Corticosteroid modulation of the mesocorticolimbic dopamine system: implications for bipolar disorder

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Abstract

There is evidence that dopaminergic dysfunction plays a role in the symptoms of bipolar disorder although the precise abnormality is unclear. In addition dysregulation of corticosteroid secretion characterised by a flattened diurnal circadian rhythm has been observed across mood states in bipolar disorder. As a result it has been hypothesised that corticosteroid hypersecretion underlies the dopaminergic dysfunction. Here the endocrine regulation of mesocorticolimbic dopaminergic neurotransmission was investigated with the hope of bettering our understanding of the pathology of bipolar disorder.

Immunocytochemical studies established that around half of dopaminergic neurones in the ventral tegmental area (VTA) express the low affinity glucocorticoid receptor (GR), whilst all of these neurones express the high affinity mineralocorticoid receptor (MR). This indicated that corticosteroids can directly modulate dopaminergic neuronal function.

By administering corticosterone to rats in their drinking water a flattened circadian profile of corticosteroid secretion similar to that seen in bipolar disorder was modelled. *In situ* hybridisation histochemistry in the VTA revealed that corticosterone treatment increased the transcription of mRNA for tyrosine hydroxylase, the vesicular monoamine transporter and the D_2 receptor, whilst decreasing expression of the 5-HT_{2C} receptor and the $GluR_1$ subunit of the AMPA receptor.

In vivo microdialysis in the medial prefrontal cortex demonstrated increased dopamine release under both basal and stimulated conditions in corticosterone treated animals. This effect did not appear to be the result of altered D_2 autoreceptor function or a change in the firing rate of dopaminergic neurones. In light of the *in situ* hybridisation data it is hypothesised that flattening the diurnal profile of corticosteroid secretion increases prefronto-cortical dopamine release by upregulating dopamine synthesis and vesicular uptake.

These studies demonstrate that corticosteroid dysrhythmia of the type seen in bipolar disorder can alter dopaminergic neurotransmission and furthermore they indicate specific aspects of dopaminergic function which might be altered. Thus circadian rhythm abnormalities in the HPA axis may play a role in the aetiology of bipolar disorder via dysregulation of dopaminergic neurotransmission.

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Abbreviations

5-HIAA – 5-hydroxyindoleacetic acid

5-HT – Serotonin

5-HTT – Serotonin transporter

7-OH-DPAT – 7-hydroxy-N,N-di-n-propyl-2-aminotetraline

AA – Arachidonic acid

ABC – Avidin-biotin complex

aCSF – Artificial cerebrospinal fluid

ACTH – Adrenocorticotropic hormone

ADX – Adrenalectomy

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ANOVA – Analysis of variance

AP – Alkaline phosphatase

AP5 – 2-amino-5-phosphonopentanoate

ATP – Adenosine triphosphate

AUC – Area under the curve

AVP – Arginine-vasopressin

BPD – Bipolar Disorder

BSA – Bovine serum albumin

cAMP – Adenosine 3', 5'-cyclic monophosphate

CNQX – 7-nitro-2,3-dioxo-1,4- dihydroquinoxaline-6-carbonitrile

CNS – Central nervous system

COMT – Catechol-O-methyl transferase

CRH – Corticotropin-releasing hormone

CSF – Cerebrospinal fluid

DA – Dopamine

DAG – Diacylglycerol

DAPI – 4',6-diamidino-2-phenylindole

DAT – Dopamine transporter

DEPC – Diethyl pyrocarbonate

dex – Dexamethasone

DHPA – 3,4,-Dihydroxyphenylacetaldehyde

DL – Dorsolateral

DM – Dorsomedial

DNA – Deoxyribonucleic acid

DOPA – 3,4-Dihydroxyphenylalanine

DOPAC – 3,4-Dihydroxyphenylacetic acid

DSM-IV – Diagnostic and Statistical Manual of Mental Health Disorders, 4th Ed.

DST – Dexamethasone suppression test

EDTA – Ethylene diamine tetraacetic acid

FG – Fluorogold

FITC – Fluorescein

GABA – γ-aminobutyric acid

GluR – Receptor subunit of the AMPA glutamate receptort

GMT – Greenwich Mean Time

GR – Glucocorticoid receptor

GRE – Glucocorticoid response element

GRU – Glucocorticoid response unit

HPA – Hypothalamic-pituitary-adrenal

HPLC – High performance liquid chromatography

HRP – Horseradish peroxidase

HVA – Homovanillic acid

ICC – Immunocytochemistry

ID – Inner diameter

In Situ – *In situ* hybridization histochemistry

IP_3 – Inositol trisphophate

ISHH – *In situ* hybridization histochemistry

LAT1 – Large neutral amino acid transporter

MAO – Monoamine oxidase

MDD – Major depressive disorder

MHPG – 3-methoxy-4-hydroxyphenylglycol

MR – Mineralocorticoid receptor

mCPP – 1-(3-Chlorophenyl)piperazine

mPFC – Medial prefrontal cortex

mRNA – Messenger RNA (ribonucleic acid)

NAcc – Nucleus Accumbens

NBQX – 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

NE – Noradrenaline

NET – Noradrenaline transporter

NMDA – *N*-methyl-d-aspartate

NMDAR – Receptor subunit of the NMDA receptor

OD – Outer diameter

OSA – Octanesulphonic acid

PBS – Phosphate buffered saline

PD – Parkinson's disease

PET – Positron Emission Tomography

PFA – Paraformaldehyde

PFC – Prefrontal cortex

POMC – Pro-opiomelanocortin

PVN – Paraventricular nucleus of the hypothalamus

Rho – Rhodamine

SCN – Suprachiasmatic nucleus of the hypothalamus

SN – Substantia nlgra

SSC – Saline-sodium citrate

TH – Tyrosine hydroxylase

VL – Ventrolateral

VM – Ventromedial

VMAT2 – Vesicular monoamine transporter 2

VTA – Ventral tegmental area

Table of Contents

Chapter 1.

Introduction

Chapter 1. Introduction

Bipolar affective disorder is an illness which has a devastating effect on the lives of those who suffer with it. The aetiology of this disorder is poorly understood and current drug therapies, in the most part discovered serendipitously, treat symptoms rather than targeting the underlying pathology. A better understanding of this disease offers the possibility of developing more effective therapies which will not only benefit individuals but reduce the burden of this chronic illness on society as a whole.

The following thesis is based on an integrative hypothesis of bipolar disorder which provides a link between observed abnormalities in the hypothalamic-pituitary-adrenal axis and changes in brain function related to dopaminergic neurotransmission. These studies have been carried out to investigate aspects of the endocrine regulation of dopaminergic neuronal systems, and the effect that alterations in HPA axis function have on these systems. Thus it is hoped to better our understanding of these systems as they relate to bipolar disorder

1.1. Bipolar Disorder

1.1.1. Symptoms and diagnosis

The affective disorders, which include bipolar disorder, are a group of illnesses primarily characterised by dysregulation of emotion. The inappropriate emotional responses characteristic of affective disorders "do not reflect a realistic appraisal of the environment at the time" (Feldman et al. 1997) and result in significant impairment to behavioural and cognitive function. Compared to other psychiatric disorders, affective disorders are highly prevalent with estimates suggesting that 5% of the UK population (~ 3.25 million people) suffer from mood disorders (Ohayon et al. 1996), whilst 10-20% of the population of the US can expect to suffer a significant affective episode at some point during their lives (Feldman et al. 1997).

According to current classifications (DSM-IV, American Psychiatric Association 1994), affective disorders fall into two broad categories, unipolar depression (with major depressive disorder as it's archetype) and bipolar disorder. Bipolar disorder is characterised by episodes of

both elevated and depressed mood, with mood states categorised according to specific diagnostic criteria (see table 1.1.). Different combinations of these mood states allows for the diagnosis of a number of related disorders classified as bipolar I disorder, bipolar II disorder, and cyclothymia (for review see Manning et al. 1998).

It is the case however, that diagnosis in bipolar disorder is difficult due to the variability in presenting symptoms and the reliance on accurate recall of previous affective episodes (see Cassano et al. 1999; reviewed by Perlis 2005). High rates of comorbidity with other psychiatric disorders (reviewed by Krishnan, 2005; Hirshfeld and Vornik, 2005; Pini et al. 2005) further complicate the picture.

Due to the variability in presenting symptoms, and the shortcomings of making diagnoses based on rigid criteria, in recent years there has been a trend towards viewing bipolar disorder as a continuum of phenotypes ranging from cyclothymia to predominantly mania (Muller-Oerlinghausen et al. 2002). Indeed some have proposed that the affective disorders as a whole represent a continuum ranging from predominantly manic bipolar disorder to major depressive disorder, with bipolar mixed states as a kind of halfway point between the two (Akiskal, 2002). Such a theory suggests a common underlying pathology across DSM-IV bipolar subtypes, however it may be the case that whilst these disorders share common symptoms they differ in their aetiology. This uncertainty makes research into the biological basis of bipolar disorder particularly difficult.

1.1.2. Prevalence and disease burden of bipolar disorder

The current lifetime prevalence of bipolar I disorder in the general population is thought to be 1-2% (Avissar and Schreiber, 2002; Muller-Oerlinghausen et al. 2002, Pini et al. 2005) and affects men and women equally (Feldman et al. 1997; Pini et al. 2005). If all bipolar spectrum disorders are included in the analysis then the prevalence increases to around 6% (Pini et al. 2005). The World Health Organisation has ranked bipolar disorder as sixth on a list of the leading causes of disability worldwide (Avissar and Schreiber, 2002; Hirshfeld and Vornik, 2005). This can be attributed to the chronic nature of the disorder (Angst, 1978; Ten Have et al.

Manic Episode	Mixed Episode	Hypomanic Episode	Major Depressive Episode
A distinct period of abnormally and persistently elevated, expansive, or irritable mood, lasting at least 1 week (or any duration if hospitalization is necessary) **During the period of mood disturbance, three (or more) of the following symptoms have persisted (four if the mood is only irritable) and have been present to a significant degree:** *inflated self-esteem or grandiosity* *decreased need for sleep (e.g., feels rested after only 3 hours of sleep)* *more talkative than usual or pressure to keep talking* *flight of ideas or subjective experience that thoughts are racing* *distractibility (i.e., attention too easily drawn to unimportant or irrelevant external stimuli)* *increase in goal-directed activity (either socially, at work or school, or sexually) or psychomotor agitation* *excessive involvement in pleasurable activities that have a high potential for painful consequences (e.g., engaging in unrestrained buying spree, sexual indiscretions, or foolish business investments)* **The mood disturbance is sufficiently severe to cause marked impairment in occupational functioning or in usual social activities or relationships with others, or to necessitate hospitalization to prevent harm to self or others, or there are psychotic features.**	**The criteria are met both for a Manic Episode and for a Major Depressive Episode (except for duration) nearly every day during at least a 1-week period.** **The mood disturbance is sufficiently severe to cause marked impairment in occupational functioning or in usual social activities or relationships with others, or to necessitate hospitalization to prevent harm to self or others, or there are psychotic features**	**A distinct period of persistently elevated, expansive, or irritable mood, lasting throughout at least 4 days, that is clearly different from the usual nondepressed mood.** **Symptoms as for a manic episode (far left)** **The episode is associated with an unequivocal change in functioning that is uncharacteristic of the person when not symptomatic.** **The disturbance in mood and the change in functioning are observable by others.** **The episode is not severe enough to cause marked impairment in social or occupational functioning, or to necessitate hospitalization, and there are no psychotic features.** **Criteria distinguishing mania from hypomania:** *Meaningful conversation is difficult to maintain for any length of time* *Euphoric or ecstatic mood deteriorates to querulous belligerence* *Affective hallucinations, frank delusions of grandiose ability or identity, delusions of assistance or persecution, delusions of reference or delusions of love are present* *Insight and judgement are lost to such a degree that frenzied expansive activity leads to serious social impairment*	**Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.** *depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g. appears tearful). Note: In children and adolescents, can be irritable mood.* *markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others)* *significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day. Note: In children, consider failure to make expected weight gains.* *insomnia or hypersomnia nearly every day* *psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down)* *fatigue or loss of energy nearly every day* *feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick)* *diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others)* *recurrent thoughts of death (not just fear of dying),* *recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide* **The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.**

Table 1.1. Symptoms of abnormal mood states in bipolar disorder (adapted from DSM-IV criteria, APA, 1994; Manning et al. 1998)

Bipolar I Disorder	Bipolar II Disorder	Cyclothymia
The patient is currently (or most recently was) in a manic, mixed, or major depressive episode. At least one previous manic, mixed or major depressive episode has occurred which is fits the criteria for a mood state different to the one the patient is currently in.	One or more major depressive episodes is or has been present. At least one hypomanic episode is or has been present. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.	For at least 2 years, the presence of numerous periods with hypomanic symptoms and numerous periods with depressive symptoms that do not meet criteria for a Major Depressive Episode. Note: In children and adolescents, the duration must be at least 1 year. During the above 2-year period (1 year in children and adolescents), the person has not been without the symptoms in Criterion A for more than 2 months at a time. No Major Depressive Episode, Manic Episode, or Mixed Episode has been present during the first 2 years of the disturbance Note: After the initial 2 years (1 year in children and adolescents) of Cyclothymic Disorder, there may be superimposed Manic or Mixed Episodes (in which case both Bipolar I disorder and Cyclothymic Disorder may be diagnosed) or Major Depressive Episodes (in which case both Bipolar II Disorder and Cyclothymic Disorder may be diagnosed) The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

Table 1.2. Criteria for diagnosis of bipolar disorder subtypes (adapted from DSM-IV criteria, APA, 1994; Manning et al. 1998)

2003; Morgan et al. 2005) and the high comorbidity with other DSM-IV axis I disorders (reviewed by Krishnan, 2005; Hirshfeld and Vornik, 2005; Pini et al. 2005). Furthermore the disease carries a significant risk of mortality due to high rates of attempted and completed suicides (Chen and Dilsaver, 1996; Baldessarini et al. 2006).

In addition to the burden for the individual, bipolar disorder represents a significant cost to society. Direct costs resulting from emergency treatment, hospitalization, psychiatric treatment and medication in the United States were estimated at $7 billion in 1991, whilst indirect costs result from impaired productivity, welfare costs, and criminality associated with the disease were estimated at $38 billion. Lost productivity due to suicide alone represented a significant proportion of this cost at $8 billion (Hirshfeld and Vornik, 2005).

1.1.3. Genetics of bipolar disorder

Studies have demonstrated a high degree of heritability in bipolar disorder with first degree relatives of patients having higher rates of bipolar and unipolar disorders than the general population (Gershon et al.1987). Further evidence comes from studies in twins where it has been shown there is much greater concordance for bipolar disorder in monozygotic twins than dizygotic twins (79% vs. 24% respectively) (Bertelsen et al. 1977). Interestingly the rate of concordance in monozygotic twins is much higher than it is for unipolar depression (where the rate of concordance is 54%) suggesting that there is a strong genetic predisposition to the disorder.

1.1.4. Environmental risk factors

Whilst there is a high degree of heritability in bipolar disorder, environmental factors play a significant role in triggering the condition. Chief amongst these factors is stress, such that adverse life events have been associated with first onset of symptoms (see reviews by Paykel, 2003 and Tsuchiya et al. 2003) whilst a positive relationship has been shown between stress and recurrent bipolar episodes. In one study patients with bipolar I disorder who experienced episodes of illness showed greater total stress levels in the preceding months than those patients without episodes (Hammen and Gitlin, 1997). Furthermore, developmental adaptations to stress induced by early life adversity (physical or sexual abuse in childhood or adolescence) have been found to correlate with earlier onset of illness, faster cycling frequencies, increased suicide rates, more co-morbidities and greater total time ill than bipolar patients without a history of early life adversity (Post et al. 2001).

1.1.5. Pharmacotherapy for bipolar disorder

Current UK guidelines for the pharmacological management of bipolar disorder recommend the use of three principal classes of drug - mood stabilisers, antipsychotics and/or antidepressants (UK NICE guidelines, 2006). The following section provides a brief introduction to these drugs and the circumstances under which they are used.

1.1.5.1. Mood stabilisers

The mood stabilisers are a group of drugs which include the monovalent ion lithium, and the anticonvulsant drugs valproate, carbamazepine and lamotrigine. These drugs are the mainstay of prophylactic treatment for bipolar disorder and are, for the most part, efficacious in the acute treatment of mania. There is evidence to suggest that while lamotrigine has prophylactic efficacy, it has greater utility for the treatment of acute depressive episodes than for acute mania (Fountoulakis et al. 2007). At the present time mood stabilisers are indicated for the first-line treatment of all phases of bipolar disorder (UK NICE guidelines, 2006).

The first drug of this class was lithium, whose mood stabilising properties were recognised during the late 1940's to early 1950's (Atack et al. 1995). The mechanism by which lithium alters neuronal function to produce its mood stabilising effect is unclear, however it has been observed that lithium is a potent inhibitor of the intracellular enzyme inositol monophophatase (Atack et al. 1995; Lenox and Frazer, 2002; Brunello, 2004). This enzyme performs a key step in the regeneration of phosphoinositide required for G-protein mediated signal transduction, with the result that lithium inhibits agonist stimulated IP_3/DAG signalling. One consequence of this is that protein kinase C (PKC) activity is reduced following long-term lithium treatment which has the potential to alter neuronal excitability, neurotransmitter release, and produce long-term alterations in gene expression and plasticity (Lenox and Fraser 2002; Brunello, 2004). Lithium also modulates the cAMP signal transduction pathway by reducing the receptor-coupled stimulation of adenylyl cyclase and increasing basal cAMP levels (Lenox and Frazer, 2002; Brunello, 2004; Montezinho et al. 2007). As cAMP modulates neuronal function via protein kinase A (PKA) this is an additional means by which lithium might modulate neurotransmission. A further property of lithium is its ability to increase Na^+/K^+ ATPase activity (Lenox and Frazer, 2002). This results in a decrease in intracellular sodium and, because calcium concentrations largely parallel those of sodium, a concomitant decrease in intracellular calcium. Given that these two ions play a critical role in neuronal excitability and neurotransmitter release, this is a further mechanism by which lithium may regulate neuronal function.

The remaining mood stabilisers valproate, carbamazepine and lamotrigine are anticonvulsants that inhibit sodium channels, particularly those with a high rate of activity (Ragsdale and Avoli,

1998). Carbamazepine and valproate have also been shown to inhibit adenylyl cyclase activity (Montezinho et al. 2007), whilst valproate has been shown to inhibit PKC activity in a similar fashion to that produced by lithium (Brunello, 2004). Thus it is likely that these drugs produce their therapeutic effect via the modulation of ion permeability ad intracellular signalling pathways in neuronal cells. Further discussion of the functional effects of these drugs on dopaminergic cells can be found in section 1.1.6.4 (c).

1.1.5.2. Antipsychotics

Antipsychotics are efficacious for both the acute treatment of mania and long-term mood stabilisation, with some atypical (see later) antipsychotics showing acute efficacy in the depressive phase of the illness (Brambilla et al. 2003; Surja et al. 2006). Current UK guidelines recommend the use of antipsychotics for the treatment of mania, although the atypical olanzapine is considered an option for long-term management of bipolar disorder (UK NICE guidelines, 2006).

Antipsychotics were discovered in the late 1940's when it was noted that various substances including promethazine produced tranquilising properties without sedation (Stip, 2002). Subsequent developments based on modifications to the phenothiazine structure of these drugs produced chlorpromazine, the antipsychotic effect of which was discovered in the early 1950's (Stip, 2002). A defining property of these early antipsychotic drugs, including haloperidol, thoridazine and flupenthixol, was their induction of so-called extra-pyramidal side-effects such as dystonia, akathisia, parkinsonism and tardive dyskinesia (Meltzer, 2002; Hood and Nutt, 2004). With the subsequent development of antipsychotics which do not produce these extra-pyramidal effects, the notion of atypical (as opposed to classical) antipsychotics was introduced. Examples of atypical drugs are clozapine, olanzapine, riperidone and quetiapine.

By the 1960's significant steps had been made towards elucidating the mechanism of action of the classical antipsychotics with the discovery that they antagonise dopamine receptors (Carlsson and Linqvist, 1963). Specifically antipsychotics bind to D_2-like receptors which are expressed at presynaptic sites on dopaminergic neurones, and at postsynaptic sites (see section 1.3.1.6.). The antipsychotic efficacy of both classical and atypical antipsychotics has

been correlated with their binding affinity at the D_2 receptor (Wilson et al. 1998; Kapur and Mamo, 2003). Whilst the lack of extra-pyramidal side-effects is the principal distinguishing feature of atypical antipsychotics, at a pharmacological level some of these drugs, such as olanzapine, risperidone and clozapine share a high affinity for the 5-HT_{2A} receptor (Stip, 2002; Meltzer, 2002). This property is not shared with the classical antipsychotic drugs and this distinction has been proposed as a further means of defining classical vs. atypical antipsychotics. Further discussion of the functional pharmacology of these drugs in relation to dopaminergic hypotheses of bipolar disorder can be found in section 1.1.6.4. (c). The role of D_2 receptors in the regulation of dopaminergic neurotransmission is also discussed in greater detail in section 1.3.5.

1.1.5.3. Antidepressants

Antidepressants as a class of drugs are useful in the treatment of the depressive phase of bipolar illness. These drugs produce their therapeutic effect by increasing monoaminergic neurotransmission in the CNS. This occurs via three principal mechanisms: 1) reuptake inhibition which has the effect of increasing synaptic monoamine concentrations and increasing post-synaptic neuronal stimulation; 2) inhibition of monoamine neurotransmitter metabolism, which has a similar effect to reuptake inhibition in that neurotransmitter inactivation is reduced; and 3) presynaptic autoreceptor inhibition resulting in increased neurotransmitter release (Artigas et al. 2002).

Some of the earliest agents to be employed as antidepressants were the tricyclic antidepressants (TCA's) such as imipramine and amitryptiline. These drugs potentiate serotonergic and noradrenergic neurotransmission by blocking nerve terminal reuptake of the neurotransmitter following release and this is thought to underlie their therapeutic efficacy (Artigas et al. 2002; Lenox and Frazer 2002). The other principal class of antidepressants discovered around this time were the monoamine uptake inhibitors (MAOIs) such as iproniazid and tranylcypromine which inhibit the oxidative metabolism of serotonin, noradrenaline, and dopamine. Again, the potentiation of monoamine neurotransmission is thought to underlie their therapeutic effect (Artigas et al. 2002; Lenox and Frazer 2002).

The discovery of these drugs contributed greatly to the monoamine hypothesis of depression, and subsequent refinements to this hypothesis led to the development of drugs which target specific monoamines such as serotonin and noradrenaline. Perhaps the best example of this would be the development during the 1970's of the selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, paroxetine and sertraline. The principal advantage of these drugs over the earlier TCA's is their improved side-effect profile such as fewer anticholinergic effects, and a better safety profile (Artigas et al. 2002; Lenox and Frazer 2002). More recently developed drugs include the selective noradrenaline reuptake inhibitors (SNRIs) such as reboxetine, the serotonin-noradrenaline reuptake inhibitors such as venlafaxine and the noradrenaline and dopamine reuptake inhibitor bupropion (DeVane 1998; Artigas et al. 2002; Tremblay and Blier 2006).

Similar refinements have been made to drugs which inhibit monoamine oxidase such that moclobemide, a drug which reversibly inhibits the MAO-A isoform of monoamine oxidase is available. This has significant advantages over the traditional irreversible MAOI's in terms of safety (Fulton and Benfield, 1996). One of the key drawbacks of monoamine oxidase inhibitors is the risk of a so-called 'cheese reaction' resulting from the inhibition of the breakdown of dietary tyramine, the symptoms of which include severe hypertension and, potentially, intracranial haemorrhage. Reversible inhibition of the enzyme greatly reduces the risk of this side-effect occurring.

The final class of antidepressant, are the drugs acting directly at neurotransmitter receptors. At the present time the only drug of this type in common clinical use is mirtazepine, an α_2 adrenergic receptor antagonist which increases noradrenergic neurotransmission via presynaptic autoreceptor inhibition (Tremblay and Blier 2006).

Current UK guidelines for the treatment of bipolar disorder recommend the use of antidepressants only in conjunction with an anti-manic drug such as a mood stabiliser (UK NICE guidelines 2006). This is due to the risk of inducing a switch to mania in patients receiving antidepressants alone. The guidelines also recommend the monitoring of bipolar patients for signs of prolonged akathisia or agitation which might be indicative of the early stages of mania.

1.1.6. Pathophysiology of bipolar disorder

The high degree of heritability in bipolar disorder indicates that there is some underlying biological deficit which predisposes individuals to the condition. Despite a huge amount of research carried out in the field of affective disorders this underlying cause is yet to be found, although a number of biological systems are currently under investigation.

1.1.6.1. Monoamine systems in bipolar disorder

One of the most enduring hypotheses regarding the aetiology of affective disorders has been the "monoamine hypothesis" which was first postulated in the mid-1960's (Schildkraut, 1965). This hypothesis was derived from the observation that drugs affecting central monoamine function can significantly alter mood. The serendipitous discovery of the monoamine oxidase inhibitors in the 1950's demonstrated that drugs which increase monoamine levels in the brain have mood elevating properties, whilst drugs which deplete central monoamines such as reserpine were found to induce depression in a manner which was, at that time, thought to be clinically indistinct from that seen in depressed patients. Initially Schildkraut postulated that noradrenaline was the neurotransmitter of crucial importance (1965), whilst others focused on the role of serotonin (Coppen, 1969). Subsequently the importance of dopamine was recognized as the clinical evidence began to be formulated into a hypothesis during the 1970's (see for example Barbeau, 1970). The vast majority of research based on the monoamine hypothesis has been carried out in major depressive disorder, and serotonin in particular has been the focus of many studies. This is principally due to the fact that the majority of effective antidepressants act on this neurotransmitter system. The study of dopamine is perhaps more pertinent to bipolar disorder due to its potential role in the distinguishing feature of this disorder, mania which can be induced by drugs which increase dopaminergic transmission (reviewed by Silverstone, 1985). In addition there has been increased interest in the role of dopamine in emotional regulation as a result of the role of the mesolimbic dopamine system in reward and motivational behaviour (reviewed by Willner, 1995), deficits of which may account for the anhedonia and amotivational states present during depressive episodes. There follows a review of the literature concerning monoamine neurotransmitter systems in bipolar disorder.

1.1.6.2. Evidence for serotonergic dysfunction in bipolar disorder

Serotonin has enjoyed a central position in the monoamine hypothesis of affective disorders in the 30 years since the discovery that selective serotonin reuptake inhibitors can be effective in the treatment of major depressive disorder. A huge body of literature exists regarding the role of serotonin in major depression and there is general agreement that deficits in central serotonergic transmission play a role in this condition (Mahmood and Silverstone, 2001). As far as the role of serotonin in bipolar disorder goes the body of evidence is much smaller with few studies looking specifically at this disease.

Some of the earliest studies concentrated on measurements of the 5-HT metabolite 5-HIAA (5-hydroxyindole acetic acid). These were, on the whole, inconclusive with one study demonstrating lower CSF levels of 5-HIAA in melancholic bipolar patients than in healthy controls (Asberg et al. 1984), a finding which a later study failed to replicate (Redmond et al. 1986). In a similar study looking at manic patients an increase in CSF 5-HIAA was found only in female subjects (Swann et al. 1983). Despite these findings decreased levels of 5-HIAA have been associated with increased suicide risk as evidenced by findings in post mortem brains of bipolar depressed suicides (Traskmann et al. 1981; Young et al. 1994) providing some evidence to support the serotonergic hypothesis.

Alterations to 5-HT transporter (5-HTT) function in bipolar patients have been demonstrated. Thus in studies using platelet 5-HT uptake as an indicator of central 5-HT uptake, decreases in 5-HT transport have been shown in both bipolar depression (Meltzer et al. 1983) and bipolar mania (Marazziti et al. 1991). More recently Oquendo et al. (2007) demonstrated decreases in 5-HTT binding in various brain regions in bipolar depressives whilst post-mortem studies have shown lower concentrations of citalopram binding in bipolar subjects who died whilst depressed (Leake et al. 1991). These findings are challenged, however, by another recent study which reports increased 5-HT transporter binding in the thalamus and a number of cortical regions, and decreased binding in the midbrain in unmedicated bipolar patients (Cannon et al. 2006) making it difficult to draw conclusions from the literature.

Changes in 5-HT receptor expression have been found in post-mortem tissue from bipolar patients, with decreases in 5-HT_{2A} receptor mRNA levels in the prefrontal cortex and increases

in 5-HT_{1B} and 5-HT_{2A} transcripts in the hippocampus (Lopez-Figueroa et al. 2004). Further evidence for changes in the expression of the 5-HT_{1A} receptor is provided by a PET imaging study of 5-HT_{1A} ligand binding in which decreases were found in the raphe and mesiotemporal cortex of bipolar depressed patients (Drevets et al. 1999). However a study looking at post-mortem hippocampal tissue from bipolar patients found no differences in 5-HT_{1A} binding (Dean et al. 2003). Due to its easy accessibility, calcium mobilization by the platelet 5-HT_{2A} receptor has been used as an indicator of receptor function in psychiatric disorders. Studies in bipolar depressed patients have shown that calcium mobilization by serotonin is markedly elevated compared to normal controls and subjects with unipolar diagnoses (Kusumi et al. 1994; Suzuki et al. 2001) and in a more recent study this was associated with increased protein kinase C activity resulting from receptor activation rather than an increase in receptor expression (Suzuki et al. 2003). Overall there is some evidence that 5-HT receptor expression and function is altered in bipolar disorder however further work is required to clarify the nature of these alterations.

The ability of mood-stabilising and antidepressant drugs to modulate the serotonergic system provides circumstantial evidence for the involvement of this system in bipolar disorder. Lithium has been shown to increase serotonin release in various regions of the rat brain (Treiser et al. 1981; Friedman and Wang, 1988; Baptista et al. 1990; Pei et al. 1995), whilst in bipolar patients lithium treatment increases plasma 5-HT levels (Artigas et al. 1989). Furthermore, the use and efficacy of selective serotonin reuptake inhibitors in combination with mood stabilisers for the treatment of severe or treatment resistant bipolar depressive episodes (see Thase, 2005) implies that there is a serotonergic component to these states. The ability of antidepressants to induce a switch to mania has been reported in around 20-40% of bipolar patients and has been reported for SSRI's as well as all other major classes of antidepressant (reviewed by Goldberg and Truman, 2003). Serotonergic dysfunction may therefore play a role in manic states, a hypothesis supported by the demonstrated efficacy of atypical antipsychotics with high affinities for the 5-HT_{2A} receptor in treating these episodes (Cousins and Young, 2007).

1.1.6.3. Evidence for noradrenergic dysfunction in bipolar disorder

Noradrenaline was originally considered to be central to the monoamine hypothesis of mood disorders as postulated by Schildkraut (1965). In more recent times its position has been supplanted by serotonin, however a limited number of studies have looked at the role of this neurotransmitter in bipolar disorder. A number of early studies demonstrated significantly lower urinary levels of the noradrenaline metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) in bipolar patients compared to unipolar or control subjects (reviewed by Schatzberg and Schildkraut, 1995), however a more recent study has failed to replicate these findings (Grossman and Potter, 1998). Studies of plasma and urinary noradrenaline levels have shown more consistent results with elevations of plasma NE in bipolar and unipolar depressed patients following orthostatic challenge being greater than those seen in controls (Rudorfer et al. 1985; reviewed by Schatzberg and Schildkraut, 1995), whilst urinary NE has been found to be greater in unipolar and bipolar patients compared to controls (Grossman and Potter, 1999).

Changes to adrenoceptor function have not been consistently demonstrated in bipolar disorder. Studies have shown a decrease in β-adrenergic receptor binding associated with bipolar disorder (Wright et al. 1984; Wood et al. 1986) although other studies have failed to replicate these findings (Berrettini et al. 1987; Kay et al. 1993; Young et al. 1994), whilst decreased β-adrenoceptor function as measured by stimulated cAMP production has been reported in lymphocytes of manic and depressed patients (Extein et al. 1979). A trend towards increased platelet α_2-adrenoceptor density has also been reported in bipolar patients (Karege et al. 1992).

The role of noradrenaline in the action of mood stabilising drugs has been investigated, and studies have demonstrated that high doses of lithium can induce the release of NE from cortical and hippocampal slices (Gross and Hanft, 1990) whilst in a peripheral model lithium has been found to decrease the release of NE (Mantelli and Ledda, 1989) although this has not been replicated (Finberg et al. 1992). Valproate has been found to have little effect on noradrenaline release in cortical slices (de Boer et al. 1982), however increases in NE in-vivo have been found following valproate treatment in the rat hippocampus and brainstem with a concurrent decrease in the hypothalamus (Baf et al. 1994a). Valproate has also been shown to increase tyrosine

hydroxylase mRNA in the locus coeruleus of rats following chronic treatment (Sands et al. 2000). Elevations in NE levels have been shown in the motor cortex and cerebellum of rats following carbamazepine treatment (Baf et al. 1994b), whilst increases in α_2 adrenoceptor sensitivity have also been demonstrated (Dilsaver et al, 1993). Overall there is some evidence that these drugs can affect noradrenergic function, but as with the rest of the literature concerning the role of noradrenaline in bipolar disorder, there is currently not enough data to draw any conclusions.

1.1.6.4. Evidence for dopaminergic dysfunction in bipolar disorder

The clinical evidence that dopamine plays a role in bipolar mood states began to be recognized during the 1970's (see Barbeau, 1970) leading to the formulation of a hypothesis implicating dopaminergic hyperfunction in mania and hypofunction in depression (Bunney and Garland,1982). There is now compelling evidence for this hypothesis with a large body of literature available implicating the mesolimbic dopamine system in motivational and reward behaviour (see later) providing a link with the anhedonic states characteristic of depression, whilst the behaviour produced by dopamine releasing drugs implicates this system in manic states. In addition the ability of dopamine to alter cognitive function via projections to the prefrontal cortex (see later) suggests that neurocognitive deficits present in the disease (see Quraishi and Frangou, 2002; Savitz et al. 2005) may be the result of dopaminergic dysfunction.

(a) Dopamine metabolites in bipolar disorder

CSF levels of the dopamine metabolite homovanillic acid (HVA) have been reported to be elevated in manic bipolar patients (Swann et al. 1983; Gerner et al. 1984; Tandon et al. 1988), and decreased in depressed bipolar patients (Asberg et al. 1984; Reddy et al. 1992, Bottiglieri et al. 2000). These studies indicate that dopamine metabolite levels are a state marker for bipolar disorder, however this may be an oversimplification. It has been suggested that CSF HVA levels are indicative of motor activity rather than mood as a result of the dense dopaminergic innervation of the basal ganglia and its close proximity to the ventricles (Willner et al. 1995). Consequently these studies may not provide an accurate measure of neuronal

function in dopaminergic systems implicated in mood and cognitive regulation such as the mesolimbic and mesocortical systems.

(b) Bipolar symptoms and dopaminergic drugs

It has been observed that a variety of drugs which stimulate dopamine release, or directly stimulate dopamine receptors can induce mania. Perhaps the most obvious example of this is amphetamine which in healthy subjects produces a behavioural syndrome very similar to that seen in mania (Silverstone, 1985) and has been shown to precipitate manic episodes in bipolar patients (Van Kammen and Murphy, 1975; Gerner et al. 1976). Further evidence for dopaminergic dysfunction in mania is provided by the observation that amphetamine produces an exaggerated behavioural response in bipolar patients compared to healthy controls (Anand et al. 2000) although the evidence for this is inconsistent (see also Nurnberger et al. 1982). Although amphetamine induces the release of both dopamine and noradrenaline it has been shown that dopamine antagonists are able to block its behavioural effects (Jonsson, 1972; Silverstone et al., 1980) implicating dopamine as the mediator of its manic effects. The idea that mania is the result of a hyperdopaminergic state is given further support by the ability of dopamine receptor agonists such as piribedil (Gerner et al. 1976), bromocriptine (Vlissides et al. 1978; Brook and Cookson, 1978; Kemperman and Zwanikken, 1987) pramipexole and ronpirole (Singh et al. 2005) to induce mania. Furthermore the dopamine precursor L-DOPA has been shown to induce mania in bipolar depressives (Goodwin et al. 1972; Murphy, 1973) whilst L-DOPA therapy in Parkinson's disease has been associated with an increased incidence of mania (Harsch et al. 1985; Black et al. 2005), an effect which is believed to occur as result of increased dopamine synthesis.

Reducing dopamine synthesis by dietary depletion of tyrosine (the amino acid precursor of dopamine) has been shown to reduce the symptoms of mania in bipolar subjects (McTavish et al. 2001), thus giving further evidence for a hyperdopaminergic state in mania. Moreover tyrosine depletion induces disrupted affect/reward characteristics in healthy subjects which parallel those seen in bipolar depressive states (McLean et al. 2004). This builds on earlier work showing that reserpine induced depletion of monoamines (including dopamine) depresses

mood, an observation which contributed to the original monoamine hypothesis of affective disorders (Schildkraut, 1965). Later analysis of the original reserpine studies revealed that reserpine did not actually produce true depressive states in the majority of individuals but a "pseudodepressive" state lacking the cognitive features of major depression (Willner et al. 1995). Despite this, there is a significant body of evidence that drugs interfering with dopaminergic transmission can induce mood states similar to those seen in bipolar disorder.

(c) Therapeutic drug effects on dopaminergic systems

A variety of drugs with efficacy in bipolar disorder interact with dopaminergic systems in the brain. In particular, the ability of antipsychotic drugs to treat the symptoms of mania and potentially depression (Brambilla et al. 2003; Surja et al. 2006; Wijkstra et al. 2007) provides good evidence for a dopaminergic mechanism in bipolar disorder. The therapeutic potency of typical antipyschotics has been correlated with their affinity for binding at D_2 receptors (reviewed by Wilson et al. 1998) and whilst atypical antipsychotics have much lower affinities for this receptor, D_2 antagonism is required for therapeutic effect (Kapur and Mamo, 2003).

Whilst all antipsychotic drugs antagonise D_2 receptors, their exact mechanism of action is currently unclear. Administration of these drugs to rats produces increases in dopamine release in the nucleus accumbens and striatum (Moghaddam and Bunney, 1990; Volonte et al. 1997; Kuroki et al. 1999; Rollema et al. 2000) an effect consistent with the blockade of presynaptic autoreceptors, but seemingly at odds with the hyperdopaminergic hypothesis of mania. Furthermore typical and atypical antipsychotics appear to differ in their effects on dopamine release in the prefrontal cortex. A number of studies have found no significant effect of acute typical antipsychotic administration on prefrontocortical dopamine release (Moghaddam and Bunney, 1990; Volonte et al. 1997; Gessa et al. 2000; Westerink et al. 2001; Ago et al. 2005), whilst others have found that typical antipsychotics are less efficacious at increasing dopamine release in the prefrontal cortex than in the nucleus accumbens or striatum (Rollema et al. 2000; Westerink et al. 2001). In contrast, acute administration of atypical antipsychotics has consistently been shown to increase dopamine release in the prefrontal cortex, nucleus accumbens and striatum (Volante et al. 1997; Kuroki et al. 1999; Gessa et al. 2000; Ichikawa et

al. 2001; Jaskiw et al. 2001). Overall it is currently unclear how the regional differences relate to therapeutic effect.

As mentioned previously, the consistent finding of increased dopamine release upon acute administration of antipsychotics would appear to be at odds with a hyperdopaminergic hypothesis of mania. One possible explanation is that blockade of postsynaptic dopamine receptors, thereby reducing dopaminergic neurotransmission, is responsible for their therapeutic effect. If this is the case, acute increases in dopamine release may well be anomalous as far as the anti-manic action of these drugs is concerned.

Whilst increased dopamine release may not explain the anti-manic properties of these drugs, it may explain the blockade of spontaneous dopaminergic cell firing in the ventral tegmental area induced by chronic administration of both typical and atypical antipsychotics (Bunney and Grace, 1978; Chiodo and Bunney, 1983; White and Wang, 1983; Todorova and Dimpfel, 1994). This effect is likely to be responsible for the decrease in dopamine release in the prefrontal cortex, nucleus accumbens and striatum which has been found to occur following chronic treatment with the atypical clozapine or the typical antipsychotic haloperidol (Morrow et al. 1999). Further evidence of changes to dopaminergic function with chronic administration of antipsychotic medication is provided by the ability of clozapine to decrease tyrosine hydroxylase (TH, the rate-limiting enzyme in dopamine synthesis) mRNA expression in the VTA and substantia nigra and TH protein levels in the nucleus accumbens and striatum (Tejedor-Real et al. 2003). These data suggest that in the long-term, antipsychotics decrease dopaminergic neurotransmission by a presynaptic mechanism, an effect which might correlate with their long-term anti-manic efficacy.

Whilst antipsychotic drugs are particularly effective in the treatment of mania, other dopaminergic drugs have shown efficacy as antidepressants in bipolar disorder. Early studies reported that the D_2/D_3 receptor agonist bromocriptine is an effective antidepressant with greater efficacy in bipolar compared to unipolar depressives (Colonna et al. 1978; Silverstone et al. 1984). Furthermore a number of studies have shown that the D_3 preferring agonist pramipexole has antidepressant efficacy in bipolar disorder (Perugi et al. 2001; Lattanzi et al. 2002; Zarate et al. 2004; Goldberg et al. 2004). The antidepressant efficacy of these agonists

suggests that dopaminergic neurotransmission is reduced in bipolar depression, however this interpretation may be oversimplistic. Systemic administration of pramipexole has been shown to decrease the release of dopamine in the striatum (Crater and Muller, 1991) presumably as a result of activating presynaptic autoreceptors. Such a mechanism of action is inconsistent with a hypodopaminergic hypothesis of depression. Another study found, however, that bromocriptine and pramipexole induce locomotor activity following intra-striatal administration but attenuate the locomotor stimulating effects of a calcium channel agonist (Maruya et al. 2003). Thus under conditions of low dopamine release these drugs may potentiate dopaminergic neurotransmission, but when dopaminergic neurotransmission is elevated, these drugs have an anti-dopaminergic effect. Thus if bipolar depression is characterised by a hypodopaminergic state bromocriptine and pramipexole may well augment dopamine neurotransmission to rectify the deficit.

A major group of drugs used in the prophylactic treatment of bipolar disorder are the mood stabilising drugs which include lithium, carbamazepine and valproate. Whilst these drugs do not interact directly with dopamine receptors, there is evidence, although inconsistent, that they modulate dopaminergic neurotransmission. Some studies in rats have shown that chronic lithium treatment increases dopamine synthetic activity (Koyama et al. 1987; Gudelsky et al. 1988) and basal levels of dopamine and its metabolites in the nucleus accumbens (Koyama et al. 1987; Baptista et al. 1993) whilst others have shown no effect on synthesis or release in this region (Reches et al. 1984; Ferrie et al. 2006). In the study of Ferrie et al. (2006) no effect of chronic lithium was seen on basal dopamine levels, however potassium stimulated dopamine release was significantly attenuated in lithium treated animals.

In the prefrontal cortex two studies have found evidence for increased dopamine synthesis following chronic lithium treatment (Koyama et al. 1987; Gudelsky et al. 1988), however measurement of levels of dopamine and its metabolites have led to conflicting results. One study found increased levels (Koyama et al. 1987), one study found a decrease (Baptista et al. 1993) and another found no effect (Reches et al. 1984).

In the striatum results have been more consistent with a number of groups finding increased dopamine and dopamine metabolite levels following chronic lithium treatment (Hesketh et al.

1978; Otero-Losada and Rubio, 1985; Koyama et al. 1987; Gudelsky et al. 1988), although again one study found no effect (Reches et al. 1984).

In a similar fashion to the effect of lithium found by Koyama et al. (1987) carbamazepine and valproate treatment have been shown to increase prefrontocortical dopamine release (Ichikawa and Meltzer, 1999; Ichikawa et al. 2005), whilst carbamazepine has also been shown to increase dopamine release in the striatum and hippocampus (Okada et al 1997).

Whilst it is evident that mood-stabilisers can modulate dopaminergic neurotransmission it is unclear how this relates to their therapeutic effect. Although the balance of evidence points to an upregulation of basal dopaminergic activity by mood-stabilisers, it is interesting that Ferrie et al. (2006) showed an attenuation of stimulated dopamine release by lithium treatment. If lithium does in fact elevate basal dopamine levels when they are low but decrease dopamine levels under stimulated conditions this may account for its mood-stabilising effect. It could thus be speculated that mood-stabilisers attenuate the hyperdopaminergic state of bipolar mania whilst potentiating dopamine release during the bipolar depressive phase.

An important point which relates to all classes of drugs used in the treatment of bipolar disorder is that our knowledge regarding their mechanism of action comes largely from studies in (presumably) healthy animals. It may be the case that the effects of these drugs on dopaminergic function differ in bipolar disorder, thus extrapolating the mechanism of their therapeutic action from these studies may not be possible. Despite this caveat there is considerable evidence that modulation of dopaminergic neuronal function may underlie their therapeutic effects.

(d) Markers of central dopaminergic function in bipolar disorder

Thus far studies investigating markers of dopaminergic function in the brain have yielded conflicting results, however the available data provide some evidence that dopaminergic function is altered in bipolar disorder.

An interesting finding, which relates directly to the results of this thesis, is the demonstration of increased vesicular monoamine transporter 2 (VMAT2) binding in the thalamus and ventral brainstem of euthymic bipolar disorder I patients compared to schizophrenics and healthy

controls (Zubieta et al. 2000; 2001). This suggests that vesicular dopamine content may be increased in bipolar disorder and this may lead to an increase in dopamine release (see section 1.3.1.2.). Conflicting somewhat with this interpretation are the results of a PET imaging study which showed that the rate constant for [^{18}F]-DOPA uptake in the striatum is unaltered in manic subjects, a finding which has been taken as evidence that dopamine synthesis and vesicular uptake (of [^{18}F]-dopamine) is unaltered in mania (Yatham et al. 2002a). It would appear however, that this is a rather indirect measure of vesicular uptake, given that this method only demonstrates the accumulation of labelled DOPA in the region of interest.

One PET study has shown that in bipolar subjects (but not unipolar subjects) DAT binding is unilaterally increased in the right striatum (Amsterdam and Newberg, 2007), suggesting that post-synaptic dopaminergic function may be decreased (as a result of greater dopamine re-uptake) in these patients.

In terms of dopamine receptors, one clinical PET study has shown a decrease in D_1 receptor binding in the frontal cortex of bipolar disorder subjects (Suhara et al. 1992) suggesting a down-regulation of receptor expression in this region, however a study of D_1 mRNA expression in post-mortem hippocampal tissue found *increased* expression in the CA2 (but not CA1 or CA3) region (Pantazopoulos et al. 2004). This suggests that there may be regional differences in dopaminergic regulation in the disease.

Studies looking at the D_2 receptor have been similarly inconsistent. Studies have shown increased D_2 receptor binding in neuroleptic-naïve psychotic manic subjects but not in non-psychotic manic subjects (Pearlson et al. 1995; Yatham et al. 2002b), whilst a further study found no difference in striatal D_2/D_3 binding between euthymic bipolar subjects (half of whom were unmedicated) and controls (Anand et al. 2000). Somewhat in contrast to these results however, are the findings of a study which examined D_2 mRNA expression in post-mortem tissue from bipolar subjects showing a decrease in expression in the prefrontal cortex (Knable et al. 2001) and another study which found that D_3 mRNA is decreased in peripheral lymphocytes of bipolar subjects (Vogel et al. 2004). Again, the available data on D_2 and D_3 receptors in bipolar disorder suggests there may be regional differences in receptor abnormalities.

Overall, it is difficult to formulate from these data a generalised hypothesis of dopaminergic

dysfunction in bipolar disorder. Increased VMAT2 binding in the thalamus and brainstem, and decreased D_1 receptor binding and D_2 mRNA expression in the frontal cortex are consistent with an upregulation of dopaminergic neurotransmission in these regions. In contrast, however, increased DAT binding in the striatum and increased D_1 mRNA expression in the hippocampus are consistent with a decrease in dopamine release in these areas. As such one could speculate that in bipolar disorder there are fundamentally different alterations in dopaminergic neurotransmission between brain areas.

(e) Linkage studies of genes related to dopaminergic function in bipolar disorder

Genetic linkage studies have failed as yet to produce any replicable results for candidate genes related to bipolar disorder and dopaminergic function. Initial studies implicating the dopamine receptor DRD2, 3 and 4 genes in bipolar disorder have not been replicated, although association with the DRD1 gene has been shown in a number of studies (reviewed by Kato et al. 2007). Importantly several studies have reported no linkage between the tyrosine hydroxylase gene and bipolar disorder (reviewed by Craddock et al. 2001).

Overall there is much evidence for a dopaminergic component in the pathology of bipolar disorder, although its exact nature remains unclear. The evidence from metabolite studies and the effects of dopaminergic drugs on behaviour suggests a hyperdopaminergic state in mania and a hypodopaminergic state in bipolar depression, however our knowledge of the effects of therapeutic drugs on dopaminergic neurotransmission is not entirely consistent with this hypothesis. Furthermore, clinical imaging and post-mortem studies in bipolar disorder suggest that the nature of the dopaminergic dysfunction may differ between the various dopaminergic projection systems and consequently a generalised hypothesis regarding dopamine is likely to be an oversimplification.

In more general terms there is no evidence that changes to monoamine systems are the underlying cause of bipolar disorder. It is likely that affective disorders, and bipolar disorder in particular, result from of a combination of genetic and environmental factors and as such hypotheses focussing on a single neurotransmitter system are an oversimplification. Progress in

understanding the aetiology of this condition requires a broadening of perspective to investigate how various bodily systems interact to produce the underlying pathology.

1.2. The HPA axis

1.2.1. Overview

The hypothalamic-pituitary-adrenal (HPA) axis is a system consisting of a number of component tissues, the overall function of which is to provide neuronal control over the release of endocrine signals from the adrenal cortex (see figure 1.1, also; McQuade and Young 2000). Activation of the HPA axis produces a cascade of events, ultimately leading to the release of corticosteroid hormones from the adrenal glands into the systemic circulation.

Corticosteroid hormones are a group of related compounds synthesized from cholesterol which includes the glucocorticoids cortisol and corticosterone, and the mineralocorticoid aldosterone (see figure 1.2.). There are species differences in the secretion of glucocorticoids such that humans predominantly secrete cortisol in a ratio of 7:1 with corticosterone, whilst rats almost exclusively secrete corticosterone (Ganong, 2001). These hormones play an important role in homeostasis and interact with a wide variety of systems in the body and CNS.

The HPA axis signaling cascade originates in a number of brain regions and is coordinated in the paraventricular nucleus of the hypothalamus (PVN). Stimulation of the PVN releases corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) into the hypophyseal portal blood via which they are transported to the anterior pituitary. CRH acts on the pituitary to induce transcription of the proopiomelanocortin (POMC) gene (Gagner and Drouin, 1985), a precursor for adrenocorticotropic hormone (ACTH), and furthermore, in concert with AVP, stimulates the release of ACTH from the pituitary (Gillies et al. 1982; Rivier et al. 1982; Donald et al. 1983; Rivier and Vale, 1983; Canny et al. 1992). ACTH is then transported in the systemic circulation to the adrenal glands where it induces the synthesis of corticosteroid hormones (Holzbauer and Vogt, 1957). Under normal physiological conditions activity within the HPA axis is tightly regulated by corticosteroid-mediated negative feedback which inhibits secretory activity in the PVN and pituitary, as well as at sites in the central nervous system (see figure 1.1.).

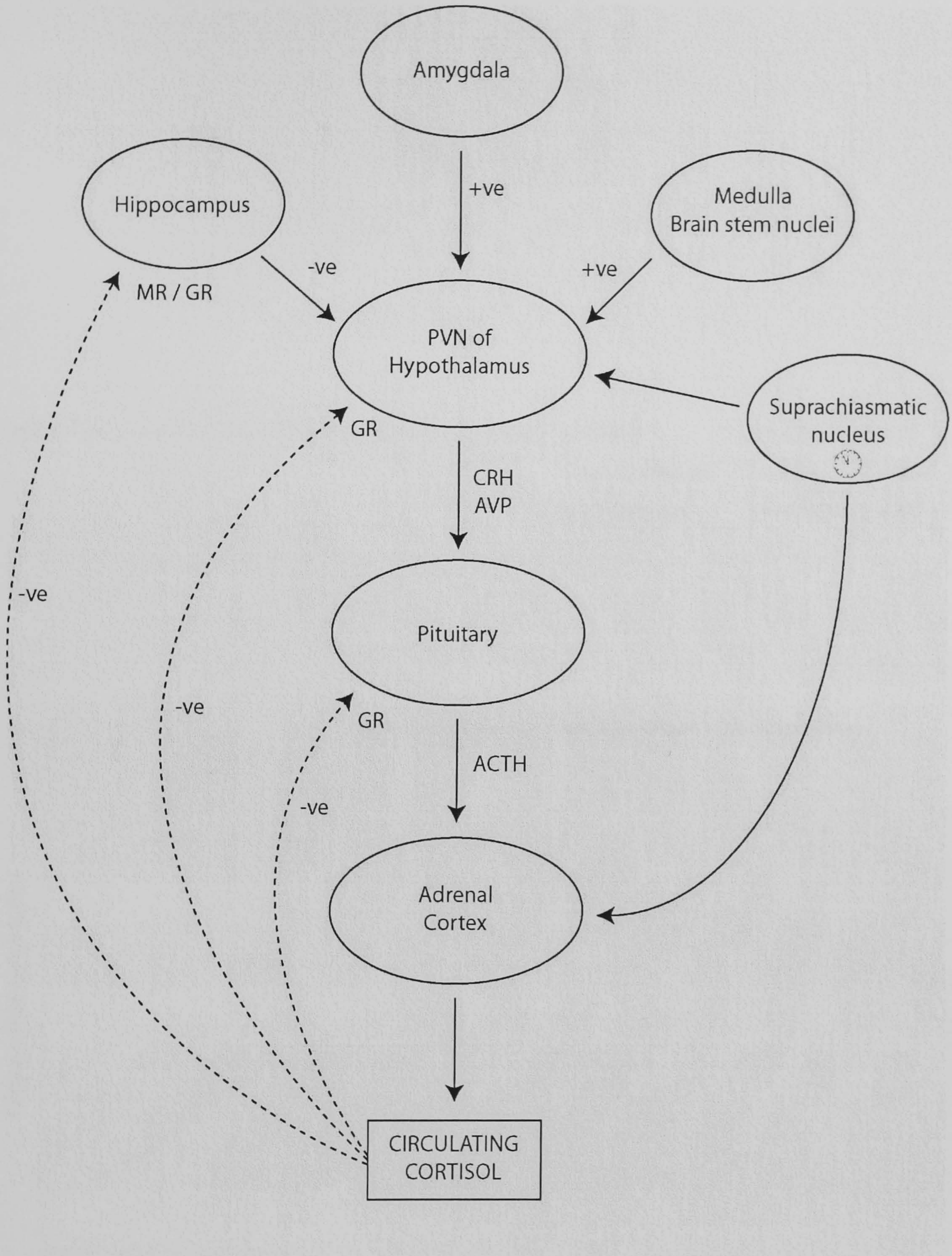

Figure 1.1. The HPA axis

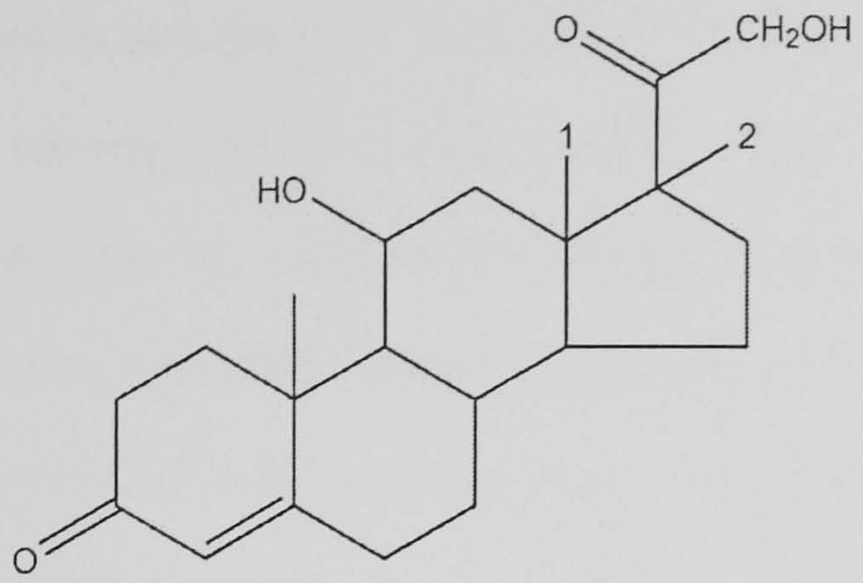

Figure 1.2. Chemical structure of the corticosteroids:

Cortisol	1 = CH_3	2 = H
Corticosterone	1 = CH_3	2 = OH
Aldosterone	1 = CHO	2 = H

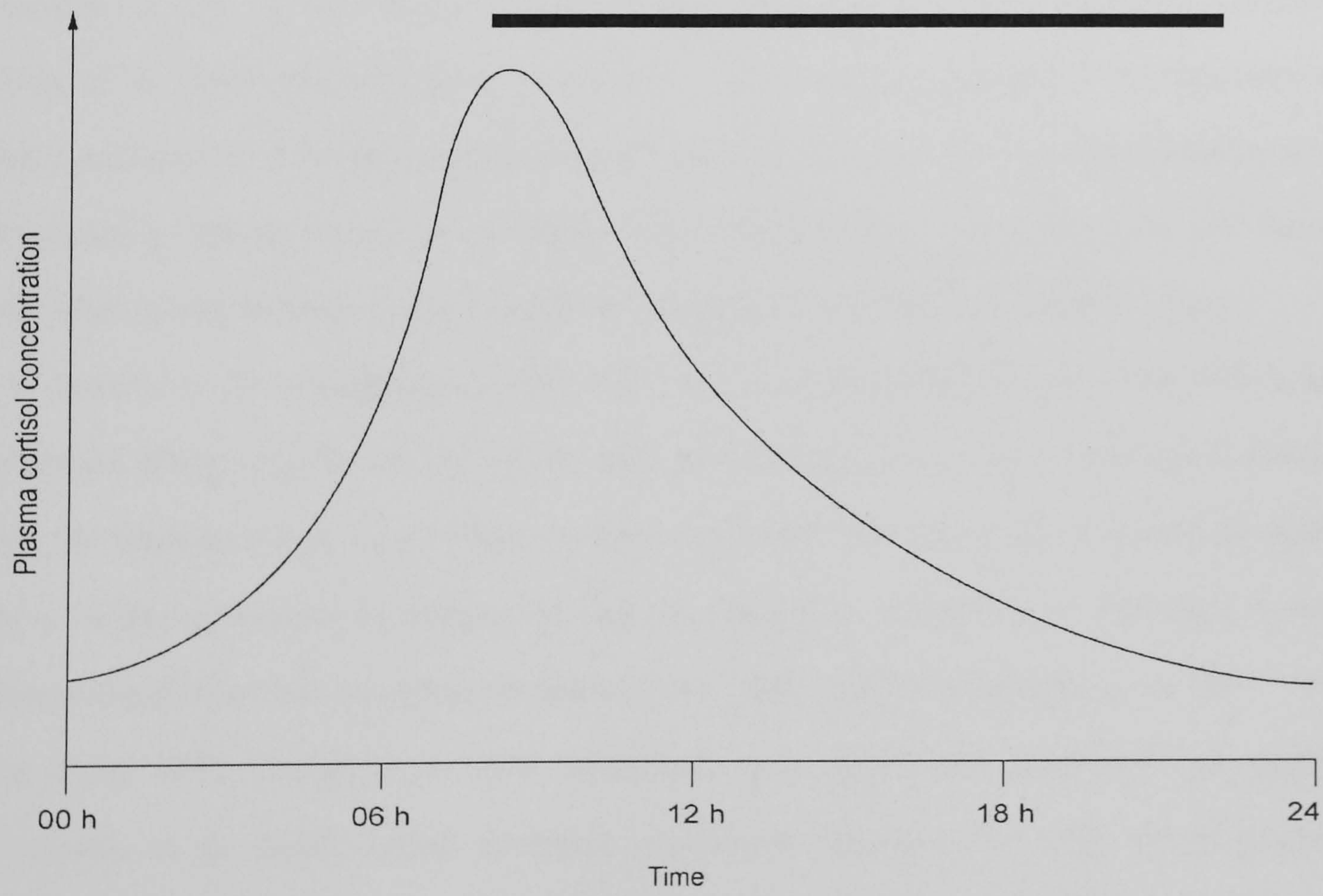

Figure 1.3. Circadian rhythm of cortisol secretion in humans. Black bar indicates waking period.

In healthy individuals the release of corticosteroids exhibits a circadian rhythm with peak cortisol levels occurring around the time of waking, and a nadir around the time of sleep onset (see figure 1.3). This pattern is true for both diurnal and nocturnal animals, thus in humans, cortisol levels peak in the morning, whilst in rats corticosterone levels peak in the evening. Disruption of the sleep-wake cycle, for example in night shift workers or airline staff, has been shown to cause major disturbances to this pattern of secretion (Hennig et al. 1998; Richardson and Tate, 2000). The corticosteroid rhythm has it's origins in the suprachiasmatic nucleus of the hypothalamus (SCN) which controls HPA axis activity directly via projections to the PVN, and indirectly via projections to GABAergic cells of the sub-paraventricular zone which in turn synapse with CRF neurones of the PVN (Pecoraro et al. 2006). In addition the SCN controls adrenal sensitivity to ACTH via autonomic projections to the adrenal gland (Buijs et al. 1999) resulting in reduced ACTH sensitivity during the nadir in plasma corticosteroid levels.

This diurnal cycle modulates the homeostatic functions of glucocorticoids to meet changing requirements throughout the day. Of particular importance is the regulation of energy metabolism with corticosteroids increasing glycogenesis and gluconeogenesis (reviewed in Rang et al. 2002) resulting in an increase in overall energy availability. The importance of glucocorticoids in this role is perhaps best demonstrated by the observation that in adrenal insufficiency, fasting produces potentially fatal hypoglycaemia. Glucocorticoids are therefore essential for maintaining energy availability during periods of restricted calorific intake.

In addition to its homeostatic role the HPA axis is intrinsically involved in the body's stress response. Plasma ACTH and glucocorticoid levels increase rapidly upon exposure to a stressor (see for example Dayas et al. 1999; Herman et al. 2005) and this stress response is regulated by a variety pathways converging on the hypothalamus. Examples of excitatory pathways include those from the amygdala (Beaulieu et al. 1986; 1987; Roozendaal et al. 1991; Van de Kar et al. 1991; Dayas et al. 1999; Bhatnagar et al. 2004) and peripheral pain pathways (Pecoraro et al. 2006), whilst inhibitory projections include those from the hippocampus (Sapolsky et al. 1984; Jacobson and Sapolsky, 1991; Herman et al. 1995; Chrapusta et al. 2003). Overall these signalling pathways provide sensory and cognitive input to the HPA axis allowing appropriate endocrine responses to perceived threats.

Peripherally, raised glucocorticoid levels following exposure to stress facilitate the ability to cope with and recover from the stress via catabolic and metabolic effects which increase energy availability. Central effects, on the other hand, promote learning and memory as well as long-term adaptive responses to stress. Thus in the short term, raised corticosteroid levels are beneficial to the health of the organism however there is increasing evidence that long-term exposure to high levels of corticosteroids can have detrimental effects on a wide variety of bodily systems.

1.2.2. Corticosteroid receptors

Corticosteroid actions are mediated by two types of receptor – the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Both are soluble intracellular proteins that act as ligand inducible transcription factors, binding to DNA to alter gene expression. They share 57% homology, with 94% homology in the DNA binding domain and bind to the same response element present in DNA consisting of the 15 nucleotide sequence AGAACAnnnTGTTCT (Funder, 1997). This is referred to as the glucocorticoid response element or GRE. The two receptors differ chiefly in their ligand binding properties, with MR binding cortisol, corticosterone and aldosterone with approximately equal, and high, affinity (K_d ~ 0.5-2 nM) whilst GR shows a lower affinity for cortisol and corticosterone (K_d ~ 10-20 nM) and little affinity for aldosterone (Reul and de Kloet, 1985; de Kloet et al. 1998). Receptor binding studies performed by Reul and de Kloet (1985) found that whilst MR is tonically activated by normal circulating levels of corticosteroids, GR is activated only during periods of elevated corticosteroid release and consequently the GR is responsible for the pathological sequelae of raised corticosteroid levels (Reul and de Kloet, 1985). This observation perhaps accounts for the far greater body of work investigating GR signalling in the central nervous system compared with that for MR, however it is not universally accepted. It has been suggested that due to their methodology, Reul and de Kloet (1985) overestimated the occupancy of the MR and that a significant proportion of MR is available for binding particularly during the circadian nadir (Pace and Spencer, 2005). Thus it may be the case that pathological sequelae of elevated corticosteroid levels result from both MR and GR binding.

It should be noted here that the term mineralocorticoid receptor is somewhat misleading in the context of the majority of tissues in which it is expressed. Mineralocorticoid activity is confined to tissues such as the kidney where MR selectivity for aldosterone is achieved by enzymatic breakdown of cortisol and corticosterone by type 2 11-β-hydroxysteroid dehydrogenase (Rang, Dale and Ritter, 1999; Buckingham, 2006). In other tissues, such as those of the CNS, glucocorticoid binding to MR predominates due to the much higher circulating levels of cortisol and corticosterone compared to aldosterone (100-1000 times higher see Ahima et al. 1991). As a result MR and GR have been referred to as the type-1 and type-2 corticosteroid receptors to avoid any suggestion of MR selectivity for aldosterone however the common nomenclature remains MR and GR.

1.2.2.1. Corticosteroid receptor signalling

An overview of corticosteroid receptor signalling can be seen in figure 1.4. Unliganded corticosteroid receptors are present in the cytoplasm where they exist in heteromeric complexes with the heat shock proteins hsp90, hsp70, hsp40, p60 and p23 (Pratt and Toft, 1997; Schoneveld et al. 2004). These complexes are essential for hormone binding, with uncomplexed receptors showing greatly diminished ligand affinity (Pratt and Toft, 1997). Upon ligand binding the heat shock proteins dissociate and conformational changes expose binding sites allowing for active transport into the cell nucleus (Nishi and Kawata, 2006). Ligand bound MR and GR form dimeric or even multimeric complexes (Savory et al. 2001) prior to DNA binding. There is some debate over where this takes place with some studies showing cytoplasmic dimerisation (Savory et al. 2001) whilst others have presented evidence that DNA binding is required for the formation of stable complexes (Truss and Beato, 1993). The traditional view has been that MR and GR form homodimers, however the co-expression of these receptors in individual cells in tissues such as the hippocampus (van Eekelen and de Kloet, 1992; Han et al. 2005) has prompted investigations revealing that functional MR/GR heterodimers are also possible (Trapp et al. 1994; Liu et al. 1995; Ou et al. 2001). This is thought to allow for greater diversity in the regulation of corticosteroid responsive genes in

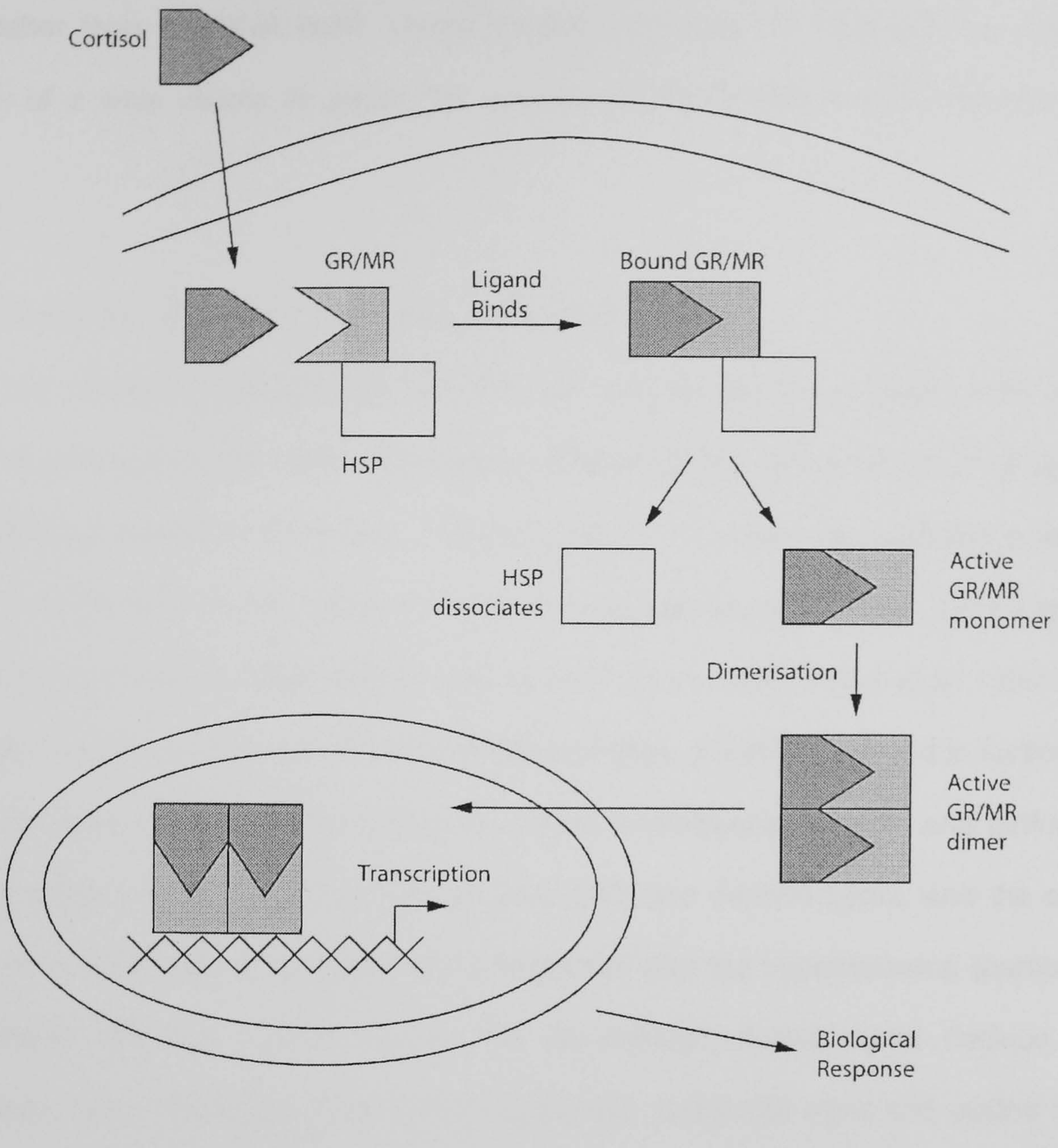

Figure 1.4. Corticosteroid receptor signalling

response to changing levels of endogenous ligand (Trapp and Holsboer, 1996; Nishi and Kawata, 2006). In some genes receptor binding to the GRE alone may be sufficient to regulate transcription. Other genes however require a glucocorticoid response unit (GRU) comprising of the bound receptor in a complex with other co-factors. This dependence on the presence of other co-factors allows for potential tissue specificity in the response of a given gene to corticosteroids. The transcriptional response to glucocorticoids can be either positive or negative, with some GRE's increasing transcription whilst others decrease transcription. Additionally, negative regulation may be achieved via a competitive mechanism at the regulatory site. In such a mechanism GR binding prevents the binding of transcriptional promoters at sites adjacent to the GRE thus decreasing gene transcription. In genes which do not have a GRE glucocorticoid receptors may regulate transcription by binding to transcriptional

factors rather than the DNA itself. These mechanisms allow for both positive and negative regulation of a wide variety of genes (for reviews see Buckingham 2006; Schoneveld et al. 2004).

1.2.2.2. Corticosteroid receptor expression in the CNS

Of the two receptors MR and GR, the GR has received the most attention as a potential mediator of pathology in the CNS. The earliest attempt to map GR in the rat CNS was carried out in a series of papers by Fuxe et al. (1985a,b; 1987). This work demonstrated a widespread distribution of GR in the CNS, whilst subsequent work allowed quantitative comparison of GR immunoreactivity between brain regions (Ahima and Harlan, 1990; Cintra et al. 1994; Morimoto et al. 1996). These studies have shown that high densities of GR are present in cortical regions such as the motor cortex, anterior cingulate cortex, somatosensory area, and piriform cortex and limbic areas such as the hippocampal CA1, CA2 and dentate gyrus, and the amygdala. High densities have also been found in the thalamus and the hypothalamus (particularly the paraventricular and dorsomedial nuclei), the serotonergic dorsal raphe nucleus and the noradrenergic locus coeruleus, whilst the dopaminergic substantia nigra and ventral tegmental area express low and moderate levels respectively.

Mapping of MR in the CNS has been less comprehensive than for GR, with only one study looking at MR expression across a large number of brain regions (Ahima et al. 1991). This work demonstrated that MR, similar to GR, has a very widespread distribution, and furthermore that regions of high MR density generally correspond with regions of high GR density as reported in previous work by the same authors (Ahima and Harlan, 1990). This is particularly true of hippocampal, thalamic and hypothalamic regions and whilst the majority of cortical regions were excluded from analysis, the piriform cortex was found to contain a high density of MR. Monoaminergic nuclei such as the dorsal raphe and locus coeruleus were also found to express 'high levels' of MR, whilst the substantia nigra and VTA contained 'low to moderate levels', again reflecting the pattern seen with GR. More recent studies looking at MR immunoreactivity have been somewhat restricted in their scope, however they confirm the presence of MR in the hippocampus and hypothalamus (Agarwal et al. 1993; Ito et al. 2000; Han et al. 2005). Of some

importance to the present work are the findings of the study by Agarwal et al. (1993) which show an absence of MR immunoreactivity in the midbrain, septum and striatum – thereby contradicting the findings of Ahima et al. (1991). Thus, given the mesencephalic location of the majority of dopaminergic cell groups, the literature is unclear on the presence of MR in these regions.

The hypothesis initially postulated by Reul and de Kloet (1985) that MR and GR act as high and low affinity binding sites for corticosteroids, with MR tonically active and GR active under conditions of elevated corticosteroids, suggests that these receptors might be expressed in the same cells. Whilst the theory stands without making this assumption, receptor co-expression offers the simplest mechanism for complex regulation of neuronal function as plasma corticosteroid levels fluctuate. Furthermore in-vivo receptor heterodimerisation would be impossible without the presence of both MR and GR. So far co-expression has been demonstrated in the hippocampus (van Eekelen and de Kloet, 1992; Han et al. 2005) and the hypothalamus (Han et al. 2005). It is likely that co-expression occurs in other regions of the brain, however at the current time there is a lack of data to support this.

1.2.3. Hypothalamic Pituitary Adrenal axis function in bipolar disorder

As previously discussed (see section 1.1.4) environmental stress plays a role in the onset of bipolar disorder in a significant number of individuals. This arouses suspicion that corticosteroids and the HPA axis are in some way involved in bipolar disorder. Further to this, therapeutic use of corticosteroids has been associated with significant psychopathology and has been associated with the onset of bipolar symptoms. One study examining records from ~2000 patients referred to a psychiatric unit found seven cases where recurrent bipolar disorder developed following corticosteroid treatment, with five of these patients initially presenting with mania or hypomania (Wada et al. 2001). This finding is supported by a number of single case reports of mania following corticosteroid therapy (Pies, 1981; Ur et al. 1992; Abouesh, 1998). A further study has shown that in ophthalmic patients treated with corticosteroids for 8-days, symptoms of hypomania occurred in about a third of patients, whilst one in ten developed depressive symptoms (Naber et al. 1996). Furthermore, chronic corticosteroid therapy has been

associated with an increase in depressive symptoms (Bolanos et al. 2004), with 60% of patients in this study meeting the criteria for a therapy-induced mood disorder. Findings such as these suggest that an underlying dysregulation of corticosteroid secretion may be involved in the aetiology of bipolar disorder.

Clinical studies have found that a common feature of unipolar affective disorders is disruption to the diurnal pattern of HPA axis activation, producing an elevated basal level, and a flattening of the rhythmic variation in plasma cortisol levels (Yehuda et al. 1996; Deuschle et al. 1997). This abnormality has also been observed in bipolar disorder (see figure 1.5.) where patients experiencing depressed, euthymic and hypomanic mood states exhibit an increased basal level of cortisol secretion, and reduced diurnal variation compared to controls, which is particularly evident during the daily nadir in cortisol levels (Cervantes et al. 2001).

Evidence for elevated cortisol secretion over 24 hours has been found in patients experiencing manic symptoms (Sachar et al. 1975; Akesode et al. 1976; Linkowski et al. 1994) compared to normal controls, with the latter two studies showing some evidence of a flattening of the diurnal rhythm. More limited studies which have examined daytime (Cookson et al. 1985) and night-time (Platman and Fieve, 1968; Carpenter and Bunney, 1971; Cookson et al. 1985; Linkowski et al. 1994) plasma cortisol levels have found increases in manic subjects compared to healthy controls. Increased free urinary and CSF cortisol levels in manic patients have also been reported (Swann et al. 1992).

In depressed bipolar patients elevations in 24 free urinary cortisol have been found (Rubinow et al. 1981) whilst more detailed studies in unipolar and bipolar depressives have shown that the 24 hour profile of plasma cortisol is elevated (Linkowski et al. 1985) whilst another study found a correlation between blunted amplitude and depression rating scores (Souetre et al. 1989). These findings are supported by data showing elevated daytime and night-time ACTH levels in melancholic depressed (including bipolar) subjects and a significant increase in morning cortisol compared to healthy controls (Moffoot et al. 1994).

Although there is evidence for a flattened cortisol rhythm in all bipolar mood states there is the question of whether relative cortisol levels vary with mood state. Whilst figure 1.5. (Cervantes et

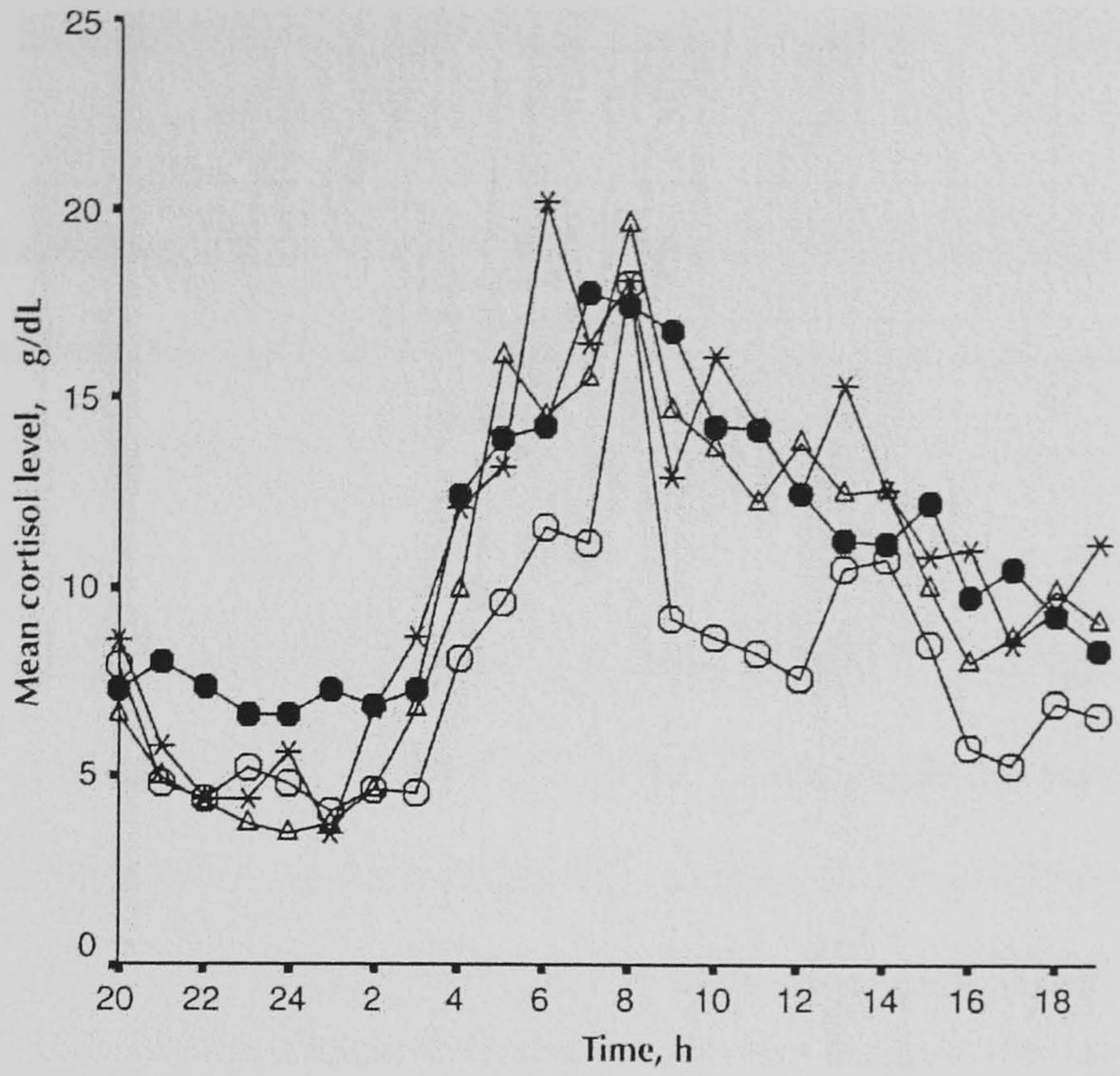

Figure 1.5. Circadian rhythm of cortisol secretion in bipolar disorder. Mean 24-h cortisol secretion of normal controls (circles) and patients with bipolar disorder in depressed (black circles), hypomanic (asterisks) and euthymic (open triangles) phases of their illness (reproduced from Cervantes et al. 2001)

al. 2001) suggests that this is not the case, data from two patients in the study for whom cortisol measurements were obtained in all of the three mood states demonstrated that levels of cortisol secretion were highest during the hypomanic phase and lowest during the euthymic phase. This is in contrast with a number of studies which have shown higher cortisol levels during bipolar depressive phases than during mania (Bunney et al. 1965; Carroll et al., 1976; Joyce et al. 1987; Gann et al. 1993; Joyce et al. 1995) and evidence that in some patients cortisol levels are higher during the euthymic phase than in mania (Joyce et al. 1987). Thus currently the evidence for a consistent correlation between mood state and cortisol secretion is lacking.

The fact that cortisol levels in euthymia (Joyce et al. 1987) were generally higher than those seen in manic phases suggests that hypercortisolaemia is a trait marker for bipolar disorder, a notion supported by the data from Cervantes (2001) which shows elevated levels compared to controls in euthymic phases of the disease. It is however unfortunate that more studies including euthymic patients and healthy controls have not been performed, leaving the question of

whether the HPA circadian profile represents a trait marker in bipolar disorder largely unresolved.

The origin of abnormalities in circadian cortisol secretion is currently unknown, however it has been suggested that they are the result of dysfunctional negative feedback control in the HPA axis, for which there is mounting evidence in bipolar disorder. The ability of dexamethasone (a synthetic glucocorticoid) to suppress the release of cortisol over a 24-hr period post-administration (the dexamethasone suppression test or DST) is used to probe negative feedback regulation in the HPA axis. Abnormalities of dexamethasone suppression have been found in a significant proportion of bipolar patients across all mood states. In manic patients non-suppression occurs in around 45% of cases (Graham et al. 1982; Cassidy et al. 1998) with a similar rate in bipolar depression (Feinberg and Carroll, 1983; Rush et al. 1996) whilst in mixed episodes non-suppression rates are as high as 86% (Swann et al. 1992; Cassidy et al. 1998). It is interesting to note that rates of non-suppression are much higher in bipolar depression than unipolar depression (Rush et al. 1996).

Another interesting finding is that in bipolar patients successfully treated with lithium, non-suppression still occurs in up to a fifth of patients (Deshauer et al. 1999) implicating non-suppression as a trait marker for the illness. Further evidence for this is the finding that euthymic patients have significantly higher post dexamethasone cortisol levels than healthy controls (Watson et al. 2006) and observations that healthy non-suppressors have a significantly higher morbid risk of affective disorders (based on their family histories), particularly for mania and hypomania (Coryell and Zimmerman, 1987). Overall the data suggest that glucocorticoid negative feedback is impaired in a significant subset of bipolar patients and may represent a trait marker for the illness. It should be noted however that successful suppression in the DST by bipolar patients does not necessarily mean that there is no abnormality in HPA axis function. A study looking at the salivary cortisol response to waking has shown that bipolar patients demonstrate an enhanced response compared to controls even when there is no abnormality in the afternoon dexamethasone response (Deshauer et al. 2003).

A further refinement of the DST is the combined dex/CRH test in which administration of dexamethasone is followed by a CRH challenge 24 hours later. In healthy subjects

dexamethasone restrains the increase in cortisol following the CRH challenge via its action at negative feedback sites. Bipolar patients have been shown to respond to post-dexamethasone CRH challenge with an increase in ACTH and cortisol levels greater than that seen in healthy controls (Watson et al. 2004) implying that negative feedback is reduced at the level of the pituitary. This abnormality appears to be independent of mood state having been observed in studies comparing both manic (Schmider et al. 1995) and depressed (Rybakowski et al. 1999) patients with healthy controls. Further to this, cortisol responses to dex/CRH remain constant in rapid cycling subjects irrespective of mood state (Watson et al. 2005). That this abnormality is a trait marker is suggested by studies demonstrating its presence in remitted patients (Schmider et al. 1995; Watson et al. 2005) and furthermore by a study showing that 20-30% of first degree relatives of patients with affective disorders (bipolar and unipolar) have an aberrant response to the dex/CRH test (Krieg et al. 2001). Overall the data from the DST and combined dex/CRH studies show that negative feedback is impaired in a significant number of bipolar patients, and this may account for the altered cortisol secretion profile which has been observed in the disorder.

Further abnormalities in the HPA axis have been found in the corticotropin releasing hormone test which measures the ability of the pituitary to respond to CRH. It has been shown that following injections of CRH, the ACTH response is blunted in remitted bipolar patients who went on to experience a depressive relapse (Vieta et al. 1997) however the same group found that euthymic patients who demonstrate a higher AUC for ACTH following CRH are at greater risk of manic episodes in the following six months (Vieta et al. 1999). This indicates that pituitary responsiveness to CRH can predict the types of relapse patients are susceptible to, however further studies are required to confirm this. Blunting of the ACTH response to CRH has been demonstrated in depression, and in some studies an inverse relationship between baseline cortisol levels and post-CRH ACTH secretion has been found. From this it has been concluded that circulating cortisol is responsible for blunting the ACTH response (Holsboer, 2000), however this hypothesis contrasts with the evidence from DST and dex/CRH studies showing reduced negative feedback in such patients. Furthermore the studies in bipolar disorder (Vieta et al. 1997, 1999) suggest that euthymic patients vary in their response to CRH challenge

despite evidence that cortisol levels remain elevated in this mood state (Cervantes et al. 2001), thus it is unclear how pituitary responsiveness and circadian cortisol secretion are related.

In conclusion, a number of abnormalities in HPA axis function have been found in bipolar disorder including cortisol hypersecretion and impaired glucocorticoid feedback regulation. The evidence for an altered cortisol rhythm is considerable having been demonstrated in euthymic, manic and depressive mood states (Akesode et al. 1976; Linkowski et al. 1985; Linkowski et al. 1994; Souetre et al. 1991; Cervantes et al. 2001) and it is hypothesised that this plays a role in the aetiology of the disease through its effects on central neuronal function.

The widespread distribution of corticosteroid receptors in the CNS (see section 1.2.2.2.) suggests that a large number of neuronal systems are likely to be prone to dysregulation by the kind of changes to HPA axis function observed in bipolar disorder. As the focus of this thesis is the mesocorticolimbic dopamine system, the mapping studies which have demonstrated GR expression in dopaminergic nuclei support the notion that cells projecting from these regions may also be affected by HPA axis circadian rhythm alterations. As far as the potential role for MR in these pathological processes, it is currently unclear whether this receptor is expressed in dopaminergic cells. The following section introduces the mesocorticolimbic dopamine system and provides evidence that corticosteroids can modulate aspects of its function.

1.3. Dopaminergic Neurotransmission in the CNS

Whilst today dopamine is well accepted as a neurotransmitter, prior to the pioneering work of Carlsson in the late 1950's it had been thought of merely as a precursor of noradrenaline and adrenaline. Carlsson postulated a role for dopamine as a neurotransmitter in 1958 having demonstrated its presence in the brain of rabbits (Carlsson et al. 1958) and based on studies showing that the akinetic effects of reserpine could be reversed by administration of the catecholamine precursor DOPA (3,4-dihydroxyphenylalanine) (Carlsson et al. 1957). DOPA treatment resulted in a recovery of dopamine but not noradrenaline levels in the brain and thus it was proposed that dopamine acted as a neurotransmitter in its own right within the central nervous system. An overview of dopaminergic neurotransmission is given in figure 1.6. and discussed in detail in the following section.

1.3.1. Dopaminergic neuronal function

1.3.1.1. Dopamine synthesis

Dopamine is a catecholamine neurotransmitter sharing a similar structure and initial synthetic pathway with noradrenaline and adrenaline (see figure 1.7.). The starting point in the dopamine synthetic pathway is L-tyrosine, an amino acid which is transported across the blood brain barrier by the large neutral amino acid transporter (LAT1) in competition with other large neutral amino acids such as tryptophan and phenylalanine (Kanai et al. 1998; Prasad et al. 1999; Hawkins et al. 2006). Tyrosine is hydroxylated in catecholamine neurons to produce L-DOPA by the enzyme tyrosine hydroxylase (TH) (see figure 1.8.), the activity of which was first characterised by Nagatsu et al. in 1964. L-DOPA is subsequently decarboxylated by the enzyme DOPA decarboxylase to form dopamine. These processes occur principally in the neuronal cytoplasm, however membrane bound forms of TH have been found in nerve terminal regions which suggest that the initial step in the pathway may take place in the vicinity of the cell membrane (Kuczenski et al. 1972).

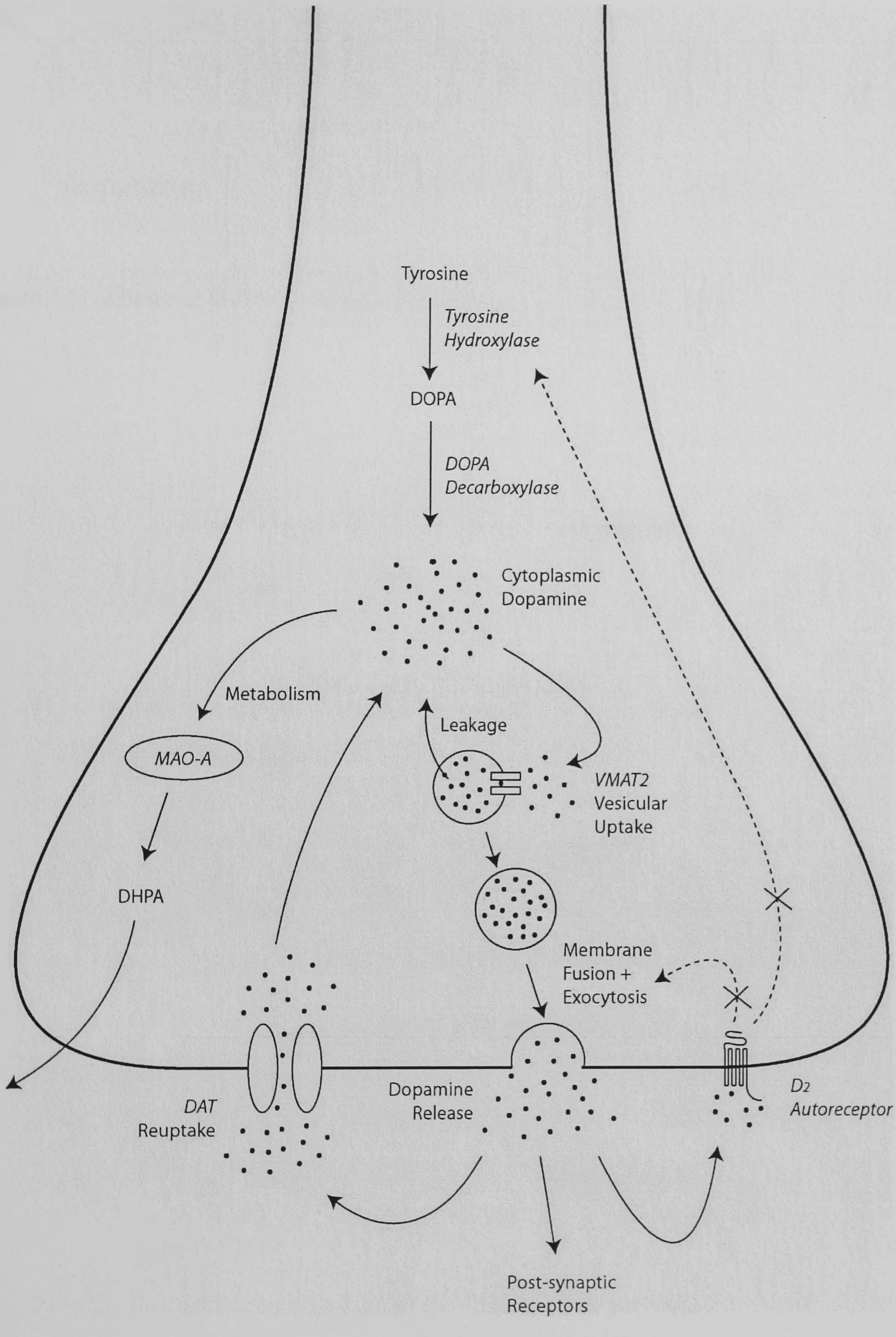

Figure 1.6. Dopaminergic nerve terminal function

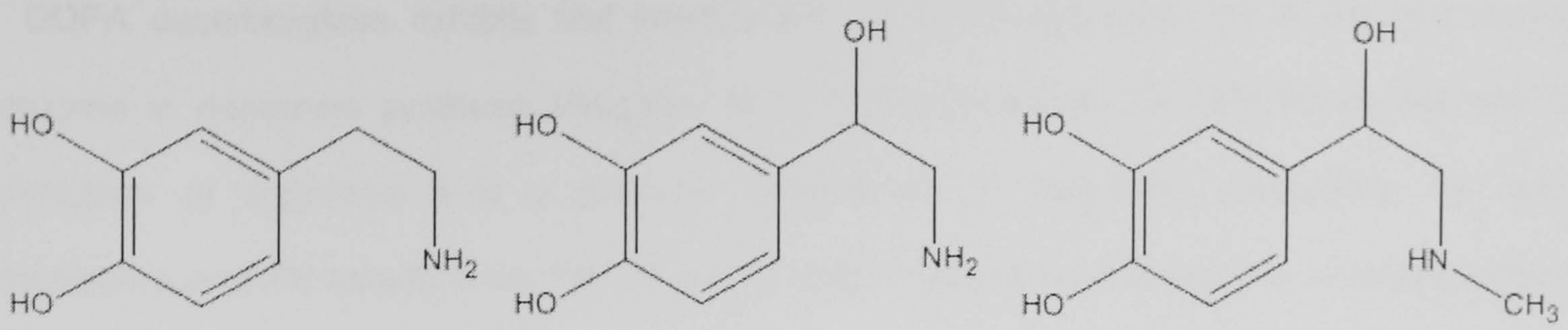

Figure 1.7. Chemical structure of the catecholamines

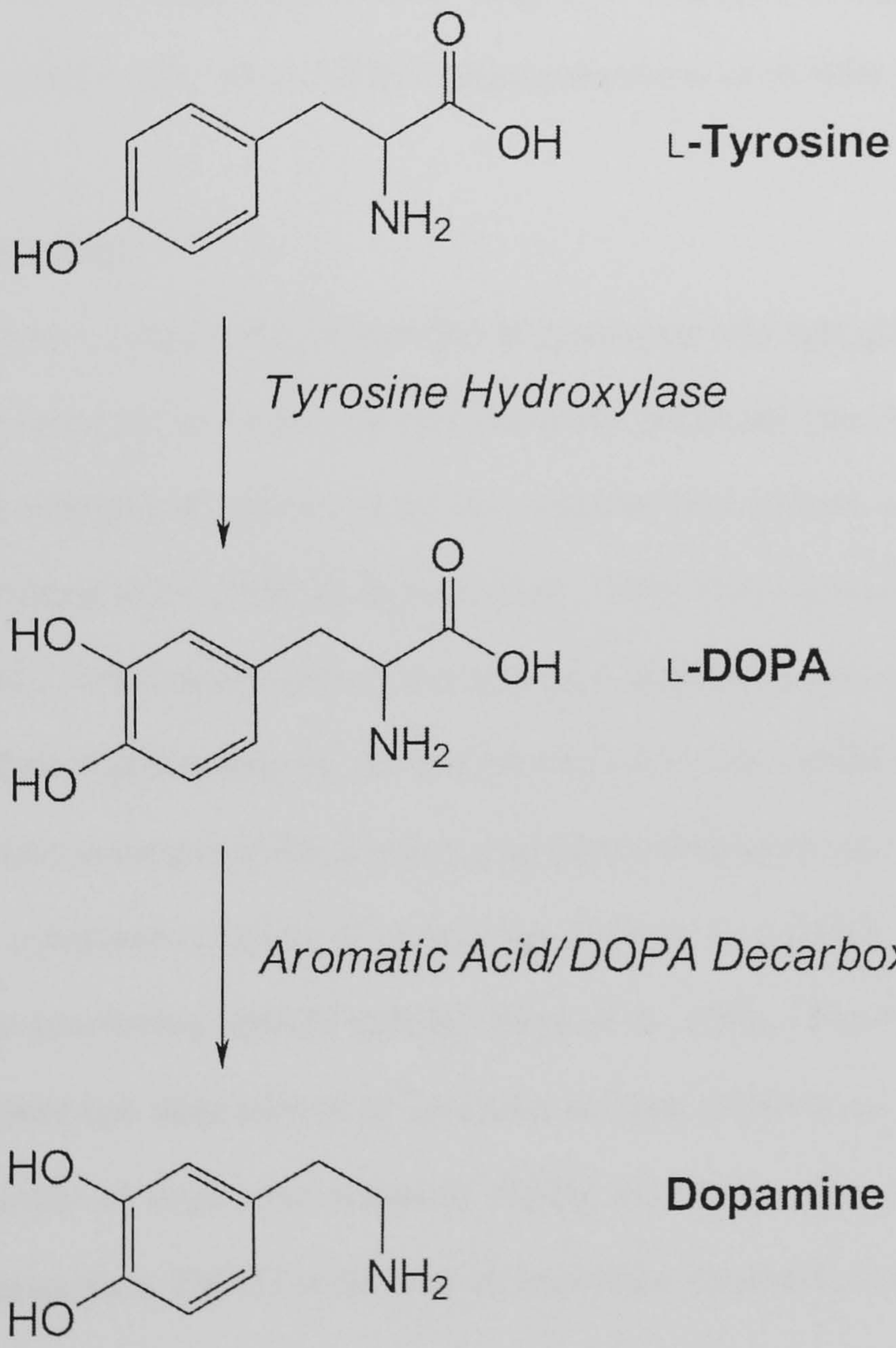

Figure 1.8. Synthetic pathway for dopamine

DOPA decarboxylase exhibits fast kinetics and as a consequence TH is the rate-limiting enzyme in dopamine synthesis (Nagatsu et al. 1964). Because TH determines the rate of formation of dopamine it is a principal determinant of dopamine availability. As such manipulation of TH activity is the key means by which regulation of dopamine synthesis occurs. This regulation can take the form of end-product inhibition (Nagatsu et al. 1964) which inhibits enzyme activity in the short term. In the medium term protein kinase dependent phosphorylation of TH can take place at a number of sites resulting in increased enzyme activity (Joh et al. 1978; Yamauchi and Fujisawa, 1979; Mallet, 1996; Lew et al. 1999) and by this mechanism a variety of receptors can control dopamine synthesis. In the long term changes in the transcription of TH mRNA affect enzyme availability and thus long term changes in dopamine synthesis can be achieved by this means (Cho et al. 1996; Hadjiconstantinou et al.1996; Young et al. 1998).

1.3.1.2. Vesicular uptake

Following synthesis, cytoplasmic dopamine is packaged into synaptic vesicles in preparation for release and to prevent its breakdown by metabolic enzymes (see figure 1.9.). The transport of dopamine into vesicles is performed by the vesicular monoamine transporter VMAT2 (Peter et al. 1995; Nirenberg et al. 1996; Hoffmann et al. 1998) utilizing a proton antiport mechanism (Eiden et al. 2004). The required proton concentration within vesicles is derived from the activity of an ATPase which translocates H^+ across the vesicular membrane to produce a pH gradient and transmembrane potential which drives the uptake of dopamine by VMAT2.

Vesicular and cytoplasmic pools of dopamine exist in a dynamic equilibrium with passive vesicular leakage countering VMAT2 activity (Floor et al. 1995; Eisenhofer et al. 2004). VMAT2 is therefore the principal determinant of vesicular content (Colliver et al. 2000) and this in turn governs the quantity of dopamine released during exocytosis (see below and Pothos et al. 2000). As a consequence VMAT2 activity is an important determinant of dopaminergic neuronal function.

1.3.1.3 Dopamine release

Release of dopamine occurs following the invasion of the nerve terminal by an action potential which causes depolarisation of the terminal membrane. This induces vesicular fusion with the terminal membrane leading to exocytosis of dopamine. The prevailing view of neurotransmitter release has been that an action potential produces fusion of an invariable number of vesicles and hence dopamine release is quantal being directly proportional to impulse activity (reviewed by Bennett and Kearns, 2000). This view is challenged however by the finding that in dopamine neurons phasic burst firing (characterized by short trains of high frequency firing) produces a supralinear increase in the release of dopamine, an effect which has been demonstrated in the nucleus accumbens and striatum (Gonon and Buda, 1985; Wightman and Zimmerman, 1990; Suaud-Chagny et al. 1992). It is thought that the supralinear increase in DA release may be a result of the increased efficiency and probability of presynaptic release in burst firing neurons compared to lower frequency firing neurons (Miles and Wong, 1986; Lisman, 1997; Snider et al., 1998). It may be the case however that saturation of uptake (Chergui et al. 1994), slow metabolism (Michael et al. 1995), or delayed autoreceptor activation (Chergui et al. 1994) contribute to the elevated extracellular levels.

A further problem with the quantal release theory is that as originally postulated, the theory requires vesicular content to remain constant (at least in the short term) – the original theory arose from measuring postsynaptic evoked potentials at the neuromuscular junction where it was found that synaptic neurotransmitter concentrations were quantal, thus vesicular content must have been constant. Recent research in dopaminergic neurones has shown that changes in vesicular content governed by VMAT2 activity allow for some variability in the amount of dopamine released despite the fact that vesicular fusion itself is quantal (Pothos et al. 2002). These findings suggest that the classic view of neurotransmitter release is an oversimplification as far as dopaminergic neurones are concerned.

1.3.1.4 Dopamine uptake

Following release into the synaptic cleft dopamine is cleared via active uptake back into the pre-synaptic terminal. The protein responsible for this is the dopamine transporter (DAT) which belongs to a superfamily of Na^+ dependent monoamine transporters including the serotonin and noradrenaline transporters (see reviews by Hoffman et al. 1998; Chen & Reith 2000). DAT is present at perisynaptic sites in the neuronal membrane and utilizes the transmembrane Na^+ electrochemical gradient maintained by the Na^+-K^+ ATPase pump to transport dopamine across the presynaptic membrane. The sodium-potassium pump is therefore an important determinant of DAT activity, which in turn is a major determinant of dopamine concentrations at post-synaptic sites. DAT activity has been shown to affect the time course of removal of dopamine from the synapse and its ability to diffuse to extrasynaptic sites (Benoit-Marand et al. 2000; Wayment et al. 2001; Cragg & Rice, 2004).

N.B. DAT transports both dopamine and noradrenaline although it has a ten-fold higher affinity for DA than NE. Conversely the noradrenaline transporter (NET) has around a four-fold higher affinity for dopamine than its proper substrate noradrenaline, and has a higher affinity for dopamine than DAT (reviewed by Masson et al. 1999). Uptake of dopamine by NET has been demonstrated in the prefrontal cortex (Moron et al. 2002) and as a consequence NET may play an important role in dopaminergic neurotransmission.

1.3.1.5. Dopamine metabolism

Dopamine metabolism occurs via two principal pathways, one intraneuronal and the other extraneuronal (see figure 1.9.). Intraneuronal metabolism involves dopamine present in the cytoplasmic pool which is composed of both newly synthesised dopamine and dopamine which has been released and undergone uptake from the synapse. The enzyme responsible for this is monoamine oxidase A (MAO-A) one of two isoforms of the monoamine oxidase enzyme in the central nervous system (the other being MAO-B) (Gesi et al. 2001; Shih 2004). Whilst dopamine is a substrate, and has equal affinity for both isoforms (Youdim and Bakhle, 2006), MAO-A is thought to be the principal isoform responsible for DA metabolism. This has been demonstrated in studies which have found that selective MAO-A inhibition leads to a reduction in levels of the

oxidative metabolite DHPA in dopaminergic terminal regions, whilst MAO-B inhibition has little effect (Kato et al. 1986; Garett and Soares-da-Silva 1990; Fornai et al. 2000). The study of Fornyai et al. (2000) also demonstrated that this enzyme is located intra-neuronally, as blockade of vesicular uptake in the striatum increased 3,4-dihydroxyphenylacetaldehyde (DHPA) levels which was presumed to result from increased metabolism of cytoplasmic dopamine. Blockade of extracellular DAT-mediated uptake failed to elicit such an effect thus ruling out extraneuronal metabolism. As the intraneuronal metabolic route is thought to be responsible for the majority of dopamine metabolism (Westerink et al 1985; see later) this further supports a preferential involvement of MAO-A in dopamine metabolism.

Regarding the relative roles of the MAO isoforms in dopamine metabolism, it is interesting to note that MAO-B inhibitors such as selegiline and rasagiline are efficacious in the treatment of Parkinson's disease (PD), a disorder characterized by deficiencies of striatal dopaminergic neurotransmission. It might therefore be suggested that MAO-B is a major determinant of striatal dopamine levels. This is contradicted by the evidence given above that MAO-B plays only a limited role in dopamine metabolism under normal conditions. It has been shown, however, that MAO-B inhibitors significantly increase dopamine levels, and reduce dopamine metabolism, following administration of exogenous L-DOPA (but not under basal conditions) suggesting a significant role for glial MAO-B metabolism during L-DOPA therapy for PD (Brannan et al. 1995). This may explain the therapeutic efficacy of these drugs in the treatment of PD, although an alternative hypothesis is that MAO-B inhibition actually reduces the generation of neurotoxic dopamine metabolites in astroglial cells thus exerting a neuroprotective effect (Nagatsu and Sawada, 2006; Youdim and Bahkle, 2006). Currently this matter remains unresolved.

Returning to the mechanisms underlying dopamine metabolism, monoamine oxidase oxidatively deaminates dopamine to produce DHPA, an aldehyde intermediate which is dehydrogenated extraneuronally to form DOPAC. Around 60% of DOPAC is then metabolised by the enzyme catechol-O-methyl transferase (COMT) to form homovanillic acid (HVA) (Westerink et al. 1985). DOPAC and HVA are then either excreted in urine or undergo

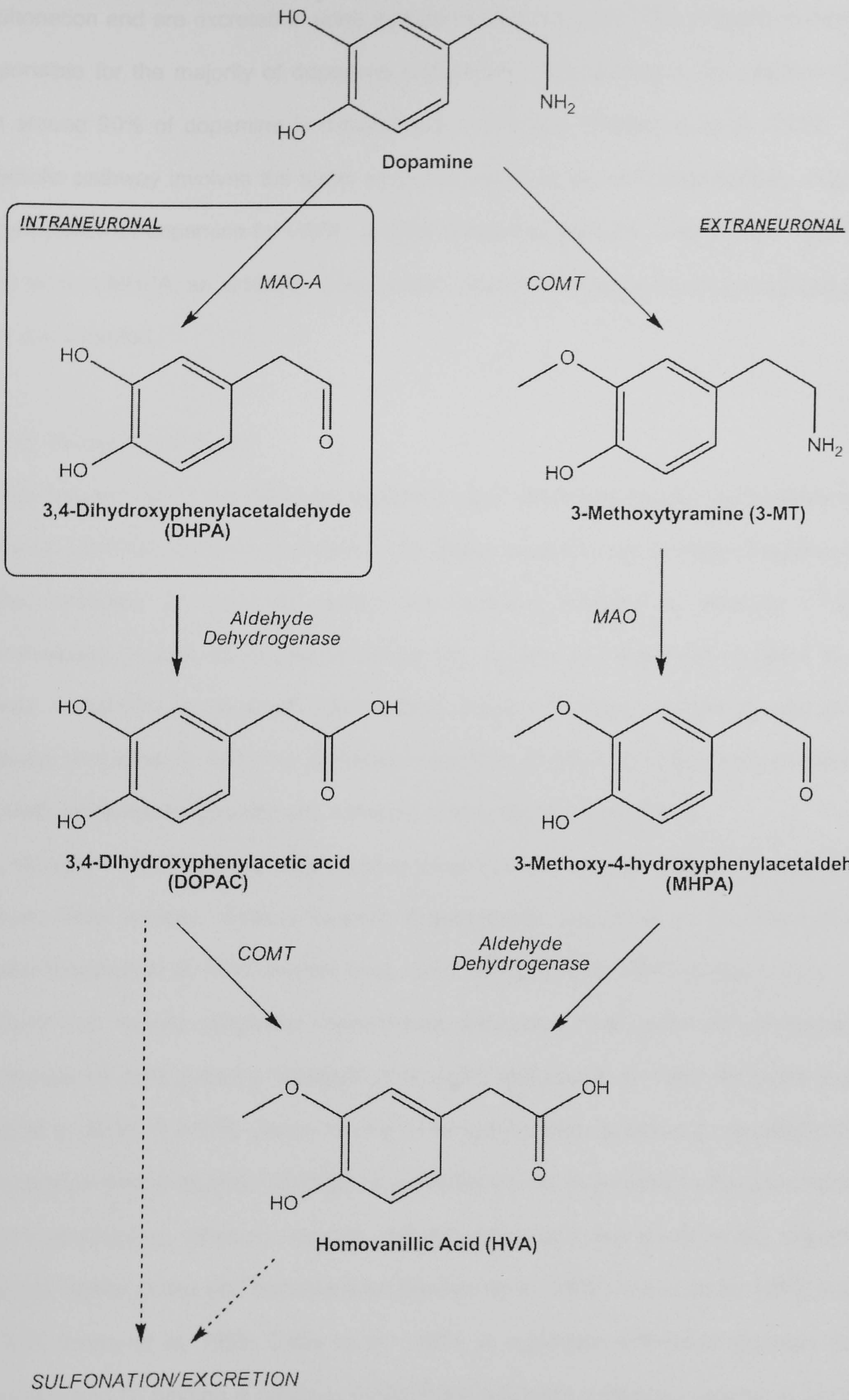

Figure 1.9. Metabolic pathways for dopamine

sulphonation and are excreted in urine as sulphated conjugates. This pathway is thought to be responsible for the majority of dopamine metabolism, with studies in the striatum suggesting that around 90% of dopamine is metabolised in this way (Westerink et al. 1985). The other metabolic pathway involves the same enzymes, however the first step involves extraneuronal O-methylation of dopamine by COMT to form 3-methoxytyramine. This is then metabolised by MAO to form MHPA, an aldehyde intermediate, which is subsequently dehydrogenated to form HVA and excreted.

1.3.1.6. Dopamine receptors

There are five known receptors for dopamine all of which are coupled to G-protein mediated signal transduction pathways (see table 1.3). These receptors are broadly categorised into the D_1-like receptors (D_1 and D_5) which are positively coupled to adenylyl cyclase and phospholipase C, and the D_2-like receptors (D_2, D_3 and D_4) negatively coupled to adenylyl cyclase. In addition D_2 receptors are positively coupled to phospholipase C, arachidonic acid synthesis and inwardly rectifying potassium channels (GIRKs) and negatively coupled to Ca^{2+} channels (reviewed by Sokoloff and Schwarz, 1995; Missale et al. 1998).

D_1 receptor mRNA is widely expressed in dopaminergic terminal regions such as the cortex, striatum, limbic system, olfactory tubercle, hypothalamus and thalamus (Fremeau et al. 1991; Meador-Woodruff et al. 1991; Weiner et al. 1991; Mengod et al. 1992; Richtand et al. 1995) but is absent from regions containing dopaminergic cell bodies such as the ventral tegmental area and substantia nigra (Meador-Woodruff et al. 1991; Mengod et al. 1992; Richtand et al. 1995; Gross et al. 2005). A similar distribution for D_1 receptor protein is shown by receptor binding and immunocytochemical studies with high to moderate levels expressed in the caudate-putamen, nucleus accumbens, olfactory tubercle and amygdala and low levels in the hypothalamus, thalamus, frontal cortex and hippocampus (Boyson et al. 1986; Ariano et al. 1989; Mansour et al. 1990; Levey et al. 1993; Caille et al. 1996). A significant difference between D_1 mRNA expression and D_1 binding is however found in the cell body containing regions of the VTA and substantia nigra, with particularly high D_1 binding in the substantia nigra and to a much lesser extent in the VTA. The lack of D_1 mRNA in these regions has been taken as evidence that the

	D_1-like		***D_2-like***		
	D_1	**D_5**	**D_2**	**D_3**	**D_4**
G-protein coupling	$G_S/G_{OLF}/G_q$	G_S	$G_{i/o}/G_q$	$G_{i/o}$	$G_{i/o}$
Second messenger pathway	↑ cAMP ↑ IP_3/DAG	↑ cAMP	↓ cAMP ↑ IP_3/DAG ↑ AA ↑ K^+ ↓ Ca^{2+}	↓ cAMP	↓ cAMP ↑ AA

Table 1.3. Dopamine receptor coupling and second messenger pathways. cAMP = adenosine 3',5'-cyclic monophosphate; IP_3 = inositol trisphosphate; DAG = diacylglycerol; AA = arachidonic acid (adapted from Jaber et al. 1996; Missale et al. 1998; Alexander et al. 2007)

D_1 receptor is located at post-synaptic sites but is not present on dopaminergic neurones themselves.

In a similar fashion to that seen with D_1 receptor mRNA, D_5 receptor mRNA is not found in the ventral tegmental area or substantia nigra, whilst its expression in terminal regions is much restricted compared to the D_1 receptor having been demonstrated only in the hippocampus, thalamus and hypothalamus of rats and certain regions of the cerebral cortex and striatum in monkeys (reviewed by Ciliax et al. 2000).

In contrast to the D_1-like receptors D_2 and D_3 receptors are expressed both pre- and post-synaptically with D_2 mRNA present in terminal regions such as the prefrontal cortex, striatum, nucleus accumbens and olfactory tubercle, and in DA cell body containing regions such as the ventral tegmental area and substantia nigra (Bouthenet et al. 1991; Weiner et al. 1991; Meador-Woodruff et al. 1992; Richtand et al. 1995). Autoradiographic binding and immunocytochemical studies show a similar distribution for D_2 receptor protein (Boyson et al. 1986; Charuchinda et

al. 1987; Ariano et al. 1989; Mansour et al. 1990; Levey et al. 1993). There is also evidence from immunocytochemical studies for the expression of D_2 receptors on dopaminergic neurones within the VTA and substantia nigra (Diaz et al. 2000).

mRNA for D_3 receptors has been found in the VTA and substantia nigra in addition to terminal regions such as the hippocampus and nucleus accumbens (Bouthenet et al. 1991) whilst immunocytochemical studies have shown expression of D_3 receptors limited to the nucleus accumbens and striatum (Levesque et al. 1992). Similar to the D_2 receptor, the D_3 receptor is expressed by all dopaminergic neurons in the midbrain (i.e. ventral tegmental area, substantia nigra etc.) (Diaz et al. 2000). The D_4 receptor also has a limited expression, with mRNA and protein detectable principally in terminal regions such as the frontal cortex, thalamus and globus pallidus and little expression in the ventral tegmental area or substantia nigra (Ariano et al. 1997) suggesting that this receptor is only expressed post-synaptically.

D_2 and possibly D_3 receptors located on dopamine neurones play an important role in the regulation of neuronal function by providing negative feedback control of cell firing (via somatodendritic receptors), and dopamine synthesis and release (via presynaptic terminal receptors) (O'Hara et al. 1996; White 1996; Centonze et al. 2002; Lindgren et al. 2003). Further discussion of the functional role of these autoreceptors can be found in section 1.3.5.

1.3.2. Dopaminergic cell groups and projections in the CNS

The development of the Falck-Hillarp formaldehyde histofluorescence technique (Carlsson et al. 1962; Falck et al. 1962) allowed the visualisation of catecholamine and serotonergic cell bodies and fibres throughout the brain. Using this technique Dahlstrom and Fuxe (1964) identified twelve catecholamine containing cell groups which they named A1-A12. Subsequent work revealed a further five catecholamine groups (A13-A17) and three adrenaline synthesising cell groups (C1-C3) (Hokfelt et al. 1984). Of the A1-A17 cell groups A1-A7 are noradrenergic whilst A8-A17 are dopaminergic. The dopaminergic cell groups are primarily located in the midbrain and thalamic/hypothalamic areas, and their cells form a number of distinct projections innervating a variety of brain regions (see table 1.4 and figure 1.10.). In terms of cell numbers it

Cell Group	Anatomical Location	Projection System	Major Anatomical Targets
A8	Retrorubral Nucleus	*Mesostriatal*	Ventral caudate-putamen
A9	Substantia Nigra	*Mesostriatal*	Caudate-putamen, globus pallidus
		Mesolimbic	Amygdala, Hippocampus
		Mesocortical	Prefrontal, cingulate and perirhinal cortices
		Mesorhombencephalic	Monoaminergic nuclei, superior colliculus, reticular formation, periaqueductal grey
		Mesodiencephalic	Various thalamic and hypothalamic nuclei
A10	Ventral Tegmental Area (VTA)	*Mesostriatal*	Anteromedial caudate-putamen
		Mesolimbic	Nucleus Accumbens, Amygdala, Hippocampus, Olfactory Tubercle, Septum
		Mesocortical	Prefrontal, cingulate, perirhinal, suprarhinal and piriform cortices
		Mesorhombencephalic	Monoaminergic nuclei, superior colliculus, reticular formation, periaqueductal grey
		Mesodiencephalic	Various thalamic and hypothalamic nuclei
A11	Periventricular Grey	*Diencephalospinal*	Spinal Cord
		Periventricular	Periaqueductal grey, medial thalamus and hypothalamus
		Incertohypothalamic	Zona incerta, anterior, medial preoptic and periventricular hypothalamus
A12	Arcuate Nucleus	*Tuberohypophyseal*	Median eminence, pituitary
A13	Zona Incerta	*Incertohypothalamic*	Anterior, medial preoptic and periventricular hypothalamus
A14	Periventricular Hypothalamus	*Incertohypothalamic*	Zona incerta, anterior and medial preoptic hypothalamus
		Tuberohypophyseal	Median eminence, pituitary
A15	Olfactory Bulb	*Periglomerular*	Olfactory glomeruli
A16	Retina		Local projections

Table 1.4. Dopaminergic projection systems in the CNS (see over for diagram) (adapted from reviews by Oades et al. 1987; Feldman et al. 1997; Bjorklund and Dunnett, 2007).

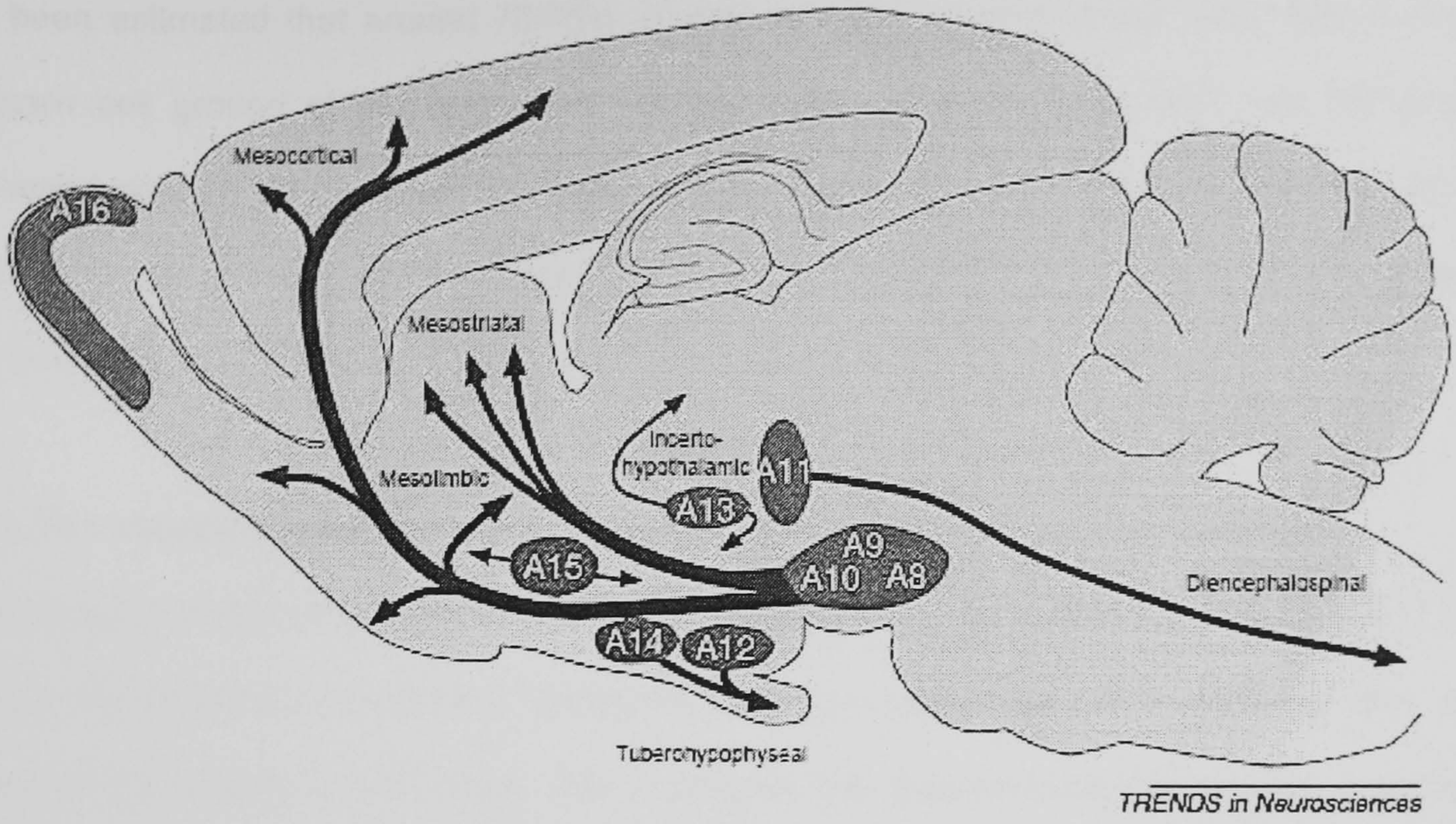

Figure 1.10. Major dopaminergic nuclei and projection pathways in the rat brain (adapted from reviews by Oades et al. 1987; Feldman et al. 1997; Bjorklund and Dunnett, 2007).

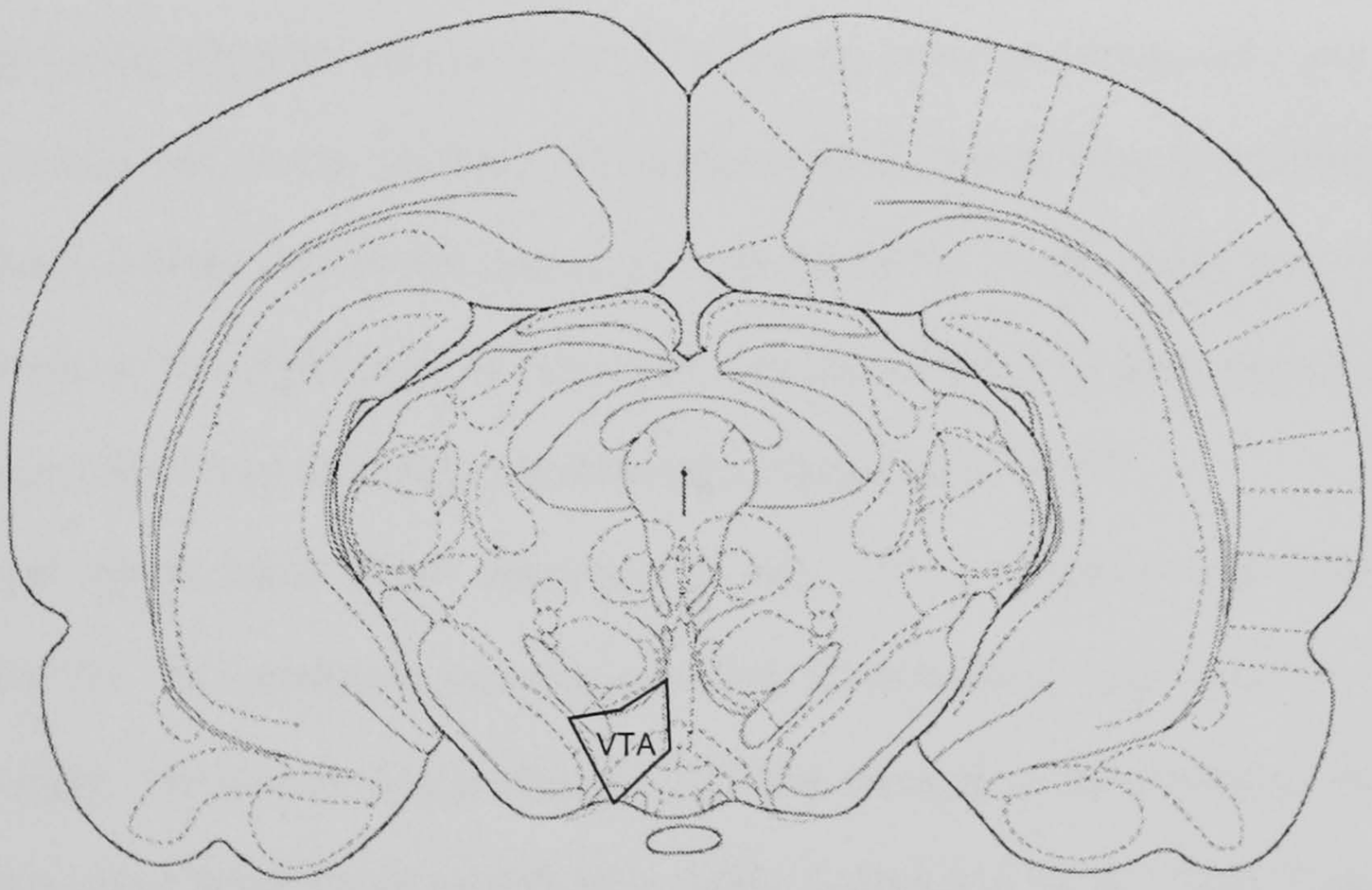

Figure 1.11. Brain atlas diagram showing the location of the VTA in a coronal section. Distance from bregma -5.6 mm (reproduced from Paxinos and Watson, 1997).

has been estimated that around 70-75% of dopaminergic neurons in the brain belong to the midbrain cell groups of the retrorubral nucleus (A8), substantia nigra (A9) and the ventral tegmental area (A10) (reviewed by Grillner et al. 2002) and as a result the vast majority of studies on dopaminergic function have been carried out either in these regions or the regions they innervate.

1.3.3. The Ventral Tegmental Area

This thesis focuses on the ventral tegmental dopaminergic neurones which form the A10 cell group in the classification system of Dahlstrom and Fuxe (1964). The VTA contains the cell bodies of the majority of neurones which comprise the mesocorticolimbic system, a system consisting of the mesocortical and mesolimbic projections. The mesocortical pathway is responsible for dopaminergic innervation of regions such as the prefrontal, cingulate, perirhinal, suprarhinal and piriform cortices, and the mesolimbic pathway innervates structures such as the nucleus accumbens, amygdala, hippocampus, olfactory tubercle and septum (see figure 1.10.).

The ventral tegmental area is located close to the midline on the floor of the midbrain (see figure 1.11.). Rostral to the VTA are the mamillary nuclei and posterior hypothalamus, with the substantia nigra lying laterally. Caudal to the VTA are the pons and hindbrain, and at its caudal extent it is situated above the nucleus interpeduncularis. Fibres from brainstem nuclei which form the median forebrain bundle (in particular from the raphe nuclei which extend dorsally from the caudal border of the VTA) pass through and dorsally to the VTA with mesocortical, nucleus ruber and oculomotor fibres situated dorsolaterally (Oades et al. 1987).

Two main cell types have been identified in the VTA – dopaminergic cells (which are immunoreactive for the synthetic enzyme tyrosine hydroxylase), and non-TH positive, non-dopaminergic cells. These non-dopaminergic cells are thought to comprise for the most part of GABAergic cells (Van Bockstaele and Pickel, 1995; Steffensen et al. 1998; Carr and Sesack, 2000; Bubar and Cunningham, 2007; Olson and Nestler, 2007), although there is evidence that the VTA contains, in addition, glutamatergic cells (Yamaguchi et al. 2007). Of the 27-29,000 cells estimated to be located bilaterally in the VTA, 18,000 stain for tyrosine hydroxylase (Oades

Neuronal Subtype	Action Potential Duration	Firing Frequency	In-vitro firing pattern
DA cell	> 2 msec	~ 0.5 - 4 Hz	Highly regular pacemaker like pattern
non-DA cell	< 1.5 msec	> 4 Hz	Variable - phasic or burst firing

Table 1.5. Electrophysiological properties of neuronal subtypes in the VTA (data from Grace et al. 1989)

et al.1987). The evidence that these TH positive cells are dopaminergic and not noradrenergic is provided not only by the fluorescence histochemistry studies of Dahlstrom and Fuxe (1964) who demonstrated the presence of dopamine in this region (dopamine is not present in detectable amounts in noradrenergic fibres, see Kitahama et al. 2000), but also by immunocytochemistry studies showing that TH positive neurones in the VTA do not express the noradrenaline synthesising enzyme dopamine-β-hydroxylase (Swanson and Hartman, 1975; Tong et al. 2000).

In addition to labelling studies, dopaminergic and non-dopaminergic neurones can be distinguished on the basis of their in-vitro electrophysiological characteristics (see table 1.5). It should be noted that the regular pacemaker-like firing of dopaminergic neurons in vitro differs significantly from the in vivo situation where firing occurs in bursts (see section 1.3.1.3).

1.3.4. Afferent regulation of VTA dopaminergic neurones

Afferent projections to the VTA arise in a number of regions and include glutamatergic, cholinergic, noradrenergic, GABAergic, and serotonergic projections (Grillner et al. 2002). Table 1.6. shows the regions of origin, and effect on VTA DA neurone firing rates, of these projections.

The following discussion focuses on the glutamatergic and serotonergic afferent systems as these have been the subject of study in this thesis. Whilst all afferent inputs have a potentially important role in regulating dopaminergic function, these two systems were selected as they were thought to be likely mediators of any observed changes in neuronal function arising from

Neurotransmitter	Region of Origin	Effect on DA cell firing
Glutamate	Prefrontal cortex, subthalamic nuclei, laterodorsal and pedunculopontine tegmental nuclei	Excitatory + Inhibitory (direct effect)
Acetylcholine	Laterodorsal tegmental nuclei (LDTg)	Excitatory (direct effect)
Noradrenaline	Locus coeruleus	Excitatory (direct effect)
GABA	Striatum, pallidum, nucleus accumbens, VTA interneurones	Inhibitory (direct effect)
5-HT	Raphe nuclei	Inhibitory (indirect effect on GABA interneurones)

Table 1.6. Afferent projections to the VTA (data from Grillner et al. 2002)

corticosteroid dysregulation. To summarise the rationale behind this, the glutamatergic system was selected due to its central role in the regulation of burst firing within the mesocorticolimbic system (see later). This functional property is critical in determining dopamine release at the terminal making glutamatergic regulation a prime candidate for investigation. The serotonergic system was selected on the basis that it is dysregulated in mood disorders (see section 1), and additionally because there is considerable evidence that corticosteroids can modulate serotonergic function (see reviews by McAllister-Williams et al. 1998; Bremner, 1999; Chaouloff, 2000).

1.3.4.1. Glutamatergic regulation of VTA dopaminergic activity

Reciprocal connections exist between the VTA, PFC, and the nucleus accumbens, consisting of GABAergic and glutamatergic projection neurons (Carr and Sesack, 1999; Moore et al. 1999; Sesack and Carr, 2002). These form a complex feedback mechanism which acts to control tonic activation of mesocortical and mesolimbic neurons (Cheramy et al. 1990; Grace, 1991). In addition to tonic regulation of dopaminergic cell firing, the phasic burst firing seen in-vivo is thought to be regulated by these projections. It has been shown that prefrontocortical afferents

play a major role in the conversion of pacemaker-like firing to phasic bursting. Application of glutamate to the PFC (Murase et al. 1993) or electrical stimulation of this region (Tong et al. 1996) results in burst-firing within the dopaminergic cells of the VTA, A study by Overton and Clark (1997) found that the latency between PFC stimulation and induction of bursting within the VTA was not always consistent with a monosynaptic connection between these two regions. It has been suggested that excitatory projections from the PFC to the subthalamic nucleus or pedunculopontine nucleus activate glutamatergic efferents from these regions which synapse onto dopaminergic cells within the VTA (Overton and Clark, 1997). Overall, the situation is currently unclear, however the PFC has been shown to be involved in the induction of burst firing and this is likely to be mediated via a mixture of monosynaptic and polysynaptic efferent projections to the VTA.

Glutamate acts on two main classes of receptor – the so called ionotropic receptors which are ligand gated ion channels and include the NMDA, AMPA and kainate receptors, and the metabotropic (G-protein linked) mGluR receptors. In terms of the receptor subtypes responsible for the regulation of dopaminergic function there is evidence for both NMDA and AMPA/kainate subtypes of ionotropic glutamate receptors on VTA DA neurons. Immunocytochemical studies have shown high levels of the AMPA receptor subunits GluR1-4 in the VTA and substantia nigra. In addition, binding studies have demonstrated the presence of NMDA, kainate, and AMPA receptors within the VTA (reviewed by Kalivas, 1993).

Microdialysis studies suggest a tonic activation of AMPA receptors within the VTA, as selective AMPA antagonists produce a decrease in dopamine release in the prefrontal cortex (Westerink et al. 1998, Takahata and Moghaddam, 2000). In the study by Takahata and Moghaddam however it was shown that the AMPA antagonist LY293558 produces an increase in dopamine release within the nucleus accumbens – the opposite effect to that seen in the PFC which suggests that these two projections may be differentially regulated.

A role for NMDA receptors in the regulation of VTA dopamine neurons is suggested by the ability of NMDA selective drugs such as the non-competitive channel blocker phencyclidine to antagonise the glutamate induced excitation of A10 neurons in a midbrain slice preparation (Wang et al. 1993). The involvement of NMDA receptors in burst firing is probably their most

important functional role within the mesocorticolimbic system. The existence of spontaneously bursting DA cells in VTA slices from immature rats has allowed the nature of burst firing to be studied in-vitro. One study has shown that spontaneous bursting can be blocked by the application of the NMDA antagonist AP-5 (Mereu et al. 1997). In addition this study found that spontaneously bursting neurons were more sensitive to NMDA induced spikes in burst firing than cells firing in a pacemaker like manner (Mereu et al. 1997). Studies in intact animals have also demonstrated that burst firing produced in VTA DA neurones by electrical stimulation of the PFC can be blocked by the NMDA antagonist CPP (Tong et al. 1996). In this study, the AMPA antagonist CNQX had no effect on bursting activity. Overall, it is clear that the NMDA receptor is involved in burst firing in these neurones, whilst the contribution of AMPA receptors is less well understood. Given the functional role of burst firing in increasing the efficiency of dopamine release, it would appear that glutamatergic signalling plays a central role in regulating dopaminergic neuronal function in the VTA.

1.3.4.2. Serotonergic regulation of VTA dopaminergic activity

Serotonergic projections to the VTA originate in the raphe nuclei and exert a tonic and phasic inhibitory effect on VTA dopaminergic neurones (reviewed by di Matteo et al. 2001). Thus studies have shown that whilst electrical stimulation of the dorsal raphe nucleus produces both excitatory and inhibitory effects on VTA DA cell firing, the inhibitory component of the response can be blocked by chemical lesion of dorsal raphe 5-HT neurones (Gervais and Rouillard, 2000). Furthermore acute systemic administration of fluoxetine, which blocks the re-uptake of serotonin, inhibits the firing rate of VTA dopaminergic cells, and this effect can be blocked by chemically lesioning raphe 5-HT neurones (Prisco and Esposito, 1995).

Functional studies suggest that the inhibitory effect of serotonin on VTA dopaminergic cell firing is mediated by the 5-HT_{2C} receptor. Thus intra-VTA application of the 5-HT_{2C} receptor antagonists SB242084 and SB206553 increases the firing rate (and burst firing) of dopaminergic neurones and increases dopamine release in the nucleus accumbens and prefrontal cortex (di Matteo et al. 1998; di Giovanni et al. 1999; Gobert et al. 2000). Furthermore the inhibitory effects of acute fluoxetine on VTA dopaminergic cell firing can be blocked by the

5-$HT_{2B/2C}$ antagonist mesulergine (Prisco and Esposito, 1995). An interesting finding in this study was that after administration of fluoxetine for 21 days, acute administration of fluoxetine did not cause any change in the firing rate of VTA DA cells compared to control animals. In addition, the ability of the 5-$HT_{2B/2C}$ agonist mCPP to reduce DA cell firing rate was abolished, suggesting a downregulation of serotonergic receptors in the VTA caused by overstimulation.

mRNA for 5-HT_{2C} receptors has been found in the VTA (Pompeiano et al. 1994; Abramowski et al. 1995; Eberle-Wang et al. 1997) and this is supported by recent work demonstrating that 5-HT_{2C} immunoreactivity is present in the VTA (Bubar and Cunningham, 2007). As the 5-HT_{2C} receptor increases IP_3/DAG via G-protein signalling and as a result is excitatory (see Feldman et al. 1997) the inhibitory action of the 5-HT_{2C} receptor on dopamine cell firing seems to be anomalous. This issue has been clarified by Bubar and Cunningham (2007) with the demonstration that this receptor is located on GABAergic interneurones. Thus it would appear that serotonin potentiates the release of GABA in the VTA via 5-HT_{2C} receptors, which in turn inhibits dopaminergic cell firing. This theory is supported by the demonstration that systemic administration of MDMA to rats increases the release of GABA in the VTA, and moreover by the fact that this effect can be blocked by local perfusion of the 5-$HT_{2B/2C}$ receptor antagonist SB206553 (Bankson and Yamamoto, 2004).

Functional studies also suggest a role for 5-HT_{2A} receptors in the regulation of VTA dopaminergic neurones as systemic administration of M100907, a 5-HT_{2A} antagonist, has been found to decrease burst firing VTA DA neurones (Minabe et al. 2001). It has been shown however that 5-HT_{2A} receptors located on glutamatergic afferents to the VTA originating in the prefrontal cortex are at least partially responsible for this effect. Prefrontocortical perfusion of M100907 attenuates the increase in prefrontocortical dopamine, and blocks the increase in glutamate in the VTA, following systemic administration of DOI (Bortolozzi et al. 2005; Pehek et al. 2006). Furthermore the presence of the 5-HT_{2A} receptor in the VTA is in question. 5-HT_{2A} receptor mRNA has not been found in the VTA (Pompeiano et al. 1994), although immunocytochemical studies have shown that 5-HT_{2A} immunoreactivity is present on dopaminergic neurones in this region (Ikemoto et al. 2000; Doherty and Pickel, 2000).

The literature suggests that serotonin is an important regulator of VTA dopaminergic function, particularly in respect of its ability to alter the burst firing properties of these cells. Current data favours a role for the 5-HT_{2C} receptor via an interaction with GABAergic interneuronal function, although there is the possibility that other receptor subtypes are involved.

1.3.5. Dopamine autoreceptor function in mesocorticolimbic neurones

The first evidence for autoreceptor control of VTA dopaminergic neurones was provided by Bunney et al. in 1973 who demonstrated that systemic application of antipsychotic drugs could increase the firing rate of these cells. This was interpreted as confirmation of a neuronal feedback hypothesis which stated that released dopamine inhibits the firing of DAergic neurones. Later work by Grace (1988) showed that application of the D_2 agonist apomorphine to an in-vitro VTA slice preparation could inhibit neuronal firing, confirming the D_2 receptor as responsible for these effects. The subsequent demonstration of somatodendritic release of dopamine in the VTA (Kalivas et al.1989) and the observation that infusion of D_2 antagonists into the VTA increases dopamine release in the PFC (Westerink et al. 1998; Chen and Pan, 2000) and the nucleus accumbens (Westerink et al. 1996; Chen and Pan, 2000) provided support for an endogenous role for these autoreceptors in regulating dopaminergic function. It is the case that the use of non-selective D_2/D_3 ligands raised the question of the role the D_3 receptor plays in somatodendritic autoreceptor function. Early in-situ hybridisation studies found little evidence for D_3 transcript in the VTA (Bouthenet et al. 1991), although more recent immunocytochemical studies have demonstrated that dopaminergic neurones in this region express both D_2 and D_3 receptors (Diaz et al. 2000). In functional studies the D_2/D_3 agonist quinpirole and the D_3 preferring ligand 7-OH-DPAT have been shown to be equipotent at inhibiting VTA cell firing (Bowery et al. 1994) suggesting that both receptor subtypes may be involved in somatodendritic autoinhibition, however work of this type has been hampered by the lack of truly selective ligands for these receptors. More recent work using knockout mice has shown that autoinhibition of VTA dopaminergic neuronal firing is dependent upon expression of the D_2 receptor (Centonze et al. 2002) whilst another study found that G-protein coupled potassium channels are not activated by D_2/D_3 ligands in D_2 knockout mice (Davila et al. 2003).

These studies therefore suggest that whilst D_3 receptors may be expressed in VTA dopaminergic neurones they are not functionally coupled to autoinhibitory effector mechanisms and may not play a significant role in the somatodendritic regulation of dopaminergic function.

In addition to somatodendritic autoreceptors there is evidence for autoreceptors on dopaminergic terminals which modulate synthesis (Hetey et al. 1985; Onali and Olianas, 1989) and release (Moghaddam and Bunney, 1990; Westerink et al. 1996; Kuroki et al. 1999), Although there is evidence that these receptors are present on mesolimbic terminals there is some controversy over the presence of these receptors at mesocortical terminals. Whilst in one study intra-PFC application of the D_2 receptor antagonist sulpiride was found to increase local dopamine release (Bean and Roth, 1991) systemic administration of sulpiride has consistently been found to have no effect on dopamine release in this region (Moghaddam and Bunney, 1990; Kuroki et al. 1999; Ago et al. 2005). Furthermore local infusion of another D_2 antagonist, haloperidol, into the mPFC has been shown to have no effect on dopamine release (Gessa et al. 2000; Devoto et al. 2001). In the study of Moghaddam and Bunney (1990) equivalent doses of sulpiride were found to significantly increase DA outflow in the nucleus accumbens whilst studies in the lab at Newcastle have produced a similar result (L. Ferrie, doctoral thesis) suggesting that autoreceptor control differs between the two projection systems. It has been suggested that in the prefrontal cortex the D_2 autoreceptor is located some distance from the synapse and therefore is only activated under conditions of elevated dopamine release (Cubeddu et al. 1990). This theory was supported by the observation that in prefrontocortical slices D_2 antagonists can augment DA release following electrical stimulation but only when very high stimulation rates are used (Agneter et al. 1994). It was suggested that these high stimulation rates release dopamine in sufficient quantities for it to diffuse to extrasynaptic sites where the D_2 receptor is located, and therefore under these conditions the receptor is active. A challenge to this theory comes from early work which found that systemic administration of the D_2 agonist apomorphine has no effect on PFC dopamine release (Chiodo et al. 1984, White and Wang 1984) from which it was surmised that mesocortical neurones do not express autoreceptors. The inconsistencies between studies using systemic and local applications of ligands suggest that in the case of systemic administration drug interactions with other neuronal

systems may cloud the picture. It does appear to be the case, however, that the mesocortical and mesolimbic systems differ in their autoreceptor function.

In terms of the mechanisms by which dopamine autoreceptors regulate neuronal function it is thought that somatodendritic receptors inhibit neuronal firing by activating K^+ channels which hyperpolarise the cell membrane (Chiodo, 1992; Zahniser et al., 1992). This mechanism may also account for the modulation of release by terminal autoreceptors, however recent studies have demonstrated that D_2 receptor activation may interfere with vesicular release via an interaction with the exocytotic machinery (Binda et al. 2005). In contrast, D_2 receptor inhibition of dopamine synthesis is thought to occur as a result of a reduction in the phosphorylation state of tyrosine hydroxylase (due to a decrease in cAMP dependent protein kinase A activity) which inhibits enzyme function (Lindgren et al. 2001). These mechanisms allow for neuronal function to be modulated in response to extracellular dopamine levels and play an important role in regulating a variety of dopaminergic systems.

1.3.6. Dopaminergic neurotransmission: physiological and psychological roles

Dopamine release in terminal regions serves a wide variety of function. The mesostriatal system arising in the substantia nigra is intrinsically involved in the control of movement with the loss of striatal dopaminergic afferents implicated in Parkinson's disease (Iversen and Iversen, 2007). The tuberoinfundibular system (originating in the hypothalamus and arcuate nucleus) controls the secretion of prolactin from the pituitary, with dopamine released into the hypophyseal portal vein inhibiting the release of this hormone (Moore, 1987). The mesocorticolimbic dopamine systems are particularly associated with reward and cognitive functions in the terminal regions of the nucleus accumbens and prefrontal cortex respectively. The following section aims to provide a brief overview of the vast literature concerning some of the most important aspects of mesocorticolimbic dopaminergic function.

1.3.6.1. Dopamine in the mesolimbic system.

The mesolimbic dopamine neurons originate in the ventral tegmental area and project to regions such as the nucleus accumbens, hippocampus an amygdala. This system is involved in emotional regulation and has been associated, in particular, with brain reward processes.

Brain reward has been the subject of intense study over the last twenty years with much evidence that dopamine plays a regulatory role. The ability of rewarding stimuli such as intracranial self stimulation, drugs of abuse, food, sex, and social interactions (reviewed by Salamone and Correa 2002) to increase dopamine release in the nucleus accumbens implicated both this brain region and the mesolimbic system in reward functions. A simplistic interpretation of these findings would be that dopamine release mediates the rewarding properties of these stimuli, however there is evidence to suggest that this is incorrect. Rather it has been suggested that dopamine is involved in anticipatory and "wanting" aspects of reward rather than the rewarding properties of the stimuli per se (Salamone and Correa, 2002). Evidence for this is provided by studies showing that dopamine depletion or administration of dopamine antagonists in the NAcc reduces the amount animals are willing to work for a rewarding stimulus but does not impair the appetite for that stimulus (Aberman et al. 1999; Nowend et al. 2001; Correa et al. 2002; Salamone et al. 2002b). This field is however controversial and others have stated that dopamine release is involved in learning and adaptive responses to reward stimuli (reviewed by Naranjo et al. 2001; di Chiara et al. 2007). This theory arose from the finding that whilst drugs of abuse increase dopamine release in the nucleus accumbens shell, natural reinforcers such as food only increase release in this region upon novel presentation (reviewed by di Chiara et al. 2007). As a consequence dopamine may be involved in learning processes involved in adaptation to novel rewarding stimuli. Overall whilst it is currently unclear what role dopamine actually plays in reward processes the ability of rewarding stimuli to alter mesolimbic dopaminergic function offers strong evidence for an involvement of this system in the brain reward circuit.

1.3.6.2. Dopamine in the mesocortical system

As the name suggests, mesocortical dopaminergic neurons from the VTA innervate a range of cortical regions including the prefrontal, cingulate, and peri-/suprarhinal cortices. The prefrontal cortex is the region of the brain associated with executive functions such as working memory, decision making, action-outcome contingency detection and visual attentional selectivity (Dalley et al. 2004). There is evidence that mesocortical release of dopamine in this region has a modulatory influence on these functions with studies showing that modest augmentation of dopaminergic activity with D_1 receptor agonists improves working memory, whilst supranormal dopaminergic activity impairs working memory (Arnsten, 1997; Robbins, 2005). This suggests a biphasic effect of dopamine on working memory, further support for which is provided by the fact that adrenalectomy induced depletions of dopamine release in this region impair working memory (Mizoguchi et al. 2004). Further involvement of dopamine in executive functions has been demonstrated by the ability of dopaminergic manipulations to alter behavioural flexibility via both D_1 and D_2 receptor mediated mechanisms (reviewed by Floresco and Magyar, 2006) and also to alter decision making processes associated with impulsivity and delayed reward (Floresco and Magyar, 2006).

Prefrontocortical cognitive functions have also been implicated in top-down regulation of emotion via sub-cortical afferents. For example lesions of the prefrontal cortex impair the ability of humans to cope emotionally with changing situations (see Davidson and Irwin, 1999; Bandler et al. 2000) and a number of imaging studies have shown that the prefrontal cortex modulates amygdala activity during conscious suppression of emotion (Beauregard et al. 2001; Levesque et al. 2003; Ochsner et al. 2004; Ohira et al. 2006; Urry et al. 2006). In rats afferents from the prefrontal cortex to the amygdala have been demonstrated (Gabott et al. 2005), whilst lesions of the prefrontal cortex have been found to impair the extinction of conditioned responses to aversive stimuli (Morgan et al. 2003). The involvement of dopamine in these processes is shown by selectively lesioning dopaminergic neurons in the PFC which delays the extinction of fear conditioning in rats (Morrow et al. 1999; Fernandez Espejo, 2003) consistent with the effect of gross lesions as demonstrated by Morgan et al. (2003). Thus dopaminergic innervation of the

mPFC appears to play an important role in emotional regulation as a result of its ability to modulate cognitive aspects of emotional processing in the PFC.

The involvement of dopamine in reward, cognition, and emotion suggests that corticosteroid modulation of dopaminergic neurotransmission may alter these brain functions and this may play a role in the symptomology of bipolar disorder. A fuller discussion regarding these functions in relation to the results of the present study and the relevance to the symptoms of bipolar disorder can be found in chapter 5.

1.3.7. Corticosteroids and mesocorticolimbic dopamine function

It is established that corticosteroids regulate mesocorticolimbic dopaminergic function, however much of the evidence comes from studies in adrenalectomised animals. This data therefore has limited utility for predicting the effects of corticosteroid dysrhythmia and hypersecretion modeled in the present study, however it gives clues as to the aspects of dopamine function which might be sensitive to corticosteroids.

Early studies showed that stereotyped behaviours in rats were increased in response to D_2 agonists such as apomorphine and LY-171555 following adrenalectomy (ADX) (Faunt and Crocker, 1988; 1989). Further evidence for alterations in dopamine receptor function was subsequently provided when it was shown that ADX decreases D_1 and D_2 receptor binding in the substantia nigra and caudate putamen (Biron et al. 1992). This decrease in D_2 receptor binding may account for an earlier observation that the apomorphine induced decrease in dopamine release in the rat striatum is attenuated following ADX (Tanganelli et al. 1990).

Following on from this early work investigations began into the role of corticosteroids in the regulation of systems involved in drug abuse. Thus in the nucleus accumbens ADX was shown to decrease basal dopamine release, and attenuate the increase in dopamine levels produced by administration of drugs of abuse, including nicotine, morphine and cocaine (Piazza et al. 1996; Shoaib and Shippenberg; 1996; Barrot et al. 2000). In the prefrontal cortex ADX has been found to reduce basal dopamine release in a similar manner to that seen in the nucleus accumbens and to induce impairments in working memory consistent with a dopaminergic role in this aspect of prefrontocortical function (Mizoguchi et al. 2004). It is interesting to note that in

contrast to the observed effect in the nucleus accumbens, ADX has been shown to increase the release of dopamine in the striatum in response to cocaine but had no effect on the response to morphine (Barrot et al. 2001). This suggests some heterogeneity in corticosteroid effects on the different dopaminergic projections.

The ADX induced attenuation of dopamine release in terminal regions innervated by the VTA may in part be explained by the ability of ADX to decrease the firing rate of VTA dopaminergic neurons (Overton et al. 1996), its ability to decrease the self-stimulation induced increase in dopamine synthesis in the nucleus accumbens (Nakahara et al. 2000), or by the evidence that dopamine metabolism is increased by ADX (although this has only been shown in the striatum; Lindley et al. 1999). One effect of ADX which is not consistent with a downregulation of dopaminergic neurotransmission, however, is the downregulation of DAT binding in the nucleus accumbens found by Sarnyai et al. (1998) although this may explain the attenuation of cocaine induced dopamine release discussed earlier (Barrot et al. 2000).

Whilst there is much evidence that adrenalectomy decreases dopaminergic neuronal function in the nucleus accumbens, with some evidence that a similar effect occurs in the prefrontal cortex, relatively few studies have looked at the effects of chronic supranormal corticosteroid levels which are more relevant to this thesis. One study by Czyrak et al. (2003) found that chronic high corticosterone increases tyrosine hydroxylase mRNA in the ventral tegmental area, and TH protein levels in the nucleus accumbens (but not the VTA). This suggests an upregulation of dopamine synthesis in the NAcc in response to corticosterone and furthermore that protein trafficking is modulated by corticosteroids. Earlier work by Pacak et al. (2002) however showed that chronic administration of cortisol reduces DOPA accumulation in the nucleus accumbens following DOPA decarboxylase inhibition, a result which indicates decreased dopamine synthesis in this region. It may be the case that tyrosine hydroxylase protein is increased in the nucleus accumbens as a result of hypercortisolaemia, but that inhibition of enzyme activity results in a decrease in dopamine synthesis.

In other dopaminergic terminal regions there is evidence that chronic administration of corticosteroids increases dopaminergic transmission. One study found an increase in striatal levels of the dopamine metabolite HVA following chronic administration of corticosterone

(Wolkowitz, 1994), whilst another found that chronic administration of corticosterone significantly increases HVA levels in the prefrontal cortex, with a concurrent non-significant increase in dopamine release in this region (Inoue and Koyama, 1996). One caveat, however, of using dopamine metabolites as a marker of dopaminergic neurotransmission is whether a change in levels represents an increase in release or an increase in metabolism.

Further evidence that chronically elevated corticosteroids can modulate dopaminergic neurotransmission was shown in the study of Czyrak et al. (2003) which demonstrated an increase in D_1 receptor mRNA in the striatum and nucleus accumbens, and an increase in VTA D_1 receptor binding. These are, however, post-synaptic receptors and consequently this effect does not suggest a direct modulation of dopaminergic neuronal function.

The use of blunt manipulations such as adrenalectomy and chronically elevated corticosteroid levels makes it difficult to predict the consequences of the more subtle corticosteroid dysrhythmia studied in this thesis. Nevertheless there is much evidence that corticosteroids modulate dopaminergic activity in mesolimbic and mesocortical systems. This in turn supports the view that corticosteroid dysregulation might alter dopaminergic neuronal function in bipolar disorder and thus may play a role in the aetiology of the disease.

1.4. Summary

To reiterate, the central hypothesis around which this thesis is based states that an underlying deficit in the hypothalamic-pituitary adrenal axis in bipolar disorder (characterized by dysregulation of the circadian pattern of secretion) produces a hypercortisolaemic state which in turn alters central mesocorticolimbic dopaminergic function. This introduction has aimed to provide an overview of biological systems implicated in this hypothesis and to review the current evidence supporting this theory.

In section 1 the evidence for altered dopaminergic function in bipolar disorder was reviewed, with data presented demonstrating elevated levels of dopamine metabolites and dopamine receptors in studies of bipolar patients. Dopaminergic hypotheses of bipolar disorder have suggested that a hyperdopaminergic state is present and this is supported by the demonstrated ability of dopamine releasing drugs to produce manic-like affective states. The ability of mood stabilizing drugs to both increase and decrease dopamine release in mesocorticolimbic terminal regions does not offer any support for a hyperdopaminergic hypothesis however it does suggest that these drugs may produce their therapeutic effects via interactions with this system. The large body of literature implicating the mesolimbic projection in reward functions offers further support for pathophysiological deficits in this system as these aspects of behaviour are altered during manic and depressive episodes. In summary it was concluded that there is considerable evidence for a mesocorticolimbic component in the pathophysiology of bipolar disorder making this system a valid candidate for study in trying to elucidate the underlying aetiology of the disorder.

In section 2 the data implicating the hypothalamic-pituitary-adrenal axis in bipolar disorder was reviewed. It was shown that there is evidence that the circadian rhythm of corticosteroid secretion is altered in bipolar disorder, with elevated hormone levels during the daily nadir in secretion. Alterations have also been found in feedback regulation of the HPA axis with a large proportion of bipolar patients exhibiting non-suppression of cortisol release in response to dexamethasone. Further evidence for dysfunction is given by the enhanced cortisol response to waking in bipolar patients and alterations in CRH responsiveness at the level of the pituitary. These data and the ability of corticosteroids to circulate throughout the body regulating a large

number of systems suggest that HPA axis dysfunction may be a primary causal factor in the aetiology of bipolar disorder. It therefore follows that modeling these deficits and investigating their effects in the CNS could increase our understanding of the disease processes at work in bipolar disorder.

In section 3 the current data regarding corticosteroid regulation of dopaminergic neurotransmission was summarised. There is significant evidence that both high doses of corticosteroids and adrenalectomy can alter mesocorticolimbic function. These effects range from altered synthetic enzyme mRNA expression to changes in dopamine release in terminal regions and altered behavioural responses to dopaminergic drugs. These studies support the general hypothesis that dopaminergic deficits may arise from HPA axis dysfunction however the fact that relatively blunt manipulations of corticosteroid function were used means that they do not accurately reflect the situation in bipolar disorder. This thesis reports on investigations which have been carried out to address these issues and to determine whether HPA axis dysfunction similar to that seen in bipolar disorder can alter mesocorticolimbic function.

Chapter 2.

Methods

Chapter 2. Methods

2.1. Animals

2.1.1. Legal status

All animal procedures were carried out in accordance with UK Home Office guidelines laid out in the Animals (Scientific Procedures) Act 1986.

2.1.2. Animal supply and housing

Experiments were carried out in male hooded Lister rats purchased from Charles River UK Ltd (Kent, UK). Male rats were used in preference to females to avoid interactions of the oestrous cycle with the DA system (Kazandjian et al. 1987; Mackenzie et al. 1988; Xiao and Becker, 1994) Animals were housed four to a cage (RC1 cages, 56 × 38 × 20 cm) with *ad libitum* access to food and water. Following delivery, animals were acclimatised for one week prior to any experimental or treatment procedures to overcome transport induced stress. All animals were kept under controlled conditions with a light cycle consisting of 12h light, 12h dark, lights on at 7am GMT; temperature 21 ± 2°C and humidity ~40%. Disturbance to the animals was kept to a minimum with cage cleaning occurring twice a week and weighing of the animals once every two days during treatment procedures.

2.2. Corticosterone treatment procedure

Corticosterone (Sigma, UK) was dissolved in 5ml of absolute ethanol (BDH) and the resultant solution diluted in one litre of tap water to yield a final concentration of 50 µg/ml corticosterone in 0.5% ethanol solution. The vehicle solution used to treat control animals consisted of 0.5% ethanol in tap water. These stock solutions were poured into water bottles and placed in the normal position on the cage with animals having *ad libitum* access. Water bottles were weighed every two days to monitor the rate of consumption and refilled with solution. Stock solution was made fresh every four days.

Animals (weight at start of treatment ~ 190-230g) were housed in cages of four as described in section 2.1.2. Treatment with corticosterone or vehicle solution was carried out for a period of 12-15 days during which animals were weighed and checked for signs of illness every two days. Adrenal glands were removed at sacrifice to confirm the effect of treatment. See chapters 4 and 5 for details of water consumption, animal weight profiles, and final adrenal weights.

The treatment procedure described above has been previously validated in experiments in our laboratory (G. Fairchild, doctoral thesis 2003). 24 hour sampling of plasma corticosterone was performed in animals treated with corticosterone and vehicle solutions for 14 days to compare the diurnal rhythm of corticosterone secretion (see figure 2.1.). Diurnal rhythms in corticosterone secretion were detected in individual animals using a single cosinor model. This analysis demonstrated that 8/8 vehicle treated animals exhibited significant diurnal rhythmicity, but only 4/7 corticosterone treated animals exhibited significant diurnal rhythmicity. Mean diurnal concentration and Area Under Curve (AUC) values were similar for both treatment groups, however there was a significant effect of treatment on the amplitude of the diurnal rhythm ($F_{2,20}$ = 4.68, $p<0.05$). When *post hoc* tests were performed, it was revealed that the amplitude of the diurnal rhythm was significantly reduced in the corticosterone treated group compared with the vehicle treated group ($p<0.05$). No significant difference between diurnal peak values was found between corticosterone and vehicle treated animals, however values at the diurnal nadir were significantly lower in the vehicle treated group compared to the corticosterone treated group. Consistent with these findings was the significantly higher ratio between peak and nadir levels in the vehicle group compared with the corticosterone group. Weight gain and water consumption in treated rats did not differ from controls during the treatment period. These results demonstrate that corticosterone administration via drinking water leads to a flattening of the diurnal rhythm in corticosterone secretion by elevating the diurnal nadir but leaving the diurnal peak intact

In a separate experiment, animals treated for 25-31 days with corticosterone in their drinking water demonstrated a 58% decrease in adrenal weight compared to their vehicle treated counterparts. Thus, as described previously, in the present experiment adrenal weight was used as a physiological measure to confirm the effect of treatment.

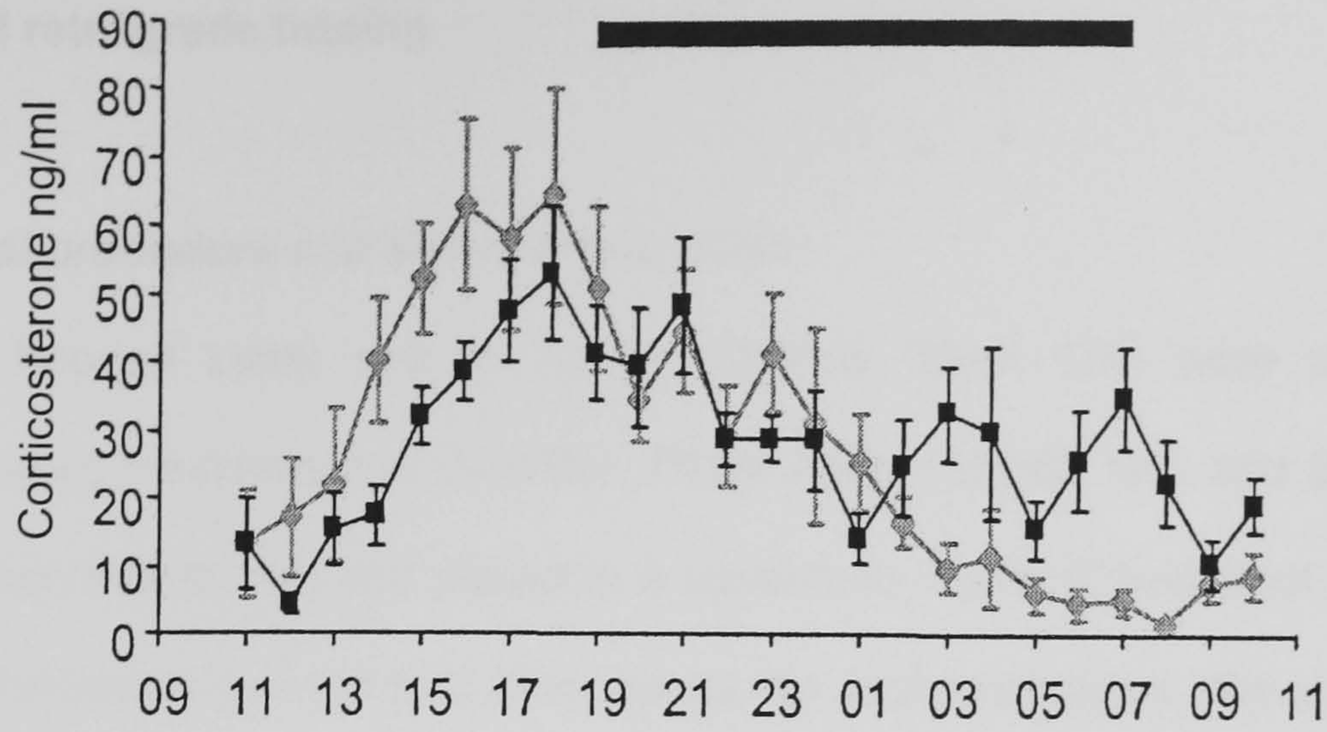

Figure 2.1. Diurnal profile of plasma corticosterone measured at hourly intervals over a 24-hour period on day 14 of treatment in vehicle treated rats (n=8, grey diamonds) and corticosterone treated rats (n=7, black squares). Solid bar indicates the dark period.

2.3. NSD1015 treatment procedure and tissue collection

Following the 14 day corticosterone treatment detailed in section 2.2., animals (8 vehicle treated, 8 corticosterone treated) were injected intraperitoneally with 100 mg/kg NSD1015 (Molekula Ltd., UK) and returned to their home cage. Either 20 min or 2 hours later the animals were sacrificed with sodium pentobarbital i.p. (0.7 ml/kg, 20% w/v solution, Dolethal Vetoquinol, UK), and the brain quickly removed. Tissue blocks containing the medial prefrontal cortex and the nucleus accumbens (according to the brain atlas of Paxinos and Watson, 1998) were rapidly dissected out and placed in pre-weighed Eppendorf tubes containing 1ml of 0.1M perchloric acid in preparation for the tyrosine hydroxylase assay detailed in section 2.6. The remaining mid- and hindbrain portion was rapidly snap-frozen in isopentane cooled on dry ice prior to storage at -70 °C to be used in *in situ* hybridization histochemistry (see section 2.5).

2.4. Neuronal retrograde tracing

2.4.1. Surgical procedure and tissue preparation

1. Male hooded Lister rats (~ 300g) (Charles River, UK) were anaesthetised with 0.25mg/kg medotomidine (Domitor, Pfizer Animal Health UK) and 58 mg/kg ketamine (Ketaset, Wyeth UK) and placed in a stereotaxic frame (David Kopf Instruments, USA) with the incisor bar set to 3.3mm below the interaural plane (flat skull position). Body temperature was maintained at ~36 °C using a thermostatically regulated heating blanket (Harvard Instruments Ltd., UK) connected to a rectal probe.
2. A longitudinal incision was made in the scalp and a trephine hole drilled in the skull at the desired rostro-caudal and medio-lateral coordinates (see 3) relative to bregma leaving the dural membrane intact. The dural membrane was then gently perforated with a fine needle
3. A glass pipette (Supracaps 709007) pulled to a fine tip (diameter ~50 µm) and containing a 2% Fluorogold (hydroxystilbamidine, Biotium UK) solution was stereotaxically implanted into either the medial prefrontal cortex or nucleus accumbens and 0.5-1.0 µl of Fluorogold was ejected pneumatically using a picospritzer (Parker Corp., USA)

Prefrontal cortex coordinates from bregma:	rostro-caudal +3.0mm
	medio-lateral -0.3mm
	dorso-ventral -4.0mm
Nucleus accumbens coordinates from bregma:	rostro-caudal +1.2mm
	medio-lateral -1.9mm
	dorso-ventral -6.9mm

4. Following surgery the medotomidine sedation was reversed with 1mg/kg atipamezole (Antisedan, Pfizer Animal Health UK) and the animals allowed to recover from anaesthesia in a recovery room maintained at ~ 25 °C

5. Following a post-operative period of one week, animals were sacrificed by transcardial perfusion (see section 2.5.1.1.) and the brains cryoprotected in sucrose. Fluorescence immunocytochemistry was used to visualise tyrosine hydroxylase immunoreactivity (see section 2.5.2.2.).

2.4.2. Visualisation and image capture

Visualisation and image capture for all experiments was carried out using a fluorescence microscope (Leica, Germany) and Metamorph image capture software (MDS Inc. USA).

2.5. Immunocytochemistry

2.5.1. Horseradish peroxidase immunocytochemistry

2.5.1.1. Tissue preparation

Adult male hooded Lister rats were injected intraperitoneally with a lethal dose (0.7 ml/kg) of sodium pentobarbital solution (Dolethal, Univet UK; 20% w/v). Once fully anesthetized a perfusion line was placed into the left cardiac ventricle and the left jugular vein was severed prior to infusion of isotonic phosphate buffered saline (PBS) at 4°C via a peristaltic pump (Gilson, France). Once ex-sanguination was complete 200-300 ml of a 4% paraformaldehyde/PBS solution was perfused and the brain removed. Tissue was then post-fixed in 4% paraformaldehyde/PBS solution for a period of 24 hours before transfer to a 30% sucrose solution for cryoprotection. Cryoprotection was deemed to be complete when the tissue was no longer buoyant in solution.

Following cryoprotection, 35 µm coronal sections of the midbrain containing the VTA (according to the brain atlas of Paxinos and Watson, 1998) were cut on a freezing microtome or a cryostat (HM 500 OM, Microm International Gmβh, Germany) maintained at -22 °C with a tissue temperature of -18 °C. Serial sections were collected in PBS in a 24 well cell-culture plate (Corning, USA) and stored at 4 °C.

2.5.1.2. Experimental procedure

TH Immunocytochemistry

1. Sections were washed in PBS for 30 minutes (2x15 min) prior to incubation with 3% H_2O_2 to quench endogenous peroxidase activity. Sections were then washed again in PBS (2x15 min).
2. Sections were incubated with 1% Triton x-100 solution for 30 min (2x15 min) to solubilise cell membranes then washed in PBS (2x15 min).
3. Tissue was incubated with 5% horse serum (Vector Laboratories, UK) for 15 minutes to prevent non-specific secondary antibody binding and then washed in PBS (2x15 min).
4. Sections were incubated for 15 min with streptavidin solution (streptavidin blocking kit, Vector Laboratories, UK), briefly washed in PBS, incubated with biotin solution (streptavidin blocking kit, Vector Laboratories, UK) for fifteen minutes and then washed in PBS (2x5 min). This step was carried out to prevent non-specific binding of the biotinylated secondary antibody and the streptavidin conjugated tertiary reagent.
5. Primary antibody (mouse anti-tyrosine hydroxylase, Sigma UK) was diluted in diluent consisting of 300mg bovine serum albumin (Sigma UK) and 180mg of lysine (BDH, UK) in 10 ml PBS.
6. Primary antibody in diluent was incubated with tissue overnight at 4°C then incubated at room temperature for two hours. Tissue was then washed in PBS (2x15 min).
7. Secondary antibody (biotin conjugated horse anti-mouse, Vector Laboratories UK) was incubated with normal rat serum at room temperature for 1 hr then centrifuged at 13,000 rpm for ten minutes. Supernatant solution was diluted 1/100 in diluent.
8. Tissue was incubated with secondary antibody in diluent for 2 hrs at room temperature and then washed in PBS (2x15 min)
9. Sections were incubated with horseradish peroxidase conjugated streptavidin diluted 1/100 in diluent for 30 min then washed in PBS (2x15 min)
10. Vector Novared HRP substrate (Vector Laboratories, UK) was applied to sections for visualization of antigen and then washed off with PBS (2x15 minutes)

GR/MR Immunocytochemistry

1. Following labeling for TH as described above tissue was incubated with 5% goat serum (Vector Laboratories, UK) for 15 minutes and then washed in PBS (2x15 min).
2. Sections were incubated for 15 min with avidin solution (avidin blocking kit, Vector Laboratories, UK), briefly washed in PBS, incubated with biotin solution (avidin blocking kit, Vector Laboratories, UK) for fifteen minutes and then washed in PBS (2x5 min).
3. Primary antibody (rabbit anti-GR primary antibody, Santa Cruz Biotechnology; or rabbit anti-MR antibody, Kawata group, Kyoto Prefectural University, see Ito et al. 2000) was diluted in diluent consisting of 300mg bovine serum albumin (Sigma UK) and 180mg of lysine (BDH, UK) in 10 ml PBS.
4. Primary antibody in diluent was incubated with tissue overnight at 4°C then incubated at room temperature for two hours. Tissue was then washed in PBS (2x15 min).
5. Secondary antibody (biotin conjugated goat α-rabbit secondary antibody, Vector Laboratories, UK) was incubated with normal rat serum at room temperature for 1 hr then centrifuged at 13,000 rpm for ten minutes. Supernatant solution was diluted 1/100 in diluent.
6. Tissue was incubated with secondary antibody in diluent for 2 hrs at room temperature and then washed in PBS (2x15 min)
7. Sections were incubated with Vector horseradish peroxidase ABC reagent (Vector Laboratories, UK) then washed in PBS (2x15 min)
8. Vector SG HRP substrate (Vector Laboratories, UK) was applied to sections for visualization of antigen and then washed off with PBS (2x15 minutes)
9. Tissue sections were mounted on subbed microscope slides and dehydrated/defatted in solvent series (70% EtOH, 95% EtOH, Abs. EtOH, Histoclear solvent) before coverslipping using Histomount mounting medium (National Diagnostics, UK).

2.5.2. Fluorescence immunocytochemistry

2.5.2.1. Tissue preparation

Animals (adult male hooded Lister rat; Charles River UK) were sacrificed by guillotine and their brains quickly removed. The whole brains were placed in 4% paraformaldehyde solution for fixation for no longer than 24 hours, before being transferred to a 30% sucrose solution for cryoprotection. Tissue was left in sucrose solution until it was no longer buoyant.

Following cryoprotection, 35 µm coronal sections of the midbrain containing the VTA (according to the brain atlas of Paxinos and Watson, 1998) were cut on a freezing microtome or a cryostat (HM 500 OM, Microm International Gmβh, Germany) maintained at -22 ± 2°C with a tissue temperature of -18 ± 2 °C. Serial sections were collected in PBS in a 24 well cell-culture plate (Corning, USA) and stored at 4 °C.

2.5.2.2. Experimental procedure

1. Sections were washed in PBS for 30 minutes (2x15 min) prior to incubation with 1% Triton x-100 solution for 30 min (2x15 min) to solubilise cell membranes then washed in PBS (2x15 min).
2. Primary antibodies (mouse anti-tyrosine hydroxylase, Sigma UK and/or rabbit anti-GR primary antibody, Santa Cruz Biotechnology) were diluted in diluent consisting of 300mg bovine serum albumin (Sigma UK) and 180mg of lysine (BDH, UK) in 10 ml PBS.
3. Primary antibodies were incubated with tissue overnight at 4°C then incubated at room temperature for two hours. Tissue was then washed in PBS (2x15 min).
4. Secondary antibodies (FITC conjugated goat α-mouse, Dako UK and/or Rhodamine conjugated bovine anti-rabbit, Santa Cruz Biotechnology) were incubated separately with normal rat serum at room temperature for 1 hr then centrifuged at 13,000 rpm for ten minutes. Supernatant solutions were then diluted 1/100 in diluent.

5. Tissue was incubated with secondary antibodies in diluent for 2 hrs at room temperature and then washed in PBS (2x15 min)
6. Sections were mounted on microscope slides using Vectashield hard-set mounting medium with DAPI for nuclear visualisation (Vector Laboratories, UK).

2.5.3. Visualisation and image capture

Visualisation and image capture for all experiments was carried out using a fluorescence microscope (Leica, Germany) and Metamorph image capture software (MDS Inc. USA).

2.6. In-situ hybridisation histochemistry

2.6.1. Tissue collection

Male hooded Lister rats (~ 276 g) which had undergone the corticosterone treatment protocol detailed in section 2.2. and the NSD 1015 treatment protocol described in section 2.3. (eight vehicle treated, eight corticosterone treated) were sacrificed with an overdose of sodium pentobarbital (0.7 ml/kg 20% w/v solution; Dolethal, Vetoquinol, UK). The brain was removed and the mid- and hindbrain portion was dissected and rapidly snap-frozen in isopentane cooled on dry ice prior to storage at -70 °C.

2.6.2. Slide preparation

Microscope slides (72 x 26 mm; Ultima, UK) were boiled in 1% Decon 90 (Decon Laboratories Ltd, UK) for 10 minutes followed by three 1 hr washes in deionised water. Slides were then heat sterilized at 180 °C for four hours and allowed to cool before immersion in a solution of 0.5% (w/v) gelatine and 0.05% (w/v) $CrK(SO_4)_2$ (Sigma, UK). Immersion solutions were prepared in RNAse-free conditions using diethylpyrocarbonate (DEPC) treated water and avoiding skin contact with treated slides. Following treatment the slides were dried overnight at 37°C then wrapped in aluminium foil for storage.

2.6.3. Tissue sectioning

Tissue was removed from storage and allowed to warm to -22 ± 2 °C in the main compartment of a cryostat (HM 500 OM, Microm International Gmβh, Germany). A block of tissue containing the midbrain was mounted rostral face down on a cryostat chuck with cryo-embedding compound (Microm International Gmβh, Germany) and the chuck affixed to the cryostat head-stage (temp. -18 ± 2 °C).

Excess tissue was trimmed until the region of the brain containing the VTA was visible. Identification of the VTA was made by comparison of the cut surface of the tissue with diagrams in the rat brain atlas of Paxinos and Watson (1997). Three 12 µm sections were then cut and thaw mounted on microscope slides such that there was 240 µm between adjacent sections on each slide. Slides were then stored at -70 °C prior to further treatment.

N.B. All solutions from this point forward made using DEPC treated water unless otherwise indicated

2.6.4. Tissue pre-treatment for storage at -20 °C

For tissue fixation and to improve retention on slides, tissue was pre-treated as follows:

1. Slides were immersed in 4% paraformaldehyde/PBS solution for 5 minutes and then washed in PBS twice.
2. Tissue was acetylated by immersion in 0.25% acetic anhydride in 0.1 M triethanolamine buffer for 10 min to reduce non-specific oligonucleotide probe binding.
3. Following acetylation sections were dehydrated in ethanol series as follows: 70% EtOH (1 min); 80% EtOH (1 min); 95% EtOH (2 min); 100% EtOH (1 min) and then delipidated in chloroform for 10 minutes.
4. Tissue was then immersed in 100% EtOH (1 min) and 95% EtOH (1 min) following which slides were air dried and stored at -20 °C.

2.6.5. Design and synthesis of oligonucleotide probes

Oligonucleotide probes were designed complementary to mRNA sequences available on the National Centre for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/sites/entrez/). Candidate sequences were tested for specificity using the BLAST search engine (http://www.ncbi.nlm.nih.gov/BLAST/). For full details of probe sequences and synthesis see Chapter 4. Probes (manufactured by MWG Biotech AG) were supplied freeze dried and dissolved in TE buffer (10mM Tris buffer [Sigma, UK]; 1mM EDTA [Sigma, UK]; pH 7.5) upon delivery to yield a concentration of 100 pmol/µl and stored at -20 °C.

2.6.6. 3' labeling of oligonucleotide probes

Oligonucleotide probes were labeled with [^{35}S]-dATP (deoxyadenosine 5'-[α-^{35}S] triphosphate) at the 3' end using the terminal deoxynucleotidyl transferase (TdT) reaction. The reaction was carried out by incubating the following at 37 °C for 1 hr:

- 5 µl molecular biology water (Sigma, UK) (Not DEPC treated)
- 4 µl 25 mmol $CoCl_2$ (Sigma, UK) solution
- 4 µl 5 × TdT forward buffer (consisting of 500 mM sodium cacodylate, 1 mM mercaptoethanol, 10 mM $CoCl_2$) (Amersham Biosciences, UK)
- 2µl (3 pmol/ul) oligonucleotide probe
- 3µl (10nmol) [^{35}S]-dATP (Perkin Elmer, UK)
- 2µl of TdT enzyme (Amersham Biosciences, UK)

When incubation was complete the reaction was stopped by adding 400 µl of a solution containing 200µl 0.5M EDTA (Sigma, UK), 1.567g Tris HCl and 100 µl triethanolamine in 100 ml DEPC water (Reagent A) and placing the mixture on ice.

Radio-labelled oligonucleotide was separated from unbound [^{35}S]-dATP by loading 400µl of the reaction mixture onto a chromatography column containing Sephadex G-50 resin (Sigma, UK) and collecting the eluate. Seven further elutions with 250 µl of Reagent A were performed and fractions collected in centrifuge tubes to give a total of eight eluted fractions. These were

placed on ice and 2 µl of each fraction pipetted into a scintillation vial containing 3 ml scintillation fluid. These were then analysed for radioactivity using a liquid scintillation counter (Beckman, UK). The vial containing fraction number 4 was generally found to contain the radiolabelled probe, with scintillation counts in the region of $7.5e^4$ CPM/µl (see Chapter 4 for further details of counts for individual probes).

2.6.7. In-situ hybridisation procedure

Hybridisation buffer solution was prepared as follows:

- 5ml deionised formamide (100% solution)
- 2ml 20 x saline sodium citrate (SSC)
- 0.5 ml 0.5 M phosphate buffer
- 0.1 ml sodium pyrophosphate
- 1 ml 50 x Denhardt's solution
- 0.2 ml denatured salmon sperm DNA
- 0.2 ml polyadenosine (5 mg/ml)
- 10 µl heparin (120mg/ml)
- 1g dextran sulphate

[^{35}S]-labelled probes were diluted in a solution consisting of 10% sodium pyrophosphate, 5% dithiothreitol and hybridisation buffer to yield a concentration of $1e^6$ cpm/200 µl in a quantity sufficient to allow the application of 200 µl to each slide. Slides were coverslipped following application of hybridisation mixture and incubated overnight at ~37 °C in humidified covered trays containing 50% (v/v) formamide:4 x SSC. On the following day coverslips were washed off the slides with 1 x SSC following which slides were washed twice in 1 x SSC at 55 °C for twenty minutes and then 1 x SSC at room temperature for 1 hr. Slides were then air dried and placed on Biomax [^{35}S] sensitive film together with a [^{14}C] microscale calibration strip covering the range 1.46-1098 Bq/g tissue (Amersham, UK) in a sealed photographic cassette. Cassettes were stored flat at room temperature (for details of exposure periods see Chapter 4).

2.6.8. Image capture and data analysis

Following an appropriate exposure period films were developed using an automated Agfa Curix Compact Plus™ processor (Agfa-Gevaert N.V., Belgium). Developed films were placed on a light box providing a constant light source and digital images captured using a monochrome digital camera (model XC-75E, Sony Corp., Japan) linked to a PC. Optical densitometry was performed on captured images using Scion Image for Windows (Scion Corp., USA). For further details see Chapter 4.

2.7. Tissue homogenate tyrosine hydroxylase assay

2.7.1. Tissue homogenization and sample preparation

Following corticosterone treatment (see section 2.2.) and NSD1015 treatment (see section 2.3.), rat brain tissue from the prefrontal cortex or nucleus accumbens was homogenised using a sonicator (Soniprep™) with the Eppendorf containing the homogenate suspended in an ice bath. Following complete homogenisation the solution was microfiltered in a centrifuge (Biofuge Pico, Heraeus Instruments GmßH) at a speed of 13,000 r.p.m. and the resultant supernatant was diluted by a factor of 1 in 10 (in the case of mPFC tissue homogenates) or 1 in 20 (in the case of nucleus accumbens homogenates) in 0.1M perchloric acid. Samples were stored at 4ºC until measurement of DOPA content was carried out using HPLC with electrochemical detection (see section 2.9).

2.8. In-vivo microdialysis

2.8.1. Probe construction

Microdialysis probes were of a single cannula concentric design with a 4.5mm dialysing window. These were constructed from stainless steel tubing (internal diameter 0.38 mm; external diameter 0.5 mm; Goodfellow, UK) according to the method of Sharp and Zetterström (1992) (see figure 2.4). Two 40 mm lengths of silica glass tubing (SGE Ltd., UK) were inserted into a 20 mm length of steel tubing with two 10 mm lengths of steel tube placed over the top of each silica tube. One of the silica tubes was retracted ~ 10 mm into the body of the main steel cannula and the three steel tubes were fixed in a Y-shape with epoxy resin (Araldite, Silmid Ltd., UK). The silica tubing protruding from the main cannula was trimmed to 4 mm and a length of tubular dialysing membrane (polyacrylonitrile/sodium methalyl sulphonate co-polymer; 20 kDA

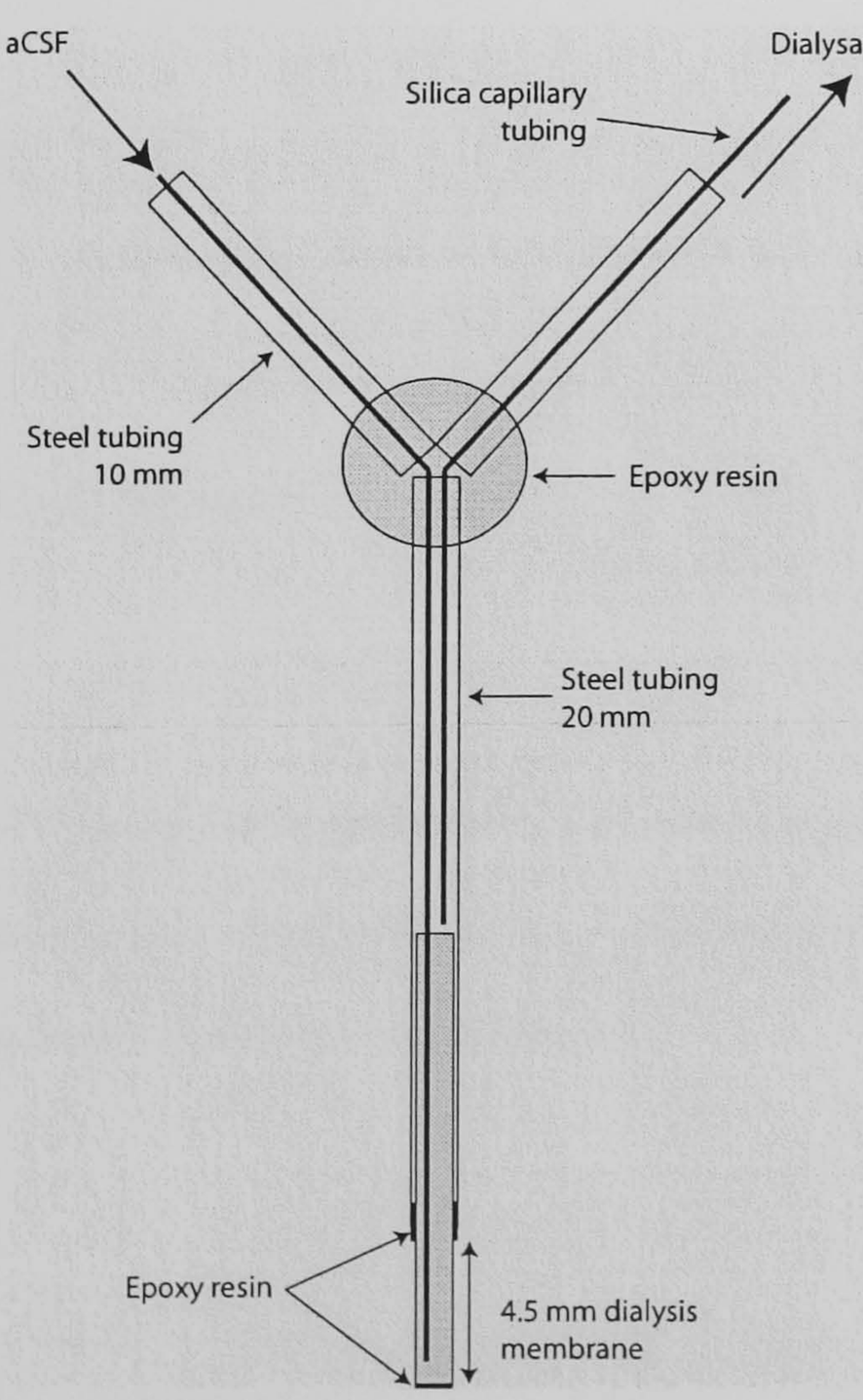

Figure 2.2. Diagram of probes used for microdialysis

molecular weight cut-off; inner diameter 0.22mm, outer diameter 0.31mm; AN 69, Hospal, Bologna, Italy) was placed over it and gently pushed inside the body of the probe. The junction of the dialysing membrane and the steel cannula was sealed with a small quantity of epoxy resin (Araldite, Silmid Ltd., UK), following which the end of the dialysing membrane was sealed with a small quantity of epoxy resin to yield a dialysing window of ~ 4.5 mm. Probes were made weekly in batches of twelve and stored in dry conditions.

2.8.2. Probe perfusion medium

During the microdialysis procedure probes were perfused with artificial cerebrospinal fluid (aCSF) consisting of:

- 140 mM NaCl
- 3 mM KCl
- 1 mM $MgCl_2$
- 1.2 mM $NaHPO_4$
- 0.27 mM NaH_2PO_4
- 7.2 mM Glucose
- 2.4 mM $CaCl_2$
- 30 µM Bupropion hydrochloride
- pH 7.4

A stock solution lacking $CaCl_2$, glucose and bupropion was prepared at the start of the study, vacuum filtered using 0.22 µm filter discs (Whatman, UK) and stored at 4 °C. $CaCl_2$, glucose and bupropion were added on the day of each experiment.

2.8.3. Surgical procedure

1. Male hooded Lister rats (~ 300 g) (Charles River UK) treated according to the corticosterone treatment protocol detailed in section 2.2. (12 vehicle treated animals; 12 corticosterone treated animals) were anaesthetised with 1500 mg/kg of a 0.5 g/ml solution of urethane injected intraperitoneally. Animals were placed in a stereotaxic frame (David Kopf Instruments, USA) with the incisor bar set to 3.3mm below the interaural plane (flat skull position). Body temperature was maintained at ~36 °C using a thermostatically regulated heating blanket (Harvard Instruments Ltd., UK) connected to a rectal probe.

2. A longitudinal incision was made in the scalp and a trephine hole drilled in the skull directly above the prefrontal cortex (Coordinates relative to bregma: rostro-caudal +3.0; medio-lateral -0.7mm) leaving the dural membrane intact. An additional hole was drilled in a caudal position and a small engineering screw (diameter 1.2 mm; length 2.6 mm; Hilco Ltd, UK) was fixed into the skull to stabilize the microdialysis probe.

3. The microdialysis probe was attached to an infusion pump (Harvard Apparatus Ltd., UK) via portex tubing (1.09 mm OD, 0.38 mm ID, Sims Portex Ltd., UK.) and perfused with aCSF at a flow rate of 2.3 ml/min. The dura membrane was then gently perforated with a fine needle and the probe inserted into the brain using a stereotaxic manipulator (David Kopf Instruments, USA) at the following co-ordinates:

Prefrontal cortex coordinates from bregma:	rostro-caudal +3.0mm
	medio-lateral -0.7mm
	dorso-ventral -5.5mm

4. Following probe implantation, dental cement (Simplex Rapid; Kemdent, UK) was applied to the exposed skull surrounding both the probe and the fixing screw to secure the probe in position.

5. Following a period >1 hr to allow neurotransmitter levels to stabilize, dialysate samples were collected into inverted polythene tubes every 20 min and placed on ice for immediate analysis using HPLC with electrochemical detection (see section 2.9.).

2.8.4. Drug administration

All drugs were administered locally via the dialysis probe by dilution in aCSF. A 1 mM stock of the dopamine reuptake inhibitor bupropion (Sigma, UK) was prepared daily and diluted in aCSF to yield a concentration of 30 µM. Bupropion was perfused throughout all experiments.

Potassium stimulated release was performed by perfusion of a hyperkalaemic aCSF which contained, in contrast to that detailed in section 2.7.2., 100 mM KCl and 43 mM NaCl. This was prepared in a stock solution, filtered and stored under the same conditions as standard aCSF.

Sulpiride was diluted in aCSF to a final concentration of 10 µM from a 1mM stock solution prepared daily.

2.8.5. Location of implanted probes

At the end of the experiment, animals were given an overdose of urethane i.p. and toluidine blue solution was perfused through the probe to allow the location of the probe to be determined. The forebrain was then sectioned on a cryostat (HM 500 OM, Microm International Gmβh, Germany) and the location of the blue dye compared to the brain atlas of Paxinos and Watson (1998). For location of implanted probes see chapter 5.

2.9. High Performance Liquid Chromatography

2.9.1. Tissue DOPA content measurement

Homogenate samples were analysed for DOPA content using reverse phase high-performance liquid chromatography (HPLC) with electrochemical (EC) detection. The HPLC-EC system consisted of the following:

- **HPLC pump**

  Gilson 302 HPLC pump/manometric module (flow rate 1 ml/min)

- **Guard Cell**

  ESA model 5020

- **Injector**

  Rheodyne model 7125, USA. Fitted with 50µl loop (Anachem Ltd, UK.)

- **Separation column**

  4.6 × 100 mm Microsorb 100-3 C18 stainless steel column (Varian Analytical Instruments, USA)

- **Analytical Cell**

  ESA Model 5011

- **Electrochemical Detector**

  Coulochem II (ESA, Analytical Ltd, UK)

- **Recorder/Integrator**

  Waters 746 data module

Mobile phase consisted of:

- 83 mM NaH_2PO_4
- 0.84 mM ethylenediamine tetraacetic acid (EDTA)
- 0.46 mM octanesulphonic acid
- 15% methanol
- pH = 4.0

Mobile phase was prepared in deionised water and filtered under vacuum using 0.22µm filter discs (Whatman, UK). Following filtration the solution was degassed by sonication for 1 hour. Mobile phase was pumped through the HPLC system at a flow rate of 1 ml/min and oxidised on a guard cell (potential difference = + 390 mV) to remove impurities.

50 µl homogenate samples were manually loaded onto the HPLC system and oxidised on a coulometric sensor comprising of two electrochemical cells. The first of these cells was set at +100 mV to partially oxidize the sample and improve the signal to noise ratio of measurements taken from the second cell set at +350 mV. Changes in current on the second cell were detected using a Coulochem II electrochemical detector and plotted on a Waters 746 recorder/integrator.

DOPA concentrations in homogenate samples were determined by measuring peak heights (se figure 2.3) compared to an external DOPA standard (10^{-9} M) (see figure 2.4) prepared daily from a 10mM stock solution (prepared weekly in 0.1M perchloric acid). The linear relationship between peak height and DOPA concentration was confirmed using a series of DOPA standards (concentrations 10^{-7} M, 10^{-8} M, 10^{-9} M). The limit of detection for this assay was estimated to be in the range of 10-20 fM.

2.9.2. Tissue DOPA data analysis

Tissue DOPA concentrations were calculated by determining total tissue sample DOPA content and dividing it by the wet mass of the dissected tissue.

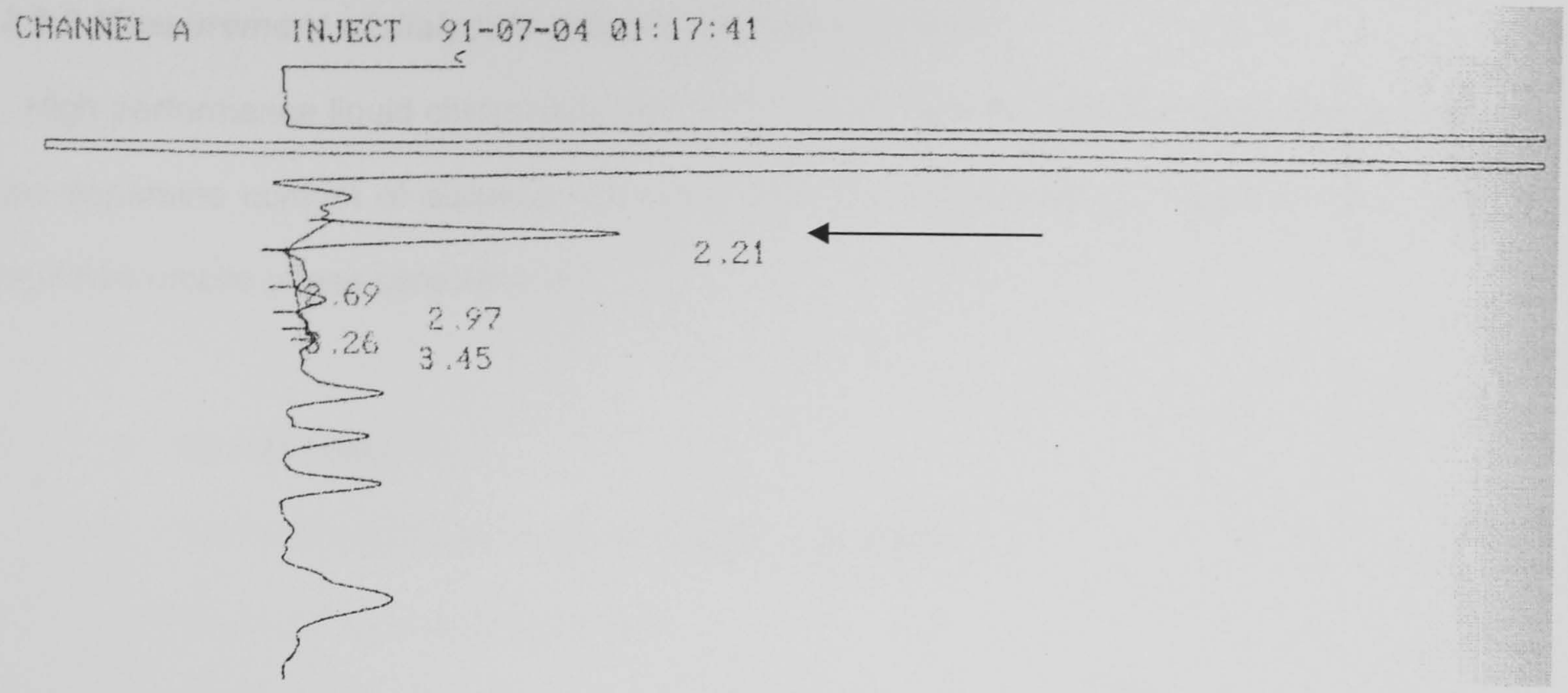

Figure 2.3. Example chromatogram showing DOPA content (indicated by arrow) in a sample of homogenized nucleus accumbens tissue.

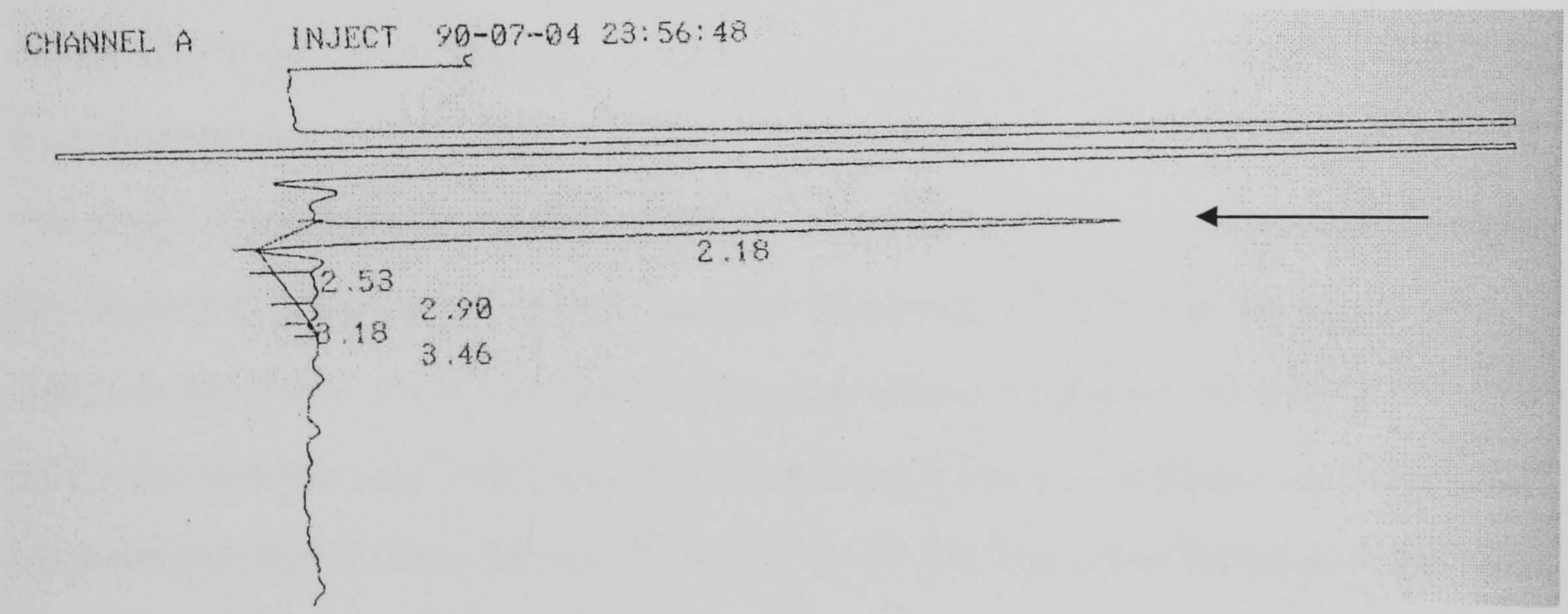

Figure 2.4. Example chromatogram showing the peak obtained for a $5e^{-8}$ M DOPA standard (indicated by arrow).

2.9.3 Measurement of dialysate sample dopamine content

High performance liquid chromatography with electrochemical detection was used to measure the dopamine content of dialysate samples. This was performed as in section 2.9.1. using a modified mobile phase consisting of:

- 83 mM NaH_2PO_4
- 1.33 mM ethylenediamine tetraacetic acid (EDTA)
- 4.14 mM octanesulphonic acid
- 12.5% methanol
- pH = 3.9

This was pumped through the HPLC system at a flow rate of 1 ml/min and oxidized on a guard cell set at +450 mV. Dialysate samples were oxidised in a two-cell coulometric sensor as detailed previously with the potential of the first cell set at -300 mV and the potential of the second cell set at +400 mV. Current fluctuations on the second cell were measured using a Coulochem III electrochemical detector (ESA Analytical, UK) and recorded on a Waters 746 recorder/integrator.

Dopamine concentrations in dialysate samples were determined by measuring peak heights (see figure 2.5) compared to external dopamine standards (10^{-9} M and $5e^{-8}$M) (see figure 2.6) prepared daily from a 10mM stock solution (prepared weekly in 0.1M perchloric acid). The linear relationship between peak height and dopamine concentration was confirmed using a series of dopamine standards (concentrations 10^{-7} M, 10^{-8} M, 10^{-9} M). The limit of detection of this assay was estimated to be in the range 2-3 fM.

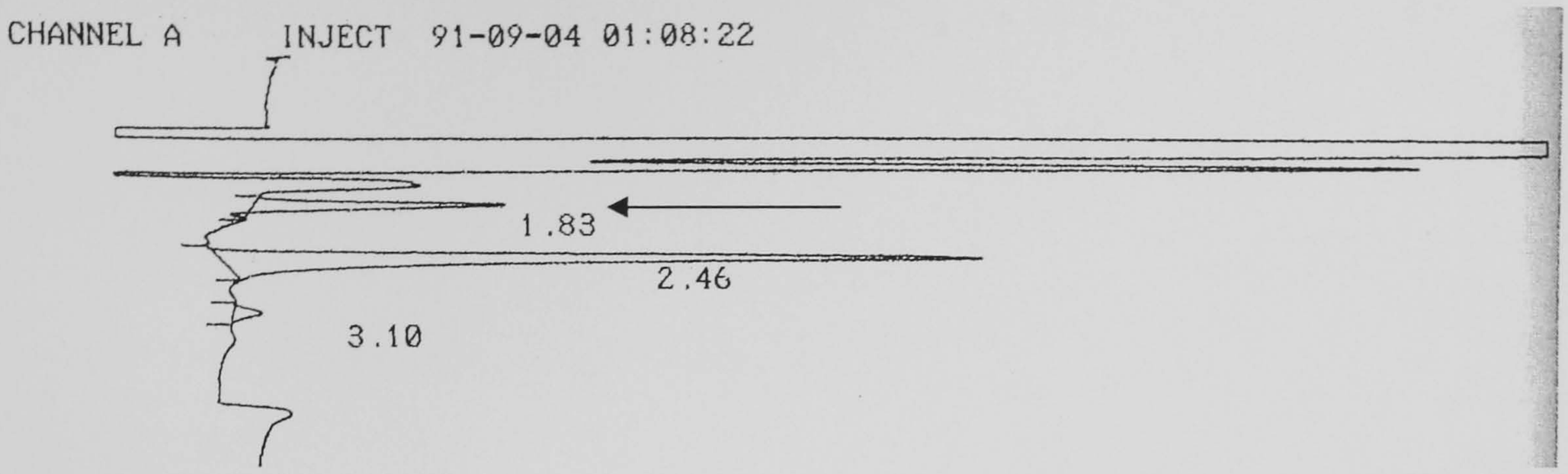

Figure 2.5. Example chromatogram showing dopamine content (indicated by arrow) in a dialysate sample obtained from the mPFC.

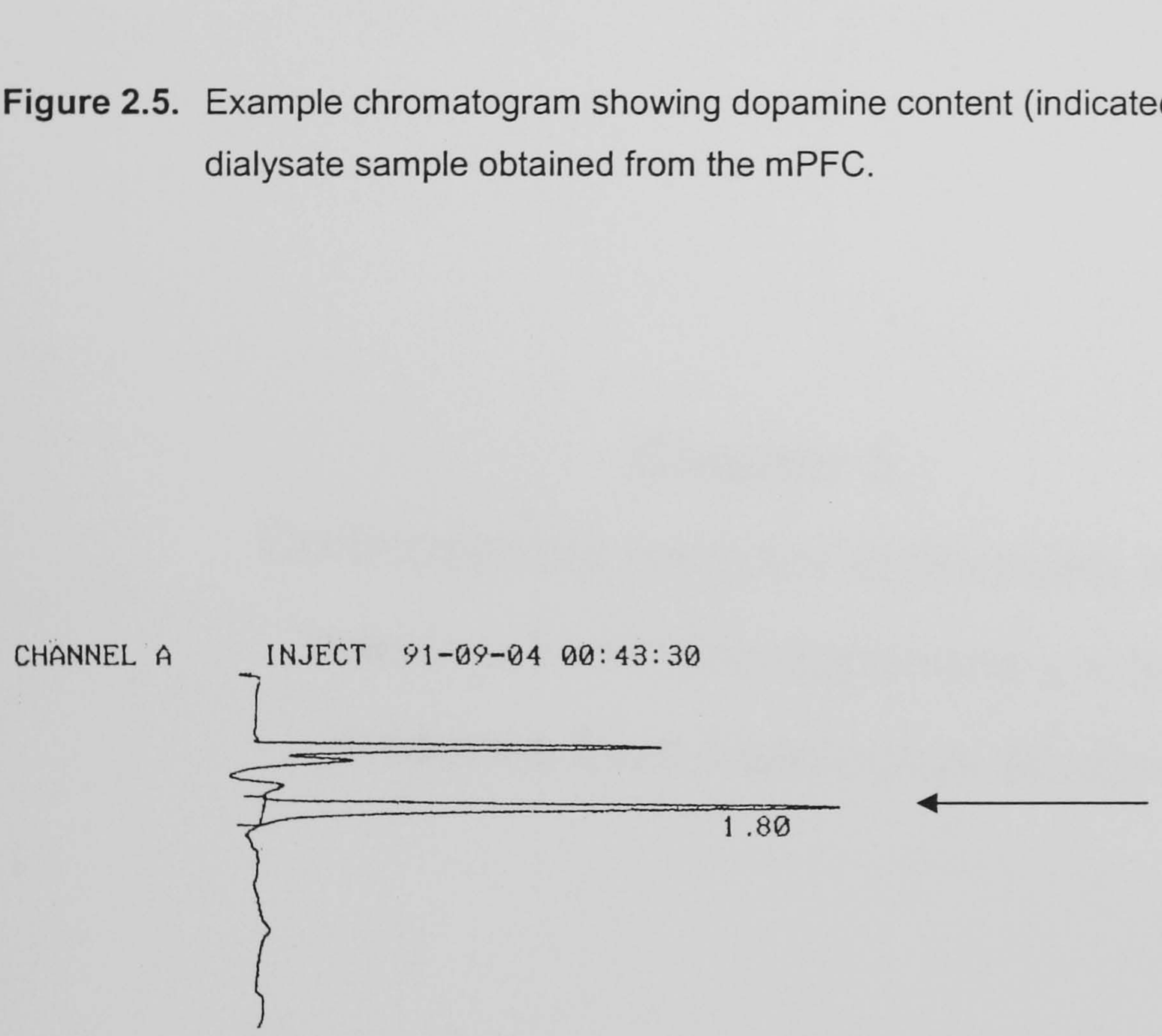

Figure 2.6. Example chromatogram showing the peak obtained from a 10^{-9} M dopamine standard (indicated by arrow).

Chapter 3.

Corticosteroid receptor expression in the mesocorticolimbic dopamine system evidence from histological studies.

Chapter 3 . Corticosteroid receptor expression in the mesocorticolimbic dopamine system – evidence from histological studies

3.1. Introduction

Underlying this PhD thesis is the theory that alterations to the diurnal corticosteroid rhythm seen in bipolar disorder are responsible for changes in mesocorticolimbic dopaminergic neurotransmission that in turn produce the symptoms of the disorder. In order to understand the effects of glucocorticoids on dopaminergic neurotransmission it is necessary to consider (a) whether dopaminergic neurones in the VTA contain corticosteroid receptors, (b) whether activation of these receptors produces changes in gene transcription that code for proteins involved in neuronal function and (c) if these changes have functional consequences in terms of dopamine release at the terminal. The first of these factors, namely whether glucocorticoid receptors are present in the neurones of interest is the subject of this chapter.

As discussed in chapter one, glucocorticoids exert their cellular actions via two types of intracellular receptor, the glucocorticoid and mineralocorticoid receptors (GR and MR respectively). When agonists bind to these receptors, chaperone heat shock proteins dissociate and the DNA binding site is exposed. Following hyperphosphorylation of the receptor protein it forms a dimer and translocates to the nucleus where it binds to glucocorticoid response elements (GREs) present on specific genes. Depending on the presence or absence of specific co-factors corticosteroids can both positively and negatively regulate gene transcription (for reviews see Schoneveld et al. 2004; Buckingham 2006).

Despite a plethora of published data showing that dopaminergic neurones originating in the VTA are sensitive to both GR and MR mediated functional alterations (see for example Overton et al. 1996; Piazza et al. 1996; Barrot et al. 2000; Mizoguchi et al. 2004) only two immunocytochemical studies have examined whether these neurones actually contain corticosteroid receptors. The first of these studies reported that around 61% of catecholaminergic neurones in the A10 cell group (which includes the VTA) express the glucocorticoid receptor (Harfstrand et al. 1986). In contrast a more recent study reported that

catecholaminergic neurones in the VTA are not immunopositive for GR (Czyrak and Chocyk, 2001). To date no studies have examined the phenotype of MR expressing cells in this region of the brain. Given the contradictory nature of the evidence regarding GR, and the lack of data on MR, it was necessary to carry out immunocytochemical studies to determine if these receptors are present in dopaminergic cells of the VTA. Tyrosine hydroxylase immunoreactivity was used as a marker for dopaminergic cells and co-reactivity with GR or MR binding markers was examined.

As the VTA constitutes a heterogenous group of dopaminergic cells projecting to various regions of the brain, the spatial distribution within the VTA of GR/MR-tyrosine hydroxylase co-reactivity was examined. To complement this, retrograde tracing from terminal regions of the mesocortical and mesolimbic projections (namely the prefrontal cortex and the nucleus accumbens) was carried out to examine whether these projections originate in topographically distinct regions of the VTA. This was to determine if there was any evidence that the projections differ in their corticosteroid sensitivity.

3.1.1. Aims

The first aim of this study was to determine whether VTA dopaminergic neurones express glucocorticoid and/or mineralocorticoid receptors and if so what proportion of neurones express the receptors. Having established the presence of the receptors in dopaminergic neurones, any spatial differences in corticosteroid receptor expression were to be compared with the distribution of neurones projecting to the prefrontal cortex or nucleus accumbens. The second aim was therefore to examine whether any differences in corticosteroid receptor expression exist between the different projections which could confer differences in corticosteroid sensitivity.

3.2. Methods

3.2.1 Tyrosine hydroxylase and corticosteroid receptor immunocytochemistry

Fluorescent immunocytochemistry protocol

Animals (adult male hooded Lister rat; Charles River UK) were sacrificed by guillotine and their brains quickly removed. The whole brains were placed in 4% paraformaldehyde solution for fixation for no longer than 24 hours, before being transferred to a 30% sucrose solution for cryoprotection. Tissue was left in sucrose solution until it was no longer buoyant (~ 3 days). In some animals nuclear translocation of GR was induced by administration of corticosterone (50mg/kg) one hour prior to sacrifice by sodium pentobarbitone. Dissection, fixation and cryoprotection were carried out as before.

Following fixation and cryoprotection a coronal section ~ 4 mm thick containing the VTA was cut by hand and coronal sections 35µm thick were cut on a freezing microtome (Microm, UK) and collected in phosphate buffered saline solution (PBS). Following incubation with Triton x-100 (1%) free floating sections were incubated overnight at 8°C with primary antibodies (Mouse α-TH, Sigma UK; Rabbit α-GR, Santa Cruz Biotechnology USA: Rabbit α-MR, Kawata group, Kyoto Prefectural University, see Ito et al. 2000) in PBS containing BSA and lysine. After incubating at room temperature for two hours primary antibodies were washed off.

Secondary antibodies (for TH, FITC conjugated goat α-mouse, Dako UK; for GR and MR, Rhodamine conjugated bovine α-rabbit, Santa Cruz Biotechnology USA) were incubated with normal rat serum for one hour and centrifuged to reduce non-specific binding. Tissue sections were then incubated with secondary antibodies diluted in BSA/lysine diluent at a concentration of 1/100 for two hours at room temperature.

Following secondary antibody incubation sections were rinsed with PBS and mounted on microscope slides using Vectashield hard-set mounting medium with DAPI for nuclear visualisation (Vector Laboratories, UK).

Horseradish peroxidise immunocytochemistry protocol

Animals (adult male hooded Lister rat; Charles River UK) were transcardially perfused with 0.9% saline solution at 4°C then perfused with 200-300ml 4% paraformaldehyde in phosphate buffered saline solution. Following perfusion the brain was dissected and post-fixed in 4% paraformaldehyde for 24 hours before transfer to a 30% sucrose solution for cryoprotection.

Following fixation and cryoprotection a coronal section ~ 4 mm thick containing the VTA was cut by hand and from this, coronal sections 35µm thick were cut on a freezing microtome (Microm, UK). Sections were collected in PBS and peroxidase activity quenched with 3% H_2O_2 before incubating with 1% Triton x-100 for 30 minutes.

TH ICC:

Sections were incubated with 5% horse serum (Vector Laboratories, UK) for twenty minutes, streptavidin solution for 15 minutes, and biotin solution for a further 15 minutes (streptavidin/biotin blocking kit, Vector Laboratories, UK). Mouse α-TH primary antibody (Sigma UK) in BSA/lysine diluent was applied and left overnight @ 8°C then room temperature for two hours and washed off. Secondary antibody (biotinylated horse α-mouse, Vector Laboratories, UK) was incubated with normal rat serum for one hour, centrifuged @ 13,000 rpm for 10 minutes and the supernatant diluted 1:100 in BSA/lysine diluent. Sections were then incubated with secondary antibody at room temperature for two hours, before application of horseradish peroxidase streptavidin (Vector Laboratories, UK) diluted 1:100 in PBS. For antigen visualisation Vector Novared HRP substrate (Vector Laboratories, UK) was applied for ten minutes and washed off.

GR ICC:

The above process was then repeated with a goat serum blocking step, either a rabbit α-GR primary antibody (Santa Cruz Biotechnology, USA) or rabbit α-MR primary antibody (Kawata group, Kyoto Prefectural University, see Ito et al. 2000), biotinylated goat α-rabbit secondary antibody (Vector Laboratories, UK), horseradish peroxidase avidin biotin complex (ABC, Vector Laboratories, UK) and Vector SG HRP substrate.

Following labelling for both TH and GR or MR sections were mounted on microscope slides and dehydrated/defatted in solvent series (70% EtOH, 95% EtOH, Abs. EtOH, Histoclear solvent) before coverslipping using Histomount mounting medium (National Diagnostics, UK).

3.2.2. Fluorescence immunocytochemistry work-up

N.B. In all immuncytochemistry experiments, labelling specificity was confirmed by omitting the primary antibody incubation step from some tissue sections and ensuring no subsequent labelling occurred. All antibodies were validated in tissue from a number of animals. In the case of the TH and GR antibodies this was in excess of 15 animals for each. Tissue from more than five animals was used to work-up the MR antibody.

TH single-labelling

Single label fluorescence for TH was carried out using established primary antibody concentrations of 1:5,000 (mouse α-TH, Sigma UK) and secondary antibody concentrations of 1:100 (FITC conjugated goat α-mouse). To establish the optimum fixation period 24 hour and 72 hour fixation times were tested and labelling was compared. At the longer fixation time immunoreactive labelling for TH was of a much lower intensity (see figure 3.1.), thus a fixation period of no longer than 24 hours was deemed to be optimal for successful labelling

TH + GR double-labelling

Combining the TH labelling with GR labelling required optimisation of the GR primary antibody concentration. Concentrations of 1:100, 1:200, 1:400 and 1:700 rabbit α-GR (Santa Cruz Biotechnology USA) were tested in dual label experiments using a rhodamine conjugated secondary antibody (1:100 bovine α-rabbit, Santa Cruz Biotechnology USA). A concentration of 1:400 was found to give optimal signal strength with the lowest background signal however problems with visualisation remained.

Autofluorescence of dopaminergic cells was found to be a problem in these dual label experiments, probably due to the formation of aldehyde catecholamine conjugates during the fixation process. Attempts were made to reduce this by treatment of tissue with 0.1% $NaBH_4$ for fifteen minutes (see Clancy and Cauller, 1998) but this was ineffective. Photobleaching of fluorescent conjugates with a mercury lamp for time periods of 30 and 60 minutes (see Neumann and Gabel, 2002) was also attempted, but again this was ineffective. At this point it was decided to try and improve the GR labelling to increase the signal to noise ratio rather than eradicate the autofluorescence.

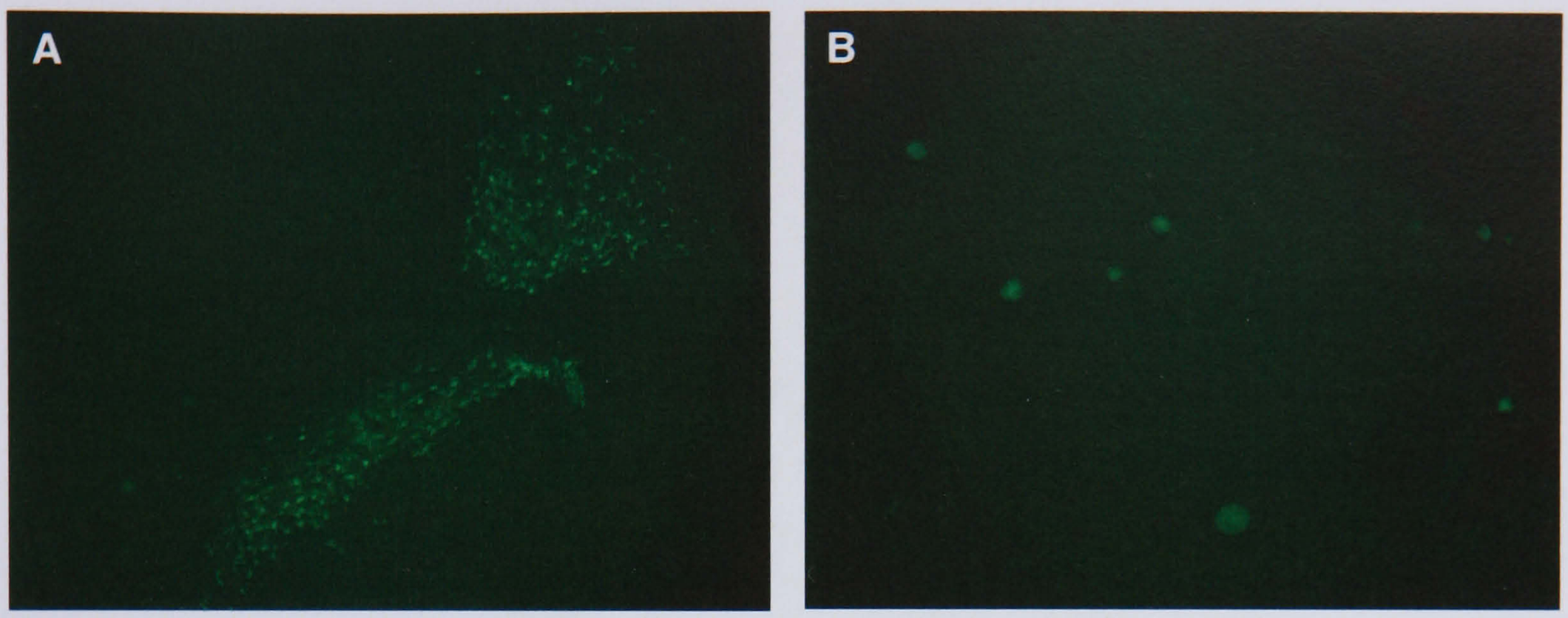

Figure 3.1. Photomicrograph of TH (FITC labelled; green) in the VTA (x10 magnification). (A) 24-hours paraformaldehyde tissue fixation. (B) 3 days paraformaldehyde tissue fixation.

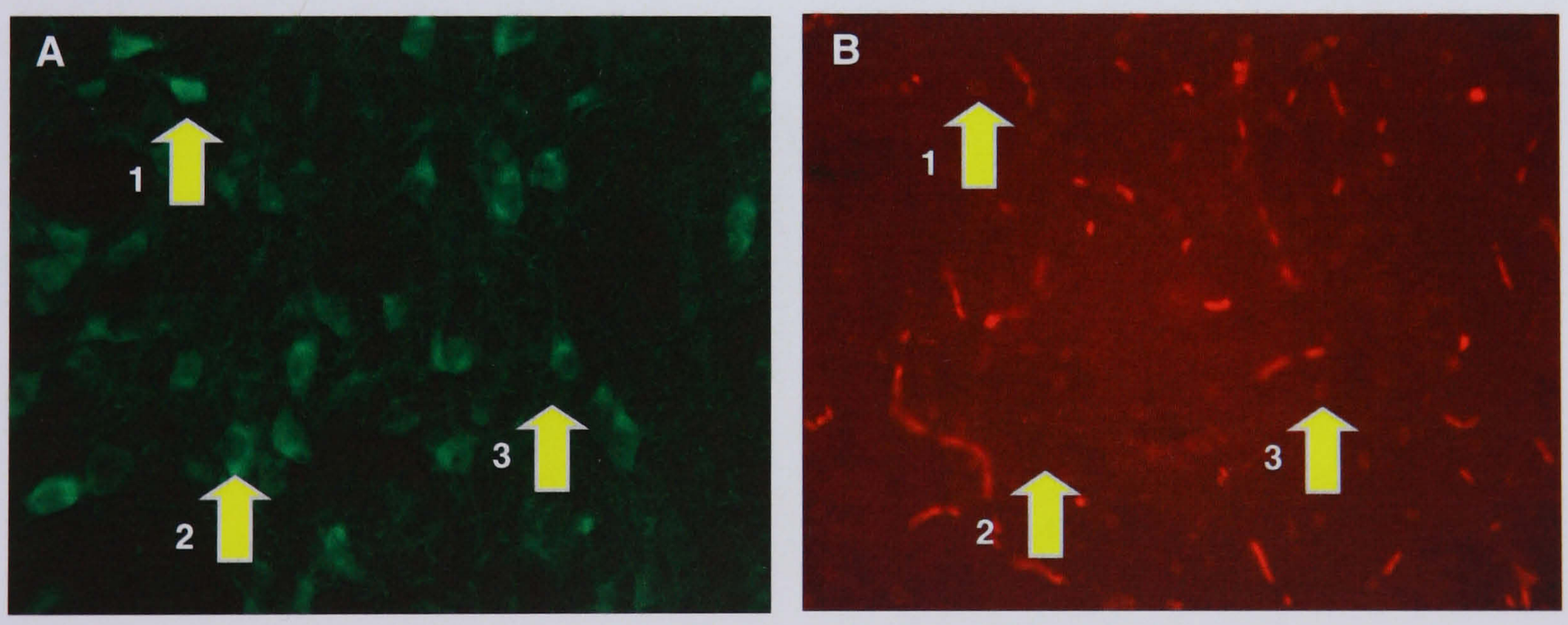

Figure 3.2. Photomicrograph (x40 magnification) of (A) TH (1/5,000 primary antibody conc.; FITC labelled; green) and (B) GR (1/400 primary antibody conc.; rhodamine labelled; red) immunoreactivity in the same VTA section. (1) TH + / GR + cell; (2) TH + / GR - cell; (3) TH - / GR + cell.

Figure 3.3. Photomicrograph (x40 magnification) of GR (HRP DAB labelled) immunoreactivity in the VTA (primary antibody concentration 1/400).

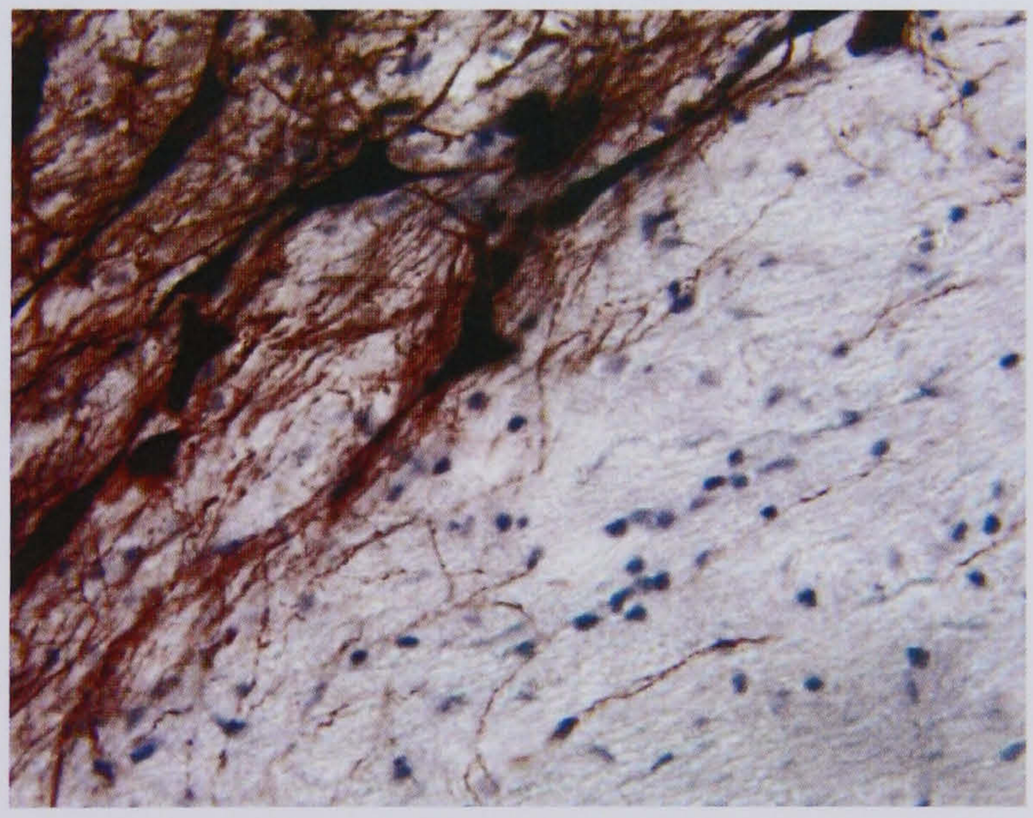

Figure 3.4. Photomicrograph of TH (x40 magnification) (HRP Vector Novared labelled; brown) and GR (HRP Vector SG labelled; blue) immunoreactivity in the VTA. TH primary antibody concentration 1/5,000; GR primary antibody concentration 1/400.

The concentration of Triton x-100 was increased from 0.1% to 1% to improve solubilisation of cell membranes and improve antibody penetration. This produced more consistent GR labelling and improved signal strength resulting in the first successful double-labelling of TH and GR (see figure 3.2.). Further attempts were made to improve the signal of labelled GR by using an amplification step in the form of biotin conjugated secondary antibodies (1:100 biotinylated goat α-rabbit, Vector Laboratories UK) and fluorescent conjugated streptavidin. Cy3 conjugated streptavidin (1:100 Extravidin Cy-3, Sigma UK) was found to give a strong GR signal but with high non-specific labelling. Texas red labelled avidin (1:100, Vector Laboratories UK) was also used as a tertiary reagent and again gave a strong signal with some non-specific background labelling, however the improvement over fluorescent conjugated secondary antibodies was minimal.

At this point an experiment had been carried out to label GR using horseradish peroxidise labelled streptavidin (1:100 Vector Laboratories UK) with a DAB chromagen. This gave very clear labelling of GR which was a great improvement over attempts to label GR with fluorescence (see figure 3.3.). Attempts to combine fluorescent labelling of TH with HRP-DAB labelling of GR were carried out but were unsuccessful. Whilst HRP labelling of GR was a success no fluorescent labelling of TH was observed. At this point it was decided to use HRP labelling for both TH and GR in future experiments.

3.2.3. HRP immunocytochemistry work-up

TH/GR double labelling

To reduce non-specific labelling all HRP-ICC was carried out on tissue from rats transcardially perfused with 0.9% saline and 4% PFA. This was to remove endogenous HRP present in blood and to provide more reliable fixation. In addition any remaining endogenous HRP activity was quenched by application of 3% hydrogen peroxide prior to the labelling procedure.

The work up on the HRP immunocytochemistry was primarily concerned with optimising the relative signal strengths between the TH and GR labelling. Initially the GR primary antibody was used at a concentration of 1:400 (visualised with Vector SG HRP chromagen, Vector Laboratories UK), and TH antibody concentrations of 1:5,000 and 1:10,000 (visualised with Vector Novared HRP chromagen, Vector Laboratories UK). Further to this Vector HRP-ABC was initially employed as a tertiary reagent which offers greater signal amplification compared to standard HRP conjugated streptavidin. At these concentrations and with this visualisation method the TH labelling was far too intense for GR immunoreactivity to be visible (see figure 3.4.). To reduce the TH label intensity the ABC reagent was replaced with HRP conjugated streptavidin and the primary antibody concentration was reduced to 1:50,000 and 1:100,000. Double labelling was evident with a TH primary antibody concentration of 1:100,000 and a GR primary antibody concentration of 1:400 (see figure 3.5.).

In some experiments cross-labelling was observed where Vector SG (for labelling GR) appeared to be reacting with TH bound HRP. This was suggested by the presence of Vector SG in the cytoplasm of labelled cells, in contrast to the distribution seen in single labelling experiments. To prevent this from occurring a different enzyme conjugated tertiary reagent (alkaline phosphatase conjugated streptavidin, Vector Laboratories UK) was used to label TH. Some cross-labelling was still seen in some experiments, and furthermore the labelling produced by AP was considered too diffuse for use in double labelling (see figure 3.6.).

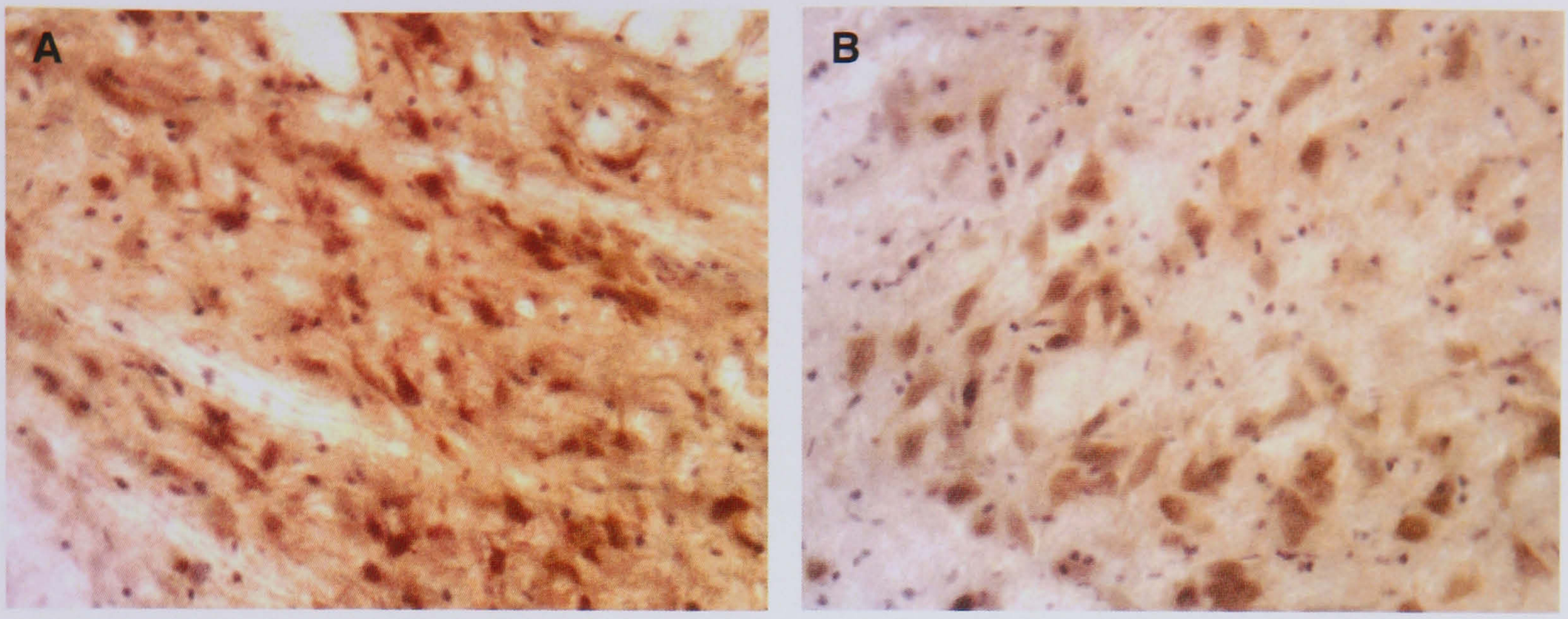

Figure 3.5. Photomicrograph of TH (x40 magnification) (HRP Vector Novared labelled; brown) and GR (HRP Vector SG labelled; blue) immunoreactivity in the VTA. (A) TH primary antibody concentration = 1/50000; GR primary antibody concentration – 1/400. (B) TH primary antibody concentration = 1/100,000; GR primary antibody concentration 1/400.

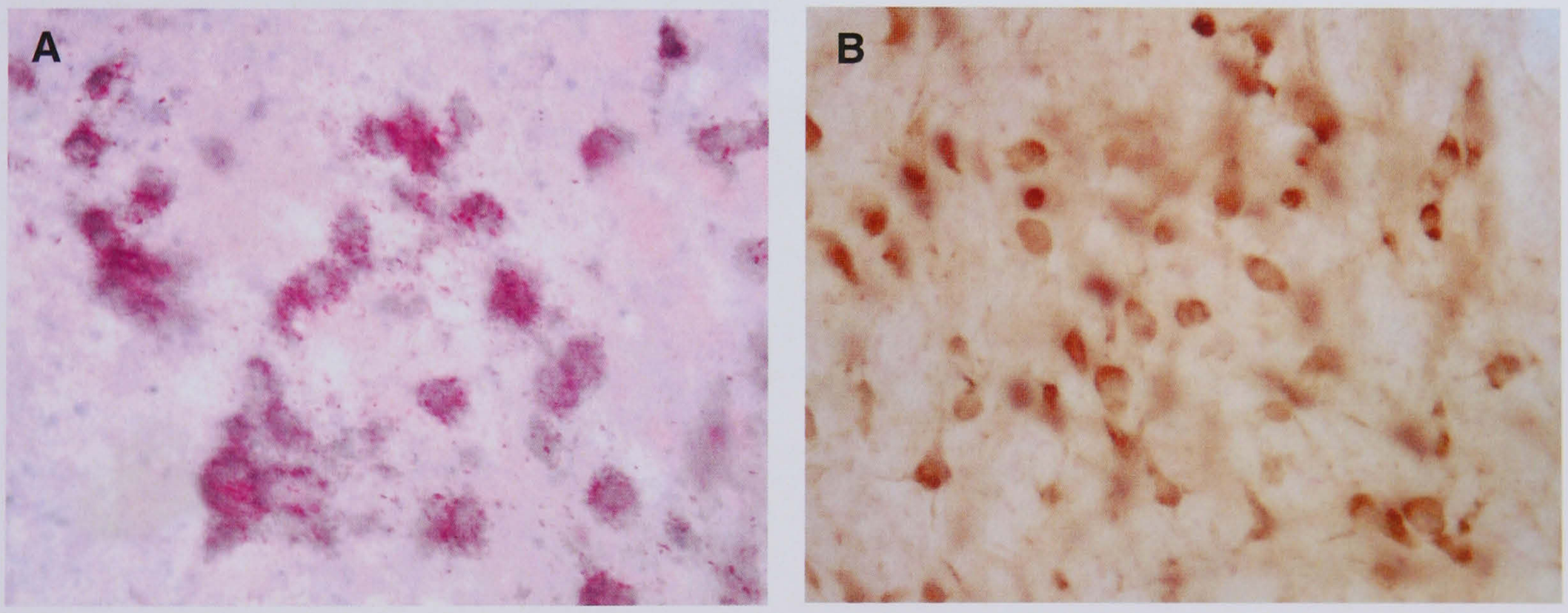

Figure 3.6. Photomicrograph (x40 magnification) of TH immunoreactivity in the VTA labelled with (A) alkaline phosphatase and Vector Red substrate (primary antibody conc. 1/100,000) (B) horseradish peroxidase and Vector Novared substrate (primary antibody conc. 1/500,000)

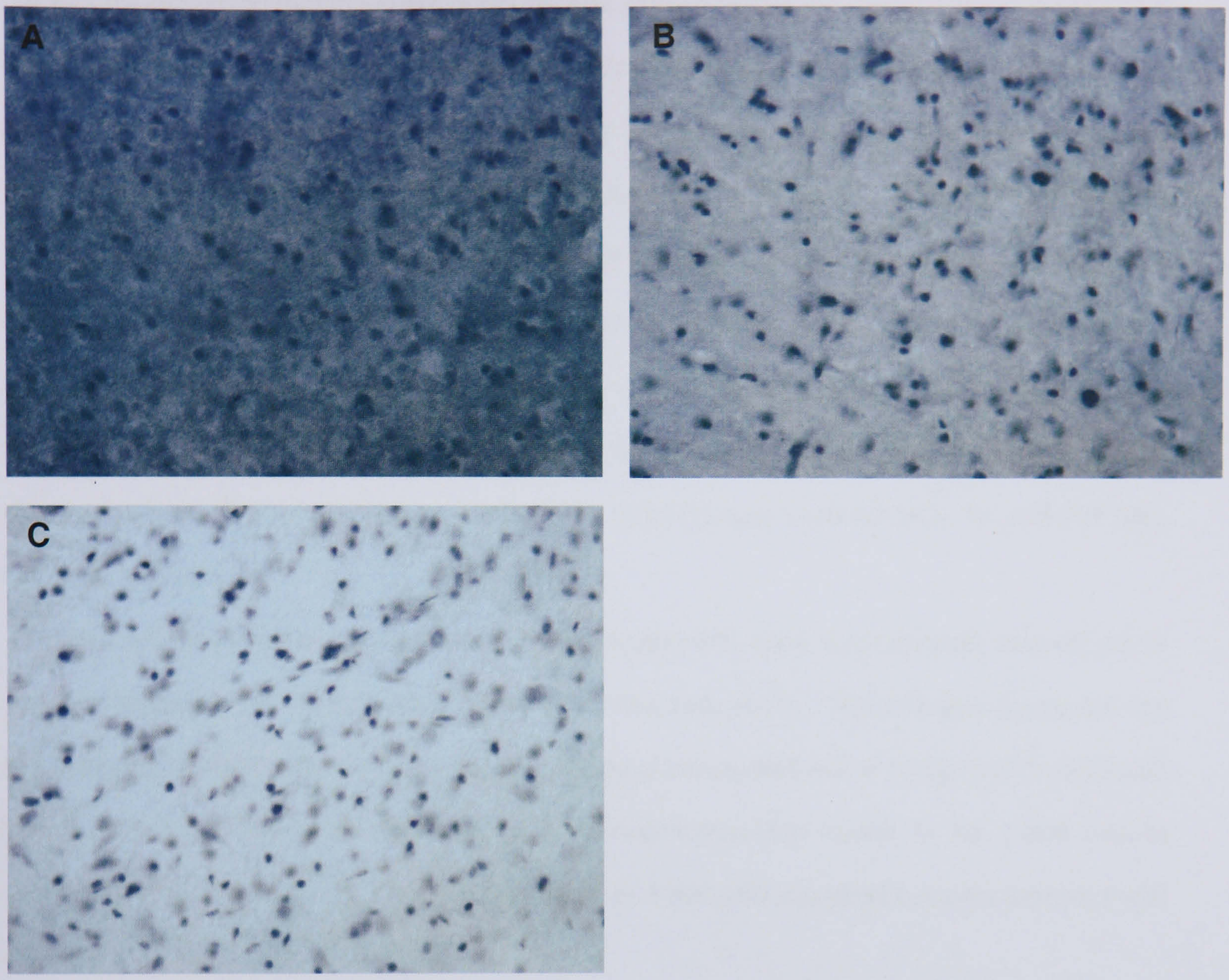

Figure 3.7. Photomicrograph (x40 magnification) of GR (HRP Vector SG labelled) immunoreactivity in the VTA. Primary antibody concentrations were (A) 1/100; (B) 1/200; (C) 1/400

It was hypothesised that the cross reactivity was caused not by the use of the same enzyme to label both proteins but by cross reaction between secondary and tertiary labels such that the HRP-ABC tertiary reagent used to label GR was in fact reacting with unused binding sites on the TH bound biotinylated secondary antibodies. To prevent this, blocking steps were incorporated into the protocol via the application of streptavidin and biotin solutions (streptavidin blocking kit, Vector Laboratories UK) to the tissue following the TH labelling to block any remaining binding sites. After the incorporation of this step no further cross reactivity was observed with the cellular distribution of labelling for both antigens corresponding to that see in single labelling experiments. Consequently HRP labelling was used for both TH and GR from this point onwards.

Further optimisations of primary antibody concentrations were subsequently carried out to improve labelling. The TH antibody concentration was reduced to 1:500,000 (see figure 3.6. B), and a dilution series performed for the GR antibody at concentrations of 1:100, 1:200 and 1:400 (see figure 3.7.). The optimal GR antibody concentration was found to be 1:200 and in combination with TH antibody at a concentration of 1:500,000 successful double labelling was achieved.

TH/MR double labelling

Using the TH primary antibody concentration (and Vector Novared HRP chromagen) from the GR/TH double labelling experiments, initial attempts to label MR were performed with an antibody concentration of 1:100 (rabbit α-MR, Kawata group, Kyoto Prefectural University, see Ito et al. 2000) and Vector SG chromagen. This resulted in a very intense MR label which obscured the TH label. MR primary antibody concentrations of 1:1000, 1:5,000, 1:10,000 and 1:50000 were tested in conjunction with TH labelling and a concentration of 1:1,000 was found to be optimal (this can be seen in figure 3.11.). At lower concentrations MR immunoreactivity was barely visible. Using an MR primary antibody concentration of 1/1,000 and a TH primary antibody concentration of 1/500,000 successful double labelling of TH and MR was achieved.

3.2.4. Fluorogold retrograde tracing of mesocorticolimbic neurones

Adult male hooded Lister rats (Charles River, UK) were anaesthetised with a mixture of 0.25mg/kg medotomidine (Domitor, Pfizer Animal Health UK) and 58 mg/kg ketamine (Ketaset, Wyeth UK) and placed in a stereotaxic frame. A glass pipette pulled to a fine tip of diameter 50 µm and containing a 2% Fluorogold (hydroxystilbamidine, Biotium UK) solution (in PBS) was stereotaxically implanted into either the medial prefrontal cortex or nucleus accumbens and a small volume of Fluorogold was ejected by means of a pressure ejection device (Picospritzer, Parker Corp. USA) and the pipette left in place for five minutes. Following withdrawal of the pipette and wound closure the medotomidine sedation was reversed with 1mg/kg atipamezole (Antisedan, Pfizer Animal Health UK) and the animals were allowed to recover from anaesthesia. Following a post-operative period of one week, animals were sacrificed by transcardial perfusion (see previous protocol) and the brains cryoprotected in sucrose. Fluorescence immunocytochemistry was used to visualise tyrosine hydroxylase immunoreactivity in sections containing the VTA (see previously) to determine co-localisation with fluorogold. Coronal sections containing the PFC and nucleus accumbens were cut, mounted on microscope slides and examined to determine the fluorogold injection site.

3.2.5. Visualisation and image capture

Visualisation and image capture for all experiments was carried out using a fluorescence microscope (Leica, Germany) and Metamorph image capture software (MDS Inc. USA).

3.3. Results

3.3.1. TH immunoreactivity

The antibody against TH labelled a large number of cells in the midbrain. These were confined to specific nuclei comprising the A8 (retrorubral nucleus), A9 (substantia nigra) and A10 (ventral tegmental area, nucleus interfasicularis, nucleus linearis caudalis and nucleus linearis rostralis) cell groups (see figure 3.8. for TH labelling in the VTA). Some TH labelled cells were seen in the rostral parts of the median and dorsal raphe nuclei (B7 and B8 cell groups). At the caudal extent of the VTA TH positive cells of the A9 and A10 groups were found to be separated by the medial lemniscus, whilst more rostrally the A9 (substantia nigra) and A10 (VTA) groups were contiguous. At the rostral extent of the VTA the A9 and A10 nuclei again become separated this time by the medial terminal nucleus of the accessory optic tract. The VTA was at all times contiguous with the nucleus interfasicularis, nucleus linearis caudalis and nucleus linearis rostralis.

In the VTA TH labelled cells were large with extensive dendrites. DAPI staining revealed that the nuclei of these cells were also large (see figure 3.9.). TH labelling was present in the somatic cytoplasm but absent from cell nuclei, and was found extensively in dendrites. Cytoplasmic labelling was most dense close to the cell membrane. This pattern of labelling was found with both fluorescence and HRP immunocytochemistry (see figure 3.2 [A] and figure 3.6 [B]).

3.3.2. GR immunoreactivity

The GR antibody labelled a very large number of cells throughout the midbrain. GR staining was evident in all monoaminergic areas present (A8, A9, A10, B7 and B8 cell groups) and throughout the dorsal midbrain. Fluorescence immunohistochemistry in conjunction with the nuclear stain DAPI revealed that GR immunoreactivity was punctate and confined to the nuclei of labelled cells whilst absent from cytoplasmic and dendritic regions. HRP labelled GR was similarly punctate with a similar sized area of labelling compared to fluorescent labelled protein (see figures 3.10. and 3.11.).

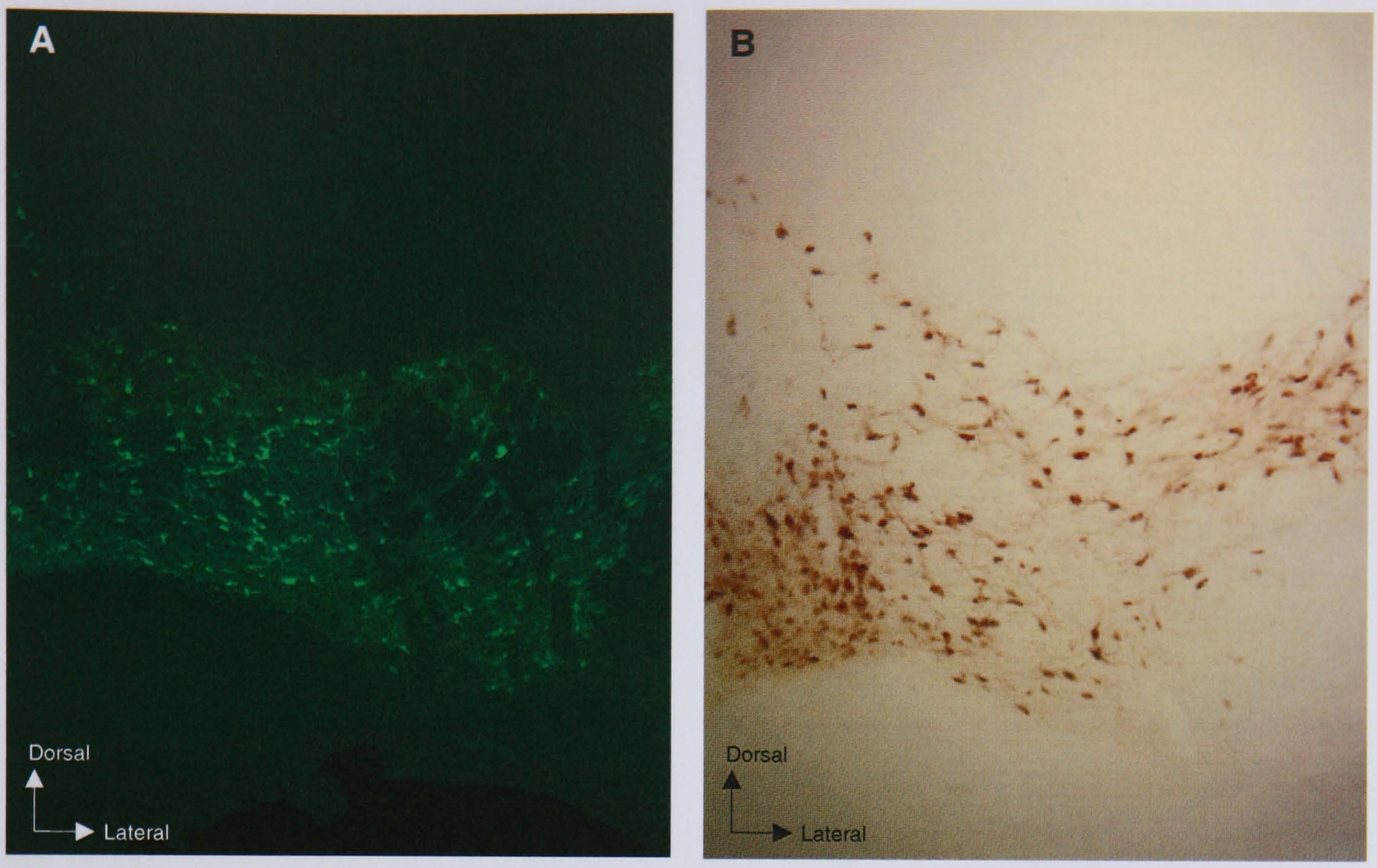

Figure 3.8. Photomicrograph of TH immunoreactivity (10x magnification) in the ventral midbrain. (A) FITC labelled; (B) HRP labelled Vector Novared

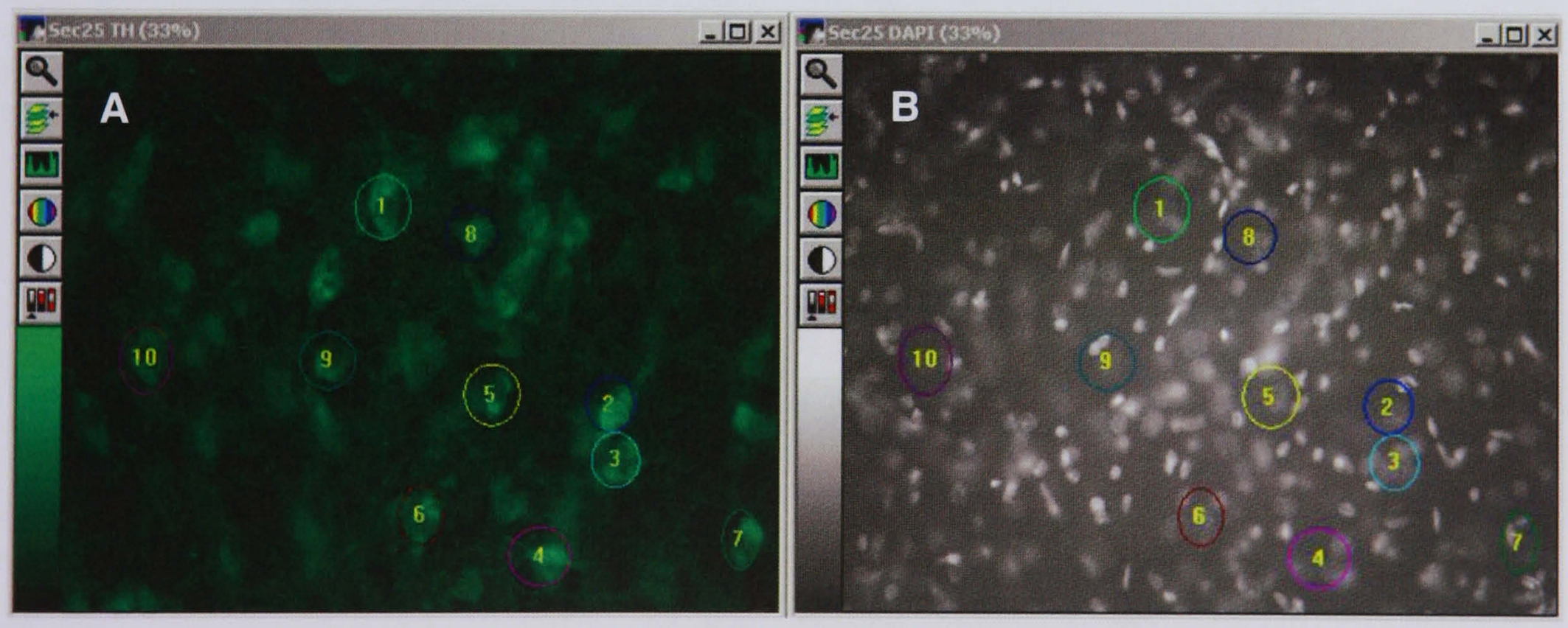

Figure 3.9. Photomicrograph (40x magnification) of FITC labelled TH immunoreactivity (A) and DAPI nuclear staining (B) in the ventral midbrain.

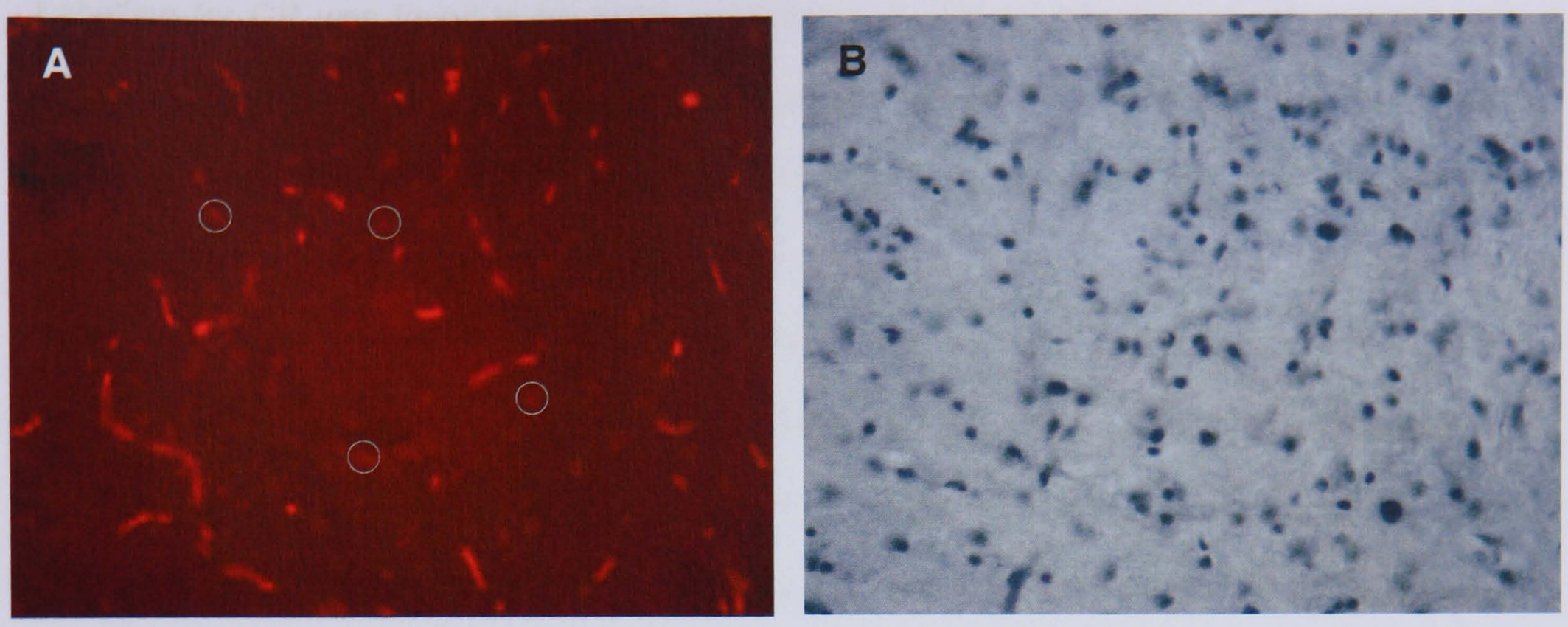

Figure 3.10. Photomicrograph of GR immunoreactivity (40x magnification) in the ventral midbrain. (A) Rhodamine labelled (examples circled); (B) HRP labelled Vector SG.

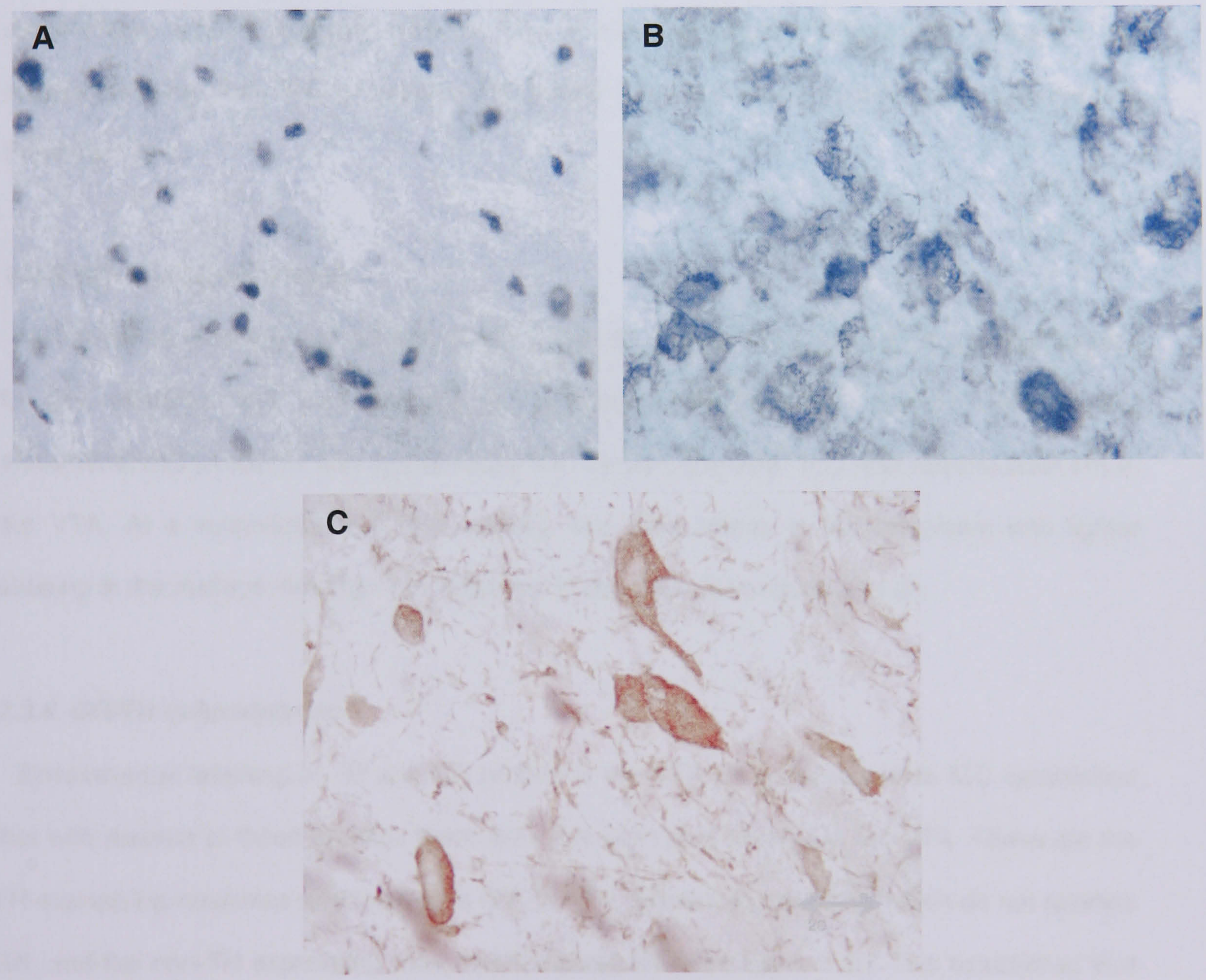

Figure 3.11. Photomicrographs (100x magnification) of (A) GR (HRP labelled Vector SG); (B) MR (HRP labelled Vector SG); (C) TH (HRP labelled Vector Novared).

Labelling for GR was found to be highly dependent on the solubilisation of cell membranes during the ICC procedure. Initially at a variety of primary antibody concentrations no GR signal was found in the VTA or adjacent monoaminergic regions, however a higher concentration of triton-X100 detergent revealed extensive labelling.

Although fluorescence immunocytochemistry successfully labelled GR, HRP immunocytochemistry was much better suited to labelling this protein as it produced a stronger signal and lower background labelling. To improve labelling specificity a number of blocking steps were incorporated into the HRP ICC protocol including the use of bovine serum albumin in the diluent (as for fluorescent ICC), preincubation of the secondary antibody with rat serum (as for fluorescent ICC), incubation of the tissue with serum from the host species in which the secondary antibody was raised, and (strept)avidin/biotin blocking. Furthermore signal amplification was incorporated with the use of HRP conjugated Vector ABC reagent in the tertiary labelling step. This provided much greater resolution of the specific labelling compared to background.

3.3.3. MR immunoreactivity

MR antibody labelling was evident in many cells throughout the midbrain. In a similar fashion to GR labelling, MR was present in all monoaminergic areas present. Due to the aforementioned problems with fluorescence ICC for GR, only HRP ICC was used to label MR in the VTA. At a subcellular level MR staining was most dense in the cytoplasm with lighter staining in the nucleus. No stain was apparent in dendrites (see figure 3.11 B).

3.3.4. GR/TH colocalisation

Simultaneous labelling of TH and GR protein in the VTA using fluorescence ICC established that with respect to these proteins there are three cell types present in the VTA. These are the TH expressing neurones which express GR, the TH expressing neurones which do not express GR, and the non-TH expressing cells which express GR (see figure 3.2.). This established that some dopaminergic cells within the VTA express the glucocorticoid receptor. More

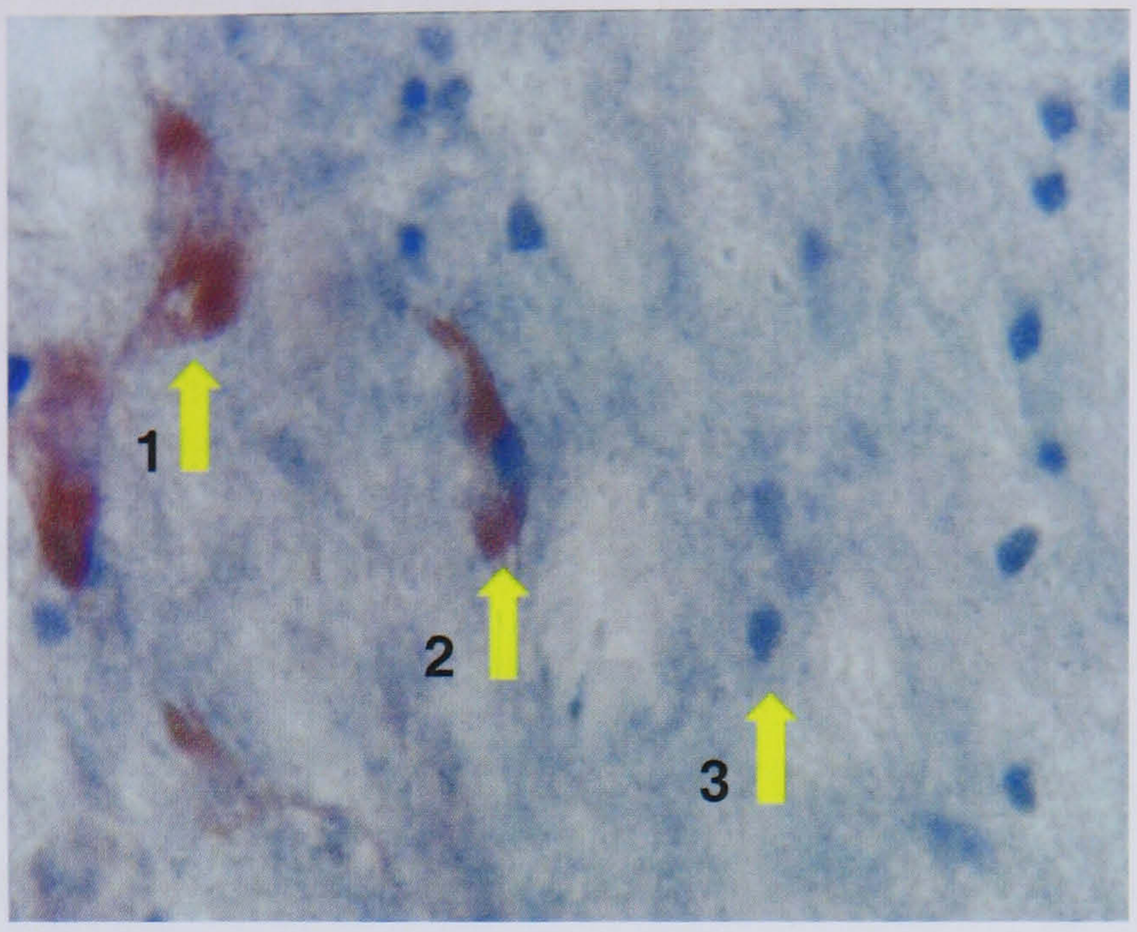

Figure 3.12. Photomicrograph (100x magnification) of GR (HRP labelled Vector SG; blue) and TH (HRP labelled Vector Novared; red/brown) in the VTA. Three types of cell are visible (1) TH + / GR - ; (2) TH + / GR + ; (3) TH - / GR + .

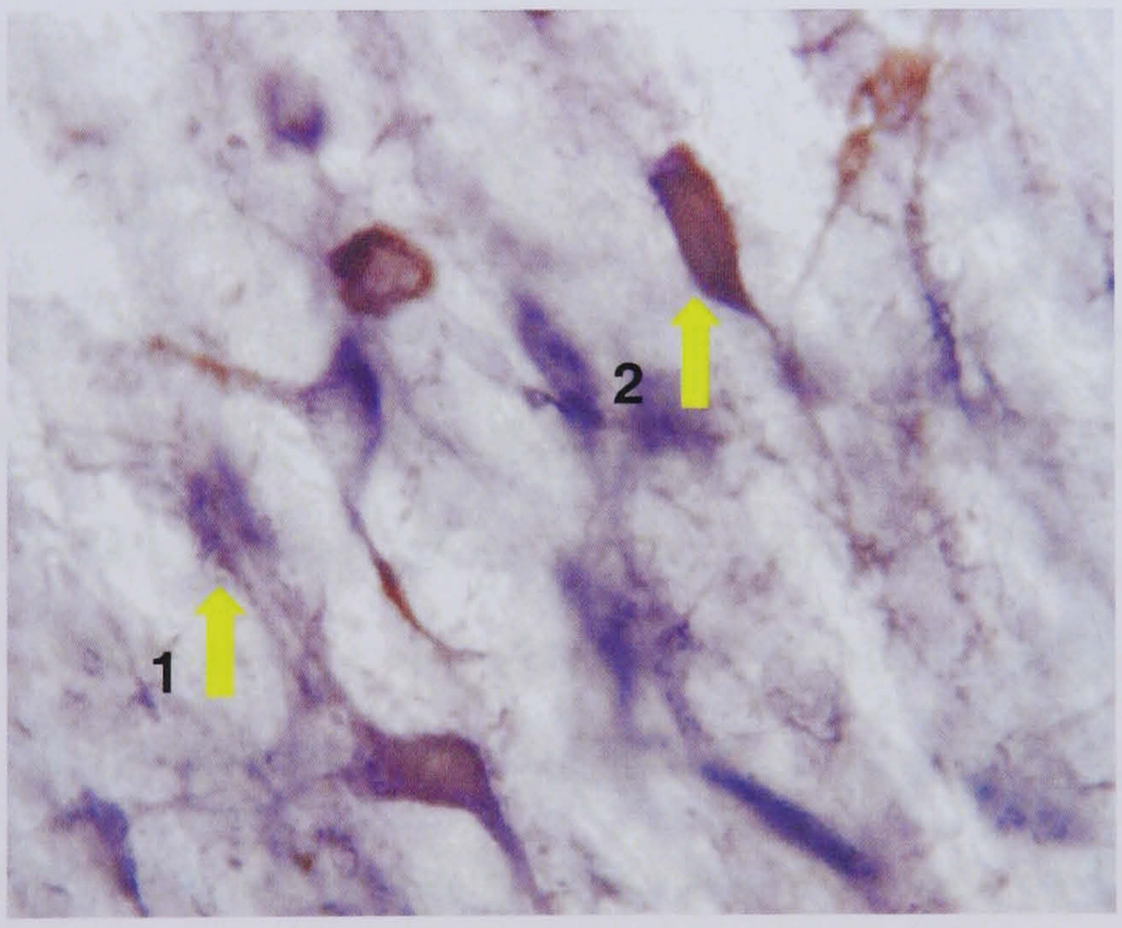

Figure 3.13. Photomicrograph (100x magnification) of MR (HRP labelled Vector SG; blue/purple) and TH (HRP labelled Vector Novared; red/brown) in the VTA. Two types of cell are visible (1) TH - / MR + ; (2) TH + / MR + .

detailed analysis was hampered by the consistently poor signal to background noise ratio of the GR signal. As a result of this it was decided to switch to a more sensitive immunolabelling method allowing for signal amplification steps to be incorporated into the procedure and utilising bright-field microscopy – namely horseradish peroxidase immunocytochemistry.

Initially when dual labelling was attempted for GR and TH using HRP ICC the experiments failed principally because the TH label was too intense. This prevented the visualisation of the GR label (see figure 3.4.). This problem was solved by reducing the TH primary antibody concentration by a factor of 100, and replacing the Vector ABC tertiary reagent used for the TH label with streptavidin conjugated HRP. This reduced the signal amplification, so through a combination of reducing the intensity of TH labelling and amplifying the GR labelling, successful visualisation of both antigens was achieved (see figure 3.12.). The results of the previous fluorescent immunocytochemistry experiments were confirmed, as three cell types were demonstrated, namely TH labelled cells expressing GR, TH labelled cells not expressing GR, and non-TH labelled cells expressing GR.

3.3.5. Analysis of GR/TH colocalisation

For analysis the VTA was divided into anatomical subdivisions according to the rat brain atlas of Paxinos and Watson (1988) (see figure 3.14.). Five representative sections of the rostral-caudal axis were selected for analysis also corresponding to the brain atlas of Paxinos and Watson (plates 39-43). Visible TH positive cells were counted in each sub-region and co-labelling for GR was recorded (see tables 3.1. and 3.2.). χ^2 analysis was performed on the data to establish whether any differences in the percentage of GR labelled TH positive cells existed either along the rostro-caudal axis or between regional subdivisions within the VTA. In both cases no significant differences were demonstrated ($p>0.05$). As a result of this it was concluded that within the VTA approximately 46% of TH expressing cells express GR with no significant regional variations in expression.

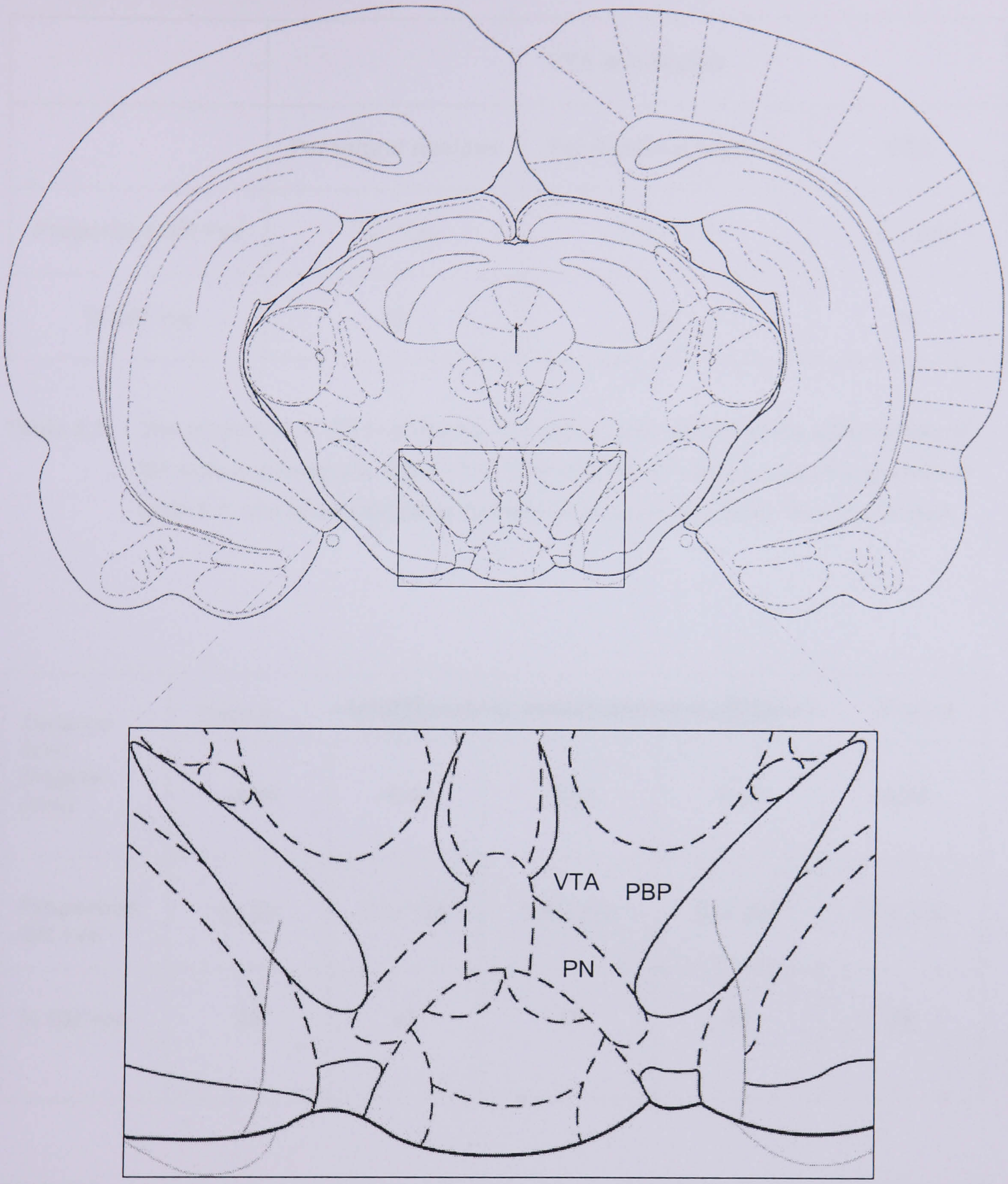

Figure 3.14. Subregions of the VTA as used for analysis of TH/GR colocalisation. PBP = parabrachial nucleus; PN= paranigral nucleus; VTA = ventral tegmental area.

	VTA sub-region		
	Paranigral nucleus	**Parabrachial nucleus**	**VTA**
Proportion GR +ve	70 / 134	176 / 377	110 / 269
% GR +ve	***52***	***47***	***41***

Table 3.1. The proportion of GR expressing TH positive cells relative to the total number of DA cells in subregions of the VTA. Cells counted in sections used for analysis in table 3.1. and totals calculated for each VTA subregion. Data from one animal.

Distance from bregma (mm)	Caudal ⟷ Rostral				
	-6.30	**-6.04**	**-5.80**	**-5.60**	**-5.30**
Proportion GR +ve	9 / 23	74 / 156	105 / 218	98 / 204	70 / 179
% GR +ve	***39***	***47***	***48***	***48***	***39***

Table 3.2. The proportion of GR expressing TH positive cells relative to the total number of DA cells in midbrain sections taken along the rostro-caudal axis of the VTA in one animal.

3.3.6. MR/TH colocalisation

The GR/TH co-labelling protocol was adapted with very little alteration to examine MR/TH colocalisation. Upon examination it was observed that MR was found in all cells expressing tyrosine hydroxylase within the VTA. In addition a large number of non-catecholaminergic cells within the VTA were found to express MR (see figure 3.13.).

3.3.7. Fluorogold retrograde tracing of mesocorticolimbic neurones

The aim of the Fluorogold retrograde tracing was to determine whether projections to the prefrontal cortex and nucleus accumbens originate in distinct regions of the VTA and whether these cells express corticosteroid receptors. The results obtained from these experiments were somewhat disappointing due to a number of problems. Due to the poor signal obtained from fluorescent labelled GR it was not possible to carry out TH and GR fluorescence immunocytochemistry on fluorogold treated rats. When HRP immunocytochemistry was attempted on fluorogold treated brains the labelling procedure resulted in loss of the TH label. Thus it was not possible to establish which terminal region GR containing catecholaminergic cells projected to.

Using single labelling for TH, an attempt was made to simply map where projections to the PFC and NAcc originate in the VTA. Fluorogold injection sites in the PFC and NAcc can be seen in figures 3.15. and 3.16. This method was again hampered by poor reliability of labelling related to tissue quality. In light of these problems the amount of tissue available for analysis was extremely limited. Furthermore the interpretation of the results was hampered by doubts regarding the accuracy of fluorogold deposits in the terminal regions. Deposits of fluorogold occurred down the track of the pipette during implantation. Furthermore, at the injection site fluorogold was observed in adjacent regions to the PFC and NAcc.

When tissue was examined to determine the distribution of cells it was found that cells labelled at their terminals in both the prefrontal cortex and nucleus accumbens were distributed throughout the VTA with no evident pattern of distribution (see figure 3.17. for FG distribution in the VTA following NAcc injection). In the case of the prefrontal cortex manual cell counting found similar numbers of cells in all four quadrants of the VTA (see table 3.3.). In the case of the

nucleus accumbens manual counting was not possible due to the large number of cells however cells were observed distributed throughout the VTA (see figure 3.17.VTA NAcc).

	Lateral VTA	*Medial VTA*	***Total***
Dorsal VTA	62	79	*141*
Ventral VTA	59	64	*123*
Total	*121*	*143*	*264*

Table 3.3. Distribution of fluorogold labelled cells in the VTA following injection into the PFC (data from one animal).

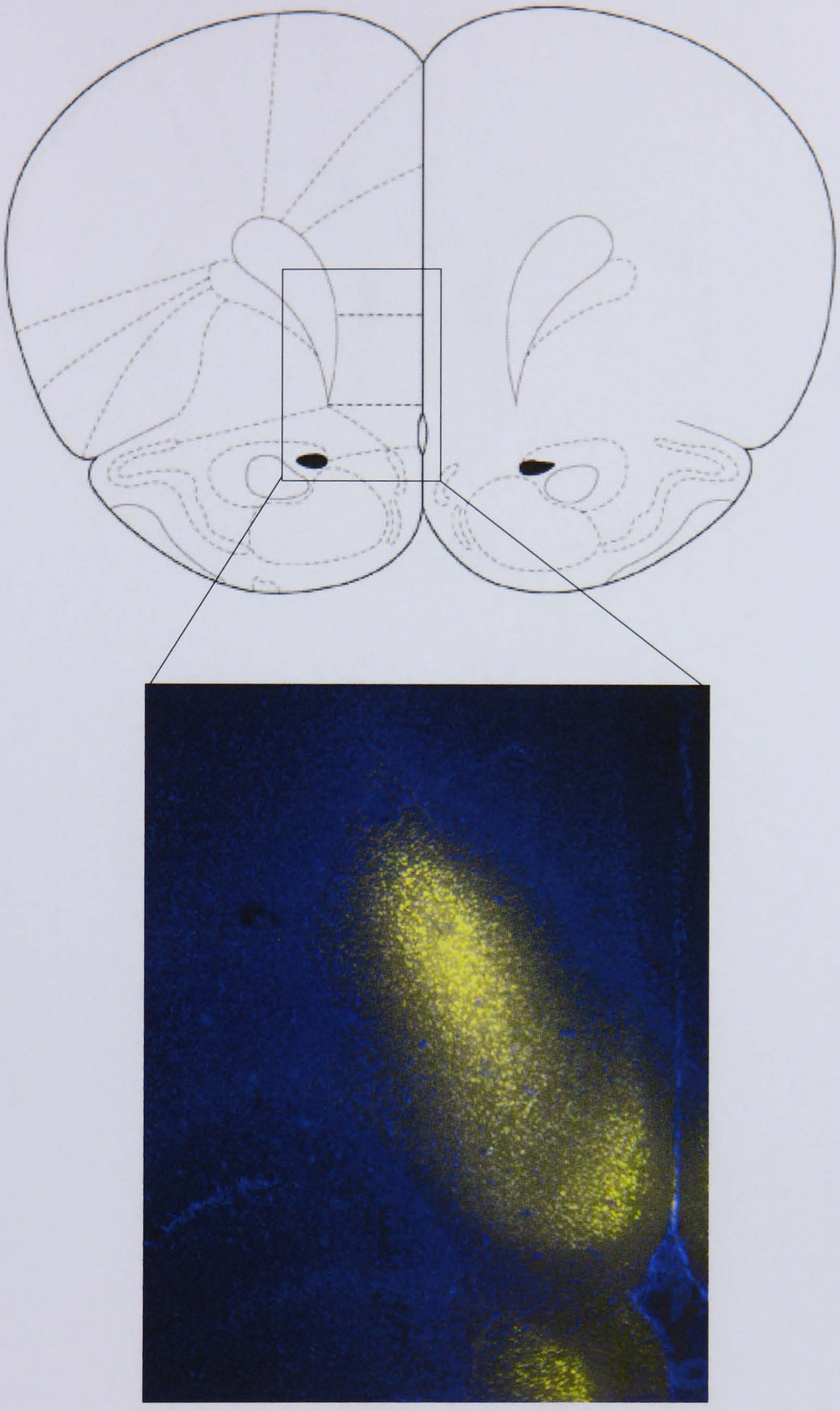

Figure 3.15. Photomicrograph (x10 magnification) of Fluorogold injection site in the mPFC.

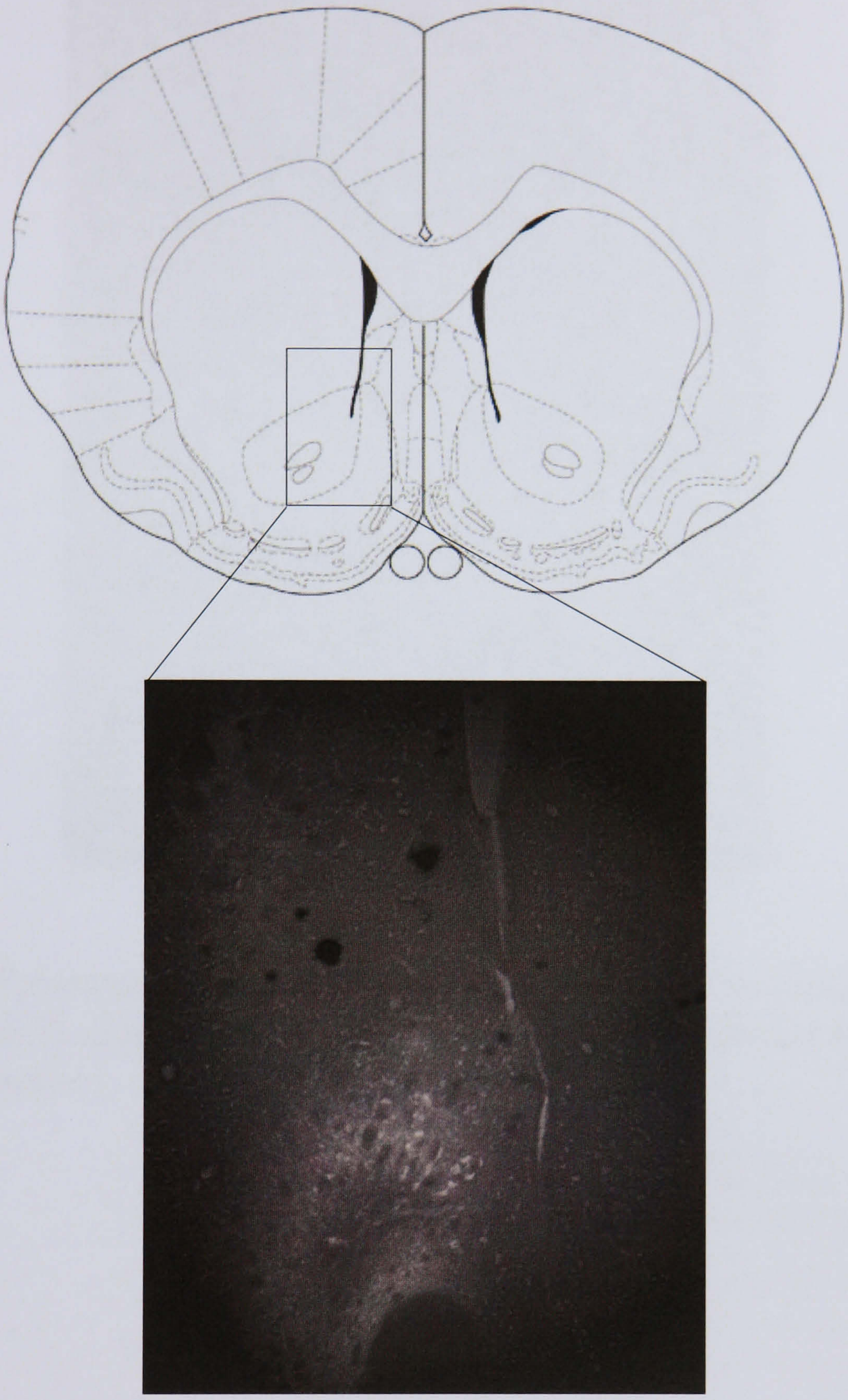

Figure 3.16. Photomicrograph (x10 magnification) of Fluorogold injection site in the NAcc.

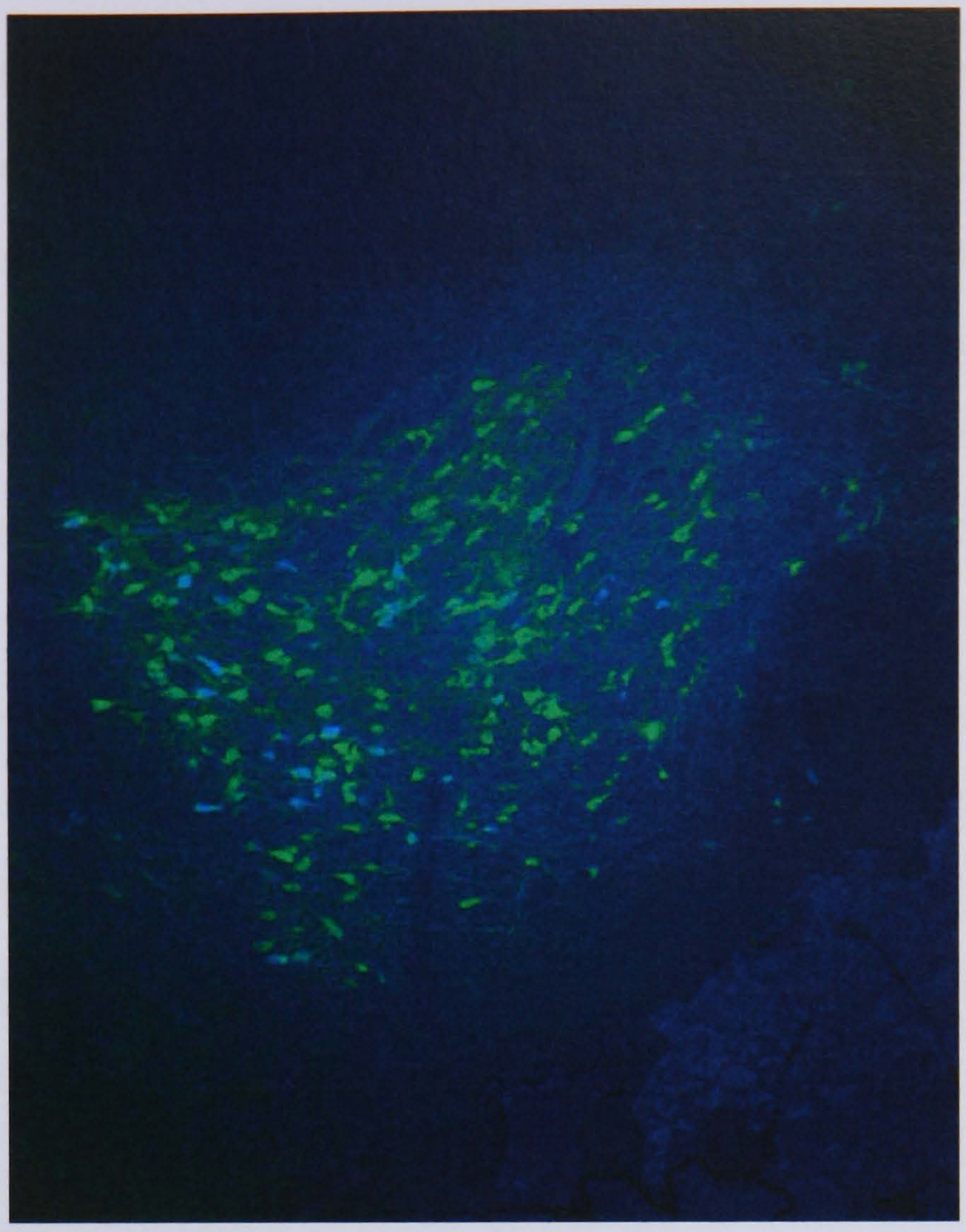

Figure 3.17. Photomicrograph (100x magnification) of cells in the VTA retrogradely labelled with fluorogold from the nucleus accumbens. Blue = FG; Green = TH (FITC labelled)

3.4. Discussion

The aims for this study were twofold: (i) to determine if corticosteroid receptors are present in catecholaminergic neurones of the VTA and if there is any pattern in the distribution of these corticosteroid receptor expressing cells, and (ii) to determine if, where a pattern of distribution is evident, whether this correlates with the projection targets of the cells in question. The results of the study demonstrated that both mineralocorticoid receptors and glucocorticoid receptors are present in cells expressing TH in the VTA. In the case of the glucocorticoid receptor, 46% of TH expressing cells co-expressed this receptor whilst all TH positive cells expressed MR. No significant pattern was seen in the distribution of the GR positive cells. It was not possible to carry out retrograde labelling in conjunction with immunocytochemistry for both TH and GR. However the lack of a discernible pattern in the GR/TH co-labelling and the fact that all TH positive cells expressed MR indicated that mesocortical and mesolimbic neurones are unlikely to differ in their expression of corticosteroid receptors.

3.4.1. Tyrosine hydroxylase expression in the midbrain

Tyrosine hydroxylase is the rate limiting enzyme in catecholamine synthesis (Nagatsu et al. 1964) and is present in catecholaminergic neurones in the CNS. Here an antibody raised against TH was used to label dopaminergic cells in the midbrain at the level of the VTA. TH immunoreactivity was seen in the A8 (retrorubral nucleus), A9 (substantia nigra) and A10 (ventral tegmental area, nucleus fasicularis, nucleus linearis) cell groups (see Dahlstrom and Fuxe, 1964; Paxinos and Watson, 1986). This is consistent with known dopaminergic regions of the midbrain. Furthermore some TH immunoreactivity was observed in the predominantly serotonergic medial and dorsal raphe nuclei. This is in agreement with reports of dopaminergic cell bodies in this region (Trulson et al. 1985; Decavel et al. 1987; Stratford and Wirtschafter, 1990; Datiche and Cattarelli, 1996; Hasue and Shammah-Lagnado, 2002). No TH immunoreactivity was seen outside of the aforementioned regions. For the purposes of this study all tyrosine hydroxylase expressing neurones in the VTA were considered to be dopaminergic. Evidence to support this is provided by both fluorescence histochemistry (Dahlstrom and Fuxe, 1964) and immunocytochemistry experiments demonstrating that

neurones in the VTA contain dopamine (Onteniente et al. 1984; Decavel et al. 1987; Hasue and Shammah-Lagnado, 2002) (dopamine is not present in detectable amounts in noradrenergic fibres, see Kitahama et al. 2000). In addition immunoreactivity against dopamine-β-hydroxylase (the enzyme responsible for the synthesis of noradrenaline from dopamine) is reportedly absent from the VTA (Swanson and Hartman, 1975).

At the subcellular level TH immunoreactivity was present in the cytosolic compartment with the highest density proximal to the cell membrane. Immunoreactivity was seen additionally in dendrites, but was not present in cell nuclei. Similar patterns of labelling have been observed in other immunocytochemical studies (see Harfstrand et al. 1986; Ford et al. 2006). This is in agreement with reports that TH exists in both cytoplasmic and membrane-bound forms (Kuczenski et al. 1972).

3.4.2. GR and MR expression in the midbrain

Widespread GR immunoreactivity has previously been reported in the brain and has been particularly well characterised in regions such as the hippocampus, cortex, hypothalamus, thalamus septum and midbrain (Ahima and Harlan, 1990; Cintra et al. 1994; Fuxe et al. 1985, 1987; Morimoto et al. 1996). The current study demonstrated that a large number of cells throughout the midbrain express GR including the dopaminergic cell groups of the VTA (and associated midline A10 nuclei) and substantia nigra, as well as in rostral portions of the serotonergic dorsal and medial raphe nuclei. Previous reports have shown similar patterns of immunoreactivity in these monoaminergic regions (Ahima and Harlan 1990; Czyrak and Chocyk, 2001; Harfstrand et al. 1986; Morimoto et al. 1996).

MR immunoreactivity in the CNS is less well characterised outside of specific regions such as the hippocampus and hypothalamus (Van Eekelen and De Kloet 1992; Agarwal et al. 1993; Ito et al. 2000; Han et al. 2005). Although one study reported that MR immunoreactivity is absent from the midbrain (Agarwal et al. 1993), an earlier study had found "low to moderate" levels of MR immunoreactivity in the substantia nigra and VTA and high levels in the dorsal raphe nuclei (Ahima et al. 1991). MR expression in the dorsal raphe nucleus has also been demonstrated by Gartside et al. (submitted, 2007). The present study supports these findings having

demonstrated MR immunoreactivity in a number of regions throughout the midbrain including the VTA, substantia nigra, and rostral portions of the dorsal and median raphe nuclei.

Subcellular distribution of labelled corticosteroid receptors

As discussed in chapter one, both MR and GR translocate to the nucleus upon binding of ligand. Given that under basal (non-stressful) conditions it has been proposed that the MR receptor is close to maximally occupied whilst the GR is mostly unbound (Reul and de Kloet, 1985) one might expect MR to be present in the nucleus, with GR predominantly in the cytoplasm. The results of this study in fact showed the reverse, with GR predominantly located in cell nuclei with MR present in both nuclei and cytoplasm.

A number of immunocytochemical studies have shown that GR is present in cell nuclei and in the majority of these studies GR was predominantly found in the nucleus compared to the cytoplasm (Gasc et al. 1989, Sanchez et al. 1990, Brink et al. 1992). Furthermore the study by Harfstrand et al. (1986) looking at GR expression in VTA catecholaminergic neurones showed a nuclear localisation of GR. It has been suggested that nuclear translocation of GR in immunocytochemical studies is in fact an artefact of the fixation and membrane solubilisation process which is carried out to allow antibodies access to GR (Nishi et al. 1999). Studies carried out in living cells using GFP conjugated glucocorticoid receptor have shown that GR is predominantly located in the cytoplasm until application of ligand (Nishi et al. 1999). An important factor to take into consideration is that in tissue taken from animals, stress and/or anaesthesia at the time of sacrifice may cause an elevation of corticosteroid levels which induces the translocation of GR i.e. the nuclear GR is in fact ligand bound.

Ito et al. (2000), who developed the MR antibody used in this study, have shown that MR is present in both the nucleus and cytoplasm of fixed rat hippocampal cells, however incubation with an MR agonist induces nuclear translocation. This result has also been shown in live cultured cells (Fejes-Toth et al. 1998) and in experiments utilising GFP-conjugated MR in COS-1 and cultured hippocampal cells (Nishi et al. 2001). The data of Ito et al. (2000) suggest that MR is not maximally occupied under basal conditions given it's presence in the cytoplasm and the ability of ligand to induce translocation. This is a possible interpretation of the results

obtained in the present study, as MR labelling was most dense in the cytoplasm with a much lower density in the nucleus. Another possibility is that the observed cytoplasmic MR is an artefact of the ICC method and that leakage of protein out of the nucleus occurred following the membrane solubilisation step. Poor compartmentalisation of the stain used is unlikely to account for labelling in the cytoplasm as the same method was used to label MR as was used to label GR. GR immunoreactivity was confined to the nucleus suggesting that the stain is localised to immunolabelled proteins and does not leach to adjacent subcellular regions.

In summary the nuclear compartmentalisation of GR was consistent with a stress induced activation of the receptor at the time of sacrifice. If this is the case, however, one would have expected the high affinity MR to be similarly translocated to the nucleus. Whilst the relative distributions of MR and GR between the nucleus and cytoplasm in the present study are consistent with similar studies in the literature, it is difficult to account for the presence of MR in the cytoplasm

3.4.3. Corticosteroid receptor expression in dopaminergic cells of the VTA

The results of this study clearly demonstrate that catecholaminergic cells in the VTA express corticosteroid receptors. In the case of the mineralocorticoid receptor this is an entirely novel finding, whilst with respect to the glucocorticoid receptor, this clarifies the confusion in the literature which arose from two contradicting papers. The earliest of these (Harfstrand et al. 1986) demonstrated that around 61% of dopaminergic cells in the VTA express the glucocorticoid receptor. A later study conducted in 2001 (Czyrak and Chocyk) reported that whilst glucocorticoid receptor was present in the VTA, it was not co-localised with TH. The results of the present study support the data published by Harfstrand et al. and offer further evidence that the glucocorticoid receptor is indeed present in catecholaminergic cells in the VTA.

In the present study GR immunoreactivity in the VTA was consistently demonstrated using both fluorescence and HRP single-label immunocytochemistry. Results were consistent between the two methods from the subcellular level right up to the distribution within the VTA, and this consistency was maintained in dual-label experiments. It is likely that there is a

methodological explanation for the failure of Czyrak and Chocyk (2001) to demonstrate the colocalisation of GR and TH in the VTA. This may result from the lack of a membrane solubilisation step in their ICC protocol. In the present study an unusually high concentration of Triton x-100 was required for successful staining of GR in the VTA, whilst it appears that no tissue solubilisation step was used in Czyrak and Chocyk's work. Although Harfstrand et al. (1986) doesn't clearly state the methodology used, they based their double staining ICC protocol on one from a group who routinely used Triton x-100 in their immunocytochemical procedures (see Oertel et al. 1982).

It is worth noting that whilst the study of Harfstrand et al. (1986) showed similar results to the present study, a slightly higher level of GR/TH co-localisation was found. In the Harfstrand study the entire A10 cell group was included in the analysis, and it is noted in the paper that the nucleus interfasicularis (IF) and nucleus linearis caudalis (CLi) showed particularly high TH/GR co-immunoreactivity. As the IF and CLi are distinct from the VTA, they were not included in the present study and this may account for the lower mean level of co-expression described here.

The presence of GR in only around half of the dopaminergic neurones in the VTA is interesting as it contrasts with GR expression in other monoaminergic cell groups. GR has been shown in all serotonergic raphe nucleus neurones (Harfstrand et al. 1986; Gartside et al. submitted, 2007) and in the vast majority of noradrenergic neurones in the locus coeruleus and nucleus tractus solitarius (Harfstrand et al. 1986; Czyrak and Chocyk, 2001). The presence of two populations of dopaminergic cells within the VTA differentiated by their expression of GR may reflect different projection targets for these neurones. Although no significant difference was found in the proportion of GR expressing dopaminergic cells across subregions of the VTA, it remains a possibility that GR expression differs between, for example mesolimbic and mesocortical dopamine neurones.

The demonstration of MR in all dopaminergic neurones suggests that all dopaminergic cells in the mesolimbic and mesocortical systems will be sensitive to MR regulation and moreover sensitivity to MR mediated regulation does not differ between projection targets.

3.4.4. Corticosteroid receptors in non-dopaminergic cells of the VTA

In the VTA large numbers of both glucocorticoid and mineralocorticoid receptors were found in cells not expressing tyrosine hydroxylase. The phenotype of these cells is unknown as no studies have as yet been carried out. It is known that the VTA contains both GABAergic (Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000; Bubar and Cunningham, 2007) and glutamatergic cells (Kawano et al. 2006; Yamaguchi et al. 2007) and it is possible that these cells express corticosteroid receptors. Additionally glial cells in other regions of the brain are known to express glucocorticoid receptors (Vielkind et al. 1990; Hwang et al. 2006), and this may also be the case in the VTA.

3.4.5. Retrograde tracing of mesocorticolimbic neurones

The results of the retrograde tracing experiments were somewhat disappointing. It had originally been envisaged that a combination of fluorescence immunocytochemistry and fluorogold retrograde tracing might be used to determine where individual GR/MR containing catecholaminergic cells project to. The poor results with fluorescent labelling of GR meant that this was not possible. When HRP immunocytochemistry was attempted with fluorogold tracing the TH stain in the VTA was lost. The reason for this is unknown, - although previous studies have successfully produced HRP reaction products from DAB in FG labelled neurones (Lee et al. 2005a; Lee et al. 2005b), other studies have failed to combine FG and HRP retrograde labelling (Shinder et al. 2001). It should be noted that the successful combination of HRP and FG labelling as carried out by Lee et al. (2005a; 2005b) was with an HRP retrograde tracer and not an HRP-conjugated immunolabel. There is the possibility that a specific incompatibility exists between HRP-ICC and FG retrograde tracing.

As single-label fluorescence immunocytochemistry could be successfully combined with FG labelling it was decided to try and map the distribution of catecholaminergic cells projecting to the medial prefrontal cortex and the nucleus accumbens, the objective being to correlate this with the distribution of GR-TH and MR-TH co-labelled cells. There is some evidence to suggest that in primates, prefrontocortical neurones originate mainly in the dorsal tier of the VTA, with the ventral PFC mapping to the dorsomedial VTA and the dorsal PFC mapping to the

dorsolateral VTA (Williams and Goldman-Rakic, 1998). Furthermore it has been shown that dopaminergic projections to the piriform cortex a preferentially located in the dorsal VTA (Datiche and Cattarelli, 1996). In contrast there is some evidence that neurones projecting to the nucleus accumbens originate in the ventral tier of the VTA along the whole medio-lateral axis (Fallon and Moore, 1978; Albanese and Minciacchi, 1983; Brog et al. 1993). It should be noted however that these studies did not show a ventral distribution of NAcc projections in all cases, my own interpretation of the data is that more localised injections of tracer in the nucleus accumbens led to better topographical resolution in the VTA. The results of the present study failed to find evidence for a distinct topography, with cells retrogradely labelled in the PFC and NAcc found throughout the VTA. A similar overlap in PFC and NAcc projections at the level of the VTA has been shown in previous studies (Carr and Sesack, 2000; Margolis et al. 2006).

Whilst it is possible that the two projections do not arise in different regions of the VTA some methodological considerations must be taken into account when considering the present results. Inspection of tissue sections taken at the site of injection revealed some leaching to neighbouring tissue – a fact which could be significant given the proximity of the ventromedial prefrontal cortex and the nucleus accumbens. In addition, in reaching these ventral injection sites some fluorogold was found to be deposited along the pipette track through the overlying tissue. Inaccuracies in the fluorogold injection may therefore explain the lack of topographical differences.

The principal aim of the retrograde tracing experiments was to determine whether mesocortical and mesolimbic projection neurones differ in their expression of corticosteroid receptors. As far as the mineralocorticoid receptor is concerned all dopaminergic neurones appear to express this receptor and so both projection systems are likely to be equally sensitive to MR mediated signalling. As no significant subregional distribution of GR/TH colocalisation was found, and it was not possible to look at individual neurones, it is unclear whether mesocortical and mesolimbic projection neurones differ in their GR expression.

3.4.6. Functional implications of GR and MR expression in dopaminergic cells

The presence of corticosteroid receptors in dopaminergic neurones allows for direct modulation of dopaminergic function by elevated gluococorticoid levels. This in turn provides evidence for a direct link between endocrine abnormalities in bipolar disorder and dopaminergic functional alterations which are characteristic of this disease. The functional consequences of elevated corticosteroid levels on dopaminergic cells are investigated and discussed in the following chapters.

The finding that MR is expressed in all dopaminergic neurones suggests that circulating corticosteroids perform a homeostatic role in regulating neuronal function. The high occupancy of these receptors by physiologically normal levels of circulating corticosteroids (Reul and de Kloet, 1985) suggests that MR mediated effects are significant when corticosteroid levels fall. Indeed adrenalectomy has been shown to markedly reduce dopaminergic neurotransmission in the nucleus accumbens (Piazza et al. 1996; Shoaib and Shippenberg, 1996; Barrot et al. 2000) and the prefrontal cortex (Mizoguchi et al. 2004), and this effect may be attributable to removal of tonic MR activity.

Based on the prevailing view that the MR is close to maximally occupied under basal conditions (as first proposed by Reul and de Kloet, 1985), disturbances to the corticosteroid rhythm which elevate circulating hormone levels would be expected to cause only minimal changes in the occupancy of MR. It has been suggested however that Reul and de Kloet overestimated the degree of MR binding particularly during the circadian nadir (Pace and Spencer, 2007). Consequently both MR and GR may be involved in the pathological consequences of elevated corticosteroid levels. Furthermore, the ability of GR and MR to form heterodimers demonstrated by Trapp et al. (1994) means that the presence of MR could be functionally important. MR/GR heterodimers can act both synergistically (Trapp et al.1994) and antagonistically (Liu et al. 1995) with GR homodimer mediated transcriptional activity in cultured cells. MR/GR heterodimers have been shown to be more efficient than homodimeric receptors at mediating the corticosteroid induced transcriptional repression of the 5-HT_{1A} gene in cultured cells (Ou et al. 2001).

Recent studies have demonstrated that changes in ligand concentration affect heterodimer formation with the degree of heterodimer formation in cultured cells increasing in response to elevated corticosteroid concentrations (Nishi et al. 2007). Consequently, corticosteroid dysrhythmia may alter the nature of corticosteroid signalling by promoting the formation of heterodimers during the elevated diurnal nadir. It may therefore be the case that these heterodimers play an important role in the pathological consequences of disturbances to circadian rhythmicity.

The finding that GR was present in approximately half of the dopaminergic cells in the VTA may have important functional consequences. As discussed above it is likely that only those cells expressing GR receptor will be sensitive to elevated circulating corticosteroid levels. Given that all GR positive cells were also MR positive, these effects could be mediated by both GR homodimers and GR/MR heterodimers but it is the presence of GR that is critical. There remains the possibility that GR expression differs between the mesolimbic or mesocortical projection systems, and as a consequence elevated corticosteroid levels could affect these systems to different degrees. This could lead to a functional "imbalance" between the two systems following disturbances to the corticosteroid rhythm. It is also possible that in both projection systems there are dopamine neurones which express GR and those that do not. This would serve to somewhat "dilute" corticosteroid effects in each system and as such could be a means of maintaining a level of dopaminergic tone in the terminal regions in spite of elevated corticosteroid levels. It is also possible that sub-regions of either the prefrontal cortex or nucleus accumbens receive specific inputs from GR expressing dopaminergic cells, however no data is available to support this theory.

3.4.7. Functional implications of GR and MR expression in non-dopaminergic cells

In addition to the presence of GR and MR in dopaminergic cells, large numbers of non-dopaminergic cells in the VTA were found to express these receptors. These may be GABAergic or glutamatergic neurones or glial cells, functional alterations of which could lead to secondary changes in dopaminergic function. As GABA and glutamate are important regulators of dopaminergic neuronal function in the VTA and glia are responsible for the uptake of a variety

of amino acid transmitters (Ganong, 2001) any changes in the function of these cells will have implications for dopaminergic neurotransmission.

As yet no one has shown whether non-dopaminergic neurones in the VTA are corticosteroid sensitive, however glial cell function has been shown to be altered by corticosteroids. In particular, glial glutamine synthetase (which is responsible for the catabolism of glutamate) is inducible by corticosteroids (reviewed by Vardimon et al. 1999). Furthermore glial glutamate transporter expression is regulated by corticosteroids, though no evidence for this has been found in the midbrain (Zschocke et al. 2005). Changes to glutamate availability would have consequences for dopaminergic transmission as DA neurones are tonically regulated by glutamate which promotes neuronal firing. Glia are also involved in glucose transport, and it has been shown that hippocampal astrocytic glucose transport is decreased by corticosteroids (Virgin et al. 1991). If this were to occur in the VTA dopaminergic function may well be altered due to changes in energy availability. Thus, there are a variety of mechanisms by which changes in glial function could affect dopaminergic neurotransmission. A link between glucocorticoid induced glial abnormalities, altered dopaminergic function and mood disorders is supported by data showing changes in glial function in a variety of psychiatric disorders including bipolar disorder (reviewed by Cotter et al. 2001). Overall these factors could be as important as direct corticosteroid effects in the regulation of dopaminergic neurotransmission.

3.5. Conclusion

The data presented in this chapter demonstrate that a variety of cells within the VTA contain corticosteroid receptors. Dysregulation of the circadian corticosteroid rhythm could therefore produce changes in VTA dopaminergic function via direct and indirect effects on dopaminergic cells. The presence of MR in VTA DAergic cells is a novel finding, whilst the clear demonstration of GR in these cells offers some clarification of the confusion which existed in the literature. Overall the findings offer a firm basis on which a hypothesis integrating endocrine and dopaminergic abnormalities observed in bipolar disorder can be constructed, a hypothesis which is examined in the rest of this thesis.

Chapter 4.

Effect of corticosterone administration on mRNA levels in the VTA.

Chapter 4. Effect of corticosterone administration on mRNA levels in the VTA

4.1. Introduction

As discussed in chapter 1, glucocorticoid receptors act as regulators of DNA transcription to produce alterations in cell function. When corticosteroids bind to GR/MR receptors chaperone heat shock proteins dissociate and the DNA binding site is exposed. Following hyperphosphorylation of the receptor protein it forms a dimer and translocates to the nucleus (Buckingham 2006; Schoneveld et al. 2004). Corticosteroid effects on a particular gene may be through direct transcriptional control (which can be positive or negative), or via transcriptional regulation of genes for transcription factors and co-activators which in turn regulate the expression of the gene in question. Furthermore the presence or absence of essential cofactors means that corticosteroid effects on a given gene can be tissue specific, varying between different cell types (for reviews see Buckingham 2006; Schoneveld et al. 2004).

Given the presence of GR and MR receptors in dopaminergic cells of the VTA as shown in Chapter 3, the ability of corticosteroids to modulate dopaminergic function (see Chapter 1), and the corticosteroid dysrhythmia in bipolar disorder, the question arises – do alterations in corticosteroid secretion affect dopaminergic transmission by modulating gene transcription within the VTA and could such changes underlie the symptoms of bipolar disorder?

A number of proteins intrinsic to dopaminergic neurones could be considered to be candidates for modulation by corticosteroids. These proteins, which include enzymes, neurotransmitter transporters and neurotransmitter receptors are described in more detail below.

<u>Tyrosine Hydroxylase</u>

- The rate limiting enzyme in dopamine synthesis (Nagatsu et al. 1964) and therefore a critical determinant of the amount of dopamine available for uptake into synaptic vesicles.

Vesicular Monoamine Transporter 2 (VMAT2)

- Responsible for the uptake of cytoplasmic dopamine into synaptic vesicles in preparation for release (Peter et al. 1995; Nirenberg et al. 1996; Hoffmann et al. 1998). VMAT2 activity determines vesicular content (Colliver et al. 2000), which in turn governs the quantal size of dopamine release following depolarisation (Pothos et al. 2000).

Dopamine Transporter (DAT)

- Following neurotransmitter release the dopamine transporter is responsible for uptake of dopamine from perisynaptic sites (see reviews by Hoffman et al. 1998; Chen & Reith 2000). Alterations in the function of this protein affect synaptic concentrations of dopamine and the time course of removal of dopamine from the synapse, as well as the amount of diffusion from the synapse to extrasynaptic sites (Cragg & Rice 2004).

Monoamine Oxidase A (MAO-A)

- Responsible for the oxidative metabolism of dopamine within dopaminergic terminals and the principle metabolic pathway for dopamine where efficient dopamine re-uptake occurs (Gesi et al. 2001; Shih 2004). More importantly MAO-A activity is a determinant of cytoplasmic dopamine content and is therefore likely to affect dopamine availability for uptake into synaptic vesicles.

D_2 Receptor

- The D_2 receptor is the inhibitory autoreceptor on dopamine neurones. It is present at somatodendritic sites where it inhibits impulse activity and at the terminal where it modulates synthesis and release of dopamine (O'Hara et al. 1996; White 1996; Centonze et al. 2002; Lindgren et al. 2003) and as such is a key determinant of dopaminergic neuronal activity.

NMDAR1

- NMDA receptors mediate excitatory input to dopaminergic neurones in the midbrain and control the switch from tonic pacemaker to phasic burst firing (Charlety et al. 1991; Chergui et al. 1993). Burst firing results in greater dopamine release, on average, per depolarisation event than the tonic pacemaker mode (Johnson et al. 1992; Chergui et al. 1994; Meltzer et al. 1997; Gonon, 1998; Adell and Artigas, 2004).
- The NMDA receptor subunit 1 (NMDAR1) is the essential subunit in NMDA receptor formation as it contains both the glutamate and glycine binding sites. Functional NMDA receptors are tetramers consisting of two NMDAR1 subunits with either two NMDAR2 subunits or an NMDAR2 and an NMDAR3 subunit (for review see Kew and Kemp, 2005).

GluR1

- AMPA receptors are excitatory on mesolimbic dopaminergic neurones, producing an increase in firing rate via increased intracellular Na^+ (Meltzer et al. 1997; Adell and Artigas, 2004). Furthermore there is evidence that mesocortical dopaminergic neurones are under tonic excitatory stimulation by this receptor subtype (Westerink et al. 1998; Takahat and Moghaddam, 2000).
- Glutamate receptor subunit 1 (GluR1) is one of four subunits which can form the tetrameric AMPA receptor. Wild-type receptors are thought to be heteromeric and the GluR1 subunit is thought to favour the formation of a Ca^{2+} permeable receptor channel (for reviews see Carlezon and Nestler, 2002; Kew and Kemp, 2005)

5-HT_{2C} Receptor

- The 5-HT_{2C} receptor is an excitatory serotonin receptor with a signal transduction pathway producing IP_3/DAG as a second messenger (Rang et al. 1999). It has been shown that serotonin potentiates the dopamine induced inhibition of VTA neurones via 5-HT2 receptors (Brodie and Bunney, 1996). Further work with selective antagonists has shown a tonic inhibition of mesocorticolimbic dopaminergic neurotransmission via

5-HT_{2C} receptors (DiMatteo et al. 1999; DiMatteo et al. 2002; Gobert et al. 2000). These receptors are found on both dopaminergic neurones and GABAergic interneurones (Bubar and Cunningham, 2007), and it would appear that the inhibitory effect of 5-HT_{2C} agonists arises from a stimulation of GABAergic signalling which inhibits dopamine nerve cell firing.

In this chapter the abnormal diurnal corticosterone rhythm seen in bipolar disorder was modelled by administering a low dose of corticosterone to male hooded Lister rats in their drinking water for two weeks. To examine the effect of this on gene transcription in the VTA *in situ* hybridisation histochemistry was performed on brain slices containing the VTA using a number of probes complementary to mRNA coding for the proteins discussed above.

4.1.1. Subregions of the VTA

A number of retrograde tracing experiments have provided evidence that dopaminergic neurones projecting to different brain structures may arise in distinct subregions of the VTA. Thus there is evidence that in primates, mesocortical prefrontocortical efferents arise in the dorsal VTA (Williams and Goldman-Rakic, 1998). Similarly in the rat, tracing studies have shown that mesocortical dopaminergic innervation of the piriform cortex arises in dorsal regions of the VTA (Datiche and Cattarelli, 1996). In terms of mesolimbic efferents to the nucleus accumbens there is some data to suggest that these arise in the ventral portion of the VTA (Fallon and Moore, 1978; Albanese and Minciacchi, 1983; Brog et al. 1983), however the data is unclear. In chapter 3 retrograde tracing with fluorogold did not show a distinct topography of prefrontal cortex and nucleus accumbens projections, however methodological problems may account for this result.

For the analysis of the present data the VTA was divided into four regions corresponding to those shown in figure 4.1. Thus it was hoped to relate any subregional differences in corticosteroid effects on mRNA transcription in the VTA to potential functional consequences in mesocortical and mesolimbic neurones projecting to the prefrontal cortex and the nucleus accumbens respectively.

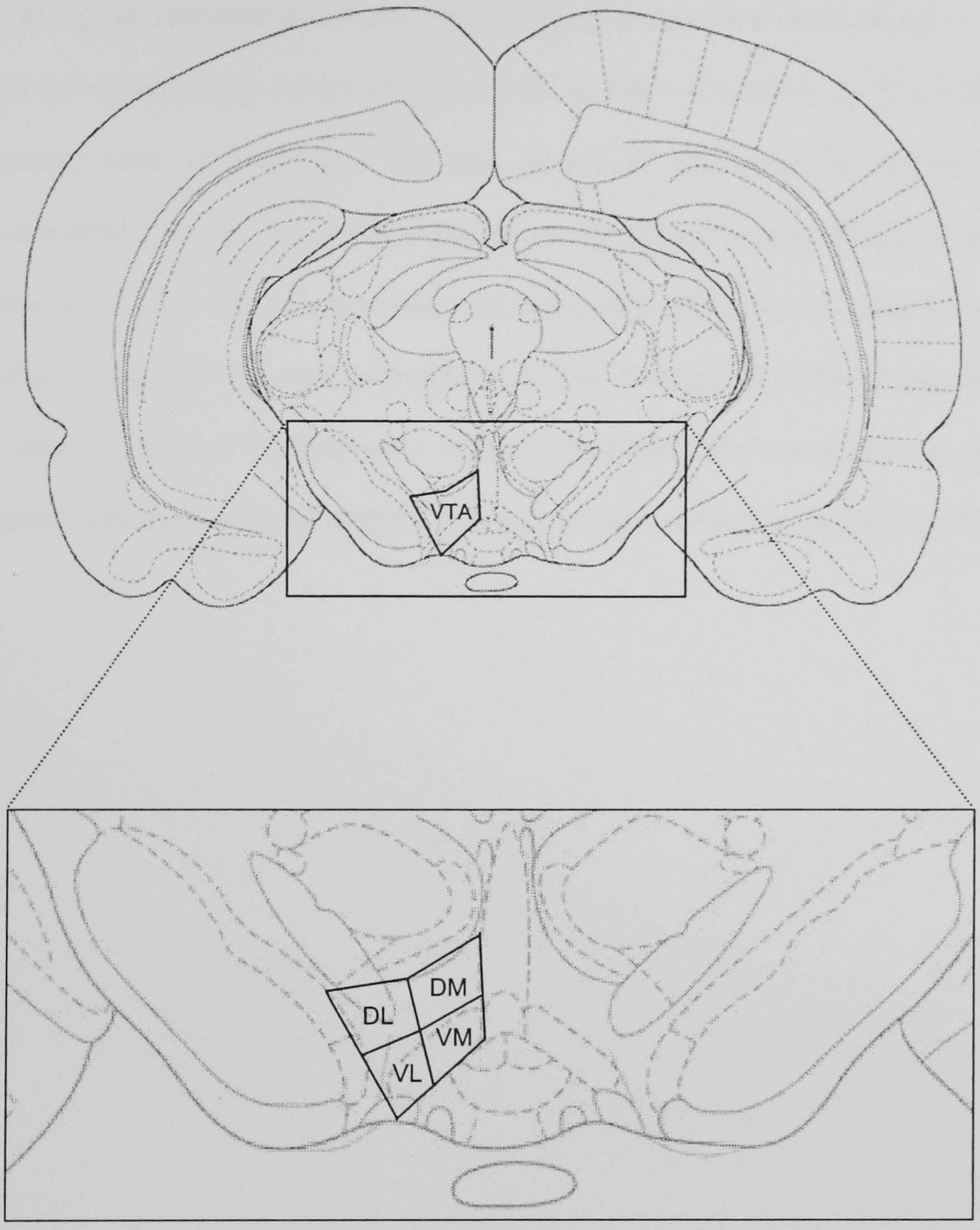

Figure 4.1. Subdivisions of the VTA used for *in situ* hybridisation histochemistry data analysis (figures from Paxinos and Watson, 1996). DL= dorsolateral; DM = dorsomedial; VL = ventrolateral; VM = ventromedial.

4.1.2. Aims

The principal hypothesis of this thesis is that the abnormalities in the diurnal corticosteroid rhythm seen in bipolar disorder modulate central dopaminergic neurotransmission, and that this is responsible for some of the symptoms observed in these disorders. In this chapter the aim was to examine how an artificially induced abnormal corticosteroid rhythm affects the expression of mRNAs coding for protein determinants of presynaptic dopaminergic neuronal activity. To this end in-situ hybridisation histochemistry was employed to compare mRNA levels in the VTA of corticosteroid and vehicle treated animals. Analysis of mRNA levels in subregions of the VTA was carried out to determine whether flattening the corticosteroid rhythm might produce different effects in subpopulations of dopaminergic neurones projecting from this brain region.

4.2. Methods

4.2.1. Animals & corticosterone Treatment

The two-week corticosteroid treatment protocol has been described in Chapter 2. Briefly, male Lister hooded rats (Charles River, UK) housed in group of four were treated for 15 days with drinking water supplemented with corticosterone (50mg/l) and 0.5% EtOH (n=8). Control animals received drinking water containing 0.5% EtOH over the same period (n=8). Animal weights and water consumption were recorded during this period. In addition, upon sacrifice, adrenal glands were removed for comparison.

Note: All animals were injected with NSD1015 (100 mg/kg i.p.) 20 minutes prior to sacrifice. This was so forebrain tissue could be assayed for tyrosine hydroxylase activity (see chapter 5).

4.2.2. Tissue collection

After sacrifice by overdose of sodium pentobarbital (0.7 ml/kg i.p., 20% w/v solution, Dolethal, Vetoquinol UK), the brain was removed and the portion containing the midbrain was snap frozen in isopentane on dry ice. Tissue was stored at -80°C prior to cutting and in-situ hybridisation treatment (see Chapter 2 for full ISH tissue preparation method).

4.2.3. Oligonucleotide probes

Oligonucleotide probes synthesised from the following nucleotide sequences were used for in-situ hybridisation histochemistry:

Tyrosine Hydroxylase (TH): 36 base probe sequence complimentary to bases 1380 to 1415 (inclusive) of the rat TH gene (L22651 Anton et al. 1994): 5′ GGG AGA ACT GGG CAA ATG TGC GGT CAG CCA ACA TGG 3′.

Vesicular Monoamine Transporter 2 (VMAT2): 48 base probe sequence complimentary to bases 271 to 318 of the rat vesicular monoamine transporter gene (NM 013031 Schwartz et al. 2003): 5' ATG CCT TTA GGT CTG GTG GTC TGG TCT CGA GCA CCA GAG GTG GAG GCT 3'

Dopamine Transporter (DAT): 43 base probe sequence complimentary to bases 1015 to 1057 of the rat DA transporter gene (M80233 Kilty et al., 1991): 5' GAG AAG GCA ATC AGC ACT CCA AAC CCA ACG CCG AGG GAG AAG C 3'.

Monoamine Oxidase A (MAO-A): 36 base probe sequence complimentary to bases 1591 to 1626 of the monoamine oxidase A gene (XM_001058993): 5' AAG ATA CGC AAA TTC CCG AGC AGT TTT TGT CCA ACA 3'

D_2 receptor: 36 base probe sequence complimentary to bases 787 to 820 of the rat dopamine D2 receptor (X17458 Monsma et al. 1989): 5' GTG TTG ACC CGC TTC CGG CAC TTC CGG AGG ACG AGT 3'

NMDA receptor subunit 1 (NMDAR1): 39 base probe sequence complimentary to bases 558 to 596 of the NMDAR1 subunit of the NMDA receptor (U11418 Sullivan et al. 1994): 5' CGG TGC CGC CCT ACT CCC ACC AGT CCA GCG TCT GGT TTG AGA 3'

AMPA receptor subunit GluR1: 39 base probe sequence complimentary to bases 1899 to 1937 of the GluR1 subunit of the AMPA receptor (X17184 Hartley et al. 1998): 5' GTC ACT GGT TGT CTG GTC TCG TCC CTC TTC AAA CTC TTC 3'

5-$HT2_C$ receptor: 36 base probe sequence complimentary to bases 697 to 732 of the serotonin 2C receptor (NM_012765 Julius et al. 1988): 5' ACG GCG CAG GAC GTA GAT CGT TAA GAA GTA GGT GAT 3'

4.2.4. In-situ hybridisation procedure

Oligonucleotide probes were labelled with ^{35}S-dATP at the 3'-tail end using terminal deoxynucleotidyl transferase (TDT) enzyme. Radiolabelled probe and unbound ^{35}S were separated using Sephadex columns and the radioactivity in column fractions was measured with a scintillation counter. The radioactivity counts for the labelled oligonucleotides are shown in table 4.1.

Oligonucleotide Probe	Radioactivity (cpm/µl)	Exposure Period
Tyrosine Hydroxylase	$9.2e^4$	2 weeks
Vesicular Monoamine Transporter 2	$7.1e^4$	4 weeks
Dopamine Transporter	$9.9e^4$	2 weeks
Monoamine Oxidase A	$8.5e^4$	4 weeks
D_2 receptor	$7.4e^4$	4 weeks
NMDA receptor subunit 1	$8.7e^4$	6 weeks
AMPA receptor subunit GluR1	$35.9e^4$	2 weeks
5-HT_{2C} receptor	$10.6e^4$	4 weeks

Table 4.1. Radioactivity counts and exposure periods for ^{35}S labelled oligonucleotide probes.

Tissue sections on microscope slides (one slide from each animal, 3 sections per slide) were incubated with radiolabelled probe overnight, washed and dried, then exposed to MR Biomax film (Amersham Biosciences, UK). A slide containing a ^{14}C microscale standard was included for the purpose of calibration. After an appropriate period of time films were developed using an automatic developer (Agfa Curix Daylight Processor) and analysed. Exposure periods for each probe can be seen in table 4.1.

4.2.5. Data analysis

Optical densitometry was used to measure mRNA expression in the VTA. The same rostrocaudal level of the VTA from each animal was used for each probe, this being determined using the tyrosine hydroxylase signal to "align" the sections between animals. In total 16 slides were incubated with each probe – one slide each from eight control animals and eight corticosterone treated animals. Separate measurements were made in the dorsolateral, ventrolateral, dorsomedial, and ventromedial regions of the VTA and optical density for each subregion was averaged between the left and right sides of the brain. Optical density was also measured across the whole VTA and averaged between the left and right sides of the brain.

Optical densitometry readings from a ^{14}C standard microscale included in each experiment were plotted to obtain a calibration graph. Optical densitometry values measured in the VTA were then converted to values expressed in nCi/g of brain tissue from this standard curve.

Statistical analysis was carried out using 2-way repeated measures ANOVA to look for effects of treatment and VTA region. Where an interaction of treatment and region was found Student's unpaired t-test was performed on data from each VTA region to determine in which regions significant effects occurred. Comparison of expression levels across the whole VTA was performed using Student's unpaired t-test.

4.3. Results

4.3.1. Corticosterone treatment

Animals were treated with vehicle or corticosterone for 15 days. Animals gained weight steadily throughout the experiment (see figure 4.2.). At 15 days animal weights did not differ significantly between treatment groups (vehicle treated 294 ± 4 g, n=8; corticosterone treated 293 ± 4 g, n=8; $p>0.05$). At 15 days water consumption per cage did not differ significantly between treatment groups (vehicle treated 245 ± 11 ml/cage/2 days, n=2; corticosterone treated 271 ± 25 ml/cage/2 days, n=2; $p>0.05$) (see figure 4.3.).

Adrenals were removed from four animals in each treatment group following sacrifice. Adrenal weight was significantly lower in corticosterone treated animals than vehicle treated animals (corticosterone treated 14.5 ± 2.2 mg, n=4; vehicle treated 28.8 ± 0.9 mg, n=4; $p<0.01$) (see figure 4.4.) consistent with the known effects of this method of corticosterone administration (see chapter 2).

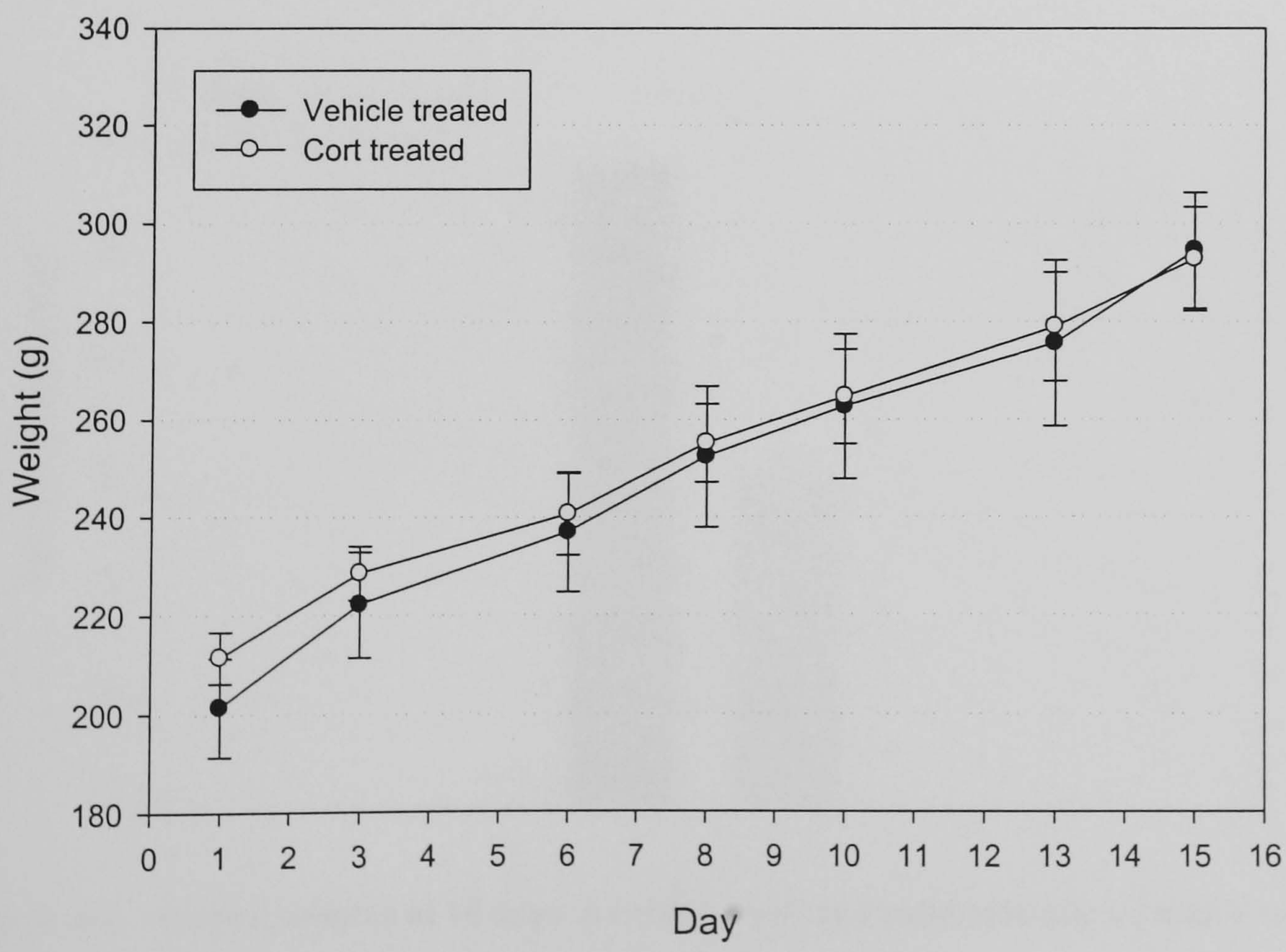

Figure 4.2. Animal weights during vehicle (n=8) and corticosterone (n=8) treatment.

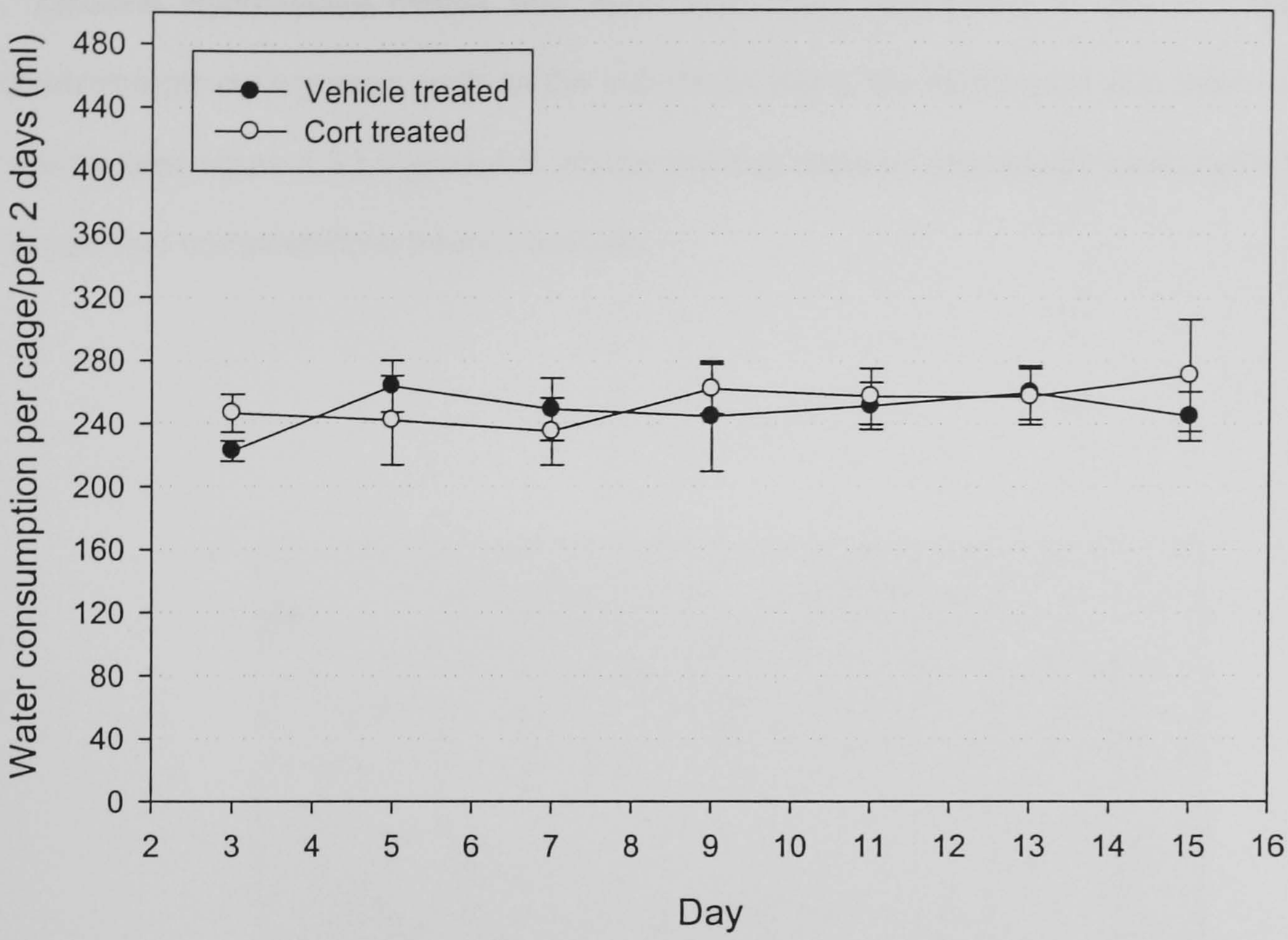

Figure 4.3. 2 day water consumption per cage of four rats treated with vehicle (n=2 cages) or corticosterone (n=2 cages).

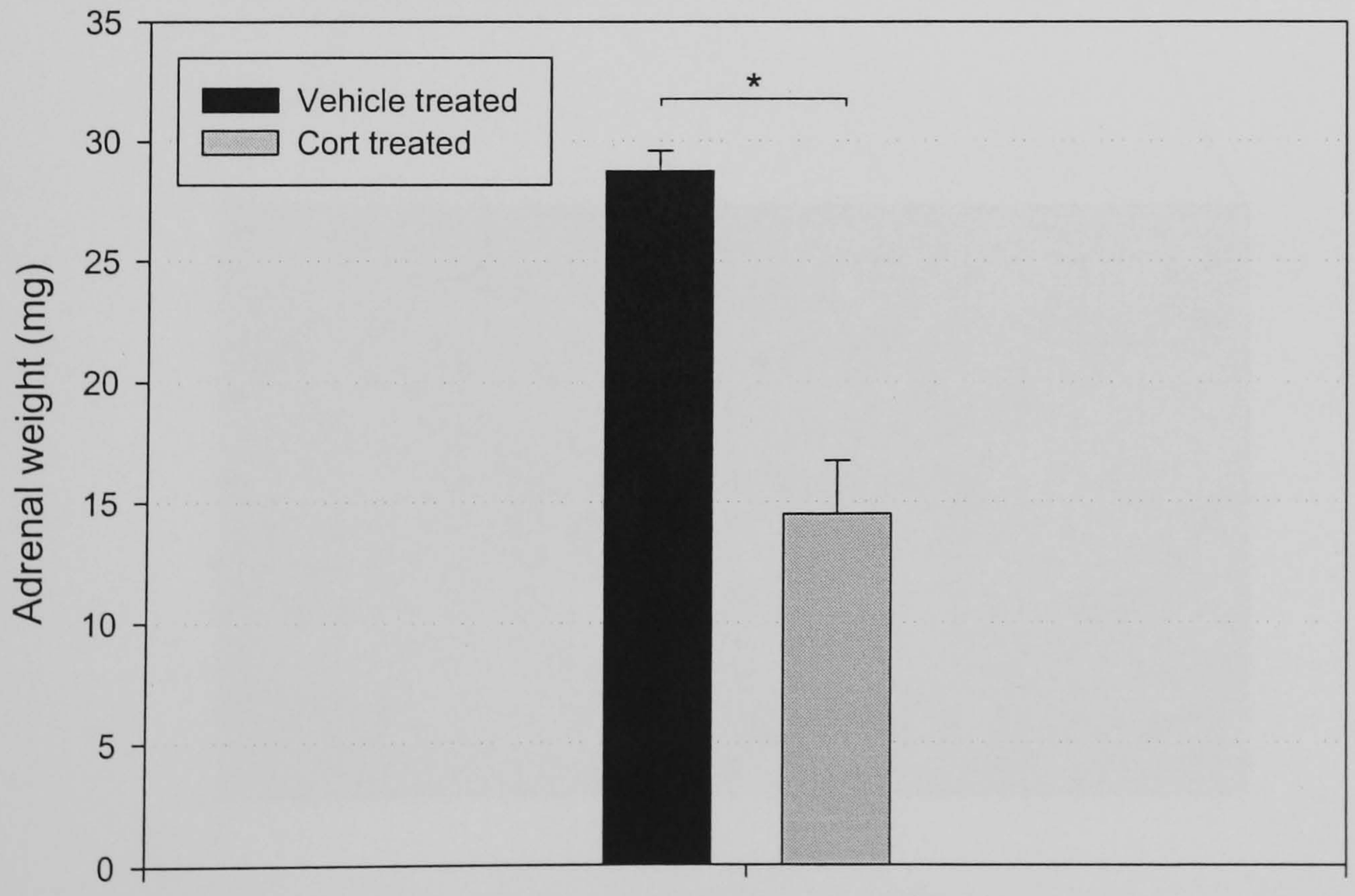

Figure 4.4. Adrenal weights at 15 days in vehicle (n=4) and corticosterone treated (n=4) animals (* $p<0.01$).

4.3.2. Tyrosine hydroxylase mRNA expression

Tyrosine Hydroxylase mRNA was seen expressed abundantly in the VTA, and adjacent dopaminergic cells groups such as the substantia nigra. No expression was seen outside these regions (see figure 4.5.). Figure 4.6. shows the sub-regional expression levels within the VTA in vehicle and corticosterone treated animals.

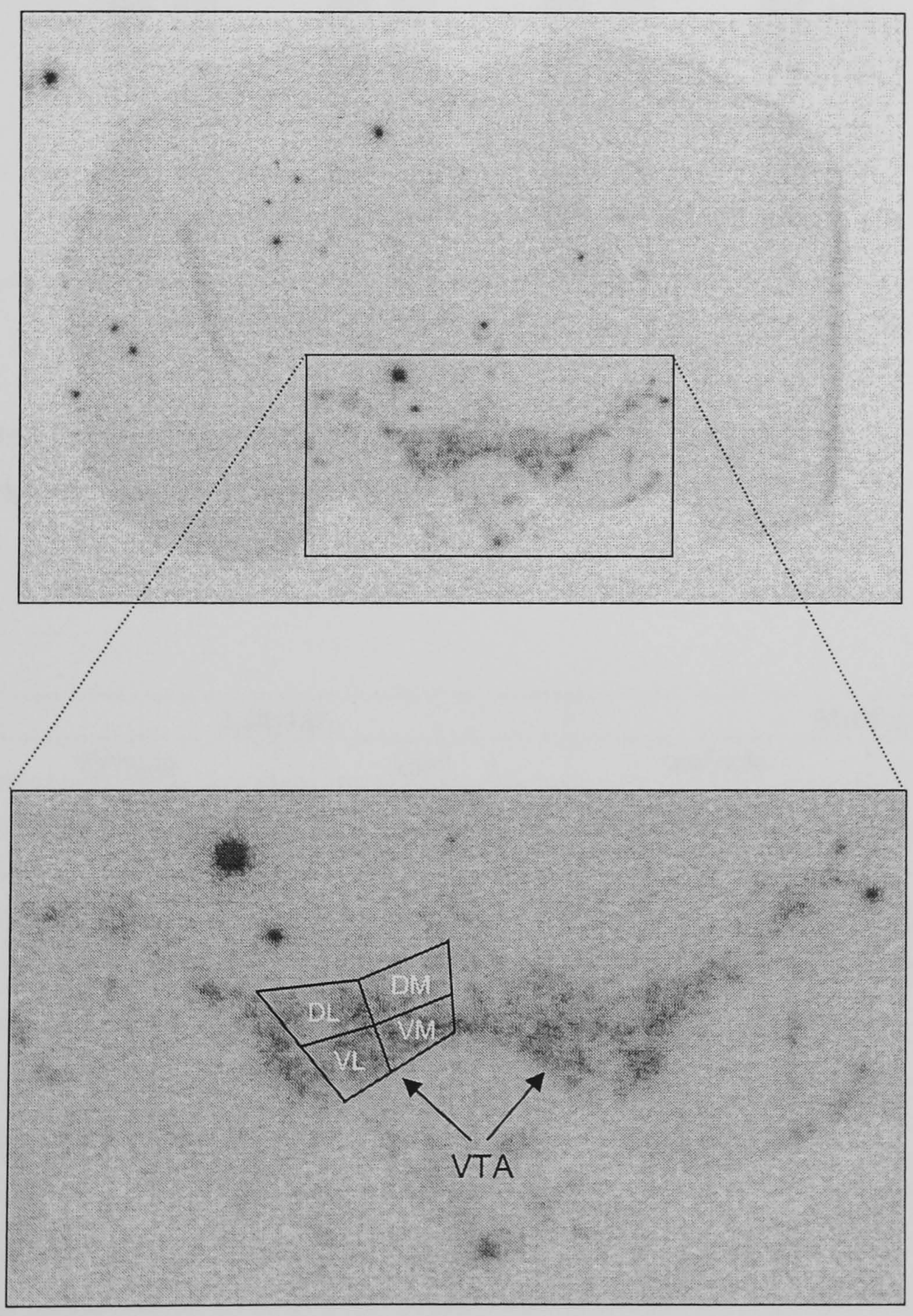

Figure 4.5. Tyrosine hydroxylase mRNA in the ventral midbrain. DL= dorsolateral; DM = dorsomedial; VL = ventrolateral; VM = ventromedial.

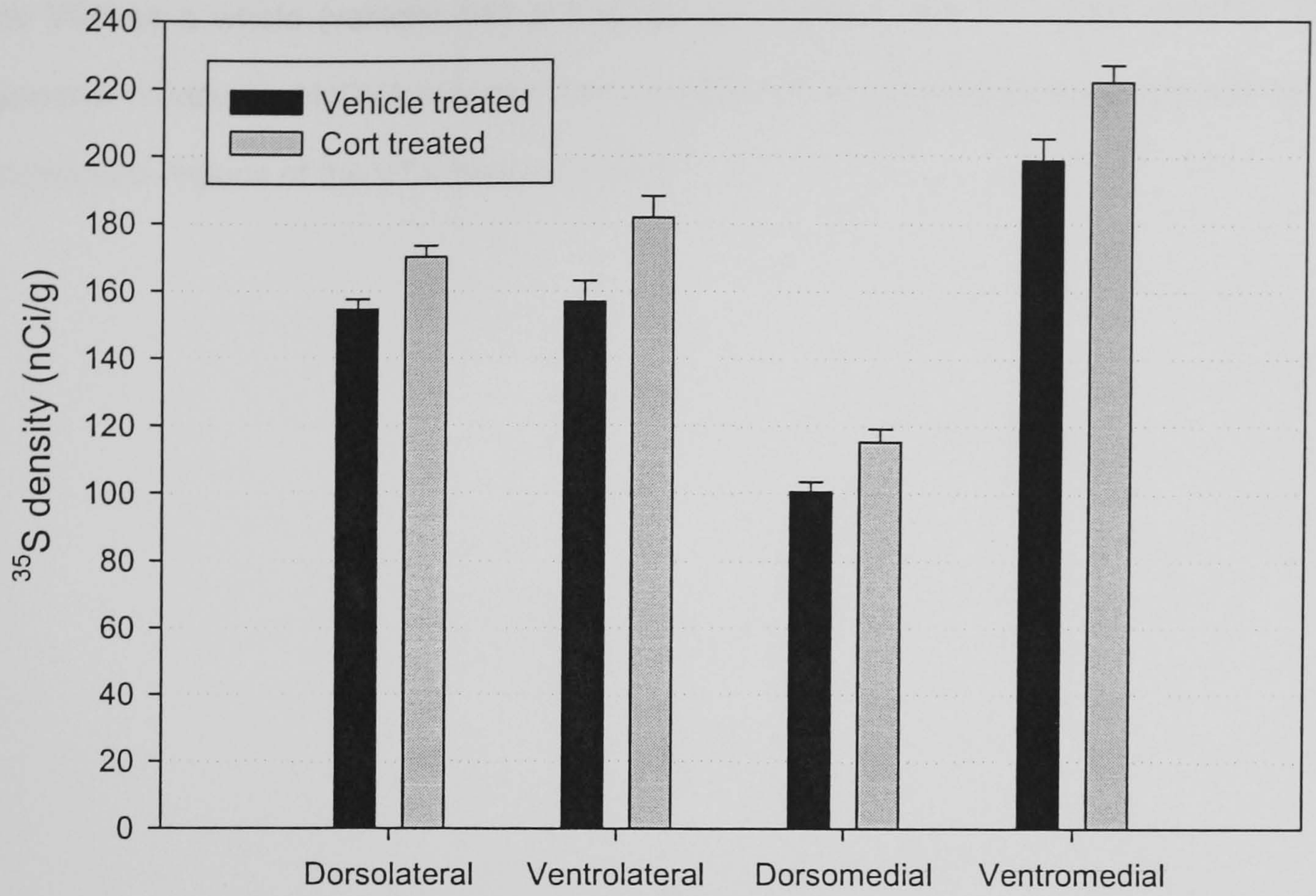

Figure 4.6. Tyrosine hydroxylase mRNA signal in subregions of the VTA in vehicle (n=8) and corticosterone (n=8) treated animals.

	Lateral		**Medial**	
	Vehicle	Cort	Vehicle	Cort
Dorsal	155 ± 3 nCi/g	170 ± 3 nCi/g	101 ± 3. nCi/g	116 ± 4 nCi/g
Ventral	157 ± 6 nCi/g	182 ± 6 nCi/g	199 ± 6 nCi/g	222 ± 5 nCi/g

Main effect of treatment	$F_{[1,14]} = 29.0$	p<0.001
Main effect of VTA sub-region	$F_{[3,42]} = 179.1$	p<0.001
Treatment x sub-region interaction	$F_{[3,42]} = 0.3$	n.s.

Table 4.2. Two-way repeated measures ANOVA of tyrosine hydroxylase mRNA signal in sub-regions of the VTA.

A 14% increase in TH mRNA expression was seen in corticosterone treated animals across the VTA as a whole (vehicle: 147 ± 3 nCi/g; cort treated: 168 ± 5 nCi/g; $p<0.01$). Two-way repeated measures ANOVA revealed that the effect of corticosterone treatment was consistent across sub-regions of the VTA (see table 4.2.).

4.3.3. Vesicular monoamine transporter 2 mRNA expression

VMAT2 mRNA was seen expressed abundantly in the VTA and adjacent substantia nigra. A high level of expression was also seen in the medial mamillary nucleus (see figure 4.7.). Figure 4.8. shows the subregional expression levels within the VTA.

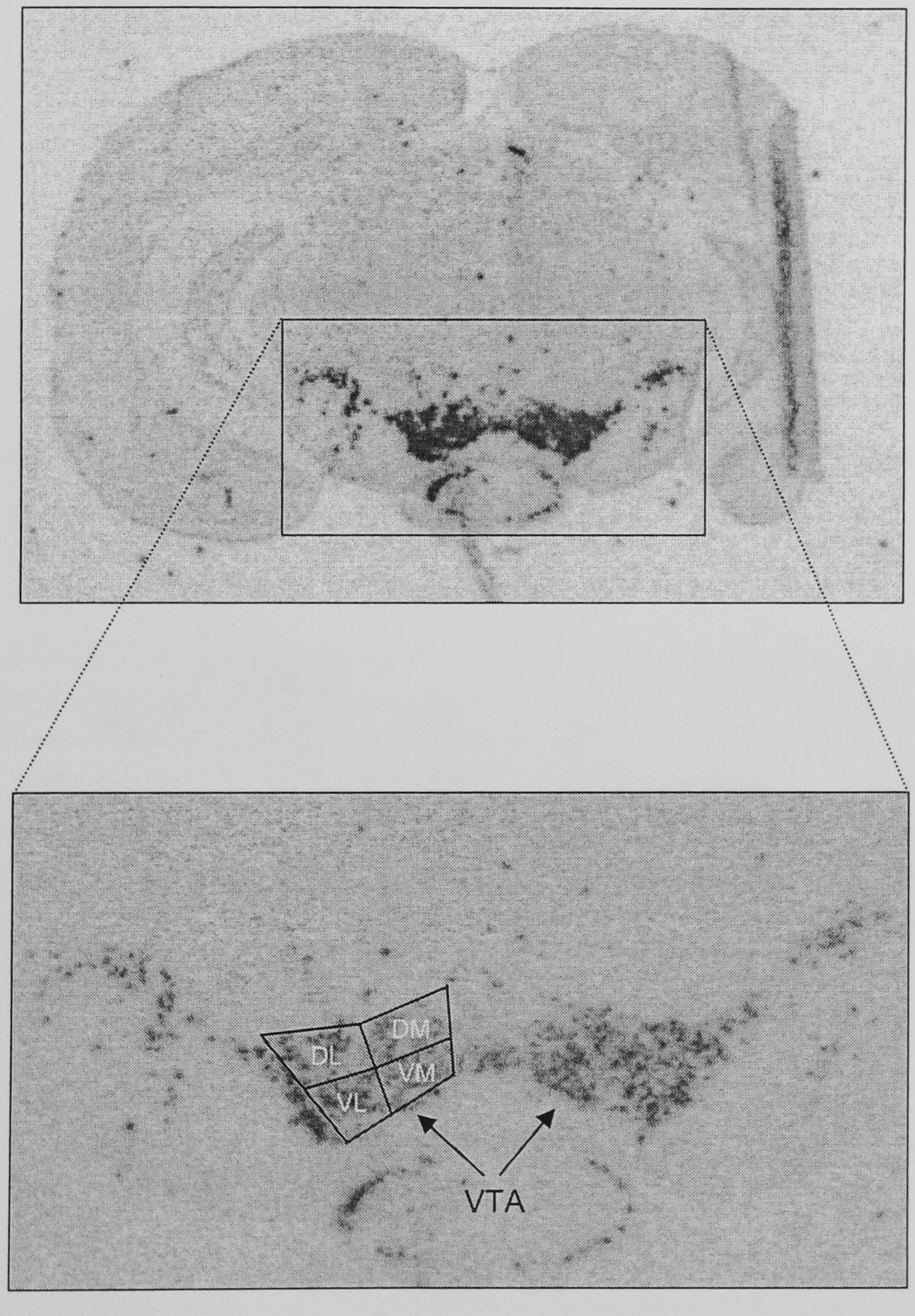

Figure 4.7. VMAT2 mRNA in the ventral midbrain. DL= dorsolateral; DM = dorsomedial; VL = ventrolateral; VM = ventromedial.

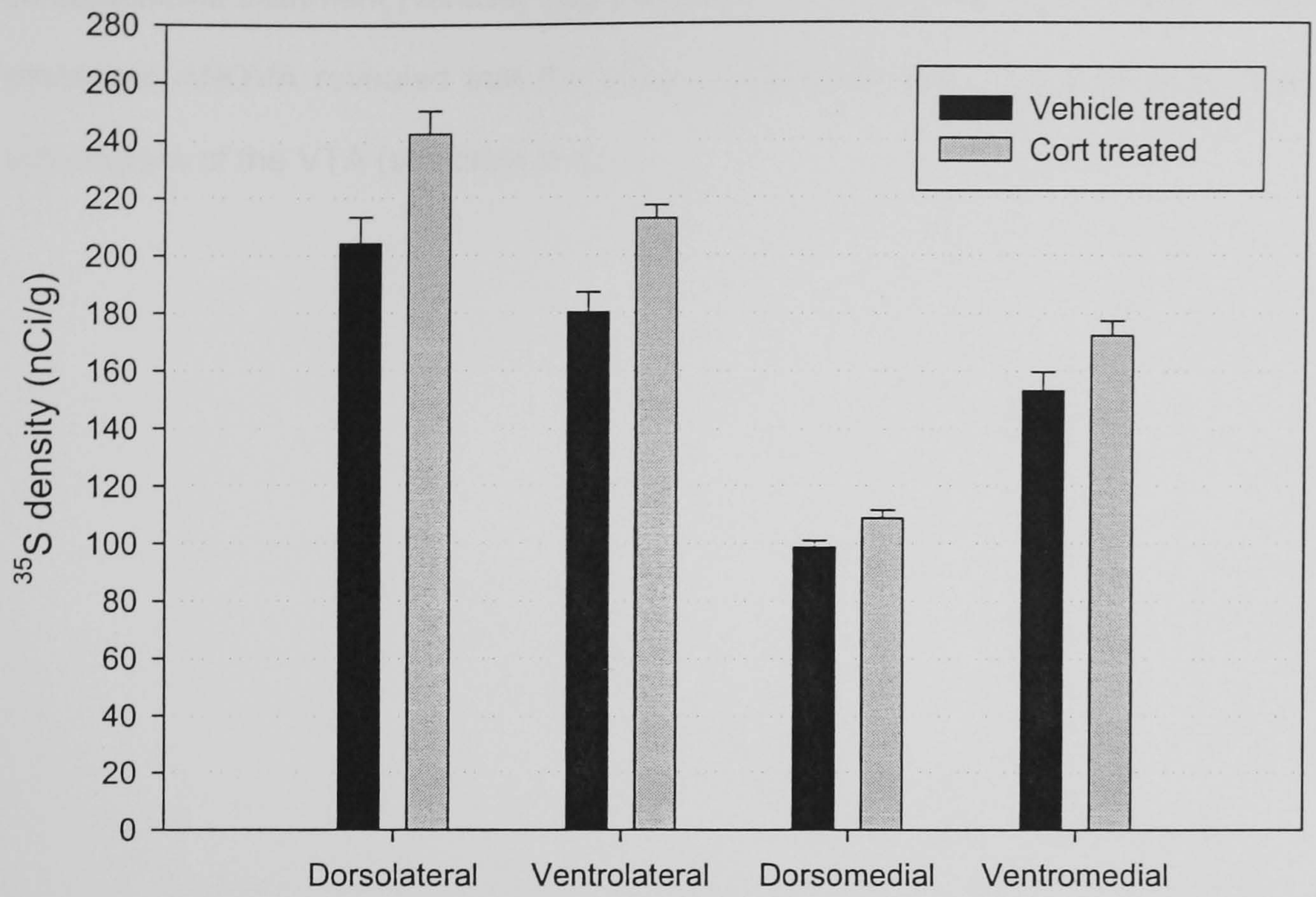

Figure 4.8. VMAT2 mRNA signal in subregions of the VTA in vehicle (n=8) and corticosterone (n=8) treated animals.

	Lateral		**Medial**	
	Vehicle	Cort	Vehicle	Cort
Dorsal	204 ± 9 nCi/g	242 ± 8 nCi/g	99 ± 2 nCi/g	109 ± 3 nCi/g
Ventral	180 ± 7 nCi/g	213 ± 5 nCi/g	153 ± 7 nCi/g	172 ± 5 nCi/g

Main effect of treatment	$F_{[1,14]} = 26.0$	p<0.001
Main effect of VTA sub-region	$F_{[3,42]} = 200.1$	p<0.001
Treatment x sub-region interaction	$F_{[3,42]} = 1.0$	n.s.

Table 4.3. Two-way repeated measures ANOVA of VMAT2 mRNA signal in sub-regions of the VTA.

There was a 13% increase in VMAT2 expression across the whole VTA following corticosterone treatment (vehicle: 158 ± 4 nCi/g, cort: 175 ± 5 nCi/g; $p<0.01$). Two-way repeated measures ANOVA revealed that the effect of corticosterone treatment was consistent across sub-regions of the VTA (see table 4.3.).

4.3.4. Dopamine transporter mRNA expression

Dopamine transporter mRNA showed strong expression and was found exclusively in the dopaminergic cell groups of the substantia nigra and VTA (see figure 4.9). Regional expression within the VTA can be seen in figure 4.10.

No significant effect of corticosterone treatment was found across the VTA as a whole (vehicle: 105 ± 4 nCi/g, cort: 104 ± 3 nCi/g; $p>0.05$). Two-way repeated measures ANOVA confirmed the lack of effect across sub-regions of the VTA (see table 4.4.).

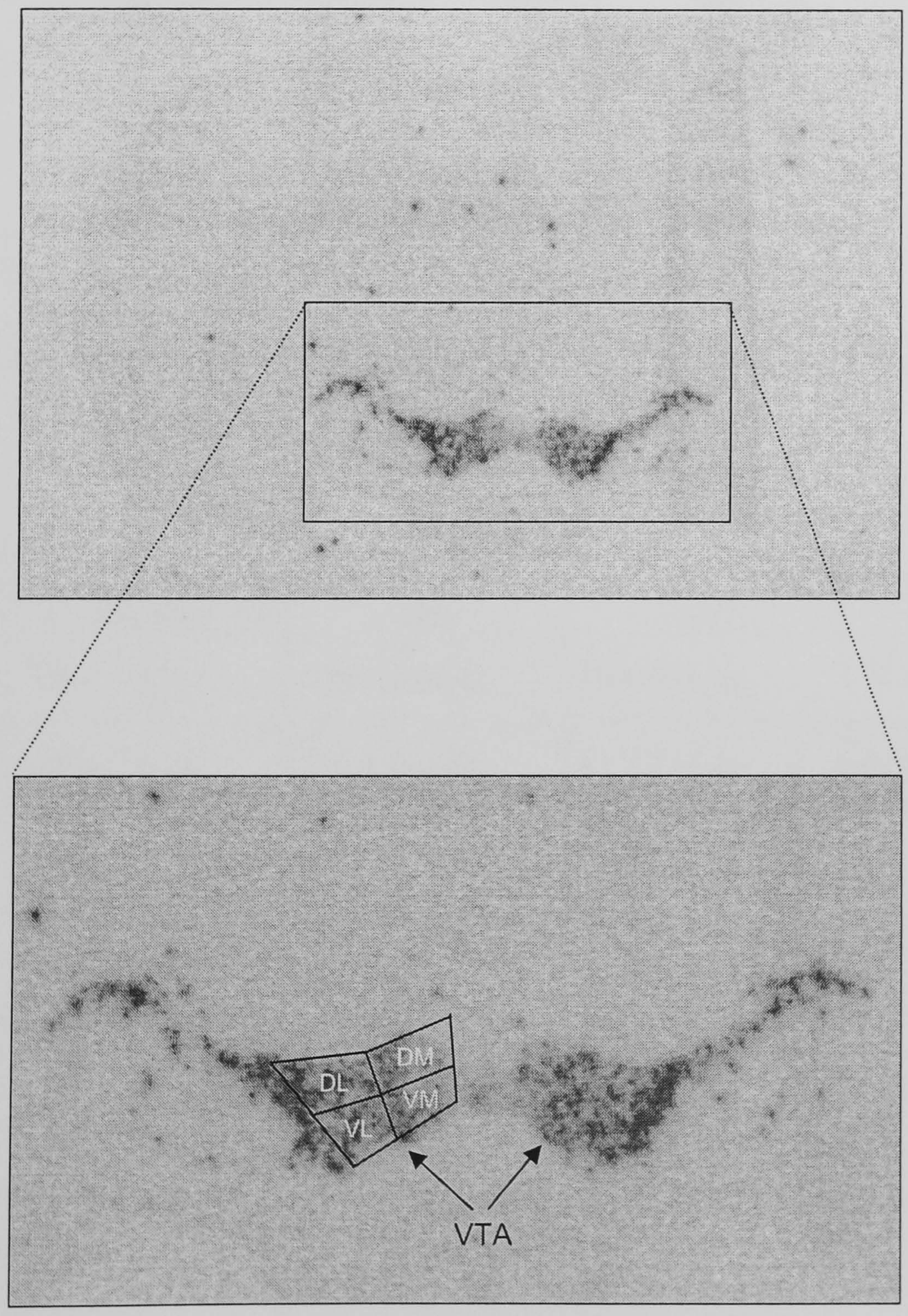

Figure 4.9. DAT mRNA in the ventral midbrain. DL= dorsolateral; DM = dorsomedial; VL = ventrolateral; VM = ventromedial.

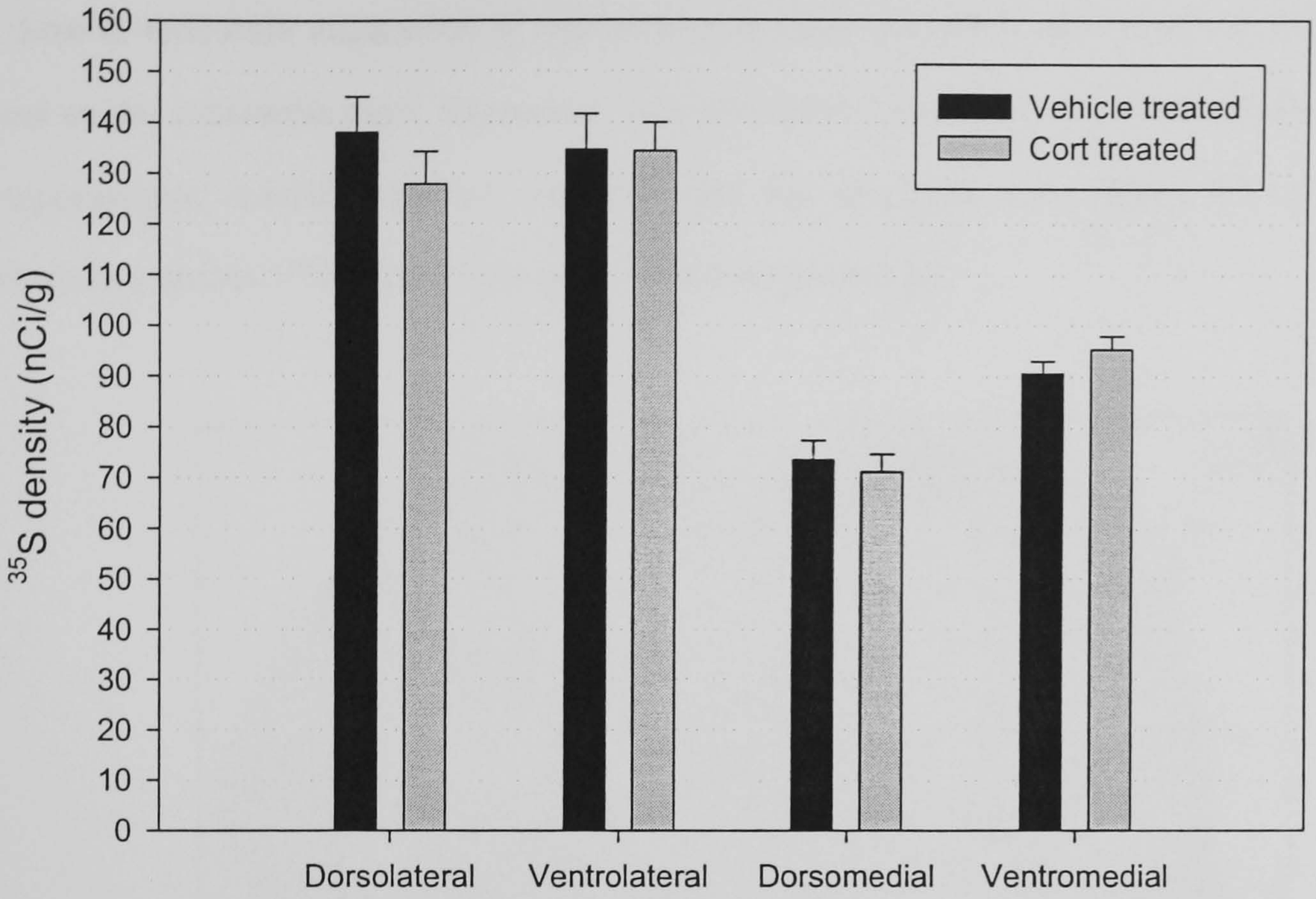

Figure 4.10. DAT mRNA signal in subregions of the VTA in vehicle (n=8) and corticosterone (n=8) treated animals.

	Lateral		**Medial**	
	Vehicle	Cort	Vehicle	Cort
Dorsal	138 ± 7 nCi/g	128 ± 6 nCi/g	74 ± 4 nCi/g	71 ± 3 nCi/g
Ventral	135 ± 7 nCi/g	134 ± 6 nCi/g	91 ± 2 nCi/g	95.4 ± 2 nCi/g
	Main effect of treatment		$F_{[1,14]} = 0.1$	n.s.
	Main effect of VTA sub-region		$F_{[3,42]} = 103.3$	$p<0.001$
	Treatment x sub-region interaction		$F_{[3,42]} = 0.9$	n.s.

Table 4.4. Two-way repeated measures ANOVA of DAT mRNA signal in sub-regions of the VTA.

4.3.5. Monoamine oxidase A mRNA expression

Low to moderate expression of monoamine oxidase A mRNA was observed in the VTA as well as the substantia nigra. Expression was also seen in the CA1, CA2 and CA3 regions of the hippocampus, medial mamillary nucleus, and the amygdala (see figure 4.11.) The signal intensities across VTA subregions can be seen in figure 4.12.

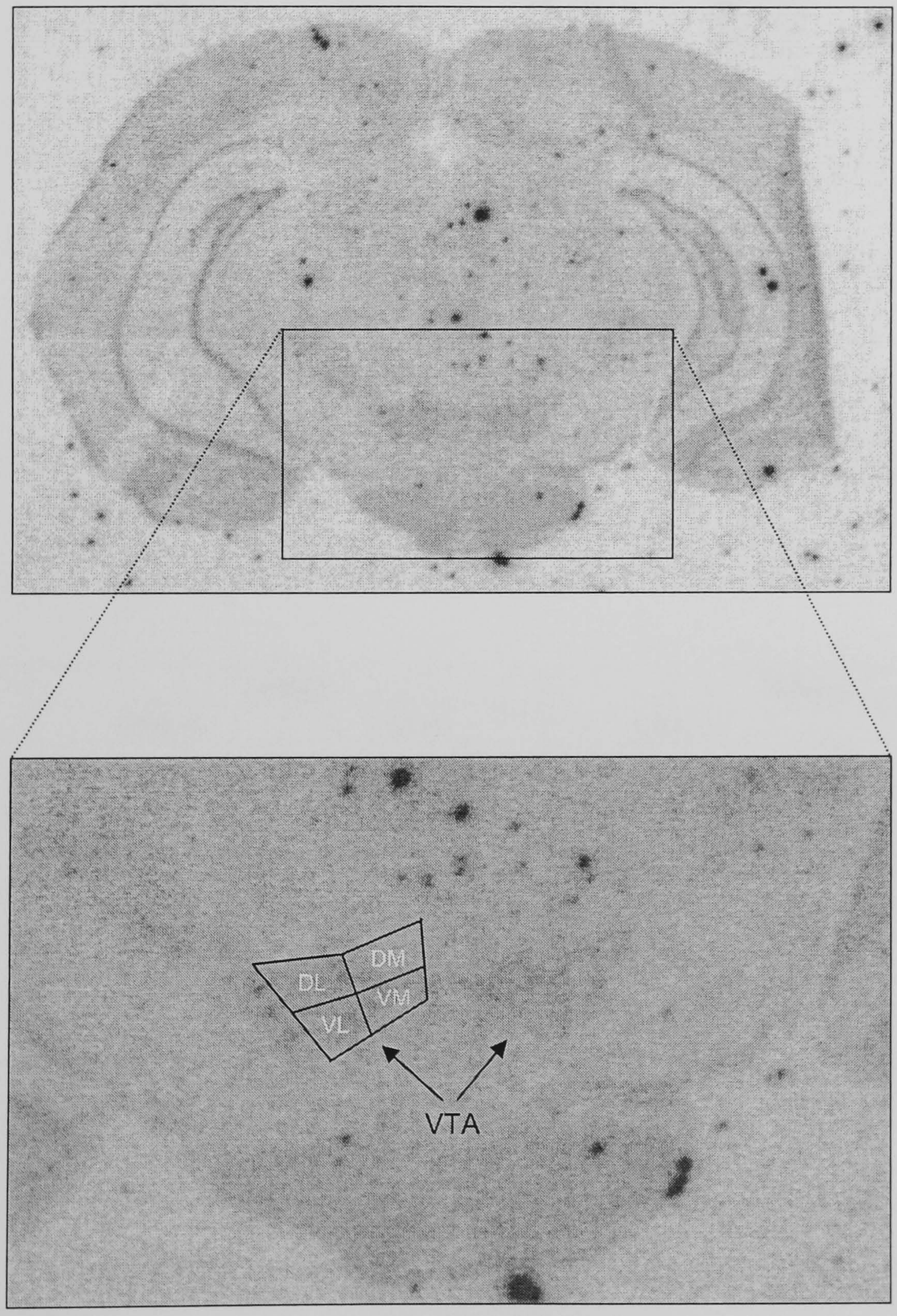

Figure 4.11. MAO-A mRNA in the ventral midbrain. DL= dorsolateral; DM = dorsomedial; VL = ventrolateral; VM = ventromedial.

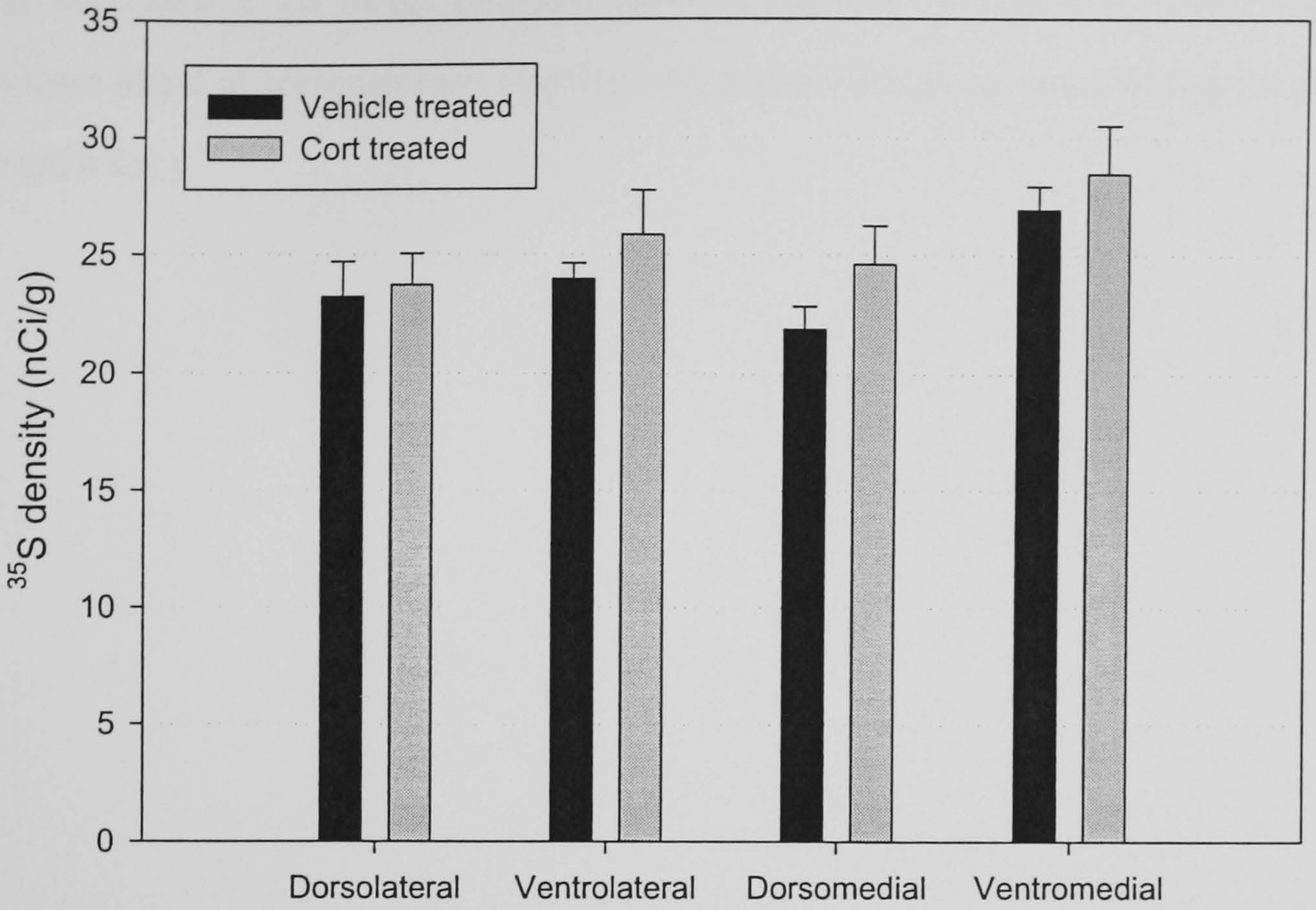

Figure 4.12. MAO-A mRNA signal in subregions of the VTA in vehicle (n=8) and corticosterone (n=8) treated animals.

	Lateral		**Medial**	
	Vehicle	Cort	Vehicle	Cort
Dorsal	23.2 ± 15.6 nCi/g	23.7 ± 1.3 nCi/g	21.9 ± 1.0 nCi/g	24.6 ± 1.6 nCi/g
Ventral	24.0 ± 0.7 nCi/g	25.8 ± 1.9 nCi/g	26.9 ± 1.0 nCi/g	28.5 ± 2.0 nCi/g
	Main effect of treatment		$F_{[1,13]} = 0.7$	n.s.
	Main effect of VTA sub-region		$F_{[3,39]} = 9.2$	$p<0.001$
	Treatment x sub-region interaction		$F_{[3,39]} = 0.5$	n.s.

Table 4.5. Two-way repeated measures ANOVA of MAO-A mRNA signal in sub-regions of the VTA.

No significant effect of treatment was found across the VTA as a whole (vehicle: 24.5 ± 0.8 nCi/g, cort: 26.5 ± 1.4 nCi/g; $p>0.05$). Two-way repeated measures ANOVA confirmed no significant effect of corticosterone treatment on signal intensity across sub-regions of the VTA (see table 4.5.).

4.3.6. D_2 receptor mRNA expression

D_2 receptor mRNA expression was found to be of relatively low intensity compared to background signal in both the VTA and substantia nigra, however, signal was selectively found in these areas (see figure 4.13.). The subregional signal intensities in the VTA can be seen in figure 4.14.

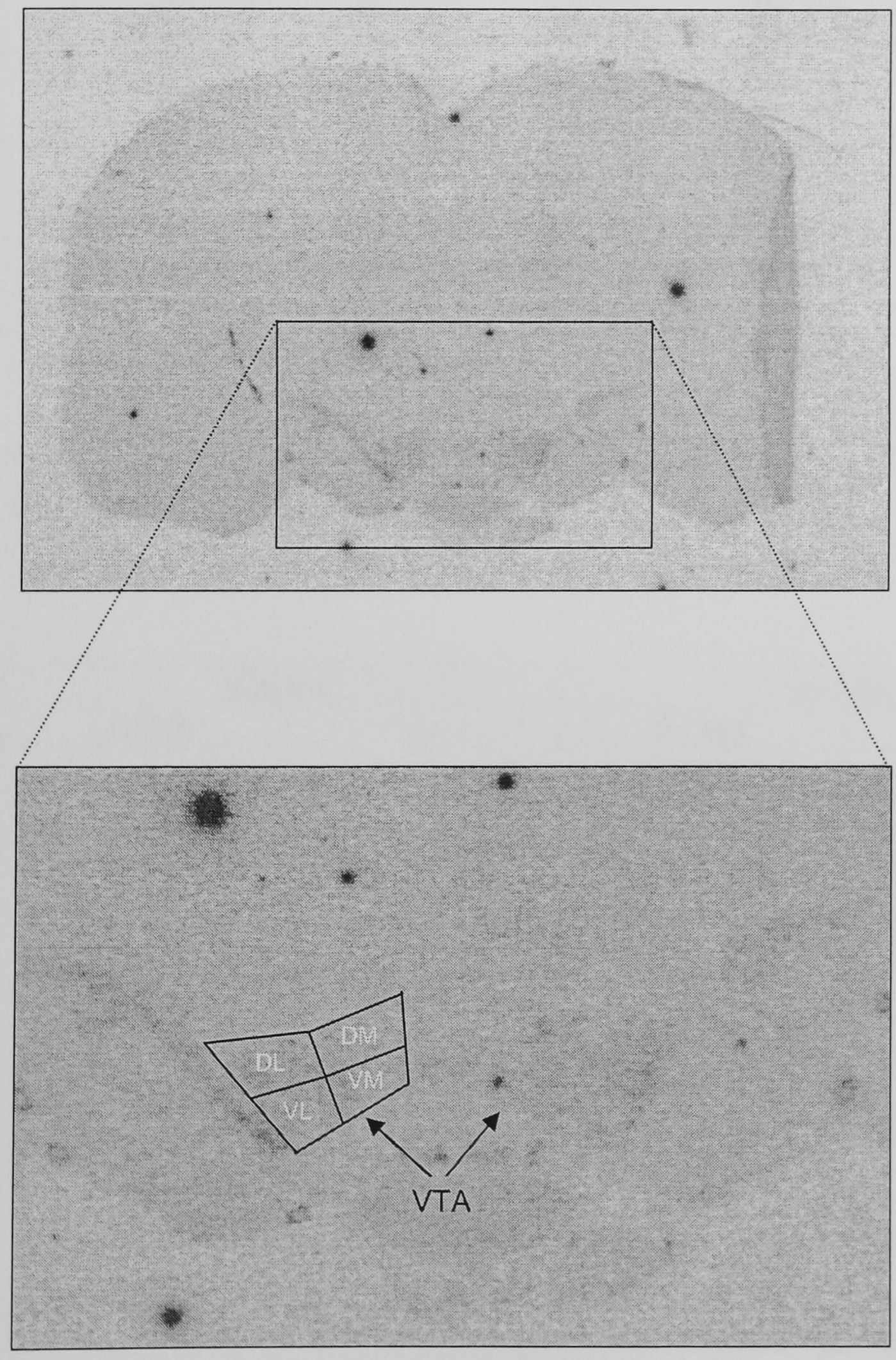

Figure 4.13. D_2 mRNA in the ventral midbrain. DL= dorsolateral; DM = dorsomedial; VL = ventrolateral; VM = ventromedial.

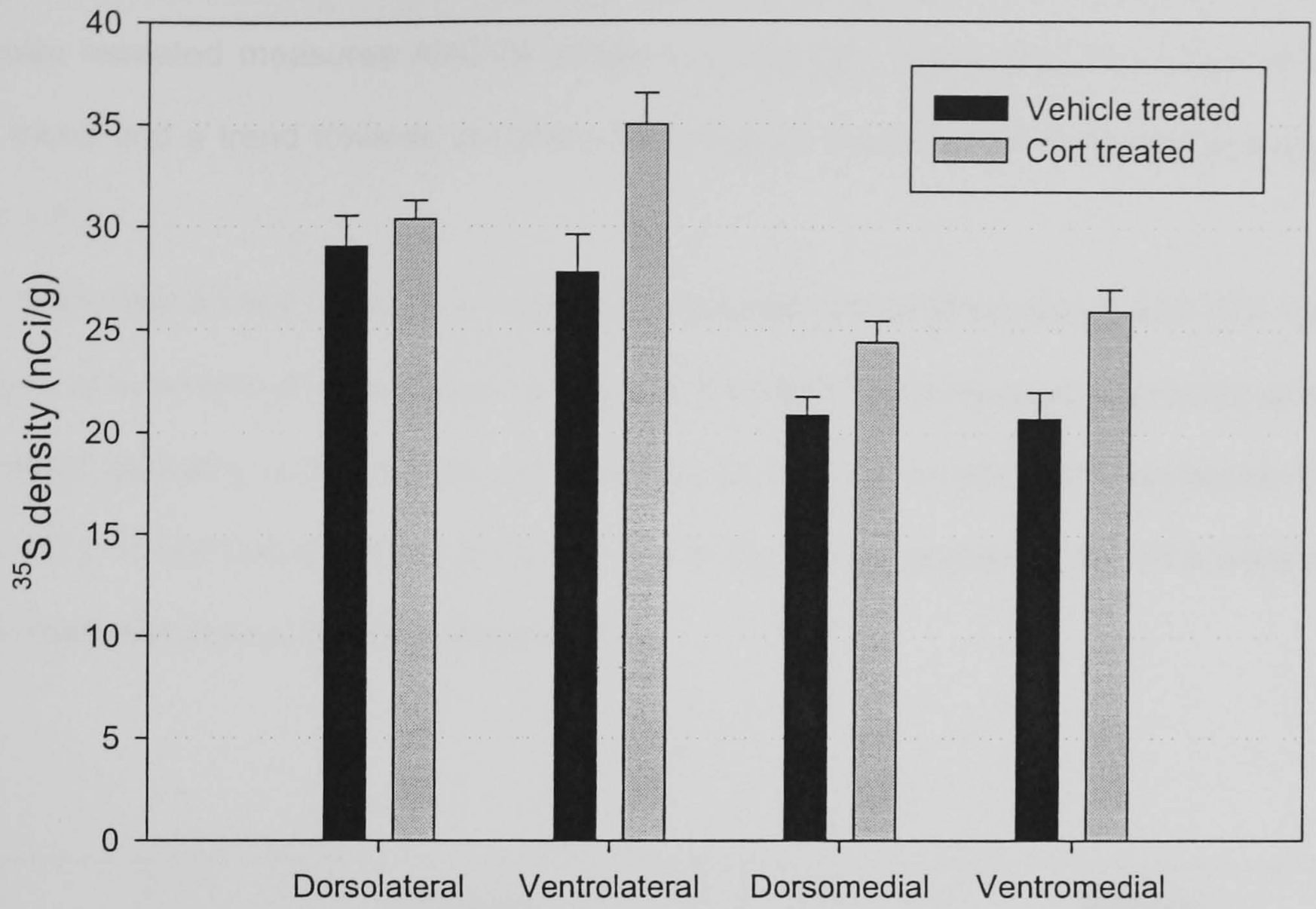

Figure 4.14. D_2 mRNA signal in subregions of the VTA in vehicle (n=8) and corticosterone (n=8) treated animals.

	Lateral		**Medial**	
	Vehicle	Cort	Vehicle	Cort
Dorsal	29.1 ± 1.5 nCi/g	30.4 ± 0.9 nCi/g	20.9 ± 0.9 nCi/g	24.4 ± 1.1 nCi/g
Ventral	27.8 ± 1.8 nCi/g	35.0 ± 1.5 nCi/g	20.7 ± 1.3 nCi/g	25.9 ± 1.1 nCi/g
	Main effect of treatment		$F_{[1,13]} = 7.8$	$p<0.05$
	Main effect of VTA sub-region		$F_{[3,39]} = 42.8$	$p<0.001$
	Treatment x sub-region interaction		$F_{[3,39]} = 2.5$	$p=0.07$

Table 4.6. Two-way repeated measures ANOVA of D_2 mRNA signal in sub-regions of the VTA.

Across the VTA as a whole there was 19% increase in D_2 mRNA in corticosterone treated animals (vehicle: 23.8 ± 1.2 nCi/g, cort treated: 28.4 ± 0.9 nCi/g; $p<0.01$). This was confirmed by two-way repeated measures ANOVA where a statistically significant main effect of treatment was found and a trend towards an interaction between the effect of treatment and region (see table 4.6.).

As there was a trend towards an interaction between effect of treatment and VTA sub-region analysis of sub-regional effects was carried out. Student's t-test revealed significant increases in D_2 mRNA following corticosterone treatment in all regions except the dorsolateral VTA (see table 4.7.). In addition increases in expression in the ventral portion of the VTA were relatively larger than that seen in the dorsomedial VTA.

	Lateral	**Medial**
Dorsal	5% increase n.s.	17% increase $p<0.05$
Ventral	26% increase $p<0.01$	25% increase $p<0.01$

Table 4.7. Regional differences in the effect of corticosterone treatment on D_2 mRNA expression in the VTA.

4.3.7. NMDA receptor subunit 1 mRNA expression

Expression of NMDAR1 subunit mRNA in the midbrain was widespread, with high expression in the CA1, CA2 and CA3 regions of the hippocampus and the amygdala, and lesser expression in the periaqueductal grey region and the cortex. Expression in the VTA was found to be relatively low compared to these regions (see figure 4.15). Signal intensities in the VTA can be seen in figure 4.16.

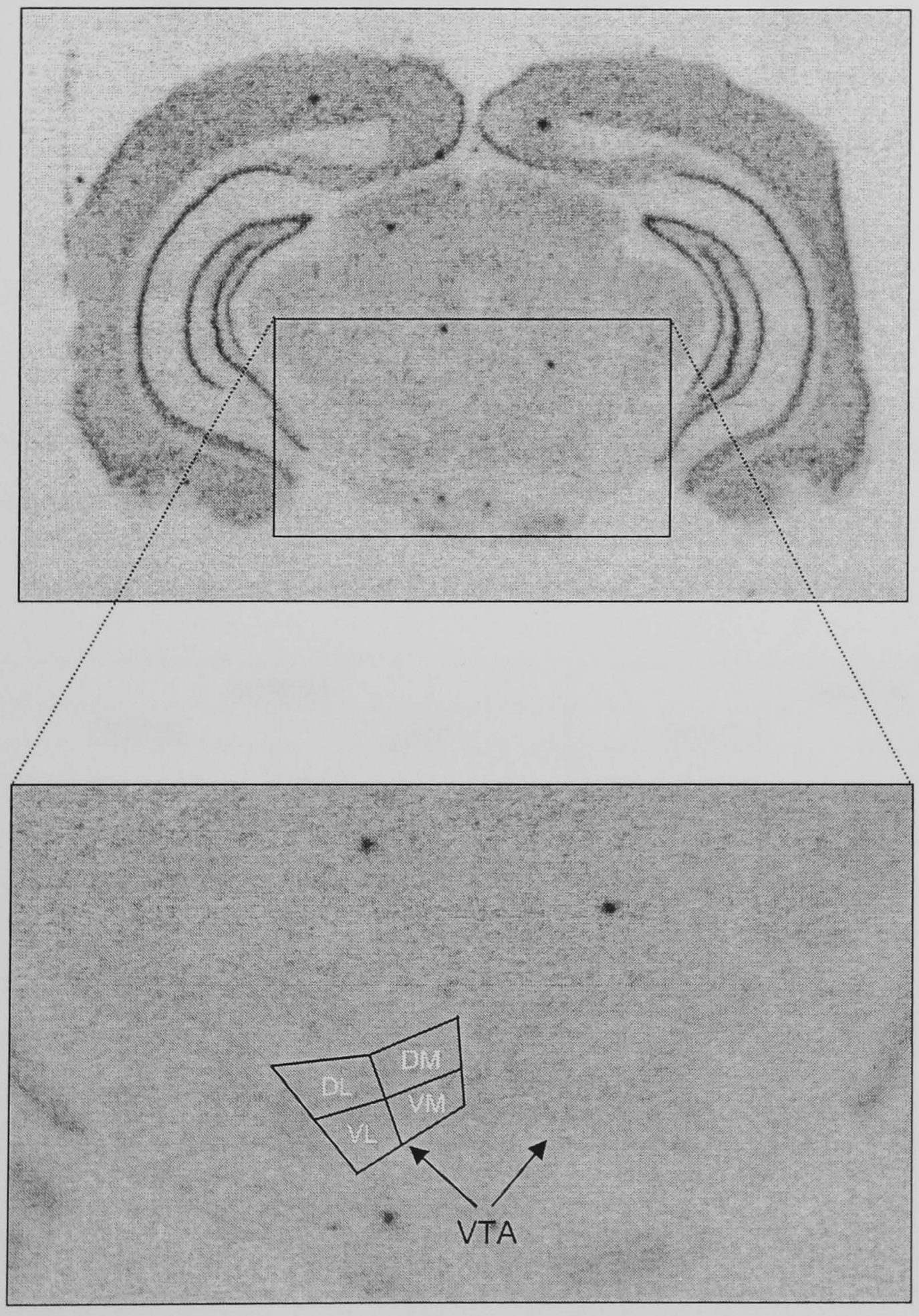

Figure 4.15. NMDAR1 mRNA in the ventral midbrain. DL= dorsolateral; DM = dorsomedial; VL = ventrolateral; VM = ventromedial.

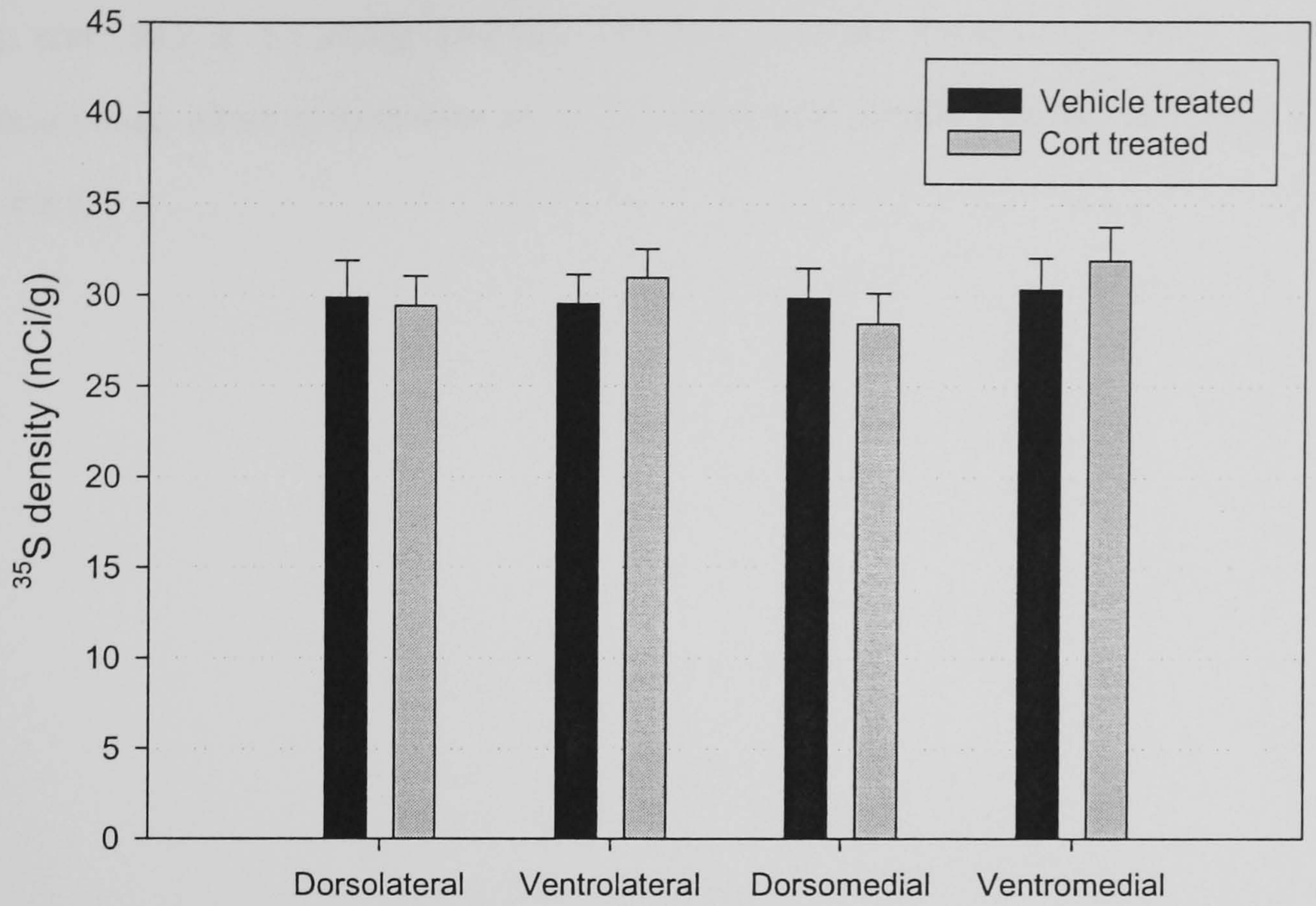

Figure 4.16. NMDAR1 mRNA signal in subregions of the VTA in vehicle (n=8) and corticosterone (n=8) treated animals.

	Lateral		**Medial**	
	Vehicle	Cort	Vehicle	Cort
Dorsal	29.98± 1.9 nCi/g	29.3 ± 1.5 nCi/g	29.7 ± 1.6 nCi/g	28.5 ± 1.6 nCi/g
Ventral	29.4 ± 1.5 nCi/g	30.8 ± 1.5 nCi/g	30.2 ± 1.6 nCi/g	31.8 ± 1.8 nCi/g
	Main effect of treatment		$F_{[1,14]} = 0.02$	n.s.
	Main effect of VTA sub-region		$F_{[3,42]} = 1.0$	n.s.
	Treatment x sub-region interaction		$F_{[3,42]} = 0.7$	n.s.

Table 4.8. Two-way repeated measures ANOVA of NMDAR1 mRNA signal in sub-regions of the VTA.

There was no effect of treatment on mRNA across the VTA as a whole (vehicle: 30.9 ± 1.4 nCi/g, cort: 30.7 ± 1.1 nCi/g; $p>0.05$). Two-way repeated measures ANOVA revealed no significant main effect of treatment on mRNA expression across sub-regions of the VTA (see table 4.8.).

4.3.8. AMPA receptor GluR1 subunit mRNA expression

Similar to the pattern of expression seen with NMDAR1, GluR1 expression was widespread and particularly intense in the hippocampal CA1, CA2 and CA3 regions and the amygdala. Lesser expression was seen in the periaqueductal grey region, the cortex, and interpeduncular nucleus, whilst in the VTA expression was of a relatively low intensity (see figure 4.17.).

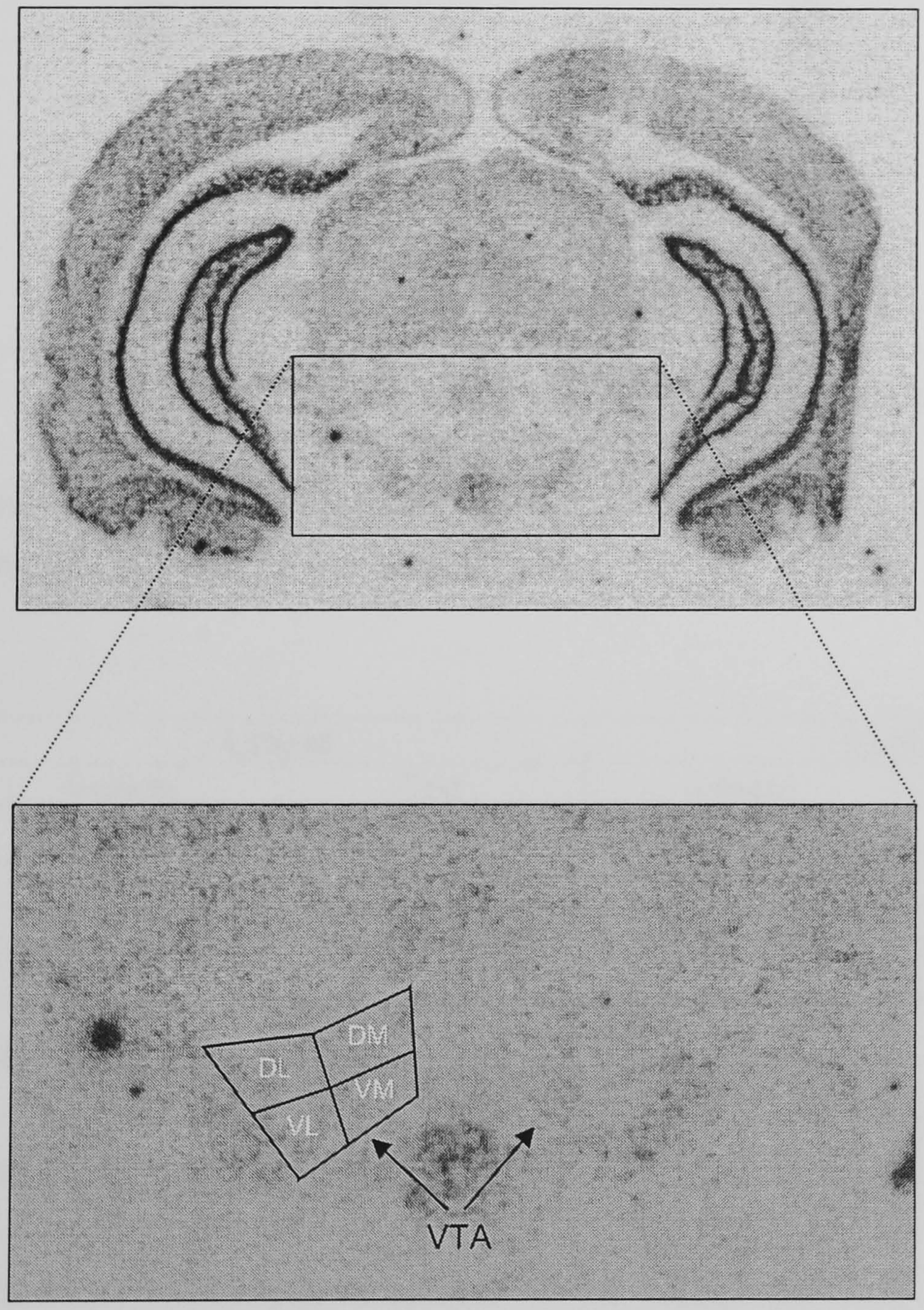

Figure 4.17. GluR1 mRNA in the ventral midbrain. DL= dorsolateral; DM = dorsomedial; VL = ventrolateral; VM = ventromedial.

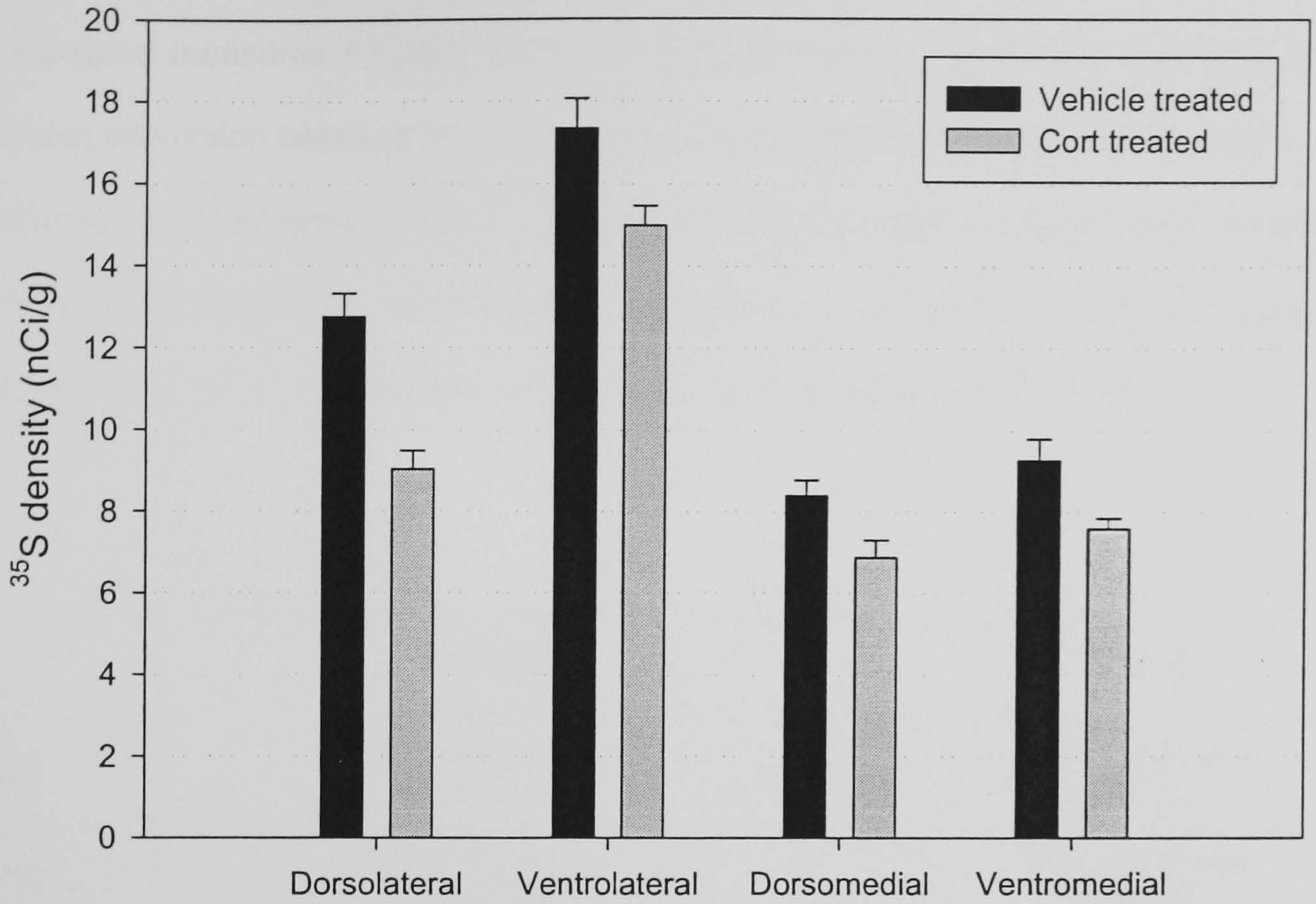

Figure 4.18. GluR1 mRNA signal in subregions of the VTA in vehicle (n=8) and corticosterone treated (n=8) animals.

	Lateral		**Medial**	
	Vehicle	Cort	Vehicle	Cort
Dorsal	12.8 ± 0.6 nCi/g	9.0 ± 0.5 nCi/g	8.4 ± 0.4 nCi/g	6.9 ± 0.4 nCi/g
Ventral	17.3 ± 0.7 nCi/g	15.0 ± 0.5 nCi/g	9.3 ± 0.5 nCi/g	7.6 ± 0.2 nCi/g
	Main effect of treatment		$F_{[1,13]} = 23.1$	p<0.001
	Main effect of VTA sub-region		$F_{[3,39]} = 120.9$	p<0.001
	Treatment x sub-region interaction		$F_{[3,39]} = 3.4$	p<0.05

Table 4.9. Two-way repeated measures ANOVA of GluR1 mRNA signal in sub-regions of the VTA.

Across the whole VTA there was a 15% decrease in GluR1 mRNA transcription following corticosterone treatment (vehicle: 10.6 ± 0.2 nCi/g, cort treated: 9.0 ± 0.2 nCi/g; $p<0.001$). Two-way repeated measures ANOVA confirmed the main effect of treatment and demonstrated a significant interaction between VTA sub-region and the effect of treatment (see table 4.9.).

Sub-regional differences in the effect of corticosterone were analysed with Student's t-test which revealed significant decreases in expression in all regions, with a relatively larger decrease in the dorsolateral region compared to other areas (see table 4.10).

	Lateral	**Medial**
Dorsal	29% decrease $p<0.001$	18% decrease $p<0.05$
Ventral	14% decrease $p<0.05$	19% decrease $p<0.05$

Table 4.10. Regional differences in the effect of corticosterone on GluR1 mRNA expression in the VTA.

4.3.9. 5-HT_{2C} receptor mRNA expression

Expression of mRNA for the 5-HT_{2C} receptor was widespread and particularly intense in the choroid plexus and in the substantia nigra pars compacta. Lesser expression was seen in the periaqueductal grey region and in the dorsomedial geniculate nucleus. In contrast to these regions expression in the VTA was of relatively low intensity (see figure 4.19.),

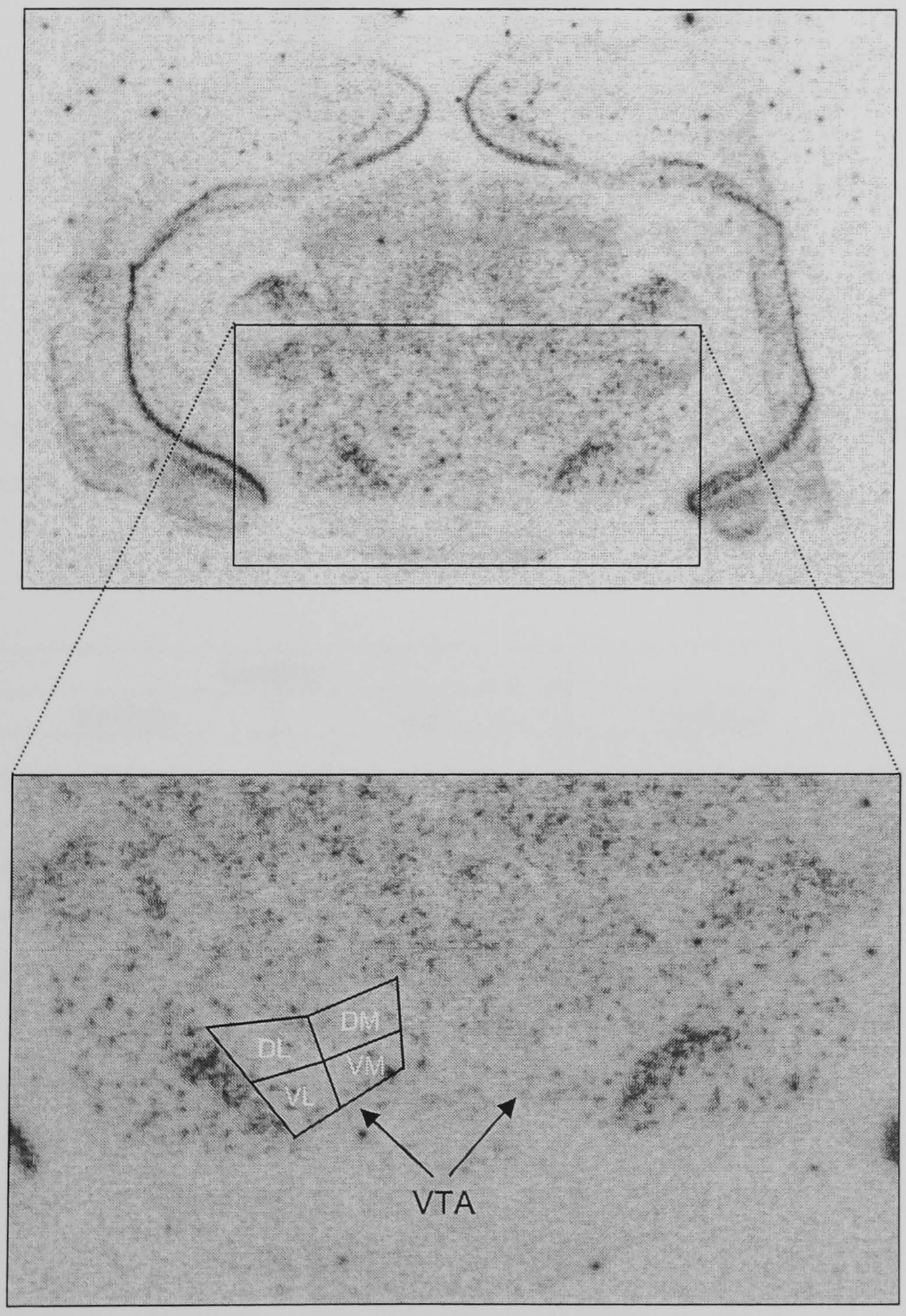

Figure 4.19. 5-HT_{2C} mRNA in the ventral midbrain. DL= dorsolateral; DM = dorsomedial; VL = ventrolateral; VM = ventromedial.

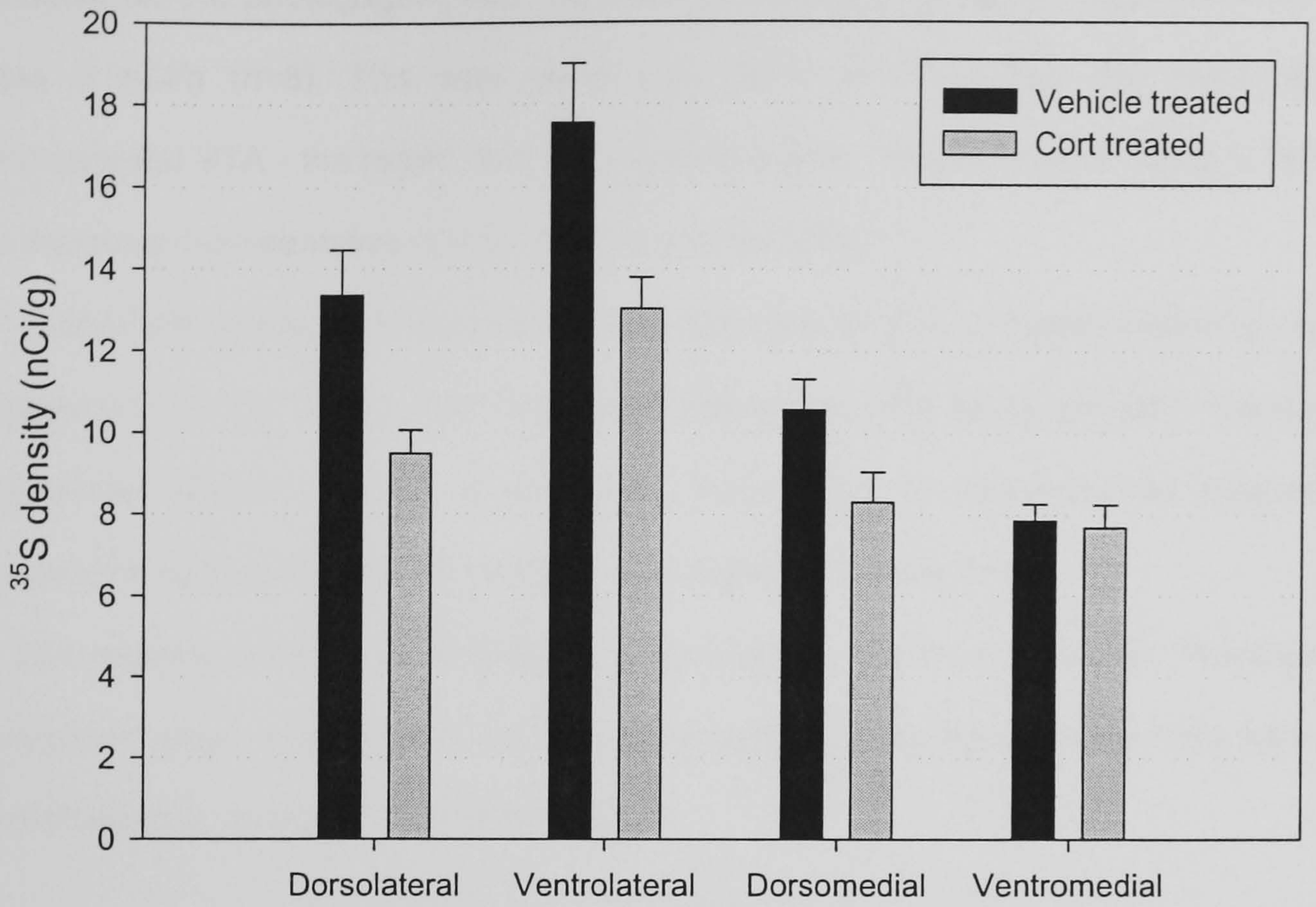

Figure 4.20. 5-HT_{2C} mRNA signal in subregions of the VTA in vehicle (n=8) and corticosterone (n=8) treated animals.

	Lateral		**Medial**	
	Vehicle	Cort	Vehicle	Cort
Dorsal	13.3 ± 9.8 nCi/g	9.5 ± 2.5 nCi/g	10.6 ± 4.5 nCi/g	8.3 ± 4.4 nCi/g
Ventral	17.6 ± 1.4 nCi/g	13.0 ± 0.8 nCi/g	7.9 ± 0.4 nCi/g	7.7 ± 0.6 nCi/g
	Main effect of treatment		$F_{[1,13]} = 9.4$	$p<0.01$
	Main effect of VTA sub-region		$F_{[3,39]} = 50.2$	$p<0.001$
	Treatment x sub-region interaction		$F_{[3,39]} = 3.2$	$p<0.05$

Table 4.11. Two-way repeated measures ANOVA of 5-HT_{2C} mRNA signal in sub-regions of the VTA.

Due to the low level of expression in the VTA measurements were made of background signal present on the photographic film. On average, background signal adjacent to tissue sections was 2 nCi/g (n=8). This was significantly lower ($p<0.01$) than the mean signal in the dorsomedial VTA - the region with the weakest signal. The measured signal in tissue sections is therefore representative of mRNA binding in the VTA.

Across the whole VTA there was 23% decrease in 5-HT_{2C} signal following corticosterone treatment (vehicle: 10.9 ± 0.4 nCi/g, cort treated: 8.4 ± 0.4 nCi/g; $p<0.05$). Two-way repeated measures ANOVA showed a significant main effect of corticosterone treatment and an interaction between treatment and VTA sub-region (see table 4.11.).

Sub-regional effects of corticosterone were tested using Student's t-test. This demonstrated a consistent effect in all regions except for the ventromedial region where there was no effect of corticosterone treatment (see table 4.12).

	Lateral	**Medial**
Dorsal	29% decrease $p<0.01$	21% decrease $p<0.05$
Ventral	26% decrease $p<0.05$	2% decrease n.s.

Table 4.12. Regional differences in the effect of corticosterone on 5-HT_{2C} mRNA expression in the VTA.

4.4. Discussion

4.4.1. Principal Findings

This chapter describes the effects of a subtle modulation of corticosteroid secretion, designed to mimic endocrine abnormalities seen in bipolar disorder, on the transcription of mRNA for key proteins involved in dopaminergic neurone function in the VTA. In-situ hybridisation histochemistry using ^{35}S labelled oligonucleotide probes and optical densitometry of exposed films was used to measure this effect. It was found that corticosteroid treated animals had significantly increased levels of mRNA for the dopamine synthetic enzyme tyrosine hydroxylase, the vesicular monoamine transporter (VMAT2) responsible for dopamine uptake into synaptic vesicles, and the dopamine D_2 autoreceptor. In addition corticosterone treated animals demonstrated lower mRNA expression for the GluR1 subunit of the excitatory AMPA receptor, and the inhibitory 5-HT_{2C} receptor (see table 4.13.).

Probe	***Effect of cort on mRNA transcription***
TH	14% ↑
VMAT2	13% ↑
DAT	-
MAO-A	-
D_2	19% ↑
NMDAR1	-
GluR1	15% ↓
5-HT_{2C}	23% ↓

Table 4.13. The effect of corticosterone on mRNA transcription in the VTA as a whole.

To confirm the specificity of the probes used, the patterns of expression were compared to those seen in other studies in the literature. In all cases mRNA distributions were consistent with previous reports (see table 4.14. for references).

mRNA Probe	
TH	Nicot et al. 1995 Lucas et al. 1998 Palkovits et al. 1999 Dietz et al. 2005 Shepard et al. 2005
VMAT2	Hoffmann et al. 1998 Cordeiro et al. 2002 Schwartz et al. 2003
DAT	Letchworth et al. 1999 Romero-Ramos et al., 2000 Lipska et al. 2003 Dietz et al. 2005 Shepard et al. 2005
MAO-A	Luque et al. 1995
D_2	Aubert et al. 1997 Stefanski et al. 2007
NMDAR1	Laurie and Seeburg, 1994 Fitzgerald et al. 1995 Paquet et al. 1997 Rafiki et al. 1998 Lu et al. 1999 Guilarte et al. 2000;
GluR1	Brene et al. 1998 Grignaschi et al. 2004
5-HT$_{2C}$	Holmes et al. 1995 Eberle-Wang et al. 1997 Huang et al. 2006

Table 4.14. Literature references for comparison of mRNA distributions.

It is interesting to note that whilst for the most part mRNA coding for proteins intrinsically involved in dopaminergic neurotransmission (specifically TH, VMAT2, DAT, and the D_2 receptor) was restricted to the dopaminergic nuclei of the VTA and substantia nigra, intense VMAT2 expression was also observed in the mamillary nucleus. This may reflect the presence of histaminergic neurones in this region (see Erickson et al. 1995) which employ VMAT2 for vesicular uptake (Erickson et al. 1995; Hofmann et al. 1998).

In contrast to the above probes, mRNA coding for the glutamate receptor subunits NMDAR1 and GluR1, and the 5-HT_{2C} receptor demonstrated a much wider distribution within the brain sections. Particularly intense expression was seen with all three probes in the hippocampus, with expression in most other regions throughout the midbrain. In the case of the NMDAR1 and GluR1 mRNAs this may be explained by the ubiquity of glutamatergic signalling throughout the brain. The widespread distribution of 5-HT_{2C} receptor mRNA also suggests that serotonergic signalling is present in a wide variety of regions at this level of the midbrain.

Differences in mRNA expression between sub-regions of the VTA were found with all probes except the NMDA receptor subunit 1. These differences in expression levels show a general pattern towards a lateral-medial gradient with higher expression in the ventrolateral VTA and lower levels in the dorsomedial VTA for most probes. An interesting exception to this was the tyrosine hydroxylase probe whose binding was highest in the ventromedial region, in contrast to all other probes. There is little evidence from this study that other characteristic dopaminergic cell components (such as VMAT2, DAT and D_2 receptor) follow a similar pattern of expression to TH in the VTA. This suggests that regional variations do not relate to dopaminergic cell density as this would have led to similar patterns of expression.

One possible explanation is that mRNA expression differs between dopaminergic cells projecting to different regions of the brain. For example, D_2 mRNA levels were lowest in the dorsomedial VTA and highest in the lateral VTA (dorsal and ventral regions). As there is evidence to suggest that projections to the nucleus accumbens arise in the ventrolateral VTA, whilst ventral prefrontocortical projections have been suggested to arise in the dorsomedial (but

not the ventrolateral) VTA, the differences in mRNA expression may relate to differences in D_2 autoreceptor function between these projections (see chapter 1 section 3.5.).

For both GluR1 and 5-HT_{2C} mRNA's there was a tendency for higher expression in lateral regions of the VTA. It is possible that for these probes in particular the differences reflect a higher density of innervation by glutamatergic and serotonergic afferents (respectively) in the lateral portion of the VTA. Alternatively, the fact that these receptors may be present on other cell types in the VTA suggests that the results could reflect cell density or expression levels in non-dopaminergic cells.

Overall the differences in mRNA expression between subregions are not discussed in detail principally because this chapter is concerned with the effect of corticosteroids on mRNA expression. Whilst it is interesting that basal levels of expression differ. it is beyond the scope of this study to explain why these differences occur or to determine the functional consequences of such differences.

For some of the probes (D_2, GluR1 and 5-HT_{2C}) sub-regional differences in the magnitude of the corticosteroid effect were found (see table 4.15.). These are discussed in greater detail in the following sections, however it is important to note that whilst the magnitude of the effect varied, an increase in one subregion and a decrease in another were never observed. Thus transcriptional regulation does not fundamentally differ between VTA subregions.

Probe	***Dorsolateral VTA***	***Ventrolateral VTA***	***Dorsomedial VTA***	***Ventromedial VTA***
D_2	5% ↑	26% ↑ *	17% ↑ **	25% ↑ *
GluR1	29% ↓ ***	14% ↓ *	18% ↓ *	19% ↓ *
5-HT2c	29% ↓ **	26% ↓ *	21% ↓ *	2% ↓

Table 4.15. Subregional effects of corticosterone administration on mRNA expression in the VTA. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

4.4.2. Effect of corticosterone administration on tyrosine hydroxylase mRNA expression

Tyrosine hydroxylase is the rate limiting enzyme in catecholamine synthesis and a major determinant of intracellular dopamine availability. The results of this study show that flattening of the corticosteroid rhythm with exogenous corticosterone elevates the expression of mRNA for tyrosine hydroxylase by 14% in the VTA.

The fact that TH mRNA transcription can be modulated by corticosteroids is supported by a number of studies in the literature. Czyrak et al (2003) claimed to find an increase in TH mRNA in the rat VTA with chronic corticosterone treatment (although as yet the original data has not been published). Perhaps more convincingly, increases in TH transcription have been demonstrated in the rat adrenal medulla and in pheochromocytoma cell lines following administration of corticosteroids (Baetge et al. 1981; Tank et al. 1986; Kumai et al. 2000). This upregulation of TH transcription by corticosteroids is consistent with a large body of work looking at the interaction between stress and TH transcription. Such studies may be taken as indirect evidence for corticosteroid regulation of transcription on the basis that corticosteroid secretion is increased by stress. The majority of this work has been carried out in peripheral adrenergic and central noradrenergic systems where a variety of different stressors have been shown to increase transcription of tyrosine hydroxylase in the adrenal medulla and sympathetic ganglia (reviewed by Sabban and Kvetnansky, 2001; Stachoviak et al. 1986) and in the rat locus coeruleus (the major noradrenergic nucleus in the brain) (Mamalaki et al. 1992; Wang et al. 1998; Serova et al. 1999; Chang et al. 2000; Sabban and Kvetnansky, 2001). Only one study has looked at stressor effects on TH transcription in midbrain dopaminergic areas. Similar to the studies discussed above, it was shown that acute and chronic immobilisation stress produces an increase in TH mRNA compared to controls in the rat VTA (Serova et al. 1999).

Although the present study did not investigate the mechanism by which corticosteroids induce TH transcription, Hagerty et al. (2001a and 2001b) have demonstrated the presence of a GRE in the promoter region of the mouse tyrosine hydroxylase gene. This GRE has been shown to be sufficient to confer maximal positive transcriptional regulation by glucocorticoids. As the mouse TH gene (L22651) and the rat TH gene (NM_009377) are homologous (BLAST search www.ncbi.nlm.nih.gov/BLAST/) this GRE is likely to be present in the rat TH gene also. This

suggests that the corticosteroid effect on TH transcription is produced directly via binding of GR to the gene.

Whilst previous studies have established the principle that TH is regulated by corticosteroids, and identified a potential mechanism, the present studies provide the first evidence that a subtle manipulation of corticosteroid levels modelling the flattened circadian rhythm seen in bipolar disorder can also alter TH mRNA transcription in the VTA.

4.4.3. Effect of corticosterone administration on VMAT2 mRNA expression

VMAT2 is the vesicular transporter responsible for the uptake of dopamine into synaptic vesicles and plays a role in determining the magnitude of dopamine release at the synapse. This study showed that elevating the nadir in the diurnal corticosteroid rhythm produced a 13% increase in VMAT2 mRNA levels in the VTA.

Although the effect of corticosteroids on VMAT2 mRNA expression has not previously been examined, one study looking at the effects of long-term repeated immobilisation stress and novel stressors showed an increase in VMAT2 mRNA in the medullary noradrenergic cell groups A1 and A2 (Rusnak et al. 2001). This provides indirect evidence that corticosteroids can increase VMAT2 mRNA and so may influence vesicular uptake. Interestingly, chronic administration of dexamethasone has been shown to increase the catecholamine content of synaptic vesicles in the carotid body (Hellstrom and Koslow, 1975), and it is possible that this increase in catecholamine storage is a result of corticosteroid induced VMAT2 transcription.

Whilst the above studies, and the present data, suggest that elevated corticosteroid levels facilitate VMAT2 transcription and function, this is put into question by published data showing a reduction in VMAT2 binding in the mesolimbic terminal regions of the nucleus accumbens and striatum following repeated restraint stress (Zucker et al. 2005). It is important to note however that stress paradigms provide only indirect evidence for corticosteroid mediated effects. Furthermore it is possible that this decrease in binding is due to a post-transcriptional effect i.e. a reduction in protein synthesis, a change in post-translational modification, or a reduction in transporter binding induced by protein phosphorylation. The discrepancy between changes in mRNA levels and levels of protein (see Gygi et al.1999) is a problem when trying to extrapolate

functional consequences from in-situ hybridisation data. In the case of corticosteroids, as it is likely that their effects are mediated by changes in gene transcription, it is of value to know that changes in mRNA levels occur.

The present data suggest that, as a consequence of flattening the corticosteroid rhythm, there is the potential for VMAT2 protein levels and VMAT2 function to be increased in dopaminergic neurones. It is the case however that further studies are needed to see whether the increase in mRNA transcription is mirrored by an increase in protein levels and transporter function (see chapter 5.).

Whilst the effects on VMAT2 gene transcription seen in this study may result from a direct effect of corticosterone, it is possible that the induction of VMAT2 transcription was secondary to increased tyrosine hydroxylase transcription and increased dopamine synthesis. It would be unsurprising to find that vesicular uptake capacity increases with synthetic capacity to allow the surplus dopamine to be utilised. Conversely increased VMAT2 activity (resulting from increased transcription) may have induced the transcription of TH. Close coupling of tyrosine hydroxylase and vesicular uptake has been demonstrated by Chen et al. (2003), who showed that disrupting the proton gradient of synaptic vesicles (required for VMAT2 activity) abolishes the ability of ATP to increase TH activity. Increased VMAT2 activity might also be expected to decrease cytoplasmic dopamine concentrations, which in turn could reduce end product inhibition of TH activity. It is possible that functional interactions between these proteins might affect transcription of their respective genes.

When the increase in VMAT2 throughout the whole VTA is compared with the effect on TH expression it can be seen that they parallel one another, with a 14% increase in TH expression and a 13% increase in VMAT2. It is therefore tempting to suggest that VMAT2 transcription is in some way linked to TH mRNA levels. It is the case, however, that in vehicle treated animals the pattern of TH expression across VTA subregions did not parallel that of VMAT2. For example, the highest subregional expression of TH occurred in the ventromedial VTA, whilst for VMAT2 the highest expression was seen in the dorsolateral VTA. Whilst this is a significant caveat, it is also true that the effect of corticosteroid treatment did not differ across VTA subregions for

either probe. Thus the effect of corticosteroid treatment on TH and VMAT2 transcription was parallel in all VTA subregions.

Concomitant changes in TH and VMAT2 protein levels in the mouse striatum have been observed following prenatal heptachlor treatment (which increased protein levels; Caudle et al. 2005) and in iron regulatory protein knockout mice (which decreased protein levels in the ventral striatum only; Salvatore et al. 2005). It is possible however that the decreases observed by Salvatore et al. (2005) were the result of dopaminergic cell loss rather than transcriptional/translational regulation. Long term immobilisation stress has been shown to increase TH and VMAT2 mRNA in the A1 and A2 noradrenergic cell groups (Rusnak et al. 2001). It is significant however that in the locus coeruleus an increase was found in TH mRNA but not VMAT2 mRNA (Rusnak et al. 2001). Similarly Bergzon et al. (1999) found that kainic acid seizures transiently increase TH mRNA in the locus coeruleus but not VMAT2 mRNA. The relevance of this last study may be limited however by the fact that the transient increase occurred 48 hours after treatment and had normalised to basal levels after two weeks. It may be the case that VMAT2 transcriptional induction requires a long term change in TH mRNA levels and thus wasn't seen by Bergzon et al. (1999). To summarise there is not currently enough evidence in the present study, or the literature to support the theory that the changes in TH and VMAT2 mRNA are directly linked. Thus whilst it is an interesting observation, further work is required to determine the degree of transcriptional and functional coupling between TH and VMAT2.

4.4.4. Effect of corticosterone administration on dopamine transporter mRNA expression

The dopamine transporter is an important regulator of dopaminergic neurotransmission, the activity of which determines the time course of dopamine removal from the synapse and its ability to diffuse to postsynaptic sites. No change in dopamine transporter mRNA expression as a consequence of corticosterone administration was seen in this study. In the literature, the effect of corticosteroids and stress on DAT mRNA expression and function appear to be complex and vary by brain region. Although one study found an increase in midbrain (no distinction made between A8, A9 and A10 cell groups) DAT mRNA in response to repeated

restraint stress (Copeland et al. 2005), GR antisense partial knockout in rats has been shown to be without effect on DAT mRNA in the substantia nigra (Cyr et al. 2001)

Of some relevance to the present study are the findings of Filipenko et al. (2001) who have shown a positive correlation between increases in TH mRNA and DAT mRNA levels in the rat VTA in winners of repeated social victory experiments, suggesting that their expression is in some way linked. The present data showing an increase in TH mRNA following corticosteroid administration but no effect on DAT mRNA, suggests that the effect on DAT mRNA seen by Filipenko et al. was not corticosteroid mediated – indeed it might be expected that circulating corticosteroid levels would be higher in the losers of social victory experiments due to the stressful nature of the defeat, and therefore Filipenko's data may not be directly comparable with the results of the present study. Studies looking at DAT protein levels in the mouse striatum have found that TH, VMAT2 and DAT increase in animals prenatally exposed to heptachlor (Caudle et al. 2005) whilst iron regulatory protein knockout mice have decreased levels of these proteins in the ventral striatum (Salvatore et al. 2005). This provides further evidence that the expression of these proteins is linked, however the present data suggests no interaction at the transcriptional level between these proteins. Furthermore as discussed in section 4.4.3. the data obtained by Salvatore et al. (2005) may reflect a loss of nigrostriatal DA neurones rather than a co-regulatory mechanism. Thus there is little evidence to suggest that TH transcription affects DAT transcription as a secondary effect of corticosterone treatment.

No evidence for a genomic modulation of VTA DAT expression was found in the present study following flattening of the corticosteroid rhythm. Furthermore the literature provides scant evidence for a corticosteroid mediated effect on DAT protein expression given that the effect differs by stress paradigm.

4.4.5 Effect of corticosterone administration on MAO-A mRNA expression

MAO-A is present within dopaminergic terminals and is the first enzyme in the intraneuronal metabolic pathway for dopamine. The majority of dopamine metabolism occurs by this intraneuronal route (see chapter 1) and as a consequence MAO-A activity critically affects cytoplasmic dopamine availability and turnover. No effect of corticosteroid administration on

MAO-A mRNA transcription was seen in this study. This is surprising as a glucocorticoid response element (GRE) has been identified in the promoter region of the human MAO-A gene which upon GR binding promotes MAO-A catalytic activity in human neuroblastoma and glioblastoma cell lines (Ou et al. 2006). Furthermore, glucocorticoid receptors promote MAO-A transcription indirectly via an interaction with R1/SP1 transcription factor binding at SP1 binding sites in neuroblastoma and UW228 cell lines (Ou et al. 2006). It is possible that the subtle corticosteroid manipulation used in the present study was insufficient to replicate these effects.

There is some evidence to suggest that glucocorticoid regulation of MAO-A transcription is tissue specific. Thus one study has shown that MAO-A transcription is increased in human skeletal muscle cells following dexamethasone treatment (Manoli et al. 2005). In contrast, work carried out by Lindley et al. (2005) in the rat brain has shown that neither adrenalectomy nor adrenalectomy followed by sustained corticosteroid administration has any effect on MAO-A expression in the medial prefrontal cortex or striatum. As MAO-A expression in the prefrontal cortex is at least partially dependent on MAO-A gene transcription in the VTA (via the mesocortical projection), with the rest likely to come from raphe serotonergic and locus coeruleus noradrenergic neurones which also contain corticosteroid receptors, the results of this work are in concordance with the results of the present study. The interaction of the glucocorticoid receptor with other transcriptional factors demonstrated by Ou et al. (2006) may offer an explanation as to how this tissue specificity could arise, as these necessary transcription factors may be absent in mesocorticolimbic dopaminergic cells.

4.4.6. Effect of corticosterone administration on D_2 receptor mRNA expression

D_2 mRNA in the VTA codes for the dopamine D_2 receptor which is localised to dopaminergic cell bodies in the VTA and terminals in forebrain areas, where it acts as an autoreceptor. D_2 mRNA is also expressed by non-dopaminergic cells in forebrain areas where it codes for post-synaptic receptors. Corticosteroid administration was found to increase the expression of D_2 mRNA in the VTA by 19% on average. This effect was greater in the ventral VTA than the dorsal VTA, and no effect was seen in the dorsolateral VTA.

There is some evidence in the literature to support a role for glucocorticoids in transcriptional regulation of D_2 mRNA. One study in which high levels of corticosterone were administered to rats via pellets (resulting in a >600% increase in plasma corticosterone levels compared to sham treated rats) found that D_2 mRNA transcription decreased in the striatum but not in the nucleus accumbens shell or core (Lammers et al. 1999). The relevance of the mRNA data in this study is limited by the fact that D_2 mRNA in terminal regions is not located in dopaminergic cells, however it does offer evidence that D_2 transcription can be regulated by corticosteroids. Indirect evidence for corticosteroid regulation of D_2 transcription in the VTA is provided by Dziedzicka-Wasylewska et al. (1997) who showed a selective decrease in D_2 mRNA in the lateral VTA following chronic stress. That the effect of chronic stress was opposite to the effect seen with administration of corticosteroids in the present study may reflect the fact that chronic stress effects are unlikely to be mediated solely by corticosteroids.

In support of the present data a GRE has been found in the proximal part of the promoter region of the D_2 gene however it appears to be under complex regulatory control (Lammers et al. 1999). The D_2 gene expressing the full endogenous promoter is not induced by dexamethasone in cultured neuroblastoma cells (Lammers et al. 1999) or primary striatal cultures (Valdenaire et al. 1998), although a truncated form of the promoter region did show an upregulation of transcriptional activity with dexamethasone in the study by Lammers et al. (1999). This truncated form of the gene did not include the GRE, however, suggesting that the effect of dexamethasone was indirect. It is possible that in the endogenous gene co-regulatory factors are responsible for inhibiting dexamethasone induced transcription in both the neuroblastoma and striatal cells. Such factors may not be present in VTA DA cells which might explain the upregulation of transcription observed in the present study.

An alternative explanation is that increases in D_2 message in the VTA are indirectly modulated by corticosteroids. If dopamine release parallels the increases in TH and VMAT2 mRNA induced by corticosteroid administration, D_2 transcription may increase as a result of elevated extracellular dopamine levels to provide compensatory feedback inhibition of dopaminergic neurotransmission. Such an increase in VTA D_2 receptor transcription has been observed following chronic treatment with the NMDA receptor antagonist MK-801 (Healy and Meador-

Woodruff, 1996) which stimulates dopamine release in mesocorticolimbic terminal regions (reviewed by Svensson, 2000).

In summary, flattening the diurnal rhythm of corticosteroid secretion resulted in an increase in D_2 mRNA in the VTA. This effect was greater in the ventral VTA than the dorsal VTA, with no effect seen in the dorsolateral VTA. Relating this to the topography of dopaminergic projections in the VTA (which is still unclear, see section 4.1.1.) suggests that mesolimbic projections originating in the ventral VTA (such as those terminating in the nucleus accumbens) will see a greater increase in D_2 transcription than mesocortical projections located in the dorsal VTA (such as those projecting to the prefrontal cortex).

4.4.7. Effect of corticosterone administration on NMDAR1 expression

The NMDA receptor is an important regulator of the firing rate of dopamine neurones in the VTA, and mediates the switch to burst firing which leads to increased efficiency of dopamine release in terminal regions (Charlety et al. 1991; Chergui et al. 1993). The NMDAR1 subunit of this receptor contains both the glutamate and glycine binding sites and is essential for the formation of functional receptors (Kew and Kemp, 2005).

No effect of corticosteroid administration was found on NMDAR1 expression in any of the regions of the VTA. Although one study has shown that NMDAR1 mRNA expression is decreased in the paraventricular nucleus following corticosteroid administration (Lee et al. 2003), the same study and others have shown no effect on NMDAR1 transcription in the hippocampus (Kamphuis et al. 2003; Lee et al. 2003; Weiland et al. 1997). This suggests that the effects of corticosteroids are regionally selective. As far as studies in the VTA go, it has been shown that voluntary wheel running, which induces increases in corticosteroid secretion, has no effect on NMDAR1 subunit expression (Makatsori et al. 2003). Thus the lack of effect on NMDAR1 transcription shown in the present study is consistent with previous data.

In the context of this body of literature, the results of the present study show that NMDAR1 subunit protein expression in the VTA is unlikely to be affected by changes to the corticosteroid diurnal rhythm seen in affective disorders, however changes in other subunits might occur which alter receptor function. The NMDAR1 subunit was chosen as it contains the ligand

recognition and glycine binding sites for the receptor complex making the functional consequences of changes in transcription somewhat more predictable than with other subunits. NMDAR2 subunit expression has been shown to be increased in the hippocampus following corticosteroid treatment (Kamphuis et al. 2003; Lee et al. 2003; Weiland et al. 1997), however without an increase in NMDAR1 subunit expression the number of functional receptors would not be expected to increase. It is possible, however, that changes in ion channel permeability or kinetic properties occur as a result of increased NMDAR2 expression. The data from the present study does not preclude the possibility that this occurs in the VTA following changes to the corticosteroid diurnal rhythm seen in bipolar disorder.

4.4.8. Effect of corticosterone administration on GluR1 expression

The GluR1 protein is one of four subunits which can form the pentameric AMPA receptor and its presence favours the formation of a Ca^{2+} permeable channel. AMPA receptors have been implicated in mediating tonic excitatory input to mesocortical dopaminergic neurones. The GluR1 subunit is therefore an important regulator of AMPA receptor activity, and hence dopaminergic function.

A 15% decrease in GluR1 expression was found in the VTA as a result of corticosteroid administration. Subregional differences in the magnitude of the effect were found such that expression in the dorsolateral VTA was decreased by a significantly greater amount than other regions. The greater decrease in this region suggests that mesocortical efferents (such as those projecting to the prefrontal cortex; see section 4.1.) may experience a greater decrease in expression of GluR1 than those which project from other regions of the VTA, such as mesolimbic nucleus accumbens projection arising in the ventral VTA.

The negative effect of corticosteroid administration on GluR1 expression seen here broadly agrees with studies in the published literature. Thus the increase in corticosteroid secretion induced by voluntary wheel running has been shown to correlate with decreased GluR1 expression in the VTA (Makatsori et al. 2003). Functional changes in AMPA receptor mediated signal transduction have also been demonstrated with corticosteroid agonists found to reduce the response to AMPA agonists and antagonists in the nucleus tractus solitarius, an effect

which was shown to be GR mediated (Shank and Scheuer, 2003). It is of note that MR mediated effects may be opposite to those mediated by GR as hippocampal GluR1 expression is reduced by adrenalectomy and this can be reversed with the MR agonist aldosterone (Watanabe et al. 2003). This positive mineralocorticoid effect on gene transcription would not be seen in studies involving levels of corticosteroids similar, or greater than, those seen under normal physiological conditions, as the mineralocorticoid receptor is thought to be close to maximally occupied under these conditions (Reul & De Kloet, 1985). As such the mineralocorticoid receptor mediated effect does not contradict the data from the present study suggesting that GR may downregulate AMPA receptor expression under conditions of excessive corticosteroid secretion. Thus in pathological conditions involving a flattened corticosteroid diurnal rhythm GluR1 expression may be downregulated, with the potential to reduce AMPA receptor mediated signalling.

4.4.9. Effect of corticosterone secretion on 5-HT_{2C} receptor expression

Corticosterone administration decreased 5-HT_{2C} receptor expression by 23% in the whole VTA. Subregional analysis revealed that this effect was confined to dorsolateral, dorsomedial, and ventrolateral regions. It may be significant that the ventromedial region showed the lowest level of expression of this mRNA. Given that levels of expression throughout the VTA were low it may be the case that the lack of effect in this region was the result of the signal being at the limits of discrimination from background. This was shown not to be the case however as background signal was much lower than the average signal in the ventromedial VTA suggesting that the result in this region was valid.

In the rat, studies have shown that adrenalectomy increases 5-HT_{2C} receptor mRNA expression in the hippocampus and this can be reversed by corticosteroid replacement (Holmes et al. 1995). In light of this work it is perhaps unsurprising that in the present study, administering corticosterone in the drinking water with the specific aim of elevating the diurnal nadir in corticosterone levels resulted in a decrease in 5-HT_{2C} receptor expression. Some work has been carried out looking at the functional effects of corticosteroids on 5-HT_{2C} receptors and the results of these studies agree with the data discussed so far. Thus in two studies short term

administration of dexamethasone (Tozawa et al. 1999) and chronic high doses of corticosterone (Berendsen et al. 1996) have been shown to reduce behavioural and biosynthetic responses to 5-HT_{2C} receptor stimulation. These data suggest that transcriptional changes in response to corticosteroids appear to be mirrored in functional changes.

As mentioned previously it appears that in the VTA the effect of corticosteroids on 5-HT_{2C} receptor transcription demonstrates some heterogeneity, with all areas but the ventromedial region showing a decrease in expression. Retrograde tracing studies (see section 4.1.) suggest that dopaminergic neurones from this region project to the nucleus accumbens but not to the prefrontal cortex. Thus corticosteroid 5-HT_{2C} transcriptional regulation may have less of an effect on this mesolimbic projection than, for example, prefrontocortically projecting mesocortical neurones originating in the dorsal VTA. Overall however the data indicate that flattening the corticosteroid rhythm decreases 5-HT_{2C} receptor mRNA transcription in most areas of the VTA.

4.4.9. Functional consequences in VTA dopaminergic projections

When predicting the consequences of alterations in mRNA transcription, as has already been mentioned, there is no linear relationship between changes in transcription and protein function (see Gygi et al. 1999). This means that any predictions are speculative and must allow for the possibility that post-transcriptional modifications and regulatory effects on protein function may occur downstream to modulate the effects of the changes in transcription. This can work to enhance the transcriptional effects so that a small increase in transcription results in a relatively greater change in protein function, or downstream events may counter the effect so that a relatively small or no change in protein function occurs. Notwithstanding this caveat, the following section discusses the possible functional implications of the mRNA results taken at face value. As discussed in section 4.1.1. an attempt has been made to relate any subregional differences in corticosteroid effects to functional consequences in mesocortical (prefrontal cortex) and mesolimbic (nucleus accumbens) projection systems.

Intrinsic Regulators

This study looked at transcription of mRNA sequences coding for proteins which can be classified into two groups. There are the proteins intrinsically involved in dopaminergic neuronal function (TH, VMAT2, DAT, MAO-A, and the D_2 receptor), and there are the proteins which mediate the modulation of dopaminergic function by external signals (the NMDA receptor, the AMPA receptor, and the 5-HT_{2C} receptor). In the first group, the effect of corticosteroid administration was an increase in tyrosine hydroxylase and VMAT2 transcription, with no effect on the dopamine transporter or MAO-A. Thus, if these transcriptional changes are mirrored by changes in protein levels the predicted result would be an increase in the intracellular dopamine concentration (as a result of increased TH activity), an increase in vesicular dopamine content (due to increased VMAT2 activity, see Pothos et al. 2000), and as a result, greater release of dopamine. In the absence of changes in DAT or MAO-A function this would lead to increased synaptic levels of dopamine. It is the case that no changes in dopamine transporter function or metabolic monoamine oxidase activity would be predicted from the present results.

In addition to changes in TH and VMAT2 transcription, corticosteroid treatment increased the transcription of D_2 mRNA. At the protein level such an increase would counter the hypothesised increase in dopamine release by upregulating somatodendritic and terminal autoinhibitory feedback mechanisms resulting in a decrease in neuronal firing rate and an inhibition of dopamine synthesis and release.

From the subregional pattern of corticosteroid effects on D_2 transcription such an increase in autoinhibtion would be predicted to occur to a greater extent in those neurones projecting from the ventral VTA (which may be mesolimbic neurones projecting to the nucleus accumbens; see section 4.1.) compared to those projecting from the dorsal VTA (which are suggested to be mesocortical neurones projecting to the prefrontal cortex). It is of particular note that no effect was seen in the dorsolateral VTA. This may "dilute" the effect of the increase in D_2 mRNA in the dorsomedial VTA as far as overall dopamine synthesis and release in cortical regions is concerned. Thus in terms of dopaminergic neurotransmission it is predicted that corticosteroid dysrhythmia increases D_2 autoinhibition in mesolimbic neurones by a greater extent than in mesocortical neurones.

Of further relevance to the dichotomy of mesocortical and mesolimbic projections is the suggestion that terminal (but not somatodendritic; see Westerink et al. 1996; 1998) D_2 autoreceptor inhibition is less efficient in the mesocortical system than in the mesolimbic system and indeed may not play a role in regulating dopamine synthesis and release under normal physiological conditions (see chapter 1 section 1.3.5.). There are therefore three possibilities regarding the effect of corticosteroid dysrhythmia on terminal autoreceptor function in these neurones:

1. The terminal D_2 receptor has no physiological role at mesocortical terminals. In this scenario dopamine synthesis and release will be restrained only by a reduced firing rate mediated by increased somatodendritic autoreceptor function in neurones originating in the dorsomedial VTA.
2. Under normal physiological conditions the terminal D_2 receptor does not function, but corticosteroid dysregulation increases D_2 mRNA transcription in mesocortical neurones bringing the terminal receptor "online". In this case the predicted increase in dopamine synthesis and release would be restrained in cortical regions by the terminal autoreceptor in conjunction with increased somatodendritic autoinhibition.
3. D_2 receptors are present and functional on mesocortical dopamine terminals. As a consequence the increase in D_2 mRNA transcription restrains the predicted increase in dopamine synthesis and release in mesocortical projection neurones via the terminal autoreceptor, and decreases the neuronal firing rate via the somatodendritic autoreceptor in a similar fashion to that predicted for the mesolimbic system.

In summary, it is hypothesised that corticosteroid dysrhythmia will increase dopamine synthesis and vesicular uptake in all dopaminergic neurones in the VTA. The tendency for these effects to increase dopamine release will be restrained by increased somatodendritic D_2 receptor inhibition of neuronal firing which will occur to a greater extent in the mesolimbic system than the mesocortical system. Furthermore the corticosteroid induced increase in terminal autoreceptor inhibition of synthesis and release is predicted to occur to a greater extent

in the mesolimbic system than in the mesocortical system, if indeed terminal autoreceptors are functional on mesocortical neurones. Thus, overall, any increase in dopamine release is likely to be greater in the prefrontal cortex than in the nucleus accumbens. Indeed it might be predicted that dopamine synthesis and release might decrease in the nucleus accumbens as a consequence of increased autoreceptor inhibition.

Extrinsic Regulators

The second group of mRNAs studied were those coding for proteins acting as receptors mediating afferent regulation of dopaminergic function in the VTA. The effects of corticosteroid administration on the transcription of mRNA for these proteins showed much subregional heterogeneity and, again, predicting the functional effects is complex.

The effect of corticosteroid administration on the GluR1 subunit of the AMPA receptor demonstrated some regional variation, with a much greater effect in the dorsolateral VTA than in all other regions. As AMPA receptors are excitatory on dopaminergic neurones (see Meltzer et al. 1997; Adell and Artigas, 2004) the inhibition of transcription is likely to result in a decrease in the firing rate, with the potential for a greater decrease in dorsolateral VTA neurones than neurones present in other areas. Thus prefrontocortical neurones present in the dorsal VTA may experience a greater decrease in tonic stimulation than, for example, mesolimbic neurones projecting from other regions of the VTA to the nucleus accumbens. Leaving aside the subregional differences, it has been suggested that prefrontocortical neurones are under tonic stimulation by AMPA receptors in contrast to neurones projecting to the nucleus accumbens (Westerink et al. 1996; 1998; Adell and Artigas, 2004) and hence the decrease observed is likely to affect mesocortical neurones to a greater extent.

The reduction in 5-HT_{2C} receptor transcription also displayed regional differences, with no effect in the ventromedial VTA and a similar effect in the dorsal and lateral regions. As this excitatory receptor is postulated to be present on GABAergic interneurones where it is under tonic stimulation (DiMatteo et al. 1999; DiMatteo et al. 2002; Gobert et al. 2000), a reduction in its transcription could result in the disinhibition of VTA dopaminergic neurones. Based on the suggestion that there may be topographical differences within the VTA regarding mesolimbic

and mesocortical DA projections, the effect of this may be to increase firing rate, and hence terminal release in the mesocortical projections arising from the dorsal tier of the VTA and the mesolimbic projections arising in the ventrolateral portion of the VTA. As the ventromedial region is populated by mesolimbic neurones projecting to the nucleus accumbens (see section 4.1.), it is likely that the decrease in firing rate would be "diluted" in mesoaccumbens neurones due to the lack of an effect in this region.

Overall the data suggest that a relatively subtle alteration of the corticosteroid rhythm could have important effects on VTA dopaminergic neurotransmission. Moreover topographical differences in transcriptional modulation suggest that on balance prefrontocortical projections are likely to experience a relatively greater increase in neurotransmission than mesolimbic projections. This has the potential to produce an imbalance in the mesocorticolimbic dopamine system which might have implications for pathophysiological states which exhibit flattened diurnal corticosteroid rhythmicity such as bipolar disorder.

4.4.10. Pathophysiological correlates of corticosteroid effects

The alterations in gene expression found in this study correlate well with a number of findings in mood disorders in which a flattening of the corticosteroid rhythm can be seen, principally bipolar disorder and major depression (particularly psychotic major depression) (see McQuade and Young, 2000; Rothschild, 2003). Regarding the observed increase in tyrosine hydroxylase expression, post-mortem analysis of brains from patients with major depression has shown elevations in tyrosine hydroxylase protein in the locus coeruleus consistent with the results of the present study (Zhu et al. 1999).

VMAT2 binding has been found to be elevated in bipolar disorder in both the thalamus and ventral midbrain compared to controls (Zubieta et al. 2002). With regards to major depression, elevations in platelet VMAT2 density have been observed with no changes in binding affinity (Zucker et al. 2002). These findings are consistent with the elevations in VMAT2 expression found in the present study.

The increase in D_2 receptor mRNA expression in the present study could be considered consistent with the therapeutic efficacy of antipsychotics as a component of combination therapies in the treatment of manic and depressive episodes (for reviews see Surja et al. 2006; Wijkastra et al. 2007). There is however little evidence that D_2 receptor expression is altered in bipolar disorder, where PET studies have shown no alterations in striatal D_2 binding in manic and euthymic bipolar subjects (Anand et al., 2000; Yatham et al. 2002). One PET study has shown increased D_2 binding in the basal ganglia of subjects with psychotic mania but no difference was found in non-psychotic manic subjects (Pearlson et al. 1995). Whilst no change in striatal D_2 binding has been found in bipolar subjects, binding in mesocorticolimbic terminal regions has not been examined. It therefore remains a possibility that receptor expression is altered in these regions in bipolar disorder.

In terms of the AMPA receptor, and specifically the GluR1 subunit, very little clinical work has been carried out. However in one study GluR1 transcripts were shown to be reduced in post-mortem brains of bipolar disorder patients but not unipolar depressives or schizophrenics (Meador-Woodruff et al. 2001).

Currently no work has been carried out to assess 5-HT_{2C} receptor binding in mood disorders however there is evidence for pre-mRNA editing of gene transcripts (via de-amination of adenosine residues) in the prefrontal cortex of depressed suicide patients (Gardiner and Du, 2006). If this effect is a consequence of elevated corticosteroid levels then the decreased in-situ binding seen in the present study may result from a lack of binding to edited transcripts rather than a decrease in transcription. As mRNA editing was not examined in this study it is unknown whether this effect takes place in corticosteroid treated animals.

Overall based on the clinical evidence the changes in mRNA transcription seen in the present study may also occur in bipolar and unipolar affective disorders. The present study offers strong evidence that the flattening of the corticosteroid rhythm seen in these affective disorders may underlie such changes.

4.5. Conclusion

The results of this study show that corticosteroid administration to mimic the flattening of the corticosteroid rhythm seen in bipolar disorder can alter transcription of a number of different genes coding for proteins in the VTA which act as important regulators of dopaminergic neurotransmission. Furthermore subregional differences in corticosteroid effects were found in the VTA which suggests there may be heterogeneous regulation of mesocortical and mesolimbic projection systems originating in the VTA. This has important implications in understanding the aetiology of mood disorders, and this is further supported by the fact that several of the observed changes in gene transcription are mirrored by altered protein function in those mood disorders which show characteristic alterations in HPA axis function, namely bipolar and unipolar depression.

The following chapter investigates the hypotheses developed here looking at tyrosine hydroxylase function in the prefrontal cortex and nucleus accumbens, and dopamine release in the prefrontal cortex following corticosterone treatment.

Chapter 5.

Effect of corticosterone administration on dopamine synthesis and release in mesocorticolimbic terminal regions

Chapter 5. Effect of corticosterone administration on dopamine synthesis and release in mesocorticolimbic terminal regions

5.1. Introduction

The results presented in chapter 4 suggest that altering the diurnal cortisol rhythm alters the transcription of genes coding for proteins involved in dopamine synthesis and release, and receptors which modulate dopamine synthesis and release. Specifically, in the rat VTA, increases were found in the transcription of tyrosine hydroxylase (the rate limiting enzyme in dopamine synthesis; Nagatsu et al. 1964), VMAT2 (the transporter responsible for vesicular uptake of dopamine; Hoffmann et al. 1998; Nirenberg et al. 1996, Peter et al. 1995) and the D_2 receptor (the impulse, release and synthesis modulating autoreceptor at dopaminergic cell somatodendritic sites and terminals; Centonze et al. 2002; Lindgren et al. 2003; O'Hara et al. 1996; White 1996).

Based on this data it was hypothesised that corticosteroid treatment would produce an increase in dopamine synthesis, vesicular uptake and release in mesocortical neurones projecting to the prefrontal cortex. The increase in D_2 receptor transcription was postulated to have limited impact on these functional effects due to its low efficacy in the prefrontal cortex and the lesser increase in D_2 mRNA found in areas of the VTA which have been suggested to project to this region. In contrast it was hypothesised that increased D_2 receptor transcription in mesolimbic neurones, induced by corticosteroid treatment, will augment autoinhibitory signalling such that the functional effects of increased TH and VMAT2 transcription on nucleus accumbens dopamine release would be attenuated.

To test these hypotheses a study was performed to investigate dopamine synthetic activity in the rat prefrontal cortex and compare it with that in the nucleus accumbens following two weeks of corticosterone administration. DOPA accumulation was measured in tissue homogenates of rats injected with a DOPA decarboxylase inhibitor prior to sacrifice to give a measure of tyrosine hydroxylase activity (see Lamensdorf et al. 1997; Lindley et al. 1999; Pacak et al. 2002; Pan et al. 2006).

Subsequently an *in vivo* microdialysis study was carried out to measure the effect of corticosterone treatment on the bupropion and potassium evoked efflux of dopamine in the rat

medial prefrontal cortex. Furthermore terminal D_2 receptor sensitivity was assessed by measuring bupropion and potassium evoked dopamine efflux in the presence of the D_2 receptor antagonist sulpiride to investigate autoreceptor function following corticosteroid treatment

Overall these experiments were designed to test the potential functional consequences on dopaminergic neuronal activity of an altered corticosterone rhythm similar to that seen in patients with bipolar disorder (see chapter 1).

5.1.1. Aims

The aims of this study were as follows:

1) To investigate whether the increase in tyrosine hydroxylase mRNA transcription produced by altering the diurnal corticosterone rhythm are reflected in changes in dopamine synthetic activity in the medial prefrontal cortex and nucleus accumbens.
2) To investigate the effect of altering the diurnal corticosterone rhythm on dopamine release and D_2 receptor sensitivity in the medial prefrontal cortex.

5.2. Methods

5.2.1. Animals

The 2-week corticosterone treatment protocol has been described in Chapter 2. Briefly, male Lister hooded rats (Charles River, UK) were treated for 14-16 days with drinking water supplemented with corticosterone (50mg/l) and 0.5% EtOH (n=8 for tyrosine hydroxylase assay, n=12 for in-vivo microdialysis). Control animals received drinking water containing 0.5% EtOH over the same period (n=8 tyrosine hydroxylase assay, n=12 for in-vivo microdialysis). Animal weights and water consumption were recorded during this period. In addition, upon sacrifice, adrenal glands were removed and weighed.

5.2.2. Drugs and chemicals

Tissue homogenate tyrosine hydroxylase assay

A solution of NSD1015 (Molekula, UK; 100mg/ml) was made up on the day of sacrifice. Pentobarbital (Dolethal, Univet, UK) was pre-prepared as a standard solution.

In vivo microdialysis

Stock aCSF and high K^+ aCSF were prepared at the start of the study with Ca^{2+} and glucose added on the day of the experiment (see Chapter 2 for full details).

A 10 mM DA (Sigma, UK) standard was prepared weekly in 0.1 M perchloric acid and stored at 4°C. Using this standard, serial dilutions were prepared daily using aCSF to gain DA standards at concentrations of 10^{-8} and 10^{-9} M. Solutions of bupropion (Sigma, UK; 3 mM) and sulpiride (Sigma, UK; 10 mM) were prepared daily using aCSF. A 50% w/v solution of urethane was prepared daily by dissolving in water for injection.

All drugs were administered via the perfused aCSF. Four types of aCSF were used during an experiment: (i) normal aCSF containing 30 µM bupropion (ii) high K^+ aCSF containing 30 µM bupropion (iii) normal aCSF containing 30 µM bupropion and 10 µM sulpiride (iv) high K^+ aCSF containing 30 µM bupropion and 10 µM sulpiride.

5.2.3. Tissue homogenate tyrosine hydroxylase assay

Following 2-weeks of corticosterone treatment, animals were injected on the morning of the fifteenth day with 100 mg/kg NSD1015 i.p. and returned to their home cage. Either 20 min or 2 hours later the animals were sacrificed with overdose of pentobarbital i.p., and the brain quickly removed. The medial prefrontal cortex and the nucleus accumbens were rapidly dissected out and placed in separate pre-weighed Eppendorf tubes containing 1ml of 0.1M perchloric acid. VTA tissue from the animals treated for 20 minutes with NSD1015 was used in the *in situ* hybridisation procedure detailed in chapter 2.

Forebrain tissue was homogenised using a sonicator (Soniprep, MSE UK) with the Eppendorf containing the homogenate suspended in an ice bath. Following complete homogenisation the solution was centrifuged and microfiltered. The resultant solution was diluted by a factor of 10 (in the case of mPFC tissue homogenates) or 20 (in the case of nucleus accumbens homogenates) in 0.1M perchloric acid. Samples were stored at 4°C for no longer than 24 hours until measurement of DOPA content was carried out using HPLC with electrochemical detection (see Chapter 2).

5.2.4. In vivo microdialysis

Following the 14 day corticosterone treatment protocol animals were anaesthetised and surgically implanted with microdialysis probes in the prefrontal cortex (see figure 5.1. for probe location and chapter 2 for full details). Dialysate samples were collected every twenty minutes for measurement of dopamine content using HPLC with electrochemical detection (see chapter 2 for full details).

Two experiments were performed, the first of which was carried out on naïve animals (n=5) to assess the repeatability of depolarisation evoked release in the mPFC using high K^+ aCSF. In this experiment, following attainment of steady dopamine levels with aCSF containing 30 µM buproprion, aCSF containing 100 mM K^+ was perfused for twenty minutes before switching back to the original perfusion medium. Following a period of 120 minutes a second switch to aCSF containing 100 mM K^+ was performed, again for twenty minutes, before returning to the original perfusion solution for the remainder of the experiment.

The second experiment was performed to investigate corticosterone modulation of dopamine release and autoreceptor function in the mPFC. Once a steady baseline was obtained with aCSF containing 30 µM bupropion, levels of dopamine were measured over a one hour period. Following this, depolarisation evoked release was induced by switching the perfusion to aCSF containing 100 mM K^+ and 30 µM buproprion for a period of 20 minutes. This was then followed by a period of no less than one hour to allow for the return of a steady baseline with the original aCSF. The perfused aCSF was then switched to one containing 10 µM sulpiride and 30µM buproprion. This was perfused for one hour and twenty minutes and was then switched for aCSF containing 100 mM K^+, 10 µM sulpiride and 30µM buproprion for a period of twenty minutes. Following this, normal aCSF containing sulpiride and buproprion was perfused for the remainder of the experiment.

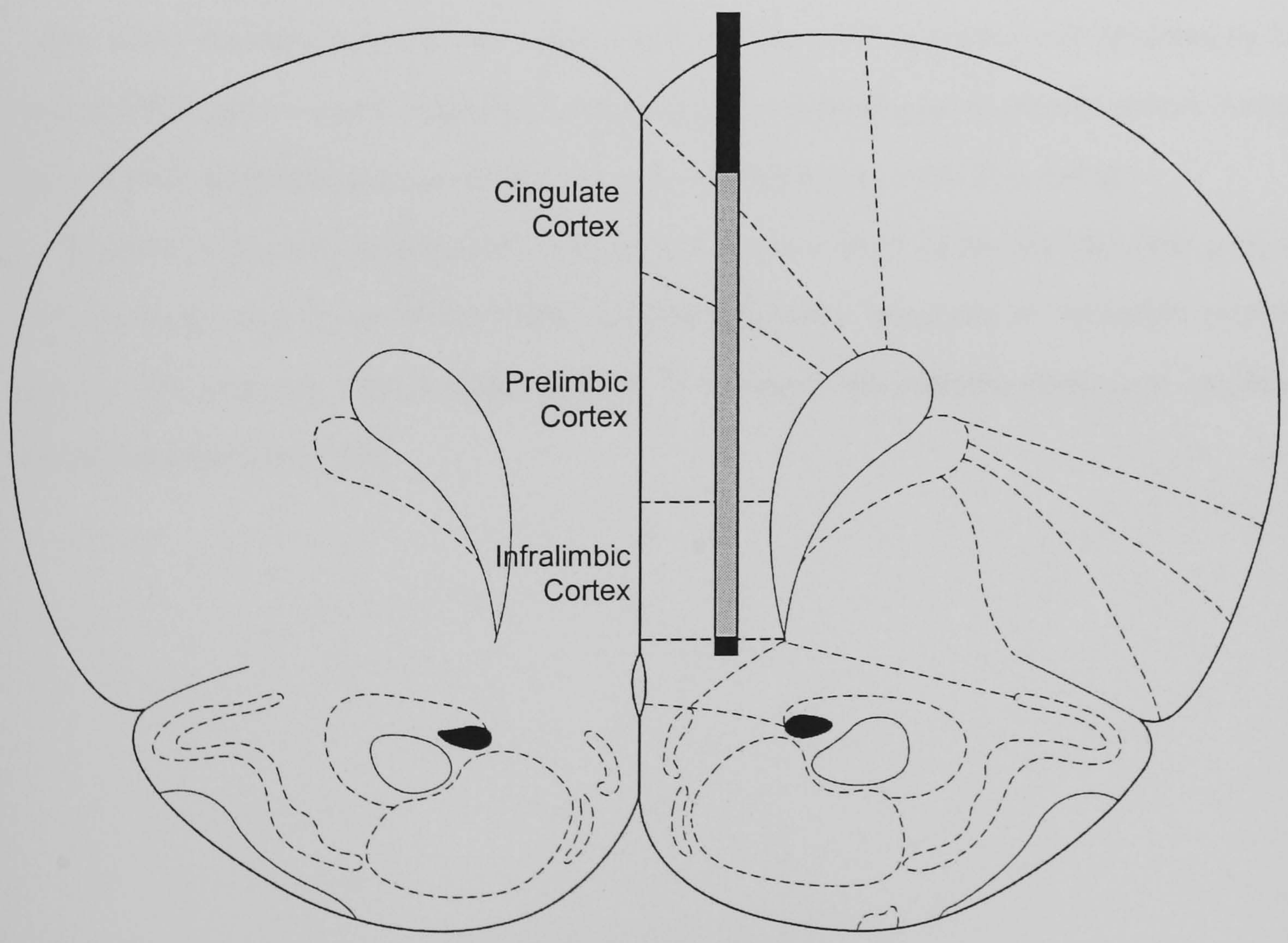

Figure 5.1. Location of dialysis probes in the medial prefrontal cortex (diagram from Paxinos and Watson, 1998). Distance from bregma; rostro-caudal = +3.0 mm, medial-lateral = -0.7 mm, dorsal-ventral = -5.5 mm. Grey region indicates dialysing "window".

5.2.5. Data analysis

Tissue homogenate tyrosine hydroxylase assay

DOPA concentrations were averaged across each treatment group in the study and Student's t-test was used to determine the statistical difference between groups. Where a non-normal distribution was found a Mann-Whitney U-test was carried out.

In-vivo microdialysis

For the first experiment assessing the repeatability of potassium evoked release, data was expressed as peak DA concentration and compared by Student's paired t-test.

To determine the effect of corticosterone on dopamine levels in the presence of bupropion data was averaged over a one hour period for each treatment group and between groups analysis performed by Student's unpaired t-test.

The effect of sulpiride on dopamine levels in the presence of bupropion was analysed by 2-way ANOVA with repeated measures comparing data averaged over treatment groups during the one hour application period with baseline data averaged over a one hour period.

The effect of sulpiride on potassium stimulated dopamine levels in the two treatment groups was analysed using 2-way ANOVA with repeated measures. Measures of potassium evoked release were peak DA concentration attained, % increase over baseline levels, and absolute increase in dopamine levels.

5.3. Results

5.3.1. Tyrosine hydroxylase activity in mesocortical and mesolimbic terminal regions following corticosterone administration

5.3.1.1. Corticosterone treatment

For details of animal weights and water consumption during corticosterone treatment and final adrenal weights see chapter 4 section 3.1.

5.3.1.2. Tyrosine hydroxylase activity in the medial prefrontal cortex

Tissue DOPA content in the medial prefrontal cortex was measured following both 20 minutes and 2 hours of NSD1015 treatment in vehicle and corticosterone-treated rats. DOPA concentrations 20 minutes after NSD1015 were undetectable by HPLC with electrochemical detection. When the experiment was repeated with 2 hours of NSD1015 treatment the mean DOPA accumulation was 93% greater in the corticosterone treated animals than control animals (1.1 ± 0.3 pmol/mg in vehicle treated animals, n=7; 2.1 ± 0.5 pmol/mg in corticosterone treated animals, n=7; see Figure 5.2.). A trend towards statistical significance was found with both Student's t-test ($p=0.07$) and Mann-Whitney U-test ($p=0.07$).

5.3.1.3. Tyrosine hydroxylase activity in the nucleus accumbens

Tissue DOPA content in the nucleus accumbens was measured following both 20 minutes and 2 hours of NSD1015 treatment in naïve and corticosterone treated rats. In both experiments DOPA concentrations were much higher than those found in the mPFC. Following 20 minutes of NSD1015, corticosterone treatment resulted in a 52% decrease in DOPA accumulation compared to control animals. Thus DOPA concentrations were 29 ± 5.3 pmol/mg in vehicle treated animals and 13.7 ± 2.6 pmol/mg in corticosterone treated animals (see Figure 5.3.). This difference was found to be statistically significant ($p<0.05$).

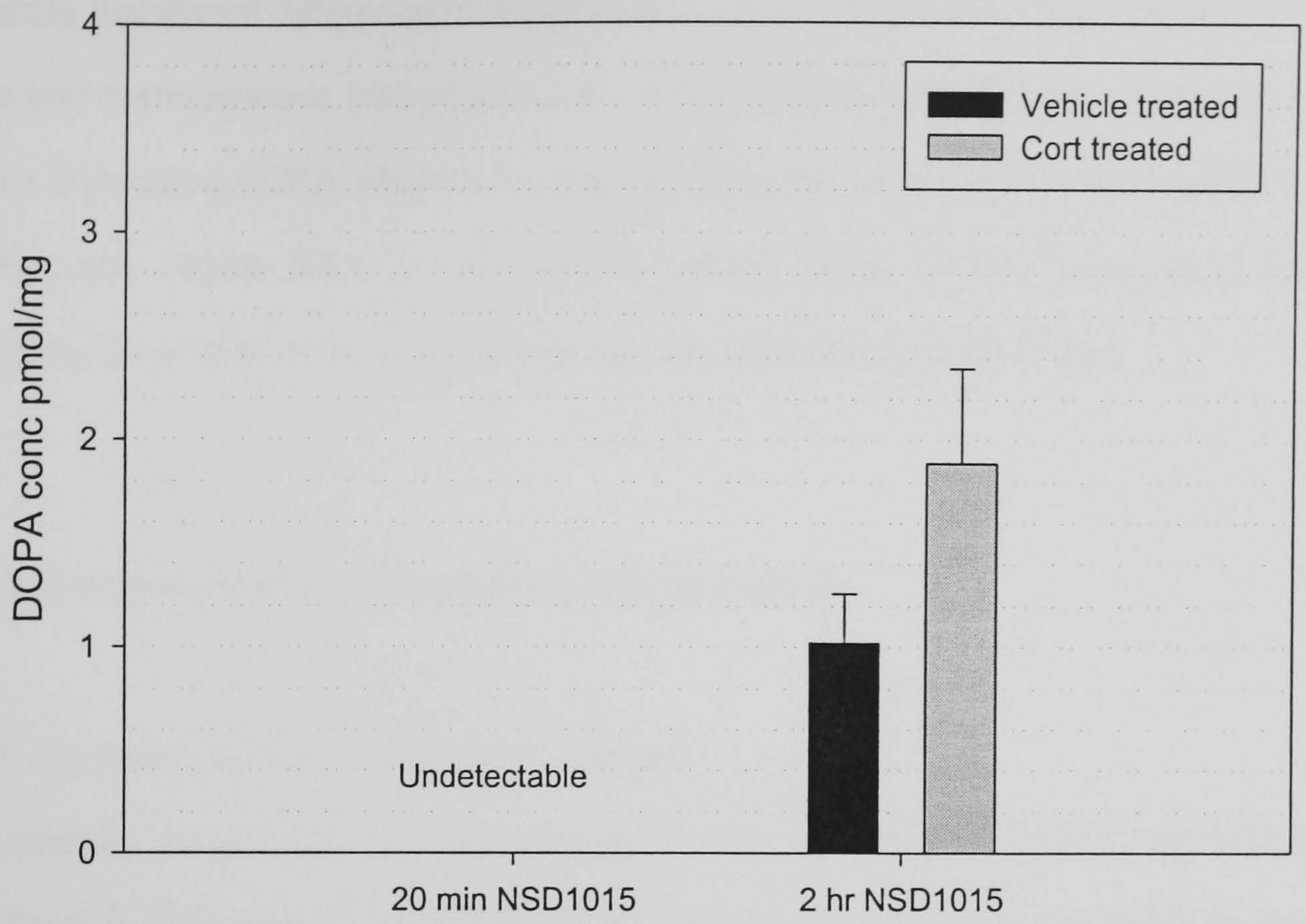

Figure 5.2. DOPA concentration in the mPFC after NSD1015 treatment in vehicle (20 min, n=8; 2hr, n=8) and corticosterone treated (20 min, n=8; 2hr, n=8) animals.

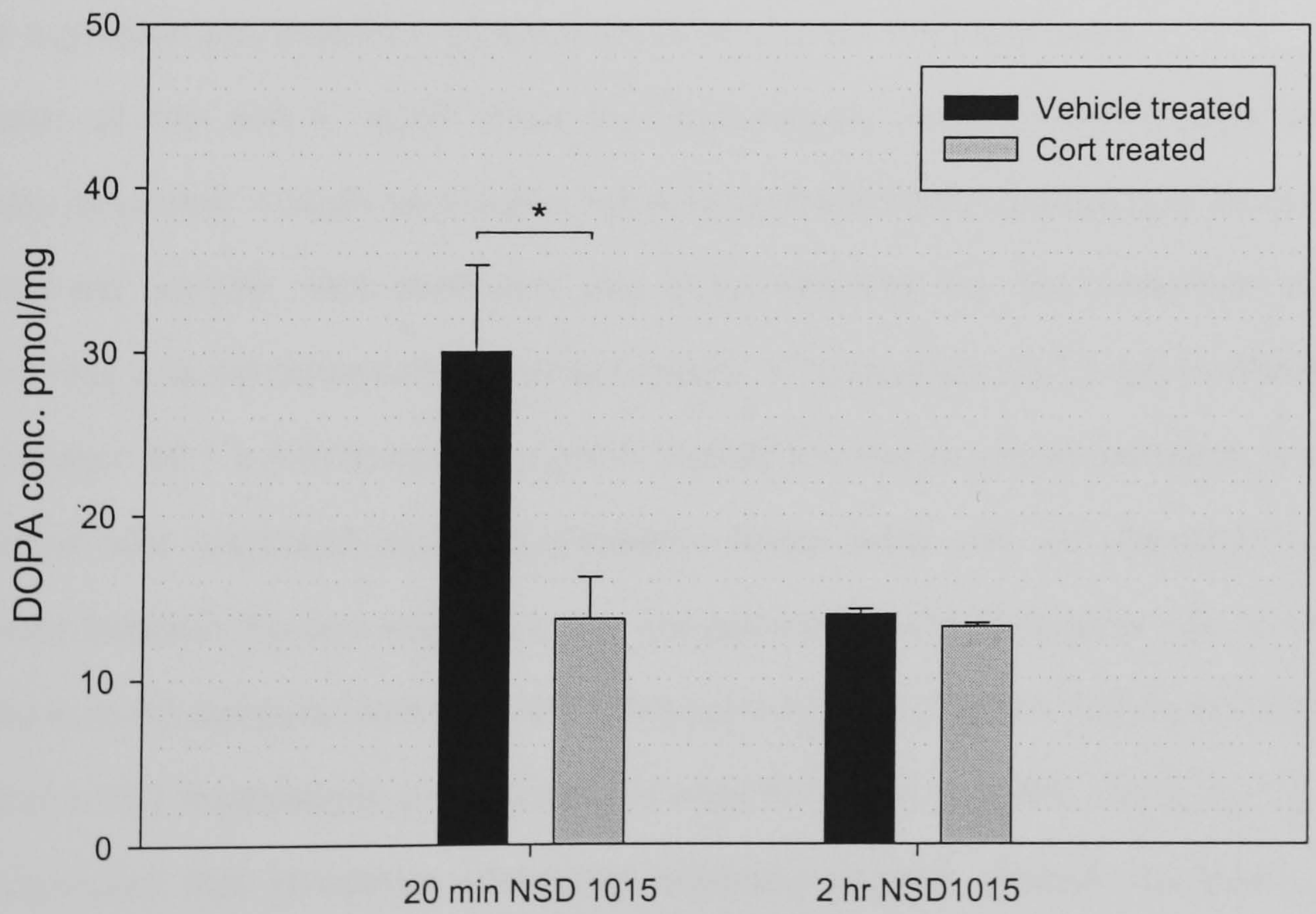

Figure 5.3. DOPA concentration in the nucleus accumbens after NSD1015 treatment in vehicle (20 min, n=7; 2hr, n=8) and corticosterone treated (20 min, n=8; 2hr, n=8) animals (* $p<0.05$).

When the experiment was repeated with a 2 hour post-NSD1015 survival period there was no statistically significant difference in nucleus accumbens tissue DOPA concentrations between vehicle and corticosterone treated animals. Vehicle treated animals had a tissue concentration of 13.9 ± 2 pmol/mg DOPA whilst corticosterone treated animals had a concentration of 13.2 ± 3 pmol/mg (see Figure 5.3.). In the vehicle treated group DOPA levels at 2 hours were significantly lower than at the 20 minute time-point ($p<0.05$ Student's t-test).

5.3.2. Dopamine release in the medial prefrontal cortex

5.3.2.1. Repeated potassium stimulated release

The planned experiments for measuring dopamine release in the mPFC required the use of two potassium stimulations during the course of an experiment, the aim being to examine the effect of a D_2 antagonist on stimulated dopamine release. To determine the validity of comparing these stimulations a preliminary study was carried out to determine if repeated stimulations are consistent. The time course of this experiment can be seen in figure 5.4. Note 30 µM bupropion was present in perfused aCSF throughout the experiment.

Infusion of 100 mM K^+ aCSF down the microdialysis probe evoked a large increase in dialysate dopamine. A small decrease in baseline (with bupropion) extracellular dopamine (- 9 ± 5.1 fmol per sample; 15% decrease) was found following the first potassium stimulation, however this was not statistically significant (before K^+ stimulation 59.1 ± 5.6 fmol/sample; after K^+ stimulation 50.1 ± 5.0 fmol/sample; $p>0.1$ Student's t- test for paired samples). A second K^+ stimulation also increased dialysate dopamine levels there was no statistically significant difference between the two responses, i.e. the potassium evoked release was no different in magnitude at K2 compared to K1 (mean difference 9.4 ± 16.6 fmol; K1 166.4 ± 6.8 fmol/sample; K2 175.8 ± 13.3 fmol/sample; $p>0.5$ Student's t-test for paired samples; see figure 5.5.). Thus it was concluded that potassium stimulated dopamine release remains consistent over two applications.

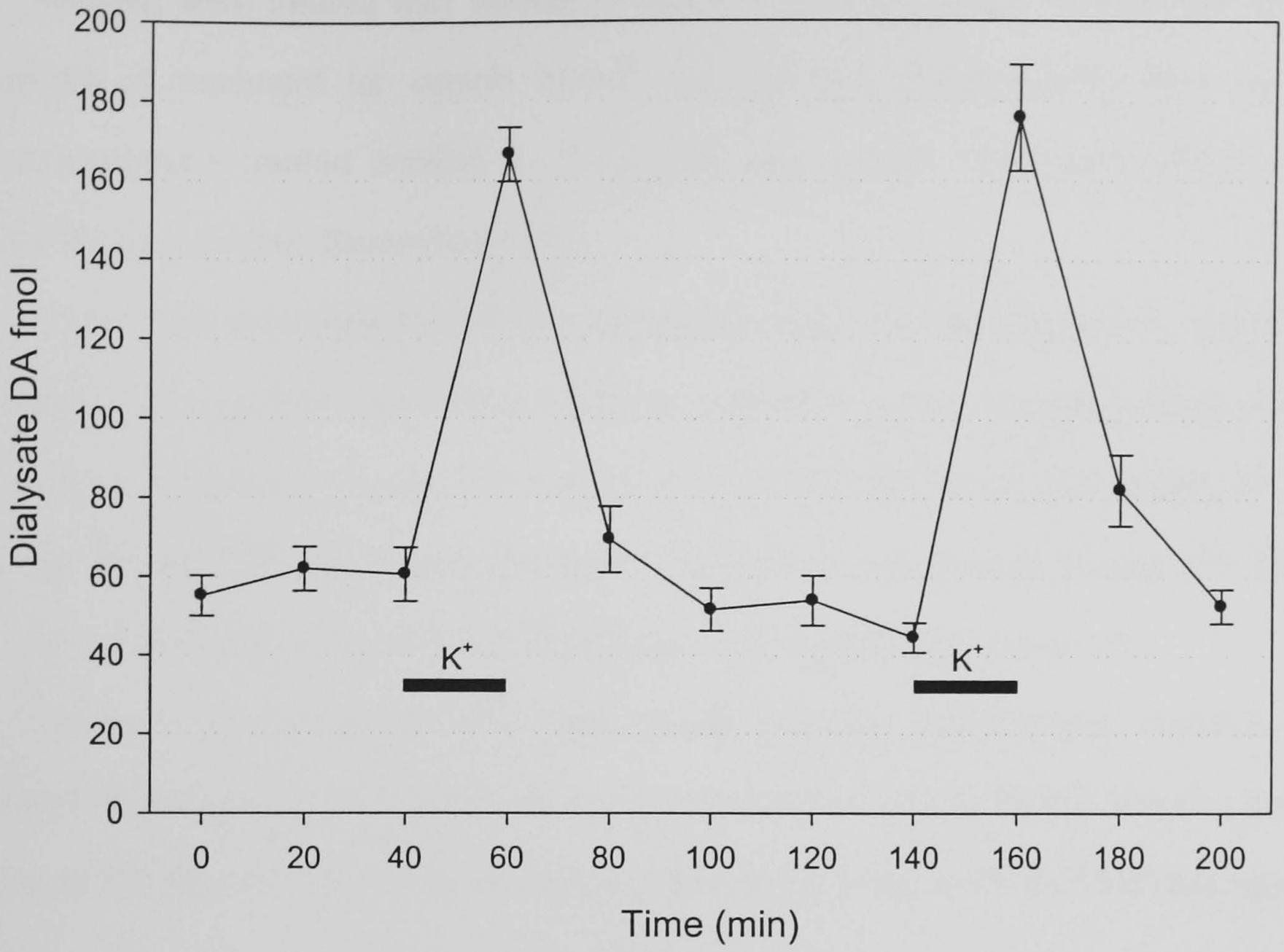

Figure 5.4. Repeated potassium stimulation of dopamine release in the medial prefrontal cortex (n=5).

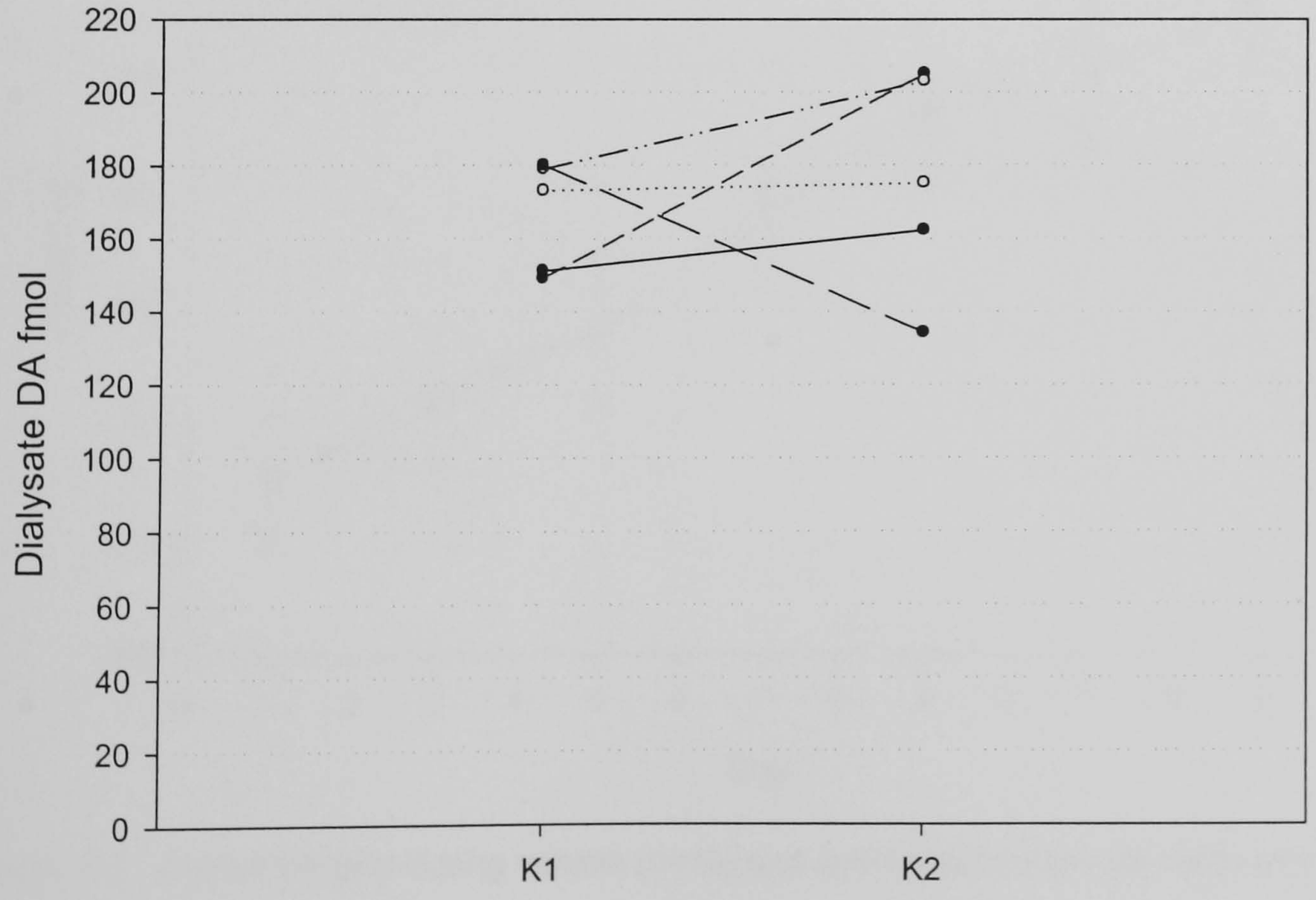

Figure 5.5. Comparison of peak dopamine levels between consecutive potassium stimulations. K1 = first potassium stimulation, K2 = second potassium stimulation (n=5).

5.3.2.2. Corticosterone treatment

Animals were treated with vehicle or corticosterone for between 14 and 16 days. Average length of treatment for vehicle treated animals was 15.25 ± 0.25 days (n=12) and for corticosterone treated animals 14.75 ± 0.22 days (n=12). Treatment lengths did not differ significantly ($p>0.05$ Student's t-test).

Animals gained weight steadily throughout the experiment (see figure 5.6.). At 13 days animal weights did not differ significantly between treatment groups (vehicle treated 300.5 ± 3.6 g, n=12; corticosterone treated 293.1 ± 3.3 g, n=12; $p>0.05$). At 13 days water consumption per cage did not differ significantly between treatment groups (vehicle treated 317 ± 20 ml/cage, n=3; corticosterone treated 263 ± 16 ml/cage, n=3; $p>0.05$) (see figure 5.7.).

Adrenals were removed from each animal following microdialysis. Adrenal weight was significantly lower in corticosterone treated animals than vehicle treated animals (corticosterone treated 20.75 ± 1.2 mg, n=12; vehicle treated 37.6 ± 1.5 mg, n=12; $p<0.01$) (see figure 5.8.).

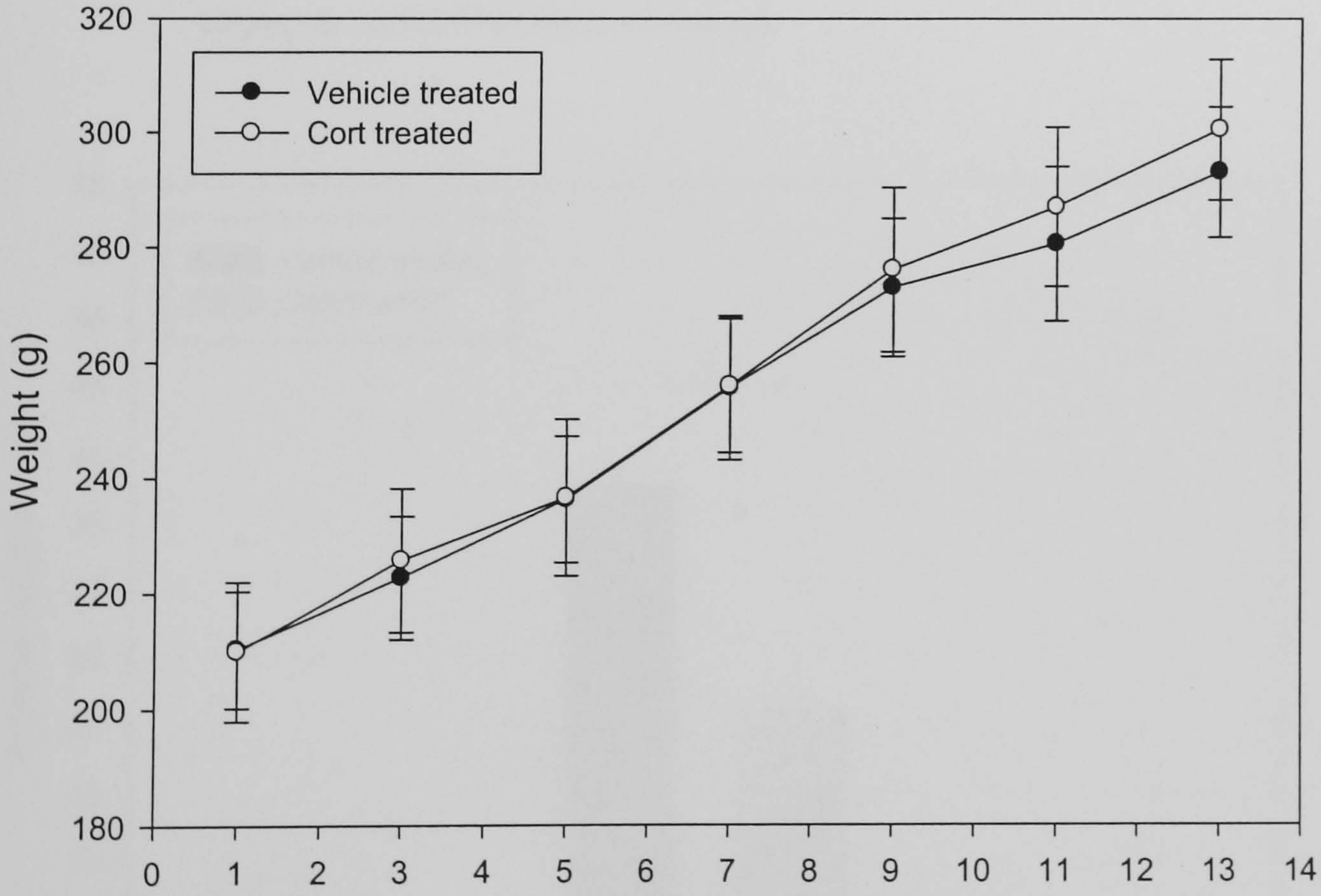

Figure 5.6. Animal weights during vehicle (n=12) and corticosterone (n=12) treatment.

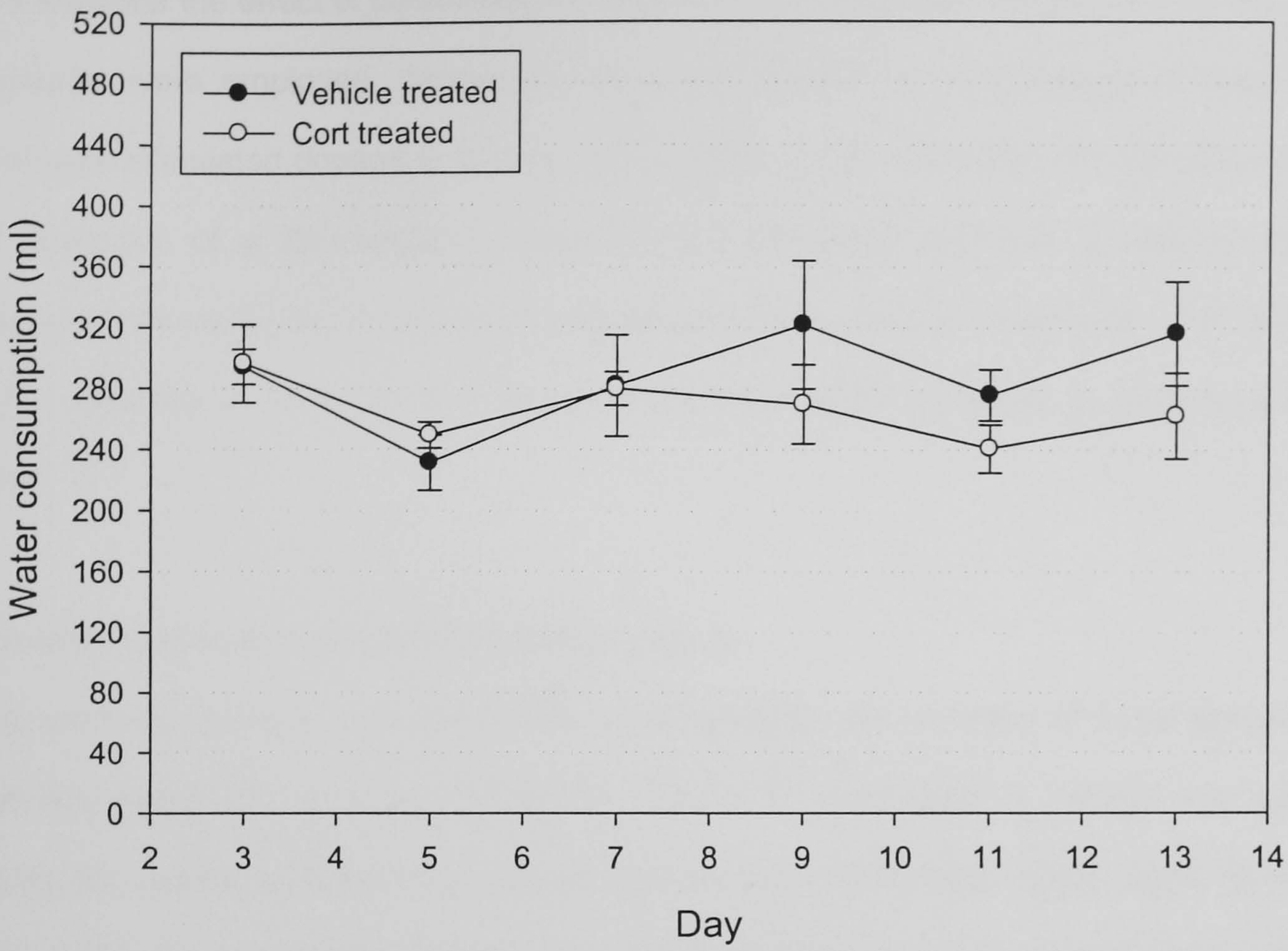

Figure 5.7. Water consumption per cage of four rats per two days treated with vehicle (n=3 cages) or corticosterone (n=3 cages).

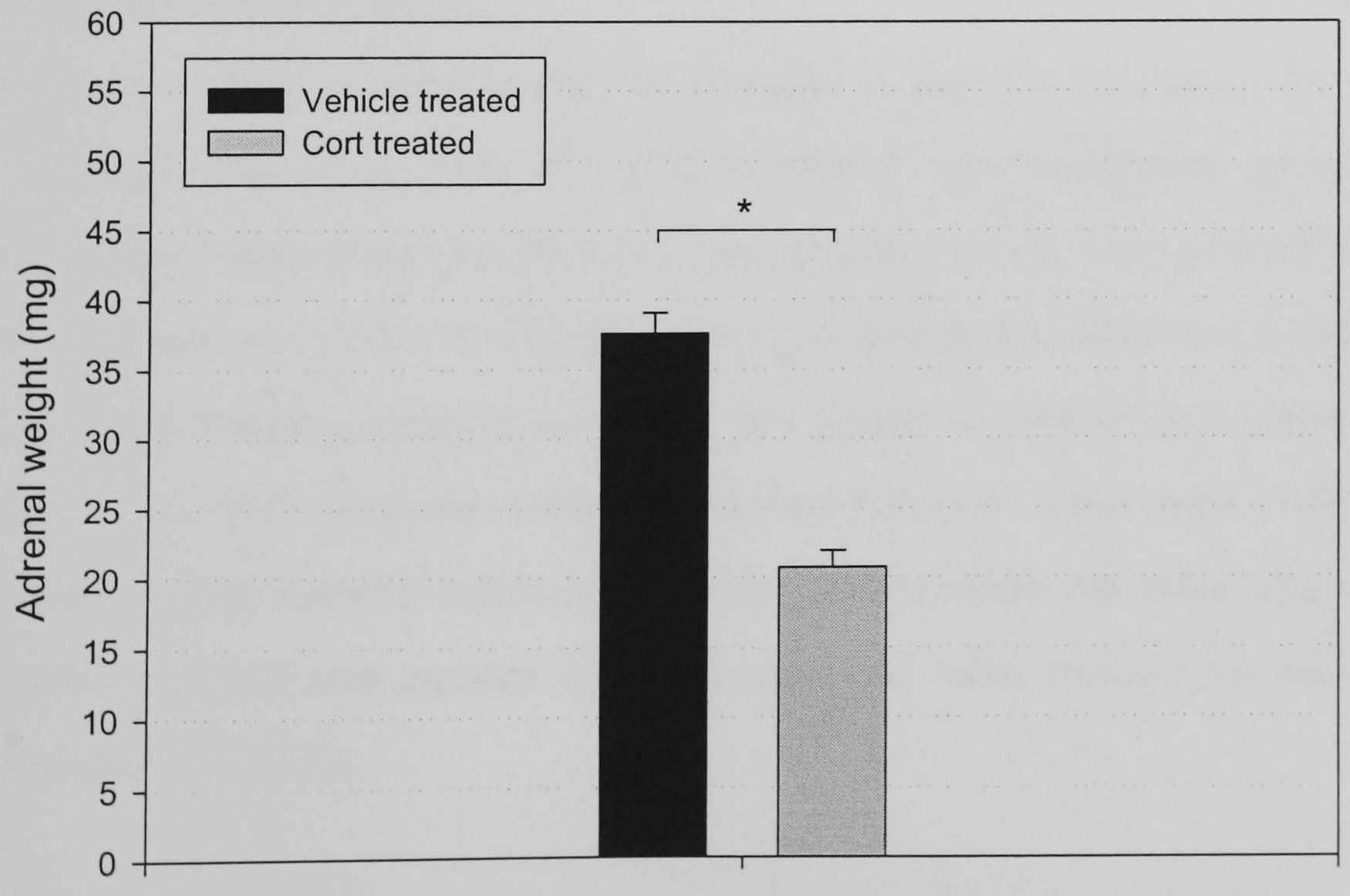

Figure 5.8. Adrenal weights in vehicle (n=12) and corticosterone treated (n=12) animals (* $p<0.01$).

5.3.2.3. Corticosterone effects on dopamine release in the medial prefrontal cortex

To examine the effect of corticosterone treatment on extracellular DA in the PFC four different measures were employed, namely (i) dopamine release in the presence of bupropion, (ii) potassium stimulated dopamine release in the presence of bupropion, (iii) dopamine release in the presence of a D_2 receptor antagonist and bupropion and (iv), potassium stimulated dopamine release in the presence of a D_2 receptor antagonist and bupropion. The time course of this experiment can be seen in figure 5.9. Perfused aCSF contained 30 µM bupropion at all times.

(i) Baseline bupropion stimulated dopamine release

To examine baseline dopamine levels with bupropion the average of three samples taken over the course of one hour immediately prior to K^+ stimulation of release was calculated. During this period corticosterone treated animals had significantly higher levels of dopamine than vehicle treated animals (vehicle 39.3 ± 5.5 fmol/sample, n=11; corticosterone 61.6 ± 5.1 fmol/sample, n=12; $p<0.01$; see figure 5.10.).

(ii) Potassium stimulated release

Perfusion of high K^+ aCSF evoked an increase in dialysate dopamine. The peak DA concentration induced by high K^+ aCSF stimulation was significantly greater in the corticosterone treated group than the vehicle treated group (vehicle 110.1 ± 15.8 fmol/sample, n=10; corticosterone 174.5 ± 18.6 fmol/sample, n=10; $p<0.05$; see figure 5.11.). The absolute increase over baseline dopamine levels was also greater in corticosterone treated animals (vehicle 72.3 ± 12.0 fmol/sample, n=10; corticosterone 112.9 ± 6.1 fmol/sample, n=10) although this failed to reach statistical significance ($p=0.058$). No statistically significant difference in the percentage increase over baseline dopamine levels was found between the two treatment groups (see figure 5.12.).

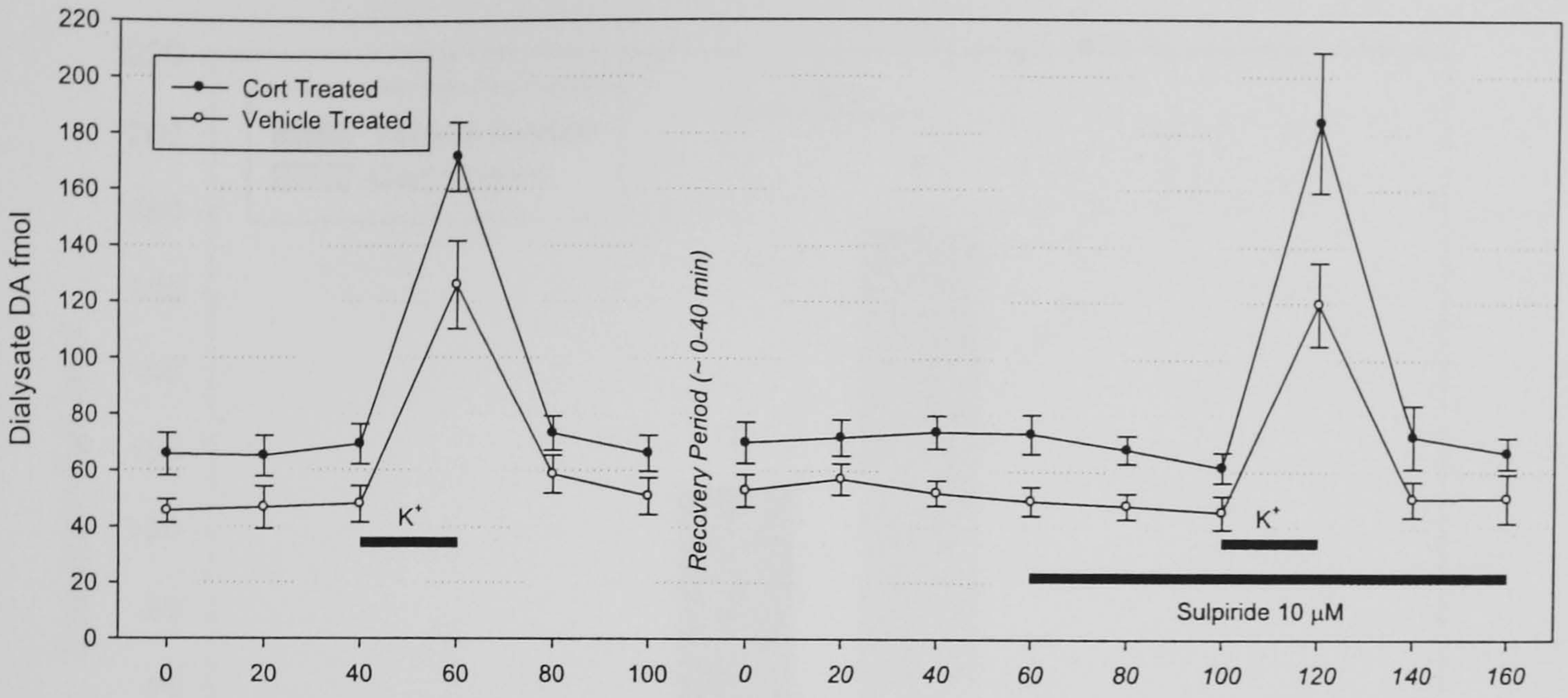

Figure 5.9. Dopamine release in the medial prefrontal cortex in vehicle and corticosterone treated animals. N.B. Data shown are only those from animals which survived the whole experiment (vehicle treatment n=6; corticosterone treatment n=7). K^+ = potassium stimulated release.

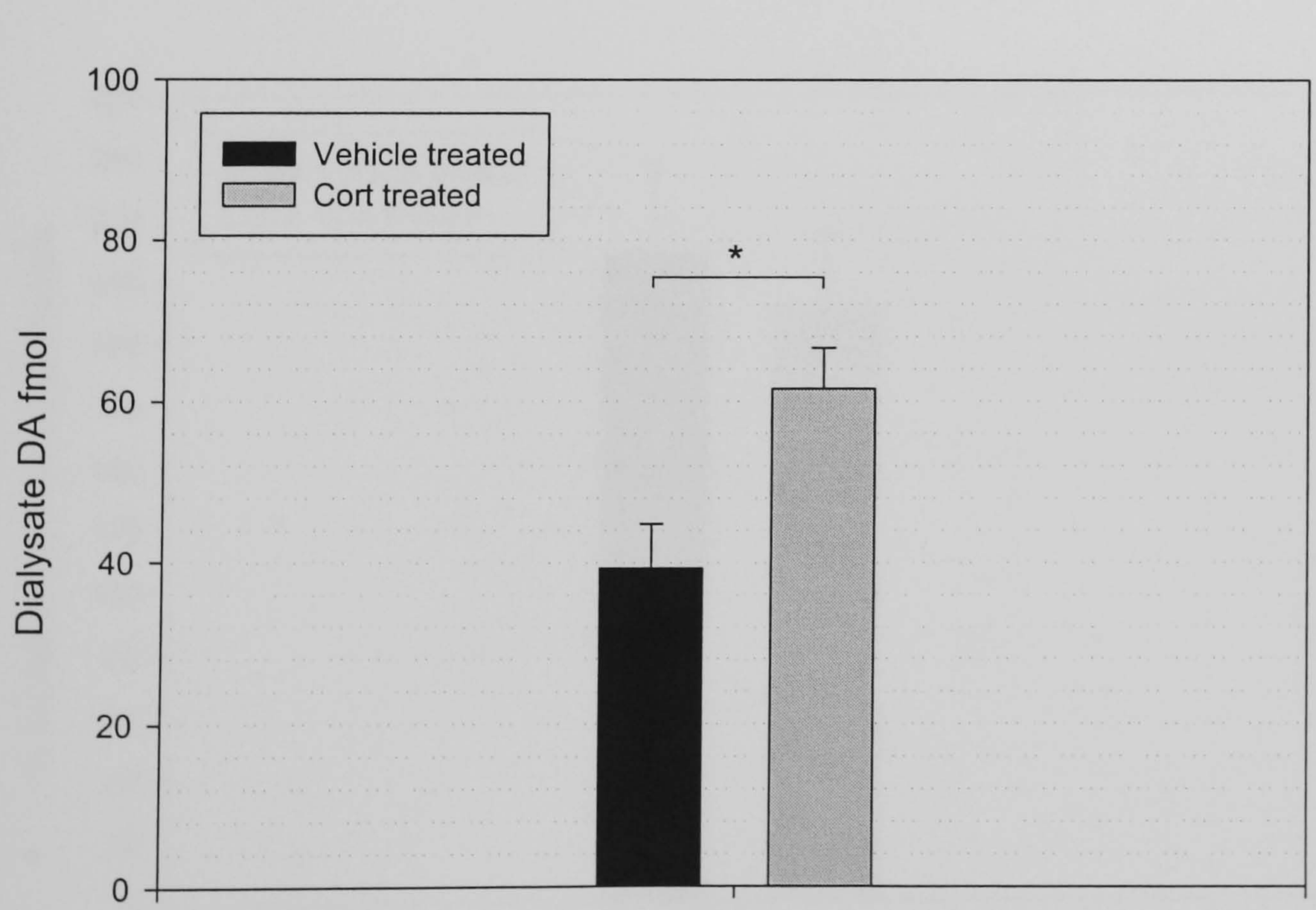

Figure 5.10. Mean baseline dopamine levels (with 30μM bupropion) in the medial prefrontal cortex in vehicle (n=11) and corticosterone treated (n=12) animals as measured by in-vivo microdialysis (* $p<0.01$).

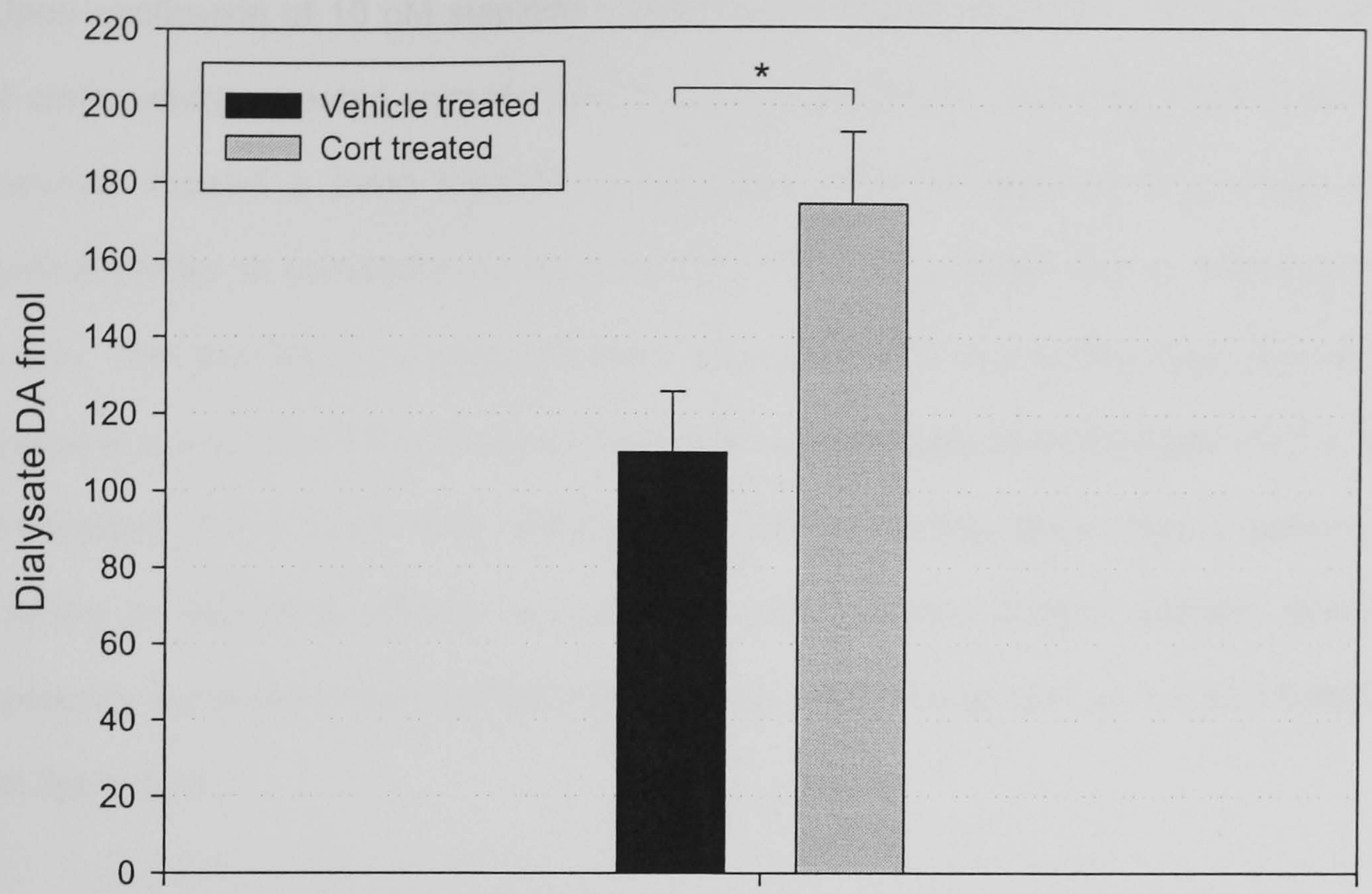

Figure 5.11. Peak dopamine release in the mPFC following potassium stimulation in vehicle (n=10) and corticosterone treated (n=10) animals (* $p<0.05$).

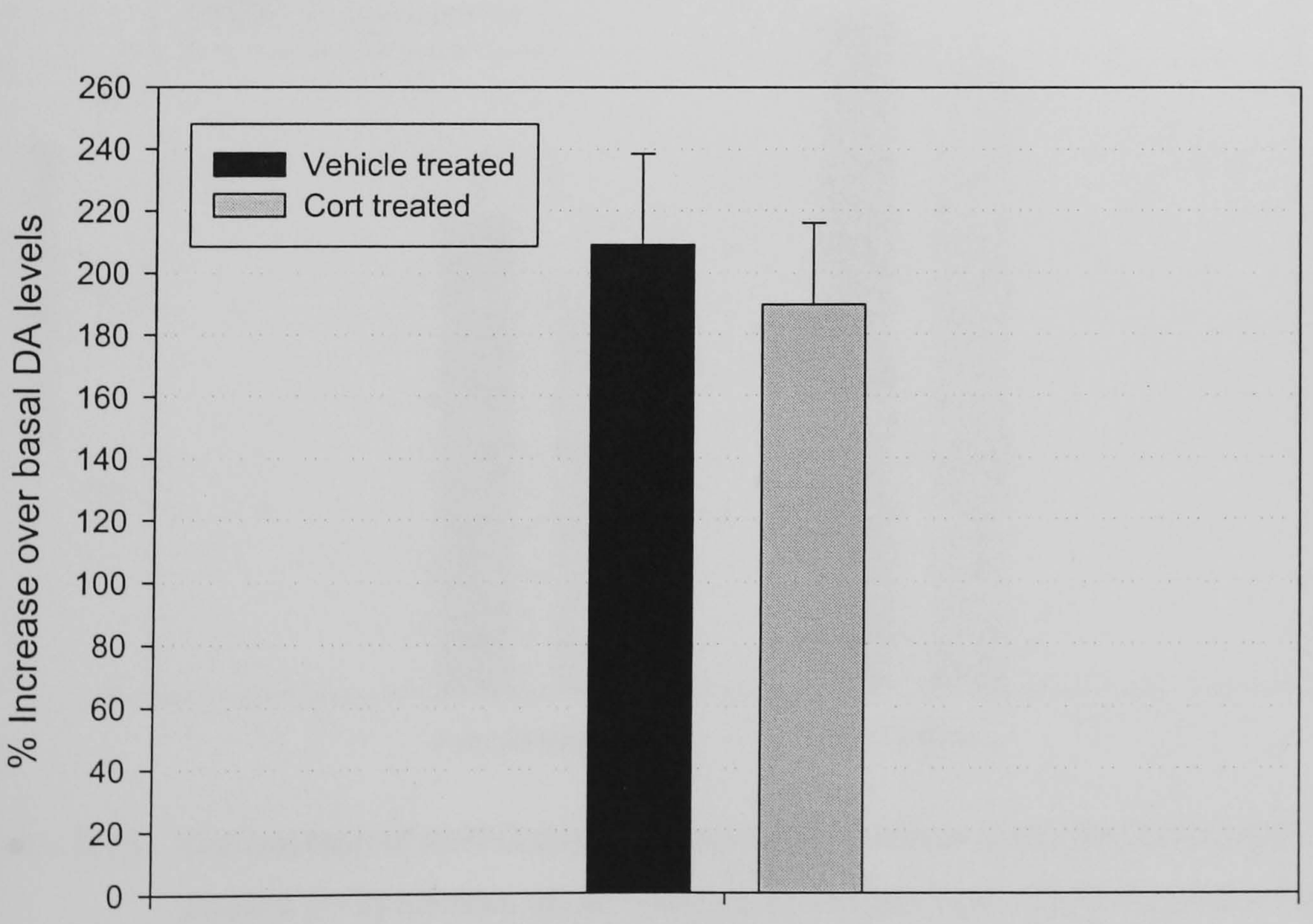

Figure 5.12. % increase in mPFC dopamine levels compared to baseline levels following potassium stimulated release in vehicle (n=10) and corticosterone treated (n=10) animals.

(iii) Effect of corticosterone administration on the dopamine response to sulpiride

Upon application of 10 μM sulpiride a decrease in dopamine levels occurred in both vehicle and corticosterone treated animals. (see figure 5.9. and 5.13.). Two-way ANOVA with repeated measures showed a trend towards a significant effect of sulpiride ($F_{[1,15]}$=3.9, p=0.07), a significant effect of corticosterone treatment (F[1,15]=6.7, p<0.05), but no interaction between the two. The percentage change between baseline levels during the hour prior to sulpiride application and levels during infusion of sulpiride was also calculated (Vehicle -10.5 ± 3.4% n=8; Cort treated -5.5 ± 7.6% n=9). Although the vehicle treated group had a greater average decrease in dopamine release compared to corticosterone treated animals there was no statistically significant difference between the two treatment groups (p > 0.05 Student's t-test) (see figure 5.14.).

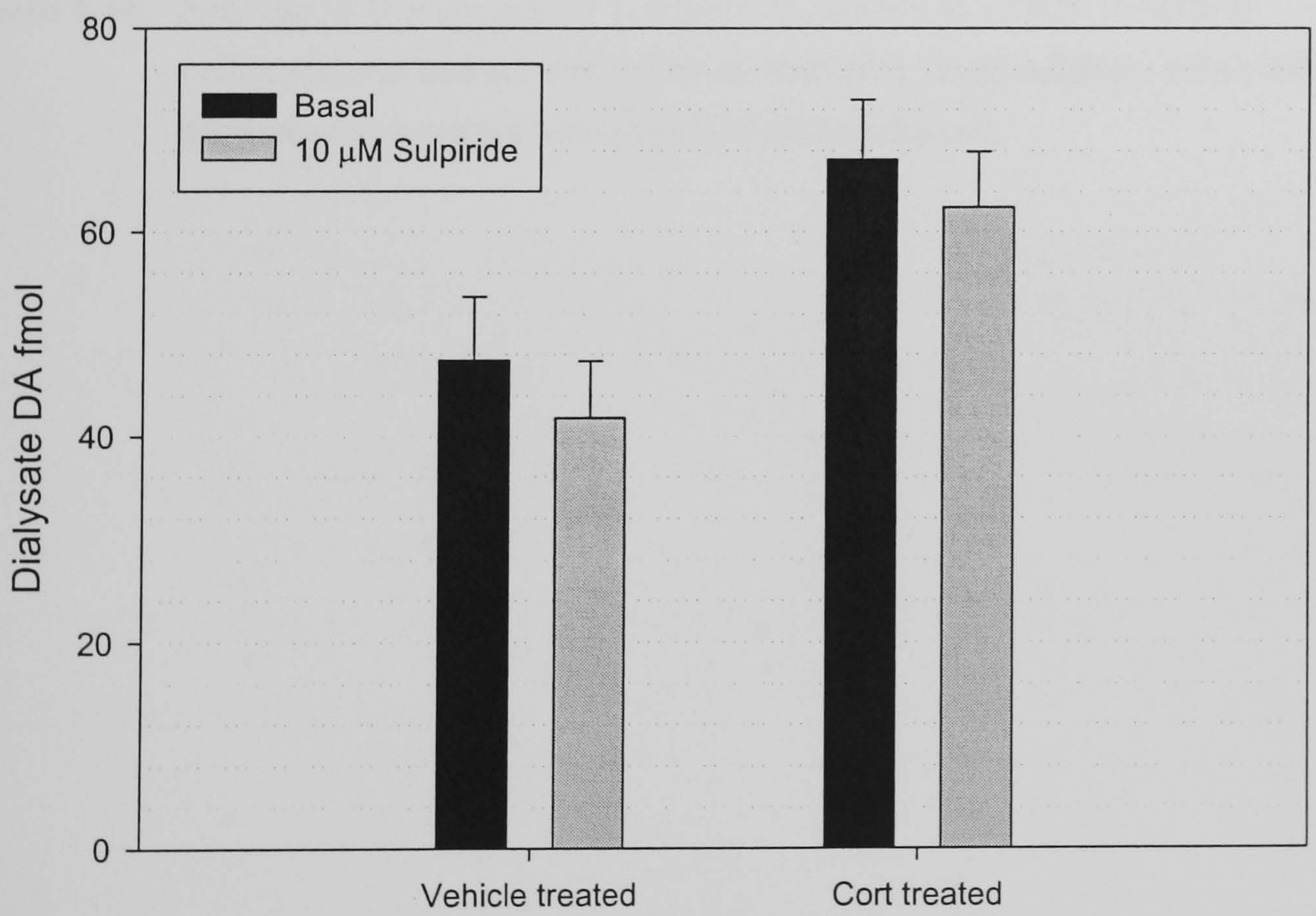

Figure 5.13. Comparison of mPFC dopamine release in vehicle (n=8) and corticosterone treated (n=9) animals under baseline conditions (with 30μM bupropion) and following intra-PFC infusion of 10μM sulpiride.

Figure 5.14. Average % change in mPFC dopamine release in vehicle (n=8) and corticosterone treated (n=9) animals following 10μM sulpiride administration compared to baseline levels (with 30μM bupropion).

(iv) D_2 receptor effects on potassium stimulated dopamine release following corticosterone administration

Table 5.1 shows data for potassium stimulated dopamine release in the absence and presence of sulpiride (10μm) in both vehicle and corticosterone treated groups of animals.

Treatment		*n*	*Peak DA level (fmol)*	*Absolute increase over baseline DA level (fmol)*	*% increase over baseline levels*
Vehicle	K^+	7	112.9 ± 18.5	71.2 ± 14.4	186.7 ± 39.1
	K^+ + Sulpiride	7	115.7 ± 13.2	72.7 ± 11.7	207.6 ± 56.6
Cortico-sterone	K^+	6	177.8 ± 12.8	114.2 ± 9.0	195.7 ± 33.3
	K^+ + Sulpiride	6	187.4 ± 28.9	121.1 ± 25.7	179.6 ± 31.4

Table 5.1. Data ± s.e.m. for repeated potassium stimulations in the absence and presence of sulpiride (10μm).

Two-way repeated measures ANOVA showed no effect of sulpiride on peak DA level ($F_{[1,11]}$=0.3, p>0.05) (see figure 5.15), absolute increase in DA from baseline level ($F_{[1,11]}$=0.1, p>0.05), or percentage increase in DA levels ($F_{[1,11]}$=0.004, p>0.05) (see figure 5.16). Corticosterone treatment had a significant effect on peak DA level ($F_{[1,11]}$=153.2, p<0.05) and absolute increase over baseline levels ($F_{[1,11]}$=5.7, p<0.05), with no effect on the percentage increase over baseline levels (see figure 5.16).

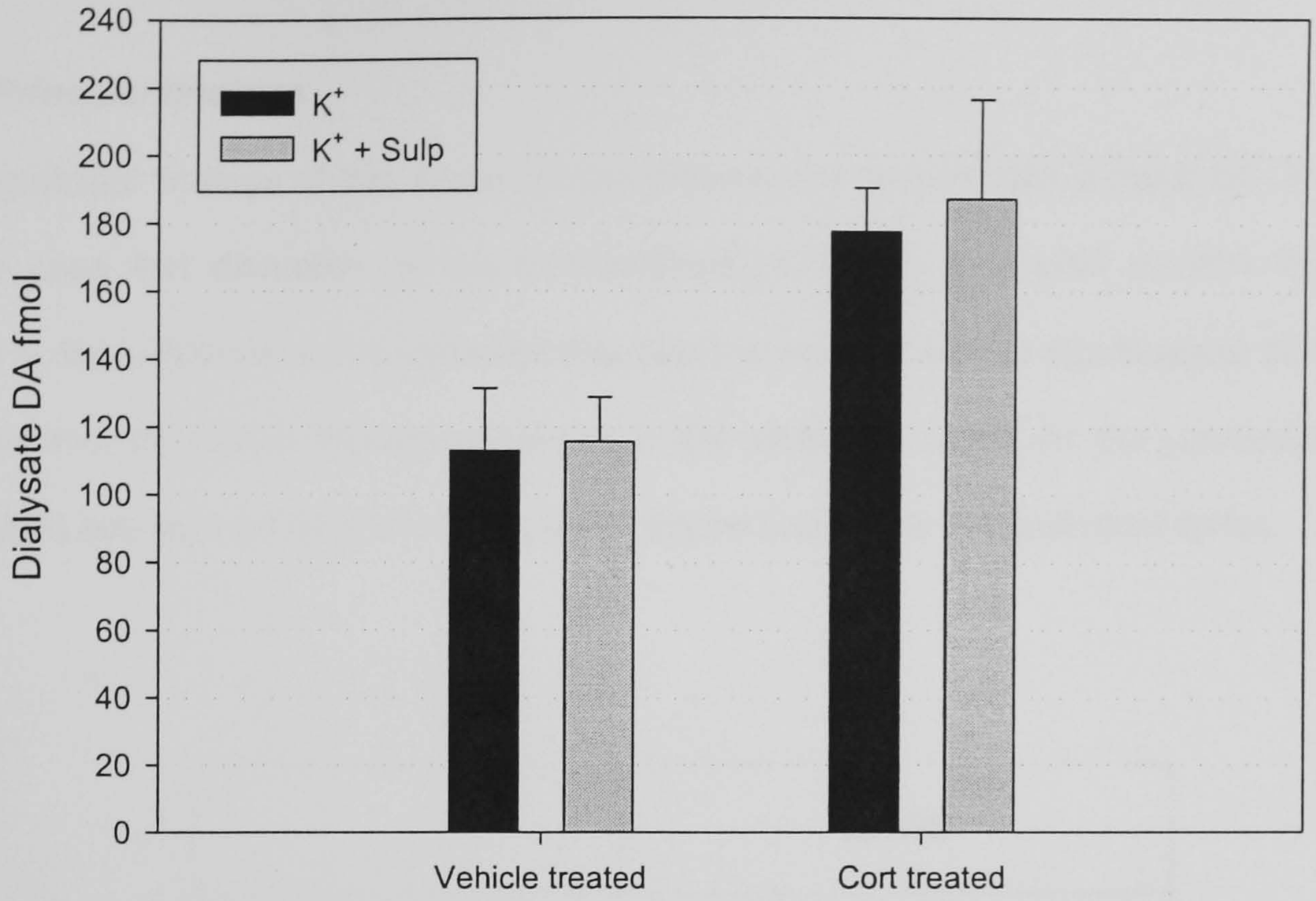

Figure 5.15. Peak mPFC dopamine release in vehicle and corticosterone treated animals following potassium stimulation in the presence and absence of 10μM sulpiride (Sulp) (see table 5.1 for animal numbers).

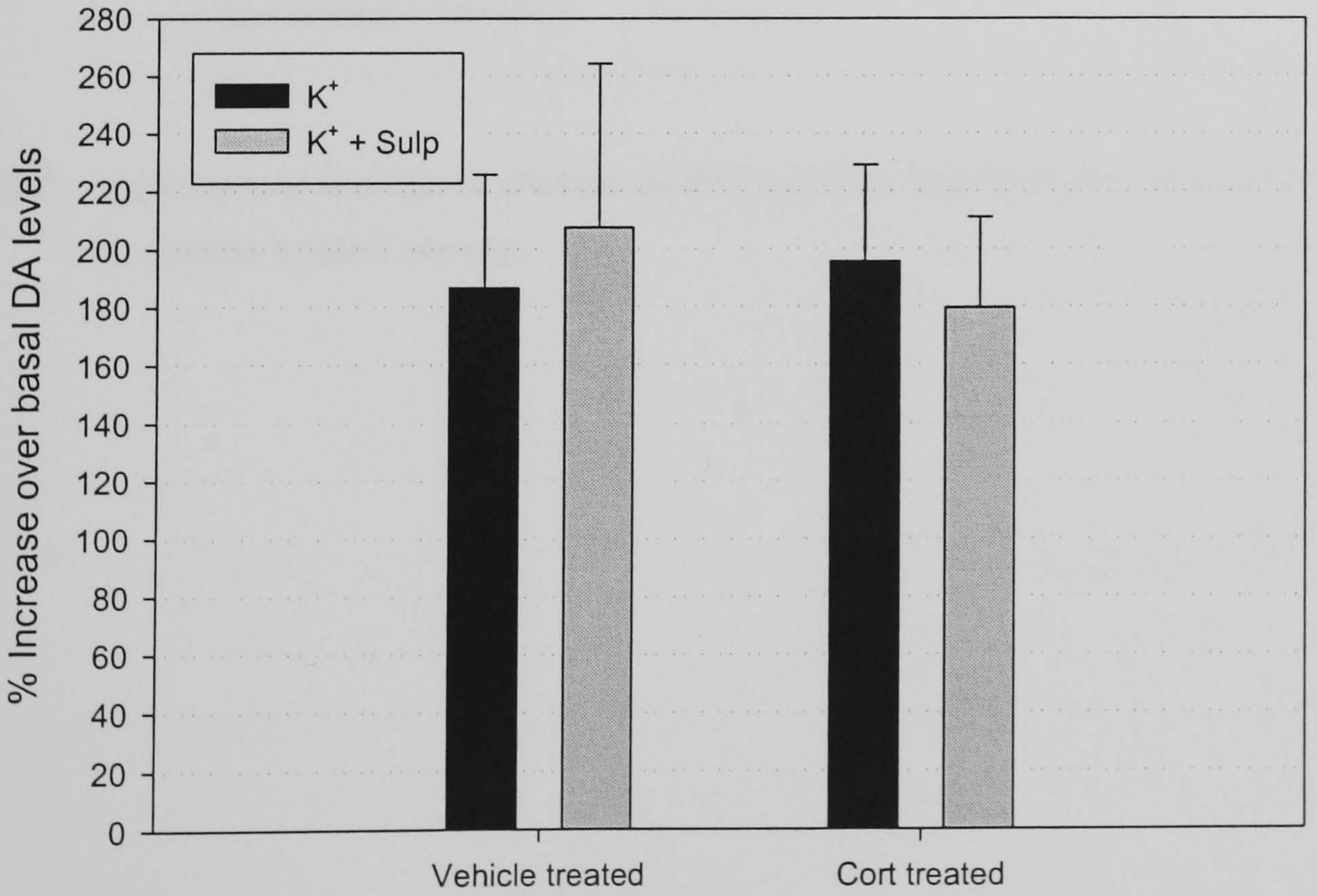

Figure 5.16. % increase in mPFC dopamine release in vehicle and corticosterone treated animals following potassium stimulation in the presence and absence of 10μM sulpiride (Sulp) (see table 5.1 for animal numbers).

5.4. Discussion

5.4.1. Principal findings

The principal findings of this series of experiments are summarised in table 5.2. From this it can be seen that disruption of the corticosteroid rhythm (i) increased tyrosine hydroxylase activity in the prefrontal cortex (although this failed to reach statistical significance) (ii) increased baseline and stimulated dopamine release in the prefrontal cortex (in the presence of 30μM bupropion) and (iv) had no effect on D_2 autoreceptor function in the prefrontal cortex.

	mPFC
Tyrosine Hydroxylase activity	↑ (93%) (non-significant trend)
Dopamine release	↑ baseline (57%) and K^+ stimulated (58%) efflux
D_2 receptor function	No change

Table 5.2. Summary of findings in animals treated with two weeks of corticosterone compared to vehicle treated animals.

5.4.2. Tyrosine hydroxylase function in corticosterone treated animals

(i) Medial prefrontal cortex

20 minutes of NSD1015 treatment prior to sacrifice failed to produce measurable levels of DOPA in mPFC tissue samples, however following 2 hours of NSD1015 pre-treatment DOPA levels were elevated such that measurement was possible. This revealed a trend towards an increase in DOPA levels in corticosterone treated animals over those found in vehicle treated animals. This result is consistent with the hypothesis that the increase in tyrosine hydroxylase gene transcription seen in the VTA (see chapter 4) results in an increase in tyrosine hydroxylase activity and therefore DOPA synthesis in this brain region.

(ii) Nucleus accumbens

The results of the two experiments looking at tyrosine hydroxylase activity in the nucleus accumbens are, at first glance, somewhat at odds with each other. In the first instance, with a 20 minute post-NSD1015 survival time, DOPA levels were significantly lower in the corticosterone treated group compared to the vehicle treated group. Such a result is consistent with a study published by Pacak et al. (2002) in which seven days of corticosterone infusion was found to produce a similar effect. It is the case however that with a longer post-NSD1015 survival time of 2 hours (which was carried out principally to elevate PFC DOPA levels to a measurable level), no effect of corticosterone was found and furthermore DOPA levels in the vehicle group were markedly lower than at the 20 minute timepoint.

Intuitively, a longer survival time after dosing with a DOPA decarboxylase inhibitor would be expected to increase rather than decrease tissue DOPA concentrations. One possible explanation comes from the finding that DOPA can not only inhibit tyrosine hydroxylase activity significantly (Nagatsu et al.1964), but is itself a substrate for tyrosine hydroxylase (Haavik, 1997). In the vehicle treated animals the high rate of tyrosine hydroxylation (as suggested at the twenty minute time point) may have depleted tyrosine levels and elevated DOPA levels. This would produce a situation in which DOPA oxidation was favoured explaining the decrease seen

at the two hour time point. In the corticosterone treated group the lower rate of TH activity possibly resulted in the maintenance of a larger pool of tyrosine, and thus DOPA oxidation may not have occurred to the same extent. As a result, at the two hour time-point DOPA oxidation in vehicle treated animals may have masked their higher rate of TH activity relative to the corticosterone treated group. Measurement of tyrosine levels would help to clarify this issue.

If the theory regarding DOPA oxidation is accepted it appears to have had little impact on the DOPA synthesis in the PFC. It is of course possible that the results have indeed been distorted by DOPA oxidation and that the rate of synthesis is in fact much higher in corticosterone treated animals than the results would indicate. Alternatively, tyrosine availability relative to synthetic rate might be such that in mPFC dopaminergic cells DOPA oxidation is competitively inhibited. From the present data it is impossible to estimate the effect of DOPA oxidation on these results. There is no reason to suspect that in the mPFC DOPA oxidation would have occurred preferentially in the vehicle treated group, where the rate of synthesis was lower and therefore tyrosine availability was likely to be greater, and so the overall result is probably valid even if the measured levels do not give a true indication of the rate of DOPA synthesis.

Overall, determination of TH activity revealed that the medial prefrontal cortex and nucleus accumbens are differentially regulated by HPA axis dysrhythmia. In the medial prefrontal cortex the corticosterone induced increase in transcription of tyrosine hydroxylase within the VTA appears to increase TH activity, whereas in the nucleus accumbens no increase was seen, and indeed there was some evidence of a decrease in TH activity in this region. Given that the results from the previous chapter suggest that tyrosine hydroxylase gene transcription is increased by hypercortisolaemia across the whole VTA, the lack of increase in TH activity in the nucleus accumbens suggests a post-translational modification of tyrosine hydroxylase function. One possible mechanism for such a post-translational modification is the increase in D_2 receptor gene transcription seen in the VTA following corticosterone treatment (see Chapter 4) as this receptor can regulate tyrosine hydroxylase activity by altering the phosphorylation state of the enzyme at the terminal (Lindgren et al. 2003). Data from the microdialysis study (see later)

suggests that the D_2 receptor is not functional in the mPFC, and furthermore in chapter 4 D_2 receptor gene transcription was upregulated to a greater extent in areas of the VTA thought to project to the nucleus accumbens than those areas thought to project to the mPFC. These findings may explain why such a post-translational effect on TH activity appears to have occurred selectively in the nucleus accumbens.

As mentioned in the previous chapter, changes in dopamine synthesis can produce parallel changes in vesicular content and therefore dopamine release (Floor et al. 1995, Pothos et al. 2000; Eisenhofer et al. 2004). The results presented here suggest that modulating the corticosteroid rhythm can result in an increase in dopamine synthesis in the mPFC but not in the nucleus accumbens. The effect of hypercortisolaemia therefore has the potential to produce an imbalance in mesocortical vs. mesolimbic dopamine release.

5.4.3. Dopamine release in the medial prefrontal cortex in corticosterone treated animals

In the second part of this chapter the consequences of corticosteroid dysrhythmia on extracellular DA levels in the mPFC were examined using microdialysis. Flattening the corticosterone rhythm increased baseline bupropion stimulated dopamine levels in the mPFC markedly. Furthermore corticosterone treatment also increased potassium stimulated dopamine release.

The increase in bupropion stimulated baseline dopamine levels seen in the corticosterone treated group could be due to a number of factors. One possibility which can be discounted is a lower rate of dopamine transporter mediated dopamine uptake in the corticosterone treated animals. First the *in situ* study showed no effect of corticosterone treatment on DAT transcription. Secondly and crucially, perfusion of the DA uptake blocker bupropion at a concentration shown to maximally inhibit uptake in the nucleus accumbens (L. Ferrie, doctoral thesis) would have masked any effects of corticosterone treatment on DAT function. As a consequence of the DAT blockade changes in DAT activity are not responsible for the elevated dopamine levels, and furthermore dialysate dopamine levels reported here are indicative of actual quantities of dopamine released.

Another possible explanation for the increase in bupropion stimulated DA release is that an increase in the basal neuronal firing rate occurred following corticosterone treatment. However, the fact that corticosterone treated animals also exhibited elevated dopamine levels in the presence of a depolarising stimulus (high K^+ aCSF) suggests this is unlikely to be the explanation. If an increase in firing rate was solely responsible for the increase in release, potassium stimulation would have produced a similar peak dopamine concentration in both treatment groups as the induction of dopamine release by this method is independent of the neuronal firing rate.

A further potential mechanism is a corticosterone induced decrease in terminal D_2 receptor function which would attenuate autoreceptor inhibition of dopamine synthesis and release. Such a mechanism is not thought to be likely in light of the *in situ* data showing an increase in D_2 receptor gene transcription in the VTA which would be expected to *increase* D_2 receptor function. Furthermore in the present study the D_2 receptor was not found to be tonically active in either vehicle or corticosterone treated rats (see section 5.4.4.)

The most credible theory based on the present data involves a corticosterone-induced upregulation of vesicular dopamine content. The trend towards an increase in tyrosine hydroxylase activity in the mPFC (see section 5.4.2.) (reflecting the corticosterone induced upregulation of TH gene transcription seen in Chapter 4) suggests that there is an increase in dopamine synthesis following corticosterone treatment. This could result in greater vesicular dopamine content as a result of altering the concentration gradient between the interior of the vesicle and the cytoplasm, resulting in a lower rate of vesicular leakage and a corresponding increase in vesicular content. Furthermore, in the previous chapter it was shown that gene transcription of the vesicular dopamine transporter (VMAT2) was upregulated by corticosterone administration. It has been shown previously that an increase in VMAT2 expression produces an increase in the amount of dopamine released per depolarisation event (Pothos et al. 2000) further supporting this as a potential mechanism. Under these conditions the number of vesicles undergoing exocytosis following depolarisation of the terminal would be the same in both treatment groups. The fact that potassium stimulation produced the same percentage increase over baseline bupropion stimulated dopamine levels in vehicle and corticosterone treated

animals is consistent with such a mechanism. The evidence therefore suggests that increased vesicular content is a likely candidate to explain the increase in dopamine release seen in corticosterone treated animals.

As calcium entry into the terminal plays a key role in neurotransmitter exocytosis it is possible that in the corticosterone treated group an upregulation of calcium signalling was responsible for the increase in dopamine release. This aspect of neuronal function was not examined in the present study but this mechanism could account for the observed increases in dopamine release under both baseline and stimulated conditions. However, the previously mentioned hypothesis involving upregulation of vesicular dopamine content successfully accounts for the observed increase in dopamine release, and *in situ* and enzyme assay data support this view. It is unknown whether, in addition, changes to calcium signalling occur, and based on the available data this possibility can not be ruled out.

5.4.4. D_2 receptor function in corticosterone treated animals

In light of in-situ data showing an upregulation of D_2 receptor gene transcription in the VTA following corticosterone treatment, (see Chapter 4), it was postulated that increased D_2 receptor activity might offset the effects of increased dopamine synthesis and release in the mPFC.

It was found that local application of the D_2 receptor antagonist sulpiride into the mPFC did not elevate extracellular dopamine levels in either corticosterone or vehicle treated animals. This suggests that there is little or no tonic inhibition of dopamine release by this receptor in the mPFC. In fact, dopamine levels appeared to decrease somewhat following sulpiride application although this failed to reach statistical significance. This trend towards a run down in dopamine release was evident in the preliminary study examining the repeatability of potassium stimulation where, in the absence of sulpiride, a non-significant decrease in dopamine release occurred following the first potassium stimulation. It is possible that this decrease is due to a "run-down" in release following the first potassium stimulation, however, the effect is relatively small and appears not to have adversely affected the results.

Sulpiride had no significant effect on the magnitude of evoked dopamine release produced by depolarisation stimulation in the two treatment groups. Given that the D_2 receptor is more likely

to be occupied under conditions of high dopamine release, the lack of effect of sulpiride following potassium stimulation further supports the notion that this receptor is inactive in the mPFC. Overall the data indicate that the D_2 receptor does not influence bupropion or potassium stimulated release in the mPFC and furthermore that the corticosterone induced increase in dopamine release is not accounted for by a decrease in receptor sensitivity.

The result of the present study is in direct contrast with microdialysis data published by Bean and Roth (1991) showing that sulpiride (at the same concentration used here) increases dopamine release by 94% in the mPFC. Their data therefore suggest that release modulating autoreceptors are present and functional in this region. In support of the present results, some studies have shown no effect of locally applied haloperidol (a D_2 antagonist) in the mPFC (Gessa et al. 2000; Devoto et al. 2001). Furthermore a variety of groups have shown no effect of systemic administration of sulpiride on mPFC dopamine release (Moghaddam and Bunney, 1990; Ago et al. 2005) and studies in slices of rabbit PFC have found that sulpiride only potentiates stimulated dopamine release when high rates of stimulation are used (Agneter et al. 1994). Thus there is some support for a lack of a physiological role for the D_2 autoreceptor in the mPFC.

Studies employing dopaminergic agonists have been similarly inconsistent with one study finding that intra-PFC infusion of apomorphine (a D_2 agonist) decreases dopamine release as measured by microdialysis (Bean et al. 1990) and another showing inhibition of DA release in slices of prefrontal cortex following local application of dopaminergic agonists (Hoffmann et al. 1998). In contrast, local application of the D_2 agonist quinpirole has been shown to have no effect on DA release in the PFC (Devoto et al. 2001). Studies with systemically administered D_2 agonists have shown no effect on DA release (Chiodo et al. 1984, White and Wang 1984). Hoffman et al. (1998) suggested that in the PFC the D_2 receptor is located some distance from the synapse as electrically evoked DA release was found to be less effective at increasing D_2 agonist efficacy than in the striatum despite a greater evoked DA outflow. Consequently it is possible that under physiological conditions in-vivo D_2 autoinhibition does not occur due to dopamine not diffusing far enough to reach receptor sites (see Cubbedu et al. 1990).

Given the lack of efficacy of systemically administered sulpiride on PFC dopamine release (Moghaddam and Bunney, 1990; Ago et al. 2005) it is interesting to note that intra-VTA application of sulpiride has been found to increase dopamine release in the PFC (Westerink et al. 1998) suggesting the presence of functional somatodendritic autoreceptors. It may be the case that following systemic administration, sulpiride modulation of afferent input to the VTA masks the autoreceptor effect whereas under normal conditions it is functional. Given that in the present study no evidence for a change in firing rate of dopamine neurones was found, it is likely that corticosterone treatment had no effect on somatodendritic autoreceptor function.

In the previous chapter an increase in D_2 receptor mRNA was found at the level of the VTA following corticosterone administration. Given that the present results (namely an increase in DA synthesis and release) are inconsistent with an upregulation of either somatodendritic or terminal D_2 receptors it is likely that corticosterone treatment did not increase D_2 receptor protein expression in mesocortical neurones.

One possible explanation for this is that the upregulation of D_2 mRNA transcription did not occur in mesocortical neurones. It has been suggested that mPFC projections arise in the dorsal VTA which exhibited a much smaller increase in D_2 mRNA following corticosterone treatment than the ventral VTA, indeed, the dorsolateral region showed no increase in D_2 transcription at all.

Another possibility is that protein synthesis and/or trafficking in mesocortical neurones is not upregulated in response to elevated levels of D_2 mRNA to increase the number of functional autoreceptors. If this were the case it might represent a further contrast with mesolimbic neurones as increased D_2 receptor function was postulated to underlie the post-translational inhibition of tyrosine hydroxylase in this region seen in the tissue homogenate TH assay.

A further possibility is that D_2 receptor protein expression is increased in the PFC following corticosterone treatment but the receptor is not functional. The lack of effect of sulpiride in vehicle treated animals supports a lack of functionality. However, if this were the case, one might expect a concomitant increase in somatodendritic D_2 receptor expression, and hence a change in neuronal firing rate. No evidence of a change in basal firing rate was suggested by comparing baseline bupropion stimulated dopamine release with potassium stimulated release.

It is more likely that corticosterone has no effect on transcription in these neurones or that some translational/trafficking deficit accounts for the lack of effect.

Overall, these results suggest that (i) terminal D_2 autoreceptors do not function in the mPFC and (ii) corticosterone treatment does not upregulate D_2 receptor expression in mesocortical dopaminergic terminals such that autoinhibition occurs.

5.4.5. Functional implications of elevated dopamine release in the PFC

The prefrontal cortex is involved in a number of executive brain functions including working memory, selective attention, goal directed behaviours, behavioural inhibition and visceromotor control (see reviews by Arnsten, 1997; Vertes, 2006). In particular the regions of the PFC looked at in the present study, namely the prelimbic and infralimbic cortices, are thought to be involved in working memory and attention regulation, with the prelimbic cortex associated with action-outcome contingency detection and visual attention selectivity (Dalley et al. 2004). Dopaminergic regulation plays a critical role in these processes with many of them sensitive to manipulation by dopaminergic agonists and antagonists. Indeed it has been suggested that dopamine is the major monoamine influencing prefrontal cortical function (Gratton et al. 1989).

The majority of the work on dopaminergic regulation of prefrontal cognitive function has been carried out in experiments investigating working memory, where it has been shown that a reduction in post-synaptic dopaminergic activity using dopamine antagonists impairs working memory, whilst modest elevation of dopaminergic activity improves cognitive function (reviewed by Arnsten, 1997; Robbins, 2005). It has been shown, however, that supranormal DA stimulation results in impairment of spatial working memory and this can be produced by both dopaminergic agonists and drugs which induce PFC dopamine release such as ketamine. Furthermore, acute stress can raise dopamine levels to a point where they are detrimental to cognitive function (reviewed by Arnsten, 1997; Robbins, 2005). Overall these data suggest that the dopamine response curve is bell-shaped with respect to cognition and that alterations in DA release can have significant effects on cognitive function.

At the cellular level the predominant action of dopamine in the prefrontal cortex is thought to be inhibitory with dopaminergic agonists having been shown to exert an inhibitory effect on

prefrontal glutamatergic pyramidal cells (Sesack and Bunney, 1989; Godbout et al. 1991). The receptor subtype responsible for the cognitive effects of post-synaptic dopaminergic signalling is thought to be the D_1 receptor subtype with working memory showing the aforementioned bell-shaped dose response curve in response to D_1 selective agonists (Arnsten, 1997; Dalley et al. 2004).

The post-synaptic D_2 receptor also appears to play a role as some groups have shown that dopaminergic inhibitory responses in the PFC are antagonised by D_2 selective but not D_1 selective antagonists (Sesack and Bunney, 1989; Godbout et al. 1991). In addition, D_2 selective agonists have been shown to be more potent than D_1 selective agonists at producing inhibitory responses in the PFC (Parfitt et al. 1990). The role of this in cognitive function is put into question, however, by studies showing that whilst a D_2 selective agonist can improve working memory function at a dose sufficient to stimulate post-synaptic receptors, D_2 antagonists do not impair cognitive function when applied directly into the PFC (Arnsten, 1997).

It has been suggested that dopamine concentrations are a critical determinant of the relative contributions of D_1/D_2 receptors to signalling in the PFC and that D_2 mediated signalling predominates at high dopamine concentrations (Trantham-Davidson et al. 2004). Overall it can be seen that whilst the exact mechanism of dopamine's postsynaptic effect is unclear, prefrontocortical function is critically regulated by dopamine levels and increased or decreased dopamine release can have detrimental effects on executive function. The functional consequences of the corticosteroid induced elevation in dopamine seen in the present study are difficult to predict, but they may result in an impairment of cognitive function due to an elevated basal dopamine level (this is discussed further in chapter 6.).

Dopaminergic prefrontocortical inhibition not only affects brain functions mediated by the PFC itself, but in addition it affects numerous other brain areas via cortical afferents. Of particular relevance to the present study is the presence of cortico-limbic afferents which feed-forward to control dopaminergic projections terminating in the nucleus accumbens. The presence of a projection originating in the PFC and targeting the nucleus accumbens was first demonstrated by Beckstead in 1979. It was subsequently shown that dopaminergic activity in the PFC exerts an inhibitory influence on dopamine release in the nucleus accumbens (Thompson and Moss,

1995; Doherty and Gratton, 1996). This suggests that another functional implication of the observed increase in dopamine release in the PFC seen in the present study could be an inhibition of meso-accumbens dopaminergic activity. This effect combined with the evidence from the tyrosine hydroxylase activity assay that dopamine synthesis is not increased in the nucleus accumbens provides further support for the hypothesis that corticosteroids can induce a functional "imbalance" between mesocortical and mesolimbic dopaminergic function.

5.5. Conclusion

The available data suggest that dysregulation of the HPA axis such as that seen in bipolar disorder and modelled in the present study produces an increase in the synthesis and release of dopamine in the prefrontal cortex, whilst producing no increase in synthesis in the nucleus accumbens. This may adversely affect cognitive function by elevating dopamine release in the prefrontal cortex. Cognitive function may also be relatively more impaired by acute stressors due to an exaggerated dopamine response caused by corticosteroid dysregulation. This suggests that a hyperdopaminergic state in the prefrontal cortex arising from dysregulation of the HPA axis could be responsible for some of the symptoms of bipolar and other affective disorders and may be implicated in the triggering of psychiatric events following exposure to stress.

Chapter 6.

General Discussion

Chapter 6. General Discussion

The aim of the studies in this thesis has been to investigate the hypothesis that changes to the diurnal corticosteroid rhythm of the type seen in bipolar disorder produce changes in mesocorticolimbic dopaminergic function. This hypothesis stems from the theory that HPA axis abnormalities are a causative factor in bipolar disorder, and from the evidence that dysregulation of dopaminergic systems plays a role in the mood states characteristic of the disorder.

HPA axis abnormalities in bipolar disorder have been demonstrated in a variety of studies looking at diurnal secretion profiles and feedback responsiveness. Of particular interest is the altered corticosteroid rhythm in bipolar patients (Sachar, 1975; Akesode et al. 1976; Linkowski et al. 1985; Souetre et al. 1989; Linkowski et al. 1994; Cervantes et al. 2001) which takes the form of an elevated level of secretion during the daily nadir which reduces the amplitude of the diurnal cycle. Of relevance to these findings are observed abnormalities in feedback function within the HPA axis in bipolar disorder with a large proportion of patients exhibiting non-suppression of HPA axis activity following administration of the glucocorticoid agonist dexamethasone. Such findings may in part explain the elevated levels of corticosteroid release during the diurnal cycle in bipolar patients, the effects of which have been investigated in this thesis.

A role for dopamine in the aetiology of bipolar disorder has been postulated based on a number of strands of evidence. A general dysregulation of dopaminergic function in the disorder has been suggested by studies looking at dopamine metabolites in the CSF of bipolar patients. These studies have shown elevations in HVA levels during manic episodes (Swann et al. 1983; Gerner et al. 1984; Tandon et al. 1988) whilst depressed bipolar patients have shown decreased levels of this metabolite (Asberg et al. 1984; Reddy et al. 1992, Bottiglieri et al. 2000). A second strand of evidence is the ability of dopaminergic drugs to replicate features of mood states from the disorder. Thus administration of drugs which increase dopaminergic neurotransmission such as amphetamine, levodopa and bromocriptine can induce manic-like states in healthy individuals (reviewed by Silverstone,

1985) whilst decreasing dopaminergic neurotransmission via tyrosine depletion (McLean et al. 2003) or reserpine administration (Schildkraut, 1965) can produce depressive symptoms. Of further relevance is the ability of mood stabilising drugs to alter dopaminergic function in pre-clinical studies. These have shown that lithium (Hesketh et al. 1978; Koyama, 1987; Gottberg et al. 1989), valproate (Ichikawa and Meltzer, 1999) and carbamazepine (Okada et al. 1997) can alter the release of dopamine in its terminal regions, whilst antipsychotic drugs which antagonize dopamine receptors are efficacious in the prophylactic and acute treatment of mania and depression (Wilson et al. 1998; Brambilla et al. 2003; Kapur and Mamo, 2003).

It is the central hypothesis of this thesis that the altered diurnal corticosteroid secretion profile in bipolar disorder is responsible for alterations in dopaminergic function in the central nervous system, and in particular the mesocorticlimbic dopaminergic neurons originating in the ventral tegmental area. Corticosteroids circulate throughout the body and readily enter the brain, and a number of studies in rats have demonstrated the ability of corticosteroids to alter mesocorticolimbic function. Whilst these studies support the hypothesis in as much as they demonstrate that corticosteroids can affect this system, their relevance is limited by the fact that they either utilised high doses of corticosterone (Wolkowitz, 1994; Inoue and Koyama 1996; Pacak et al. 2002; Czyrak et al. 2003) or adrenalectomy (Piazza et al. 1996; Shoaib and Shippenberg, 1996; Lindley et al. 1999; Barrot et al. 2000; Nakahara et al. 2000; Barrot et al. 2001; Mizoguchi et al. 2004) – manipulations which do not model the more subtle changes in corticosteroid levels observed in bipolar disorder. A more clinically relevant manipulation was used in the present study by modelling the pattern of corticosteroid secretion observed in bipolar disorder through administration of low doses of corticosterone to rats in their drinking water. This method has previously been shown to produce a flattened corticosteroid rhythm with many of the features observed in bipolar disorder (G. Fairchild, doctoral thesis 2004) and by measuring the effects of this manipulation on mesocorticolimbic function it was hoped to gain some insight into the neurobiological mechanisms of bipolar disorder.

6.1. Summary of results

6.1.1. Corticosteroid receptor expression in the mesocorticolimbic system

The central hypothesis, in its simplest interpretation, requires corticosteroids to act directly on receptors within dopaminergic cells to modulate their function. In this context the existing literature lacked clarity regarding the presence of corticosteroid receptors in dopaminergic neurons of the VTA and whilst mapping studies had demonstrated the presence of GR (Ahima and Harlan, 1990; Cintra et al. 1994; Morimoto et al. 1996) and MR (Ahima et al. 1991) in this region their cellular location remained unclear. Two studies with conflicting results have been published demonstrating the presence (Harfstrand et al. 1986) and absence (Czyrak and Chocyk, 2001) of GR within dopaminergic cells, whilst MR expression within these cells has not been examined. This prompted the initial studies in this thesis which utilised immunohistochemistry to demonstrate the presence of both MR and GR in VTA dopaminergic cells.

The present study showed that both GR and MR are expressed in dopaminergic neurones within the VTA. Close to half of the sampled tyrosine hydroxylase positive cells in this region were found to express GR, whilst all of tyrosine hydroxylase expressing cells co-expressed MR. This presence of GR and MR in dopaminergic cells of the VTA supports the central hypothesis as it allows for direct corticosteroid modulation of dopaminergic neurotransmission, via alterations in the transcription of regulatory genes and their proteins within dopaminergic cells. This is certainly true of the subset of dopaminergic cells which express GR and MR, where the presence of the low affinity GR suggests that these cells would be responsive to supranormal levels of corticosteroids. In addition, the ability of GR and MR to form heterodimers as suggested by a variety of studies (Trapp et al. 1994; Liu et al. 1995; Ou et al. 2001) allows for some diversity in corticosteroid receptor signalling within these cells, although the implications of this are unknown. Studies investigating corticosteroid receptor occupancy have found that the MR is close to fully occupied by normal physiological levels of corticosterone (Reul and de Kloet, 1985) although this has been questioned (Pace and Spencer, 2005). If Reul and de Kloet are correct, the subset of

dopaminergic neurones expressing MR but not GR are unlikely to play a role in pathophysiological processes resulting from elevated corticosteroid levels. It is likely, however that the MR exerts a tonic regulatory role within VTA dopaminergic neurones, and that decreases in corticosteroid secretion may adversely affect the functioning of these cells.

It had been hoped that combining retrograde tracing with the aforementioned immunohistochemistry would reveal whether there are differences between mesocortical and mesolimbic neurones in their expression of these receptors. This was not possible, and a further attempt to compare the spatial distribution of retrogradely labelled cells from the two projections with the pattern of GR expression also failed. This failure was due to the fact that retrogradely labelled cells from both terminal regions were found intermingled throughout the VTA. This result was somewhat disappointing as data from other groups suggests that these cells may originate in distinct regions of the VTA (Albanese & Minciacchi, 1983; Fallon et al. 1978; Brog et al. 1993; Datiche and Cattarelli , 1996; Williams & Goldman-Rakic, 1998; Hasue & Shammah-Lagnado, 2002). It is possible that this was due to a methodological error, however other groups have shown similar results to those presented here (Margolis et al. 2006). If mesolimbic and mesocortical neurones are intermingled within the VTA the present results do not preclude the possibility that GR is preferentially expressed in either mesocortical or mesolimbic cells. However on the basis of the available data no firm conclusions can be drawn.

6.1.2. Effects of corticosteroid rhythm dysregulation on gene transcription in the VTA

Following the finding that corticosteroid receptors are expressed in VTA dopaminergic cells it was hypothesized that altering the corticosteroid rhythm would alter the transcription of genes within these cells. *In situ* hybridization histochemistry was used to compare mRNA levels within the VTA in tissue from animals which had undergone corticosterone treatment and untreated animals. A number of genes were selected based on their role in DA synthesis (tyrosine hydroxylase), release (VMAT2), reuptake (DAT) and metabolism. In addition gene expression of the D_2 autoreceptor, subunits of the NMDA and AMPA receptors and the 5-HT_{2C} receptor were investigated. The proteins coded for by these genes play important roles

in regulating dopaminergic neurotransmission and were thought to be likely candidates to mediate corticosteroid effects on dopaminergic function.

The results of these experiments demonstrated that modulating the corticosteroid rhythm in rats resulted in increased mRNA expression of the dopaminergic synthetic enzyme tyrosine hydroxylase, the vesicular monoamine transporter VMAT2, and the D_2 autoreceptor. Decreases were found in the GluR1 subunit of the glutamatergic AMPA receptor and the 5-HT_{2C} receptor. These results suggested that both upregulation and downregulation of dopaminergic transmission might occur following corticosteroid induced transcriptional changes. If changes in mRNA levels are reflected at the protein level then both dopamine synthesis, and vesicular transport (and therefore vesicular content) would increase leading to an increase in dopamine release in terminal regions. Further to this the decrease in expression of the 5-HT_{2C} receptor which inhibits dopaminergic neurotransmission would augment this effect. This would likely be a result of a decrease in the firing rate of the GABAergic interneurons on which this receptor is thought to be located, and thus disinhibiting dopaminergic neuronal firing.

In contrast to these results, the increase in D_2 receptor transcription suggests a greater degree of autoreceptor inhibition of neuronal firing and dopamine release which would not only counter the any increase in neuronal firing resulting from the decrease in 5-HT_{2C} receptor transcription via somatodendritic receptors, but also the increase in tyrosine hydroxylase expression via receptors at the terminal. The terminal D_2 receptor regulates the phosphorylation state of tyrosine hydroxylase and inhibits enzyme activity. Thus even with an increase in TH protein as suggested by these results, the concomitant increase in D_2 protein would act to limit increases in dopamine synthesis. This would also limit the effect of VMAT2 upregulation due to lower neurotransmitter availability, and additionally an increased concentration gradient between dopamine containing vesicles and the cytoplasm, thus favouring leakage of dopamine to the cytoplasm. Furthermore the decrease in transcription of the GluR1 subunit of the AMPA receptor would lead to a decreased excitatory drive on dopaminergic neurons decreasing their firing rate.

Analysis of sub-regional differences in mRNA expression within the VTA was performed in an attempt to determine whether there are differences in transcriptional regulation between mesolimbic and mesocortical neurones. Whilst the retrograde tracing experiment in the initial study had failed to find evidence that mesolimbic and mesocortical projections arise in different regions of the VTA, there is some evidence in the literature to support this notion (Albanese & Minciacchi, 1983; Fallon et al. 1978; Brog et al. 1993; Datiche and Cattarelli , 1996; Williams & Goldman-Rakic, 1998; Hasue & Shammah-Lagnado, 2002). The principal findings here were that D_2 receptor transcription was increased to a greater degree in the ventral region of the VTA, a region from which it has been suggested that the majority of dopaminergic neurons project to limbic areas. This suggests that the type of counter-regulatory effects of increased D_2 transcription discussed in the previous paragraph occur to a greater degree in mesolimbic cells compared to mesocortical cells. Further support for this hypothesis is given by reports that the terminal synthesis regulating autoreceptor is less effective in mesocortical cells compared to mesolimbic cells (Hoffmann et al. 1988; Moghaddam and Bunney, 1990; Agneter et al. 1994; Kuroki et al. 1999; Gessa et al. 2000; Devoto et al. 2001; Ago et al. 2005) suggesting that increases in D_2 receptor transcription would have less of an impact on DA synthesis in this projection. This does not however preclude the possibility that the increase in transcription of D_2 mRNA brings the terminal receptor "on-line" in the mesocortical system or that there is an increase somatodendritic D_2 mediated inhibition of firing.

Sub regional analysis of the effect of corticosterone on 5-HT_{2C} receptor expression revealed that in the ventromedial VTA no effect of cortiosterone was found. As the ventromedial region contains mesolimbic neurons projecting to the nucleus accumbens, it might be expected that the decreased inhibitory tone predicted for neurons in other regions of the VTA may not occur in a subset of mesolimbic neurons. This was taken as further evidence that neurotransmission increases by a greater extent in mesocortical neurons following corticosterone treatment.

Another finding of the sub-regional analysis was a significantly greater decrease in GluR1 mRNA in the dorsolateral VTA than other regions. This part of the VTA has been suggested

to contain a high density of mesocortical cells, and the consequence of this could be a relatively greater loss of excitatory tone in this group of neurons compared to mesolimbic cells. It would appear that this finding is anomalous in the broader context of these results as far as they concern the mesocortical system, with the balance of evidence pointing to an upregulation of neurotransmission in this projection, however some attenuation of this effect may occur as a result of AMPA receptor downregulation.

Overall it was hypothesized that in the mesocortical system the effects of corticosteroid dysrhythmia on gene transcription in the VTA would lead to an increase dopamine release as a result of increased synthesis, vesicular content, and decreased 5-HT_{2C} mediated inhibitory tone, with some amelioration of these effects by the decreased excitatory drive resulting from AMPA receptor downregulation and increased somatodendritic D_2 mediated inhibition.

In contrast it was hypothesized that mesolimbic neurons would undergo an increase in TH transcription but the functional effects of this would be limited by an increase in terminal D_2 receptor expression, which would also serve to limit the effects of increased VMAT2 expression and neurotransmitter release. The lesser decrease in 5-HT_{2C} receptor expression in regions of the VTA containing mesolimbic neurons, the decrease in AMPA receptor function and the increased somatodendritic D_2 receptor expression would lead to an overall decrease in the excitatory drive of these cells and reduce the firing rate. Thus the evidence suggests that mesolimbic neurotransmission would decrease following corticosteroid dysrhthymia of the type seen in bipolar disorder.

6.1.3. Effects of corticosteroid rhythm dysregulation on dopaminergic function in mesocorticolimbic terminal regions.

Based on the hypotheses generated by the *in situ* hybridization experiments, the functional effects of corticosteroid dysrhythmia were investigated. The first experiment was an assay of tyrosine hydroxylase activity in the nucleus accumbens and the prefrontal cortex to investigate the theory that this enzyme is differentially regulated by corticosteroids in the mesolimbic and mesocortical systems. The results provided some support for this hypothesis

however they were not conclusive. Tyrosine hydroxylase activity as measured by DOPA accumulation was almost doubled in the prefrontal cortex following corticosterone treatment however the results failed to reach statistical significance. In the nucleus accumbens the results were again inconclusive with a 50% decrease in DOPA accumulation in corticosterone treated animals following 20 minutes of DOPA decarboxylase inhibition (a finding consistent with a previous study, Pacak et al. 2002), but no effect following 2 hours of treatment. Despite the lack of conclusive results these data provided some evidence that TH activity is differentially regulated in mesolimbic and mesocortical neurons. They also gave some support to the hypothesis that the increase in TH mRNA within the VTA would be reflected in an increase in function in the prefrontal cortex but not the nucleus accumbens as a result of differential D_2 receptor function in the two systems. However the nature of these differential effects is yet to be fully elucidated.

The second investigation employed *in vivo* microdialysis to evaluate dopamine efflux in the prefrontal cortex following corticosteroid dysrhythmia to determine whether changes in mRNA transcription in the VTA do, as hypothesized, increase dopamine release in this region. The results demonstrated that dopamine efflux was elevated by ~50% in the presence of bupropion in corticosterone treated animals and additionally that potassium stimulated release increases dopamine levels to a greater degree following corticosterone treatment, with stimulated DA levels ~50% greater than those seen in untreated animals. This was interpreted as evidence for an increase in dopamine release occurring without a change in the neuronal firing rate. Thus it would appear that as far as mesocortical neurons are concerned, the corticosterone induced alterations in GluR1, 5-HT_{2C}, and somatodendritic D_2 receptor transcription seen in the *in situ* study did not affect the neuronal firing rate as had been originally hypothesized.

In contrast, the demonstration of corticosterone induced increases in TH and VMAT2 transcription in the *in situ* study provide a possible explanation for the observed increase in dopamine release. By increasing dopamine synthesis and vesicular uptake, it was hypothesized that vesicular content would increase in corticosterone treated animals. Increased vesicular content would account for the elevated release of dopamine seen in

treated animals in the microdialysis study and could increase release in the absence of changes to neuronal firing rate.

D_2 receptor function in the prefrontal cortex was also probed to determine whether the increase in D_2 mRNA following corticosterone treatment affects terminal autoreceptor function. It was found that intra-PFC application of a D_2 receptor antagonist had no effect on either bupropion or potassium stimulated dopamine release in treated and control animals. This replicates the findings of previous studies which have found little evidence for functional D_2 terminal autoreceptors in this region (Moghaddam and Bunney, 1990; Kuroki et al. 1999; Gessa et al. 2000; Devoto et al., 2001; Ago et al. 2005). Furthermore it confirms the theory that the observed increase in D_2 mRNA would have little impact on terminal receptor function.

This is consistent with the tyrosine hydroxylase assay results where evidence for an increase in enzyme activity was found in the PFC but not the nucleus accumbens following corticosterone treatment. To summarize, the results can be taken as supporting the theory that in mesocortical neurones increased TH transcription following corticosterone treatment led to increased dopamine synthesis. Furthermore the lack of functional D_2 receptors at the terminal meant that this increase in synthesis was not restrained by autoinhibition. The lack of an increase in TH function in the nucleus accumbens suggests that the concomitant increase in D_2 receptor transcription following corticosterone treatment led to an increase in autoinhibition. This in turn attenuated synthesis in these neurons. The result of this was that increased TH transcription in mesolimbic neurons did not lead to an increase in dopamine synthesis.

Whilst the hypothesis regarding mesocortical function which developed from the results of the *in situ* experiments is supported by the present data, mesolimbic function was not fully investigated. Ideally microdialysis would have been performed in the nucleus accumbens to compare dopamine release and autoreceptor function with the prefrontal cortex however this was not possible due to time constraints. The tyrosine hydroxylase assay did provide some evidence to support the theory that dopamine transmission is inhibited due to reduced dopamine synthesis, and it could be argued that as a result the increase in VMAT2

transcription would have limited effect, especially in the long term where dopamine availability is reduced. Furthermore, if it is indeed the case that increased terminal D_2 activity was responsible for the lack of an increase in TH function, this would inhibit neurotransmitter release as well, thus negating the effects of increased VMAT2 transcription. There is some evidence in the literature suggesting that chronic administration of corticosteroids may attenuate dopamine release in the nucleus accumbens (Pacak et al. 2002). This attenuation of dopamine release may result not only from transcriptional changes but also from corticolimbic afferents which inhibit dopamine release in the nucleus accumbens in response to increases in dopamine in the pre-frontal cortex (Thompson and Moss, 1995; Doherty and Gratton, 1996). Consequently it may well be the case that dopaminergic function is differentially regulated by corticosteroids in the mesocortical and mesolimbic systems.

Overall the findings of this study provide evidence that corticosteroid dysrhythmia of the type seen in bipolar disorder increases mesocortical dopamine release and that this probably occurs as a result of increased neurotransmitter synthesis and vesicular packaging. Furthermore there was some evidence that mesolimbic and mesocortical neurons are differentially regulated by this dysrhythmia in terms of their tyrosine hydroxylase activity possibly as a consequence of their differential regulation by the terminal D_2 autoreceptor. These findings are of potential importance in our understanding of mesocorticolimbic dopaminergic function in bipolar disorder.

6.2. Methodological considerations

6.2.1. Immunocytochemistry and retrograde tracing

Immunocytochemistry was used in this study to determine whether corticosteroid receptors are present on dopaminergic cells in the VTA. This is a powerful and reliable technique for the detection of proteins within tissue provided appropriate steps to maintain labeling specificity are used. In the protocol used here a number of methods including peroxidase treatment and blocking steps with rat serum, BSA, and biotin/(strept)avidin were used to ensure labelling was specific to the protein of interest. This was confirmed by carrying out experiments in the absence of primary antibody in which labeling was not seen. As a consequence it is believed that the results obtained here are reliable.

The principle criticism of the immunocytochemistry study would be the limited number of animals used. Whilst it was demonstrated that both MR and GR are expressed in VTA dopaminergic cells, and this result is not in question, there is less certainty over the proportion of cells expressing these receptors. It is possible that GR/TH co-expression varies between animals, hence using more animals would have provided more accurate data. It is the case however that the proportion of GR/TH co-expressing found here was similar to that reported previously (Harfstrand et al. 1986).

In the case of the GR, the fact that it was only found in a proportion of DA cells was interpreted as meaning that those cells where it was not observed do not express GR. These cells may, however, express this protein at a much lower level than those cells where labeling was evident. Labelling of GR required high concentrations of primary antibody compared to labeling for TH and signal amplification steps were required to improve visualization. Given this lack of sensitivity low levels of GR may not have been detectable in our experiments, and as a consequence it may not be accurate to state that those cells in which it was not observed do not express this receptor.

Retrograde tracing was used in an attempt to determine the origin within the VTA of cells projecting to the prefrontal cortex and nucleus accumbens. The results of this study disagreed with a body of literature suggesting that these projections arise in different regions

of the VTA (although two published reports agreed with the results). Consequently no conclusions were drawn from these experiments, principally because there was some doubt over the accuracy of the tracer injections given the proximity of the PFC and NAcc at their respective caudal and rostral extents. These results were not used to derive any hypotheses for later work and are not thought to be detrimental to the rest of the study or any of the conclusions drawn.

6.2.2. In situ hybridization

In situ hybridization was used to quantitavely determine changes in levels of mRNA coding for specific proteins in the VTA. The probes used are of high specificity being complimentary to the mRNA of interest and a significant advantage of this technique is that probes can be designed for any known gene sequence. In this study measuring changes to mRNA levels was considered particularly appropriate given that corticosteroid receptors act as transcriptional regulators.

There are a number of types of probe and various labels which can be used in *in situ* hybridization. In the present study oligonucleotide probes labeled with [^{35}S]-dATP at the 3' tail were used to visualize mRNA. A drawback of this technique is the relatively low signal obtained from the probe, and as a consequence low abundance mRNAs are difficult to detect. Furthermore, where signal is detectable, if mRNA expression is low then changes in expression are more difficult to detect. An alternative to the use of an oligoprobe is to use a riboprobe which can be labeled at multiple sites and gives a better signal, however it has been reported that oligoprobes are more accurate at determining relative levels of mRNA in different brain regions (Broide et al. 2004). Furthermore oligoprobes are easier to label and cheaper to produce making them the better choice for this study.

A major, and often cited, drawback of *in situ* hybridization is that it does not give an accurate measure of protein expression. Wide discrepancies have been demonstrated where changes in mRNA levels have been compared with protein expression (Gygi et al.1999) and this makes extrapolating functional consequences from *in situ* hybridization results difficult. Further to this, the absence of changes to mRNA expression does not offer

any evidence that protein levels are not altered as a consequence of a particular treatment as demonstrated by studies in our laboratory (L. Ferrie, doctoral thesis 2005). In defence of the use of this technique in the present study, the fact that corticosteroid receptors are transcriptional regulators means that their primary effects are via changes in mRNA expression. Measures of mRNA are therefore of value in demonstrating that these receptors are present and functional in the VTA in support of the results from the immunocytochemistry experiments.

A further drawback of *in situ* hybridization is that it does not give any information regarding the phenotype of the cells in which a particular mRNA is expressed. In the present study it was, with one exception (the 5-HT_{2C} receptor), assumed that the changes in mRNA expression occurred in dopaminergic neurons, however the presence of corticosteroid receptors in other cells within the VTA as demonstrated by ICC offers the possibility that other types of cell were involved. In fact this is only likely to be true for the GluR1 subunit of the AMPA receptor and possibly the D_2 receptor (see Pickel et al. 2002). All other probes targeted mRNA coding for proteins specifically related to dopaminergic function (TH, VMAT2, DAT). In the case of the 5-HT_{2C} receptor it was assumed based on evidence from the literature that this receptor was present on GABAergic interneurons, although the evidence to support this is indirect.

6.2.3 Tyrosine Hydroxylase assay

Tyrosine hydroxylase enzyme function was measured in the nucleus accumbens and prefrontal cortex of rats by inhibiting DOPA decarboxylase activity for a period of either 20 minutes or 2 hours prior to sacrifice and subsequently measuring DOPA accumulation in homogenised tissue from the regions of interest. This method gives a measure of the rate of dopamine synthesis as TH is the rate limiting enzyme in this pathway.

DOPA concentrations were calculated by measuring total DOPA tissue content with high performance liquid chromatography. A limitation of this method was the sensitivity of the HPLC assay. In tissue from the prefrontal cortex, 20 minutes of DOPA decarboxylase inhibition did not result in measurable levels of DOPA, whilst after 2 hours levels were

measurable but very low. The results indicated that TH activity was increased following corticosterone treatment in this region but statistical significance was not reached and this is likely to be a direct consequence of the extremely low levels present. As such, limitations in the assay are thought to be responsible for the inconclusive results obtained.

A further reason for the variability in the data, which was also seen in the nucleus accumbens, was the method used to dissect the tissue. The dissection was relatively inaccurate in both regions consisting of tissue blocks containing the region of interest. This offers an explanation for the variable tissue levels recorded, given that the tissue blocks might have contained varying amounts of the adjacent regions. Overall limited conclusions could be drawn from the data although some indication of differential effects of corticosterone in the two brain areas was evident.

6.2.4. In-vivo microdialysis

In vivo microdialysis is a useful method for measuring neurotransmission in the brains of intact animals. It allows measurement of basal neurotransmitter release, as well as the effects of local application of drugs and solutions to probe neuronal function. In this thesis microdialysis was used to measure dopamine efflux under a variety of conditions in rats treated with corticosterone and control animals.

An important factor in interpreting microdialysis data is the invasive nature of the technique. Insertion of a microdialysis probe into the brain causes damage to a number of brain regions en-route to the region of interest. In this study the probe was placed in the infralimbic cortex and passed though more dorsal regions of the PFC during implantation. There is the possibility that this may have affected afferent input to the infralimbic cortex with potential consequences for neurotransmission in this region. In addition there is evidence that probe implantation damages the blood brain barrier (Groothuis 1998). It has been reported that between one and two hours after probe implantation neurotransmitter levels are restored to their normal levels (Benveniste and Huttemeier 1990) and as a consequence in the present studies samples were not taken for at least one hour after surgery.

Another important factor is the use of anaesthetized animals in the present study. Urethane has been reported to decrease neurotransmission in dopaminergic terminal regions such as the striatum (Warenycia and McKenzie, 1988, West, 1998) and as a consequence it has been suggested that anaesthetics are a confounding factor when extrapolating data from studies in anaesthetized animals. The converse argument to this is that anaesthetized animals have reduced sensory input, are not moving around, and show no behavioural responses to drug administration, factors which could complicate the interpretation of results from awake animals. Whilst it is true that the function of the dopaminergic neurons examined in this study might be significantly different to that in an awake animal, the fact that differences were seen in dopamine efflux between treated and control animals remains a valid finding. Given that the changes observed are likely to be the result of increased synthesis and vesicular uptake of dopamine rather than a change in neuronal firing rate (which is more likely to be affected by anesthesia due to alterations in afferent input), one would expect a similar effect of corticosterone on dopamine release in awake animals. The findings do therefore provide evidence that corticosterone dysregulation produces an increase in dopaminergic neurotransmission within the prefrontal cortex.

6.2.5. Corticosterone treatment model

The corticosterone treatment model used in this thesis, in which corticosterone was administered to animals via their drinking water, has been validated in previous studies carried out in our laboratory (G. Fairchild, doctoral thesis 2004). Repeated blood sampling over a twenty four hour period has shown that this treatment leads to an increase in corticosterone levels during the diurnal nadir (approximately 5-8 fold greater than those seen in vehicle treated animals) whilst leaving the diurnal peak relatively unchanged. This pattern of secretion is similar to that seen in bipolar disorder patients with HPA axis abnormalities (Akesode et al. 1976; Linkowski et al. 1985; Linkowski et al. 1994; Souetre et al. 1991; Cervantes et al. 2001). Corticosterone administration via drinking water offers several advantages over administration via osmotic minipump or corticosterone pellet. First it is non-invasive avoiding the stress of surgery and recovery and the confounding influence of

anaesthetic treatment. Furthermore it is highly flexible allowing the withdrawal and reinstatement of treatment at any time and as such can be adapted to model episodic as well as chronic dysregulation of corticosterone treatment. It should be noted, however, that this model does not mimic other aspects of HPA axis dysregulation seen in bipolar disorder (see 1.2.3.) including reduced negative feedback regulation, ACTH secretion hypo-responsiveness to CRH, or ACTH hyper-responsiveness to combined dexamethasone/CRH. As such what is modeled is not the HPA axis dysfunction itself, but rather the corticosterone secretion profile seen with this dysfunction. Whilst it is believed this has validity for the study of corticosteroid effects on CNS function in bipolar disorder, the limitations of the model must nevertheless be taken into consideration.

6.2.6. Rodent modelling of human disease states

The use here of a rodent model to investigate mechanisms which might be responsible for bipolar disorder in humans has two principal limitations. The first of these is the validity of the model i.e. what is actually being modeled and how can that be interpreted in a clinical context. The second limitation is the question of whether a rat model has validity for the study of human disease processes.

The issue of what is actually being modeled is extremely important, and has to a certain extent been discussed in section 6.2.5. Here it was stated that the corticosterone treatment paradigm only models the pattern of corticosterone secretion seen in bipolar disorder, but not the HPA axis dysfunction itself. More fundamentally, this treatment does not produce a model of bipolar disorder. Rather, it may model a vulnerability towards bipolar disorder in individuals with HPA axis dysfunction, which with the right interplay of genes and environment can trigger the condition in some individuals. The fact that very similar HPA axis abnormalities are found in some patients with unipolar depression (Yehuda et al. 1996; Deuschle et al. 1997) further argues against this as a model of bipolar disorder specifically but possibly a vulnerability model for affective disorders in general. A common aetiological mechanism for affective disorders would fit with the notion that bipolar disorder and depressive disorders are not distinct but exist on a continuum with mood states ranging from

pure mania to outright depression (see Akiskal, 2002; Muller-Oerlinghausen, 2002). In the context of more categorical definitions of affective disorders, the HPA axis abnormalities are likely to interact with environmental and genetic factors which determine the specific type of affective disorder triggered in a susceptible individual, with bipolar disorder representing one of many possible outcomes. In light of these considerations it can be seen that simply administering corticosterone will not produce a cage full of bipolar rats any more than corticosterone administration to humans will induce bipolar disorder. In defence of the model it was never the intention to replicate bipolar disorder but to investigate the effects of corticosteroid dysrhythmia on dopaminergic function. This has relevance to bipolar disorder due to the HPA axis and dopaminergic abnormalities which have been observed in the illness and is likely to have some value in aiding our understanding of dopaminergic aspects of the disease process.

The reason a model of the disease itself was not attempted stems from the incredibly complex nature of the illness and our lack of understanding of it. In the past, attempts have been made to model mania in rats using stimulant drugs, sleep deprivation, brain lesions and electrical stimulation, whilst models of depression have utilised stress paradigms, brain lesions, neurochemical manipulation, and rats bred to exhibit depressive behaviours (reviewed by Machado-Vieira et al. 2004). Although these models produce certain behaviours which are thought to be analogous to those seen in human mania or depression they have consistently failed to replicate anything like the full spectrum of affective and cognitive disturbances characteristic of the illness. Furthermore there has never been a model which produces one of the most important characteristics of bipolar disorder which is mood cycling. As a consequence whilst these models might provide insights into the generation of mood and its dysregulation, they cannot be described as models of bipolar disorder.

A more fundamental problem with any animal model of bipolar disorder is how closely cognitive and emotional dysregulation, as measured by relatively primitive behaviours, can be related to the symptoms of the disease in humans. This is particularly true of rodent models where cognitive and emotional processing is far less advanced than in humans –

indeed it is questionable whether rodents can develop bipolar disorder at all, and if they can would we recognize it as such? In the present study these problems were avoided by attempting to model what may be a causative factor or vulnerability trait for bipolar disorder, looking at the effects of this on neuronal function, and attempting to relate the results to neuronal abnormalities which are thought to contribute to bipolar disorder in humans. At the neuronal level a rodent model can provide insight into human brain function given the broad similarities in basic function and anatomy between mammalian brains. Even at this level however there are questions over the validity of rodent models.

In the present study increases in dopamine release were found in the medial prefrontal cortex of rats following corticosterone treatment and in the following section the potential clinical implications for humans will be discussed. It is the case, however, that the presence of a rat prefrontal cortex analogous to and serving the same functions as the human/primate prefrontal cortex has been questioned (Preuss, 1995). Anatomically, the prefrontal cortex is defined as the regions of the frontal lobe receiving afferents from the mediodorsal thalamic nucleus (Rose and Woolsey, 1948). In primates this includes the dorsolateral, medial and orbital frontal cortices, whilst in rats only the medial and orbital cortices receive these projections. As a consequence of this it has been suggested that the primate dorslateral cortex is functionally unique, which, were it to be true, would make the extrapolation of data from the rat PFC to humans somewhat difficult. There is evidence to suggest, however, that dorsolateral functions in humans are carried out in other regions of the PFC in rats. In humans the dorsolateral prefrontal cortex is associated with so called “executive” cognitive processes including working memory, attentional control, reasoning and decision-making, and the temporal organization of behaviour (Brown and Bowman, 2002). In rats, similar functions have been found associated with the medial prefrontal cortex (reviewed in Brown and Bowman, 2002), suggesting that despite the lack of a dorsolateral PFC, its cognitive functions are preserved. A good demonstration of this is the role of these regions in the formation of higher order rules for guiding behaviour. In humans this can be tested using the Wisconsin Card Sort Test which measures attentional set-shifting and is dependent on visual-processing, attention, and working-memory. Studies in humans have shown that

lesions of the frontal lobe, including dorsolateral PFC lesions, impair performance on this test with a greater number of perseverative errors due to an inability to shift attention from one aspect of a complex stimulus (e.g. colour) to another (e.g. shape) (reviewed by Alvarez and Emory, 2006). In rats an analogous test has been devised which involves the animals digging for food in bowls where stimuli vary in terms of odour, digging medium or surface texture (Birrell and Brown, 2000). In this test, lesions of the medial prefrontal cortex result in impairments of set-shifting analogous to those seen with dorsolateral prefrontal cortex lesions in humans. The similarities in cognitive function between human dorsolateral PFC and rodent mPFC suggest that changes to PFC function in rats can be related to PFC function in humans including those processes associated with the dorsolateral PFC. As a consequence it can be argued that any discussion of mPFC dysfunction as it relates to cognition in rats can be extrapolated to cognitive function in humans, and furthermore that the results from the present study have clinical relevance as far as the effect of HPA axis dysregulation on prefrontocortical function is concerned.

6.3. Clinical Implications

The results presented in this thesis demonstrate that flattening of the corticosteroid rhythm results in an increase in the release of dopamine in the prefrontal cortex. This is likely to result from corticosteroid action at receptors within VTA dopaminergic cells which increase the transcription of genes coding for tyrosine hydroxylase and the vesicular monoamine transporter in response to elevated cortisol levels during the diurnal nadir in secretion. An increase in dopamine release in this region is consistent with the current monoamine hypothesis of bipolar disorder which states that dopaminergic hyperfunction is in part responsible for the emotional and cognitive impairments characteristic of the disease (see chapter 1). The following section considers in more detail how excess dopamine release in the prefrontal cortex might be involved in these impairments and examines the evidence that HPA axis dysfunction is a pre-morbid vulnerability trait for affective disorders.

6.3.1. Prefrontocortical cognitive function

In chapter 5 the implications of increased dopamine release in the prefrontal cortex were discussed in relation to the known functions of this part of the brain. The microdialysis study measured dopamine levels in the pre- and infralimbic cortices which have been associated with "executive functions" such as working memory, attentional regulation, action-outcome contingency detection and visual attention selectivity in rats (Dalley et al. 2004). In studies of working memory in rodents modest elevations in dopaminergic activity using DA agonists were shown to improve working memory, whilst supranormal DA activity impaired working memory (reviewed by Arnsten, 1997; Robbins, 2005). Furthermore, stress induced elevations in dopamine release produce impairments of working memory in a similar fashion to those induced by DA agonists (reviewed by Arnsten, 1997; Robbins, 2005). The increase in dopamine levels seen in the present study was relatively modest (~50% increase) compared to the levels which have been shown to produce working memory impairments (~150-250% increase; Murphy et al. 1996; Verma and Moghaddam, 1996) and it is possible that cognitive function improves with such an increase. It is currently unclear, however, at what level of dopaminergic stimulation cognition is impaired and it could equally be the case

that the increase in DA release seen here is detrimental to working memory and other executive functions.

An impairment of prefrontal cognitive function as a consequence of elevated dopamine levels would be consistent with neuropsychological findings in bipolar disorder. A number of studies have demonstrated impairments to executive function in bipolar patients (reviewed by Quraishi and Frangou, 2002; Savitz et al 2005) and these deficits are present in depressed, manic, and hypomanic phases of the illness (Martinez-Aran et al. 2004; Malhi et al. 2007) as well as during the euthymic phase when mood symptoms are absent (Martinez-Aran et al. 2004; Thompson et al. 2005; Kolur et al. 2006). The presence of neurocognitive deficits in euthymic patients suggests that the impairment is independent of mood state and may represent a trait marker for bipolar illness. Further evidence for this is given by the fact that whilst the degree of neurocognitive impairment correlates with severity and length of illness (Robinson and Ferrier, 2006), significant deficits are present at first hospitalization (Gruber et al. 2007) making it possible that the impairment was present prior to the onset of affective symptoms. As circadian corticosteroid secretion is abnormal in all phases of bipolar illness (Akesode et al. 1976; Linkowski et al. 1985; Linkowski et al. 1994; Souetre et al. 1991; Cervantes et al. 2001) such persistent neurocognitive deficits support the theory that HPA axis abnormalities play a role in their generation. Further to this the ability of corticosteroids to modulate neurocognitive function in healthy individuals has been demonstrated in studies where corticosteroid administration was found to impair verbal and working memory (Lupien et al. 1999; Newcomer et al. 1999). It may be the case that elevated dopamine levels caused by chronic HPA axis dysrhythmia are responsible for deficits in executive function which are entirely independent of mood state and may precede the onset of affective symptoms, thus implicating both HPA axis abnormalities and dopamine induced neurocognitive deficits as vulnerability factors for bipolar disorder.

Such a theory depends on the elevation of dopamine levels being sufficient to impair cognitive function in itself, and, as discussed previously, this may not in fact be the case. Under these circumstances an alternative interpretation of the present results is that the threshold for cognitive impairment is reached more readily under conditions of stimulated

dopamine release in people with HPA axis abnormalities due to an elevated basal level of dopamine. In addition the fact that potassium stimulated release produced a greater peak concentration of DA in treated animals indicates that there is a greater capacity for dopamine release in these animals than in controls. As a consequence it is possible that not only is the threshold for cognitive impairment reached more readily in people with a flattened corticosteroid rhythm, but that the cognitive impairment produced is more severe due to more dopamine being released following an appropriate stimulus.

These types of effects are likely to manifest themselves during stress induced increases in prefrontal dopamine. The ability of acute stress to increase dopamine release in the prefrontal cortex has been hypothesised to be of benefit in the "fight or flight" response where fast habitual responding is required to deal with perceived threats. Elevated dopamine levels which produce an impairment of PFC cognitive function actually serve to take the PFC "offline" via inhibition of cortical pyramidal cells during stressful events, and it has been shown that these elevations of dopamine in the PFC improve performance at simple well-rehearsed tasks which are presumably controlled by lower order brain functions (Arnsten, 1997). It could be suggested therefore that the elevated basal DA levels put the PFC into a state where it is primed to over-respond to stressors, increasing the likelihood of triggering a "fight or flight" response where it is inappropriate. Such a priming mechanism might play a role in the well documented ability of stressful events to trigger both first-onset and repeated episodes of bipolar disorder. Thus whilst corticosteroid dysrhythmia may not be directly responsible for neurocognitive deficits, the dopaminergic hyper-responsiveness it produces may predispose individuals to this type of impairment, again implicating it as a vulnerability factor in bipolar disorder.

6.3.2. Prefrontocortical regulation of emotion

Whilst HPA axis dysfunction might be responsible for neurocognitive deficits in bipolar disorder, or act as a priming mechanism for such deficits, linking it to emotional aspects of the illness is more problematic. The fact that the pattern of corticosteroid secretion is not predictive of mood state indicates that other mechanisms are involved in the generation of

the fundamentally different symptoms characteristic of mania and depression. Despite this there is evidence that the prefrontal cortex is involved in the regulation of emotion although the processes involved are poorly understood.

Lesions of the medial prefrontal cortex in humans impair the ability of subjects to anticipate future negative and positive consequences of their actions and to cope emotionally with different situations (reviewed in Davidson and Irwin, 1999; Bandler et al. 2000) and it has been suggested that damage to the PFC impairs the ability to sustain emotion so it can be used to guide future behaviour (Davidson and Irwin, 1999). Adaptive responding to emotional experiences is dependent on the ability to regulate emotion via cognitive processes (Urry et al. 2006) and a number of imaging studies have shown that the prefrontal cortex is involved in this regulatory mechanism via modulation of amygdala activity during conscious suppression of emotion (Beauregard et al. 2001; Levesque et al. 2003; Ochsner et al. 2004; Ohira et al. 2006; Urry et al. 2006). Afferents from the medial prefrontal cortex to the amygdala have been demonstrated in both rats (Gabott et al. 2005) and primates (Stefanacci and Amaral, 2002) and lesions of the medial prefrontal cortex in rats impair the extinction of conditioned responses to aversive stimuli (Morgan et al. 2003). The role of dopamine in this process has been demonstrated by selectively lesioning DA neurones in the prefrontal cortex, the result of which is to delay the extinction of fear conditioning (Morrow et al. 1999; Fernandez-Espejo, 2003) in a similar fashion to that seen with gross lesions of the mPFC. These effects parallel the findings in the human imaging studies mentioned previously in as much as the ability of the animal to suppress the fear response to the conditioned stimulus once it is no longer presented with a painful stimulus is impaired following prefrontal lesions. The elevated dopamine levels seen in the present study may have an analogous effect to gross lesions as a result of their ability to reduce prefrontocortical function (Arnsten, 1997) and this may impair emotional processing in a similar fashion. This makes it possible that the changes to prefrontocortical function seen in the present study affect the ability of the PFC to regulate emotional adaptation and this implicates such changes in the affective dysregulation characteristic of bipolar disorder.

Evidence for prefrontocortical-amygdala dysfunction in bipolar disorder to support this theory is provided by studies which have examined facial emotion recognition in bipolar patients, a process associated with activity in the prefrontal cortex and the amygdala (O'Doherty et al. 2003; Ochsner, 2004). Abnormalities in facial emotion processing have been found in remitted (Bozikas et al. 2006; Pavaluri et al. 2007), depressed (Lawrence et al. 2004) and manic (Lennox et al. 2004) bipolar subjects suggesting that this may be a trait marker for bipolar illness. The evidence that this deficit is present in all mood states supports the theory that HPA axis dysfunction may play a causative role, given that the pattern of circadian cortisol secretion in bipolar patients is abnormal across mood states (Akesode et al. 1976; Linkowski et al. 1985; Linkowski et al. 1994; Souetre et al. 1991; Cervantes et al. 2001). As such it is possible that HPA axis dysregulation adversely affects prefrontocortical function, in turn impairing emotional processing in the amygdala.

Such an involvement of the HPA axis in these processes is given further credence by the previously mentioned imaging study by Urry et al. (2006) which looked at suppression of negative emotions in human subjects. By taking salivary cortisol samples throughout the day it was demonstrated that subjects with a flattened cortisol secretion profile performed less well in their ability to suppress negative emotions than subjects with a normal profile, an intriguing finding given that the data in this thesis could provide the "missing link" between the flattened cortisol profile and the PFC-amygdala dysfunction associated with negative affect suppression. It is possible that flattening of the cortisol rhythm increases PFC dopamine (as shown in the present study), which in turn alters prefrontocortical regulation of the amygdala to produce the impairments of emotion seen in bipolar disorder.

6.3.3. Implications of potential changes to dopaminergic transmission in the nucleus accumbens

Dopamine release in the nucleus accumbens is thought to play a central role in the brain reward circuit and increases in dopamine release in this region are associated with the rewarding properties of intracranial self stimulation, drugs of abuse, food, sex, and social interactions (reviewed by Salamone et al. 2002a). The exact role dopamine plays in reward

related processes is somewhat controversial, but there is evidence that dopamine is involved in anticipatory and "wanting" aspects of reward rather than the rewarding properties of a stimulus per se (Salamone and Correa, 2002). Evidence for this is provided by studies which have shown that dopamine depletion or administration of dopamine antagonists in the NAcc reduces the amount animals are willing to work for a rewarding stimulus but do not impair the appetite for that stimulus (Aberman et al. 1999; Nowend et al. 2001; Correa et al. 2002; Salamone et al. 2002). The results of the tyrosine hydroxylase assay in chapter 5 suggest that dopamine synthesis in the nucleus accumbens may be decreased by flattening the corticosteroid rhythm and this is supported by experiments performed by Pacak et al. (2002) showing that chronic hypercortisolaemia inhibits dopamine synthesis and turnover in this region. Furthermore there is evidence that an increase in dopamine release in the prefrontal cortex, such as that seen in the present study, can inhibit dopamine release in the nucleus accumbens (Thompson and Moss, 1995; Doherty and Gratton, 1996). A role for glucocorticoids in regulating reward processes is suggested by studies showing that adrenalectomy attenuates the release of dopamine in the NAcc in response to cocaine (Barrot et al. 2000) and that this occurs selectively in the nucleus accumbens., whilst administration of corticosteroids to replicate stress levels promotes the self administration of drugs of abuse (Piazza et al. 1991; Mantsch et al. 1998). Although the effects of flattening the corticosteroid rhythm on dopamine release in the NAcc are unknown, the literature suggests that a functional consequence might be a disruption of dopamine mediated motivational processes in the reward pathway. Disruption of systems involved in the generation of reward has been implicated in anhedonic and motivational aspects of major depression (Nestler and Carlezon, 2006) and deficits in these functions accord with the symptoms of depressive states in bipolar disorder. The high comorbidity of substance abuse disorders with bipolar disorder (see Krishnan, 2005; Hirshfeld and Vornik, 2005; Pini et al. 2005) further supports the notion that dopaminergic aspects of the reward system are dysfunctional in the illness increasing the susceptibility of individuals to self administer drugs of abuse which activate this system. This active seeking of rewarding stimuli is also consistent with the symptoms of mania in which there is compulsive reward seeking

behaviour in conjunction with a disregard or inability to predict the consequences of such behaviour. Without knowing the effect of flattening the corticosteroid rhythm on dopamine release in the nucleus accumbens, and given the complex role of dopamine in these processes, it is difficult to predict the consequences for human reward functions. The evidence suggests, however, that the flattening of the corticosteroid rhythm seen in bipolar disorder may induce changes in nucleus accumbens dopaminergic function that play a role in the affective symptoms of the illness via interfering with the brain reward system.

6.4. Conclusion

In conclusion the present thesis demonstrates that flattening of the corticosteroid rhythm results in changes to mRNA transcription in the VTA which produces differential effects on dopamine synthesis in the mesocortical and mesolimbic pathways whilst upregulating dopaminergic neurotransmission in the prefrontal cortex. Furthermore the finding that MR and GR receptors are present in dopaminergic cells of the VTA suggests that these effects result from a direct modulation of gene transcription in these cells rather than indirect interactions with other brain systems.

This increase in dopamine release in the prefrontal cortex is consistent with alterations to neurocognitive function and emotional processing which are thought to be trait markers for bipolar illness which, when taken into consideration with the evidence that the cortisol profile is altered in euthymic bipolar individuals and across all mood states, suggests that flattening of the 24 hour cortisol profile may be responsible for such deficits and may in itself be a trait marker for bipolar disorder.

The implications for nucleus accumbens dopaminergic function were consistent with affective dysregulation resulting from decreased dopaminergic activity in this region. This is certainly consistent with anhedonic aspects of depressive phases of the illness and may also account for the pleasure seeking aspects of mania which may be a compensatory strategy for such a deficit.

Overall the data presented in this thesis suggests that a flattened cortisol rhythm may be a vulnerability factor for bipolar disorder which disrupts dopaminergic neurotransmission and may account for alterations in brain function associated with deficits in cognitive and emotional processing present in bipolar individuals.

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