 ± 0.09	0.44 ± 0.16	0.81 ± 0.03	0.58 ± 0.11
Turnover	0.42 ± 0.003	0.52 ± 0.04	0.44 ± 0.009	0.47 ± 0.01	0.41 ± 0.005	0.58 ± 0.09	0.45 ± 0.01	0.49 ± 0.04

Table 3.4. Effect of prenatal DEX treatment and / or adult GC treatment on striatal DA and m etabolite levels in res ponse to 6 -OHDA lesi oning. Striatal DA, DA metabolite and DA turnover were assesed by HPLC at 6 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) GC treatment. At 5.5 months rats received an injection of 5µg 6-OHDA to the left MFB. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of DA to it's metabolites (DOPAC and HVA) in pmol/mg. Statistical significance was assessed by three way anova with tukeys post hoc multiple comparison tests.*p<0.05, **p<0.01, ***p<0.001 vs controls, # vs unlesioned hemisphere. Males:: n = 7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT). Females:: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

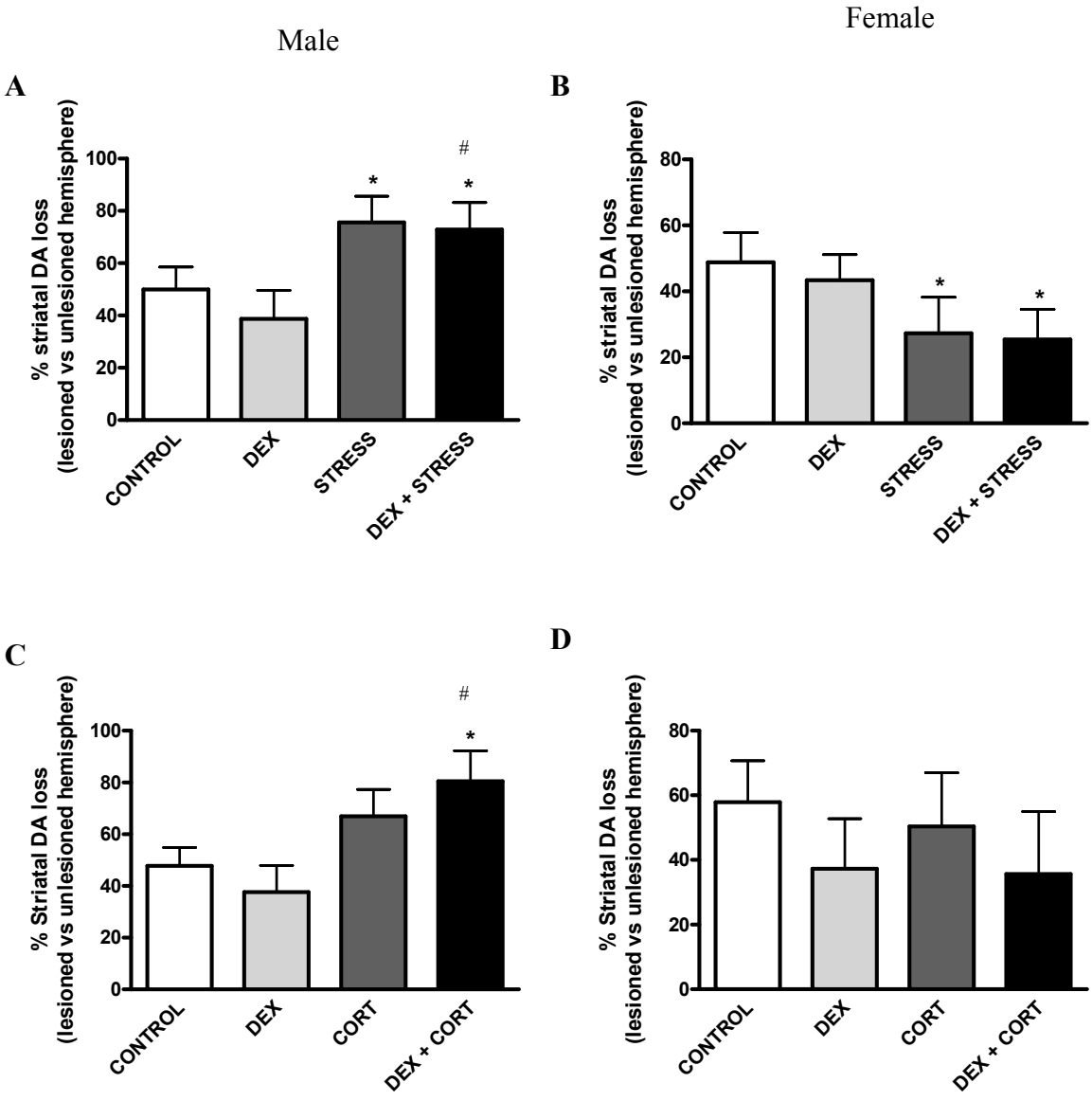


Figure 3.12. Effect of prenatal DEX treatment and/or adult stress or GC treatment on percentage striatal DA loss following 6-OHDA lesioning. Striatal DA loss in the ipsilateral striatum versus contralateral striatum in male (A,C) and female (B,D) animals exposed to a 6-OHDA. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effects of prenatal (GD16-19) DEX treatment and / or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and/or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. . A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

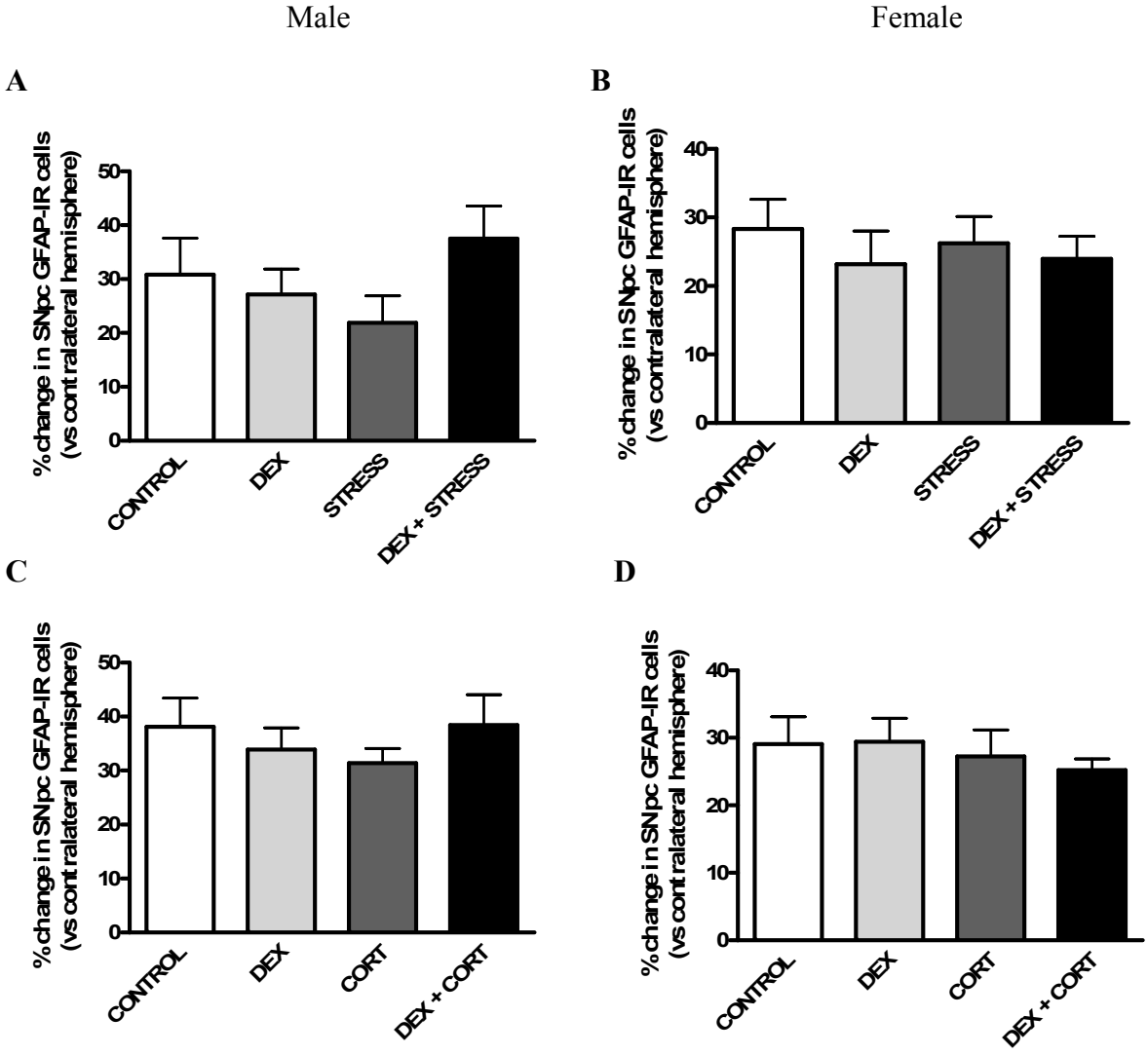


Figure 3.13. Effect of prenatal DEX treatment and/or adult stress or GC treatment on GFAP-IR cells within the SNpc following 6-OHDA lesioning. Stereological estimates of the number of GFAP-IR cells within the SNpc of male (A,C) and female (B,D) animals exposed to a 6-OHDA were determined by immunohistocemistry. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effects of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 6 (DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

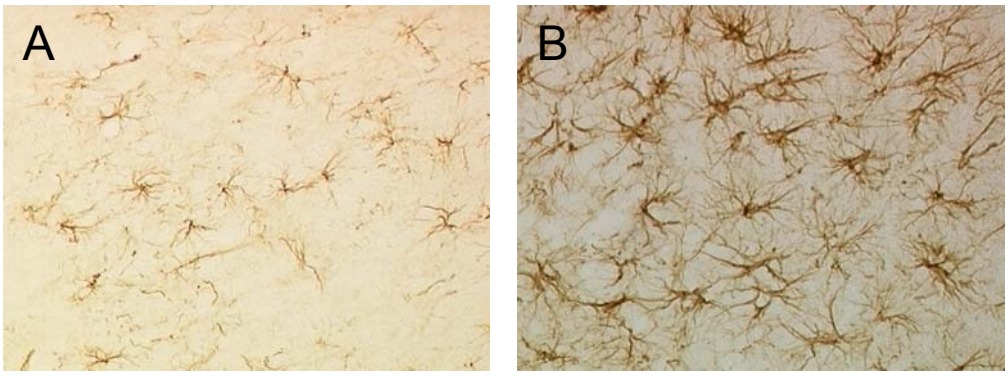


Figure 3.14. Representative photomicrographs of GFAP-IR staining within the unlesioned (A) and lesioned (B) SNpc. Images were taken at x100 magnification.

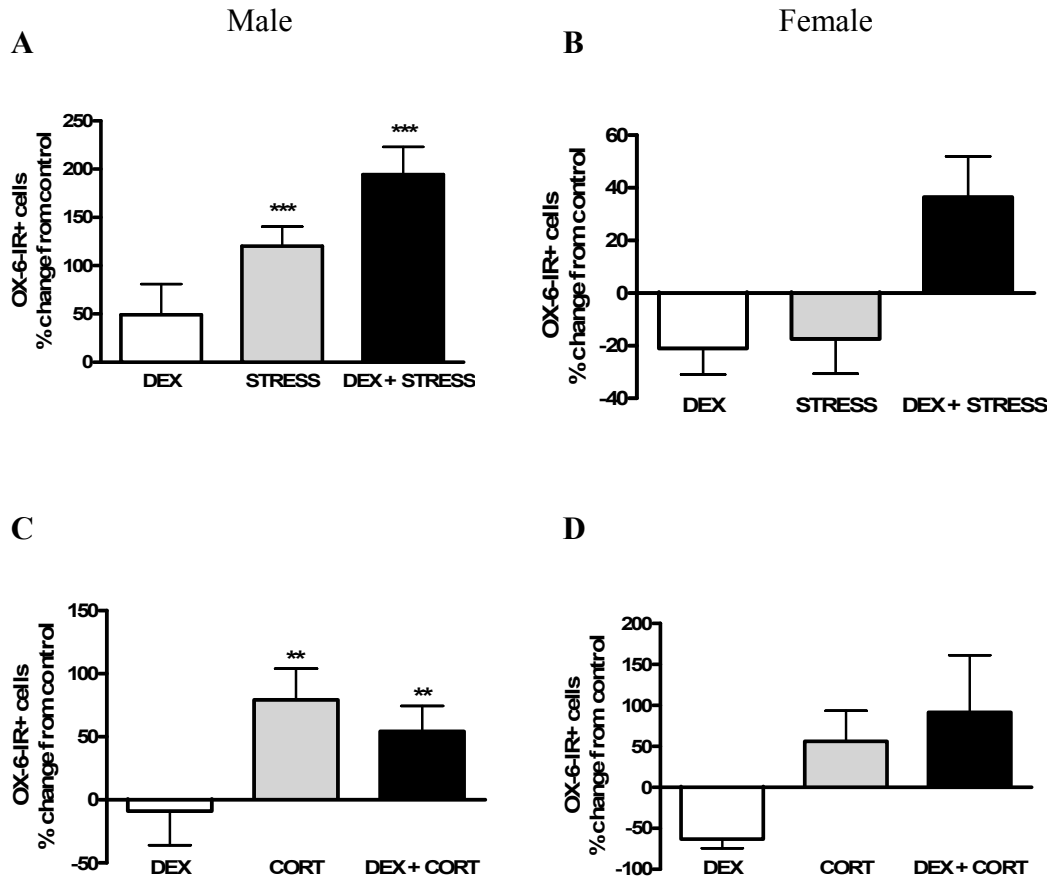


Figure 3.15. Effect of pr enatal DEX treatment and/ or adult stress or GC treatment on OX-6-IR cells after 6-OHDA lesioning. Stereological estimates of the total number of ox-6 positive microglia within the left SNpc of male (A,C) and female (B,D) animals were obtained and expressed as a percentage of controls. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the left MFB. A and B indicate the effects of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001, # vs DEX, + vs adult treatment. A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 6 (DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

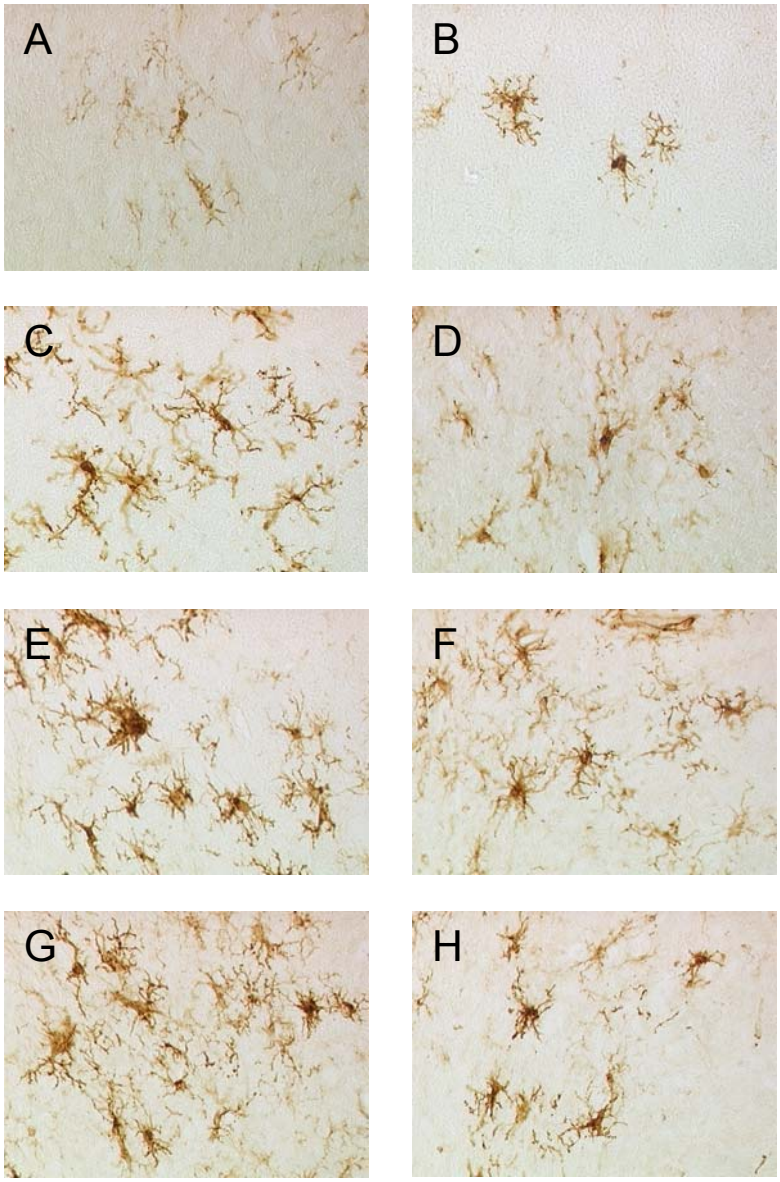


Figure 3.16. Representative photomicrographs of OX-6 staining within the 6-OHDA lesioned SNpc of male (A,C,E,G) and female (B,D,F,H) rats. Controls (A,B), stressed (C), DEX + stress (D,E), CORT (F,G), DEX + CORT (H). Images were taken at x200 magnification.

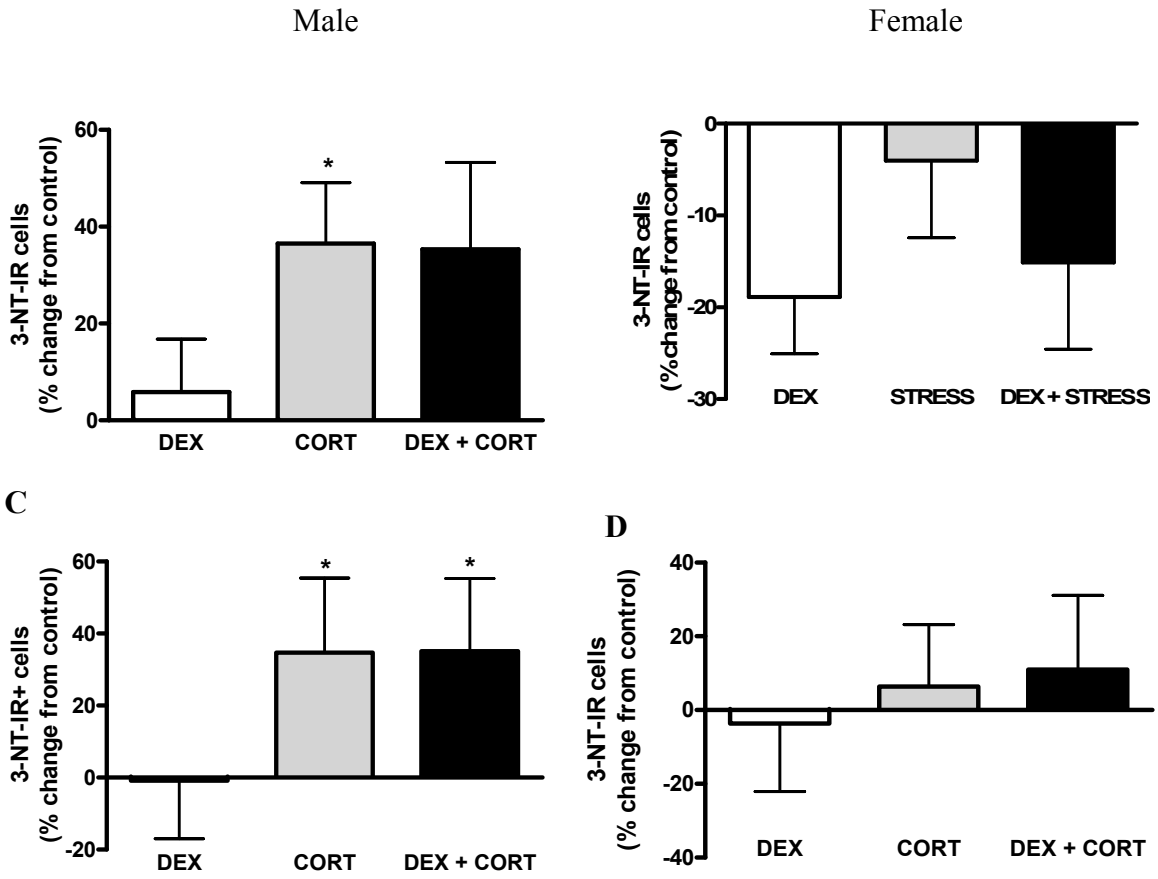


Figure 3.17. Effect of pr enatal DEX treatment and/ or adult stress or GC treatment on 3-NT-IR cells after 6-OHDA lesioning. Stereological estimates of the total number of 3-NT positive positive cells within the left SNpc of male (A,C) and female (B,D) animals were obtained and expressed as a percentage of controls. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the left MFB. A and B indicate the effects of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001, # vs DEX, + vs adult treatment. A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 6 (DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

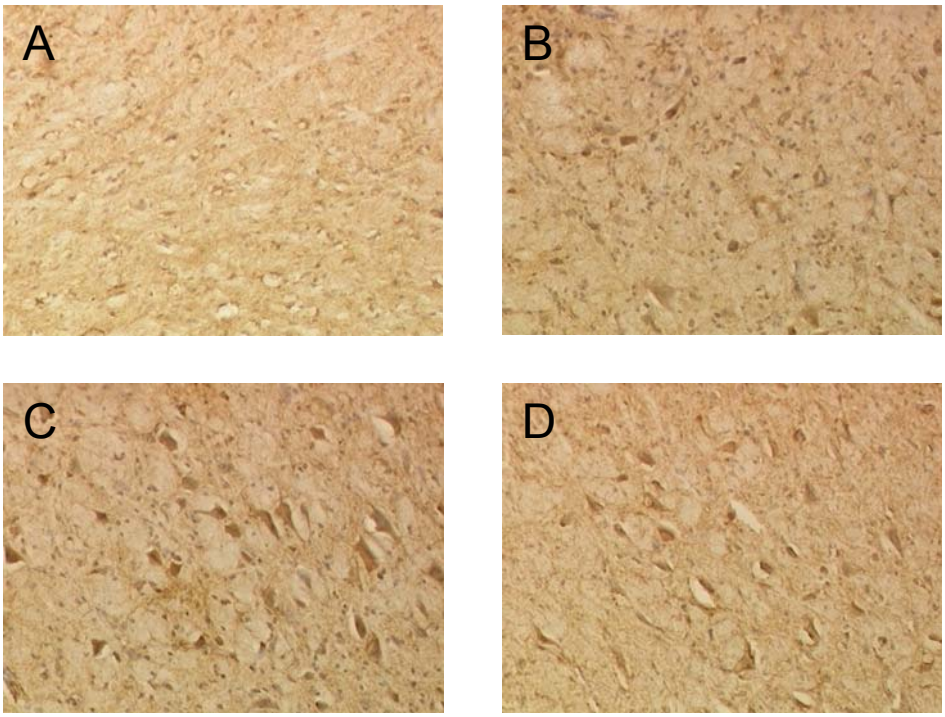


Figure 3.18. Representative photom icrographs of 3-NT staining within the SNpc of male rats. Unlesioned SNpc (A) and lesioned SNpc (B) of controls and lesioned SNpc of stressed (C) and GC treated (D) males. Images were taken at x 100 magnification.

3.4 DISCUSSION

The data presented within this chapter demonstrate that chronic GC treatment and exposure to repeated stress in adulthood have complex but different effects on NSDA integrity that are sexually dimorphic. Specifically, repeated stress in adulthood causes up-regulation of NSDA measures (TH-IR and striatal DA) in females but not males. Conversely chronic GC treatment in adulthood produces up-regulation of the NSDA in males but has no effect on the female NSDA. Furthermore, prenatal GC treatment permanently alters both the male and female NSDA by increasing TH-IR within the SNpc but renders the male NSDA only susceptible to a negative effect of adult challenge (repeated stress or GC treatment), resulting in a significant down-regulation of both TH-IR and striatal DA. Finally, adult challenge but not prenatal GC treatment, sex-dependently altered the NSDA response to a neurotoxic insult, where both adult stress and GC treatment are detrimental to a 6-OHDA induced lesion in males but protective in females. Together these findings increase our understanding of the sexually dimorphic nature of NSDA responses and provide compelling evidence for a potential role of stress and/or GCs in the pathogenesis of PD.

3.4.1 The Intact NSDA

Effect of repeated adult stress alone: Although the male NSDA appeared totally unaffected by adult stress, both TH-IR and striatal DA content were increased after repeated restraint in females compared with controls. These data indicate marked sex differences in the capacity of the pathway to adapt to repeated stress. Other groups have reported elevated striatal DA levels in male mice and increased DA uptake by the DA transporter (DAT) within the striatum in response to restraint stress (Kim et al., 2005)

(Copeland et al., 2005). Although there are discrepancies between these studies they could be attributed to differences in strain and the timing or duration of stress. However, together with our data they indicate that repeated stress is capable of manipulating the NSDA pathway.

Repeated intermittent restraint provides a model of mild psychogenic stress. Animals exposed to repeated homotypic stressors show habituation to that stress which was readily observed in the behaviour of our animals. This induces plastic changes in the HPA axis that are partly due to GC negative feedback inhibition of the HPA axis (Jaferi et al., 2003). Evidence for a detrimental effect of stress and GCs on the brain comes mainly from work on the hippocampus where chronic stress causes degeneration of pyramidal neurons and subsequent deficits in memory and cognitive function in rats (Sapolsky et al., 1986; Lupien et al., 2007a). Hippocampal damage results in a reduction in GC negative feedback with the overall effect of further increasing GC levels. This is known as the GC cascade hypothesis which is implicated in brain aging (Sapolsky et al., 1986) and therefore of relevance to diseases associated with aging such as PD.

The data also show that there is an increased number of OX-42-IR microglia within the male SNpc in response to repeated stress but these microglia did not show any changes in morphology, indicating proliferation but not activation of microglia. Small increases in protein nitration within the male SNpc were also reported in response to repeated stress. It has previously been hypothesised that elevations in DA levels may lead to increased oxidative stress as the autoxoidation and normal metabolism of DA result in the production of reactive oxygen and nitrogen species as well as toxic DA quinones (Sulzer and Zecca 2000).

Effect of chronic GC treatment alone: Unlike our repeated stress paradigm animals treated with chronic hydrocortisone would not undergo habituation to treatment although they would adapt to treatment. Treatment would raise circulating GC levels but presumably suppress endogenous corticosterone release via HPA negative and result in a downregulation of GR. Treatment would also elicit a circadian profile of GC levels due to the increase in activity and therefore drinking behaviour in the evening. The dose of hydrocortisone that we used was based on a study (Magarinos et al., 1998) that showed chronic GC administration via the drinking water produced morphological changes within the hippocampus similar to that produced by repeated restraint stress. The dose reduced adrenal and thymic weights (indicative of a suppressed HPA axis) and increased circulating levels of GCs (Magarinos et al., 1998). They also found that animals failed to gain weight, a finding that was replicated in the present study (section 2.6.2). In contrast to the pattern of responsiveness for repeated adult stress, chronic GC treatment did not affect measures of the NSDA system, whereas in males this treatment significantly increased both TH-IR and striatal DA, without a significant effect on turnover. The number of NeuN-IR cells was not altered in the same animals suggesting that chronic GC treatment results in increased TH expression/phenotype rather than an increase in the number of neurons within the SNpc. However, it is shown here and by others (Finklestein 2000) that only ~10% of neurons within the SNpc are not TH-IR neurons. Therefore changes in the expression of TH may not fully account for this. Although the SNpc is thought to have neurogenic potential throughout adulthood (Lie et al., 2002; Zhao et al., 2003; Shan et al., 2006), this remains controversial (Frielingsdorf et al., 2004). On the other hand a putative GC response element has been found in the regulatory region of the TH gene (Hagerty et al., 2001) and both TH protein and

mRNA expression are increased by GCs (Kumer and Vrana, 1996). Elevated striatal DA levels observed in males could be due to an increased synthesis of DA as a result of increased TH expression / activity or could be due to effects on DA metabolism and clearance. The main metabolising enzymes of DA, monoamine oxidase and catechol-o-methyltransferase are unaffected by GC treatment (Lindley et al., 2005). However, stress increases DAT expression and binding in the male striatum (Copeland et al., 2005; Lucas et al., 2007) and recently it has been shown that the organic cation transporter 3 (OCT-3, uptake 2) is present within the NSDA (Gasser et al., 2009) and that this transporter can be inhibited by GCs (Gasser et al., 2006) thus it may contribute to the effects of stress and GCs on the NSDA by reducing the clearance of DA. As with repeated stress chronic GC treatment resulted in elevated levels of protein nitration within the SNpc which could be a result of elevated DA levels leading to elevated oxidative stress as a result of enhanced DA metabolism.

Together, these results show that the effects of repeated stress and GC treatment in adulthood on the NSDA pathway are clearly dissociated in both sexes. Responses are also sexually dimorphic, with males exhibiting an ability to respond/adapt to GCs (increase in NSDA parameters) but failing to respond to repeated restraint, and vice-versa in females. Our observation that GC treated rats failed to gain weight over the treatment period whereas stressed rats gained weight normally further highlights the differences between the two treatments.Thus, the effects of repeated stress on the NSDA in adulthood are unlikely mediated by GCs.

Effect of prenatal DEX on the 'resting' adult NSDA*:* The observed effects of DEX on the NSDA were likely due to the action of DEX on the fetus, as at the time of

treatment (GD16-19) it is known that DEX is able to cross the placenta and blood brain barrier (Owen et al., 2005). Moreover GR are present within the developing brain from E15.5 (Diaz et al., 1998) which supports a direct effect of DEX on the developing NSDA. Furthermore, the effects of DEX were not confounded by any observable changes in maternal behaviour. Treatment via the maternal drinking water has the distinct advantage that it avoids the stress which would be associated with parenteral treatments and associated handling, and available pharmacokinetic data allow us to estimate that the plasma concentrations achieved with this regime of DEX treatment which exposes the fetus to GC levels approximating those seen in the physiological stress range (McArthur et al., 2006). There is a vast literature on the programming effects of stress and GCs on the developing fetus and the purpose of this plasticity early in life is thought to be to program the fetus for survival in a presumptive detrimental environment. Prenatal stress or GC exposure produces a hyperactive HPA axis in adulthood (Kapoor et al., 2006) and epidemiologic evidence suggests that this can increase susceptibility to disease later in life (Barker, 2002). More recently, adverse environments early in life have been linked to disorders of the CNS such as addictive behaviour (Meaney et al., 2002) and affective disorders (Seckl, 2008). There is now an emerging interest in the role of the early environment in the development of neurodegenerative diseases such as PD (Carvey et al., 2003; Barlow et al., 2007; Pienaar et al., 2008a).

The data show that prenatal DEX significantly increased TH-IR cell numbers in males and females, without affecting striatal DA levels. This corroborates our earlier findings (McArthur et al., 2005; McArthur et al., 2007a) and suggests that adaptive mechanisms in the NSDA system permit maintenance of striatal DA levels in the face of changing

cell numbers. Although the underlying mechanisms are unclear, they appear not to involve major changes in neuronal activity because DA turnover is not significantly altered by prenatal DEX. Prenatal DEX also increased the number of SNpc cells IR for a general neuronal marker (NeuN) suggesting that DEX produces an increase in the number of cells rather than an increase in TH enzyme expression/phenotype. The processes of neurogenesis and migration of TH-expressing cells into the midbrain nuclei are thought to be complete around E16 (Bayer et al., 1995; Lieb et al., 1996) and are therefore unlikely to be the major targets of GC influences between E16-19. However, this is a period of programmed cell death in the brain via apoptotic mechanisms and it has been proposed that GCs play a significant role in this process. Although often thought of as inducers of apopotosis, GCs can also promote cell survival in the developing brain (Gould and Cameron, 1996; Abraham et al., 2001) suggesting a process which could influence DA cell counts in the adult SNc.

There are limited studies exploring the functional consequences of prenatal GC programming on the NSDA, but reports that prenatal GC treatment can increase spontaneous motor activity in male and female rats and exerts sexually dimorphic effects on apomorphine induced motor behaviour (Diaz et al., 1997) as well as data reporting that that postnatal treatment with GCs results in a deficit in motor coordination in adulthood (Benesova and Pavlik, 1989), support our findings that this has significant impact upon the adult NSDA system. By comparison prenatal exposure to lipopolysaccharide (LPS) during pregnancy results in offspring with a reduced number of DA neurons within the SNpc that exhibit greater levels of neuroinflammation (Ling et al., 2002; Carvey et al., 2003). Low dose GCs have potent anti-inflammatory actions (Sorrells and Sapolsky, 2007) therefore it could be hypothesised that decreased

neuroinflammation in our prenatally treated animals could account for the increased number of DA neurons. However, OX-42 immunohistochemistry demonstrated that prenatal DEX treatment resulted in a larger microglial population in adulthood. Although GCs are known to suppress microglia it has also been shown that stress via elevated corticosterone can induce microglia proliferation (Nair and Bonneau, 2006). During development amoeboid microglia are likely responsible for the phagocytosis of dying neurons and dendritic debris, however microglia are not always associated with dying cells and can display a ramified morphology (typical of resting microglia). These microglia are present within the developing brain from E14 (Ashwell, 1991; Jonakait, 2007) and have been shown to produce neurotrophic factors. This extra neurotrophic support provided by proliferating microglia may result in an increased number of DA neurons within the SNpc that is seen in DEX treated animals. Astrocytes are not present in the rodent brain until the first postnatal week when they proliferate and play a significant role in synaptogenesis (Ullian et al., 2001; Ullian et al., 2004). Our regime would therefore not directly impact upon the astrocyte population. However the data presented here suggest that DEX treatment results in a reduced astrocyte population which presumably is an indirect effect of prenatal DEX treatment. Further study is required to determine the mechanism behind this.

Effect of prenatal dexamethasone on the challenged adult NSDA pathway

Adult stress challenge*:* In males prenatal DEX had a profound effect on the adult response to repeated stress. Thus, although prenatal DEX increased TH-IR numbers in the unchallenged male SNpc compared with controls (as discussed above), it inhibited

the NSDA response to repeated stress such that TH-IR numbers and striatal DA were significantly suppressed in the prenatal DEX + adult restraint group compared with controls after exposure to repeated adult restraint. This clearly contrasts with the failure of the normal adult male NSDA system to respond to stress and indicates the potential of the male NSDA to suffer long term consequences of prenatal stress exposure. In striking contrast, the ability of the normal female NSDA pathway to up-regulate both TH-IR and striatal DA in response to adult stress was completely unaffected by prenatal DEX exposure. This may indicate an ability of normal females to adapt positively, whereas males cannot, possibly making them more vulnerable. It also suggests a robustness of the female NSDA to early intervention, but a susceptibility in males, where the normal failure to adapt may even be worsened, as indicated by suppressed TH-IR cell numbers and striatal DA.

Adult GC challenge: As was the case for stress in adulthood, prenatal DEX profoundly altered the response of the male, but not female, NSDA to chronic adult CG treatment. Thus, in males chronic stress ablated the up-regulation of the NSDA induced by prenatal DEX treatment (increased TH-IR in SNpc) or adult GC treatment (increased TH-IR and striatal DA) and both TH-IR and striatal DA were reduced to levels below those of the untreated control. This was accompanied by an increase in DA turnover, which may indicate an increase in activity in an attempt to compensate for the reduction in TH-IR and striatal DA.

It appears, therefore, that prenatal DEX treatment may compromise the ability of the adult male NSDA system to respond appropriately to either repeated stress or chronic GC treatment in adulthood. By contrast, the resistance (upregulation of NSDA

measures) of the female NSDA to adult stress or GCs, respectively, were unaffected by prenatal DEX. The fact that there were similar effects (suppression of NSDA measures) of prenatal DEX on adult stress and GC treatment suggests that this novel programming effect in males is HPA mediated. We propose that this down-regulation is the result of the failure of prenatally programmed males but not females to adapt to an adult challenge (Bhatnagar et al., 2005). The premise of early life experience influencing adult NSDA structure and function is supported by a study demonstrating that prenatal LPS exposure followed by adult LPS resulted in progressive DA neuron loss within the SNpc where pre-existing neuroinflammation prolonged the inflammatory response to the second exposure (Ling et al., 2006). There are also data supporting the idea that prenatal GC treatment can suppress the response to adult challenge in adult rats that had been treated prenatally with DEX failed to display a reduction in pituitary ACTH release in response to GCs (Theogaraj 2005, 2006).

3.4.2 The injured NSDA system

It is important to consider that the process of producing a 6-OHDA lesion is in itself a stressful procedure. Surgical stress results from a combination of anaesthesia, tissue injury and post-operative pain and is characterised by activation of the HPA axis (Desborough 2000). It has also been shown that volatile anaesthetics such as isofluorane can stimulate the HPA axis (Karuri 1998). As we have shown that chronic GCs can increase TH-IR in the SNpc we hypothesise that surgical stress may produce an increase in TH-IR via elevated GC levels.

Adult repeated stress had marked and opposite effects on susceptibility to 6-OHDA-induced lesions in males and females, which were harmful and protective, respectively, at both DA cell bodies in the SNpc and terminals in the striatum. This is a striking sex dimorphism which indicates that stress has a major influence on susceptibility to neurodegenerative stimuli. This may correlate with the failure of the normal (unlesioned) male NSDA to respond/adapt to chronic stress, whereas a positive response was seen in females. A recent study (Smith et al., 2008) examined the effects of chronic stress on 6-OHDA neurotoxicity in female rats. The authors did not observe any changes in TH-IR loss within the SNpc but did not assess effects in males. Sex differences in response to 6-OHDA are discussed further below.

Chronic adult GC treatment also had sexually dimorphic effects at SNpc level on susceptibility to 6-OHDA-induced lesions in males (harmful) and females (protective), providing further evidence for sex differences in the response of this pathway to stressful stimuli. These results therefore identify certain similarities in the effects of adult exposure to stress and GCs with respect to survival of the SNpc DA cell populations in the injured NSDA, albeit in opposite directions in males and females. These data indicate that GCs could be important mediators of this effect of stress. However, unlike stress, GCs were without effect on DA depletion at striatal level in both sexes, demonstrating that changes in DA cell numbers can occur independently of striatal DA content. It is not possible to determine the underlying mechanisms from the present study, but we have recently shown that although manipulation of circulating sex steroid hormone levels can significantly modify striatal DA depletion in response to neurotoxins, there is no concomitant effect on SNpc DA cell survival, further illustrating dissociation of effects of steroid hormones at perikarya and terminal level

(McArthur et al., 2007a). In order to explain this, we have proposed that sex hormones influence the activity of the surviving DA neurons, which would compensate for cell loss in the partially injured NSDA pathway, rather than affecting cell survival *per se*. Conversely, it would appear that GCs predominantly influence cell survival without affecting the compensatory mechanisms.

Prenatal treatments alone and in combination with adult treatments.

In male and female rats which received no adult treatments, prenatal DEX was without effect on 6-OHDA-induced toxicity at SNpc or striatal level. However other studies have found that perinatal manipulations such as maternal separation or prenatal LPS exaggerated the toxic effects of 6-OHDA (Ling et al., 2004; Pienaar et al., 2008b) highlighting the importance of the nature and timing of the perinatal manipulation on the impact they have on the NSDA. Presentation of the adult animals with stress or GC treatment had striking, sexually dimorphic and treatment-specific effects.

Prenatal dexamethasone plus challenge with adult stress: The damaging effects of adult stress in males on 6-OHDA-induced SNpc neuron loss and striatal DA depletion were unaffected by prenatal DEX exposure. The protective effect of repeated adult stress in females, as detected by the attenuation of striatal DA depletion by prenatal DEX treatment, was also not affected, but the protective effect at SNpc level was lost. It would therefore appear that early exposure to GCs may render the female, but not male, SNpc more vulnerable to the effects of stress on neurotoxic injury, without affecting the degree of striatal DA depletion.

Prenatal dexamethasone plus challenge with adult GC treatment*:* In males the ability of adult GC treatment to exacerbate 6-OHDA-induced SNpc DA cell loss

was not influenced by prenatal dexamethasone treatment, but it did render adult GCs capable of enhancing striatal DA depletion, which they were unable to do in the absence of early GC exposure. In contrast, in females the effects of adult GCs on 6-OHDA toxicity at either SNpc (reduced cell loss) or striatum (no effect) were unaffected by prenatal dexamethasone. It would therefore appear that early exposure to GCs renders the male, but not female, striatum more vulnerable to the effects of glucocorticoids, without affecting the SNpc. The turnover of DA within the striatum was increased in both stressed and GC treated males that were prenatally treated with DEX indicating an increase in DA activity. Increased DA metabolism would result in an increase in the production of reactive oxygen species, elevating levels of oxidative stress and potentially further endangering neurons.

3.4.3 Mechanisms of toxicity

As aforementioned stress can increase DAT expression and binding in the male striatum. By increasing the uptake of 6-OHDA into neurons this may underlie increased neurodegeneration. The exacerbation of the 6-OHDA lesion by repeated stress and chronic GC treatment in male animals was associated with elevated levels of microglial activation and increased protein nitration within the lesioned SNpc. Both inflammation and oxidative stress are heavily implicated in the pathogenesis of PD (Olanow, 2007) and in the 6-OHDA model (Simola et al., 2007). Evidence to suggest that these processes might underlie the increased toxicity we see in response to repeated stress and GCs is provided by findings that chronic stress elevates oxidative stress and antioxidant status within the rodent brain (Fontella et al., 2005; Kim et al., 2005; Zafir and Banu, 2008). Furthermore, although GCs are traditionally thought of as anti-inflammatory, stress and GCs can promote a pro inflammatory environment within the brain (Garcia-Bueno et al., 2008), an area of research that is currently gaining significant attention.

Our finding that both these processes are potentiated by our repeated stress and chronic GC treatment paradigms suggest that they may contribute to the associated increased toxicity of 6-OHDA. Microglia can produce a number of neurotoxic molecules including proinflammatory cytokines and prostaglandins as well as reactive oxygen and nitrogen species. Conversely elevated oxidative stress can stimulate microglial activation. However, the effects on inflammatory and oxidative markers may merely be a result of increased toxicity rather than being a causative factor. Another process thought to play a role in PD that may underlie the effects of stress and GCs on the NSDA is glutamate excitotoxicity. This was not investigated in the present study but stress and GCs have previously been found to elevate extracellular glutamate concentration within the male striatum (Moghaddam, 1993; Moghaddam et al., 1994). Excessive glutamate signaling may lead to excitotoxicity and subsequent neurodegeneration.

3.4.4 Role of the environment on the integrity of the NSDA

Repeated restraint is an example of a psychogenic stress where there is no actual threat to the animal's well-being. The effect of systemic stressors on the NSDA have also been evaluated. Systemic administration of bacterial endotoxin (LPS) or TNF-α administration results in neuroinflammation and a progressive loss of SNpc dopaminergic neurons (Qin et al., 2007) caused by elevations in brain levels of TNF-α and the subsequent induction of proinflammatory mediators within the brain. As there are relatively high numbers of microglia in the SNpc compared to other brain regions (Kim et al., 2000) an increased susceptibility to inflammation has been hypothesised to underlie the SNpc's sensitivity to degeneration. Further studies revealed that a non toxic dose of LPS administered directly to the SNpc exacerbated the effects of 6-OHDA and

increased degeneration in response to paraquat exposure (Koprich et al., 2008; Mangano and Hayley, 2008). Furthermore, systemic IL-1 administration also exaggerates 6-OHDA neurotoxicity (Godoy et al., 2008). Moreover, LPS has also been found to decrease glutathione and increase levels of lipid peroxidation within the brain (Zhu et al., 2007) that are indicative of elevated oxidative stress. The fact that the effects of LPS and other inflammogens are similar to those of repeated stress and GC treatment reported here and may indicate a similar mode of action. This hypothesis is further supported by our evidence of elevated microglial activation and oxidative stress in the SNpc in response to stress and GCs. In addition viruses have also been linked to an enhanced susceptibility to PD. In particular the influenza virus has been indentified as a predisposing factor. Although it probably does not directly cause PD, some strains of the influenza virus are able to enter the brain where they can produce cytokine induction and cell death (Jang et al., 2008) and may therefore contribute to a number of hits, precipitating PD.

Further evidence for an effect of the environment on the NSDA is provided by experiments that study the impact of environmental enrichment or exercise. Environmental enrichment confers resistance to MPTP (Bezard et al., 2003; Faherty et al., 2005) by decreasing DAT expression and increasing neurotrophic factors within the striatum. In 6-OHDA rats exercise suppresses NSDA neuron loss (Mabandla et al., 2004; Yoon et al., 2007) and provides some beneficial effects to PD patients (Goodwin et al., 2008). It is currently thought that exercise and environmental enrichment confer neuroprotection by decreasing levels of oxidative stress (Radak et al., 2008). Interestingly and of significant relevance to the present study, stress reversed the

neuroprotective effect of exercise in the 6-OHDA rat (Howells et al., 2005) providing further evidence for our hypothesis that stress and GCs can endanger the NSDA.

3.4.5 Sex differences in the susceptibility to 6-OHDA

Unlike other studies we do not report a sex difference in the size of 6-OHDA lesion in control animals. However, there are data to suggest that sex differences are lost when the lesion size exceeds a certain limit (Gillies et al., 2004). Additionally our animals were significantly older than those used in most other studies and thus may also impact upon both lesion size and sex differences (Tamas et al., 2005). Furthermore the stage of estrous in our female animals may impact on lesion size. There are intrinsic sex differences within the NSDA. For example there is greater expression of and uptake by the DA transporter (DAT)(Walker et al., 2000; Bhatt and Dluzen, 2005) and recently it has been demonstrated that although females have a more highly active DAT (and VMAT2) they are less sensitive to the toxic effects of methamphetamine (Dluzen and McDermott, 2008). They suggest that although females express a more active DAT, this can be downregulated by estrogen (Disshon et al., 1998), allowing for fine control of the system and more efficient sequestering of DA or neurotoxin. There are also differences in the response to stress where females generally exhibit a greater HPA response to stressors than males but are more able to cope with subsequent stressors (Dalla et al., 2005). Recent studies support our findings that stress produces opposing effects in males and females. Chronic stress was shown to produce sexually dimorphic effects on DA transmission (Dalla et al., 2008) and skilled movement in response to restraint stress was better in females than in males study (Jadavji and Metz, 2008). Furthermore females used a different strategy to overcome motor disturbances caused by stress and are able to recover faster.

It is thought that sex differences arise due to the organisational effects (during development) or the activational effects (during adulthood) of sex steroid hormones. Interestingly our previous work has shown that systemic estrogen and gonadal factors have opposing effects on striatal susceptibility to 6-OHDA where they are protective in females but are detrimental in males (Gillies et al., 2004). Thus, stress may be acting indirectly through an influence on sex steroids. Stress is known to suppress both the male and female reproductive systems and both CRH and GCs can prevent the release of GnRH. GCs also decrease the production of sex hormones via a direct action on the testes and ovaries (Swaab et al., 2003). Conversely sex steroids also have an impact on HPA function.

Resistance to stress induced elevated neurotoxicity could partly account for the sex differences observed in the susceptibility to 6-OHDA (Gillies et al., 2004) and to PD (Schrag et al., 2000). It has been proposed that estrogen underlies the sex difference in the higher prevalence of PD in males (Shulman, 2007) and the lower toxicity of 6-OHDA, MPTP and LPS in female animals (Dluzen, 2000; Murray et al., 2003; Liu et al., 2008). Estrogen decreases the expression of DAT which is elevated by stress (Copeland et al., 2005) and it has been reported that estrogen has antioxidant properties (Sawada et al., 1998; Nilsen, 2008), indeed administration of supraphysiologic levels of estradiol prior to MPTP treatment in male mice reduced levels of nitrotyrosine immunoreactivity and increased levels of antioxidant enzymes (Tripanichkul et al., 2007) within the SNpc. Estrogen can also exert anti-inflammatory actions for example; pre-treatment with estradiol (supraphysiological) was found to down regulate microglial activation and attenuate DA neuron loss in MPTP male mice (Tripanichkul et al., 2006).

Furthermore, estrogen can attenuate glutamate excitotoxicity (Behl, 2002a). Further study is required to determine exactly what is conferring neuroprotection in our female animals.

3.4.6 Clinical Implications

In this study plasma concentration of CORT in DEX treated pups was estimated to be ~ 30ng/ml. This is a lower dose than that used in perinatal medicine (Theogaraj et al., 2005) therefore our findings are of considerable interest to the use of GCs in cases of preterm labour in which GCs are used to promote lung maturation. Human prenatal GC treatment has been shown to cause impaired childhood cognition and long term behavioural implications (Sloboda et al., 2005) but at present there is a lack of information on the long term implications of perinatal GC treatment in humans highlighting the need for long term follow up studies. In addition these results have implications for the use of inhalational low dose corticosteroids in pregnancy and in children where the HPA axis has been shown to be suppressed (Zollner, 2007).

PD is increasingly thought to be a multifactorial disease (Sulzer, 2007) whereby a number of genetic and/or environmental insults act in concert to cause disease onset and progression. Our data support a role for stress and GCs in accelerating the onset or progression of PD and provide further evidence for the multiple hit hypothesis of PD (Carvey et al., 2006). The sexually dimorphic responses of the NSDA to stress may to some degree contribute to the higher prevalence of PD in males. As GCs are often used to treat autoimmune and inflammatory conditions, it may be of benefit to limit their use in PD patients. In patients with elevated GC levels, measures to control GC levels may

confer some beneficial effect however there is a need for epidemiological studies to assess this.

3.4.7 Conclusions

In summary the data within this chapter show that adult chronic GC treatment or repeated stress produce opposing sex-dependent effects on the NSDA. Furthermore although prenatal DEX treatment increases the DA neuron population of both the adult male and female SNpc we present a novel programming effect of prenatal GCs whereby males, but not females, are rendered susceptible to a down-regulation of both TH-IR cells and striatal DA in response to an adult challenge (repeated stress or chronic GC treatment). Our data also reveal striking sexually dimorphic effects of adult challenge on the susceptibility of the NSDA to 6-OHDA where exposure to an adult challenge exacerbates the neurotoxic lesion in males but confers neuroprotection in females. The data also indicate that oxidative stress may be involved in the detrimental effects of the stress/GCs on the NSDA. The study highlights important sexual dimorphisms in the effects of stress and GCs on the NSDA and provides novel insights into the possible involvement of stress and GCs in PD.

CHAPTER 4

Sex-specific effects of stress and glucocorticoid treatments on the integrity of the mesolimbic and mesocortical dopaminergic pathways.

4.1 INTRODUCTION

PD is primarily considered a disease of motor function but there is a high prevalence of disabling non-motor symptoms including psychiatric complications such as depression (Allain et al., 2000; Wermuth and Bech, 2006), anxiety (Richard, 2005), apathy (Dujardin et al., 2007), anhedonia (Isella et al., 2003) and compulsive behaviour (Torta and Castelli, 2008). Although cell loss in PD is most severe in the SNpc there is also significant degeneration of DA neurons, albeit to a lesser extent, within the VTA (German et al., 1989). These neurons form two major DA projections, the mesolimbic (MLDA) and mesocortical (MCDA) pathways. Degeneration of these pathways in PD patients is now thought to contribute to the neuropsychiatric symptoms of PD. Growing attention for the non motor symptoms of PD mirrors the desire for improved quality of life for PD patients. The MLDA system consists of dopaminergic neurons within the VTA that primarily project through the medial forebrain bundle to the NAcc which is found in the ventral striatum, as well as innervating the amygdala and hippocampus. NAcc DA input from the VTA modulates the activity of GABAergic medium spiny neurons within the NAcc that form part of the cortico-striato-thalamo-cortico-loop. The MLDA forms the brains major reward pathway (Schultz, 2000) and as such has been implicated in depression and addiction. On the other hand the MCDA is composed of DA neurons that primarily innervate the PFC. This pathway is involved in motivation and emotional responses and has been associated with the negative symptoms of schizophrenia.

There is considerable evidence supporting a role of midbrain DA in depression (Martin-Soelch, 2009) and although there are numerous difficulties in diagnosing major depression in PD patients, meta analysis of published data suggest that 31% of PD

patients suffer from depression compared to 16% of the general population (Kessler et al., 2003). Additionally there is greater degeneration of VTA neurons in depressed PD patients than non depressed PD patients (Mann and Snowden, 1995) supporting a role of the MLDA and MCDA pathways in depression in PD. Stress and GC's both in early life and adulthood have been linked to depression (Heim et al., 2008; Yu et al., 2008). Similarly, stressful events both early in life and as an adult have been implicated in the pathogenesis of schizophrenia (Cotter and Pariante, 2002; Bennett, 2008). The dopamine hypothesis of schizophrenia suggests that excess DA activity in the MLDA system contributes to the positive symptoms of the disease that are mediated by D2 receptors, whilst reduced DA in the MCDA system underlie the negative symptoms of schizophrenia via D1 receptors . Futhermore D2 receptors are increased in post mortem brains of schizophrenics (Seeman and Kapur, 2000). Most evidence for an effect of stress and GCs on DA pathways comes from studies on drugs of abuse. Current data suggest that stress induced elevations in GCs, increase vulnerability to drug abuse via alterations in the MLDA that mimic the effects of psychostimulant drugs (Marinelli and Piazza, 2002). Moreover GC suppression by adrenalectomy reduces NAcc levels of DA (Piazza et al., 1996; Barrot et al., 2001) which can be reversed by corticosterone supplementation. These effects are mediated by GR (Marinelli et al., 1998). Together, these observations illustrate the prime importance of both stress and glucocorticoids on the mesocorticolimbic pathways in the most common psychiatric disorders. However, the actual consequence of stress or GC exposure on these pathways is poorly defined.

4.2 EXPERIMENTAL DESIGN

The aims of the work presented in chapter 4 were to firstly elucidate the effects of prenatal GCs and adult stress or chronic GC treatment on the MLDA and MCDA pathways and secondly whether these treatments compounded with the effects of 6-OHDA induced lesions, affect these pathways. To achieve this, 2 studies were performed. The first examined the effects in adulthood of prenatal DEX treatment that was administered via the maternal drinking water on GD16-19 and/or repeated intermittent restraint stress (immobilisation for 1 hour every other day) at 3.5 months for a period of 6 weeks. The second study examined the effects of prenatal DEX treatment as well as adult (3.5 month) chronic GC treatment (via the drinking water for 6 weeks). To determine how stress and GC treatments affected MLDA and MCDA responses to insults an injection of 6-OHDA was administered to the MFB of a subset of rats from each treatment group.

Integrity of the DA systems was assessed by the analysis of the number of neurons within the VTA which was achieved by immunohistochemistry for TH (DA neurons) and NeuN (all neurons). The number of astrocytes and microglia within the VTA were also evaluated by GFAP and OX-42/OX-6 immunohistochemistry respectively. 3-NT immunohistochemistry measured the amount of protein nitration within the VTA that would be indicative of levels of oxidative stress. HPLC was used to measure DA and its metabolites within the PFC and NAcc as indicators of the dynamics of DA transmision. For detailed methodology please refer to chapter 2.

4.3. RESULTS

4.3.1. The unchallenged MLDA and MCDA

Controls: On combination of data from study 1 and study 2 the number of TH-IR cells within the VTA was estimated to be 16000 ± 837 in adult male rats. Female rats had ~20% more TH-IR cells than males (19620 ± 688; $p<0.01$) which agrees with previous findings of a sex difference (McArthur et al., 2006). The number of NeuN-IR cells within the VTA was significantly higher in both males and females (34120 ± 1775 in males and 39710 ± 1583 in females). Thus, TH-IR neurons represented ~50% of the total number of VTA neurons. To establish the size of the astrocyte population in our control (unchallenged) adult (5.5month old) VTA, immunohistochemistry for GFAP was performed. The estimated number of GFAP-IR cells in the male VTA was 4226 ± 192 and 3885 ± 141 in the female. Similarly OX-42 immunohistochemsitry was used to assess the VTA microglial population. In controls there were 2187 ± 200 and 2127 ± 148 OX-42-IR cells in the male and female VTA respectively. We also established that the number of VTA cells positive for protein nitration in adult controls was estimated to be 4100 ± 270 in males and 3747 ± 261 in females. The two main dopaminergic efferent pathways of the VTA project to the NAcc and PFC. DA levels within the adult NAcc were 6.75 ± 0.59 ng/mg in adult males and 12.87 ± 0.51 ng/mg in females ($p<0.001$; Table 4.1) again this supports previous findings of sex differences within the MLDA (Becker, 2009). Additionally, DA turnover within the NAcc was higher in males than in females ($p<0.001$; 1.65 ± 0.26 in males and 0.62 ± 0.04 in females; table 1). The VTA also contains DA neurons that project to the PFC, here average adult male DA levels were 0.14 ± 0.04 ng/mg . Adult female PFC DA levels in females were increased but not significantly different in study 1 but in study two female DA levels were found to be significantly higher ($p<0.001$; Table 2). As in the NAcc DA turnover within the PFC

was higher in males ($p<0.001$). HVA levels within the female PFC were negligible in study 1.

***Adult treatments in male rats*:** In adult males repeated stress increased the number of TH-IR cells in the VTA by ~20% ($p<0.05$; Figure 4.1A). DA levels within the NAcc were elevated by ~50% ($p<0.01$) in male rats that had been subjected to repeated restraint stress (Table 4.1). Unlike repeated stress, adult GC treatment did not affect TH-IR in the VTA but did produce comparable changes in the NAcc where DA levels were increased by ~60% in ($p<0.05$; Table 4.1). By comparison PFC DA levels were not significantly altered by repeated stress or chronic GC treatment (Table 4.2). The number of OX-42-IR cells within the VTA were increased in both stressed (~60%) and GC treated (~40%) animals ($p<0.05$; figure 4.4). An effect on GFAP-IR cells was only seen in stressed males where the GFAP population was increased by ~25% ($p<0.01$; Figure 4.3). Neither repeated stress nor chronic GC treatment exerted an effect on the number of 3-NT-IR cells in the VTA (Figure 4.5).

***Prenatal treatment in male rats*:** Prenatal DEX treatment resulted in an elevation of TH-IR cell numbers within the VTA of adult male rats that was only significant in study 1 ($p<0.05$; Figure 4.1A). Interestingly, DA levels within the male NAcc were elevated in study 2 ($p<0.05$; Table 4.1) but not study 1 and within the PFC prenatal DEX treatment increased adult DA turnover in study 1 only ($p<0.01$; Table 4.2). The number of GFAP-IR cells in the adult VTA was reduced by ~20% in rats exposed to gestational DEX ($p<0.05$; Figure 4.3A,C). Male rats that had been treated prenatally with DEX exhibited a higher number of OX-42-IR cells ($p<0.001$ in study 1 and $p<0.01$ in study 2; Figure 4.4A,C) where the average number of OX-42-IR cells was increased

approximately two fold to 4875 ± 358 in males. Prenatal DEX treatment did not affect 3-NT-IR cell number within the VTA (Figure 4.5A,C).

Prenatal DEX treatment in combination with adult treatment in male rats: Interestingly a history of prenatal DEX treatment attenuated adult NAcc DA levels ($p < 0.05$; Table 4.1) in both stressed and GC treated males but did not alter their effect on TH-IR cells in the VTA (figure 4.1). The combination of prenatal DEX treatment and adult repeated stress or GC treatment did not elicit any interactive effect on GFAP, OX-42 or 3-NT-IR cell counts (Figure 4.3-4.5).

Adult treatments in* female *rats*:* As in males repeated stress in adulthood increased the number of TH-IR cells within the female VTA ($P<0.01$, Figure 4.1B) but in contrast to the effect of stress in males DA levels within the NAcc were reduced by ~45% ($p<0.001$; Table 4.1). Additionally in females, DOPAC and DA turnover were increased by repeated restraint ($p<0.05$). The number of OX-42-IR cells within the female VTA were also increased by repeated stress ($p<0.05$). Adult GC treatment didn't alter the number of TH-IR cells within the VTA but did reduce NAcc DA levels by ~20% ($p<0.05$; Table 4.2) and elevate the number of OX-42-IR cells within the VTA. DA levels within the female PFC were not altered by either adult treatment (Table 4.3). There was no effect of adult treatment on GFAP or 3-NT-IR cell numbers.

Prenatal treatment in female rats: In contrast to its effects in the male prenatal DEX did not exert an effect on TH-IR cells within the adult female VTA but reduced female NAcc DA levels ($p<0.05$ in study 1, $p<0.001$ in study 2; Table 4.1) and

increased DA turnover in study 2 ($p<0.05$). By contrast, DA levels within the adult female PFC were unaffected by DEX treatment (Table 4.2). As for males prenatal DEX reduced the number of GFAP-IR cells ($p<0.05$; Figure 4.3 B,D) and dramatically increased the number of OX-42-IR cells ($p<0.01$; Figure 4.4 B,D) but didn't affect 3-NT-IR.

Prenatal treatment in combination with adult treatment in female rats: Again in contrast to the effect seen in males NAcc DA levels in stressed and GC treated females were not changed by prenatal exposure to DEX. However, there was an increase in NAcc DA turnover in stressed females that had also been treated prenatally with DEX ($p<0.05$). In the same group of animals DA and DOPAC levels within the PFC were reduced (Table 4.2).

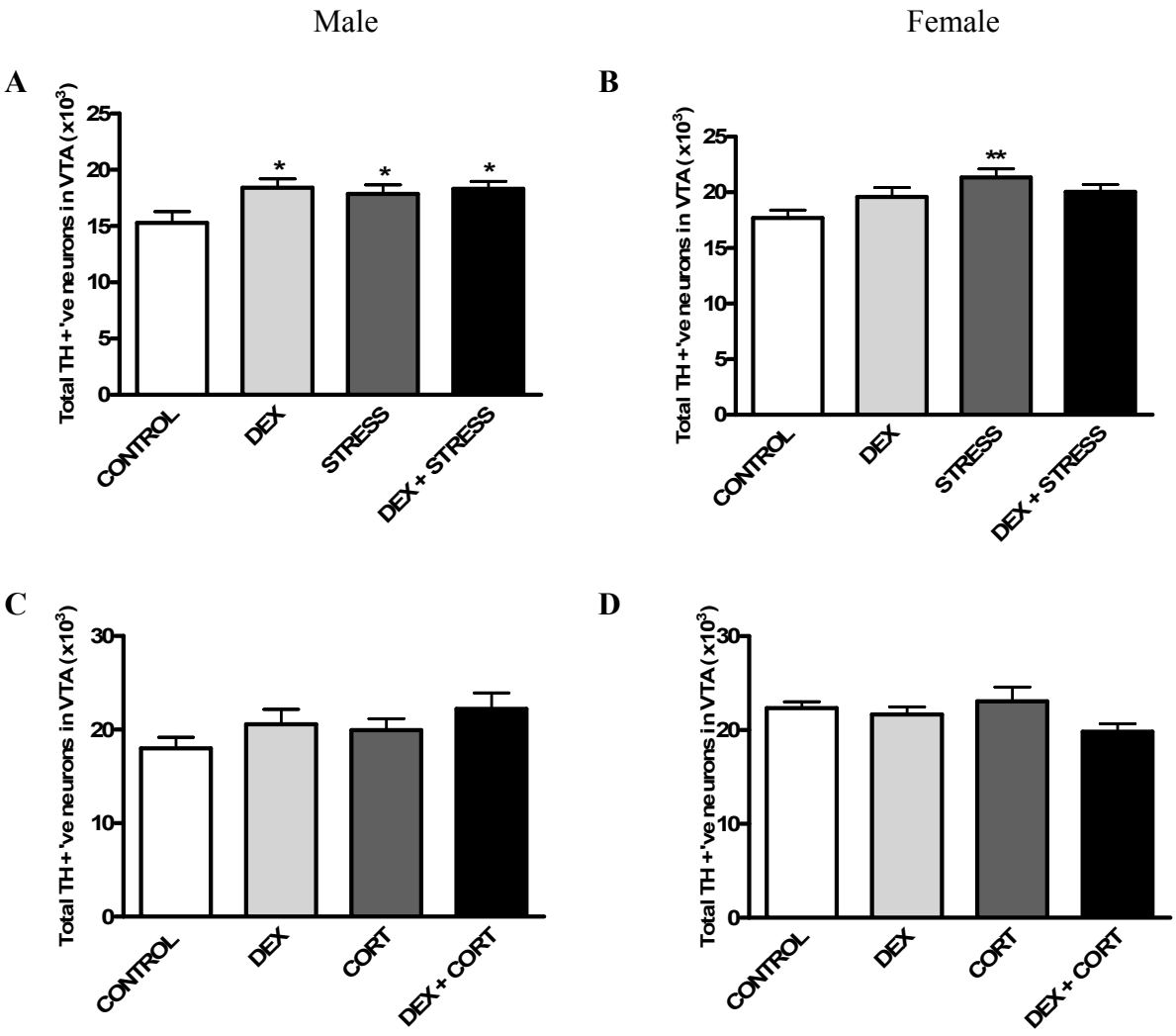


Figure 4.1. Effect of prenatal dexam ethasone an d/or adult stress or GC treatment on TH-IR cells w ithin the VTA. Stereological estimates of total TH-IR+ cells within the VTA in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by TH immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

Male Female

A B

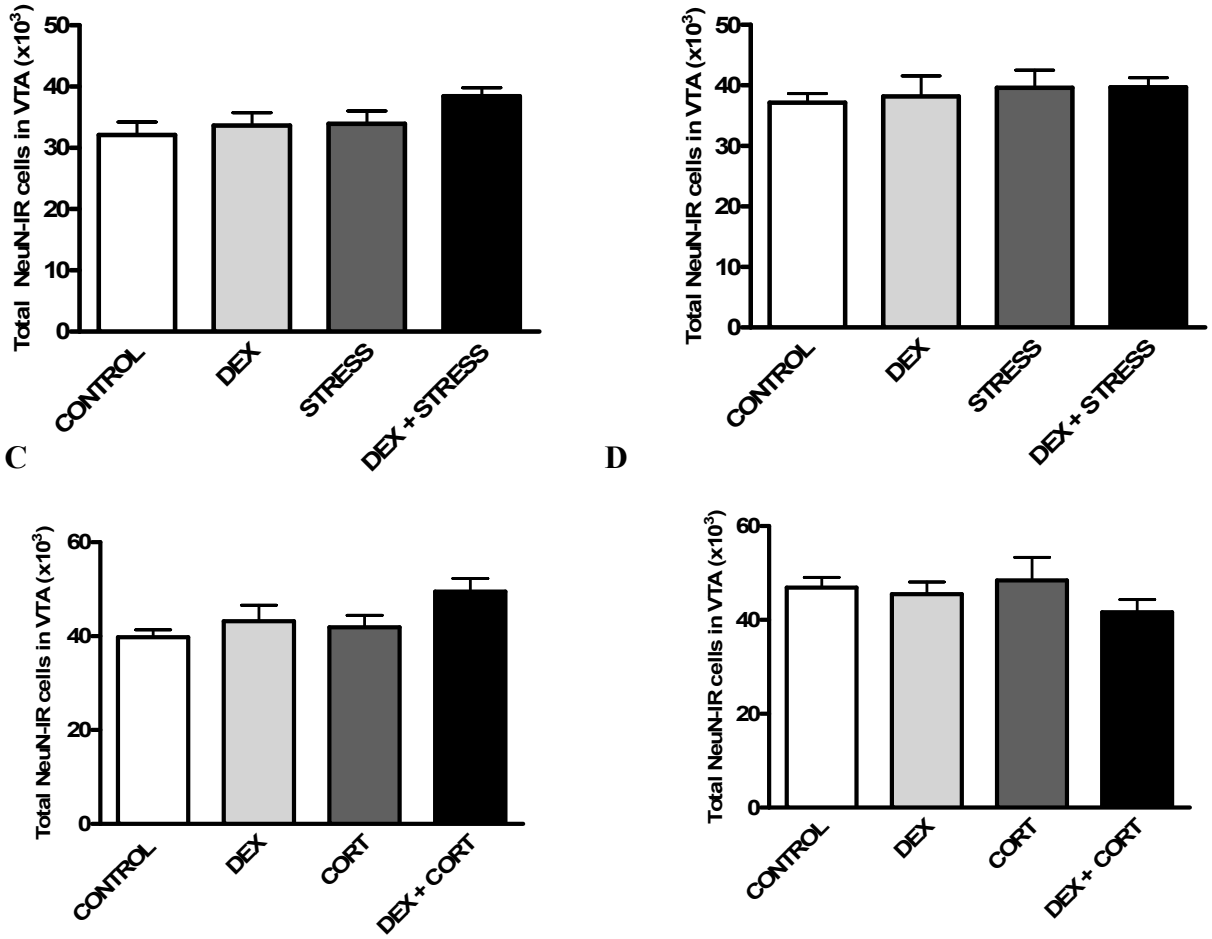


Figure 4.2. Effect of prenatal dexam ethasone an d/or adult stress or GC treatment on NeuN-IR cells w ithin the VTA. Stereological estimates of total NeuN-IR cells within the VTA in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by TH immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls. N numbers can be found in figure 2.11. A: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

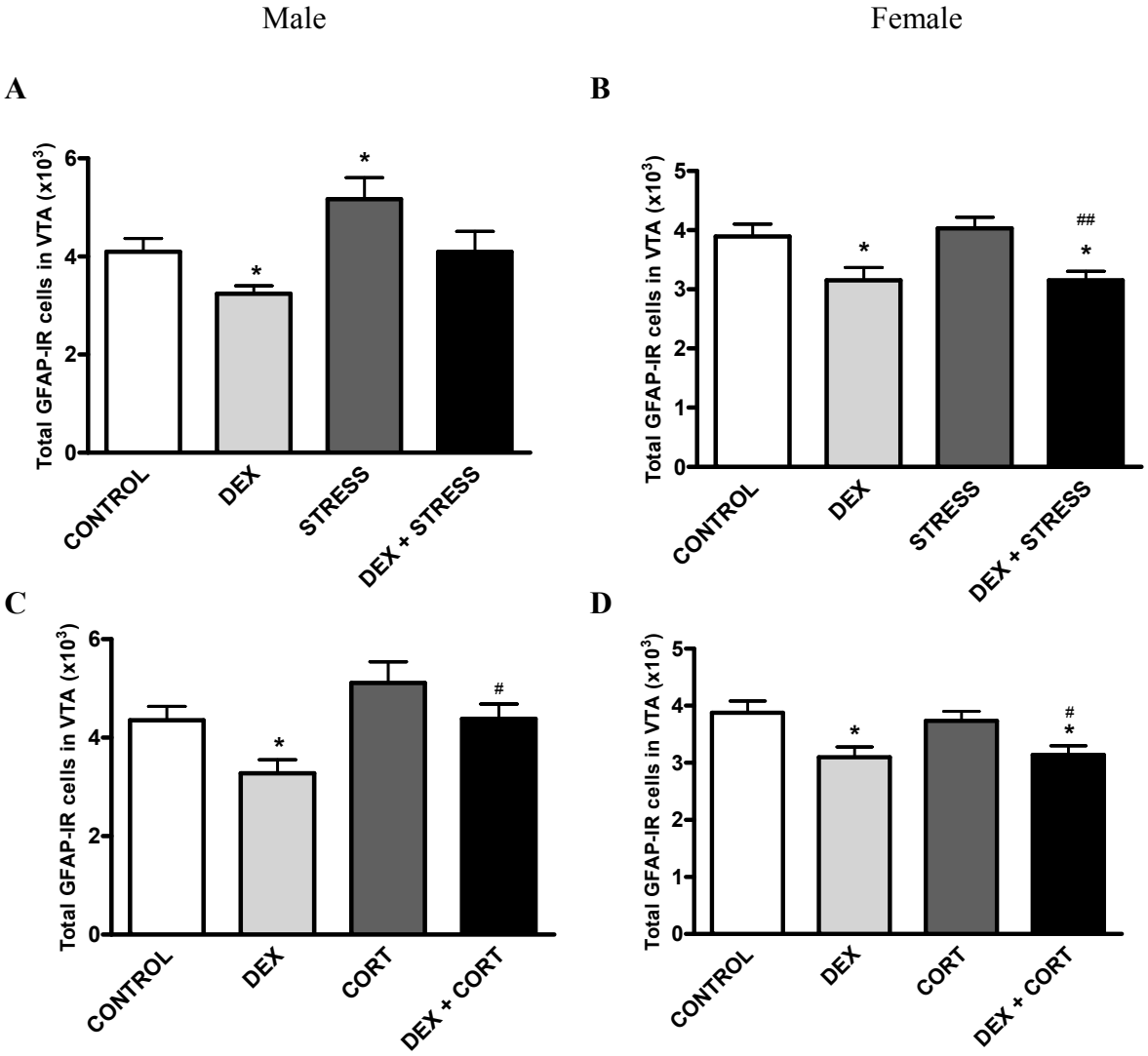


Figure 4.3 . Effec t of prena tal dexamethasone and/ or adult stress o r GC treatment on GFAP-IR cells within the VT A. Stereological estimates of total GFAP-IR cells within the VTA in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by GFAP immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

Male Female

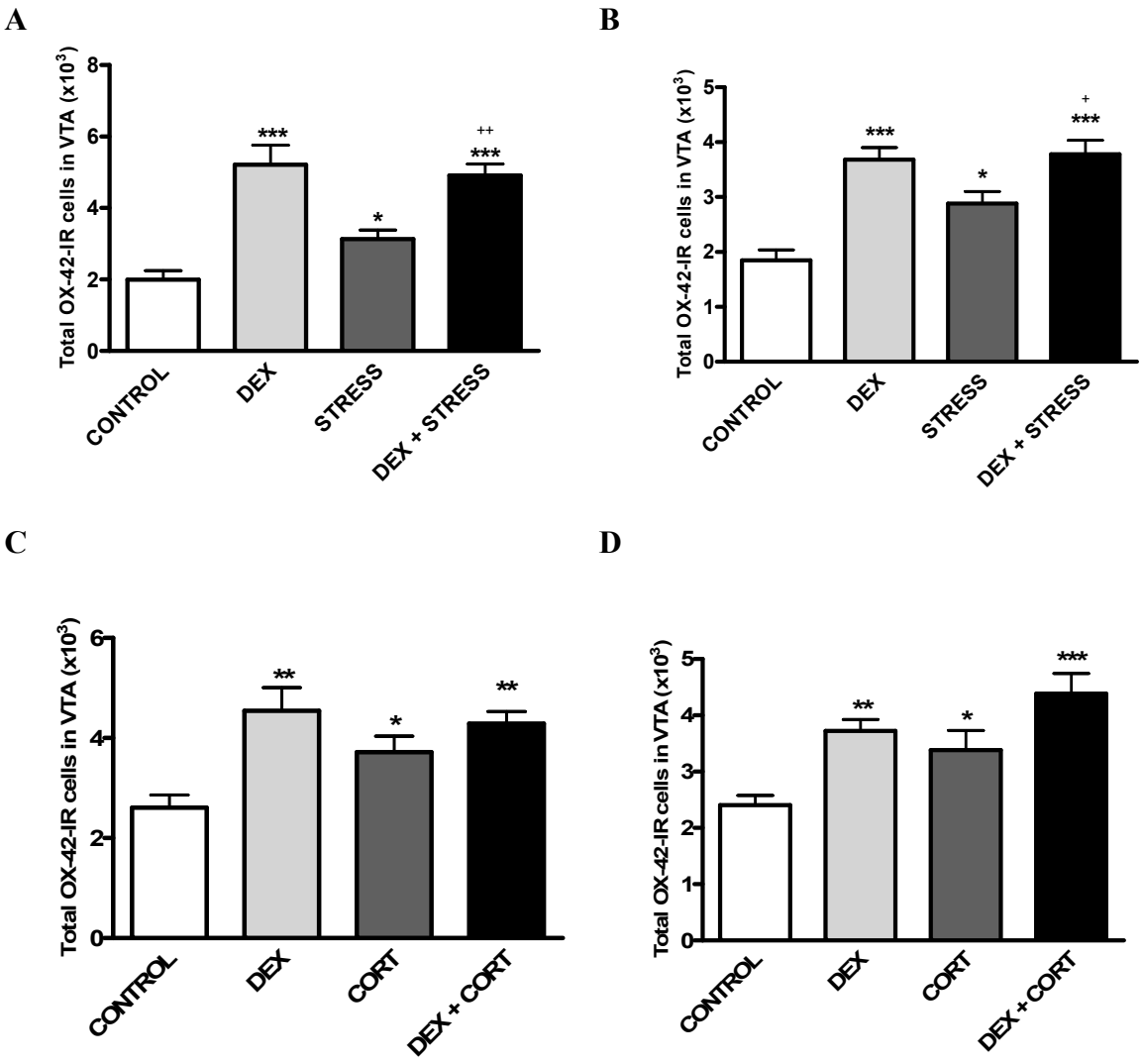


Figure 4.4. Effect of pr enatal dexameth asone and or adult stress or GC treatment on OX-42-IR cells within the VTA. Stereological estimates of total OX-42-IR cells within the VTA in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by OX-42 immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and / or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT

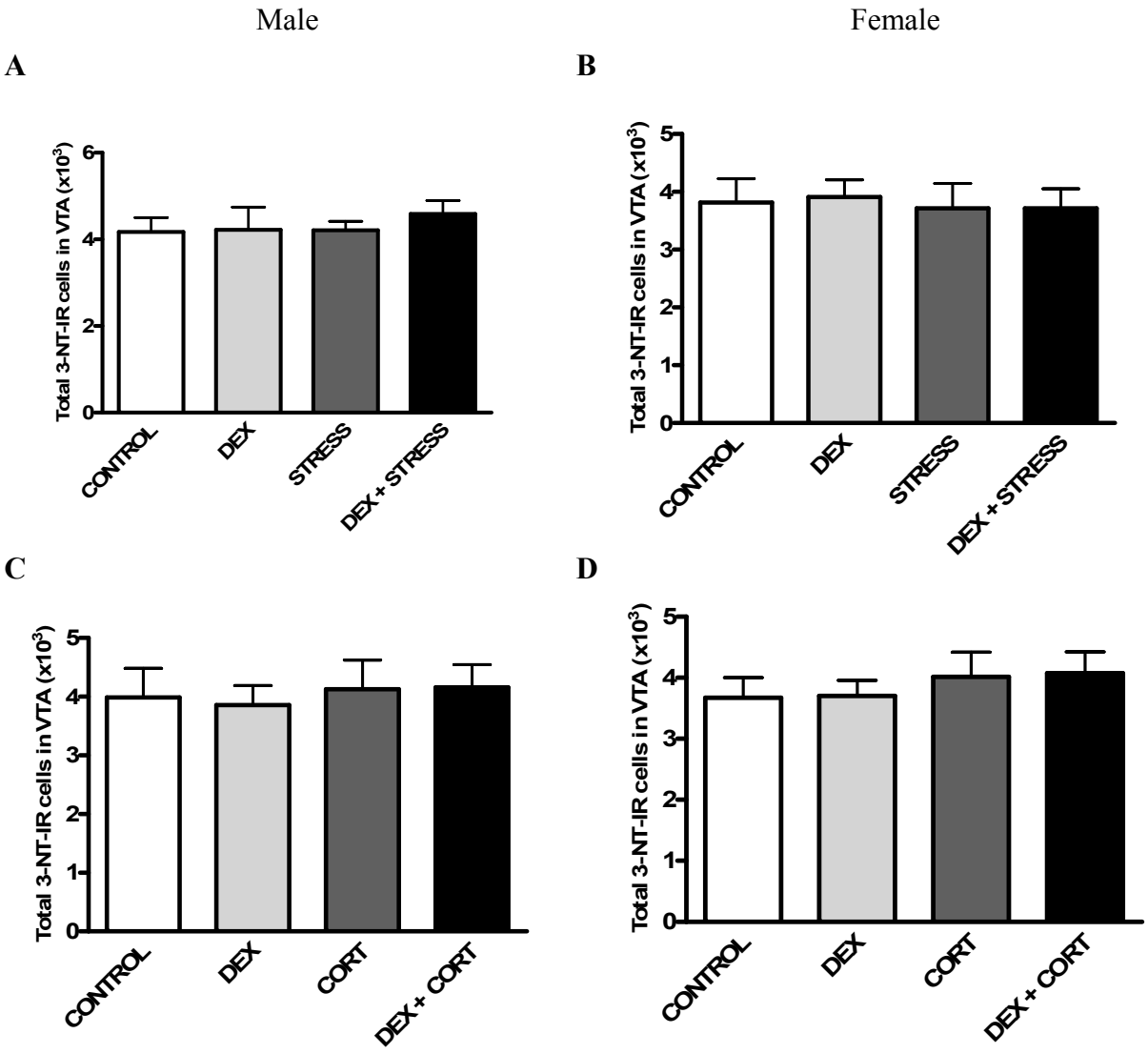


Figure 4.5. Effect of pr enatal dexameth asone and or adult stress or GC treatment on 3-NT-IR cells within the VTA. Stereological estimates of total 3-NT-IR cells within the VTA in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by 3-NT immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and / or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). B: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). C: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

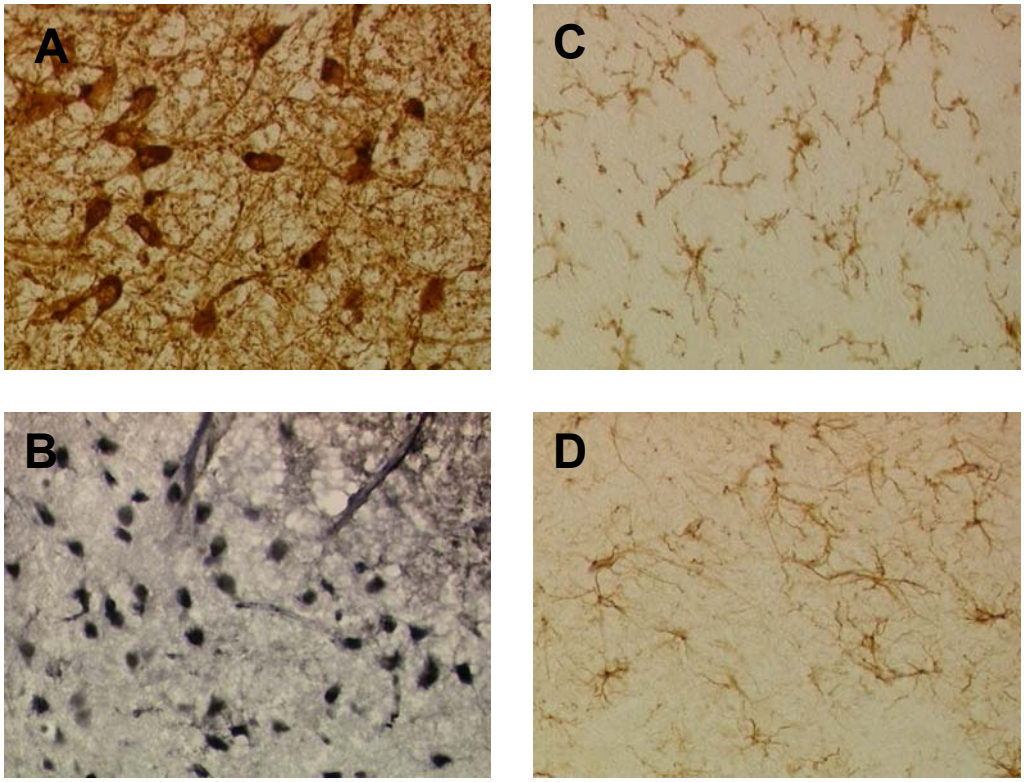


Figure 4.6. Representative photomicrographs of TH (A), NeuN (B), OX-42 (C) and G FAP (D) w ithin the VTA of control males. Pictures taken at 200x magnification.

DA, DOPAC and HVA levels (ng/mg)				
STUDY 1	**CONTROL**	**DEX**	**STRESS**	**DEX + STRESS**
Male Nucleus Accumbens				
DA	**7.70 ± 0.64**	**8.21 ± 0.56**	**11.54 ± 1.00 ****	**8.86 ± 0.67**
DOPAC	**7.72 ± 0.85**	**7.65 ± 0.48**	**6.37 ± 0.47**	**6.59 ±0.42**
HVA	**0.54 ± 0.04**	**0.50 ± 0.05**	**0.40 ± 0.03**	**0.44 ± 0.003**
Turnover	**1.47 ± 0.18**	**1.15 ± 0.10**	**0.71 ± 0.10 *****	**0.92 ± 0.08 ***
Female Nucleus Accumbens				
DA	**14.41 ± 0.79**	**10.48 ± 0.95 ***	**7.72 ± 0.66 *****	**8.08 ± 1.32 *****
DOPAC	**8.384 ± 0.23**	**7.69 ± 0.57**	**8.13 ± 0.57**	**8.46 ± 0.48**
HVA	**0.62 ± 0.04**	**0.61 ± 0.05**	**0.64 ± 0.06**	**0.85 ± 0.07 ***
Turnover	**0.72 ± 0.04**	**0.95 ± 0.13**	**1.40 ± 0.19 ***	**1.70 ± 0.38 ***

STUDY 2	**CONTROL**	**DEX**	**CORT**	**DEX + CORT**
Male Nucleus Accumbens				
DA	**5.52 ± 0.86**	**8.84 ± 0.72 ***	**8.93 ± 0.84 ***	**6.29 ± 0.62**
DOPAC	**4.52 ± 0.67**	**4.03 ± 0.13**	**5.89 ± 0.98**	**5.98 ± 0.98**
HVA	**0.67 ± 0.07**	**0.76 ± 0.05**	**0.86 ± 0.05**	**0.78 ± 0.04**
Turnover	**1.79 ± 0.44**	**0.71 ± 0.09**	**1.16 ± 0.42**	**1.31 ± 0.16**
Female Nucleus Accumbens				
DA	**11.78 ± 0.49**	**7.89 ± 0.37 *****	**9.54 ± 0.67 ***	**7.15 ± 0.46 *****
DOPAC	**4.56 ± 0.59**	**5.10 ± 0.40**	**7.07 ± 0.69 ***	**5.20 ± 5.20**
HVA	**1.05 ± 0.02**	**0.98 ± 0.04**	**1.14 ± 0.06**	**1.02 ± 0.05**
Turnover	**0.54 ± 0.06**	**0.89 ± 0.07 ***	**1.03 ± 0.12 ****	**1.03 ± 0.09 ****

Table 4.1. Effect of prenatal DEX and or adult stress or GC tre atment on DA levels within the NAcc. NAcc DA, DA metabolite and DA turnover were assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeated restraint stress or GC treatment. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of DA metabolites (DOPAC and HVA) to DA in pmol/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. Study 1: Males: n=8 (control), 8 (DEX), 8(stress), 8 (DEX + stress). Females: n= 9 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). Study 2: Males: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). Females: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

DA, DOPAC and HVA levels (ng/mg)				
STUDY 1	**CONTROL**	**DEX**	**STRESS**	**DEX + STRESS**
Male Frontal Cortex				
DA	**0.21 ± 0.03**	**0.15 ± 0.01**	**0.16 ± 0.008**	**0.18 ± 0.02**
DOPAC	**0.06 ± 0.01**	**0.07 ± 0.008**	**0.06 ± 0.006**	**0.09 ± 0.02**
HVA	**0.14 ± 0.009**	**0.16 ± 0.01**	**0.15 ± 0.009**	**0.17 ± 0.007**
Turnover	**1.16 ± 0.09**	**1.67 ± 0.12 ****	**1.46 ± 0.73**	**1.65 ± 0.11 ****
Female Frontal Cortex				
DA	**0.31 ± 0.05**	**0.22 ± 0.04**	**0.20 ± 0.03**	**0.15 ± 0.007 ***
DOPAC	**0.14 ± 0.02**	**0.17 ± 0.05**	**0.10 ± 0.02**	**0.07 ± 0.008 *#**
HVA	**-**	**-**	**-**	**-**
Turnover	**0.54 ± 0.07**	**0.64 ± 0.05**	**0.59 ± 0.04**	**0.65 ± 0.09**

STUDY 2	CONTROL	DEX	CORT	DEX + CORT
Male Frontal Cortex				
DA	**0.10 ± 0.06**	**0.09 ± 0.12**	**0.06 ± 0.05**	**0.11 ± 0.04**
DOPAC	**0.41 ± 0.08**	**0.47 ± 0.03**	**0.36 ± 0.02**	**0.50 ± 0.05**
HVA	**0.04 ± 0.02**	**0.04 ± 0.003**	**0.03 ± 0.001**	**0.05 ± 0.009**
Turnover	**8.30 ± 1.92**	**6.69 ± 0.70**	**7.77 ± 0.70**	**5.58 ± 1.98**
Female Frontal Cortex				
DA	**4.03 ± 0.96**	**1.73 ± 0.70**	**2.42 ± 0.95**	**3.33 ± 1.36**
DOPAC	**0.56 ± 0.07**	**0.56 ± 0.07**	**0.64 ± 0.10**	**0.74 ± 0.05**
HVA	**0.29 ± 0.06**	**0.38 ± 0.05**	**0.43 ± 0.06**	**0.35 ± 0.04**
Turnover	**0.39 ± 0.13**	**0.84 ± 0.18**	**0.86 ± 0.20**	**0.73 ± 0.18**

Table 4.2. Effect of prenatal DEX and adult stress or GC treatment on DA levels within the PFC. PFC DA, DA metabolite and DA turnover were assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeated restraint stress or GC treatment. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of DA metabolites (DOPAC and HVA) to DA in pmol/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. Study 1: Males: n=8 (control), 8 (DEX), 8(stress), 8 (DEX + stress). Females: n= 9 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). Study 2: Males: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). Females: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

4.3.2. 6-OHDA challenged MLDA and MCDA

Controls: On combination of data from study 1 and study 2, administration of 5µg 6-OHDA to the MFB resulted in a ~15% loss of VTA TH-IR cells in males ($p<0.05$) and ~25% loss in females ($p<0.01$). The size of the lesion was calculated as a % loss in the lesioned versus unlesioned hemisphere (figure 4.8, 4.10). The sex difference previously reported in VTA TH-IR cell number (section 4.3.1) was lost in response to 6-OHDA lesioning. No significant difference in NeuN-IR cells within the VTA in response to 6-OHDA was found in either sex (figure 4.9) but there was a concomitant fall in DA levels within the NAcc (Table 4.3/4.4). In the male, DA levels in the contralateral NAcc were 5.29 ± 0.51, which were reduced by ~25% to 3.92 ± 0.95 ng/mg in the ipsilateral NAcc ($p<0.05$; table 4.3). Similarily, in response to 6-OHDA there was a ~55% reduction in female NAcc DA (13.84 ± 0.71 versus 7.06 ± 1.29; $p<0.01$) Please note that there was only significant DA loss in the male on combination of the data sets - for individual study values see tables 4.3 and 4.4. In the adult male, 6-OHDA had no effect on DA metabolite levels or turnover but in the female 6-OHDA caused significant depletion of NAcc HVA ($p<0.05$). Within the PFC, 6-OHDA lesioning resulted in depletion of DA levels ($p<0.05$) in males but not females (Table 4.5). There was no effect on GFAP-IR cells within VTA after 6-OHDA lesioning when compared to the contralateral hemisphere. However, there was more intense staining and a higher proportion of cells showing a reactive morphology in the VTA of the lesioned hemisphere. In response to 6-OHDA we also observed microglial activation in the lateral portion of the VTA of the lesioned but not unlesioned hemisphere of both male and female rats. The number of OX-6-IR cells was 594 ± 67 and 586 ± 67 in males and females respectively. Minimal OX-6-IR cells were present in the contralateral VTA. 3-

NT immunoreactivity within the VTA of lesioned rats was not significantly different to that of naïve rats.

Repeated stress in 6-OHDA treated males: Previous exposure to repeated stress caused the TH-IR but not NeuN-IR 6-OHDA lesion to become significant ($p<0.05$; figure 4.7). In contrast to effects on TH, stressed males do not exhibit significant NAcc DA depletion in response to 6-OHDA. In the adult male NAcc, repeated stress did not significantly affect DA or metabolite levels but did increase DA turnover in the contralateral NAcc ($p<0.05$; table 4.3). Stress had no effect on DA levels within the PFC of 6-OHDA treated males. Furthermore previous repeated stress did not alter GFAP immunoreactivity (Figure 4.11) of lesioned rats but did increase the number of OX-6-IR cells (Figure 4.12) in the VTA in response to a 6-OHDA lesion. These were primarily located in the area of VTA adjacent to the SNpc. In males the number of OX-6-IR cells rose from 720 ± 94 to 1494 ± 153 ($p<0.01$; figure 4.12). There were no alterations in 3-NT-IR cells in stressed males subjected to 6-OHDA (Figure 4.13).

Repeated stress in 6-OHDA treated females: In stressed females that were subjected to 6-OHDA there was no longer a significant TH lesion (Figure 4.7B). Repeated stress resulted in decreased DA levels ($p<0.05$) and DOPAC levels ($p<0.001$; Table 4.3) in the contralateral NAcc that were inline with the effects seen in naïve females but there was no effect on the ipsilateral hemisphere. Similarly within the PFC adult stress reduced DA levels in 6-OHDA females ($p<0.05$; table 4.5). As in males repeated stress resulted in an elevated number of OX-6-IR cells within the ipsilateral VTA ($p<0.05$; figure 4.12B) but had no effect on GFAP or 3-NT levels in the ipsilateral VTA of 6-OHDA treated females.

Chronic GC treatment in 6-OHDA treated males: In male 6-OHDA treated rats, a history of chronic GC treatment had no effect on TH-IR cell counts in the VTA of either hemisphere. Chronic GC treatment had no effect on DA levels in either the ipsilateral or contralateral NAcc but did increased DOPAC and HVA levels in both ($p<0.05$; table 4.4). Dopamine turnover in the male NAcc was also increased but only significantly in the contralateral NAcc ($p<0.05$). In the male, chronic GC treatment resulted in increased DA ($p<0.05$) and HVA ($p<0.01$) levels within the PFC of 6-OHDA males (table 4.5). Similarly to repeated stress, chronic GC treatment resulted in a greater number of OX-6-IR cells in the VTA of 6-OHDA males ($p<0.05$; figure 4.12C) and females (although not significant $P=0.0564$; figure 4.12D) but didn't affect the number of GFAP-IR cells (figure 4.11)

Chronic GC treatment in 6-OHDA treated females: As with repeated stress previous chronic GC treatment eliminates the TH-IR lesion in female 6-OHDA rats (Figure 4.7D). In addition although DA levels were reduced in the contralateral hemisphere of female rats that had been treated with GCs ($p<0.05$), there was no longer significant DA depletion in the ipsilateral verus contralateral NAcc (table 4.5). DA metabolite levels and turnover in lesioned females were unaffected by a history of GC treatment. There was also a trend for reduced DA levels in the PFC of lesioned females that had received chronic GC treatment. Although this was not significant, there was a significant increase in DA turnover (Figure $p<0.05$; table 4.5).

Prenatal DEX in 6-OHDA treated males: In 6-OHDA males that had received prenatal DEX treatment a significant TH-IR lesion within the VTA was seen in study 1

only ($p<0.05$; figure 4.7A,B). Prenatal DEX treatment did not significantly alter the number of TH-IR (Figure 4.7A,C) or NeuN-IR (Figure 4.9A,C) cells in the VTA of either hemisphere. The level of DA in both the ipsilateral and contralateral NAcc of animals treated with prenatal DEX was no different to that of the controls, and there was no significant hemispheric difference in NAcc DA levels (tables 4.3,4.4). DA metabolite levels and turnover within the adult NAcc of 6-OHDA rats were similarly unaffected by DEX. Within the PFC prenatal DEX increased DA turnover in 6-OHDA (DA and HVA levels were increased in study 1 only; table 4.5). In study 1 prenatal DEX decreased the number of GFAP-IR cells in the VTA of lesioned male rats but this wasn't significant in study 2 ($p<0.05$, figure 4.11A,C). OX-6 and 3-NT-IR cell number were not altered by prenatal DEX in male 6-OHDA rats (Figure 4.12-4.13).

Prenatal DEX in 6-OHDA treated females: In female 6-OHDA rats prenatal DEX treatment eliminated the lesion in TH-IR within the VTA (figure 4.7B,D). In study 2, prenatally DEX treated females had less DA in the contralateral NAcc compared to controls ($p<0.05$; tables 4.3, 4.4) which is comparable to the effect reported in section 4.2.2 but did not impact upon DA levels in the ipsilateral NAcc (9.615 ± 1.38; 6.41 ± 0.69; $p<0.05$) meaning that there was no longer significant DA loss as a result of 6-OHDA lesioning . NAcc DOPAC levels of 6-OHDA rats were also decreased in DEX treated females in study 2 ($p<0.05$) but not significantly in study 1. Within the PFC of 6-OHDA females prenatal DEX treatment increased DA turnover in study 1 only. Furthermore, DEX treatment did not alter GFAP-IR, OX-6-IR or 3-NT-IR within the VTA of female lesioned rats (Figures 4.11-13)

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Prenatal DEX in combination with adult treatment in 6-OHDA males: Prenatal DEX treatment in stressed or GC treated rats did not alter the effect of 6-OHDA on TH-IR cells and NeuN-IR cells within the VTA. Interestingly, male rats that received both gestational DEX and repeated stress or chronic GC treatment in adulthood exhibit significantly less DA in the lesioned versus unlesioned hemisphere ($p<0.05$; table 4.3,4.4; figure 4.10 AC). In stressed males there was also significantly less DA in the NAcc of both hemispheres ($p<0.05$; tables 4.3,4.4) when compared to control males. In GC treated males there was less DA, DOPAC and HVA in the ipsilateral NAcc when compared to the contralateral NAcc ($p<0.05$; table 4.4) and in stressed males that had been exposed to gestational DEX there was decreased DA and DA turnover in the ipsilateral versus contralateral NAcc ($p<0.05$; table 4.3). Within the PFC prior DEX treatment didn't alter the effect of stress or GC treatment on lesioned males.

Prenatal DEX in combination with adult treatment in 6-OHDA females: In stressed females that had received DEX prenataly there was more significant fall in DA levels of the contralateral NAcc ($p<0.01$; table 4.3) and significant depletion of HVA (contralateral versus ipsilateral; $p<0.05$). Comparably GC treated females that had a history of DEX treatment also showed a more significant reduction in DA levels of the unlesioned hemisphere ($p<0.01$; table 4.4). By contrast to males prenatal DEX treatment resulted in a significant fall in DA levels and an increase in HVA levels in 6-OHDA females (table 4.5). There was also an interactive effect of DEX and repeated stress in lesioned females on GFAP-IR cells which were reduced in these animals ($p<0.05$; figure 4.11).

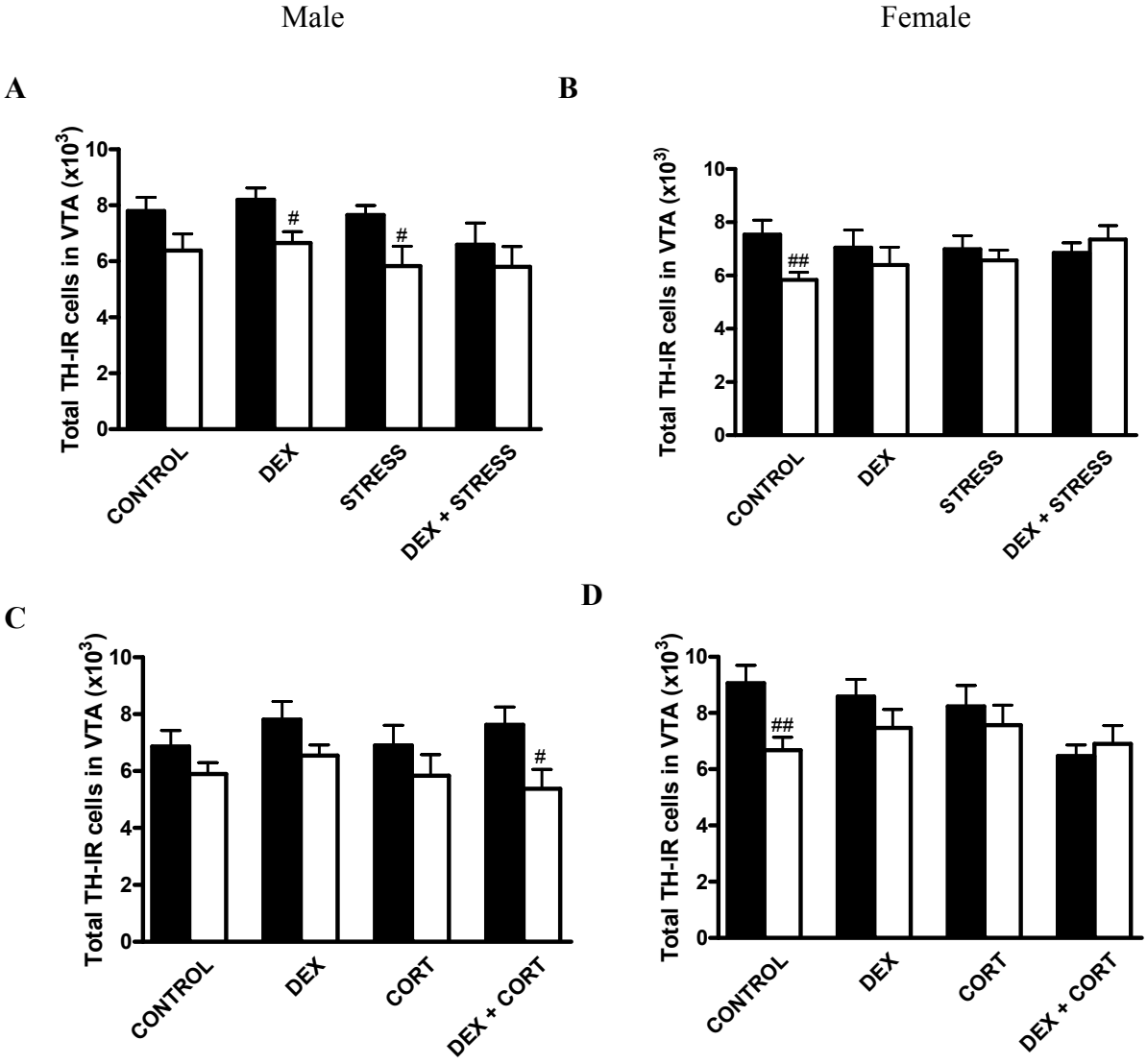


Figure 4.7. Effect of prenatal DEX treatment and/or adult stress or GC treatment on TH-IR cells within the VTA following 6-OHDA lesioning. Stereological estimates of the number of TH-IR cells within the VTA of male (A,C) and female (B,D) animals exposed to a 6-OHDA were determined by TH immunohistocemistry. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs unlesioned hemisphere. A: n=8 (control), 8(DEX), 8(stress), 7 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 7(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

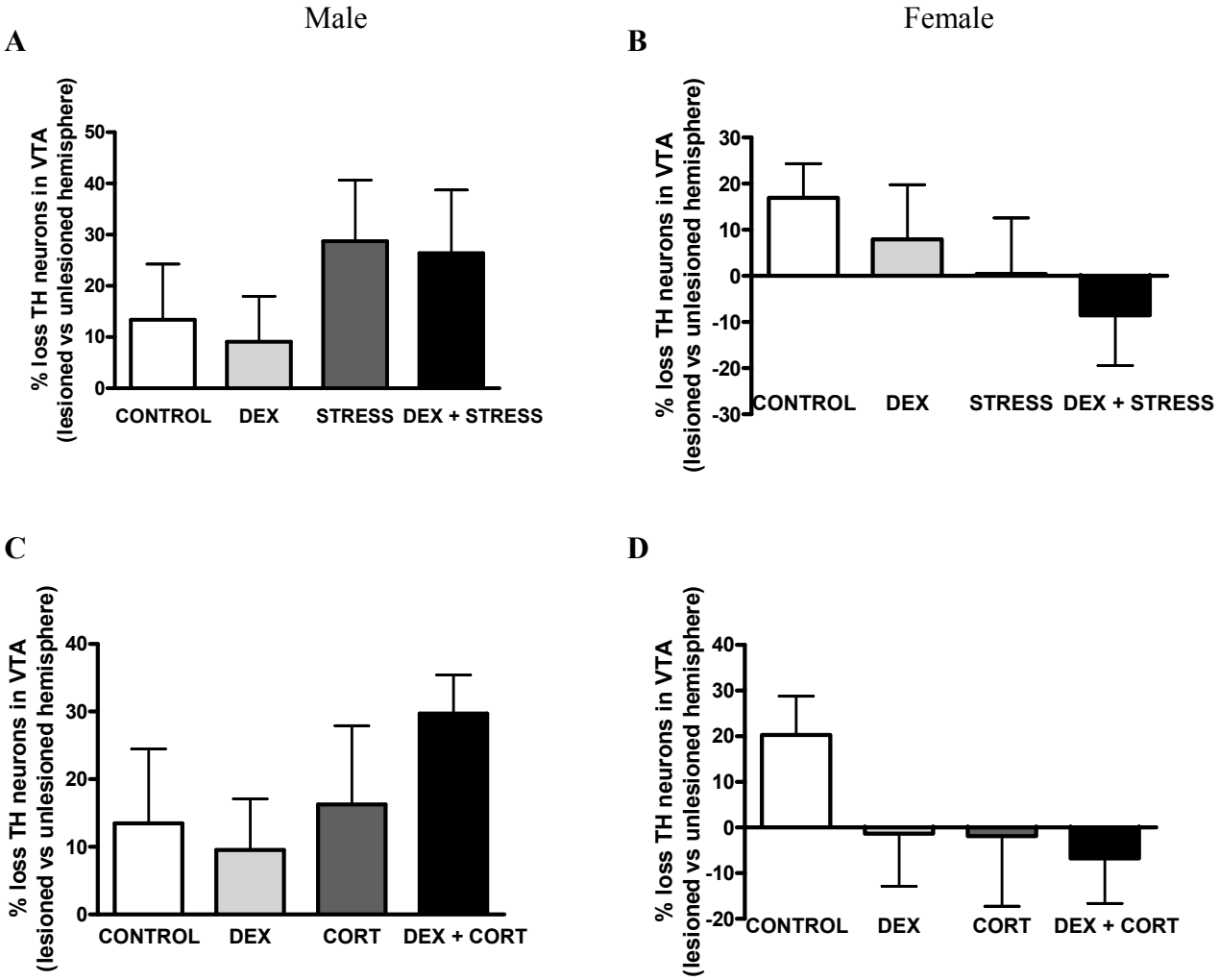


Figure 4.8. Effect of prenatal DEX treat ment and/or adult stress or GC treatment on percentage loss of TH-IR cells in the VTA follow ing 6-OHDA lesioning. The percentage loss of TH-IR cells in the lesioned VTA versus the unlesioned VTA in male (A,C) and female (B,D) animals exposed to a 6-OHDA. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effects of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. . A: n=8 (control), 8(DEX), 8(stress), 7 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 7(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

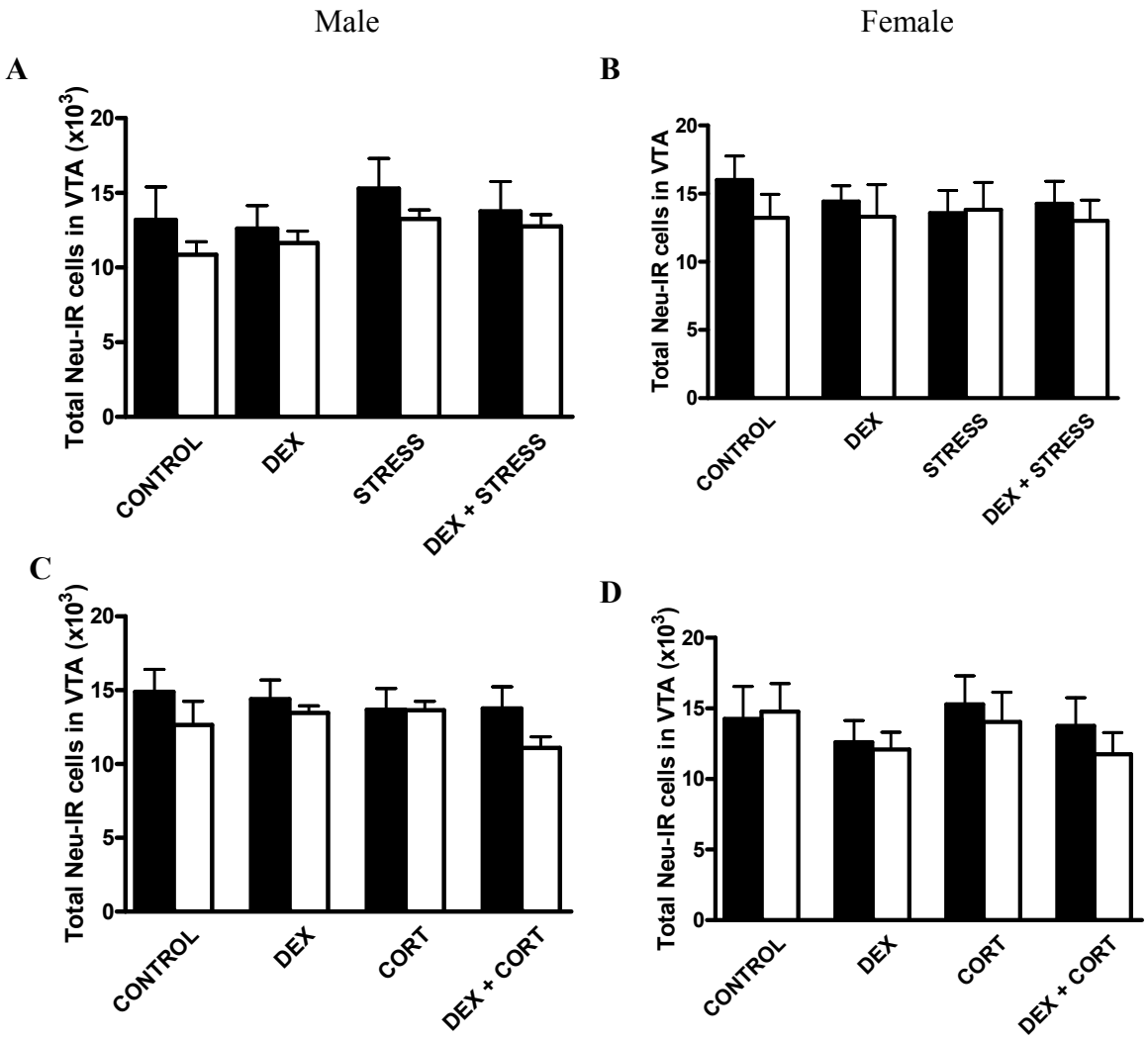


Figure 4.9. Effect of prenatal DEX treat ment and/or adult stress or GC treatment on NeuN-IR cells within the VTA following 6-OHDA lesioning. Stereological estimates of the number of NeuN-IR cells within the VTA of male (A,C) and female (B,D) animals exposed to a 6-OHDA were determined by NeuN immunohistocemistry. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. . A: n=8 (control), 8(DEX), 8(stress), 7 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 7(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

NAcc DA, DOPAC and HVA in 6-OHDA lesioned animals								
	CONTROL		DEX		STRESS		DEX + STRESS	
	Right	Left	Right	Left	Right	Left	Right	Left
Male NAcc								
DA	**5.45 ± 1.06**	**4.19 ± 1.85**	**5.20 ± 1.42**	**4.79 ± 2.02**	**3.99 ± 0.59**	**3.35 ± 0.88**	**2.81 ± 0.54 ***	**1.12 ± 0.38 #**
DOPAC	**4.91 ± 0.75**	**3.04 ± 0.47**	**3.30 ± 0.42**	**2.89 ± 0.65**	**6.18 ± 0.87**	**4.61 ± 0.62**	**4.17 ± 0.47**	**5.00 ± 0.27**
HVA	**0.51 ± 0.20**	**0.87 ± 0.16**	**0.75 ± 0.20**	**0.63 ± 0.24**	**0.90 ± 0.10**	**1.12 ± 0.12**	**0.50 ± 0.17**	**0.89 ± 0.15**
Turnover	**1.23 ± 0.28**	**1.03 ± 0.59**	**0.81 ± 0.19**	**0.78 ± 0.44**	**2.18 ± 0.21 ***	**1.99 ± 0.32**	**2.13 ± 0.27***	**5.35 ± 0.27 #**
Female NAcc								
DA	**14.10 ± 1.40**	**7.25 ± 2.11 #**	**11.01 ± 2.30**	**6.26 ± 1.14**	**9.05 ± 1.92 ***	**7.11 ± 2.38**	**6.46 ± 0.76 ****	**4.06 ± 1.4**
DOPAC	**5.11 ± 0.61**	**6.40 ± 0.35**	**4.29 ± 0.15 *****	**3.56 ± 0.23**	**3.76 ± 0.45 *****	**3.82 ± 0.28**	**3.69 ± 0.37 *****	**3.68 ± 0.42**
HVA	**1.32 ± 0.17**	**0.73 ± 0.12 #**	**1.22 ± 0.09**	**1.64 ± 0.29**	**1.16 ± 0.22**	**0.99 ± 0.21**	**1.51 ± 0.14**	**0.93 ± 0.25**
Turnover	**0.65 ± 0.09**	**0.93 ± 0.23**	**0.59 ± 0.09**	**0.87 ± 0.13**	**0.82 ± 0.19**	**1.04 ± 0.32**	**0.85 ± 0.2**	**0.97 ± 0.11**

Table 4.3. Effect of prenatal DEX and adult stress on NAcc DA levels in 6-OHDA unilaterally lesioned rats. NAcc DA, DA metabolite and DA turnover were assesed by HPLC at 6 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeated restraint stress. At 5.5 months rats received an injection of 5µg 6-OHDA to the left MFB. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of DA metabolites to DA in pmol/mg. Statistical significance was assessed by three way anova with tukeys post hoc multiple comparison tests.*p<0.05, **p<0.01, ***p<0.001 vs controls, # vs unlesioned hemisphere. A: n=8 (control), 8(DEX), 8(stress), 7 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 7(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

NAcc DA, DOPAC and HVA in 6-OHDA lesioned animals								
	CONTROL		DEX		CORT		DEX + CORT	
	Right	Left	Right	Left	Right	Left	Right	Left
Male NAcc								
DA	**5.13 ± 1.01**	**3.65 ± 1.93**	**7.59 ± 1.06**	**6.93 ± 0.55**	**5.15 ± 0.45**	**4.35 ± 1.09**	**4.40 ± 0.39**	**1.39 ± 0.70 #**
DOPAC	**4.72 ± 0.85**	**2.48 ± 0.57**	**3.78 ± 0.61**	**3.60 ± 0.55**	**8.22 ± 0.87 ***	**6.01 ± 0.45 ***	**6.55 ± 0.40**	**3.09 ± 0.80 #**
HVA	**0.81 ± 0.14**	**0.42 ± 0.17**	**0.74 ± 0.26**	**0.81 ± 0.20**	**1.49 ± 0.11 ***	**1.20 ± 0.09 ***	**1.37 ± 0.11**	**0.61 ± 0.20 #**
Turnover	**1.33 ± 0.19**	**1.89 ± 0.76**	**0.80 ± 0.17**	**0.82 ± 0.17**	**2.55 ± 0.32 ***	**2.58 ± 0.55**	**2.02 ± 0.06 ***	**2.71 ± 0.22**
Female NAcc								
DA	**13.59 ± 1.64**	**6.86 ± 1.75 #**	**8.22 ± 1.54 ***	**6.56 ± 0.88**	**9.86 ± 1.14 ***	**7.88 ± 2.80**	**6.93 ± 0.56 ****	**4.11 ± 1.27**
DOPAC	**6.76 ± 1.12**	**5.05 ± 0.88**	**3.48 ± 0.29**	**3.97 ± 0.52**	**4.90 ± 0.77**	**3.84 ± 0.45**	**4.21 ± 0.62**	**3.89 ± 0.38**
HVA	**1.04 ± 1.50**	**0.72 ± 0.13 #**	**0.74 ± 1.76**	**1.06 ± 1.64**	**1.29 ± 0.16**	**1.05 ± 0.26**	**1.65 ± 0.11**	**0.97 ± 0.24 #**
Turnover	**0.66 ± 0.09**	**0.98 ± 0.31**	**0.59 ± 0.09**	**0.84 ± 0.12**	**0.82 ± 0.19**	**1.11 ± 0.38**	**0.98 ± 0.13**	**0.96 ± 0.13**

Table 4.4. Effect of prenatal DEX and GC treatment on NAcc DA levels in 6-OHDA unilaterally lesioned rats . NAcc DA, DA metabolite and DA turnover were assesed by HPLC at 6 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) chronic GC treatment. At 5.5 months rats received an injection of 5µg 6-OHDA to the left MFB. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of DA metabolites to DA in pmol/mg. Statistical significance was assessed by three way anova with tukeys post hoc multiple comparison tests.*p<0.05, **p<0.01, ***p<0.001 vs controls, # vs unlesioned hemisphere. A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 7 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 67(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

PFC DA, DOPAC and HVA levels (ng/mg) in 6-OHDA lesioned rats				
	CONTROL	DEX	STRESS	DEX + STRESS
Male PFC				
DA	0.11 ± 0.02	0.08 ± 0.008	0.08 ± 0.007	0.09 ± 0.009
DOPAC	0.03 ± 0.006	0.03 ± 0.004	0.03 ± 0.004	0.04 ± 0.005
HVA	0.07 ± 0.003	0.07 ± 0.003	0.07 ± 0.002	0.08 ± 0.002
Turnover	1.17 ± 0.09	1.67 ± 0.11 **	1.46 ± 0.07	1.65 ± 0.11 **
Female PFC				
DA	0.40 ± 0.08	0.36 ± 0.11	0.20 ± 0.04 *	0.15 ± 0.01 *
DOPAC	0.15 ± 0.02	0.20 ± 0.07	0.10 ± 0.03	0.07 ± 0.01 *
HVA	0.01 ± 0.004	0.02 ± 0.008	0.004 ± 0.003	0.01 ± 0.006
Turnover	0.54 ± 0.07	0.64 ± 0.05	0.59 ± 0.04	0.65 ± 0.09

	CONTROL	DEX	CORT	DEX + CORT
Male PFC				
DA	0.02 ± 0.002	0.04 ± 0.006 *	0.07 ± 0.02 *	0.04 ± 0.01 *
DOPAC	0.58 ± 0.07	0.45 ± 0.03	0.50 ± 0.03	0.45 ± 0.02
HVA	0.02 ± 0.002	0.04 ± 0.004 *	0.05 ± 0.007 **	0.03 ± 0.006
Turnover	39.19 ± 8.90	15.58 ± 1.93 *	15.13 ± 2.37 *	14.66 ± 3.04 *
Female PFC				
DA	4.26 ± 0.99	2.67 ± 1.07	2.68 ± 1.00	1.69 ± 0.30 *
DOPAC	0.55 ± 0.04	0.64 ± 0.05	0.62 ± 0.06	0.55 ± 0.08
HVA	0.15 ± 0.02	0.24 ± 0.03	0.23 ± 0.03	0.28 ± 0.03 *
Turnover	0.25 ± 0.06	0.65 ± 0.16 *	0.52 ± 0.09 *	0.56 ± 0.15

Table 4.5. Effect of prenatal DEX and or adult stress or GC treatment on PFC DA levels in 6-OHDA unilaterally lesioned rats. PFC DA, DA metabolite and DA turnover were assesed by HPLC at 6 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeated restraint stress or chronic GC treatment. At 5.5 months rats received an injection of 5µg 6-OHDA to the left MFB. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of DA metabolites to DA in pmol/mg. Statistical significance was assessed by three way anova with tukeys post hoc multiple comparison tests.*p<0.05, **p<0.01, ***p<0.001 vs controls, # vs unlesioned hemisphere**.** A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 7 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 67(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

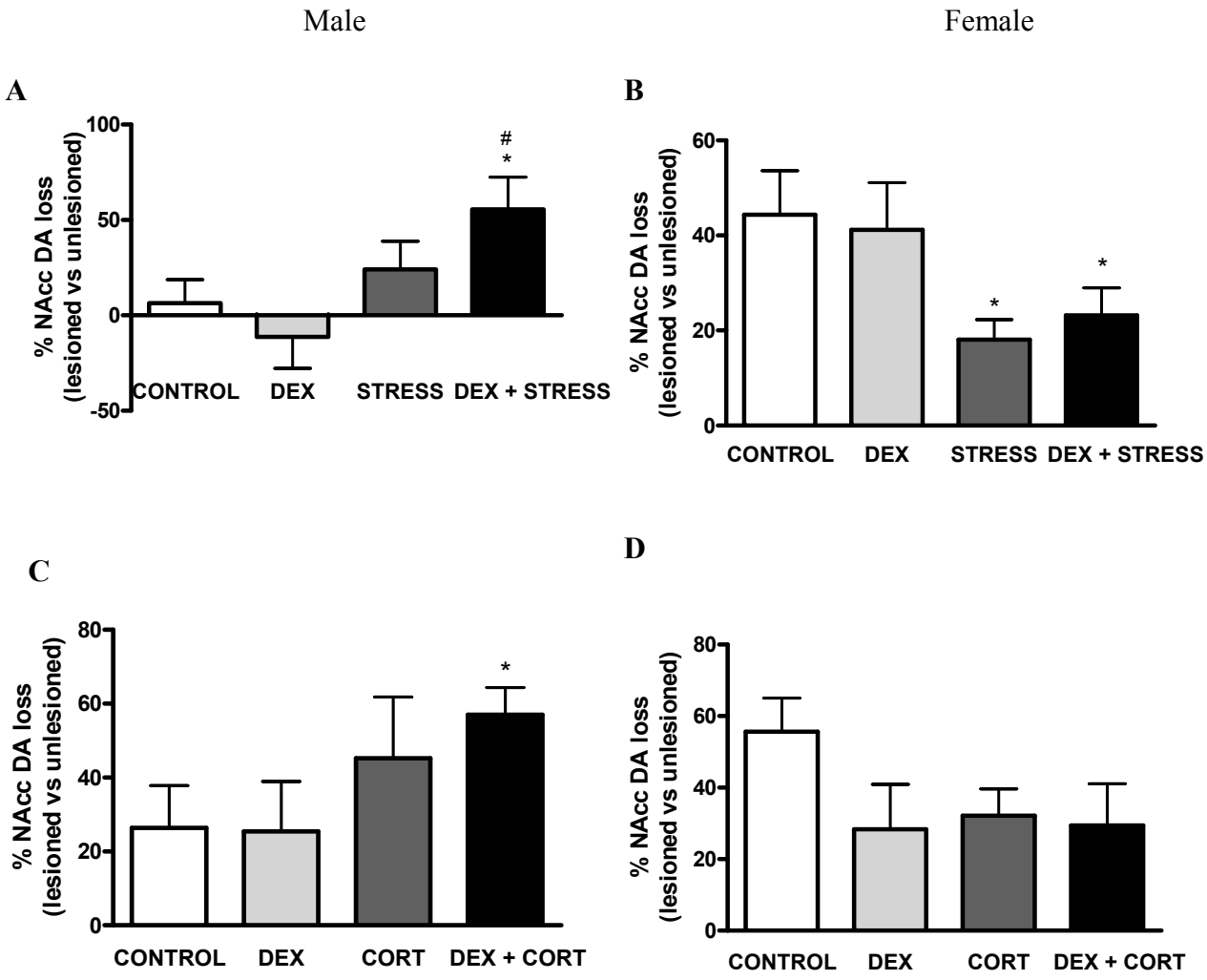


Figure 4.10. Effect of prenatal DEX treatment and/or adult stress or GC treatment on percentage NAcc DA loss following 6-OHDA lesioning. NAcc DA loss in the lesioned versus unlesioned hemisphere in male (A,C) and female (B,D) animals exposed to a 6-OHDA. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effects of prenatal (GD16-19) DEX treatment and / or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and/or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 8(DEX), 8(stress), 8 (DEX + stress). B: n= 8 (control), 8 (DEX), 7 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 67(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

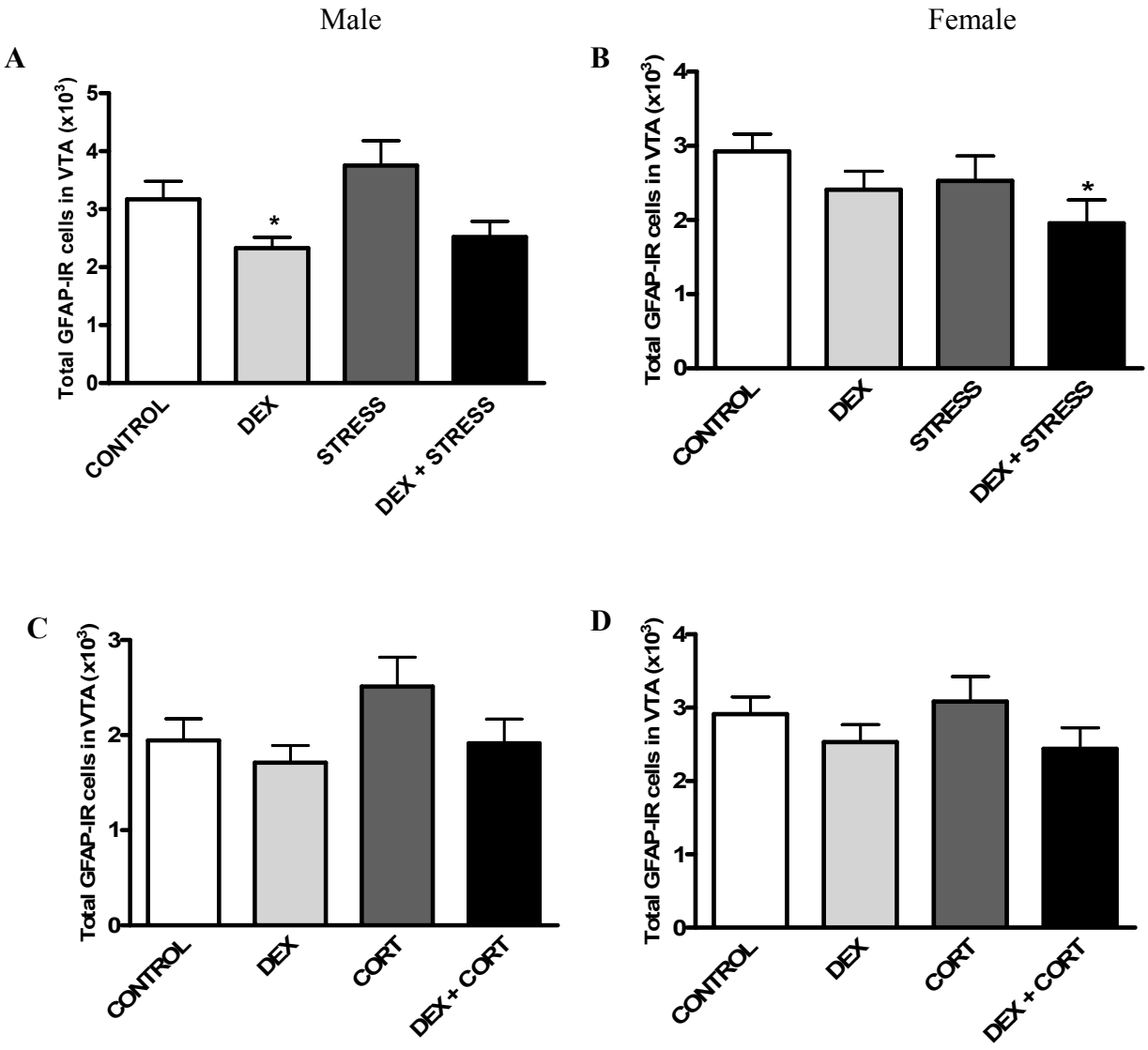


Figure 4.11. Effect of prenatal DEX treatment and/or adult stress or GC treatment on GFAP-IR cells within the VTA following 6-OHDA lesioning. Stereological estimates of the number of GFAP-IR cells within the ipsilateral VTA of male (A,C) and female (B,D) animals exposed to a 6-OHDA lesion were determined by GFAP immunohistocemistry. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 8(DEX), 8(stress), 7 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 67(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

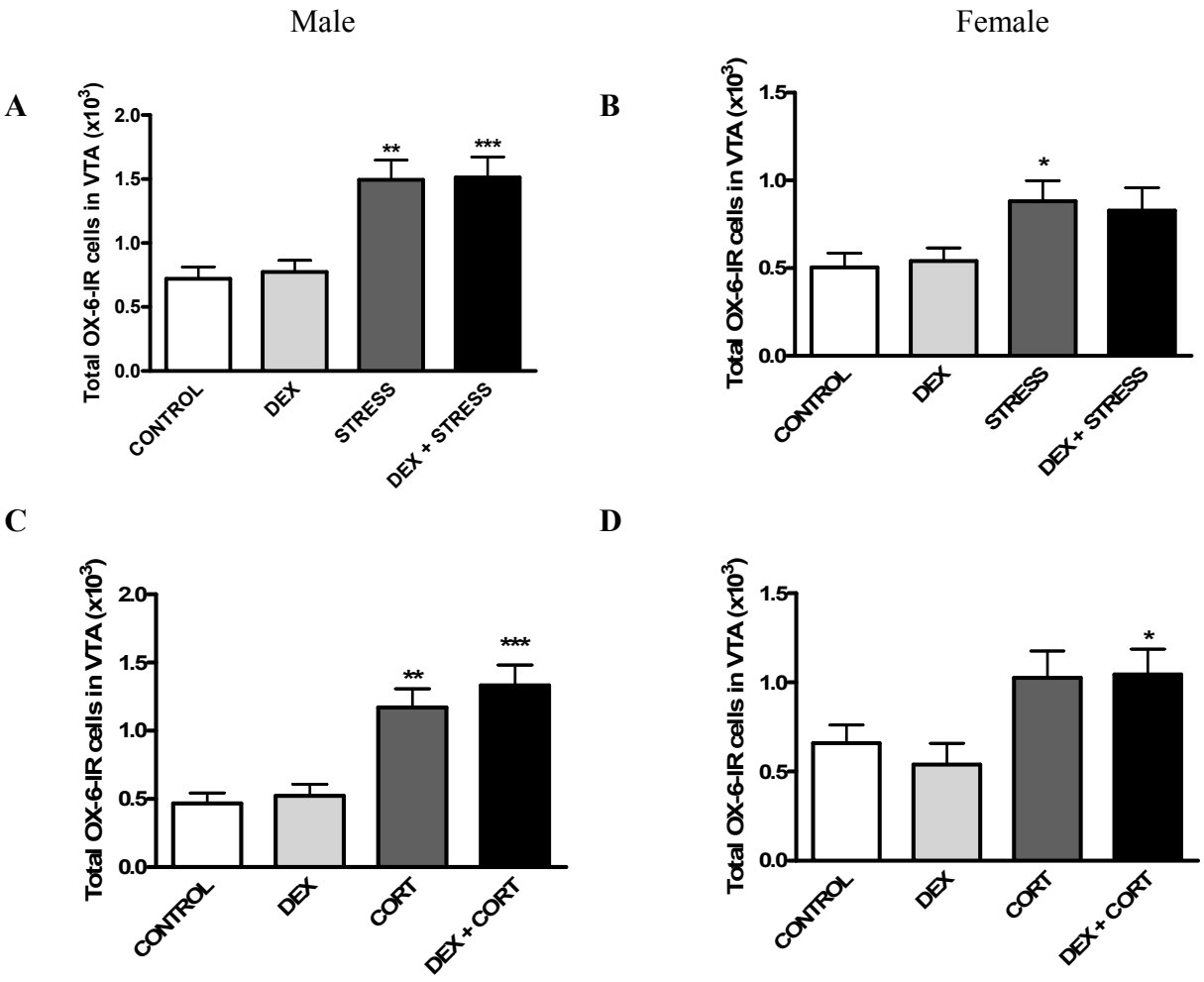


Figure 4.12. Effect of prenatal DEX treatment and/or adult stress or GC treatment on OX-6-IR cells within the VTA following 6-OHDA lesioning. Stereological estimates of the number of OX-6-IR cells within the VTA of male (A,C) and female (B,D) animals exposed to a 6-OHDA were determined by OX-6 immunohistocemistry. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 8(DEX), 8(stress), 7 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 67(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

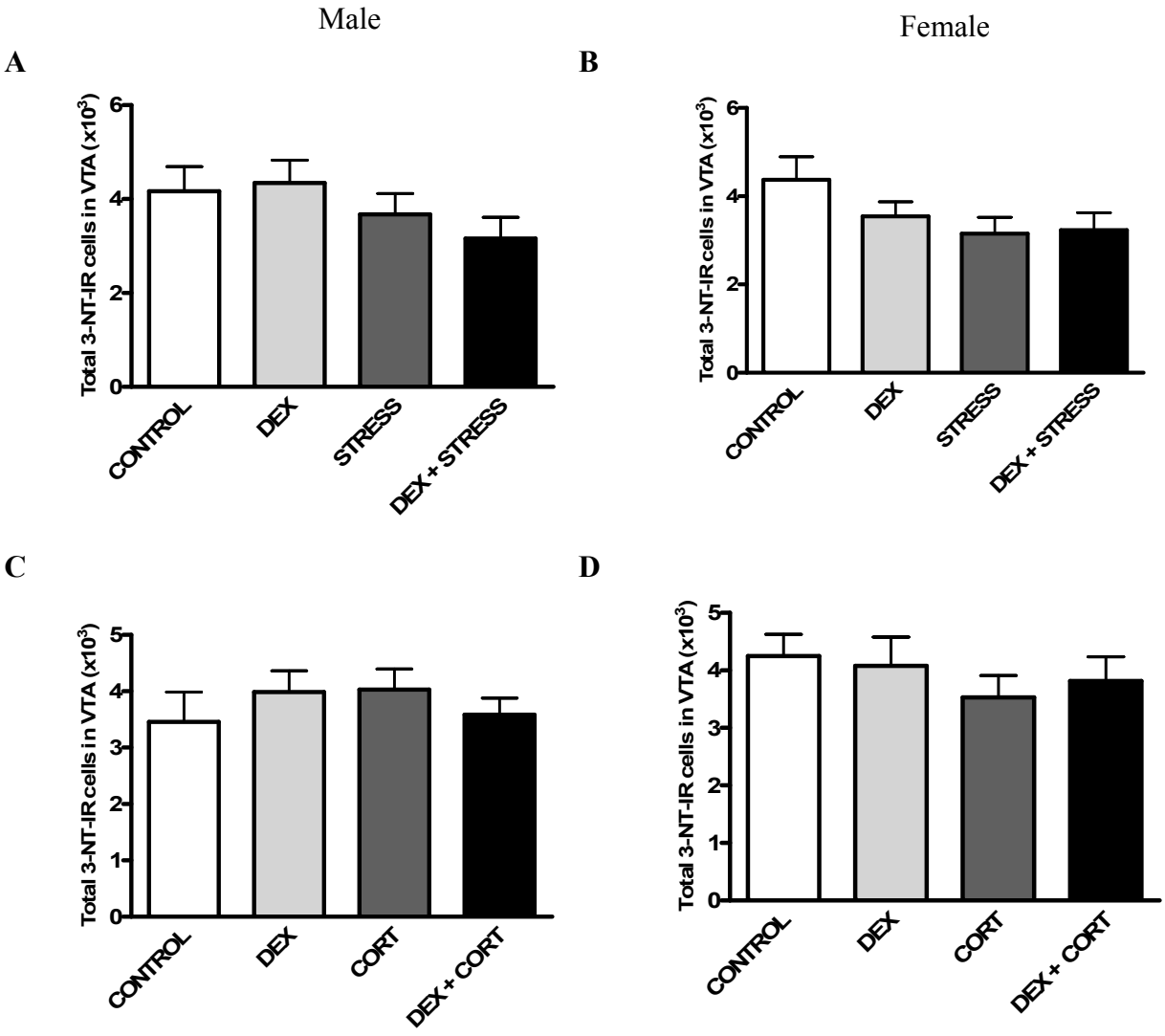


Figure 4.13. Effect of prenatal DEX treatment and/or adult stress or GC treatment on 3-NT-IR cells within the VTA following 6-OHDA lesioning. Stereological estimates of the number of TH-IR cells within the VTA of male (A,C) and female (B,D) animals exposed to a 6-OHDA were determined by 3-NT immunohistocemistry. Measurements were made in 6 month old animals, 2 weeks after exposure to 5µg 6-OHDA injected into the MFB. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukey's post hoc comparison tests *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=8 (control), 8(DEX), 8(stress), 7 (DEX + stress). B: n= 8 (control), 8 (DEX), 8 (stress), 8 (DEX + stress). C: n = 7 (control), 7 (DEX), 7 (CORT), 67(DEX + CORT). D: n=7 (control), 7 (DEX), 7 (CORT), 7 (DEX + CORT).

4.4. DISCUSSION

The data presented within this chapter demonstrate that stress and GC treatments exert persistent sexually dimorphic effects on the MLDA pathway. Significant emerging patterns include the ability of adult exposure to either repeated stress or chronic GC treatment to suppress DA content in the female NAcc but to enhance it in males. These changes appear not to be direct consequences of changes in the total numbers of DA neurons in the VTA, and not to be influenced by treatment-induced changes in the patterns of glial cell numbers in the VTA, which follow a similar trend in males and females. It is likely, therefore, that the MLDA pathway itself, as well as input pathways that regulate its activity, are targets for the actions of GCs and stress. Notably, prenatal DEX treatment eliminates the effect of adult stress and GCs on NAcc DA content in males, but not females. This indicates an important role for the MLDA in GC programming and adds to the evidence in the literature that programming is sexually dimorphic. The work also provides the first evidence that at the level of the NAcc, the MLDA system in females is considerably more susceptible to a neurodegenerative stimulus (6-OHDA) than males. Although the degenerative response of the male system appears resistant to stress or GC treatment in adulthood, both these factors exacerbate the loss of TH-IR cells in the VTA and NAcc DA in adult males previously exposed to DEX. In complete contrast, all treatments (DEX, stress, GCs, DEX + stress, DEX + GCs) suppress 6-OHDA-induced toxicity in the female NAcc. On balance, the MCDA system, as assessed by PFC DA levels, appears relatively unresponsive to treatments. Overall, these data add to the body of evidence supporting the premise that stress and GCs modify DA pathways and support the view that the effects of adult stress on MLDA are due, at least in part, to GCs. They also highlight novel interactive effects of

early life and adult treatments that could have implications for the development of neuropsychiatric disease.

4.4.1 Adult treatments

The effects of adult stress and GC exposure on the MLDA and MCDA have been widely studied and exert similar effects to that of drugs of abuse. Microdialysis studies revealed that like acute exposure to drugs of abuse, acute exposure to stress increases extracellular DA within the ventral striatum of humans and that repeated stress or repeated drug exposure results in the sensitisation (Sorg and Kalivas, 1993) that underlies addiction. In the present study repeated stress in adult male rats increased the number of TH-IR, GFAP-IR and OX-42-IR cells in the VTA. Similarly, chronic drug exposure has previously been shown to produce increased TH and GFAP levels and reduced levels of neurofilament proteins in the VTA (Ortiz et al., 1995). A study by the same group later revealed that chronic unpredictable stress produced similar effects within the VTA (Ortiz et al., 1996). In support of these findings the present data show that repeated restraint stress also produces a persistent elevation in DA levels and DA turnover in the NAcc suggesting that increases in the number of dopaminergic neurons results in elevated DA levels and activity in the male NAcc .

Repeated exposure to stress enhances behavioural responses to subsequent stress but also to drugs of abuse (Robinson and Becker, 1986; Sorg and Kalivas, 1991; Stewart and Badiani, 1993) that are associated with augmented DA release in the NAcc (Doherty and Gratton, 1992). Cross sensitisation of stress and drugs of abuse is associated with their similar effects that are characterised by enhanced DA release in the NAcc, decreased DAT expression and reduced D3 receptor mRNA in the NAcc shell

(Kalivas and Stewart, 1991; Doherty and Gratton, 1992; Brake et al., 2004). Although acute drug abuse increases MLDA activity, repeated use followed by withdrawal down regulates MLDA activity resulting in reduced basal and stimulated DA levels (Di Chiara et al., 2004). This may be due to autoregulation whereby increased DA levels reduce DA neuron firing by increasing activation of somatodendritic autoreceptors. Interestingly these autoreceptors are not present on projections to the PFC.

The study that examined the effects of chronic variable stress within the VTA also investigated the effects of chronic GC exposure and although this produced increases in TH, neither GFAP or neurofilament levels were altered (Ortiz et al., 1996). The present data do not indicate alterations in either GFAP-IR or TH-IR in response to chronic GC treatment suggesting that in this instance, mediators other than GCs may be involved in the effects of stress on the VTA. Nevertheless, suppression of GCs by adrenalectomy resulted in reduced extracellular DA levels within the NAcc shell both basally and in response to psychostimulants (Piazza et al., 1996; Barrot et al., 2000). Adrenalectomised animals also show reduced psychomotor stimulant effects of cocaine (Marinelli et al., 1997) and amphetamine (Cador et al., 1993) which can be reversed by administration of corticosterone. In addition to these effects, the reinforcing effects of drugs of abuse, as measured by self administration, are reduced in situations of low GC levels (Goeders and Guerin, 1996).

Chronic GC treatment also resulted in greater NAcc DA levels. Human studies have also reported that elevations in cortisol are correlated with DA accumulation within the ventral striatum (Oswald et al., 2005). Together these data imply that stress induced elevations of GCs result in increased DA levels and DA release that may increase the

reinforcing effects of drugs of abuse. This increase in NAcc DA content may be a result of changes in release, synthesis or metabolism. Drugs of abuse, stress and elevations in CRF and GCs have been shown to potentiate glutamate synapses on DA neurons within the VTA, enhancing the activity of DA neurons (Overton et al., 1996; Saal et al., 2003; Ungless et al., 2003; Wang et al., 2005). This activation of the MLDA by stress may represent a coping mechanism by which stress can increase motivation and drive.

GC's may impact on DA systems via their genomic actions through GR activation. GR is a transcriptional regulator that through binding to a GRE or interference with other transcription factors such as NF-kB, AP-1 or CREB can influence gene expression (De Bosscher et al., 2003). Promoter regions of genes encoding DA-synthesising enzymes, DA receptors and the DAT all have motifs for transcription factors such as AP-1 and CREB (Van Craenenbroeck et al., 2005). Additionally GCs could exert effects on DA systems via epigenetic mechanisms or non genomic actions.

4.4.2 Prenatal DEX treatment

Stressful experiences early in life are thought to predispose individuals to psychiatric disorders such as addiction, depression and schizophrenia which have been linked to dysfunction in the MLDA and MCDA pathways. Here we report that prenatal DEX exposure increased TH-IR and NeuN-IR within the adult male VTA, supporting previous findings by (McArthur et al., 2007a) but not (Leao et al., 2007) who report a reduced number of TH-IR cells in the adult VTA of prenatally DEX treated animals. These discrepancies may be due to the route of administration of DEX, which was via the maternal drinking water in the McArthur study and the present study but by sub cutaneous injection, which would cause additional stress to the mother in the Leao

study. Additionally the dose of DEX received in the Leao study was higher than in the McArthur study and the present study. Together however, these studies confirm that prenatal GCs are capable of exerting programming effects on the VTA that persist into adulthood, although this may depend upon the dose and method of administration. As extensive neurogenesis and cell death take place during development it is possible that alterations in GC levels during brain development may interfere with this delicate balance. Indeed, GCs are known to impact upon both neurogenesis and apoptosis (Sapolsky, 1996). However, further studies are required to determine how these processes are affected by GCs in the developing midbrain DA systems. In addition to their effects on cell survival, it is possible that GCs could affect other factors such as the nuclear orphan receptor nurr1. Nurr1 is present in 95% of TH-IR neurons within the VTA (Backman et al., 1999) and is essential for the development and survival of midbrain DA neurons (Vitalis et al., 2005). Intriguingly it has recently been determined that GR is a transcriptional regulator of nurr1 (Carpentier et al., 2008), thus activation of GR by the GR agonist DEX may modify the activity of nurr1 influencing the development and survival of DA neurons. DEX treatment not only affected neurons within the VTA but it also modified astrocyte and microglial populations. Within the adult male VTA the astrocyte population was reduced but the microglial population increased. These changes may alter the integrity of the MLDA and MCDA systems.

In addition to alterations at the level of the VTA, levels of DA within the adult NAcc were increased in males that had received DEX prenatally. Although studies have not previously reported elevated basal levels of NAcc DA, there is compelling evidence to suggest that early life exposure to stressors is associated with an increase in activity within the MLDA. For example anoxia (Boksa and El-Khodor, 2003), maternal

separation (Meaney et al., 2002) and prenatal exposure to LPS (Fortier et al., 2004) all lead to increased DA release in response to stress or amphetamine. The fact that prenatal exposure to GCs also modifies the MLDA and MCDA systems implies that they may underlie the effects of stress early in life. Early life stress has been shown to alter DA receptor expression and decrease DAT availability later in life (Berger et al., 2002) (Diaz et al., 1998) (Meaney et al., 2002). Reduced DAT expression may result in enhanced post-synaptic DA activity as less DA is cleared from the synapse.

4.4.3 Prenatal DEX in combination with adult treatments: Curiously, preceding perinatal GC exposure attenuated DA levels in both stressed and GC treated adult male rats. This novel finding suggests that the male MLDA is susceptible to early life programming where it results in an altered response to an adult challenge in male animals. Early life exposure to stress and GCs results in an increased HPA response to stress (Plotsky and Meaney, 1993; Liu et al., 1998) and reduced negative feedback sensitivity (Ladd et al., 2000), thus one would expect prolonged elevation of GC levels in GC or stress treated rats that received DEX prenatally. As TH-IR was not altered in these animals it is possible that increases in DA release, possibly by excessive DA action on autoreceptors or increases in uptake, by alterations in DAT expression or function may underlie these changes. As there was no change in DA turnover and there is no evidence to suggest metabolising enzymes are under GC control (Lindley et al., 2005) it is unlikely that the effects seen are due to altered metabolism of DA. Further studies are required to determine the mechanism behind this interactive effect.

4.4.4 Effect of stress and GC treatments on the MCDA

The HPLC data presented within this chapter for PFC DA levels show some important inconsistencies which make it difficult to interpret the data unless these experiments are repeated. In particular female DA levels in study 2 look extremely high (Table 4.2 and 4.5). Although we didn't find a consistent effect on DA levels in the PFC, it has previously been shown that prenatal and postnatal stress result in decreased dopaminergic drive to the PFC (Brake et al., 2000). The PFC is dependent upon environmental experiences for maturation (Zhang et al., 2005b) thus is particularly susceptible to the effects of GCs. Previous studies have shown that early life stress results in increased synaptic densities within the PFC and altered densities of DA and 5-HT terminals (Braun et al., 2000). In human studies child maltreatment as well as causing neuroendocrine changes also results in alterations in the size and volume of the PFC that correlate with the initiation of addictive behaviour (De Bellis et al., 2002).

Chronic GCs exposure has been shown to cause structural changes within the PFC (Wellman, 2001) (Brown et al., 2005; Cerqueira et al., 2005) and it has been established that there is an increase in DA activity in the PFC in response to a variety of stressors (Gresch et al., 1994; Finlay et al., 1995; Cuadra et al., 1999; Cabib et al., 2002; Dalla et al., 2008). It has been proposed that this is an adaptive mechanism that results in inhibitory control of the NAcc. Our data did not show any effect of adult stress or GC treatment on PFC DA in naïve animals with the exception of increased DA turnover in DEX treated males.

4.4.5 Inflammation and neuropsychiatric disorders

The HPA axis and immune axes are inextricably linked and there is bi-directional crosstalk between the two systems. Indeed, in depressed patients there is activation of the innate immune system as well as the presence of elevated GC levels (Herbert and Cohen, 1993; Maes, 1999). Immunologic alterations in depression occur as a result of an impaired balance of Th1 and Th2 inflammatory mediators both of which can be increased by circulating GC levels. Potentially activation of microglia may increase glutamate levels resulting in neurotoxicty. Importantly evidence of neurotoxicity has not yet been confirmed in depression. The present data show that both prenatal DEX treatment and adult stress result in an elevated number of microglial cells within the VTA of adult animals. This increased population of microglia may increase vulnerability to this proposed neurotoxic pathway. Additionally in DEX treated animals we consistently found a reduced population of astrocytes in adulthood. As astrocytes are responsible for mopping up excess glutamate (Danbolt, 2001) and promote neuronal survival via the release of growth factors, this reduction in astrocyte population may further endanger DA systems. Schizophrenia may also have an inflammatory component. It has been shown that there is a decreased production of Th1 cytokines such as IFN-gamma (Wilke et al., 1996; Muller et al., 2000). There is a neurodevelopmental risk for schizophrenia associated with obstetric complications and pre and post natal infections that has been suggested to be associated with glutamate excitotoxic damage within the fetal brain (Fearon 200; Farker 1995). Type-1 cytokines can result in stimulation of NMDA receptors through the accumulation of kynurenic acid that has been reported to be elevated in the PFC of patients with schizophrenia. Thus, a deficit in glutamate transmission may result in DA dysfunction and an uncontrolled flow of sensory information to the PFC resulting in psychotic problems

(Muller 2008). Alterations in HPA function as well as the altered populations of astrocytes and microglia that were seen in response to treatment could well contribute to immune dysfunction that in turn may contribute to the onset of disorders such as schizophrenia and depression.

4.4.6 Sex differences

Here we confirm previous reports of innate sex differences in the MLDA system where females have a higher number of dopaminergic neurons in the VTA and greater NAcc DA content. It is known that males and females have different susceptibilities to addictive behaviour and mood disorders (Simon and Volicer, 1976). They also differ in GC secretory profiles and in GC responses to stress (Patchev et al., 1997) as well as in hormonal and behavioural responses to prenatal stress (Bowman et al., 2004). It is perhaps therefore unsurprising that the effects of stress and GC treatments result in significantly different effects within the female rat MLDA and MCDA systems. Unlike McArthur et al., 2005, 2007a) we did not see an increase in adult TH-IR in the VTA of females exposed to prenatal DEX. Interestingly in contrast to the elevated DA levels in the NAcc of male animals treated with prenatal DEX, levels of DA within the adult female NAcc were reduced in prenatally DEX treated animals. This reduction in DA was independent of changes in TH-IR in the VTA

Human studies show that women are more vulnerable to addiction (for review see (Lynch et al., 2002). Furthermore animal studies show that female rats are more sensitive to the psychomotor activating effects of psychostimulants than males and show greater psychomotor sensitisation (Hu et al., 2003) . Evidence that estrogen is involved in this sex difference comes from studies demonstrating that ovariectomy attenuates amphetamine induced DA release and that estrogen agonists augment DA

release (Robinson et al., 1981; Xiao and Becker, 1998). The MLDA is also influenced by the stage of estrus. Becker and Cha (1989) showed that amphetamine induced DA release was higher during estrus than at any other stage of the cycle. Our finding of attenuated DA levels in response to stress or GC treatments contrasts significantly with their effect in males and appears to contradict these findings. However, DA activity as measured by DA turnover was increased in our female treatment groups. This increased DA activity in females and subsequent depletion of dopamine may result in an increased propensity to addictive behaviour, although this remains to be tested.

The ability of prior DEX treatment to eliminate the effects of adult stress in males was not seen in females. This adds to the literature supporting the idea that programming effects are sexually dimorphic (Bale, 2009). If correct these effects may contribute to sex differences in prevalence of neurodevelopmental disorders. This is further supported by evidence in the MCDA system. Although data from the PFC is inconsistent there was decreased DA turnover within the PFC of DEX treated females that were subjected to stress in adulthood. This provides evidence that prenatal exposure to GCs endangers the female MCDA rendering it susceptible to adult stress, thus corroborates the idea that early life stress may predispose to depressive behaviour in adult females. It is possible that interactions between GC and sex hormone signalling may underlie this vulnerability, indeed estrogen has been shown to amplify stress induced PFC dysfunction in female rats (Shansky et al., 2004). Additionally, recent reports suggest that the male and female MCDA pathway have different proportions of DA neurons (Kritzer and Creutz, 2008). Again, it is possible that interplay between organisational hormones such as GCs and gonadal hormones may impact upon this during development resulting in a vulnerable phenotype. (Dalla et al., 2008) compared the

effects of two types of stress in males and females. They reported either a lack of, or reduced PFC DA activity in females compared to males. This would reduce inhibitory control of the NAcc thus resulting in a failure to cope that may be related to the higher prevalence of depression in females.

4.4.7 6-OHDA model

6-OHDA administered to the MFB resulted in a loss of TH-IR and depleted NAcc and PFC DA but to a lesser magnitude to that seen in the NSDA. Previous studies show that the VTA is less susceptible to degeneration than the SNpc (Matzuk and Saper, 1985; Sullivan and Szechtman, 1995) The lesion and subsequent DA loss in the female MLDA was greater than that in the male for both TH-IR cells and NAcc DA levels suggesting that the female MLDA is more susceptible to 6-OHDA neurotoxicity. Although prenatal DEX had no effect in males it was shown to be neuroprotective in the female MLDA in 6-OHDA treated rats. Similarly adult stress or GC treatment had little effect on DA or TH loss in males but attenuated them in females again suggesting that stress and GCs are neuroprotective in females. Interestingly DEX treatment exacerbated TH-IR loss in GC treated and DA loss in both stress and GC treated males. Within the contralateral hemisphere effects on TH-IR that were seen in naïve animals were lost whereas effects on NAcc DA levels were maintained in female but not male animals. This provides further evidence that effects on DA levels are independent of changes in neuronal numbers. Within the male contralateral NAcc, prenatal DEX treatment in combination with either adult treatment resulted in reduced DA content. This correlates with increased DA loss in these animals suggesting that prenatal DEX treatment can render the male MLDA susceptible to future insults. The effects of stress and GCs on the MLDA are largely comparable with the effects seen within the NSDA where stress and GCs are detrimental in males but protective in females. Furthermore sex differences

in susceptibility to 6-OHDA are unlikely due to changes in glial populations as stress and GC treatments produce similar effects on glia in males and females and these changes differ to those seen within the NSDA.

4.4.8 Conclusions

The data presented within this chapter adds to a wider body of evidence supporting crosstalk between steroids and neurotransmitter systems. Dopamine and stress related disorders are not always easy to study as they are multifactorial, involve complex pathways and often have both genetic and environmental components. Our data support growing evidence that females display a differential response and adaptation to stress**.** Stressful experiences early in life predispose individuals to neuropsychaitric disorders such as addiction, schizophrenia and major depression. Here it is reported that the MLDA and MCDA systems are susceptible to sex-specific programming effects of GCs. These alterations in the MLDA and MCDA systems that we report as a result of elevations of prenatal GC levels, improve our understanding of the effects of early life stress and GC exposure on the developing brain. Furthermore the data presented here along with earlier studies also provide evidence that adult stress and GC treatment can modify the MLDA and MCDA systems in a highly sexually dimorphic manner and that these modifications may result in sex differences in susceptibility to neuropsychiatric disorders such as depression and addiction later in life. In addition, as degeneration of these pathways occurs in PD they may well contribute to the development and the subsequent progression of a variety of neuropsychiatric disturbances that are present in PD patients.

CHAPTER 5

The sex dependent effects of stress and glucocorticoids on non-dopaminergic pathways affected in Parkinson's disease.

5.1 INTRODUCTION

In addition to the degeneration of DA systems, other ascending neurotransmitter systems are affected in PD. The noradrenergic (LC), serotonergic (dorsal raphe nucleus; DRN) and cholinergic (NBM) systems are all affected to a varying degree in PD (Jellinger, 1999). Disturbances in cognition and mood that are present in PD patients may be a result of complex interactions of these neurotransmitter pathways. This chapter therefore aims to determine the effects of stress and GCs on these transmitter pathways with a view to their involvement in PD.

5.1.1 Noradrenergic system

The locus coeruleus (LC), is located on the dorsal wall of the rostral pons and is the principal site of NA synthesis in the brain, with extensive projections throughout the entire CNS. NA shares the same biosynthetic pathway as DA with DA being converted to NA by DβH. The LC mediates arousal and is highly sensitive to alerting or stressfull stimuli. Many brain regions involved in the stress response are innervated by the LC and mediate alertness, focus and cognition (Foote 1995). There are complex interactions between the CRH neurons of the PVN and the neurons of the LC. CRH acts as a neurotransmitter within the LC, which receives projections from the PVN (Rassnick et al., 1998). Conversely central NA stimulates CRH neurons within the PVN (Sawchenko and Swanson, 1982) and lesions of the LC decrease stress induced ACTH and CORT release (Maccari et al., 1990; Ziegler et al., 1999). Thus it is thought that there is a positive feedback loop between the PVN and LC (Dunn et al., 2004). The LC also mediates autonomic effects during stress to increase sympathetic and decrease

parasympathetic outflow. Disruptions in the LC have been associated with clinical depression, panic disorder and anxiety, where there is evidence for excessive NA activity (Young et al., 2005).

The LC nearly always shows cell loss or lewy body inclusions (most often both) in PD patients (Forno et al., 1993) and there is evidence to suggest that neuronal loss in the LC may precede SN degeneration and even surpass it (German et al., 1992; Del Tredici et al., 2002; Braak et al., 2003; Zarow et al., 2003). The loss of NA innervation is also thought to facilitate the onset of dyskinesias in PD patients during DA replacement therapy (Fornai et al., 2007). Interestingly, PD patients with depression show more pronounced PD symptoms that can be alleviated by use of the NET (norepinephrine transporter) blocker reboxetine (Papapetropoulos et al., 2006; Pintor et al., 2006) again suggesting an involvement of NA in PD. Furthermore, α2 antagonists provide relief from L-dopa induced dyskinesias(Grondin et al., 2000; Archer and Fredriksson, 2003). NA neurons directly innervate both midbrain DA neurons and the striatum with stimulation of LC NA neurons facilitating the burst firing of SNpc neurons (Grenhoff et al., 1993).

5.1.2 The Cholinergic system

There are four groups of cholinergic neurons within the basal forebrain consisting of the medial septum, nucleus basalis of meynert (NBM), the vertical diagonal band and the horizontal diagonal band. The medial septum and vertical diagonal band form the medial pathway that innervates the hippocampus. The lateral pathway is composed of the horizontal diagonal band that innervates the olfactory bulb and the nucleus basalsis of meynert that innervates the neocortex and amygdale (Rye et al., 1984; Saper, 1984).

Cortical cholinergic transmission is involved in the mediation of arousal, attention and memory. Neurodegenerative diseases and age-related cognitive decline have partly been attributed to defects in cholinergic transmission (Bartus et al., 1982; Coyle et al., 1983; Kasa et al., 1997). For example during aging there is a loss of basal forebrain cells and cholinergic markers (Baxter et al., 1999). Cholinergic neurons have a large impact on cognitive processes (Myhrer, 2003) and a decreased activity of the cholinergic system can affect attention, learning, memory and contributes to the cognitive deficits observed in AD (McGaughy et al., 2000).

Cholinergic degeneration and the presence of Lewy bodies in the NBM has also been identified in PD patients (Perry et al., 1985; Tiraboschi et al., 2000). Subsequently, a cholinergic deficit has been hypothesised to play a role in the development of dementia and psychosis in PD that occurs in 20-39% of patients. Indeed, the degree of cholinergic deficit is related to the severity of cognitive impairment in PD patients (Mattila et al., 2001).

5.1.3 Serotonergic system

5-HT is synthesised by a small group of nuclei in the brain stem known as the raphe nuclei (Dahlstrom and Fuxe, 1964). These can be split into two groups: the inferior group that mainly project to the grey matter of the spinal cord and a superior group composed of the dorsal raphe nucleus (DRN) and medial raphe neucleus (MRN) which together provide about 80% of the 5-HT input to the forebrain. Although there is considerable overlap between the projection fields of these two nuclei the MRN preferentially innervates the hippocampus, preoptic area, suprachiasmatic nucleus,

olfactory bulb and medial septum with the DRN preferentially innervating the basal ganglia circuitry including the corpus striatum, globus pallidus, STN, SN, PPN, VTA, NAcc and most innervation to the PFC. Despite this discrete population of serotonergic nuclei there is an almost ubiquitous distribution of 5-HT containing terminals. There are 7 classes of 5-HT receptor with 15 subtypes (Hoyer et al., 2002) leading to diverse effects of 5-HT signalling. 5-HT is known to play a role in the control of mood, emotion, sleep and appetite.

Biochemical evidence suggests 5-HT transmission is abnormal in the basal ganglia of PD patients (Hornykiewicz, 1998) and is involved in the resting tremor (Doder et al., 2003) and L-Dopa induced dyskinesias (Bezard et al., 2001; Carta et al., 2007). A loss of 5-HT cell bodies in the DRN of PD patients has been reported (find ref). Post-mortem studies show that there is decreased 5-HT in the cortex and striatum (Scatton et al., 1983) (Birkmayer and Birkmayer, 1987) and a reduction in all 5-HT markers in the caudate (Kish et al., 2008). In-vivo studies show that there is reduced 5-HIAA in csf and reduced 5-HT activity in the caudate, putamen, thalamus and mPFC of PD patients (Haapaniemi et al., 2001; Kim et al., 2003).

5.2 EXPERIMENTAL DESIGN

In order to investigate the effects of stress and GCs on non dopaminergic neurotransmitter systems affected in PD we assessed the morphology of the LC and NBM using immunohistochemical markers for NA and ACh neurons. NA cell number within the LC was assessed using immunohistochemistry for DβH and NeuN along with stereological quantification techniques. The number of neurons within the NBM was similarly assessed by stereological quantification of cells immunoreactive for choline-acetyl-transferase (ChAT) and NeuN. Furthermore the glial populations within these two regions were quantified by immunohistochemical identification of GFAP and OX-42. 3-NT immunoreactivity was also assessed within both the LC and NBM. The effects of stress and GCs on neurotransmitter levels were also investigated. HPLC was used to determine the concentration of DA, 5-HT and the 5-HT metabolite 5-HIAA within the PFC, NAcc and striatum. Study 1 investigated the effects of prenatal treatment with DEX and/or adult repeated restraint stress and study 2 the effects of prenatal DEX and/or adult chronic GC treatment. For more detailed methodology see chapter 2.

5.3 RESULTS

5.3.1 Noradrenergic system

Innate sex differences: Female controls had a significantly greater number of DβH-IR cells in the LC than male controls (487.7 ± 32.49 in males vs 641 ± 32.97 in females; $p<0.01$; Figure 5.1). The same was true for NeuN-IR cells where males had 1532 ± 66 and females 1744 ± 61.4 ($p<0.05$) NeuN-IR cells within the LC (Figure 5.2). There was no significant effect of sex on GFAP, OX-42 or 3-NT immunoreactivities within the LC (Figures 5.3-5.5). Within the striatum and PFC levels of NA were higher in females than males ($p<0.01$ and $p<0.001$ respectively; Table 5.1, 5.3). There was no sex difference in NAcc NA levels (Table 5.2)

Adult treatments: Adult repeated stress or adult chronic GC treatment had no effect on the NA system at the time point examined with the exception of a doubling of striatal NA in female rats subjected to adult GC treatment ($p<0.05$; Table 5.1), which was further increased 4-fold compared with controls after Dex ($p<0.001$; Table 5.1).

Prenatal DEX treatment: Adult male and female rats that had received DEX treatment prenatally exhibited fewer DβH-IR cells than controls (Figure 5.1). In study 1 the number of DβH cells was reduced by ~34% from 454.5 ± 48.2 to 298.8 ± 41.9 in males ($p<0.05$) and by 25% from 642.8 ± 47.9 to 480.0 ± 46.9 in females ($p<0.05$). Similarly in study 2 the number of cells in prenatally DEX treated rats compared to controls fell by ~35% in males ($p<0.05$) and ~22% in females ($p<0.05$) (Figure 5.2). DEX treated males also exhibited fewer NeuN-IR cells in the LC (-19%) in both study 1

and 2 ($p<0.05$). In the female, NeuN-IR cell number was reduced by 16% in the prenatal DEX group in study 1 ($p<0.05$) but this was not significant in study 2 ($p=0.07$). Prenatal DEX treatment reduced the number of GFAP-IR cells by ~26% in adult males and females (although this did not reach significance in study 1 males where $p=0.09$; Figure 5.3). However, prenatal DEX treatment had no effect on adult numbers of OX-42-IR or 3-NT-IR cells within the LC and had no influence on NA levels in the projection areas studied.

Prenatal DEX treatment in combination with adult treatments: Animals that were subjected to stress in adulthood and had received prenatal DEX treatment had a significantly lower number of DβH-IR LC cells compared to stressed or control groups in both males and females ($p<0.05$; Figure 5.1). In these animals the number of NeuN-IR cells within the LC was no longer significant but in females was significantly lower than the group that received adult stress only ($p<0.05$; Figure 5.2). Males subjected to both prenatal DEX and adult GC treatment had a DβH-IR cell number not significantly different to controls but significantly lower than the GC treated group ($p<0.05$) whereas females had fewer DβH-IR cells compared to controls and the GC treated group ($p<0.05$). Both male and females subjected to prenatal DEX and adult GC treatment had fewer NeuN-IR cells in the LC (Figure 5.2). Interestingly, females exposed to the combination of prenatal DEX and adult GC treatment had greater levels of striatal NA when compared to controls or the GC treated group ($P<0.001$/$p<0.05$; Table 5.1).

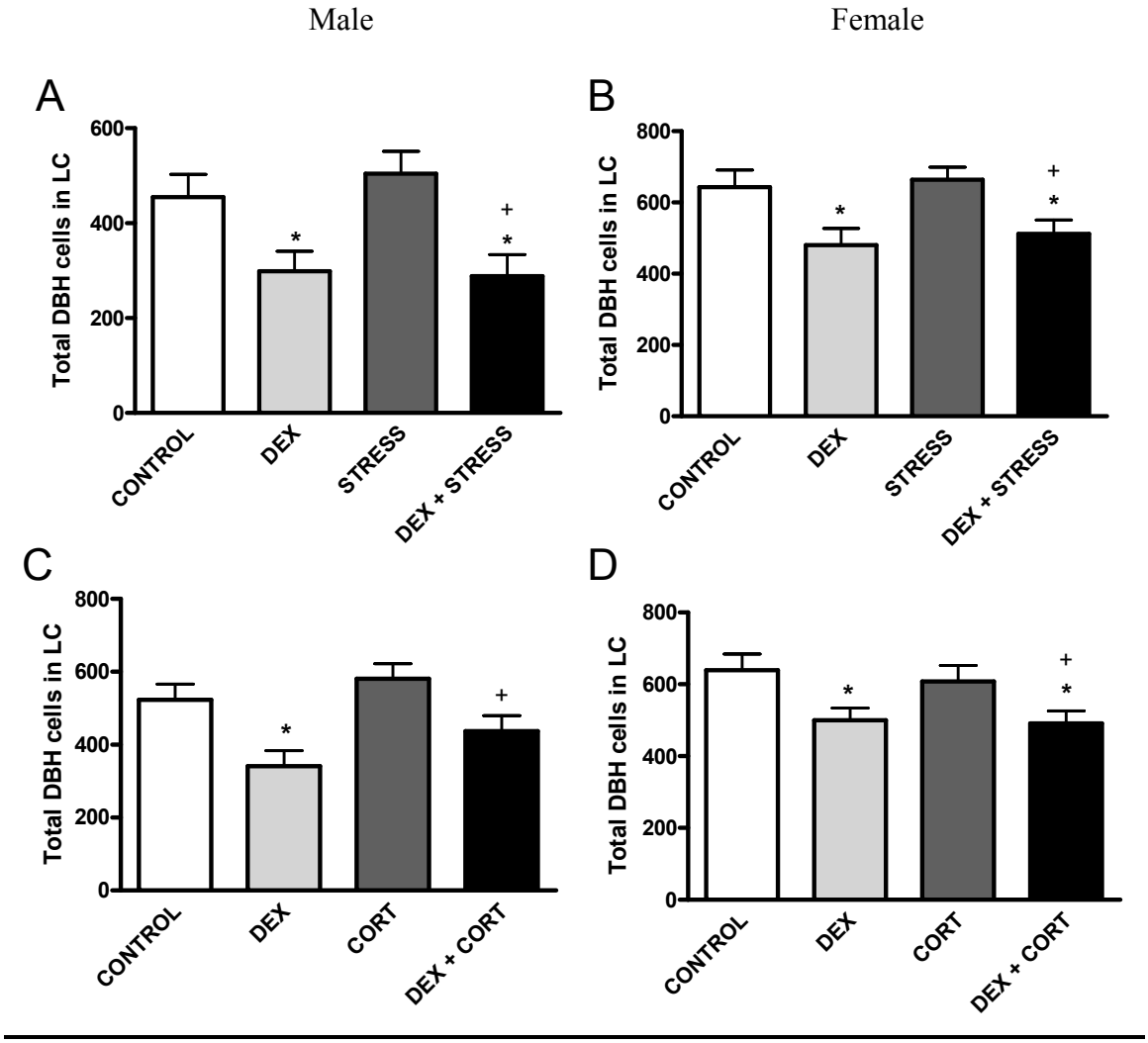


Figure 5.1. Effect of prenatal dexamethasone and/or adult stress or GC treatment on DβH-IR cells within the LC. Stereological estimates of total DβH-IR cells within the LC in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by DβH immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=6 (control), 7 (DEX), 8(stress), 7 (DEX + stress). B: n= 7 (control), 8 (DEX), 7 (stress), 8 (DEX + stress). C: n = 7 (control), 6 (DEX), 5 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 6 (CORT), 7 (DEX + CORT).

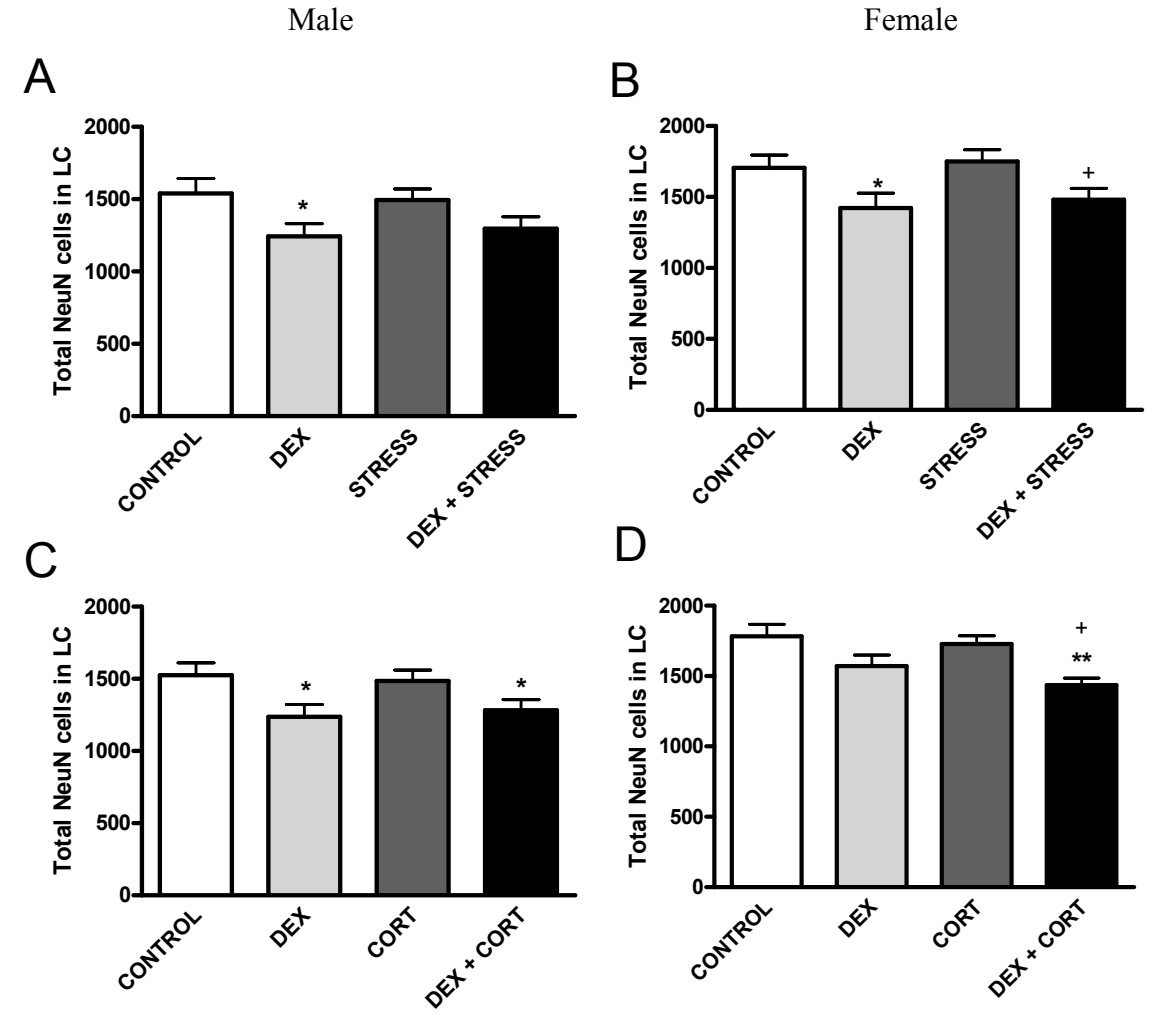


Figure 5.2. Effect of prenatal dexamethasone and/or adult stress or GC treatment on NeuN-IR cells within the LC. Stereological estimates of total NeuN-IR cells within the LC in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by NeuN immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls # vs DEX, + vs adult treatment. A: n=6 (control), 7 (DEX), 8(stress), 7 (DEX + stress). B: n= 7 (control), 8 (DEX), 7 (stress), 8 (DEX + stress). C: n = 7 (control), 6 (DEX), 5 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 6 (CORT), 7 (DEX + CORT).

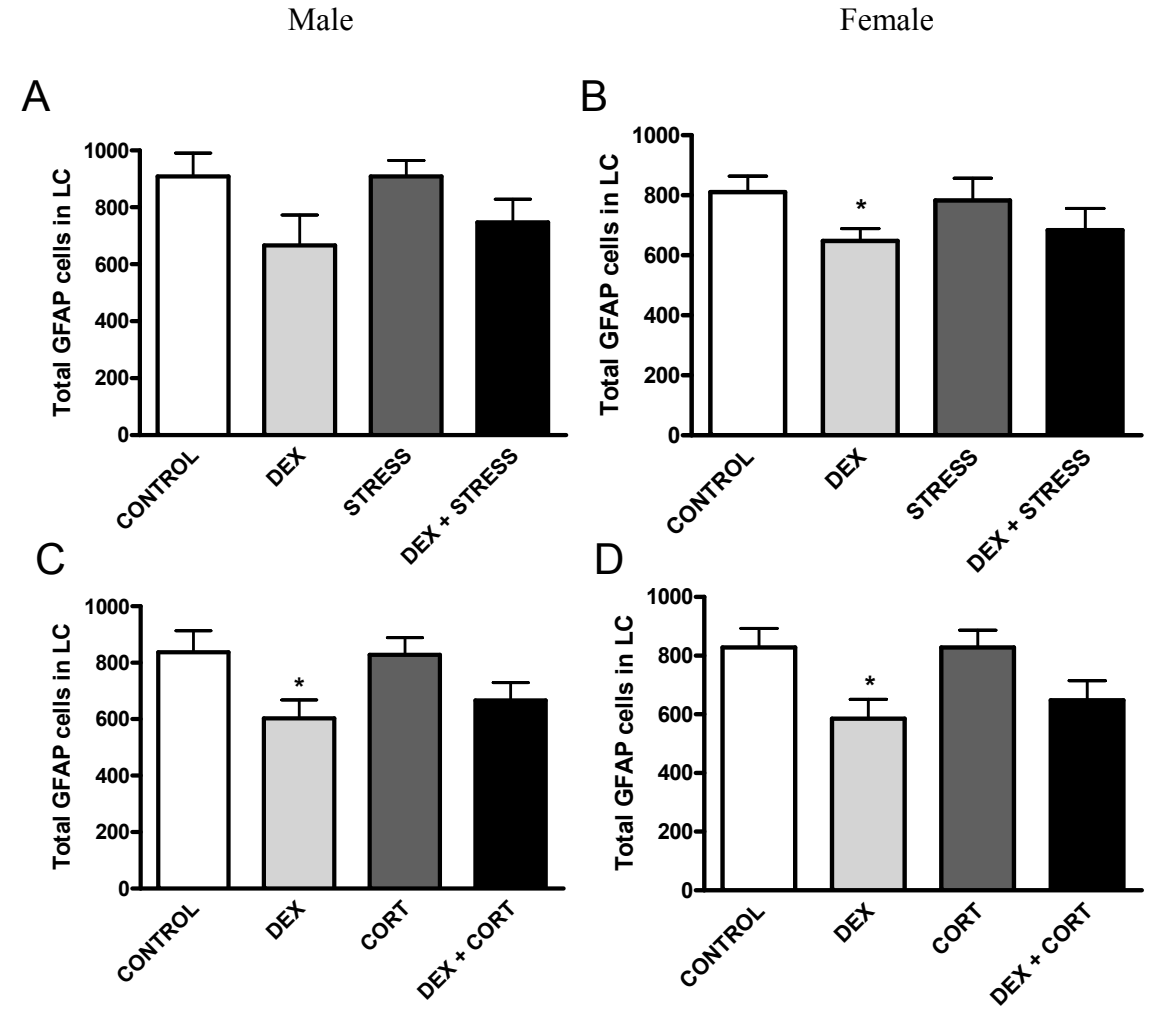


Figure 5.3. Effect of prenatal dexamethasone and/or adult stress or GC treatment on GFAP-IR cells within the LC. Stereological estimates of total GFAP-IR cells within the LC in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by GFAP immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=6 (control), 7 (DEX), 8(stress), 7 (DEX + stress). B: n= 7 (control), 8 (DEX), 7 (stress), 8 (DEX + stress). C: n = 7 (control), 6 (DEX), 5 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 6 (CORT), 7 (DEX + CORT).

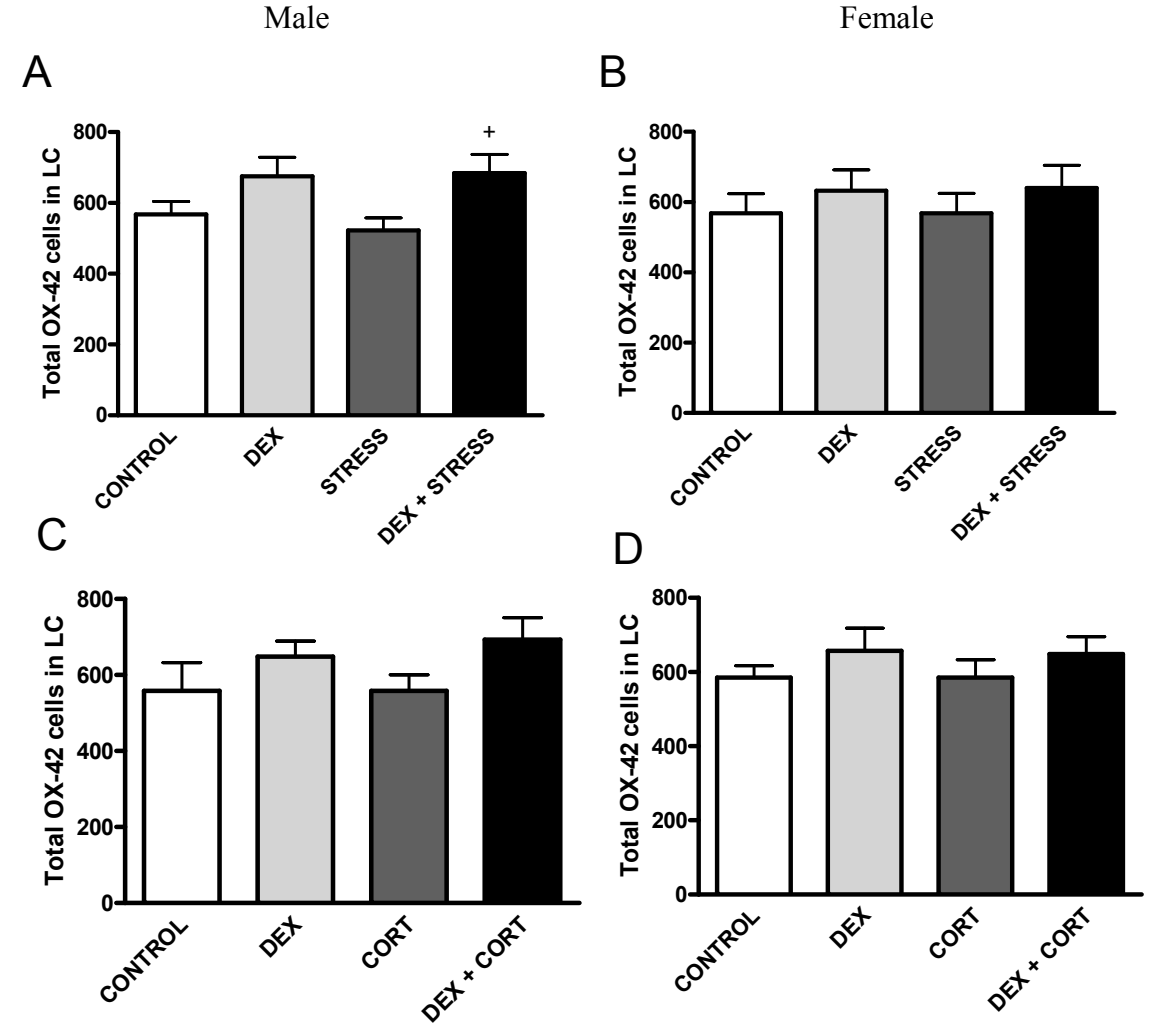


Figure 5.4. Effect of prenatal dexamethasone and/or adult stress or GC treatment on OX-42-IR cells within the LC. Stereological estimates of total OX-42-IR cells within the LC in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by OX-42 immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and/or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks). Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. A: n=6 (control), 7 (DEX), 8(stress), 7 (DEX + stress). B: n= 7 (control), 8 (DEX), 7 (stress), 8 (DEX + stress). C: n = 7 (control), 6 (DEX), 5 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 6 (CORT), 7 (DEX + CORT).

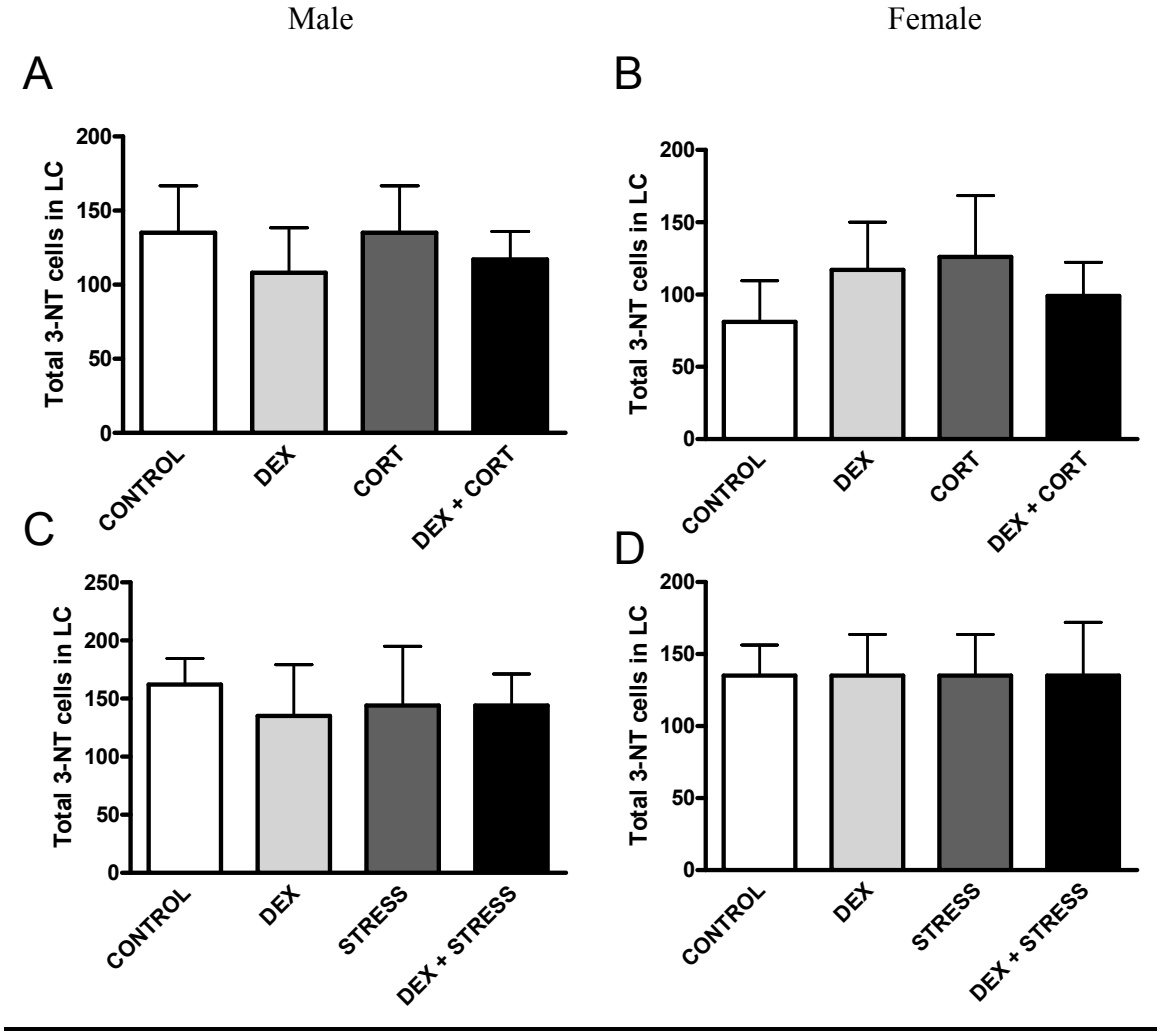


Figure 5.5. Effect of prenatal dexamethasone and or adult stress or GC treatment on 3-NT-IR cells within the LC. Stereological estimates of total DβH-IR cells within the LC in adult (5.5 month) male (A,C,) and female (B,D,) animals as assessed by DβH immunohistochemistry. A and B indicate the effect of prenatal (GD16-19) DEX treatment and / or adult (3.5 month) stress (repeated restraint for 1 hour every other day for 6 weeks). C and D depict the effects of prenatal DEX treatment and or adult GC treatment (hydrocortisone via the drinking water for 6 weeks) Data are expressed as means ± SEM. Statistical significance was assessed by two way ANOVA with tukeys post hoc comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls #p<0.05 vs adult treatment.. A: n=6 (control), 7 (DEX), 8(stress), 7 (DEX + stress). B: n= 7 (control), 8 (DEX), 7 (stress), 8 (DEX + stress). C: n = 7 (control), 6 (DEX), 5 (CORT), 5 (DEX + CORT). D: n=7 (control), 6 (DEX), 6 (CORT), 7 (DEX + CORT).

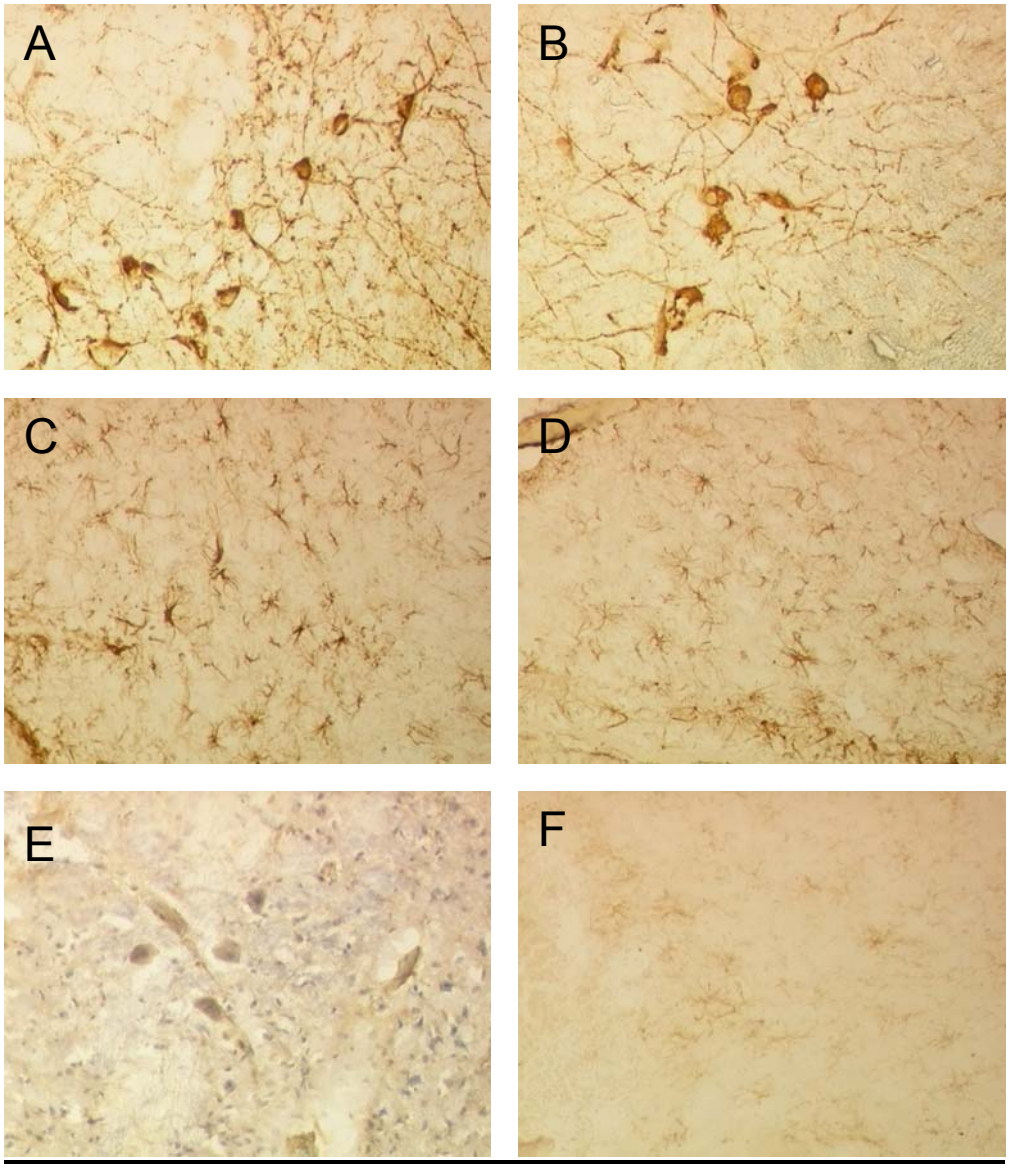


Figure 5.6. Representative photomic rographs of immunohistochemical staining within the LC. DβH in male control(A) and DEX treated(B) rats. GFAP in male control(C) and DEX treated(D) rats. OX-42(E) and 3-NT(F) in male controls. Images taken at x200 magnification.

Adult striatal NA levels				
STUDY 1	**CON**	**DEX**	**STRESS**	**DEX + STRESS**
Male				
NA	0.12 ± 0.01	0.11 ± 0.005	0.09 ± 0.007	0.12 ± 0.01
Female				
NA	0.19 ± 0.02	0.26 ± 0.05	0.14 ± 0.03	0.18 ± 0.04

Adult striatal NA levels				
STUDY 2	**CON**	**DEX**	**CORT**	**DEX + CORT**
Male				
NA	0.09 ± 0.01	0.06 ± 0.009	0.08 ± 0.007	0.07 ± 0.01
Female				
NA	0.17 ± 0.03	0.32 ± 0.04	0.36 ± 0.08 *	0.69 ± 0.12***

Table 5.1 Effect of prenatal DEX an d/or adult repeated stress or GC treatment on NA levels within the striatum. Striatal NA was assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeated restraint stress or chronic GC treatment. Data are means ± SEM in ng/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. Study 1 male: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). Female:: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). Study 2: male:: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). Female: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

Adult nucleus accumbens NA levels				
STUDY 1	**CON**	**DEX**	**STRESS**	**DEX + STRESS**
Male				
NA	0.22 ± 0.06	0.23 ± 0.02	0.22 ± 0.03	0.22 ± 0.01
Female				
NA	0.17 ± 0.03	0.26 ± 0.05	0.14 ± 0.03	0.18 ± 0.04

Adult nucleus accumbens NA levels				
STUDY 2	**CON**	**DEX**	**CORT**	**DEX + CORT**
Male				
NA	0.33 ± 0.05	0.25 ± 0.06	0.19 ± 0.02	0.43 ± 0.11
Female				
NA	0.21 ± 0.02	0.25 ± 0.02	0.31 ± 0.06	0.27 ± 0.04

Table 5.2 Effect of prenatal DEX an d/or adult repeated stress or GC treatment on NA levels w ithin the NAcc. NAcc NA was assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeated restraint stress or chronic GC treatment. Data are means ± SEM in ng/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. Study 1: male: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). Female:: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). Study 2: male:: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). Female: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

Adult frontal cortex NA levels				
STUDY 1	**CON**	**DEX**	**STRESS**	**DEX + STRESS**
Male				
NA	0.61 ± 0.01	0.71 ± 0.04	0.77 ± 0.06	0.69 v ± 0.009
Female				
NA	0.82 ± 0.02	0.77 ± 0.03	0.74 ±	0.76 ± 0.04

Adult frontal cortex NA levels				
STUDY 2	**CON**	**DEX**	**CORT**	**DEX + CORT**
Male				
NA	0.58 ± 0.10	0.75 ± 0.11	0.70 ± 0.03	0.62 ± 0.05
Female				
NA	1.10 ± 0.05	1.05 ± 0.02	0.94 ± 0.03	0.99 ± 0.05

Table 5.3 Effect of prenatal DEX an d/or adult repeated stress or GC treatment on NA levels with in the PFC. PFC NA was assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeated restraint stress or chronic GC treatment. Data are means ± SEM in ng/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. Study 1: male: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). Female:: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). Study 2: male:: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). Female: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

5.3.2 Cholinergic system

No sex differences were observed in the number of neurons or glia within the NBM. Furthermore, prenatal DEX treatment or adult exposure to repeated stress or chronic GC treatment had no effect on the morphology of the NBM in either sex.

5.3.3 Serotinergic system

Innate sex differences: 5-HT levels were significantly higher in control females than in males within the striatum ($p<0.01/p<0.001$; Table 5.4), NAcc ($p<0.05/p<0.01$; Table 5.5) and PFC ($p<0.05$; Table 5.6) with male 5-HT turnover significantly exceeding that of females within the NAcc ($p<0.05/p<0.01$; Table 5.5) and PFC ($p<0.001$; Table 5.6).

Adult repeated stress: In the male repeated restraint stress in adulthood exerted no effect on the serotinergic system in the striatum or NAcc but increased 5-HT levels and reduced 5-HT turnover within the PFC (Table 5.6). In the female adult, stress increased 5-HIAA levels ($P<0.01$) and 5-HT turnover ($p<0.01$) in the striatum (Table 5.4) and within the NAcc reduced 5-HT levels ($p<0.05$) and increased 5-HT turnover ($p<0.05$; Table 5.5). In contrast to the male, repeated stress reduced 5-HT ($p<0.01$) and elevated 5-HT turnover ($p<0.01$) in the female PFC (Table 5.6).

Adult chronic GC treatment: Adult treatment with hydrocortisone did not alter 5-HT parameters in the male striatum or NAcc but reduced 5-HT turnover within the male

PFC ($p<0.05$). In females adult GC treatment increased 5-HIAA ($p<0.001$) and 5-HT turnover ($p<0.05$) in the striatum, elevated 5-HT turnover ($p<0.05$) in the NAcc and reduced 5-HT ($p<0.01$), increased 5-HIAA ($p<0.01$) and increased turnover ($p<0.001$) in the PFC.

Prenatal DEX treatment: Prenatal DEX treatment did not affect 5-HT measures in the male striatum or NAcc but did increase 5-HT levels within the male PFC in study 1 ($p<0.05$ vs control) which did not reach significance in study 2 ($p=0.059$; Table 5.6). However, prenatal DEX treatment reduced 5-HT turnover in the male PFC in both studies ($p<0.01$/$p<0.001$ vs control). Conversely, in females prenatal DEX treatment increased 5-HIAA levels ($p<0.01$ vs control – s1 only) and 5-HT ($p<0.05$/$p<0.001$) turnover within the striatum (Table 5.4). Within the female NAcc, prenatal DEX resulted in reduced 5-HT and 5-HIAA levels and increased 5-HT turnover ($p<0.001$) in study 2 (Table 5.5). These parameters were altered in a similar fashion in study 1 but did not reach significance. Interestingly and in contrast to the male prenatal DEX exerted no effect on 5-HT within the PFC.

Prenatal DEX treatment in combination with adult treatments: Although prenatal DEX, adult repeated stress and chronic GC treatment exerted no effect on striatal 5-HT on their own, a history of prenatal DEX treatment resulted in increased 5-HT turnover in male rats exposed to repeated stress ($p<0.05$) or chronic GCs ($p<0.05$) showing a significant interaction of the two treatments (insert stats). In the female, combination of prenatal DEX and adult stress had no interactive effect on 5-HT within the striatum. However, in females treated prenatally with DEX and then GCs in adulthood 5-HIAA levels ($p<0.001$) and 5-HT turnover ($p<0.05$) were significantly

attenuated . Within the NAcc and PFC there is no interaction between prenatal DEX and adult treatment in either se

Adult striatal 5-HT and 5-HIAA levels				
STUDY 1	**CON**	**DEX**	**STRESS**	**DEX + STRESS**
Male				
5-HT	1.43 ± 0.10	1.41 ± 0.07	1.26 ± 0.10	1.32 ± 0.11
5-HIAA	1.08 ± 0.04	1.11 ± 0.05	0.99 ± 0.06	1.24 ± 0.04
Turnover	1.58 ± 0.09	1.59 ± 0.04	1.61 ± 0.06	1.98 ± 0.13 *
Female				
5-HT	1.93 ± 0.11	1.97 ± 0.08	1.80 ± 0.20	1.76 0.18
5-HIAA	1.17 ± 0.04	1.50 ± 0.04 **	1.51 ± 0.07 **	1.37 ± 0.09 *
Turnover	1.27 ± 0.08	1.56 ± 0.06 *	1.82 ± 0.16 **	1.63 ± 0.09 *

Adult striatal 5-HT and 5-HIAA levels				
STUDY 2	**CON**	**DEX**	**CORT**	**DEX + CORT**
Male				
5-HT	0.31 ± 0.04	0.34 ± 0.06	0.25 ± 0.04	0.33 ± 0.10
5-HIAA	0.36 ± 0.05	0.30 ± 0.03	0.34 ± 0.02	0.42 ± 0.039
Turnover	2.09 ± 0.91	2.10 ± 1.18	3.27 ± 1.21	3.74 ± 1.72 *
Female				
5-HT	0.53 ± ± 0.14	0.40 ± 0.12	0.55 ± 0.10	0.55 ± 0.19
5-HIAA	0.42 ± 0.02	0.48 ± 0.02	0.55 ± 0.01 ***	0.42 ± 0.02 #
Turnover	1.661 ± 0.08	2.65 ± 0.12	2.09 ± 0.10 *	1.76 ± 0.17 #

Table 5.4 Effect of pre natal DEX and/or adult repeated stress or GC treatment on 5-HT and 5-HIAA level s within the striatum. Striatal 5-HT, 5-HIAA and 5-HT turnover were assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeaterestraint stress. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of 5-HIAA to 5-HT in pmol/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. Study 1 male: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). Female:: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). Study 2: male:: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). Female: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

Adult nucleus accumbens 5-HT and 5-HIAA levels				
STUDY 1	**CON**	**DEX**	**STRESS**	**DEX + STRESS**
Male				
5-HT	0.69 ± 0.16	0.85 ± 0.08	0.85 ± 0.14	0.89 ± 0.06
5-HIAA	1.28 ± 0.24	1.41 ± 0.06	1.04 ± 0.05	1.35 ± 0.05
Turnover	5.36 ± 1.13	3.54 ± 0.28	3.35 ± 0.64	3.24 ± 0.21
Female				
5-HT	1.13 ± 0.14	0.99 ± 0.18	0.64 ± 0.07 *	0.74 ± 0.14 *
5-HIAA	1.79 ± 0.15	1.78 ± 0.04	1.74 ± 0.09	1.84 ± 0.10
Turnover	3.40 ± 0.25	4.27 ± 0.65	6.01 ± 0.71 *	7.15 ± 1.80 *

Adult nucleus accumbens 5-HT and 5-HIAA levels				
STUDY 2	**CON**	**DEX**	**CORT**	**DEX + CORT**
Male				
5-HT	0.47 ± 0.07	0.38 ± 0.03	0.42 ± 0.03	0.35 ± 0.02
5-HIAA	0.67 ± 0.07	0.76 ± 0.05	0.86 ± 0.05	0.78 ± 0.04
Turnover	3.62 ± 0.68	4.21 ± 0.25	4.40 ± 0.36	4.63 ± 0.35
Female				
5-HT	0.77 ± 0.06	0.47 ± 0.08 **	0.56 ± 0.03	0.43 ± 0.02 ***
5-HIAA	0.55 ± 0.02	0.47 ± 0.01 *	0.55 ± 0.02	0.42 ± 0.03 *
Turnover	1.53 ± 0.08	2.37 ± 0.19	2.06 ± 0.11 *	2.03 ± 0.10 *

Table 5.5 Effect of pre natal DEX and/or adult repeated stress or GC treatment on 5-HT and 5-HIAA levels within the NAcc. NAcc 5-HT, 5-HIAA and 5-HT turnover were assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeaterestraint stress. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of 5-HIAA to 5-HT in pmol/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. . Study 1 male: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). Female:: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). Study 2: male:: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). Female: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

Adult frontal cortex 5-HT and 5-HIAA levels				
STUDY 1	**CON**	**DEX**	**STRESS**	**DEX + STRESS**
Male				
5-HT	0.48 ± 0.17	0.91 ± 0.04 *	0.89 ± 0.07 *	0.85 ± 0.13
5-HIAA	0.34 ± 0.04	0.38 ± 0.03	0.45 ± 0.02	0.44 ± 0.02
Turnover	2.91 ± 0.21	0.84 ± 0.09 ***	1.03 ± 0.08 **	1.15 ± 0.15 **
Female				
5-HT	0.92 ± 0.04	0.79 ± 0.06	0.64 ± 0.05 **	0.70 ± 0.05 *
5-HIAA	0.28 ± 0.01	0.30 ± 0.02	0.33 ± 0.01	0.32 ± 0.01
Turnover	0.60 ± 0.04	0.81 ± 0.12	1.06 ± 0.08 **	1.01 ± 0.10 **

Adult frontal cortex 5-HT and 5-HIAA levels				
STUDY 2	**CON**	**DEX**	**CORT**	**DEX + CORT**
Male				
5-HT	0.47 ± 0.18	0.98 ± 0.15	0.81 ± 0.04	0.78 ± 0.07
5-HIAA	0.37 ± 0.05	0.39 ± 0.05	0.42 ± 0.02	0.41 ± 0.03
Turnover	1.58 ± 0.18	0.85 ± 0.09 **	1.06 ± 0.08 *	1.13 ± 0.15 *
Female				
5-HT	0.82 ± 0.04	0.81 ± 0.01	0.64 ± 0.03 **	0.70 ± 0.02 *
5-HIAA	0.25 ± 0.009	0.30 ± 0.01	0.34 ± 0.01 **	0.34 ± 0.02 **
Turnover	0.62 ± 0.04	0.76 ± 0.04	1.09 ± 0.09 ***	0.98 ± 0.09 **

Table 5.6 Effect of prenatal DEX and/or adult repeated stress or GC treatment on 5-HT and 5 -HIAA levels within the prefrontal cortex. PFC 5-HT, 5-HIAA and 5-HT turnover were assesed by HPLC at 5.5 months of age. Male and female rats were treated prenatally with DEX via the maternal drinking water and were subjected to adult (3.5month) repeaterestraint stress. Data are means ± SEM in ng/mg. DA turnover was calculated as the ratio of 5-HIAA to 5-HT in pmol/mg. Statistical significance was assessed by two way anova with tukeys post hoc multiple comparison tests. *p<0.05, **p<0.01, ***p<0.001 vs controls, # vs DEX, + vs adult treatment. Study 1 male: n=8 (control), 9 (DEX), 8(stress), 8 (DEX + stress). Female:: n= 10 (control), 8 (DEX), 10 (stress), 10 (DEX + stress). Study 2: male:: n = 7 (control), 6 (DEX), 7 (CORT), 5 (DEX + CORT). Female: n=7 (control), 6 (DEX), 7 (CORT), 7 (DEX + CORT).

5.4 DISCUSSION

This chapter reveals some salient effects of stress and GC treatment, at different times in life, on non-dopaminergic systems. Notably, although the adult LC was largely resistant to adult stress or GC treatment, prenatal DEX treatment resulted in a dimished number of noradrenergic cells within both the adult male and female LC. Interestingly the adult astrocyte population was similarly reduced in this group of animals. In contrast the effects of stress and GCs on 5-HT neurotransmission were found to be sexually dimorphic. For example within the PFC, prenatal DEX treatment resulted in reduced 5-HT activity in males but had no effect on females. Adult treatments also reduced 5-HT activity in males but in females 5-HT levels in the PFC were reduced and 5-HT activity increased. Serotonergic and noradrenergic pathways are altered in PD thus these data may have relevance to the hypothesis that stress and glucocorticoids may be involved in the development of PD.

5.4.1 Noradrenergic system

The present data are consistent with previous findings of sex differences within the LC NA system (Guillamon et al., 1988; Luque et al., 1992). It has been suggested that more apoptosis early in life in the male and an extended postpuberal neurogenetic period in the female are responsible for the sex difference in neuronal number within the LC (Pinos et al., 2001). Sex differences in the NA system have been implicated in the higher prevalence of depression in women . One could speculate that increased NA input to the NSDA may also contribute to reduced prevalence of PD and the resistance to NSDA toxins in females (see section 3.4.4).

This chapter presents the novel finding that prenatal DEX treatment in the last week of gestation permanently reduces the number of noradrenergic neurons within the LC of both male and female rats. The effect of prenatal GCs on LC neuronal number has not previously been explored, however studies of prenatal stress have shown that prenatally stressed rats have reduced NA content and turnover within the hippocampus and PFC (Takahashi et al., 1992; Muneoka et al., 1997) and exhibit increased anxiety and depressive behaviour in adulthood (Vallee et al., 1997) (Morley-Fletcher et al., 2003; Morley-Fletcher et al., 2004). Interestingly perinatal food restriction has also been found to reduce the number of LC neurons (Morgane et al., 1993; Pinos et al., 2004). As prenatal malnutrition has been shown to reduce placental 11βHSD2 activity and activate the maternal HPA axis, the developing fetus would be exposed to higher levels of maternal GCs (Langley-Evans et al., 1996). The present data thus support a role for GCs in the effects of prenatal malnutrition on LC neurons. GRs are present within the LC from E15 (Cintra et al., 1993) so DEX administered on GD16-19 could have a direct effect on developing neurons within the LC. Increased cell death during development may underlie the observed reduction in cell number however cell death does not underlie the decrease in LC neurons of malnourished rats (Pinos et al., 2004). It remains to be determined if apoptosis plays a role in the effect of GCs on the LC neuron population. An alternative possibility is that GCs may limit or slow neurogenesis within the developing LC. GCs influence both apoptosis and neurogenesis, however most work has been carried out on the hippocampus. GCs also have profound effects on sex hormone status as well as having a strong influence on the development of the rat LC (Guillamon et al., 1988; Pinos et al., 2001) thus GCs may indirectly act on the LC through changes in levels of sex hormones. In addition to neuronal loss there was also a decrease in the size of the astrocyte population in the LC in both sexes. This may occur

through similar mechanisms to those described above and could possibly contribute to neuronal loss, due to a lack of neurotrophic support. Prenatal DEX treatment didn't alter NA levels in projection areas therefore one could assume that despite the neuronal deficit the adult noradrenergic system is able to function normally. Further studies examining the activation and firing of LC neurons and NA turnover would be necessary to confirm this.

Adult repeated stress or GC treatment largely did not produce persistent effects on the noradrenergic system. However, in females exposed to chronic GCs in adulthood an increase in striatal NA was observed. Whereas stress would result in activation of LC neurons due to excitatory input from the PVN, chronic GCs would not elicit the same effect. Essentially, GCs would decrease PVN activity through GC negative feedback leading to reduced activation of LC neurons and reduced NA release in projection areas. Since this is in contrast to the rise in striatal NA observed it may be that GCs are having a direct effect on noradrenergic transmission. GCs can alter the transcription of TH, DβH and the NAtransporter (NET) (references) that might alter levels of NA within the striatum. Intriguingly, there is evidence that NA has neuroprotective actions within the NSDA (Fornai et al., 2005; Pintor et al., 2006) thus an increase in striatal NA in GC treated females may be responsible for the neuroprotection observed in these animals in response to 6-OHDA (chapter 3). NA is critical for the firing of NSDA neurons and the striatal release of DA (Rousselet et al., 2003). Furthermore NA has anti-inflammatory (Feinstein et al., 2002) and anti-oxidant properties (Liu et al., 2003) and NA neurons can release neuroprotective modulators such as galanin and BDNF (Xu et al., 1998) Conner 1997; Elliott-hunt 2004; sun 2004). Moreover, depression is associated with dysregulation of the NA system and elevated levels of NA (Frazer, 2000) thus this data

provides further evidence that increased GC levels may be responsible for an increased risk of depression in females. The fact that increased striatal NA in GC treated females was potentiated if they had been exposed to prenatal DEX suggests that prenatal GCs may sensitise the female NA system to the effects of adult GCs.

5.4.2 Cholinergic system

Although we found no effect of stress or GCs on the NBM, there is evidence to suggest that that stress and GCs can alter cholinergic neurotransmission. Repeated restraint stress has previously been shown to reduce acetylcholinesterase activity (Kaufer et al., 1998), presumably increasing levels of ACh within the cortex, which wasn't measured in the present study. Indeed, handling stress increases the extracellular concentration of ACh within the PFC which can be reversed by environmental enrichment (Segovia et al., 2008). GRs are present within the NBM and PFC (Cintra et al., 1994; Morimoto et al., 1996) and GR mediated transactivation can result n the manipulation of signalling pathways involved in cholinergic transmission (Maroder et al., 1993) (Nordeen et al., 1994). This evidence suggests that it is likely that our treatment regimes may have altered functioning of the cholinergic system that wasn't assessed in the present study. Interestingly high or low levels of chronic corticosterone administration in adrenalectomised rats result in increased susceptibility to NMDA induced neurodegeneration within the NBM via a GR mediated mechanism (Abraham et al., 2000) thus although we didn't observe an effect on neuron number within the NBM, stress or GC treatment may render the NBM more vulnerable to further insults.

Prenatal DEX exposure has previously been shown to impair spatial learning and memory in rats (Brabham et al., 2000) (Rayburn et al., 1997; Welberg et al., 2001), functions that are associated with cortical cholinergic transmission. Moreover, school age children that were treated prenatally with DEX have a lower IQ (Yeh et al., 2004). Prenatal DEX treatment can render cholinergic neurons vulnerable to challenge with prenatally DEX treated animals exhibiting a larger lesion in response to the immunotoxin ^{192}IgG-saporin. Postnatal handling also results in a higher loss of ChAT neurons in the NBM in response to an NMDA excitotoxic lesion (Horvath 2004). In the present study prenatal DEX treatment did not alter the effects of an adult insult (repeated stress or chronic GC treatment) on the NBM.

5.4.3 Serotinergic system

There are numerous reports of sex differences in 5-HT neurotransmission (Cosgrove et al., 2007). In general, females have higher levels of 5-HT than men (Ortiz et al., 1988) and have higher levels of the 5-HT transporter (SERT) (Staley et al., 2001). On the other hand men synthesise 5-HT faster than women (Kessler et al., 1991). In line with these findings the present data show females have greater levels of 5-HT in all three of the regions examined with males showing greater 5-HT activity in the NAcc and PFC. It is thought that sex differences within the brain can be attributed to the effects of sex hormones, both during development and in adulthood. Indeed estrogen is known to modulate the serotinergic system by increasing the density of 5-HT receptors and SERT (Fink et al., 1998).

Serotonin in detectable in the rat brain from E13 and plays a trophic role in neuronal development (Wallace and Lauder, 1983) thus prenatal exposure to synthetic GCs may act upon the developing serotinergic system. Prenatal DEX treatment did not affect male striatal or NAcc 5-HT measures but reduced 5-HT activity within the male PFC. Reduced 5-HT activity, as indicated by a reduction in 5-HT turnover has previously been detected in cortex, hippocampus, hypothalamus and midbrain of neonates exposed to DEX during the last week of gestation (Muneoka et al., 1997). This study demonstrates that for at least the PFC this effect is maintained into adulthood. Furthermore, prenatal GCs can reduce SERT mRNA expression in the postnatal male rat brain (Fumagalli et al., 1996) (Slotkin et al., 1996). By contrast prenatal DEX increases 5-HT activity in the NAcc and striatum of females but has no effect on the PFC. These findings further highlight the sexually dimorphic response of the brain to GC programming. Further evidence for sex differences in the GC programming of the 5-HT system is provided by evidence that that prenatal GCs exert sexually dimorphic effects on SERT expression within the DRN and MRN (Andrews and Matthews, 2004). Neonatal handling that permanently reduces the HPA axis response to stress due to increased maternal care (Smythe et al., 1994) (Liu et al., 1997) (opposite effect of prenatal GCs) has been shown to increase 5-HT and turonover within the hippocampus (Smythe et al., 1994) resulting in upregulation of GR within the hippocampus. However this increase in 5-HT did not persist into adulthood (Smythe et al., 1994).

Similar to the effects of prenatal DEX, adult stress or GC treatment do not affect 5-HT measures within the striatum or NAcc but reduce 5-HT activity in the PFC. Conversely in the female adult stress or GC treatment potentiates 5-HT activity in all three regions. It is well known that there is significant interplay between the serotinergic system and

the HPA axis. For example the loss of 5-HT terminals potentiates stress induced elevations of cort (Richardson, 1984), 5-HT agonists elevate ACTH and cort levels (Wetzler et al., 1996) (Klaassen et al., 2002) and increased 5-HT activity can result in upregulation of GRs within the hippocampus (Smythe et al., 1994). Conversely, in response to stress there is an increase in DRN activity as indicated by elevated c-fos expression (Greenwood et al., 2003) and an increased release of 5-HT in projection areas (Bland et al., 2003; Amat et al., 2005). Corticosterone has a stimulatory effect on tryptophan hydroxylase activity (Singh et al., 1990) the rate limiting enzyme in 5-HT synthesis. However, chronic stress or GC treatment reduces 5-HT levels as well as reducing 5-HT_{1A} receptor expression and binding (Mendelson and McEwen, 1991; Crayton et al., 1996) (Luine et al., 1993). Furthermore the transcription of 5-HT_{1A} receptors is negatively regulated by glucocorticoids (Meijer et al., 1997; Ou et al., 2001). The present data suggest that repeated stress produces region and sex specific effects on 5-HT neurotransmission that are likely mediated by GCs.

Interestingly several effects of prenatal DEX on adult treatments were seen in the striatum whereby prior DEX treatment altered the response to adult insult by increasing 5-HT turnover in males. Furthermore, in the female prenatal DEX altered the adult response to GC treatment, but not stress, by attenuating 5-HT activity. These findings suggest a sensitivity of the striatum to the programming effects of DEX and that there is an altered 5-HT response to stress and GCs in DEX treated animals. Interestingly these effects are strikingly sexually dimorphic and may underlie sex differences in susceptibility to disease states associated with the serotinergic system.

Reduced 5-HT levels are associated with an increased risk of depression (van Praag and de Haan, 1979) which has a 25-40% comorbidity with PD {Leentjens, 2004 #726}. Furthermore, altered 5-HT transmission may also influence cognitive symptoms such as memory consolidation (Schmitt et al., 2000). Moreover, in addition to its influence on the non-motor symptoms of PD, 5-HT can also influence dopaminergic neurotransmission within the basal ganglia. It is generally thought that 5-HT has an inhibitory effect on striatal DA release, however different receptor subtypes can differentially influence DA release (Lucas et al., 2000). Stimulation of 5-HT_{1A} receptors increases locomotor activity (Mignon and Wolf, 2002) and has a neuroprotective effect in the 6-OHDA rat (Matsubara et al., 2006). Importantly 5-HT may not directly influence DA neurons but may modulate glutamate neurons, reducing glutamatergic drive to the STN or striatum, ultimately resulting in disinhibition of the thalamus(Mignon and Wolf, 2007) or may interact with cholinergic interneurons that have inhibitory control over striatal MSN's (Pakhotin and Bracci, 2007). Indeed 5-HT action at 5-HT_{2c} receptors increases activity of cholinergic interneurons (Blomeley and Bracci, 2005).

5.4.4 Summary

The present chapter provides confirmation of previous findings of innate sex differences within neurotransmitter systems. Importantly the data present compelling evidence that prenatal DEX treatment alters the development of the LC, resulting in a reduced neuron and astrocyte population in adulthood that may have important implications for the adult stress response. As an adult, chronic GC treatment but not repeated stress resulted in elevated levels of NA within the female but not male striatum that was potentiated by

prior DEX treatment. Stress and GCs also produced wide ranging effects on serotonergic neurotransmission that were region and sex specific. Prenatal DEX reduced 5-HT activity (turnover) in the adult male PFC but increased activity in the female NAcc and striatum. Adult treatments reduced 5-HT activity in the adult male PFC but increased 5-HT activity in the female PFC, NAcc and striatum. By contrast the NBM was insensitive to stress and GC treatments. Modulation of these neurotransmitter systems in response to stress and GCs may affect the development of both motor (via interactions with the DA system) and non motor symptoms of PD.

CHAPTER 6

Discussion

6.1 Summary of main findings

The data presented within this thesis provides confirmation that stress and GC treatment at different times in life are capable of producing persistent changes within brain regions that are known to degenerate in PD. Furthermore, novel evidence illustrating that stress or GC treatment can sex dependently potentiate neurotoxicty in an animal model of PD is presented. The principal findings of this study are as follows:

- Confirmation of previous reports that prenatal DEX treatment exerts long term changes in midbrain DA pathways.
- Prenatal DEX treatment consistently reduces the number of noradrenergic neurons within the adult LC.
- Prenatal DEX treatment results in persistent changes in glial populations largely by increasing microglial numbers and reducing astrocyte numbers in DA and NA nuclei.
- Adult stress and chronic GC treatment exert differential sex and region specific changes in neuron and glial populations.
- Adult stress and chronic GC treatment elevate levels of nitrosative stress in the male NSDA.
- The highly novel finding that exposure to prenatal DEX treatment results in an altered response to adult challenge within the male midbrain DA pathways, whereby the number of SNpc neurons and striatal and NAcc DA levels are attenuated.
- Adult repeated stress or chronic GC treatment exacerbates neuron loss and the neurochemical deficit in the 6-OHDA rat model of PD in males but affords neuroprotection in females.

- Prenatal DEX does not alter susceptibility to 6-OHDA within the NSDA. However, in the male MLDA where 6-OHDA did not produce a significant lesion in males exposed to a single treatment - males treated prenatally with DEX who went on to receive either stress or GC treatment as adults did exhibit a significant lesion. This provides further evidence for a programming effect of prenatal DEX on the DA midbrain resulting in a vulnerable phenotype.
- Sex differences in the unchallenged brain as well as in the effects of stress and GCs are repeatedly reported highlighting the importance of sex within this area of research.

6.2 Methodological considerations

This thesis provides significant evidence that stress and exposure to elevated levels of GC hormones have the potential to affect PD pathogenesis. The use of an in-vivo PD model is suggestive of significant clinical relevance to the human condition. Unfortunately there is currently no animal model of PD that recapitulates all features of the human disease. Despite this they do provide us with useful insights into the possible pathogenesis of PD (section 1.1.12). In the present study a rat model of PD was created by unilateral injection of 6-OHDA to the MFB. This regime was used as preliminary data (section 2.1.7) showed that it produced a partial lesion of the NSDA that would allow the determination of any detrimental or protective effects of treatment regimes on lesion size, as well as providing an internal control. Importantly, the 6-OHDA model was chosen as the lesions produced are highly reproducible. Based on the present findings and those of others in future studies it may be useful to use a model that is firstly more progressive so that the effects of stress and GCs and stress on disease

progression can be assessed. For example stress and GCs have recently been shown to accelerate neuron loss in a 6-OHDA model (Metz, 2007) and data from PD patients report that stress worsens PD symptoms (Macht et al., 2005). Unfortunately the 6-OHDA model is relatively acute, although there is some evidence to suggest that degeneration is progressive (Marinova-Mutafchieva et al., 2009) it is likely maximal at the time point used in this study (2 weeks post lesion). Secondly, in light of the findings that stress and GCs alter not only DA pathways but also NA and 5-HT pathways, a model that recapitulates the multi-system degeneration observed in PD, would be especially useful. The proteosome inhibitor model of PD would be a highly attractive model for further study of the effects of stress and GCs on brain regions implicated in PD as not only does it elicit neurodegeneration in non-dopaminergic pathways but also exhibits protein aggregates - a key feature of the human disease. However there is still considerable controversy around the use of this model with some groups reporting that there is no lesion at all. As we did not predict that stress or GC exposure would elicit such a striking effect on the size of the 6-OHDA lesion behavioural testing was not employed. In the future this would enable further characterisation of the effects of stress and GC's on the model. The large lesion produced in males that had received adult restraint stress or adult hydrocortisone treatment may also have prevented any additive effects of prenatal DEX treatment from being detected. Indeed an effect of DEX in combination with adult stress or GCs did further endanger the MLDA system where there was a much smaller lesion size, owing to the MLDA being less susceptible to 6-OHDA induced neurotoxicity. A lower dose or altered delivery of 6-OHDA may enable interactive effects of treatments to be identified more clearly.

As discussed in previous chapters one can not conclude that the effects of stress and the effects of GC treatments are one and the same as the two paradigms employed involve activation of different pathways and have a different severity. Importantly the HPA axis is suppressed in GC treated animals therefore CRF or ACTH may be involved in their differential effects. Furthermore animals are able to habituate to repeated stress. The daily rhythm of CORT secretion is also known to be disrupted in the GC treated group (Magarinos et al., 1998). Although many similarities between their effects have been identified, presumably due to elevated levels of GCs we have reported important differences. Other studies confirm that repeated restraint stress and chronic cort treatment do not elicit the same effects. For example chronic cort treatment decreases the volume fraction occupied by mitochondria in the CA3 region of the hippocampus whereas repeated restraint stress increased mitochondrial profiles in mossy fibre terminals (Coburn-Litvak et al., 2004) (Magarinos et al., 1997). Moreover GC treatment and restraint stress resulted in CA3 remodelling alone but when given in combination abolished the change (Magarinos et al., 1998) which is suggestive of the activation of different mechanisms.

Despite the fact that estrus cycle is affected by GCs and that it affects 6-OHDA lesioning estrus cycle stage was not taken into account in the present studies as although smears were taken on the morning of 6-OHDA surgery, grouping animals according to estrus cycle stage would have resulted in very small n numbers which would not enable the determination of any statistical significance. In order to account for estrus stage it would be necessary to have 4 groups of females as well as a group of males which would have significant cost implications and would have made organisation of the study

very difficult owing to the extremely large number of animals that would be required. Additionally, although circadian rhythm may have affected the cort concentration the animal was exposed to at the time of surgery, we were unable to fully take this into account due to the practicalities of using surgery facilities. These factors may contribute to some variability within the data although they are unlikely to produce highly significant effects as it has been shown that estrus stage and circadian stage only produce small changes in the effects of 6-OHDA (Datla et al., 2003). It is important to note that there are some discrepancies between data sets. However, the salient results are consistently confirmed across the studies. These discrepancies may have arisen due to the highly plastic nature of the pathways that were being studied. Indeed as we have shown relatively mild stress to have a significant impact on the pathways, it is possible that small differences in environment owing to the fact that studies were carried out 6 months apart may underlie these differences, despite every effort being made to ensure conditions within the animal house were maintained. Furthermore regarding HPLC data, it was impossible to run all samples in the same run thus data from different runs/studies can not be directly compared. This is likely to account for some base line differences between and within studies.

The experimental design and the model system in which the effects of stress or cort exposure are measured, dramatically affect the outcome. For example the effects of restraint stress, chronic variable stress, psycho-social stress and novelty stress all differ. Therefore it is important for future work and the advancement of the field that the effects of different stress paradigms are characterised so that we can gain an understanding of how the nature of the stressor also affects outcome. Chronic stress procedures are also nearly always different between laboratories. It would be extremely

useful if standard experimental protocols for chronic stressors were created to enable easier and more useful comparison of the literature. There is also a tendency to group together early life manipulations when one is talking about programming. However, there are very different outcomes in terms of the type of manipulation and especially in the timing of the manipulation. Manipulations carried out prenatally, postnatally and in adolescence have very different outcomes as they impact on different stages of brain development.

Although many neurochemical and histological changes were identified, the functional consequences of these changes were not examined. This makes it difficult to determine whether the results presented within this thesis are clinically meaningful or whether the changes identified have little impact on overall physiological function. However the data presented provides a novel insight into several stress induced changes within the brain that may or may not be relevant to pathologies known to have a stress component. This is discussed further in section 6.8.

6.3 Stress as a risk factor for Parkinson's disease

Stress was first implicated in PD in the 1960's when Schwab and England reported cases of emotional and physical trauma that had directly preceded the onset of Parkinsonian symptoms. More recently in light of the discovery that GCs could result in the loss of neurons within the hippocampus this hypothesis started to regain attention (Smith et al., 2002; Metz, 2007) although very few studies have looked at this in any depth. One of the major findings within the present study is that repeated stress or chronic treatment with GCs can render adult male rats more susceptible to 6-OHDA induced neurodegeneration. GCs have the potential to impact upon most pathological

pathways that have been identified as important in PD. These processes are often intricately interlinked which is depicted in figure 6.1, thus elevated or dysregulated GC levels may result in the initiation of a deleterious cascade that may promote or exacerbate neurodegeneration. This is discussed further below.

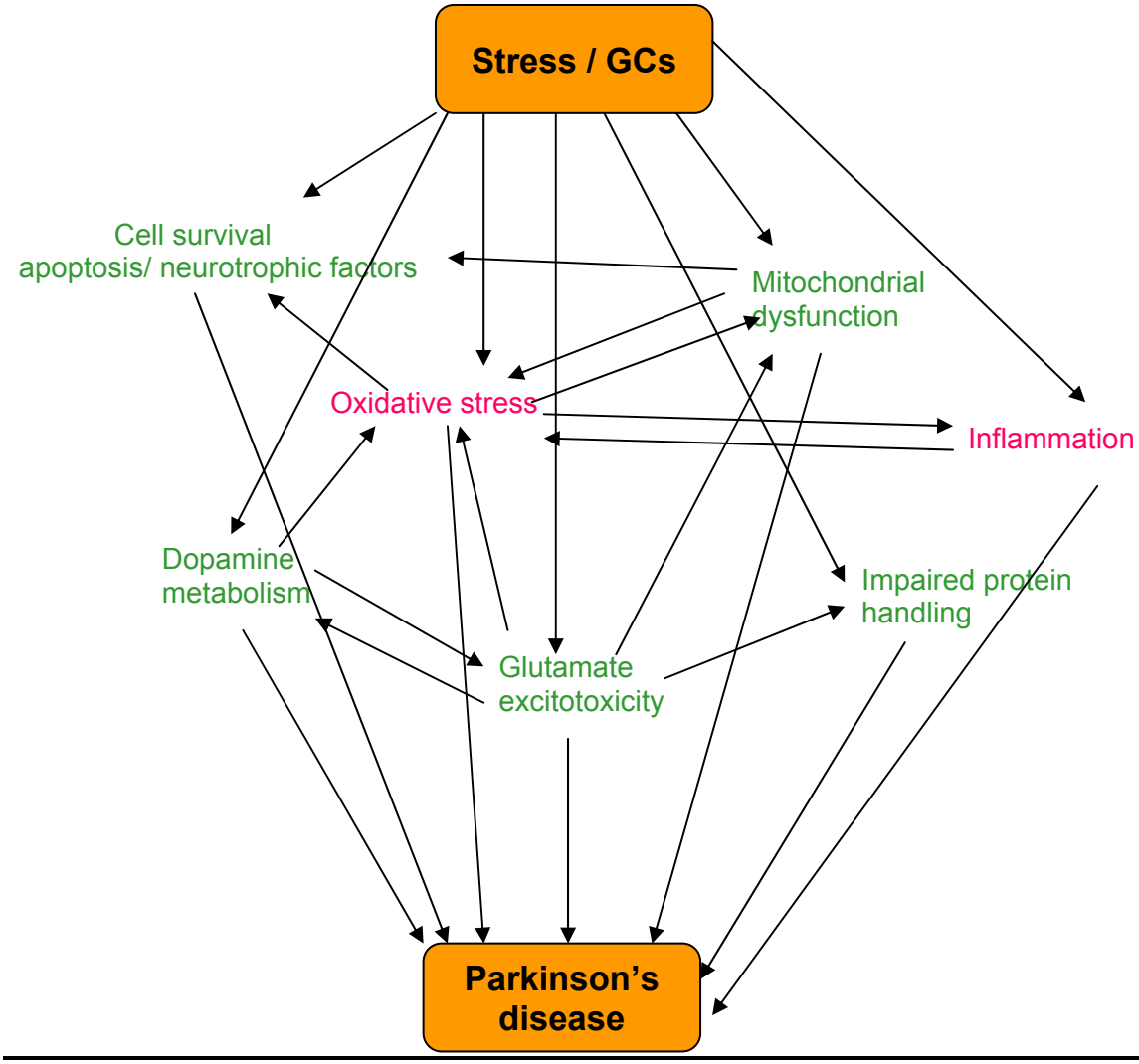


Figure 6.1. The complex interactions between GCs and the pathogeneic mechanisms involved in PD.

Dopamine

GCs are known to elevate DA levels. This rise in DA has the potential to cause neurotoxicity. Auto-oxidation of DA results in the production of superoxide anion as well as a highly reactive quinones that can readily modify proteins by covalently modifying cysteine residues, and irreversibly alter or inhibit protein function resulting in cytotoxicity (Asanuma et al., 2003). The formation of DA-quinones was found to be important in methamphetamine induced neurotoxicity (Miyazaki and Asanuma, 2008). In addition, DA metabolism itself may have deleterious consequences. Metabolism of DA by MAO results in the production of a toxic intermediate 3,4-dihydroxyphenylacetaldehyde (DOPAL) which is subsequently converted to DOPAC by aldehyde dehydrogenase. However, it has been shown that oxidative stress products such as 4HNE and MDA inhibit aldehyde dehydrogenase thereby increasing levels of the intermediate DOPAL which has been shown to be toxic to DA cells (Burke et al., 2004) and involved in the cross linking of α-synuclein (Burke et al., 2008). In addition DA metabolism by MAO also produces hydrogen peroxide which can be broken down by iron, which is abundant within the SN, to hydroxyl free radicals. These pathways may be responsible for the greater susceptibility of DA neurons to degeneration in PD.

Mitochondrial dysfunction

Mitochondrial dysfunction has been identified in PD and both the 6-OHDA and MPTP models of PD work to some degree via mitochondrial inhibition. GRs have been shown to be able to translocate to mitochondria (Koufali et al., 2003) as well as the nucleus where they have an impact on the role of mitochondria in the regulation of intracellular calcium levels and neural plasticity. A recent report shows that chronic high doses of

GCs attenuate mitochondrial function and result in reduced mitochondrial GR and Bcl-2 (anti-apoptotic protein) expression levels (Du et al., 2009). This would have detrimental consequences for the cell thus may promote degeneration. Du et al., 2009 suggest that by enhancing mitochondrial function you may be able to counteract the effects of excessive GCs (Du et al., 2009).

Oxidative stress

There are many reports that stress and GCs elevate levels of oxidative stress within the brain (Madrigal et al., 2006). We confirm this in our male but not female animals where stress and GCs result in elevated levels of protein nitration within the NSDA in both naïve males and 6-OHDA treated males. Within DA systems oxidative stress could exacerbate intrinsic DA toxicity (see above) and contribute to pathology in PD.

Glutamate excitotoxicity

In response to stress and HPA activation a large amount of glutamate is released into the extracellular space (Moghaddam, 1993). Through excessive activation of its receptors, most notably the NMDA receptor, glutamate results in elevated intracellular calcium, with the subsequent activation of calcium dependent enzymes, the generation of oxygen free radicals and protein misfolding. It is also interesting that stress reduces glutamate transporter expression and increases NR1 and NR2 subunits of the NMDA receptor (ref). Furthermore DA and glutamate act synergistically and can potentiate each others neurotoxic effects (Hoyt et al., 1997). As the SNpc receives gluatamatergic input from the STN, excessive glutamate signalling could be detrimental to the NSDA.

Inflammation

Although GCs are classically considered the anti-inflammatory drug of choice, there is now a wider appreciation of their proinflammatory effects within the CNS. Stress or high GC levels has been shown to induce the release and accumulation of inflammatory mediators such as TNF-α, IL-1β, IL-6, IFNγ and the activation of NFκB. Furthermore stress can induce microglial activation (Sugama et al., 2007; Kwon et al., 2008). Our data confirm previous reports that stress and GCs can result in proliferation of microglial within the brain (Nair and Bonneau, 2006). It has been proposed that stress induced microglial proliferation is due to a GR, glutamate and NMDA mediated mechanism. Stress induced microglial activation on the other hand has been shown not to be accompanied by neurotoxic factors such as cytokines and NO (Sugama et al., 2007) therefore it is unlikely that stress directly triggers microglia induced neuron degeneration but rather that it is detrimental in situations where there are dying neurons, propelling a vicious cycle. This may indeed be the case in the 6-OHDA model where stress and GC treatment exacerbate neuronal loss which is accompanied by increased microglial activation. The preceding chapters highlight the potential involvement of oxidative stress and microglial activation in the involvement of stress and GCs in PD, this is summarised in figure 6.2.

Neuroinflammation can also compromise the integrity of the blood brain barrier (BBB) thereby facilitating the activation and infiltration of peripheral immune cells into the brain. There is evidence of BBB leakage following treatment with DA neurotoxins and the vascular changes that have been identified in PD patients are suggestive of BBB dysfunction (Monahan et al., 2008). There is now an emerging hypothesis that PD has an autoimmune component based on evidence that animals exposed to DA neurotoxins

exhibit BBB dysfunction. This may allow peripheral immune cells to enter the brain where they may contribute to the progressive nature of the disease (Monahan et al., 2008). The immune system-brain interface is crucial for communication between the brain in health and disease and pathogens and toxins present in the environment. We require a better understanding of the routes of communication between the peripheral immune system and the brain including the mechanisms by which microglia can switch their phenotype.

Cell survival

Through regulation of transcription GCs can also reduce expression of anti-apoptotic proteins (as in mitochondria) and neurotrophic factors and increase pro-apoptotic proteins.

Protein deposition

Stress has been shown to increase deposition of amyloid-beta in an AD model (Green et al., 2006). Further investigation could establish whether a similar phenomenon occurs with α-synuclein in PD.

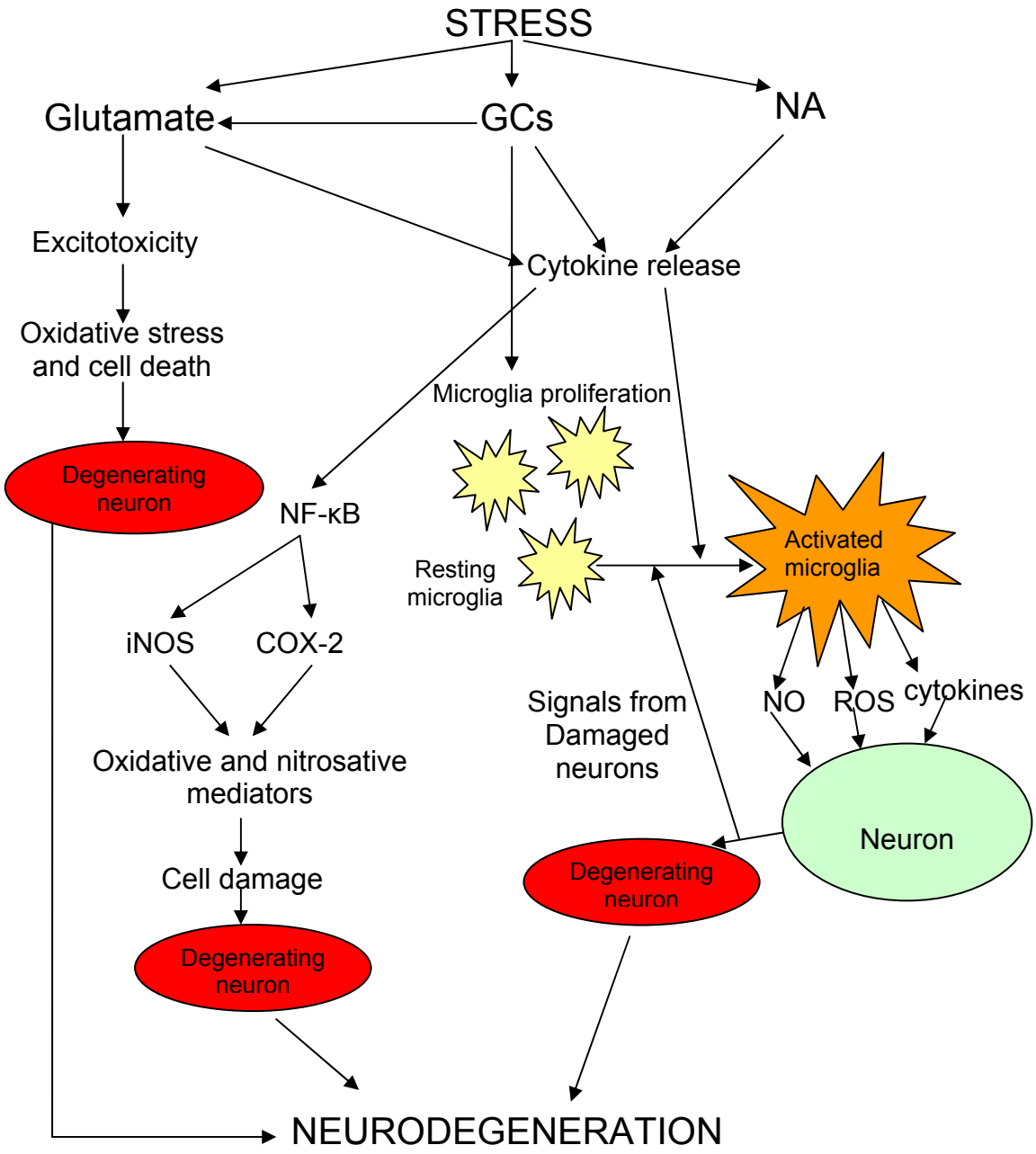


Figure 6.2. Schematic diagram to illustrate the potential involvement of oxidative and inflammatory pathways in stress induced neurodegeneration.

Although the present data doesn't allow us to infer that stress or GC treatment causes NSDA damage on its own, it does show that stress and elevated levels of GCs increase susceptibility to a subsequent toxic insult. An involvement of stress in PD is supported by clinical studies showing that acute or chronic stress worsens the symptoms of PD and accelerates the progression of the disease (Treves, 1990) (Gage and Storey, 2004) (Goetz et al., 1990). In addition acute stress appears to unmask symptoms in the preclinical stage of the disease (Schwab and Zieper, 1965; Snyder et al., 1985). I propose that although it is unlikely that stress alone can result in PD, it is probable that stress accelerates the onset and subsequently the progression of the disease via the above mechanisms in predisposed individuals. Alternatively stress may act as a trigger in predisposed individuals and may initiate the neurodegenerative cascade of events. PD is now appreciated as a multifactorial disease whereby a number of environmental insults (such as chronic stress or GC treatment) in combination with genetic factors result in precipitation of the disease. Based on the evidence presented in this thesis and within the literature the pathway that I believe to be most likely and would like to investigate further is depicted in figure 6.3. Here adult stress and subsequent raised GC levels result in increased activation of GR. Increased transcriptional activity via GR may result in alterations in the expression of TH, DAT, VMAT, uptake 2 or metabolising enzymes. Altered uptake, synthesis or metabolism of DA subsequently results in increased DA content. Due to its ability to auto-oxidise this would result in elevated levels of oxidative stress within the NSDA. Oxidative stress can then trigger microglial activation which in turn can further escalate oxidative stress. This mechanism would leave the NSDA in a vulnerable oxidative state so that when presented with an oxidative challenge such as 6-OHDA, its toxicity is enhanced.

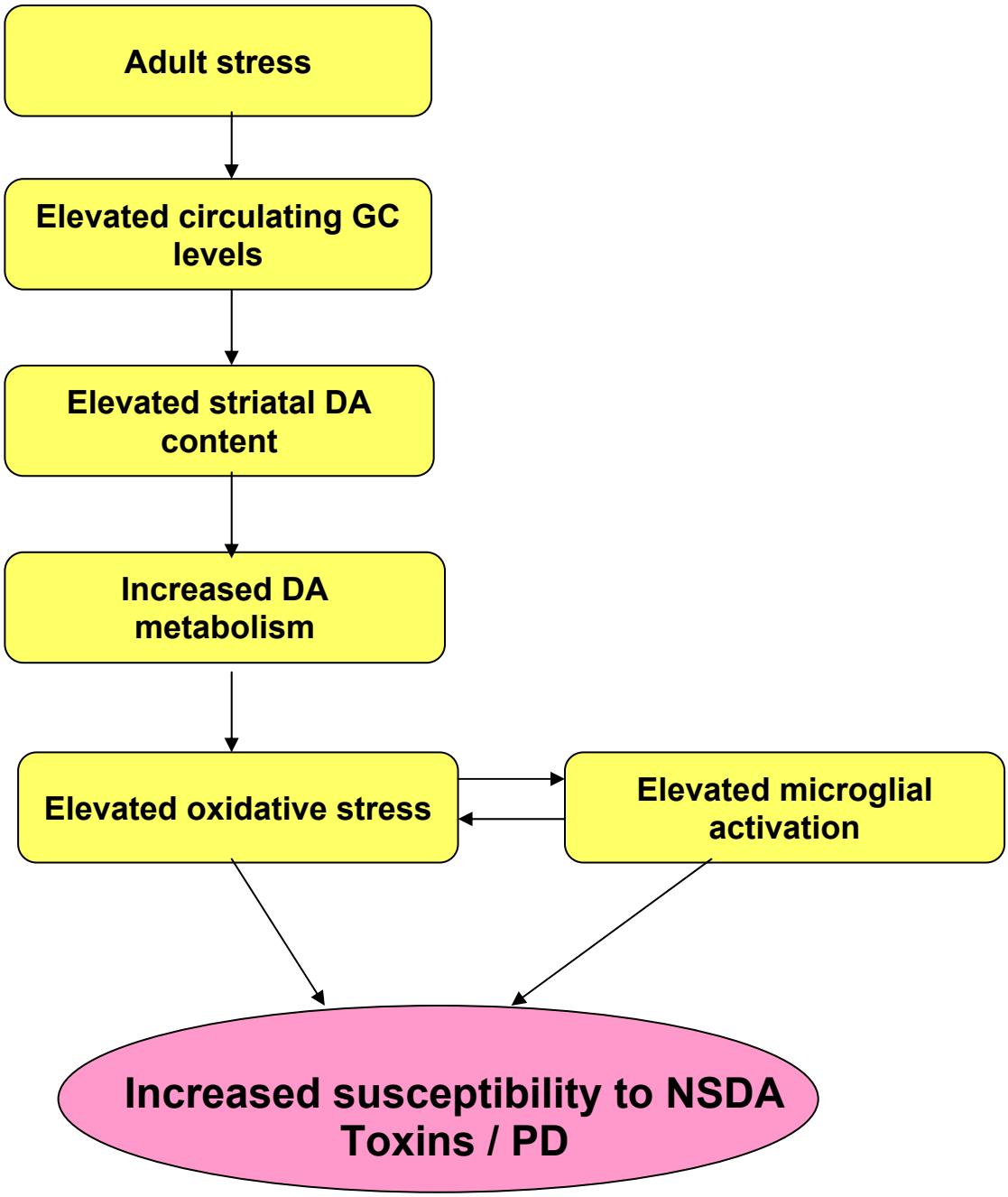


Figure 6.3. Potential mechanism for stress-induced enhanced neurotoxicity within the NSDA.

Age is by far the biggest risk factor for PD and GCs have long been linked to the aging process. The HPA axis promotes survival by enabling adaptation to environmental stressors. This is known as 'allostasis'. A severe, prolonged or inadequate stress response means that it is more difficult to maintain homeostasis – in this case the organism has an increased 'allostatic load' (McEwen and Lasley, 2003). During aging, coping and adaptation are less efficient and there is an increase in allostatic load that has been associated with increased and prolonged CORT secretion. It has been shown that Cort levels increase with age and that this correlates with age associated cognitive decline. These rises in CORT levels that occur with aging may further accelerate the aging process in a self perpetuating cycle - known as the GC cascade hypothesis. The GC cascade hypotheses was proposed before the identification of two corticosteroid receptors and it is now thought that it is the MR/GR balance that is key to health and disease. MR expression is known to decrease with age and alterations in the balance of MR and GR due to either genetics or environmental factors, particularly within the limbic system, would result in a dysregulated HPA axis that may ultimately increase vulnerability to disease. Moreover, gene variants of MR and GR have been identified (DeRijk and de Kloet, 2005) which may go some way to explaining individual variability to stress related disease and further highlights the importance of genetic as well as environmental impact. For example a loss of function MR gene variant has been associated with the development of depression (Kuningas et al., 2007). As aging is the single most important risk factor for PD, accelerating the aging process, by HPA dysregulation may result in an earlier onset of PD.

6.4 Developmental programming and its relevance to PD

Animal studies have shown that within the hippocampus prenatal GC's can delay the maturation of neurons, myelination, glia and vasculature in offspring, can alter neuronal structure and synaptogenesis and inhibits neurogenesis (Seckl, 2008). Human data has also shown that prenatal GC treatment has long term neurodevelopmental effects (Kapoor et al., 2008). The underlying mechanisms of the detrimental effects of GCs on brain development are still unclear and could be due to direct actions on neurons and glia or though dysregulation of the HPA axis. The present studies confirm previous reports that prenatal DEX permanently alters midbrain DA pathways. In addition it is shown that prenatal DEX also alters the noaradrenergic LC as well as influencing adult glia populations. As these changes have been shown to persist into adulthood, it is likely that they have long term consequences for brain function and disease.

It perhaps seems unlikely that elevated developmental GC levels would produce opposing effects on NA and DA neurons. However they have distinct origins and projection fields. Hindbrain NA neurons of the LC have diffuse projections whereas midbrain DA neurons have delineated projection fields. Moreover although NA and DA share the same biosynthetic pathway the development of hindbrain NA neurons and midbrain DA neurons are under different transcriptional control. LC neurons arise in the dorsal isthmic region of the hindbrain and are formed early in development (E9-10.5 in the mouse)(ref). The transcription factors Mash1, Phox2a, Phox2b and Rnx/Tlx3 are required for early LC development. On the other hand, the first midbrain DA neurons are detected at E10 and the correct specification and maintenance of midbrain DA neurons is dependent on the activity of the transcriptional regulators Nurr1, Lmx1b and Pitx3 (ref). Although in adults GCs negatively influence neurogenesis (Gould and

Tanapat, 1999) (Karishma and Herbert, 2002), the effects of GCs in the developing brain are essentially unknown. However, Sundberg et al., 2006 (Sundberg et al., 2006) have shown that dexamethasone inhibits the proliferation of embryonic neural stem cells isolated from the striatum on E17. It is yet to be determined whether early life programming is controlled by MR and GR although recent evidence suggests that the effects of early life programming are partly due to epigenetic changes in the GR promoter (Weaver et al., 2004; Seckl and Holmes, 2007; Oberlander et al., 2008; Szyf et al., 2008). Interestingly human hippocampal GR expression was found to be decreased in suicide victims that had a history of childhood abuse (McGowan et al., 2009) suggesting that epigenetic mechanisms may also be relevant to the human scenario. The importance of epigenetic changes induced by early life programming is only just emerging but it may be of significant relevance to the data presented here.

Oitzl et al. (2009) introduce the idea that it is not simply a case that an adverse event in early life results in a vulnerable phenotype, but that it is a case of whether the early environment matches the organism's future environment. This idea is supported by findings that although reduced maternal care is typically thought to be detrimental to future health, in the case of cognition this was true for the control state but when stressed, maternally separated animals performed better in cognitive tests than controls (Champagne et al., 2008). A similar phenomenon appears to occur within the NSDA where increases in striatal DA and TH-IR neurons in the SNpc that are seen in stressed or cort treated males are prevented by prenatal DEX exposure. Depending on the environment the animal is in this could be either an adaptive response or a maladaptive response. Figure 6.4 illustrates how the early life environment can contribute to adult disease. The present data support the hypothesis that early life manipulations produce

long term changes in pathways implicated in PD that in combination with other environmental, genetic and adult challenges probably contribute to PD pathogenesis.

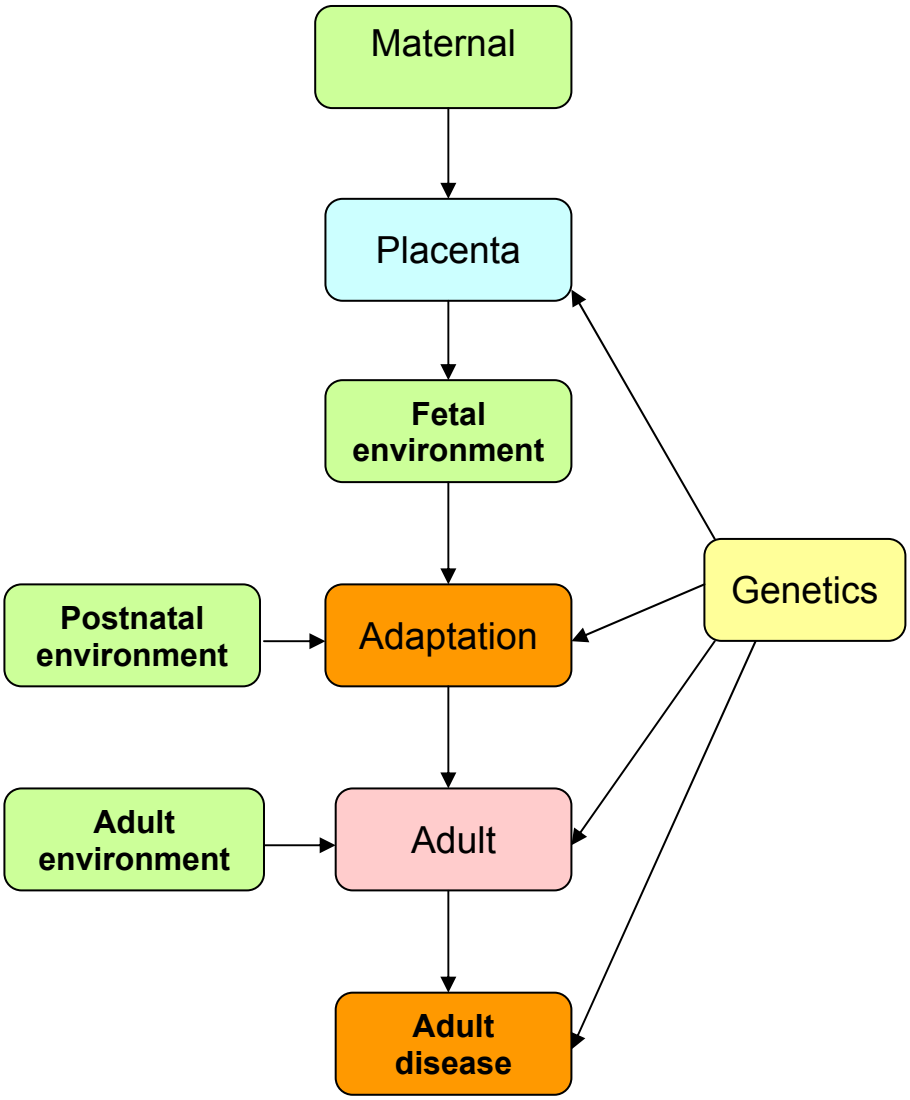


Figure 6.4. Develop mental programming results in adaptive responses to promote survival in future environmen t. Mismatching of the earl y life and adult environments results in maladaptation and a vulnerable phenotype

6.5 Stress, GCs and the non-motor symptoms of PD

Non-motor symptoms are common in PD patients and have important clinical consequences for patients, caregivers and society. Aarsland et al., 2009 (Aarsland et al., 2009) found that neuropsychiatric symptoms were present in early unmedicated PD cases, the most common symptoms being depression, anxiety, sleep disturbances and apathy. This study is important as it is the first to look at unmedicated PD patients as medication may have confounding effects on many neuropsychiatric symptoms. The aetiology of neuropsychiatric symptoms in PD as with motor symptoms is likely multifactorial with contributions from psychological stress and brain changes associated with PD. Although the diagnosis of a chronic debilitating disease may induce mood symptoms it has previously been described that neuropsychiatric symptoms can precede motor symptoms (Ishihara and Brayne, 2006) suggesting that these symptoms are related to the disease process. There is a consistent relationship between motor severity and neuropsychiatric symptoms in PD suggesting that motor and neuropsychiatric symptoms develop in parallel and therefore may have similar underlying pathology. Stress and GCs have already been heavily implicated in symptoms such as depression and anxiety therefore may be a significant contributory factor to pathogenesis.

6.6 The importance of sex differences

Nearly all tissues show sex differences in both health and disease and an increasing number of sexual dimorphisms have been identified within the brain. Moreover sex differences are found in the prevalence of many neurological disorders including PD, AD, PTSD, anxiety, stroke, MS, autism, addiction, ADHD and eating disorders. The purpose of studying sex differences is two fold. Firstly to further our knowledge of the

underlying biology of the system to be studied and secondly to determine whether the observed sex difference may have significant clinical relevance and should therefore be considered in development of preventative, therapeutic or diagnostic interventions. The sexually dimorphic nature of the data presented within this thesis emphasises the importance of studying sex differences within the brain and in particular with reference to PD. The innate sex differences in neurotransmitter systems identified by us and others as well as the sex differences in neuronal systems in response to stress and elevated GCs identified here may underlie the sex differences we see in susceptibility to different disease states. Furthermore the finding that stress and GCs produce sexually dimorphic responses in pathways affected in PD may suggest a contribution to the sex difference in the prevalence of PD. An understanding of these differences may be crucial in developing our knowledge of the disease process as well as in the development of appropriate therapeutics.

The assumption that sex differences arise as a consequence of altered exposure to sex steroid hormones is probably false as manipulation of the adult sex steroid environment has little impact on brain circuitry. It is more likely therefore that innate sex differences predominately arise due to organisational effects of sex steroids during development. Therefore sex differences in endocrine-neurotransmitter circuitry may underlie the altered responses to stress and GCs that was observed in males and females. All biological sex differences are encoded by genes encoded on the sex chromosomes. In rats the critical period of brain masculinisation and defeminisation occurs from GD18 to PND10 (Arnold and Gorski, 1984). Interestingly this coincides with our DEX treatment regime which thus has the potential to interfere with the masculinisation/defeminisation of the brain produced by testosterone during development.

Large sex differences in neuronal number are probably not a result of effects on the birth of neurons (Breedlove and Arnold, 1983) (Cooke et al., 1998) although other steroids such as GCs have been shown to modify neurogenesis (Zhang et al., 2008) (Ahmed et al., 2008; Galea, 2008). Sex steroids can however influence the outgrowth of axons and dendrites, the amount of cell death and can regulate synapse formation (Forger, 2006; McCarthy et al., 2008). There is also a recent interest in their epigenetic effects whereby steroids can alter the chromatin in the region of specific genes, permanently altering transcription. For example histone acetylation has been shown to be sexually dimorphic within the hypothalamus (Tsai et al., 2009). The potential of GCs to alter sex steroid levels during this critical period of development may have long term implications for the individual either making future responses more masculine or feminine in their nature. For example it could be hypothesised that the changes in neurotransmitter systems that occur as a result of prenatal DEX treatment may be due to enhanced masculinisation of the brain.

In adults both animal and human studies indicate that gonadal steroid hormone secretion is markedly altered by stress and glucocorticoids (See chapter 1). Conversely sex steroid hormones can differentially affect stress responsiveness. Figure 6.5 illustrates the interactions between the HPA and HPG axes. Stress and GCs have largely been shown to suppress the HPG axis. Acute stress inhibits GnRH and LH secretion presumably via direct actions of CRH on GnRH neurons (Gambacciani et al., 1986). Whilst chronic stress reduces the release of GnRH , decreases pituitary sensitivity to GnRH and interferes with LH stimulation of sex steroid hormones (Rivier and Rivest, 1991). Stress inhibition of the HPG axis occurs both in males and females. Furthermore physical

stressors (e.g. infection) also suppress HPG axis via a GC mediated mechanism (Debus et al., 2002). As is seen in the present work stress can have opposite effects in males and females in some systems. These differences may be due to sex differences in the stress response rather than the parameter being measured. Both basal and stress-induced corticosterone secretion is more pronounced in females than in males (Carey et al., 1995). Incidental data and data from Cushings patients and long term prednisolone treatment also show that stress inhibits the HPG axis in humans. (Sakakura et al., 1975; Reame et al., 1985). Furthermore, stress also results in inhibition of testosterone secretion in men, secondary to inhibition of the central HPG axis (Morgan et al., 2000).

As previously discussed estrogen has neuroprotective properties. Estrogen regulates growth factor supply (Cardona-Gomez et al., 2001; Ivanova et al., 2002), attenuates inflammation (Kipp et al., 2007; Vegeto et al., 2008); promotes anti-apoptotic signalling (Nilsen and Brinton, 2004); facilitates neuronal activity (Woolley, 2007); can act as a free radical scavenger (Behl, 2002b); influences mitochondrial function (Arnold 2009) and may even maintain the integrity of the BBB (Sohrabji, 2007). Furthermore estrogen can downregulate DAT (McArthur et al., 2007b). These potentially neuroprotective effects of estrogen may account for the reduced susceptibility to 6-OHDA in females and their lack of stress or GC enhancement of neurodegeneration.

Current literature suggests that some aspects of brain organisation are very similar in males in females and in other aspects are remarkably different. This may have an evolutionary origin and be explained by the fact that males and females are subjected to both similar and markedly different selective pressures. The present work highlights the significant importance of sex differences in the field as often opposing effects were

observed suggesting that the neuroendocrine-neurotransmitter circuitry is completely different in males and females. This implies that it can not be assumed that similar processes occur in men and women and therefore that therapeutics will not necessarily produce the same effect. Parallel research in males and females is required to further our understanding of the implications of sex differences within the brain and may lead to tailored therapeutic strategies that are dependent on sex.

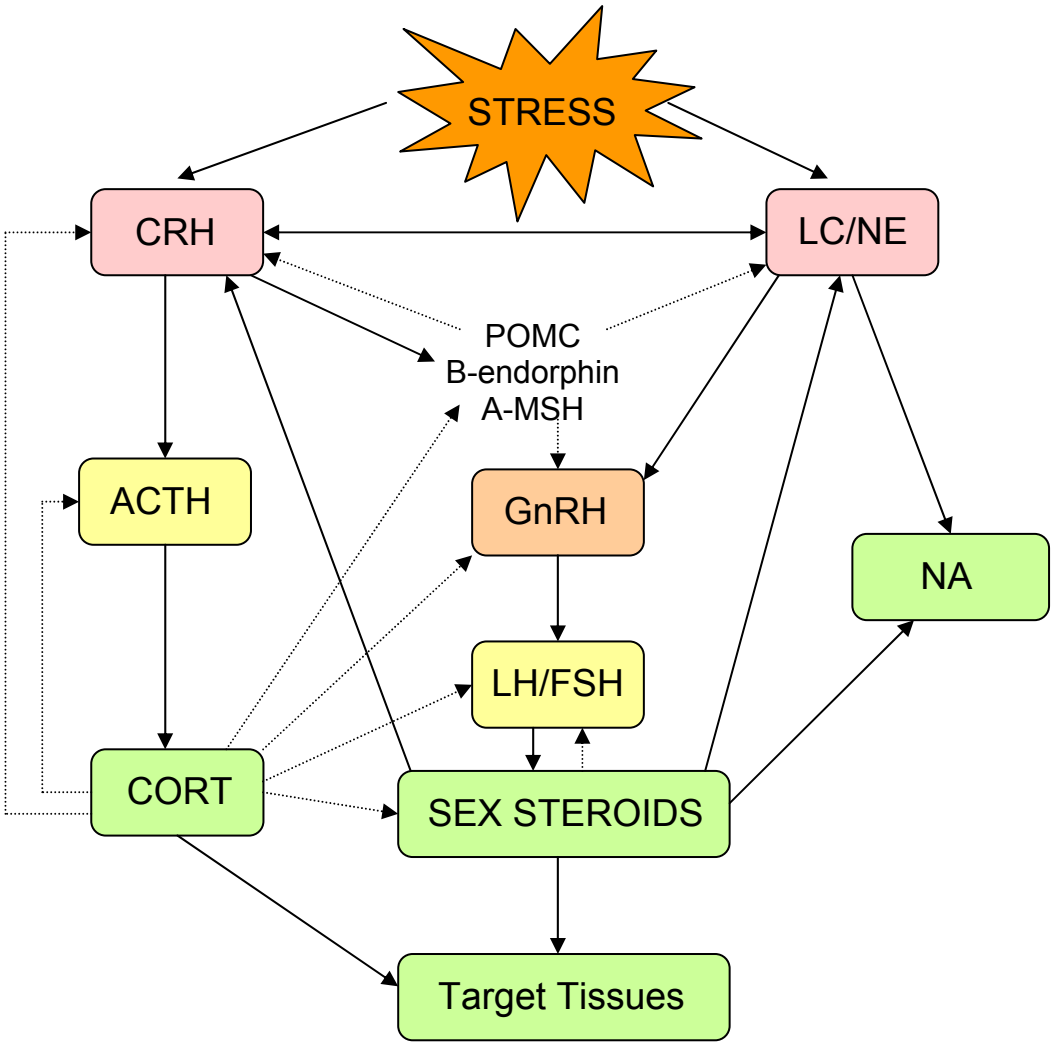


Figure 6.5. Summary of the interactions between the HPA and HPG axes.

6.7 Potential therapeutic and clinical applications

Although it is impossible to eliminate stress from our daily lives, effective stress management may be of significant benefit in the preclinical stage of the disease. This would only be possible with the development of a reliable biomarker for PD but would have the potential to extend the preclinical stage of the disease, delaying the onset of symptoms. Stress management may also be important in slowing the progression of the disease. The development of social policies and environmental enrichment may aid this. A positive outlook on life and good-self esteem have been shown to have long lasting health consequences (Pressman and Cohen, 2005). Furthermore good social support and having 3 or more regular social contacts has a positive effect on allostatic load (Seeman et al., 2002) whereas poor self esteem can result in a reduced ability to habituate and elicits greater cortisol responses to stress (Kirschbaum et al., 1995; Pruessner et al., 2005). In cases of early life abuse or stress, intervention performed early on may prevent the deleterious effects of chronic stress or abuse on the developing brain and could also help protect the brain against effects of chronic stress on the brain in adulthood/aging.

GCs are commonly used to treat a variety of inflammatory diseases. It may be sensible to limit their use in PD patients (no epidemiological evidence for this is currently available) due to their potential to accelerate disease progression. It is thought that transrepression of transcription factors such as AP-1 and NF-κB by GR is responsible for reducing the expression of pro-inflammatory genes. Therefore GC receptor ligands that could dissociate tranactiavtion from transrepression may confer less deleterious side effects. Several agonists and ligands have been developed that bind to GR cause a

conformational change favoring receptor- protein interaction rather than receptor DNA interaction, these ligands have been shown to have fewer side effects (Catley, 2007; McMaster and Ray, 2007).

The use of GCs in perinatal medicine should only be used where there is a significant risk of pre-term labour. A recent review advises against repeat doses of corticosteroids as there is no evidence to suggest any further beneficial effect (Newnham and Jobe, 2009). Improved methods of predicting pre-term birth would limit unnecessary treatment and research into the prevention of pre-term birth should be made a priority.

Further understanding of the detrimental effects of stress and GCs within the brain may highlight potential avenues for therapeutic intervention in the treatment of stress related diseases. The current research suggests that anti-inflammatory and anti-oxidative strategies may be beneficial in combating the deleterious effects of stress and GCs in PD. Furthermore future investigation into the mechanism behind glucocorticoid and stress induced neuroprotection in females may reveal novel potential therapeutic targets. Due to the multi-factorial nature of the disease, with stress and GCs possibly constituting one factor, logical thought suggests that a combination of therapeutic strategies are likely to be the most successful approach to disease management. One such strategy could involve stress management and the prevention of glucocorticoids excess.

6.8 Future work

There are many potential avenues for future research stemming from the present work and it would be impossible to highlight them all. Below I have detailed some areas for future work that I feel would be most interesting and beneficial to the field.

Firstly it is of paramount importance that we follow up children who are enrolled in randomized controlled trials of antenatal steroid use. This will provide us with vital information as to the long term consequences of their use and determine whether the large amount of literature based on animal models is relevant to humans.

In regard to the effect of stress and GCs and their relevance to PD, once the reproducibility problems associated with the proteosome inhibitor models are ironed out, it would be an ideal model for further study of the effects of stress and GCs on PD. Repeating the current studies in this model may provide further confirmation or could disprove the relevance of the present data to PD. As the model is progressive it would enable us to gain information on the effects of stress and GCs on both the onset and progression of degeneration as well providing further information on extra-nigral areas of degeneration and the presence of lewy-bodies. It would be especially interesting to look at whether stress or GC treatments have an effect on alpha-synuclein deposition as there is evidence to suggest that stress can result in increased amyloid-beta in an AD model. To further elucidate the role of stress in PD a similar study to the present one could be carried out in which the adult manipulation consists of chronic variable stress for a period of 6 weeks. This is a more chronic stress model than repeated restraint and would allow us to determine whether habituation of the stress response is important in the effects of stress on pathways implicated in PD and would indicate whether the

involvement of other stress related symptoms such as the autonomic nervous system are involved in the effects of stress on these regions. Moreover the effects of chronic variable stress may be more applicable to the human population. It would also be important to add in functional behavioural testing to any future experiments to determine the functional consequences of any neurochemical or histological changes that were identified. Furthermore alterations in motor function have been observed in the absence of any changes in NSDA measures (Metz, 2007).

It would be very interesting to look at changes in GR (and MR) expression both within the limbic system and within our brain regions of interest in response to our treatment regimes. This could be investigated using in-situ hybridization on sections containing the regions of interest. This would enable us to establish any changes in mRNA expression that may contribute to the effects of stress and GCs on these systems. Taking this further a very recent study has shown that selective ablation of the GR gene in dopaminoceptive neurons of the MLDA facilitated cocaine seeking behavior (Ambroggi et al., 2009). This approach could be utilized within the NSDA to determine the function of GR and whether the effects of stress are due to HPA axis regulation (circulating GC levels) or are GR mediated as I have suggested in figure 6.3. In figure 6.3 I also indicate that GR activation may initiate changes in gene transcription that ultimately result in elevated DA levels. To investigate what these underlying changes might be I would look at the effect of our treatment regimes on the expression of TH, DAT, VMAT, COMT and MAO.

We identified sex differences but this doesn't tell us what hormones, if any underlie these differences. This could be achieved by repeating the experiments in

gonadectomised animals to determine whether the gonads are important in the sex difference and then hormone replacement to see whether this returns the difference to control. If this is not the case i.e. gonadectomy does not eliminate the sex difference, it may be that the sex difference arose due to the action of testosoterone during development that caused permanent alterations. This could then be investigated by manipulating hormone levels during development. For example the administration of physiological levels of testosterone during fetal and early post-natal life and then measuring whether the female is more masculine in terms of the sex difference identified. Alternatively administration of an androgen blocker in males could determine whether this blocks normal masculinisation. If the sex difference could not be explained by the activational effects of gonadal hormones in adulthood or by organisational effects during pre and postnatal development another potential explanation may be that genes encoded on the sex chromosomes act within cells to sexually differentiate XX and XY cells.

6.9 Conclusions

Glucocorticoids have the potential to impact upon virtually all systems thus are able to exert wide ranging effects. Although these effects normally exert a protective function allowing us to cope with and adapt to changing environments, severe, prolonged or dysregulated exposure to GCs can be detrimental. This was initially shown within the hippocampus and there is now a wider appreciation for the detrimental effects of GCs in other brain regions as well as systemic systems. The work presented within this thesis along with the work of others suggests that stress and GCs have extremely wide ranging effects on the structure and function of the brain and that these effects vary significantly

in a region, dose, and sex-specific manner. The current data along with the work of others suggest that stress and GC exposure at different times in life do indeed have the potential to interfere with pathways involved in PD and affect the outcome of a PD animal model, suggesting that stress may be a significant risk factor for the disease. This is extremely timely research as our increasingly stressful life styles combined with our aging population mean that the health and economic burden of PD is likely to increase over the coming years. Thus the management of stress and GC levels may be important in the prevention and management of PD as well as many other stress-related and age-related disorders.

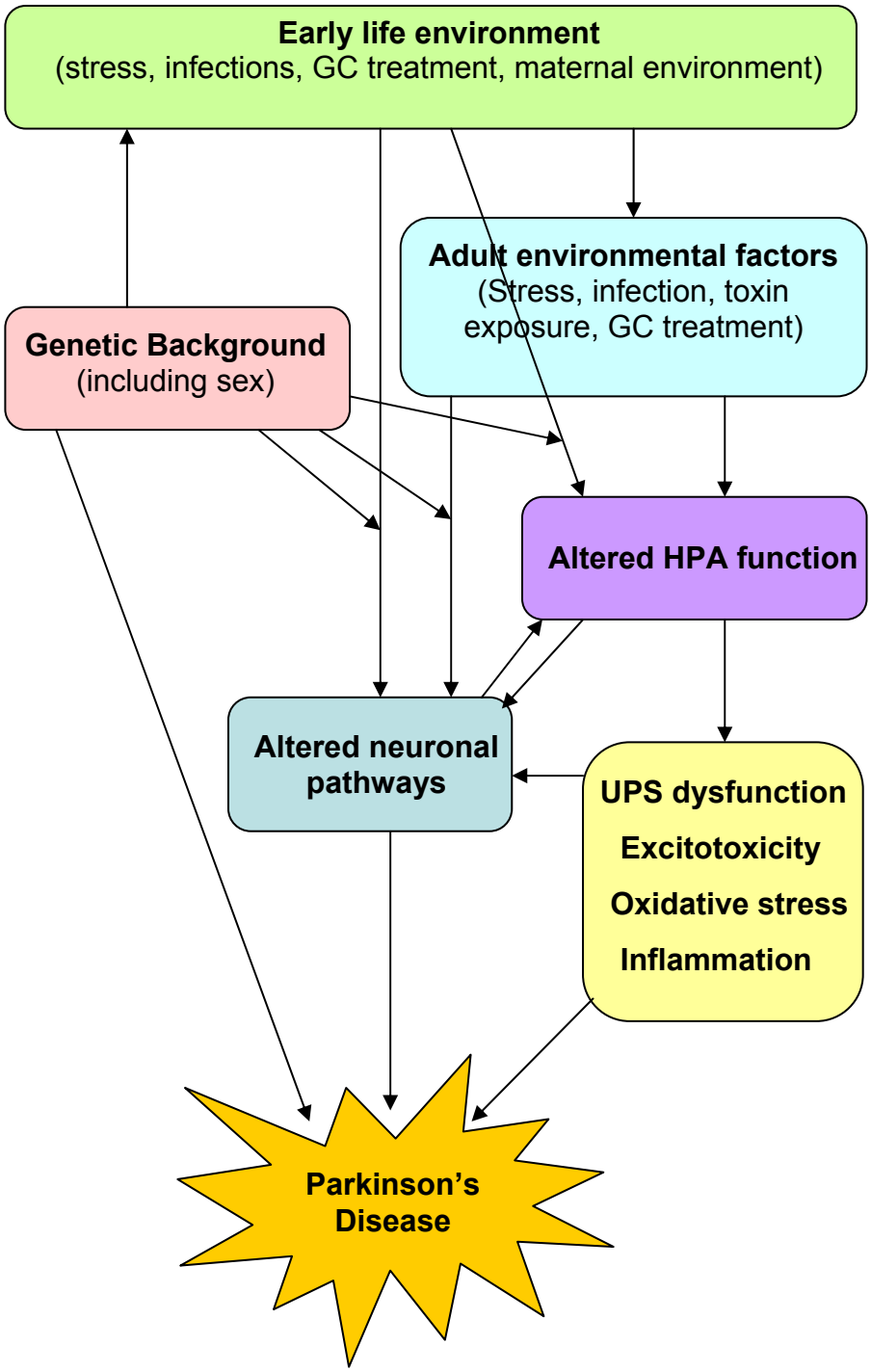


Figure 6.6. Simplified diagram to show proposed involvement of stress and GCs in the development and progression of PD showing potential direct and indirect effects of stress/GCs. PD is widely accepted to be a multifactorial disease whereby a combination of genetic and environmental insults

result in disease onset and progression. Exposure to stress or elevated GCs during early life can result in altered neuronal pathways and altered HPA function that may have persistent effects on adult physiology that contribute to disease onset. Similarily adult stress and GC exposure can alter the functioning of neuronal pathways and alter HPA function, elliciting inflammatory and oxidative responses that could contribute to neurodegeneration. Additionally our data show that early life experience may alter the response to adult challenge which may represent another contributory factor to disease progression. Although not included in this diagram for simplicity the HPG axis may also have a significant impact on neurodegeneration.

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APPENDIX 1: Summary of statistical analyses

Two-way ANOVAs

STUDY 1	DEX		STRESS		INTERACTION	
	F	p	F	p	F	p
Males SNpc						
TH	5.782	0.023	0.246	0.234	14.431	0.012
NeuN	6.272	0.032	0.376	0.496	16.254	0.002
OX-42	1.103	0.432	7.907	0.036	0.834	0.876
GFAP	1.023	0.562	0.768	0.765	0.987	0.865
3-NT	1.346	0.765	3.268	0.049	0.876	0.235
Female SNpc						
TH	6.862	0.010	14.109	0.000	0.872	0.352
NeuN	7.100	0.009	6.95	0.010	1.396	0.240
OX-42	0.966	0.547	0.002	0.968	0.178	0.444
GFAP	4.002	0.036	0.214	0.765	0.198	0.322
3-NT	0.988	0.775	1.134	0.432	0.122	0.988
Male Striatum						
DA	0.622	0.278	0.982	0.568	17.812	0.000
DOPAC	1.123	0.110	0.976	0.577	1.432	0.102
HVA	1.028	0.714	0.725	0.868	5.640	0.032
Female Striatum						
DA	1.838	0.180	52.456	0.000	8.173	0.006
DOPAC	0.527	0.471	0.482	0.491	23.43	0.000
HVA	0.835	0.365	0.342	0.561	6.426	0.014
Male VTA						
TH	4.332	0.032	3.022	0.044	0.332	0.865
NeuN	0.136	0.714	15.203	0.000	15.270	0.000
OX-42	21.210	0.000	15.786	0.000	0.654	0.785
GFAP	9.243	0.002	1.245	0.436	1.436	0.243
3-NT	0.988	0.666	1.130	0.543	1.670	0.123
Female VTA						
TH	0.151	0.699	7.288	0.010	4.405	0.041
NeuN	1.234	0.788	2.034	0.102	0.236	0.563
OX-42	19.662	0.000	7.250	0.001	0.025	0.985
GFAP	8.674	0.000	0.326	0.788	0.024	0.927
3-NT	0.927	0.340	0.149	0.702	1.120	0.660
Male NAcc						
DA	3.358	0.079	2.345	0.569	12.023	0.000
DOPAC	1.002	0.236	0.567	0.543	1.027	0.320
HVA	0.615	0.331	1.110	0.678	0.245	0.898
Female NAcc						
DA	6.078	0.032	12.320	0.003	0.206	0.603
DOPAC	0.604	0.890	1.245	0.320	1.203	0.220
HVA	0.998	0.335	0.276	0.333	0.778	0.453
Male LC						

DβH	7.632	0.001	1.345	0.120	1.003	0.520
NeuN	5.980	0.020	0.276	0.633	0.678	0.953
OX-42	0.902	0.892	0.668	0.725	0.984	0.764
GFAP	0.304	0.990	1.246	0.371	1.223	0.320
3-NT	0.234	0.677	0.311	0.471	1.242	0.481
Female LC						
DβH	6.459	0.033	0.768	0.825	0.954	0.664
NeuN	4.896	0.043	0.166	0.686	0.544	0.525
OX-42	1.675	0.218	1.34	0.721	0.721	0.411
GFAP	0.232	0.798	0.461	0.653	0.205	0.949
3-NT	0.140	0.843	0.565	0.782	0.313	0.771
Male PFC						
NA	0.166	0.851	0.556	0.462	1.560	0.222
Female PFC						
NA	0.123	0.728	2.141	0.154	0.081	0.778

STUDY 2	**DEX**		**CORT**		**INTERACTION**	
	F	**p**	**F**	**p**	**F**	**p**
Males SNpc						
TH	0.040	0.843	0.505	0.482	13.3	0.001
NeuN	0.136	0.714	15.203	0.000	15.270	0.000
OX-42	0.866	0.537	0.102	0.996	0.278	0.431
GFAP	0.345	0.444	11.022	0.000	1.325	0.443
3-NT	0.031	0.861	6.307	0.016	0.071	0.791
Female SNpc						
TH	0.002	0.968	0.166	0.686	5.544	0.025
NeuN	7.140	0.009	6.95	0.010	1.396	0.240
OX-42	0.863	0.682	0.856	0.678	1.431	0.262
GFAP	0.124	0.782	0.120	0.531	1.063	0.103
3-NT	0.001	0.977	2.359	0.055	0.055	0.816
Male Striatum						
DA	1.688	0.143	20.622	0.000	8.108	0.032
DOPAC	2.083	0.112	5.664	0.003	1.235	0.930
HVA	0.898	0.476	1.123	0.768	8.765	0.009
Female Striatum						
DA	0.133	0.678	0.210	0.371	1.342	0.471
DOPAC	0.923	0.782	0.756	0.778	1.233	0.162
HVA	1.345	0.215	4.089	0.056	1.402	0.092
Male VTA						
TH	2.815	0.114	1.560	0.231	0.010	0.920
NeuN	0.104	0.749	0.120	0.731	10.063	0.003
OX-42	26.330	0.000	16.291	0.000	0.876	0.100
GFAP	8.768	0.001	1.430	0.102	0.991	0.231
3-NT	1.436	0.457	0.777	0.336	1.432	0.118
Female VTA						
TH	1.675	0.218	1.34	0.721	0.721	0.411
NeuN	0.132	0.718	0.361	0.553	4.205	0.049

OX-42	25.701	0.000	20.021	0.000	0.987	0.765
GFAP	7.451	0.024	1.672	0.099	1.267	0.789
3-NT	1.123	0.110	0.976	0.577	1.432	0.102
Male NAcc						
DA	0.177	0.676	0.279	0.60	13.452	0.001
DOPAC	0.863	0.582	0.956	0.378	1.231	04262
HVA	0.797	0.324	0.122	0.453	0.602	0.960
Female NAcc						
DA	4.056	0.039	3.087	0.051	0.021	0.977
DOPAC	1.223	0.170	0.774	0.476	1.232	0.107
HVA	4.745	0.487	6.012	0.044	0.034	0.125
Male LC						
DβH	3.900	0.043	0.874	0.775	1.331	0.707
NeuN	3.372	0.050	0.656	0.461	1.660	0.254
OX-42	0.978	0.428	0.123	0.983	0.802	0.460
GFAP	4.273	0.046	0.156	0.542	0.893	0.342
3-NT	0.140	0.843	0.975	0.682	1.023	0.801
Female LC						
DβH	6.235	0.034	1.775	0.118	1.640	0.721
NeuN	3.675	0.059	0.677	0.728	0.023	0.983
OX-42	4.823	0.039	1.167	0.564	1.230	0.432
GFAP	1.476	0.428	1.630	0.721	0.221	0.314
3-NT	0.232	0.798	0.461	0.653	0.205	0.949
Male PFC						
NA	0.240	0.643	0.765	0.882	0.363	0.972
Female PFC						
NA	1.785	0.209	0.998	0.786	6.983	0.028

Three way ANOVAs

STUDY 1	DEX x Lesion		Stress x Lesion		DEX x Stress x Lesion	
	F	**p**	**F**	**p**	**F**	**p**
Males SNpc						
TH	4.166	0.051	0.556	0.462	1.560	0.222
NeuN	0.123	0.728	2.141	0.154	0.081	0.778
Female SNpc						
TH	4.674	0.044	2.173	0.049	0.586	0.345
NeuN	0.245	0.765	1.256	0.267	1.132	0.432
Male Striatum						
DA	1.301	0.444	7.510	0.018	0.876	0.635
DOPAC	1.123	0.110	0.976	0.577	1.432	0.102
HVA	0.797	0.324	0.122	0.453	0.602	0.960
Female Striatum						
DA	0.978	0.328	0.022	0.883	0.002	0.960
DOPAC	0.174	0.483	0.120	0.431	1.063	0.103
HVA	0.301	0.777	1.359	0.555	1.055	0.416
Male VTA						
TH	0.745	0.728	1.630	0.221	0.210	0.714
NeuN	1.232	0.298	0.562	0.453	1.205	0.243
Female VTA						
TH	1.675	0.218	1.34	0.721	0.721	0.411
NeuN	1.132	0.180	0.761	0.537	0.205	0.949
Male Striatum						
DA	0.233	0.478	1.210	0.771	0.442	0.471
DOPAC	0.923	0.782	0.756	0.778	1.233	0.162
HVA	0.345	0.825	1.088	0.156	1.002	0.192
Female Striatum						
DA	1.648	0.180	1.654	0.230	1.173	0.606
DOPAC	0.528	0.471	0.472	0.491	1.334	0.850
HVA	0.835	0.365	0.342	0.561	6.426	0.014

STUDY 2	DEX x Lesion		CORT x Lesion		DEX x CORT x Lesion	
	F	**p**	**F**	**p**	**F**	**p**
Males SNpc						
TH	1.797	0.424	2.142	0.098	0.502	0.760
NeuN	0.360	0.552	3.725	0.061	1.164	0.287
Female SNpc						
TH	2.648	0.064	1.554	0.130	1.174	0.406
NeuN	0.217	0.644	0.027	0.870	0.984	0.327
Male Striatum						
DA	1.325	0.043	3.244	0.039	0.987	0.675
DOPAC	0.832	0.765	0.220	0.978	2.668	0.062

HVA	0.898	0.476	1.123	0.768	8.765	0.009
Female Striatum						
DA	1.702	0.190	1.246	0.220	1.603	0.219
DOPAC	0.798	0.635	0.236	0.433	0.788	0.753
HVA	0.002	0.968	0.166	0.686	1.544	0.625
Male VTA						
TH	1.123	0.110	0.976	0.577	1.432	0.102
NeuN	0.797	0.324	0.122	0.453	0.602	0.960
Female VTA						
TH	0.978	0.428	0.123	0.983	0.802	0.460
NeuN	0.217	0.644	1.844	0.182	71.890	0.000
Male NAcc						
DA	1.675	0.218	1.34	0.721	0.721	0.411
DOPAC	0.232	0.998	0.461	0.653	0.205	0.949
HVA	1.576	0.326	1.620	0.821	0.421	0.714
Female NAcc						
DA	0.628	0.471	0.472	0.491	1.334	0.850
DOPAC	0.835	0.765	0.242	0.561	1.426	0.214
HVA	0.223	0.728	0.141	0.754	0.181	0.978