The Effect of Antidepressant Treatment on Brain-Derived Neurotrophic Factor Expression in the Rat Hippocampus

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In Loving Memory Of My Father,
Dr. Abdul-Majid Khundakar.

Abstract

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Enhanced BDNF expression has been implicated in the mechanisms of action of antidepressant treatment. Previous findings have shown that chronic antidepressant drug treatment produces a 'bi-phasic effect' on total BDNF gene expression, i.e. down-regulation at 4h and up-regulation at 24h. The earlier effect is common to acute administration, while the latter is unique to chronic treatment. To clarify the involvement of differential BDNF transcripts in the bi-phasic effect, this thesis aims to investigate this effect by examining the effect of a range antidepressant drugs on individual BDNF exon transcripts. For comparison, the effect of electroconvulsive shock application on BDNF exon transcripts was also studied. In addition, the thesis investigated the mechanisms behind the reduction in BDNF expression at 4h. It was hypothesised that GABA plays an inhibitory role on BDNF mRNA, thus various GABA enhancing drugs were examined. Finally, to investigate if BDNF mRNA changes correlated with changes the corresponding protein, BDNF protein expression was studied after chronic antidepressant treatment. *The main findings were as follows:*

- Acute antidepressant drug treatment inhibited total BDNF expression and exon IV, but not exon I mRNA in the dentate gyrus (DG) at 4h.
- Acute $GABA_B$, but not $GABA_A$ receptor stimulation inhibited total BDNF mRNA in the DG at 4h
- Chronic antidepressant treatment increased total BDNF and exon I, but not exon IV mRNA in the DG at 24h

- Acute ECS increased total BDNF, exon I and exon IV mRNA; chronic ECS increased total BDNF and exon I, but not exon IV mRNA
- Chronic ECS increased BDNF immunoreactivity in the parietal cortex and mossy fibre zone, CA3 and CA1 of hippocampus.

This thesis has thus shown a differential effect of acute and chronic antidepressant treatment on total BDNF mRNA, an effect mediated by differential use of the variable exons that comprise the gene.

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Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (Serotonin)
5-HTP	5-hydroxytryptophan
AADH	Aromatic L-amino decarboxylase
ABC	Avidin-biotin horseradish peroxidase complex
AC	Adenylate cyclase
ACTH	Adrenocorticotrophin
ANOVA	Analysis of variance
ATF	Anti-activating transcription factor 1
BDI	Beck Depression Inventory
BDNF	Brain-derived neurotrophic factor
bp	Base pair
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
COMT	Catechol-o-methyltransferase
CRE	Cyclic adenosine monophosphate response element
CREB	Cyclic adenosine monophosphate response element binding protein
CREM	Cyclic adenosine monophosphate response element modulator
CRF	Corticotrophin releasing factor
CSF	Cerebrospinal fluid
DA	Dopamine

DAG	Diacylglycerol
dATP	Deoxyadenosine 5'-Triphosphate
DEPC	Diethyl pyrocarbonate
DG	Dentate gyrus
DHMA	3, 4 dihydroxymadelic acid
DHPG	Aldehyde 3, 4 dihydroxyphenyglycol
DNA	Deoxyribonucleic acid
DRN	Dorsal raphe nucleus
DSM-IV™	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition™
DTT	Dithiothreitol
ECS	Electroconvulsive Shock
ECT	Electroconvulsive therapy (treatment)
EDTA	Ethylenedinitro tetraacetic acid
ELISA	Enzyme-linked sorbent assay
E-LTP	Early phase long-term potentiation
GABA	Gamma aminobutyric acid
G-protein	Guanine nucleophile binding protein
HAMD	Hamilton Rating Scale for Depression
HPA	Hypothalamic pituitary axis
IC_{50}	Inhibitory concentration 50%
ICC	Immunocytochemistry
ICD-10	International Statistical Classification of Diseases and Related Health Problems (10th Revision)
IDD	Inventory for Diagnostic Depression
IEG	Immediate early gene
IgG	Immunoglobulin G

IgY	Immunoglobulin Y
i.p.	Intraperitoneal
IP_3	Inositol-1, 4, 5-triphosphate
IPSP	Inhibitory post-synaptic potential
ISH	In-situ hydridisation
JNK	Jun-N terminal kinase
LC	Locus coeruleus
L-DOPA	Levadopa
L-LTP	Late phase long-term potentiation
LTP	Long-term potentiation
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MAPK	Mitogen activated protein kinase
MF	Mossy fibres
MFB	Medial forebrain bundle
MFZ	Mossy fibre zone
Mg/kg	Milligrams per kilogram
MHPG	3-methoxy-4-hydroxyphenylglycol
MRI	Magnetic resonance imaging
MRN	Median raphe nucleus
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
NARI	Noradrenaline reuptake inhibitor
NGF	Nerve growth factor
NMDA	N-methyl-D-asparate
NT-3	Neurotrophic factor-3

NT-4/5	Neurotrophic factor-4/5
NT-6	Neurotrophic factor-6
Par ctx	Parietal cortex
PBS	Phosphate buffered saline
pCA	p-chloroamphetamine
PCPA	p-chlorophenlalanine
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PET	Positron emission tomography
PFA	Paraformaldehyde
PI	Phosphoinositide
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphoinositide 4, 5- biphosphate
PLC	Phospholipase C
PKC	Protein kinase C
PVN	Paraventricular nucleus
RIMA	Reversible monoamine oxidase-A inhibitor
RN	Raphe nucleus
S.E.M.	Standard error of the mean
SERT	Serotonin transporter
SNRI	Serotonin/Noradrenaline reuptake inhibitor
SPECT	Single photon emission computed tomography
Shc	Homologous and collagen-like adaptor protein
SSC	Standard saline citrate
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant

TCP	Tranylcypromine
TdT	Terminal deoxynucleotidyl transferase
TEA	Trietyhlamine
TH	Tyrosine hydroxylase
THIP	4,5,6,7-tetrahydroisoxazolo{5-4-C]pyridin-3-0
T_m	Melting temperature
TPH	Tryptophan hydroxylase
TRIS	TRIS (hydroxymethyl) aminomethane
TrkB	Tyrosine kinase B
TRP	L-tryptophan
TTX	Tetrodotoxin
VMA	3- methoxy-4-hydroxy-mandelic acid

Publications

The publications listed below have arisen from the data presented in this thesis.

Khundakar AA, Mazumdar A & Zetterström TSC. (2002). Gene expression for brain-derived neurotrophic factor in rat hippocampus is reduced by baclofen, tranylcypromine and fluoxetine, but not by flunitrazepam. *Br J Pharmacology* **136** 57.

Khundakar AA & Zetterström TSC. (2002). Differential expression of brain-derived neurotrophic factor expression after paroxetine and tranylcypromine. *Br J Pharmacology* **137** 122.

Khundakar AA, Marsden CA & Zetterström TSC. (2003). Gene expression of BDNF exons in the rat brain after repeated antidepressant drug administration. *Meeting abstract for British Neuroscience Association*, April 2003.

Khundakar AA, Marsden CA & Zetterström TSC. (2003). Differential effects of acute and chronic antidepressant administration on BDNF exon mRNA distribution in rat hippocampus. *Meeting abstract for British Association for Psychopharmacology*, July 2003.

Khundakar AA, Marsden CA & Zetterström TSC. (2003). Differential effects of acute and chronic antidepressant administration on BDNF exon mRNA distribution in rat hippocampus. *Meeting abstract for Society for Neuroscience*, November 2003.

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Chapter One

General Introduction

1.1. Scope of the thesis

Depression is among the most prevalent forms of mental illness and a major cause of morbidity worldwide. Despite limited understanding into the pathophysiology of the disorder many treatments with varying degrees of effectiveness are available.

The serendipitous discovery of two classes of antidepressant drugs: tricyclics and monoamine oxidase inhibitors indicated the facilitation of 5-HT and/or noradrenaline neurotransmission in the mechanism of action of antidepressant drugs and this has led to the development of numerous second-generation antidepressants designed specifically to augment 5-HT (SSRIs) or noradrenaline (NARIs). However, despite rapid augmentation in extracellular 5-HT and/or noradrenaline levels by antidepressant drug application, several weeks of chronic treatment are required in order for full therapeutic efficacy to occur. This has led to ongoing research focusing on events beyond the monoamine receptor. Various 5-HT and noradrenaline receptor subtypes are known to selectively regulate several intracellular pathways which activate numerous target genes, prolonged activation of

which may be instrumental in eventual therapeutic efficacy. One such downstream target gene implicated is the neurotrophin brain-derived neurotrophic factor (BDNF).

Chronic antidepressant drug treatment has been shown to increase BDNF mRNA expression in the (Nibuya et al, 1995) and human (Chen et al, 2001) brain. In addition, direct infusion of BDNF protein has been shown to produce significant antidepressant effects in animal models of depression (Siuciak et al, 1997). Contrary to this, acute (single) antidepressant administration has been shown to inhibit BDNF mRNA acutely (Coppell et al, 2003). An explanation to this phenomenon may lie in the existence of multiple BDNF transcripts, which may respond differentially following short- and long-term antidepressant drug administration, and thus contribute to an overall change in the full BDNF gene.

BDNF gene transcripts are comprised of either of four variable 5′ exons (exons I to IV) each induced with individual promoters by alternative splicing of the total gene sequence, and an invariable 3′ exon (exon V), which codes for the corresponding BDNF protein (Timmusk et al, 1993). These transcripts are expressed differentially

across brain areas and are regulated by different manipulations. As the BDNF gene is variably manipulated by acute and chronic antidepressant drug treatment and BDNF facilitation is associated with the mechanism of action of antidepressants, investigating the effect on the variable BDNF exon transcripts could further elaborate on the role of BDNF in antidepressant drug treatment. This may be particularly useful in understanding the reasons and mechanisms behind the acute inhibition in BDNF mRNA after acute antidepressant administration. Thus, by using oligonucleotide probes specific to total BDNF mRNA, as well as two variable exon-specific probes, a series of *in-situ* hybridisation (ISH) experiments were conducted. This was to assess the effect of acute administration of numerous antidepressant drugs on two variable BDNF exon transcripts (exons I and IV), as well as total BDNF mRNA (exon V). The drugs were chosen for their varying effect on 5-HT or noradrenaline reuptake sites or metabolism in order to determine whether 5-HT or noradrenaline facilitation played a prominent role in the acute inhibitory response. For comparison, the effect of acute administration of two drugs with little effect on 5-HT transmission: the mixed noradrenaline/dopamine reuptake inhibitor methylphenidate and the selective dopamine reuptake inhibitor GBR 12909 on

BDNF exon transcripts was also investigated. The results from this series of experiments are presented in *Chapter 3.*

It was further hypothesised that the acute inhibitory effect of antidepressants on hippocampal BDNF mRNA occur as a result of the activation of stimulatory 5-HT receptors localised on GABAergic interneurones in the hippocampus. In order to test this series of ISH experiments, the effect of agonists selective to $GABA_A$ or $GABA_B$ receptor complexes on BDNF mRNA was assessed. In addition, the effect of selective GABA receptor antagonists administered prior to antidepressant drugs was assessed to clarify $GABA_B$ receptor involvement in the acute inhibition of BDNF mRNA in the hippocampus *(See Chapter 4)*.

The up-regulatory BDNF response to chronic antidepressant treatment was then investigated. By employing oligonucleotide probes specific to two variable exons (exons I and IV), as well as total BDNF mRNA (exon V), a series of ISH experiments were performed to assess the effect of chronic antidepressant administration in the hippocampus *(See Chapter 5)*.

Electroconvulsive therapy (ECT) is one of the most effective antidepressant treatments for severe or resistant forms of depression. Repeated administration of electroconvulsive shock (ECS) in rats has been shown to elicit profound increases in BDNF mRNA in the hippocampus. A series of ISH experiments were conducted to assess the effect of acute and chronic ECS on two variable exon transcripts as well as total BDNF mRNA *(See Chapter 6)*.

Though many studies have examined the effect of antidepressant treatment on BDNF mRNA, few have studied the corresponding protein product. The effect on BDNF protein is vital as the protein represents the functional correlate of BDNF signalling. Thus, *Chapter 7* studied the effect of ECS and monoamine oxidase inhibitor antidepressant tranylcypromine on BDNF protein levels in the hippocampus using immunocytochemistry techniques.

The aim of this work is to examine the effect of antidepressant drug treatment and ECS on the BDNF gene and its protein product within the hippocampus of the adult rat, with the overall objective of improving understanding of the mechanisms of action of antidepressant treatment.

1.2. Depression

Depression is a common, debilitating, at times life-threatening psychiatric illness. The term encompasses many symptoms and varies in severity. Many people will at some point experience a transient state of depressed mood (anhedonia), especially in times of adversity. This feeling usually passes with new experiences and emotions and will not require any form of psychiatric intervention. However, if the depressed mood is prolonged and begins to greatly impede a person's ability to lead a normal life, intervention may be necessary. The core symptom of depression is anhedonia. An individual may also experience 'subsidiary symptoms', such as feelings of worthlessness or guilt, suicidal tendencies, disturbed bodily functions such as weight loss, psychomotor retardation and reduced cognitive function, fatigue, loss of sexual appetite and disturbed diurnal patterns (Arbabzadeh-Bouchez et al, 2002). In addition the persons appearance may deteriorate, dressing and grooming for example may be neglected. Generally, diagnosis of depression will occur in a person experiencing one core symptom as well as four subsidiary symptoms every day over a two-week period impairing social

and occupational functioning in the absence of psychotropic drugs or bereavement (DSM-IV™, 1994).

1.3. Antidepressant Treatment

1.3.1. Antidepressant drug treatment

Antidepressant treatment by pharmacological means began in the 1950s with the serendipitous discovery of two main classes of antidepressant drugs: tricylic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs). TCA antidepressants were derived from phenothaizides, which were originally intended for use as antihistamines and sedatives (Zeller et al, 1952), whereas the MAOI isoniazid was initially intended for the treatment of tuberculosis (Loomer et al, 1957. Both drugs showed antidepressant qualities and both had the ability to enhance or prolong the actions of the monoamines 5-hydroxytrytamine (5-HT; serotonin) and noradrenaline (NA). The recognition of depression as a biochemical disorder led to the monoamine theory of depression, which hypothesised that depression was due to a deficiency of brain monoaminergic activity and thus may be treated by pharmacological means accordingly (Schildkraut, 1965).

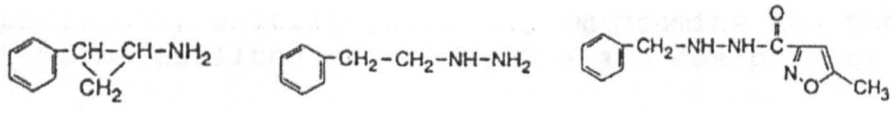
Among the monoamines, 5-HT has been suggested to be the primary target of antidepressant drugs. Thus, the last 20 years has seen the introduction of a class of antidepressant aimed directly at selectively enhancing the transmission of 5-HT. Selective serotonin reuptake inhibitors (SSRIs) achieve this by potently inhibiting 5-HT uptake. These drugs were widely accepted as safer and better tolerated, though no more efficacious than TCAs and MAOIs (Anderson et al, 2000), thereby questioning the role of 5-HT as the primary target for antidepressant drugs.

1.3.1.1. Monoamine oxidase inhibitors

Monoamine oxidase (MAO) was first described by Hare (1928). The enzyme deaminates monoamine neurotransmitters and exogenous amines to form aldehydes, which are then converted to acids or alcohols (Yu, 1994). Deamination terminates the action of the neurotransmitter, as well as detoxifying the exogenous amines. MAO exists as two isoenzymes: MAO-A and MAO-B. Under physiological conditions MAO-A is primarily found in the brain, gut and liver (but not platelets), and deaminates NA and 5-HT (Sandler and Youdim, 1972). Whereas MAO-B predominates in the brain and

platelets, and deaminates dopamine (Murphy et al, 1987). MAOIs are often classed by their relative selectivity for sub-forms of MAO (type A, type B or mixed; Mann et al, 1984; Yamada and Yasuhara, 2004).

The acute effects of MAOIs are to decrease the degradation of monoamines stored in pre-synaptic neurones, thus causing increased availability of monoamines at the synapse (Schildkraut, 1965; Klein and Davis, 1970).



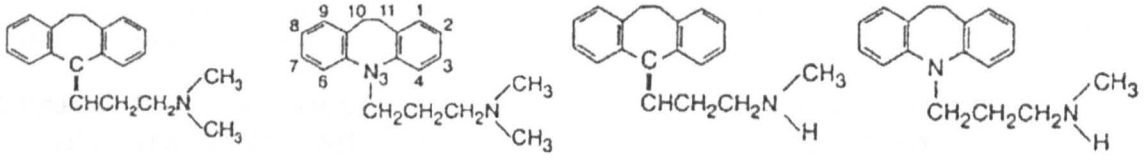
Tranylcypromine Phenelzine Isocarboxazid

Figure 1.1. Chemical structures of the monoamine oxidase inhibitors tranylcypromine, phenelzine and isocarboxazid

1.3.1.2. Tricyclic antidepressants

Unlike MAOIs, TCAs have no effect on monoamine metabolism. Their primary mode of action is via the inhibition of pre-synaptic reuptake of monoamines by the blockade of neuronal transporters (Sigg, 1959; Axelrod, 1962). TCAs act with varying degrees of potency and selectivity towards NA and

5-HT, for example clomipramine is the most 5-HT selective and desipramine the most noradrenaline selective, with amitriptyline being relatively non-selective (Tatsumi et al, 1997; Frazer, 1997; *See Figure 1.2*).



Amitriptyline Imipramine Nortriptyline Desipramine

Figure 1.2. Chemical structures of tricyclic antidepressants, amitriptyline and imipramine and their respective metabolites nortriptyline and desipramine

1.3.1.3. Selective serotonin reuptake inhibitors

Reuptake inhibitors can be divided into categories based on their relative potency to block serotonergic or noradrenergic reuptake sites. Selective serotonin reuptake inhibitors (SSRIs) are potent to the 5-HT reuptake pump *(See Figure 1.3)*.

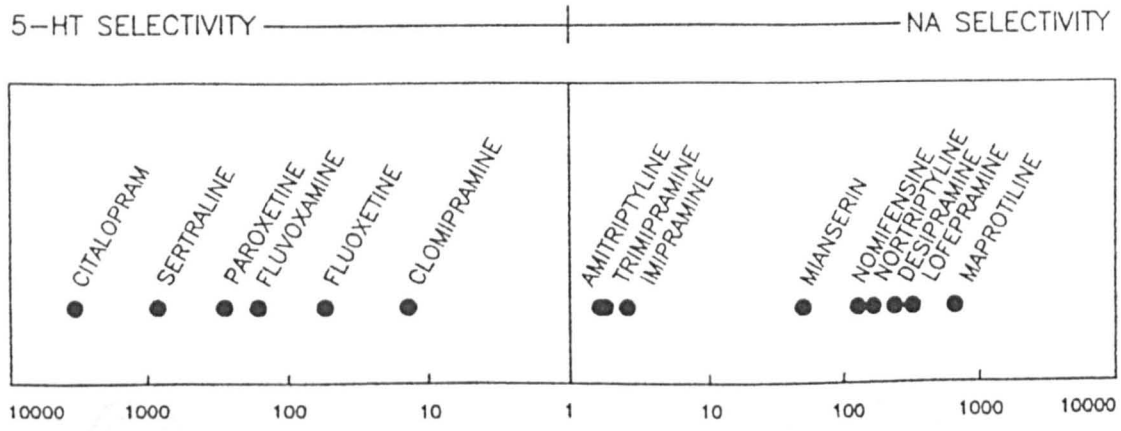


Figure 1.3. Selectivity ratios for a series of uptake inhibitors measured *in-vitro*. To the left 5-HT selective drugs are shown by the IC_{50} NA uptake/IC_{50} 5-HT uptake ratio, whereas to the right NA selective drugs are shown by the IC_{50} 5-HT uptake/IC_{50} NA uptake ratio. The higher the value, the more selective for 5-HT respective NA uptake. (Adapted from Hytel, 1994).

SSRIs were the first class of antidepressant to confirm the inhibition of neurotransmitter uptake as an important therapeutic principle (Vaswani et al, 2003). Reuptake is the major inactivating mechanism for monoamines after their release into the synaptic cleft (Lane et al, 1995). The mode of action of SSRIs therefore is to prolong monoamine activation by blocking 5-HT reuptake. Negative allosteric modulation of the 5-HT transporter (SERT) results in increased concentration of 5-HT in the extracellular space (Fuller and Wong, 1987). Inhibitory pre-synaptic auto-receptors are activated in response, thus decreasing the turnover of 5-HT. However when given chronically, the persistent increase in synaptic concentration has been

shown to desensitise 5-HT_{1A} auto-receptors after various SSRI treatment, thereby reducing auto-inhibition of 5-HT release (Chaput et al, 1986; Blier et al, 1987; Rutter et al, 1994; Invernizzi et al, 1996).

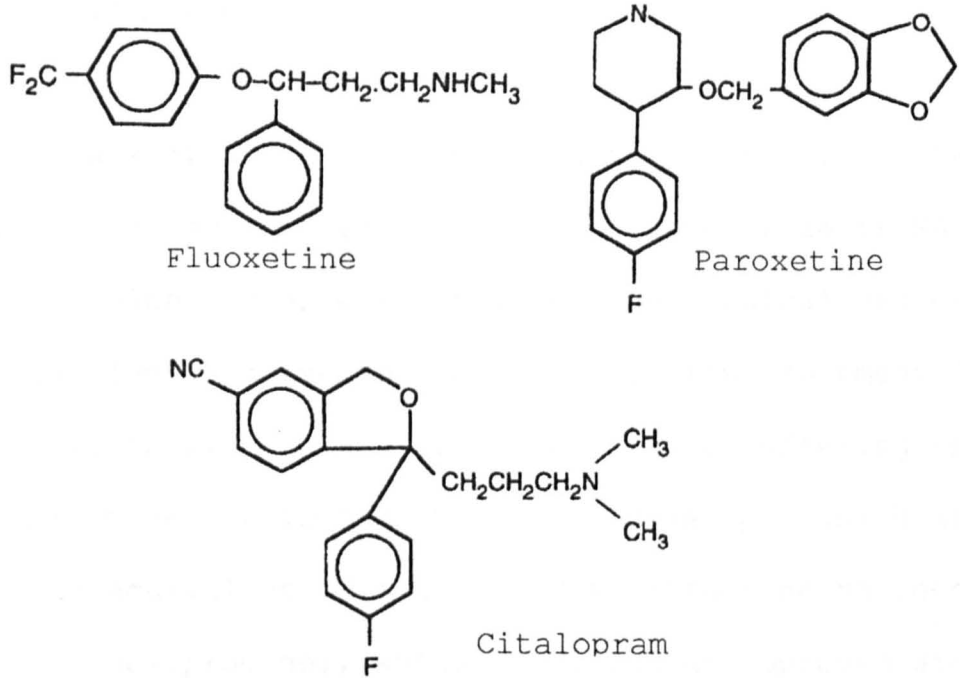


Figure 1.4. Chemical structures of SSRI antidepressants fluoxetine, paroxetine and citalopram

1.3.1.4. Serotonin/noradrenergic reuptake inhibitors

Following the emergence of SSRIs, the past few years has seen a renewed interest in the development of dual action antidepressants designed specifically to block both 5-HT and NA reuptake. Serotonin/Noradrenaline reuptake

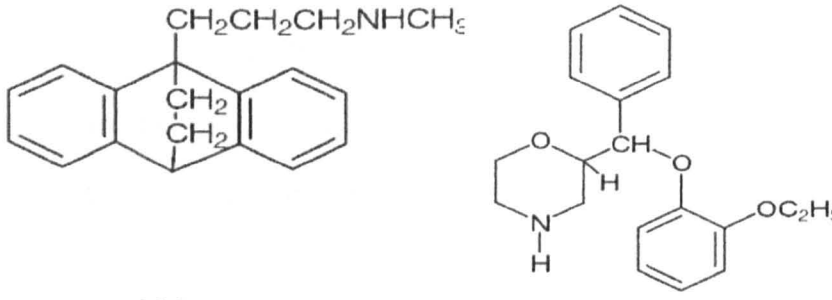
inhibitors (SNRIs) have shown successful levels of efficacy and tolerability (Thase et al, 2001; Smith et al, 2002). The pharmacology of SNRIs depends on their relative affinities for 5-HT and NA reuptake blockade.

1.3.1.5 . Selective noradrenergic reuptake inhibitors

The lack of distinction between the efficacy of SSRIs and TCAs has led to a re-examination of the role of NA in depression and an evaluation of the clinical use of selective NA reuptake inhibition in its treatment (Brunello et al, 2002). A new generation of drugs offering specific NA reuptake inhibition have been developed which aim to offer equivalent efficacy as TCAs affecting NA reuptake (e.g. desipramine), whilst offering an improved side-effect profile. Selective NA reuptake inhibitors (NARIs) include the tetracyclic compound maprotiline and reboxetine.

Maprotiline has been shown to exhibit an antidepressant action. It strongly inhibits the uptake of NA in the brain and peripheral tissues, though it is notable in its lack of inhibition of serotonergic uptake. Maprotiline has also been shown to produce effects at histaminergic and cholinergic receptors (Ruhdorfer and Potter, 1987).

Reboxetine selectively inhibits NA uptake without inhibition of 5-HT, DA or MAO isoforms (Kent, 2000).



Maprotiline Reboxetine

Figure 1.5. Chemical structures of NARI antidepressants maprotiline and reboxetine.

1.3.2. Electroconvulsive therapy

The antidepressant properties of convulsive therapy were first demonstrated by the application of a seizure-inducing dose of camphor, which produced a rapid improvement in depressive symptoms in a number of patients (Meduna, 1935; 1936). Subsequently, chronic electroconvulsive therapy (ECT) has been shown to be one the most effective treatment of severe depression and bipolar disorder (Greenberg et al, 1988; Fink, 1990; Mann, 1998). Despite its major limitations such as high relapse rate and possible profound effects on memory (Weeks et al, 1980), it is still a popular option for drug resistant patients. However, the

mechanisms that underlie the therapeutic actions of chronic ECT remain unclear (Fink, 1990). The application of the animal model of ECT, referred to as electroconvulsive shock (ECS), has been shown to elicit widespread effect of numerous neurotransmitters and signalling components within the brain (Nomikos et al, 1991; Ozawa and Rasenick, 1991).

1.4. The pathophysiology of depression

1.4.1. Involvement of stress and the HPA axis in depression

Chronic exposure to stress and the body's stress response has long been implicated for a role in the pathophysiology of depression (Board et al, 1956). The prominent mechanism by which the brain counteracts stressors is the activation of the hypothalamic-pituitary-adrenal (HPA) axis (McEwen, 2000; Nestler et al, 2002), which receives and integrates inputs indicative of stress (Dunn and Berridge, 1990; Chrousos and Gold, 1992). Corticotrophin releasing factor (CRF) is released from neurones in the paraventricular nucleus (PVN) of the hypothalamus, which acts on anterior pituitary CRF receptors. This consequently stimulates the release of adrenocorticotrophin (ACTH), which causes the secretion of glucocorticoids (cortisol in humans;

corticosterone in rodents) from the adrenal cortex (Dinan, 2001). Glucocorticoids have a profound effect on metabolism, such as increasing the availability of glucose (Holsboer, 2001), as well as behavioural effects via direct actions in other brain areas (Nestler et al, 2002). The hippocampus projects forward to the PVN of the hypothalamus, where it plays an inhibitory role on HPA activity (Herman et al, 1989; McEwen and Brinton, 1987; Smelik, 1987). Glucocorticoids regulate hippocampal and PVN neurones and therefore exert feedback effects on the HPA axis activity (Nestler et al, 2002). Glucocorticoids also act as inhibitory transcription factors, by antagonistically occupying a promoter site that could otherwise be bound by a positive regulator (Drouin et al, 1989).

Hypercortisolaemia is often a key feature of depression (Gibbons and McHugh, 1962; Carpenter and Bunney, 1971;). This manifests as enhanced serum cortisol, non suppression of dexamethasone and adrenal gland hyperplasia (Rubin et al, 1995) and a blunted ACTH response to CRF challenge (Gold et al, 1986; Holsboer et al, 1986). Cushing's syndrome patients (who exhibit abnormal cortisol levels) have a higher prevalence of depression, however depressive

symptoms can often be reversed following reductions in cortisol levels (Holsboer, 2003). Centrally administered CRF also produces behavioural effects in rats, which closely resemble many key features of depression, including increased arousal and vigilance, decreased appetite, decreased sexual behaviour and increased heart rate (Arborelius et al, 1999; Holsboer, 2001). Post-mortem studies on depressed patients have revealed increased CRF cerebrospinal fluid (CSF) levels in depressed patients (Nemeroff et al, 1984; Banki et al, 1992). Whereas decreased CRF binding sites were found in suicide victims (Nemeroff et al, 1988; Arato et al, 1989). In addition, hippocampal atrophy has been shown to occur in the presence of high levels of corticosterone (Magarinos and McEwen, 1995).

Despite the large quantity of data supporting a role for the HPA axis in depression, it is not clear whether HPA dysfunction is the primary cause of depression or a consequence of underlying abnormalities exerting influence on the axis. Novel drugs aimed at rectifying HPA system dysfunction include steroid synthesis inhibitors, CRF antagonists and glucocorticoid inhibitors (McQuade and

Young, 2000). Initial trials indicate varying degrees of success (Manji et al, 2003).

1.4.2. Involvement of monoamines in depression

1.4.2.1. Noradrenaline

The initial finding that led many to believe that NA played a significant role in the pathophysiology of depression came from the effect of the reserpine, which rapidly depletes amines in the CNS and periphery and subsequently induced depressive symptoms (Fries, 1954). A compound with similar amine-depleting actions: tetrabenzine had a similar effect and this effect was reversed when treatment was discontinued (Bartonicek et al, 1964). Furthermore, supplementation with the catecholamine pre-cursor L-DOPA reversed the psychological effect of reserpine (Schildkraut, 1965). The main NA metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) has been used as an index of brain NA turnover in depressed patients. Initial studies showed a decrease in MHPG levels in the urine of depressed patients (Maas et al, 1972). Subsequently however, increases as well as decreases in urinary MHPG have been reported in patients, with a lack of correlation occurring

between patients and studies (Potter et al, 1984; Roy et al, 1986; Potter and Manji, 1993). Correlations between increases in urinary MHPG and anxiety have been more frequent (Sevy et al, 1989). Hyper-secretion of NA in plasma and CSF have been reported in patients suffering unipolar depression and anxiety states (Wyatt et al, 1971; Roy et al, 1988; Sevy et al, 1989).

1.4.2.2. 5-hydroxytryptamine (Serotonin)

The main 5-HT metabolite 5-HIAA has been widely used as an index of 5-HT turnover. Numerous findings have implicated decreases in 5-HIAA in the plasma and CSF of depressed patients, as well as 'violence-impulsivity' behaviour and in suicide victims (Dencker et al, 1966; Mendels et al, 1972; Van Pragg and de Hann, 1979; Asberg et al, 1976; Faustmann et al, 1991; Mann et al, 1996; Mann and Malone, 1997). However, the degree of the reduction in CSF 5-HIAA level does not generally correlate with the severity of the depression (Martensen et al, 1989; Mann, 1999).

Plasma prolactin has been used as an indicator of central 5-HT function in response to 5-HT releasing agents in numerous studies. Generally, a blunted plasma prolactin

response has been found after fenfluramine (a 5-HT releasing agent) administration in depressed patients, compared with controls (Mitchell and Smythe, 1990; Maes et al, 1989; 1991; Lichtenberg et al, 1992). Inhibition of TPH by parachlorophenylalanine (pCPA), which causes a reduction in 5-HT synthesis and subsequent turnover, has been shown to produce a relapse in depressive symptoms in patients receiving TCP treatment (Shopsin et al, 1976). Likewise, depletion by dietary means using an amino acid mixture drink lacking the 5-HT precursor TRP has been shown to cause a relapse in depressive symptoms in drug-free patients with a history of depression (Delgado et al, 1994).

1.4.3. The monoamine hypothesis

The discovery of MAOI and TCA classes of antidepressants in the early 1950s suggested a fundamental role for the monoamines (and possibly DA) in the pathology of depression. This led to the relatively simple and concise theory of the aetiopathology of depression: the "monoamine hypothesis", which implicated a deficiency of monoamine neurotransmission in depressed individuals (Schildkraut, 1965; Bunney and Davis, 1965). Thus, it was postulated

that drugs facilitating activity could correct the perceived deficiency in brain monoamines.

In the years since the monoamine hypothesis was proposed it has become obvious that the theory is insufficient in itself due to the failure to explain numerous findings. For instance, drugs that also rapidly increase brain monoaminergic (and DA) activity such as cocaine and amphetamine are not effective as antidepressants. Also, patients receiving the same antidepressant regimen do not always respond equally. The theory can also not explain why the agent tianeptine, which enhances 5-HT uptake is an effective antidepressant (Loo et al, 1999; Pineyro and Blier, 1999). However, administration of a typical antidepressant causes an increase in synaptic monoamines and monoamine facilitation very rapidly, however there is a 2-3 week lag period before measurable therapeutic onset (Oswald et al, 1972; Heninger and Charney, 1987). Despite its obvious drawbacks the monoamine hypothesis still provides a benchmark behind which rationales are formed in the development of new generations of antidepressants.

1.4.3.1. The antidepressant therapeutic latency period

A major drawback in the monoamine hypothesis of depression is its inability to explain the apparent gap between the widespread acute biochemical changes and the onset of psychological therapeutic change (Oswald et al, 1972; Heninger and Charney, 1987). Antidepressants must be taken over a prolonged period of time in order to gain therapeutic response, thus exposing the patient to deleterious side effects for a longer period of time, without any apparent mood-enhancing effect. Interestingly, ECT also requires chronic administration (6-12 treatments) for antidepressant efficacy to occur (Pearlman, 1991).

1.5. Intracellular signaling transduction pathways

The apparent latency period between the acute antidepressant action (i.e. the blockade of uptake or metabolism of synaptic levels of 5-HT or NA) and the onset of therapeutic effects has led to research focusing on possible monoamine receptor-linked intracellular signal transduction pathways. A prominent hypothesis predicts that the therapeutic delay is a result of the period required

for targeted changes to occur in gene expression initiated by 5-HT and/or NA-receptor mediated activation (Nestler et al, 1989; Duman et al, 1994, 1997; Manji et al, 1995). The various 5-HT and NA receptor subtypes are known to selectively couple and regulate several intracellular transduction pathways. The long-term activation of such receptor coupled second messenger cascades could lead to adaptations via specific target genes that result in therapeutic efficacy.

1.5.1. G-proteins

All NA and 5-HT receptors are linked to coupling guanyl nucleotide-regulated (G)-proteins. G-protein coupled (metabotropic) receptors trigger intracellular pathways that control enzyme activity usually via phosphorylation, or dephosphorylation of serine or threonine residues (Gerber, 2002). Four main categories of G-protein have been identified: G_s, G_i, G_q and G_{12} (Gilman, 1989; Gould and Manji, 2002). G_s stimulate the enzyme AC and regulate Ca^{2+} and K^+ channels. G_s proteins dissociate after transmitter-receptor interaction and in turn activate effector systems e.g. AC, PLC, phospholipase A, ion channels (Gilman, 1987; Birnbaumer, 1990). G_i stimulation results in inhibition of

AC, whereas G_q interact with PLC (Gilman, 1989). The function of G_{12} remains unknown, however G_{12} activation has been linked with the release of the transcriptional activator β-catenin (Meigs et al, 2002).

1.5.2. cAMP signaling pathway

The cAMP signaling cascade represents a common target for several classes of antidepressant (Ozawa and Rasenick, 1991; Nestler et al, 1989; Tinelli et al, 1989; Thome et al, 2000). Blunted cAMP signalling through decreases in stimulated AC has been recorded in patients with major depressive disorder (Stewart et al, 2001).

Activation of numerous receptor subtypes including β-AR, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7, lead to the stimulation of AC by the G_s-protein, which results in cAMP generation. Increased levels of cAMP result in the activation of cAMP-dependent protein kinase (PKA). PKA regulates cellular function by phosphorylation of many types of regulatory proteins including receptors, ion channels, enzymes and transcription factors (Duman, 1998). Amongst the substrates affected is the transcription factor cyclic AMP response element binding protein (CREB).

1.5.3. Phosphoinositide signaling pathway

Another well-characterised G-protein-linked signaling pathway involves the breakdown of the cell membrane component: phosphoinositide 4,5-biphosphate (PIP2; Majerus, 1992). The phosphoinositide pathway (PI) is coupled to noradrenergic α_1, 5-HT_2 as well as muscarinic M1, M3 and M5 receptors via the G_q subunit (Raulli et al, 1989; Gould and Manji, 2002). Following the binding of a ligand to its extracellular receptor, GTP binding induces hydrolysis of PIP2 to form diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (IP3). DAG activates phosphorylation enzyme protein kinase (PKC) and increases the affinity for the enzyme to Ca^{2+} (Nishizuka, 1992). Once activated, PKC phosphorylates specific proteins including CREB, which are critical in cellular and physiological functions e.g. transcription and long-term potentiation (LTP; Berridge, 1993; Jakobs et al, 1986). The second product of PIP2 breakdown, IP3 binds to the IP3 receptor which facilitates the release of calcium reservoirs in the endoplasmic reticulum (Gould and Manji, 2002). The released calcium interacts with various proteins including calmodulin receptors (CaM). Calmodulin activates ion channels,

signalling molecules and transcription factors (Ishidu et al, 1978; Soderling et al, 2000).

Several studies have implicated PI signalling abnormalities in patients with mood disorders and suicide victims (Jope et al, 1996; Pacheco et al, 1996; Karege et al, 1996). For example, 5-HT and thrombin-stimulated formation of IP_3 has been found to be increased in the platelets of depressed patients (Mikuni et al, 1991; Karege et al, 1996). It has also been reported that PKC binding sites and PLC expression are down-regulated in the pre-frontal cortex of teenage suicide victims (Pandey et al, 1997).

1.5.4. cAMP response element binding protein

As mentioned earlier, a common target for both the cAMP (via β-NA, 5-HT_4, 5-HT_5 5-HT_6 5-HT_7 receptor activation; Duman, 1997; Saxena, 1995; Pandey et al, 1995) and PI (via α_1-NA, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2c} receptor activation; Mori et al, 1991; Berg and Clarke; 2001; Kurrasch-Orbaugh, 2003) cascades is the transcription factor Ca^{2+}/cAMP response element binding protein (CREB). CREB therefore regulates gene response due to stimulation of the cAMP and PI

cascades (Mayer and Habener, 1993; Ghosh and Greenberg, 1999).

CREB is a member of the ATF_1, leucine zipper family of transcription factors (Lee and Masson, 1993). Phosphorylation of CREB occurs at serine-133 and serine 119 sites, which leads to the dimerisation and dramatically increases the functional and transcriptional potential of CREB.

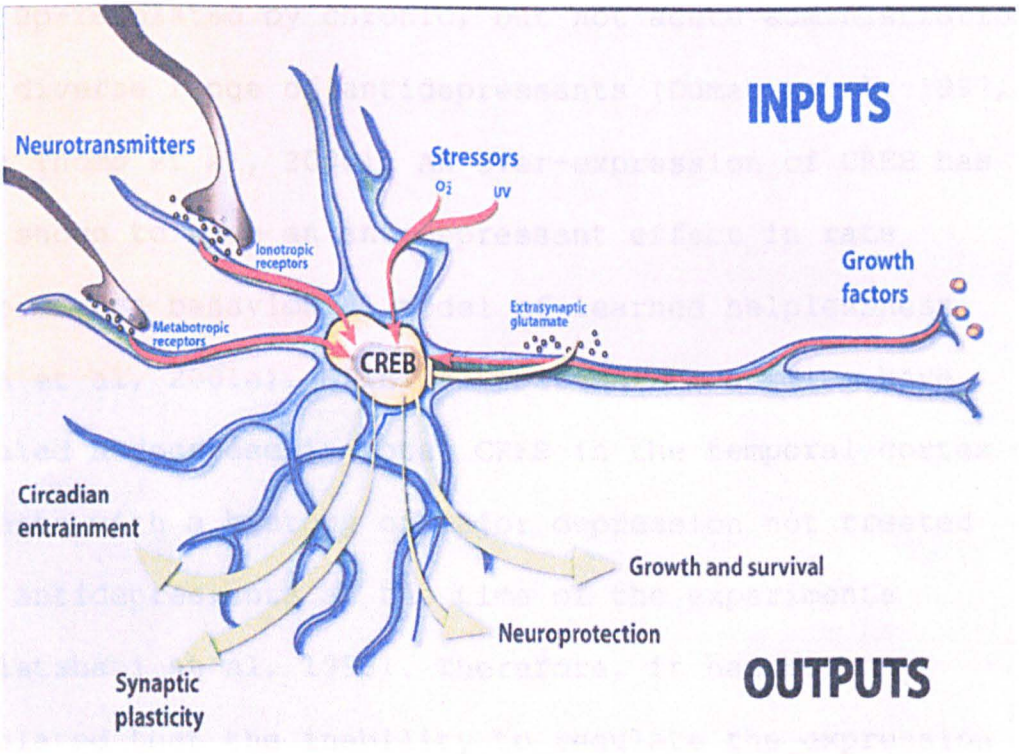


Figure 1.6. Diagram showing various stimuli and conditions implicated in influencing CREB gene expression (INPUTS) and postulated physiological and pathological consequences of CREB activation (OUTPUTS; Adapted from Lonze and Ginty, 2002).

1.5.4.1. Evidence for enhanced CREB signaling in antidepressant function

Nibuya et al (1996) demonstrated that chronic, but not acute administration of several diverse types of antidepressant drugs (fluoxetine, TCP, desipramine and sertraline), as well as ECS caused an increase in expression of CREB protein and mRNA in the rat hippocampus. The phosphorylation and transcriptional activity of CREB is also up-regulated by chronic, but not acute administration of a diverse range of antidepressants (Duman et al, 1997, 1999; Thome et al, 2000). An over-expression of CREB has been shown to have an antidepressant effect in rats performing a behavioural model of learned helplessness (Chen et al, 2001a). Clinical post-mortem studies have revealed a decrease in total CREB in the temporal cortex of patients with a history of major depression not treated with antidepressants at the time of the experiments (Dowlatshahi et al, 1998). Therefore, it has been postulated that the inability to regulate the expression and function of CREB and henceforth induce adaptive gene expression may contribute to the aetiopathology of depression (Vaidya and Duman, 2001). Thus, recent research has focussed on CREB and its target genes in response to

antidepressant challenge. One such heavily implicated gene is that of Brain-Derived Neurotrophic Factor (BDNF) (Condorelli et al, 1994; Nibuya et al, 1995; Siuciak et al, 1996; Duman, 1998; Duman et al, 1999).

1.6. Brain-Derived Neurotrophic Factor

BDNF is part of the family of neurotrophins. These proteins are structurally and functionally related and also include nerve growth factor (NGF; Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980), neurotrophin 3 (NT-3; Maisonpierre et al, 1990; Rosenthal et al, 1990); neurotrophin 4/5 (NT 4/5; Ip et al, 1992; Halbrook et al, 1991; Widmer and Hefti, 1994) and neurotrophin 6 (NT-6; Gotz et al, 1994).

Neurotrophins have long been known as endogenous signalling molecules, which provide extracellular control over the development and maintenance of neurones. This control occurs due to the selective inhibition of apoptosis through the binding of neurotrophins to the cell surface receptors on specific populations of neurones in adulthood (Davies, 1994). Recent findings however expanded the profile of neurotrophin function, suggesting roles in synaptic

transmission and neuronal plasticity (Lohof et al, 1993; Stoop and Poo, 1996; Liou and Fu, 1997; Mcallister et al, 1996). In essence, neurotrophins are now being heralded as a new class of neuromodulators that mediate activity-dependent modifications of neuronal connectivity and synaptic efficacy.

1.6.1. The structure of BDNF

BDNF, like all members of the neurotrophin gene family is synthesised as a pre-cursor and is processed at classical dibasic cleavage sites into a biologically active neurotrophin which contains approximately 50% conserved domains (Jungbluth et al, 1994). The structure comprises of seven β-strands, which contribute to three anti-parallel pairs of twisted β-strands (Robinson, 1995). These strands are locked by a 'cysteine knot' of three disulfides (McDonald and Hendrickson, 1993).

1.6.2. BDNF receptors

BDNF function is mainly mediated through interaction with the tropomyosin-related kinase B (trkB) receptor. trkB is a member of the tyrosine kinase receptor family, which also

includes trkA, which binds to NGF and trkC, whose ligand is NT-3. BDNF may also exert some actions through the p75 neurotrophin receptor (Rodriguez-Tebar et al, 1990; 1992), though this receptor may not play a direct role in neurotrophic function.

1.6.2.1 trkB receptors

Structure

trkB receptors occur as splice variants of the trkB gene. trkB receptors comprise of two cysteine-rich domains separated by a leucine-rich domain, two extracellular immunoglobulin (IgG) domains and two intracellular tyrosine kinase domains (Lamballe et al, 1991; Klein et al, 1991; Tsoulfas et al, 1993). All trkB isoforms share a common extracellular domain and are thought to be produced as alternative splice variants encoded by the same gene (Fryer et al, 1996). There is one apparent full-length trkB receptor possessing a (full) trkB domain and two identified variants of truncated, physiologically inactive trkB receptor lacking the intracellular kinase domain (Middlemas et al, 1991).

Distribution

trkB receptor mRNA is expressed in high levels in the brain cortex, striatum and hippocampus and a high degree of co-localisation has been reported between trkB and BDNF mRNA (Merlio et al, 1993; Salin et al, 1995). Morphologically, catalytic trkB appears to be expressed primarily in neurones, however truncated trkB is expressed in both neurones and glia.

Signalling

As BDNF binds to the trkB receptors, the receptor dimerises (Marsh et al, 1993) leading to the autophosphorylation of specific tyrosine residues within the intracellular domains (Kaplan et al, 1991, Lamballe et al, 1991; Soppet et al, 1991). The phosphorylated tyrosine residues act as protein interaction sites for the src homologous and collagen-like (shc) adaptor protein (Stephens et al, 1994). The shc adaptor protein links the activated trk receptor to two distinct intracellular pathways (Chao, 2003; *See Figure 1.7*). One pathway, which promotes neuronal survival, involves the activation of phosphatidylinositol 3-kinase (PI3K) and its putative effector the serine and threonine kinase AKT (Burgering and Coffer, 1995; Franke et al, 1995). trk phosphorylation of the shc adaptor also leads to

the activation of GRB2/SOS causing ras activation. Activation of Ras leads to a chain of phosphorylation reactions including that of raf, mek, erk and mitogen activated protein kinase (MAPK; Segal and Greenberg, 1996; Grewal et al, 1999; Ballif and Blenis, 2001). This in turn leads to the activation of transcription factors such as CREB, which may ultimately influence cell survival, neurite outgrowth and synaptic plasticity (Lonze and Ginty, 2002; Chao, 2003).

In addition to src adaptor linked pathways, PLC may bind to phosphorylated TrkB residues. The activated PLC cleaves phosphatidylinositol 4, 5-biphospate to generate Inositol-1, 4, 5-triphosphate (IP3; which induces Ca^{2+}) and diacylglycerol (DAG; which activates PKC).

1.6.2.2. p75^{NTR} Receptors

p75 receptors bind with all neurotrophins with a similar affinity (Rodriguez-Tebar et al, 1990; 1992; Squinto et al, 1990). However, several investigators have suggested that the receptor may not play a direct role in neurotrophic actions of cells (Taniuchi et al, 1986; Barbacid, 1993; Bothwell, 1995). Alternative functions such as buffering

the concentration of available extracellular neurotrophin, which would maintain a high concentration of neurotrophin near the site of release have been suggested (Taniuchi et al, 1986). This would allow neurotrophin bound to p75 on one cell to be presented to trk receptor on another cell (Barbacid, 1993), or facilitating trk signalling by acting as an accessory subunit to trk receptors (Bothwell, 1995).

One established function of p75 receptors is to promote cell death. p75 receptor activation causes increases in pro-apoptotic jun-N terminal kinase (JNK; Roux and Barker, 2002). This may be useful for refining incorrect neuronal innervation during development, thus eliminating unwanted connections (Majdan and Miller, 1999).

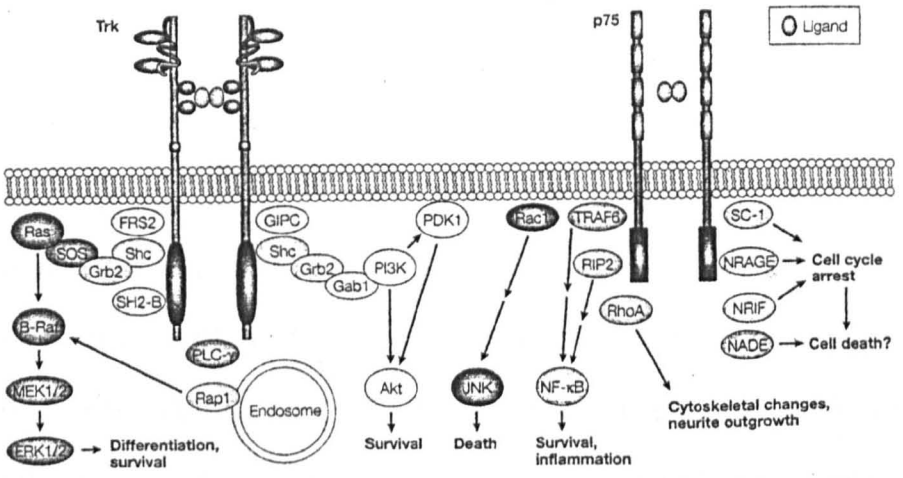


Figure 1.7. BDNF receptor signaling. (Adapted from Chao, 2003).

1.6.2.3. Functional consequences of BDNF signaling

During development in vertebrates BDNF is vital for survival, maintenance and growth of neurones in the central nervous system (Barde et al, 1982; Leibrock et al, 1989). BDNF has been demonstrated to promote survival for serotonergic (Altar et al, 1994), dopaminergic (Knusel et al, 1991; Altar et al, 1994), cholinergic (Nonner et al, 1996) and GABAergic (Altar et al, 1994; Ventimiglia et al, 1995) neurones.

Role in LTP and synaptic plasticity

In addition to the normal development of the nervous system, it has been widely reported that neurotrophins (including BDNF) are capable of modulating transmission at central synapses by pre- and post-synaptic mechanisms (Mcallister et al, 1999) in the adult CNS. LTP can be separated into distinct phases: the early phase (E-LTP) and the late phase (L-LTP). E-ETP is short lasting (1-3 hours) and independent of protein synthesis mechanisms, whereas L-LTP requires induction of cAMP and protein synthesis mechanisms (Frey et al, 1988; Nguyen et al, 1994). Evidence exists that BDNF is capable of influencing both phases. BDNF has the ability to act rapidly at pre- and post-synaptic receptors to modulate LTP (Xu et al, 2000; Kovalchuk et al, 2002). Indeed, with respect to synaptic function, BDNF is capable of depolarising neurones as rapidly as glutamate via the trkB receptor (Kafitz et al, 1999). Acutely, BDNF is capable of enhancing glutamate synaptic transmission (Lohof et al, 1993; Lessmann et al, 1994) and phosphorylating subunits of NMDA receptors in the hippocampus (Suen et al, 1997). In addition to acute effects, more stable synaptic changes occur that may involve altered gene expression and protein synthesis (Kang and Schuman, 1996; Finkbeiner et al, 1997), which may

subsequently modulate the strength of synaptic transmission. Prolonged treatments with neurotrophins have been demonstrated to promote LTP and maturation of synaptic sites (Wang et al, 1995; Thakker-Varia et al, 2001). This observation is reinforced in mice with targeted deletion of the BDNF gene, which display impaired synaptic plasticity (Korte et al, 1995).

Role in axonal sprouting

BDNF has been shown to have trophic effects on 5-HT neurones (Mamounas et al, 1995) and the gene for the trkB receptor has been identified on 5-HT neurones (Madhav et al, 2001). Local BDNF infusion is capable of producing sprouting in mature, uninjured 5-HT axons as well as those damaged by *p*-chloroamphetamine (*P*CA) pre-treatment (Mamounas et al, 1995; 2000). Indirect BDNF induction via repeated ECS induction (Nibuya et al; 1995; Zetterström et al, 1998) has also been demonstrated to greatly enhance 5-HT axonal sprouting in rats with lesioned hippocampi (Madhav et al, 2000).

1.6.2.4. Evidence linking BDNF with depression

Evidence implicating BDNF with both aetiopathology and the treatment of depression has been slowly accumulating from numerous lines of investigation.

Antidepressant effect of BDNF infusion

A seminal study showed direct infusion of the BDNF protein into the rat midbrain produced an antidepressant effect in two models of depression in rats: the forced swim test and the learned helplessness model (Siuciak et al, 1997). The site of injection in the midbrain was in the proximity of the periaqueductal grey matter, DRN and MRN, thus allowing BDNF exposure to the highest number of 5-HT cell bodies in the brain, suggesting a possible augmentation of 5-HT activity. Indeed, central administration of BDNF has been shown to exert widespread changes in 5-HT activity (Altar et al, 1994; Maumounas et al, 1995; Pellymounter et al, 1995; Siuciak et al, 1994; 1998). Infusion of BDNF into the dentate gyrus (DG) region of the hippocampus also exerted antidepressant affects in forced swim and learned helplessness models (Shirayama et al, 2002).

Neurotrophic effects of BDNF

BDNF has been demonstrated to have widespread trophic effects on existing 5-HT (Maumounas et al, 1995; 2000) and noradrenergic (Sklair-Tavron et al, 1995) neurones following neurotoxic lesions. Enhanced neurogenesis has also been shown in numerous areas of the rat brain following over-expression (Benraiss et al, 2001) and infusion (Pencea et al, 2001) of BDNF.

Effect of stress on BDNF levels

BDNF is thought to play a role in cellular and behavioural responses to stress. Chronic stress models in rats have been shown to lower BDNF mRNA in the hippocampus. Immobilisation and inescapable exposure to noxious stimuli diminish hippocampal BDNF mRNA (Smith et al, 1995; Ueyama et al, 1997), as does elevation of exogenous corticosterone levels (Schaaf et al, 1998), an effect which is blocked by subsequent antidepressant treatment (Nibuya et al, 1995). The apparent atrophy of hippocampal neurones in animals exposed to experimental stressors has been hypothesised to be a result of decreased BDNF expression (Duman et al, 1997; 1999).

Effect of antidepressants on BDNF levels

Numerous studies have indicated that chronic, but not acute administration of various antidepressant drugs increase BDNF gene expression in the hippocampus and cortex in rats (Nibuya et al, 1995; Zetterström et al, 1998; Russo-Neustadt, 1999; 2000; Coppell et al, 2003), over a time period similar to that experienced when administering antidepressants clinically. In addition, electroconvulsive seizure (ECS), the animal model of ECT, strongly increases BDNF mRNA in the cortex and the areas of the hippocampus (Nibuya et al, 1995; Zetterström et al, 1998b).

Voluntary physical exercise (wheel running) has also been shown to produce a rapid increase in BDNF mRNA in the hippocampus and cortex of the rat (Neeper et al, 1996; Russo-Neustadt et al, 2000), as well as augmenting monoaminergic function (Dunn et al, 1996; Dishman et al, 2000). Physical exercise in humans has been shown to enhance mood and psychological coping mechanisms (Emery et al, 1992; Hill et al, 1993), as well as cognitive functioning (Blomquist et al, 1987). In rats exercise has been shown to counteract the decreased BDNF effect of forced swim tests (Russo-Neustadt et al, 2001)

1.6.3. BDNF transcripts

The structure of the rat BDNF gene was elucidated by Timmusk's group (Timmusk et al, 1993). A rat genomic library was screened using sequences from three different regions of BDNF cDNAs. Following hybridisation of rat genomic sequences to rat brain mRNA and reverse transcription-assisted polymerase chain reaction (PCR), the presence of five exons within the rat BDNF gene was discovered (Timmusk et al, 1993). The rat BDNF gene includes four short 5′ exons (exons I-IV) that are each associated with a separate promoter, and one 3′ exon (exon V) that encodes for the mature BDNF protein (Timmusk et al, 1993; Nakayama et al, 1994). As any exon can be polyadenylated in two positions, four different promoters give rise to eight predominant transcripts, each of these transcripts will contain one of the four variable 5′ exons, in addition to an exon V segment (Lauterborn at al, 1996; Timmusk et al, 1993; *See Figure 1.8*).

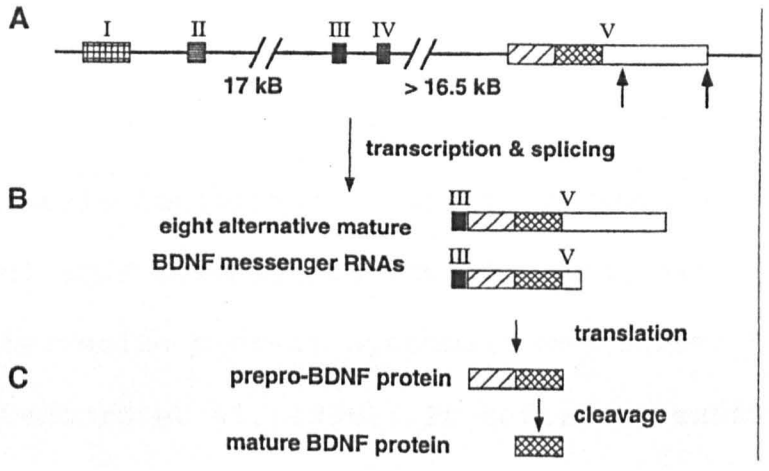


Figure 1.8. The organisation of the BDNF gene. (A) The BDNF gene spans over 40KB and contains five exons (I-V). Each of the four exons contains a unique promoter and one or more transcription initiation site. (B) Each BDNF message can be transcribed from any of exons I to IV (exon III in this example). (C) A precursor form of BDNF protein (prepro-BDNF) is encoded within exon V and undergoes cleavage to yield mature BDNF protein (Adapted from Finkbeiner, 2000).

The transcripts are expressed differentially across different brain areas (Timmusk et al, 1993; Bishop et al, 1994) and regulated differentially from a variety of manipulations (Nakayama et al, 1994; Kokaia et al, 1995). In the hippocampus ischemia has been shown to selectively increase expression of the transcript form containing exon III, while kainate injections increase all of the transcript forms (Falkenberg et al, 1993; Metsis et al, 1994; Timmusk et al, 1995). The apparent presence of multiple transcript forms of BDNF, translating to one identical protein is unique amongst neurotrophins and is

potentially a means of multiple levels of regulation (Russo-Neustadt et al, 2000).

Protein synthesis inhibition on activity-dependent expression of BDNF transcripts revealed that exon I and II required intervening protein synthesis mechanisms to alter levels (Lauterborn et al, 1996). In contrast, exons III and IV are fairly inductable in the presence of protein synthesis blockers (Lauterborn et al, 1996), suggesting that these transcripts are immediate early gene (IEG)-like in function.

1.7. The hippocampus

The hippocampus is one of the most commonly examined areas of the brain in relation to the effect of depression and antidepressant actions. It also has been shown to contain the greatest expression of BDNF mRNA and protein in the CNS.

1.7.1. Evidence linking the hippocampus with depression

MRI studies

Using high-resolution magnetic resonance imaging (MRI), significant bilateral hippocampal atrophy has been reported in patients with a history of recurrent, major depression against matched controls (Sheline et al, 1996; 1999; Bremner et al, 2000). Shah et al (1998) also reported hippocampal atrophy in patients with chronic depression, but found no atrophy in patients with "remitted" depression over controls, the clinical characteristics of remitted depression were however not described, making comparison difficult. Two further studies (Swayze et al 1992; Axelson et al 1993) found no hippocapmpal atrophy in depressive patients, however a less sensitive MRI methodology was used that was unable to differentiate between the hippocampus and amygdala (Sheline, 2000).

Animal models of stress

Animals subjected to recurrent episodes of stress have been shown to possess damaged hippocampal neurones. Chronic restraint stress produced significant atrophy of dendrites of CA3 neurones (Watanabe et al, 1992). Multiple stressors (shaking in addition to restraint) produced dendritic

atrophy and a robust increase in corticosterone (Magarinos and McEwen, 1995). After repeated stressor episodes, ultra-structural changes in mossy fibre projections have been demonstrated (Magarinos and McEwen, 1995). These changes however are reversible and may be prevented by antidepressant (tianeptine) treatment (Watanabe et al, 1992; Czeh et al, 2001). Lesioning of the hippocampus has been shown to eliminate the behavioural deficits induced by the learned helplessness model of animal depression (Elmes et al, 1975).

1.7.2. Anatomy

The mammalian hippocampal formation comprises of two main multi-layered components: the hippocampal gyrus and dentate gyrus (DG; area dentata).

The synaptic connections within the hippocampal formation are described as the 'trisynaptic circuit' (Andersen et al, 1966; Witter et al, 1989). The first connection is formed by fibres of the perforant pathway, layers II and III of the entorhinal cortex project into the DG to terminate on granule cell dendrites in the molecular cell layer (Hjorth-Simonsen, 1972; Steward, 1976). The second connection

arises from the granule cell layer, which sends axons (mossy fibres) to form synapses with the CA3 pyramidal cells of the hippocampal gyrus. The axons of the pyramidal cells form the main output for the hippocampus (Hinoi et al, 2002). However pyramidal cells also project collaterals, which make contact with pyramidal cells in the CA1 (Andersen et al, 1971; Ishizuka et al, 1990), thus completing the loop *(See Figure 1.12)*.

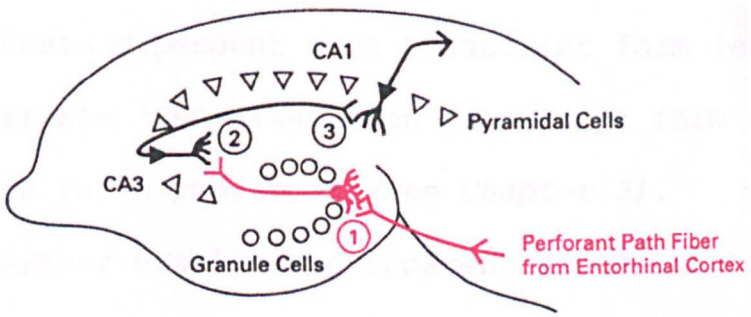


Figure 1.9. Simplified schematic diagram showing the pathways comprising the 'tri-synaptic loop' (adapted from Levitan and Kaczmarek, 1997)

All the neurones comprising the tri-synaptic loop architecture are glutamatergic. These principle cells represent 90% of hippocampal neurones (Vizi and Kiss, 1998). The remaining 10% non-principal cells are interneurones and are thought to use gamma aminobutyric acid (GABA) as a neurotransmitter (Gaiarsa et al, 2001).

They form well-organised neural networks, which control and regulate the operation of principal cells (Freund and Buzsaki, 1996).

1.8. Aims of the Present Study

- To assess the effect of acute administration of various antidepressant drugs on total (exon V) BDNF mRNA, as well as a representative form of a protein synthesis dependent exon transcript form (exon I) and a variable 'IEG-like' exon transcript form (exon IV) in the rat hippocampus *(See Chapter 3)*.
- To further examine the apparent acute inhibitory effect of antidepressant drugs on BDNF mRNA by the application of various GABAergic receptor compounds in the rat hippocampus *(See Chapter 4)*.
- To assess the effect of chronic administration of various antidepressant drugs on total (exon V) BDNF mRNA, as well as a representative form of a protein synthesis dependent exon transcript form (exon I) and a variable 'IEG-like' exon transcript form (exon IV) in the rat hippocampus *(See Chapter 5)*.

- To assess the effect of acute and chronic ECS application on total (exon V) BDNF mRNA, as well as a representative form of a protein synthesis dependent exon transcript form (exon I) and a variable 'IEG-like' exon transcript form (exon IV) in the rat hippocampus *(See Chapter 6)*.
- To examine the effect of chronic administration of an antidepressant drug and chronic ECS application on BDNF protein expression within the rat hippocampus *(See Chapter 7)*.

Chapter Two

Materials and Methods

2.1. Animals

All animal procedures were performed according to guidelines set by the Ethics and Animal Welfare Committee, De Montfort University, in accordance with the Animals (Scientific Procedures) Act, 1986. Animals were kept in 12h light: 12h dark conditions, with food and water available *ad liberatum*. Male Sprague-Dawley rats (Charles River, UK) were purchased 10 days before drug administration to minimise environmental impact and housed four per cage. Animals weighed between 225g and 250g upon drug administration. Injections were administered interperitoneally (i.p) in the lower left or right quadrant of the abdomen, with the needle angled at 45° to the skin. Electroconvulsive shock (ECS) was administered under halothane-induced anaesthesia via earclips placed bilaterally *(See Section 2.1.1.2)*.

Animals were killed according to Schedule 1 of the Animals (Scientific Procedures) Act, 1986 by rapid dislocation of the neck. Every effort was made to ensure a quick and humane sacrifice.

2.2. Experimental protocol

2.2.1. Drug administration

2.2.1.1. Acute administration

Groups of six animals were injected i.p once with the relevant compound and observed every 30min for 4h for any behavioural changes, prior to sacrifice. Drug doses are listed in *Sections 3.2 and 4.2.*

2.2.1.2. Chronic administration

Groups of six animals were injected i.p once daily with the relevant compound for 21d and observed periodically for behavioural changes and illness. Animals were sacrificed 24h after the last injection. Drug doses are listed in *Section 5.2.*

2.2.2. ECS administration

ECS was administered under halothane-induced anaesthesia. ECS induction was elicited using a small animal electroplexy unit (Theratronics Ltd, Guernsey). Induction

was set at 150V, 50 Hz for 1 second via earclip electrodes, resulting in tonic-clonic seizures lasting 15 to 20 seconds. Sham ECS involved the induction of halothane anaesthesia and the placement of earclips for a comparable length of time, without seizure induction.

2.2.2.1. Acute ECS administration

Animals were exposed to either a single ECS or sham treatment, before being sacrificed at 4h.

2.2.2.2. Chronic ECS administration

Animals were exposed to repeated ECS or sham treatment (5 treatments over a 10d period, i.e. one ECS every 48h). They were then sacrificed 24h after the last application.

2.3. *In-situ* hybridisation

2.3.1. Tissue pre-treatment

Brains were rapidly removed post-mortem, frozen in cooled isopentane and stored at -80°C until further use. Coronal sections (10μm) were cut using a cryostat (Bright, UK) set to -20°C, and thaw-mounted onto gelatinised microscope

slides (3 per slide). Sections were then post-fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4) for 5 min, then rinsed twice in PBS. Sections were then acetylated by 0.25% acetic anhydride in triethanolamine buffer (0.1M triethanolamine in 0.9% saline, pH 8) for 10 minutes, then dehydrated through a series of graded ethanol solutions (70, 80, 95 and 100%), delipidated in chloroform for 10 min and further dehydrated with 100% and 95% ethanol. Sections were air-dried and stored at -20°C, prior to use in labelling procedures.

2.3.2. Oligonucleotide probe labelling

2.3.2.1. Oligonucleotide probes

Oligonucleotide DNA probes complimentary to BDNF mRNA, BDNF exon I mRNA, BDNF exon II, BDNF exon III and BDNF exon IV mRNA were obtained (Eurogentec DNA Service Ltd. Southampton, UK). No significant homology between any probe and other previously identified gene sequences was found using a basic BLAST search, present on the National Institute for Health website (www.ncbi.nlm.nih.gov/blast/blast.cgi). Probe sequences were as follows:

BDNF - GGT CTC GTA GAA ATA TTG CTT CAG TTG GCC TTT TGA (Sequence code F75377; Eurogentec, UK. G C content 41.7%).

BDNF (SENSE STRAND) - CCA GAG CAT CTT TAT AAC GAA GTC AAC CCG AAA ACT (Sequence code F75378; Eurogentec, UK. G C content 41.7%)

BDNF (Exon I) - ACC CAA AGC AAT ATC GCA AGC TTC AAC TCT CAT CCA CTT (Sequence code F56636, Eurogentec, UK. G C content 45%).

BDNF (Exon II; PROBE A) - ATG AAG TAC TAC CAC CTC GGA CAA ATC CGC TGG (Sequence code G28478, Eurogentec, UK. G C content 52%)

BDNF (Exon II; PROBE B) - AGT AAC AGA CCT CAC TAA AGC CAT ATG CTT CCC AGC AGA (Sequence code F56637, Eurogentec, UK. G C content 50%)

BDNF (Exon III) - TAA TAC TCG CAC GCC TTC AGT GAG AAG CTC CAT (Sequence code G282479, Eurogentec, UK G C content 48%)

BDNF (Exon IV) - TTC CTT TAG GAA TGT CTC AAG TAC CAT TCC CCA CCT CCA T (Sequence code F67353, Eurogentec, UK. G C content 45%)

2.3.2.2. Labelling

Oligonucleotide probes were radiolabelled at the 3′ end with [α-[^{35}S] thio]-dATP (specific activity 1250 Ci/mmol; NEN life sciences, Stevenage, UK) using terminal deoxynucleotidyl transferase (TdT, Promega, UK). A reaction mixture was made containing 6pmol oligonucleotide, 30-60 units TdT, 60 pmol [^{35}S]-dATP in sterile water, 100 mmol potassium cacodylate, 1mM $CoCl_2$ and 100nM dithiothreitol (DTT) in diethyl pyrocarbonate (DEPC; 1%) treated purified water (final volume 35µl). After incubation at 37°C for 30 minutes, the reaction was stopped by adding 400µl of Reagent A solution (0.1M Tris hydrochloride, 10mmol trietyhlamine (TEA), 1mmol ethylenediaminetetraacetic acid (EDTA, pH 7.7). The reaction mixture was passed through a spin column containing Sephadex G-50 in order to separate the labelled probe from unincorporated nucleotide. Two more applications of 500µl Reagent A were passed through the column, resulting in three end solutions. The specific activity of each solution was determined using a scintillation counter (Beckman L55000ce, USA). A labelling procedure was deemed successful if the majority of the activity was present in the second tube and 50% of the radioactivity had been successfully incorporated.

2.3.2.3. Hybridisation

The microscope slides containing the previously thaw-mounted sections *(See Section 2.1.2)* were removed from the freezer, before being thawed and placed on RNA-ase free filter paper soaked in 4x standard saline citrate (SSC) (equivalent to 600mmol sodium chloride, 60mmol sodium citrate) in bioassay dishes. Labelled probe ($2x10^6$ cpmol/slide) which comprised of 50% deionised formamide, 20% 20 x SSC (4 x SSC), 5% sodium phosphate buffer (0.5mol), 1% sodium pyrophosphate buffer (0.1mol), 10% v/v 50x Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone and 0.2% bovine serum albumin in sterile water), 10mg/ml herring sperm DNA, 5mg/ml polyadenelic acid, 120mg/ml heparin and 10% dextran sulfate powder and 50mM dithiothreitol (DTT) was applied to thawed sections (3 per slide). After application of the reaction mixture the sections were cover-slipped, the bioassay boxes covered and incubated in humid chambers at the relevant calculated temperature for incubation of the probe (*See Section 2.1.6).*

2.3.3. Washing of sections

Cover slips were washed off in room temperature 1 X SSC. Sections were then washed in 1 x SSC at a pre-calculated washing temperature for a specific probe *(See Section 2.1.6)* for 3x20min, then at room temperature for 2 x 60min. To remove excess salt present, the sections were rinsed in sterile water and air-dried at room temperature. The slides were then placed into X-ray cassettes with a ^{14}C micro-scale standard (Amersham, UK) and exposed to Bio-Max film© (Kodak) in darkness for 7d at room temperature.

2.3.3.1. Hybridisation and washing temperature calculations

Hybridisation depends on the ability of the DNA to re-anneal to a complementary strand, just below its melting point. The melting temperature (T_m) of DNA-RNA hybrids (in this series of experiments the oligonucleotide DNA probe-target mRNA hybrids) is influenced by many factors, which in turn contribute to the stringency of hybridisation. The stringency of hybridisation determines the degree to which mismatched hybrids are permitted to form. The factors which affect stringency and therefore (T_m) include: (i) the guanine and cytosine percentage content the base

composition, (ii) the length of the probe, (iii) the concentration of monovalent cations (the Na^+ concentration) and (iv) the concentration of formamide present in buffer. The (T_m) was calculated using the following formula (Meinkoth and Wahl, 1984).

(T_m) = 81.5°C + 16.6 log ([monovelent cations]) + 0.41 (%G-C content of probe) - 0.61 (% formamide) - (675/probe length)

All incubation and washing procedures were carried out at 15°C below the T_m.

(i) A, Total BDNF (exon V) oligo probe

HYBRIDISATION
G C content 41.7%, Number of bases 36

$$T_m = 16.6\log(4\times0.165) + 0.41(41.7) + 81.5 - 0 - \frac{675}{36} - 0.65 \times 50$$

$$= -2.99 + 17.1 + 81.5 - 18.75 - 32.5$$
$$= 44.36$$
$$= T_i = T_m - 15 = \mathbf{29.3°C}$$

WASHING

$$T_m = 16.6\log(0.165) + 0.41(41.7) + 81.5 - 0 - \frac{675}{36}$$

$$-12.99 + 17.1 + 81.5 - 18.75$$
$$= 66.86$$
$$T_w = 66.9 - 15 = \mathbf{51.9°C}$$

(ii) BDNF exon I oligo probe

HYBRIDISATION
G C content 45%, Number of bases 40

T_m = 16.6log (4x0.165) +0.41(45) +81.5 -0 $-\frac{675}{40}$ -0.65 x50

= -2.99 +18.5 +81.5 -16.88 -32.5
= 47.63
= $T_i = T_m$ -15 = **32.6°C**

WASHING
T_m = 16.6log (0.165) +0.41(45) +81.5 -0 - $\frac{675}{40}$
-12.99 +21.32 +81.5 -16.88
= 72.95
T_w = 72.95 -15= **57.9°C**

(iii) BDNF exon II oligo probe (PROBE A)

HYBRIDISATION
G C content 52%, Number of bases 33
T_m = 16.6log (4x0.165) +0.41(52) +81.5 -0 $-\frac{675}{33}$ -0.65 x50

= -2.99 +21.3 +81.5 -20.45 -32.5
= 46.86
= $T_i = T_m$ -15 = **31.8°C**

WASHING
T_m = 16.6log (0.165) +0.41(52) +81.5 -0 - $\frac{675}{33}$
-12.99 +21.32 +81.5 -20.45
= 69.36
T_w = 69.4 -15 = **54.4°C**

(PROBE B)

HYBRIDISATION
G C content 50%, Number of bases 36
T_m = 16.6 (4x0.165) +0.41(50) +81.5 -0 $-\frac{675}{36}$ -0.65 x50

= -2.99 +20.5 +81.5 -18.75 -32.5
= 45.24
$T_i = T_m$ - 15 = **30.2°C**

WASHING
T_m = 16.6log (0.165) +0.41(50) =81.5 -0 $-\frac{675}{}$

-12.99 +20.5 +81.5 - 18.75
= 70.26
T_m = 70.26 -15 = **55.3°C**

(iv) BDNF exon III oligo probe

HYBRIDISATION
G C content 48%, number of bases 33
T_m = 16.6log (0.165x4) +0.41(48) +81.5 -0 $-\frac{675}{33}$ -0.65 x50
= -2.99 +19.68 +81.5 -20.45 -32.5
= 45.3
= T_i = T_m -15 = **30.3°C**

WASHING
T_m = 16.6log (0.165) +0.41(48) +81.5 -0 - $\frac{675}{33}$
-12.99 +19.68 +81.5 -20.45
= 67.76
T_w = 67.76 -15 = **53°C**

(V) BDNF exon IV oligo probe

HYBRIDISATION
G C content 45%, Number of bases 40
T_m = 16.6log (4x0.165) +0.41(45) +81.5 -0 $-\frac{675}{40}$ -0.65 x50
= -2.99 +18.4 +81.5 -16.9 -32.5
= 47.51
= T_i = T_m -15 = **32.5°C**

WASHING
T_m = 16.6log (0.165) +0.41(45) +81.5 -0 - $\frac{675}{40}$
-12.99 +18.4 +81.5 -16.8
= 70.11
T_w = 69.4 -15= **55.1°C**

2.3.4. Autoradiogram Analysis

2.3.4.1 Film development

Following exposure to the tissue slices, the Bio-max film was developed by immersion in developer for 1 min 30 s, rinsed in tap water, then immersed in a fixing solution for 5 min, followed by a further rinse in tap water. The Kodak GBX developer and fixer were obtained from Sigma-Aldrich Co. UK.

2.3.4.2. Film analysis

Densitometric analysis of the radioemissions was conducted using the NIH Image (version 1.61) program on a Macintosh™ G4 computer. Autoradiogram images were captured via an Epson™ Stylus Photo scanner set to 1200 x 1200 pixel optical resolution. The optical densities of specific brain regions were quantified and converted to nCi/g tissue weight using a standard curve generated by autoradiographic [^{14}C] microscale standards (Amersham Biotech Ltd. UK). A 9 x 9 pixel sampling area was chosen and placed over the corresponding area to be analysed. 100 readings were taken for each region assessed in each slice (three slices per animal) and a mean value was calculated to give a single

mean for each particular area in an animal *(See Figure 2.1)*.

2.3.4.3. Statistical analysis

Statistical analyses of the effects of drug/ECS intervention were made using either Student's t-test for two sets of data (vehicle control vs. drug-treated), or one-way/two-way analysis of variance (ANOVA) for multiple sets of data, with Bonferoni's multiple comparison post-hoc test, using GraphPad Prism Version 3.00 (GraphPad Software Inc., Califronia, USA). Significance was determined at $p<0.05$, all data is presented as mean $\pm$ standard error of mean (S.E.M.).

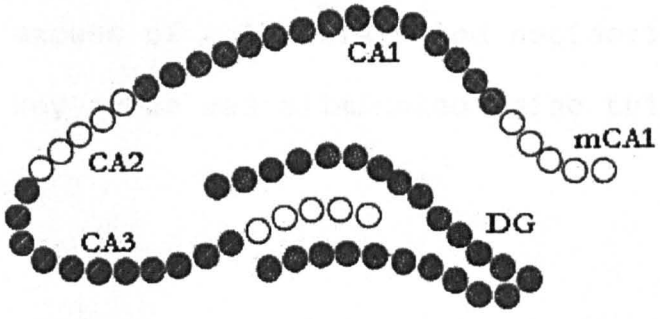


Figure 2.1. Schematic diagram of coronal section through the rat hippocampus showing approximate location of principal cell layers where optical densities were studied (adapted from Gibbs, 1998)

2.3.5. Control sections

Two methods of controls were employed to check for specificity of the anti-sense probes used in this study. For the total (exon V) BDNF, a cDNA sense sequence was obtained, corresponding to the parallel anti-sense sequence. The sense control was used to detect a background signal indicating the level of non-specific interactions. Minimal background signal was present for any control experiment, confirming the specificity of the oligonucleotide probe used. In addition, sections were hybridised with radiolabelled anti-sense probes corresponding to the total BDNF sequence used, as well as BDNF exon I and BDNF exon IV probe used, in the presence of over 100-fold excess of cold unlabelled nucleotide. Expression in key areas was eliminated using this technique.

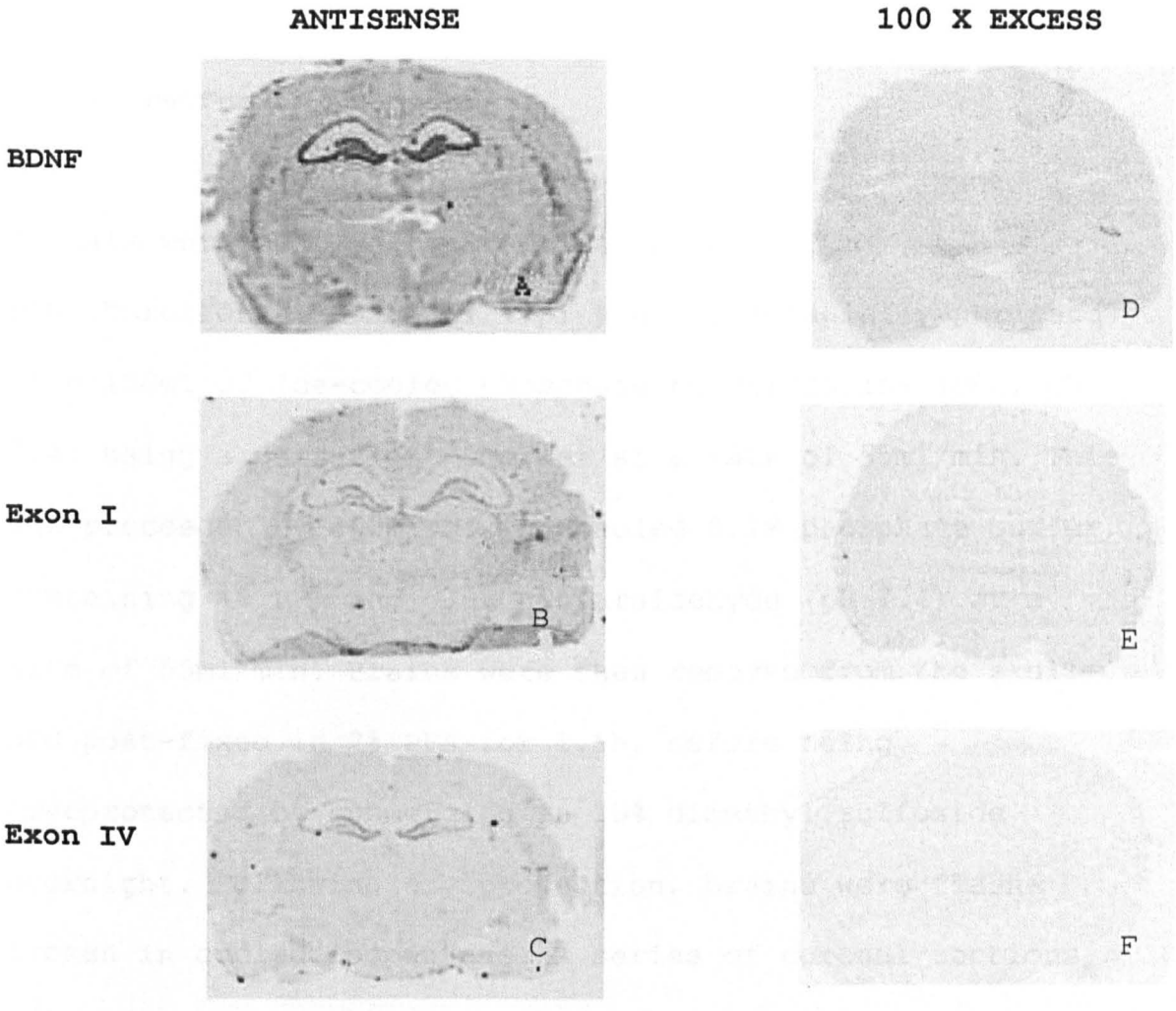


Figure 2.2. **Control sections.** *In-situ* hybridisation images demonstrating BDNF labelling with A, BDNF antisense probe; B, BDNF exon I antisense probe; C, BDNF exon IV antisense probe D, 100-fold excess BDNF; E, 100-fold excess exon I probe; F, 100-fold excess exon I probe.

2.4. Immunocytochemistry

2.4.1. Perfusion procedure

Animals were deeply anaesthetised with sodium pentobarbitone (200mg/kg, i.p) and transcranially perfused with 150ml of ice-cooled phosphate buffer saline (PBS, pH 7.4) using a perfusion pump set at a rate of 55ml/min. This was proceeded by 450ml of ice-cooled 0.1M phosphate buffer, containing 4% PFA and 0.1% glutaraldehyde (pH 7.4) at a rate of 55ml/min. Brains were then removed from the skull and post-fixed in 2% PFA for 1.5h, before being cryoprotected by submerging in 10% dimethyl sulfoxide overnight. Following cryoprotection, brains were flash-frozen in cooled isopentane. A series of coronal sections (30μm thick) through the frontal cortex, striatum and hippocampus were then cut on a cryostat. Free-floating sections were collected in cold PBS in 24-well plates.

2.4.2. Immunostaining

Pre-incubation

Sections were placed in sterile well plates covered in a pre-incubation of PBS and 0.25% Triton X-100 for 15mins then washed twice in PBS and placed in a pre-blocking agent of PBS, 0.25% Triton X-100 and 5% bovine serum albumin for 1h.

Primary incubation

Sections were washed twice in PBS then transferred by a glass tool into sterile well plates containing primary antibody (pAb) solution consisting of 10µg/ml chicken anti-hBDNF IgY pAb and 0.25% Triton X-100 in PBS for 24h at room temperature.

Secondary incubation

The sections were washed three times in PBS and transferred to well plates containing a secondary antibody solution consisting of 5µg/ml rabbit biotinylated anti-chicken IgY IgG in PBS for 2h at room temperature.

Avidin-biotin-horseradish peroxidase complex and diaminobenzine reactions

Sections were then covered in Vector elite avidin-biotin-horseradish peroxidase complex (ABC) reagent™ for 2h at room temperature, before being washed twice in PBS and washed once in 50nM TRIS (hydroxymethyl) aminomethane (TRIS) buffer (pH 7.7). Each section was then incubated in 3,3′-diaminobenzidine (DAB, 0.5mg/ml) in 50mM Tris saline buffer, containing 0.009% hydrogen peroxide. In addition 2xdrops of nickel stain were added to the mixture. After 5 mins the reaction was terminated by removing the sections from the solution. The sections were the rinsed three times in 50mM TRIS buffer, then stored in PBS, before being mounted on gelatinised microscope slides.

Mounting sections

Individual sections were placed on gelatinised slides and delipidated though immersion in graded 2min ethanol washes (50%, 70%, 90%, 100% in distilled water), then 100% xylene for 8mins. Sections were then mounted using DPX Histoclear mountant and cover-slipped, lightly applying pressure to ensure against bubbles.

Photomicrography

Sections were examined using a Leitz-Diaplan photomicroscope and images were captured with a digital camera using Openlab software (Improvision, UK).

Image and data analysis

Relative abundance was measured in selected areas using a computerised analysis system (MCID-4, Imaging Research, St Catherine's, Ontario, Canada) and expressed as an optical density unit, which was corrected for background (non-specific) signal by subtracting white matter (corpus callosum) from the raw figures. The immunoreactivity of BDNF protein was measured in four areas, the CA1, CA3 and DG of the hippocampus, and the parietal cortex. Differences between saline/sham and treatment groups were assessed using Student's *t*-test.

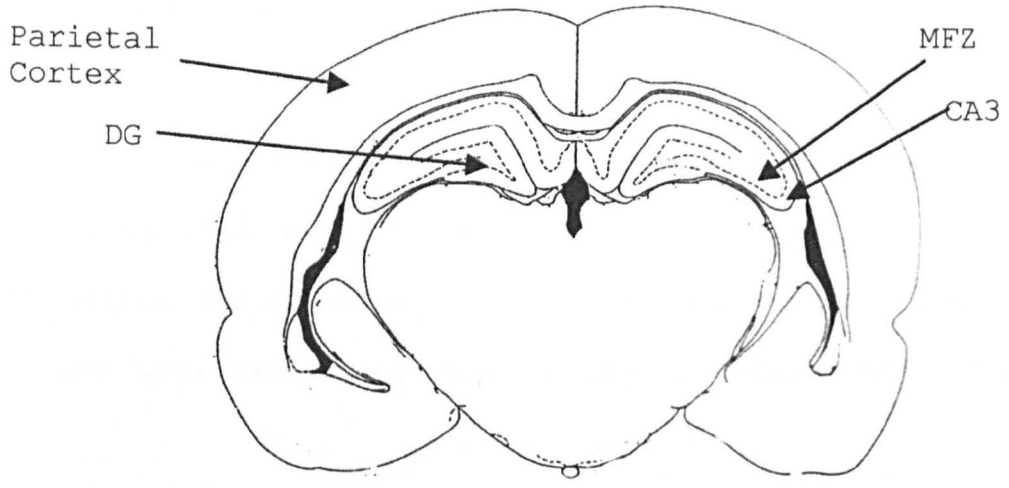
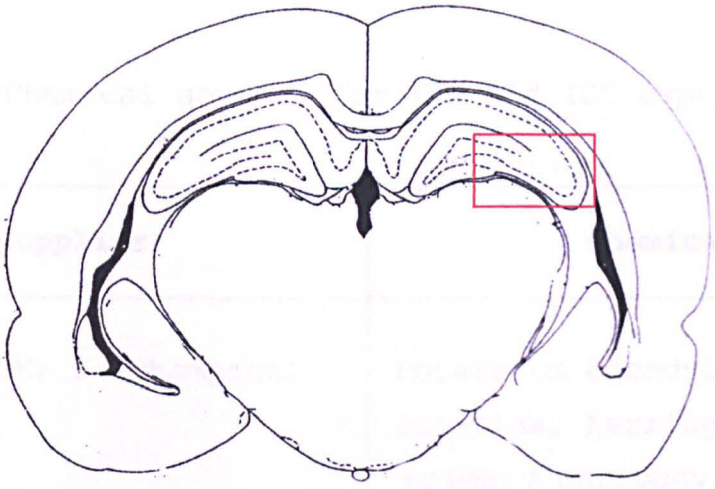


Figure 2.3. Anatomical locations of regions studied in the immuncytochemistry experiments (Adapted from Paxinos and Watson, 1997; Plate 33).

2.4.3. Control sections

Controls were performed on sections in each corresponding group by following the same procedure except for incubating in Triton X-100 instead of primary antibody. All other stages were performed identically to experimental sections.



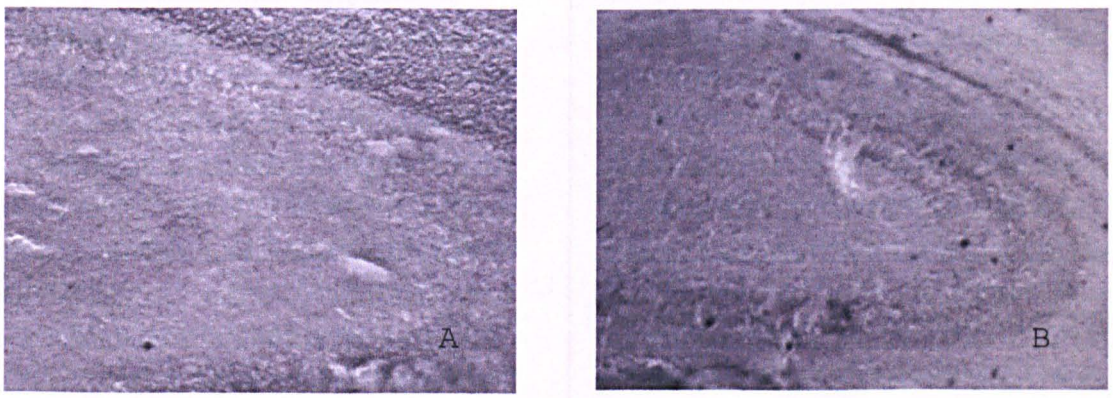


Figure 2.4. ICC experiment coronal hippocampal control section image (A), compared with section with BDNF primary antibody present (B). The anatomical location is highlighted on a schematic diagram above (Adapted from Paxinos and Watson, 1997; Plate 34).

2.5. Chemicals and drugs

2.5.1. Chemicals

The chemicals used (with supplier) used in ISH and ICC experiments are tabulated below.

Table 2.1. Chemical sources for ISH and ICC experiments.

Supplier	Chemical
Promega UK; Southampton)	Potassium cacodylate, cobalt chloride, herring sperm DNA, primary antibody, secondary antibody
Sigma UK; Poole, Dorset).	Phosphate buffer saline tablets, triethanolamine, acetic anhydride, diethylpyrocarbonate, dithiothreitol, Sephadex G-50, triethylamine, Trizma hydrochloride, deionised formamide, formamide, sodium citrate, Denhart's solution, polyadenylic acid, heparin, dextran sulphate, bovine serum albumin, TRIS buffer

Fisher Scientific UK; Loughborough, Leicestershire	Glacial acetic acid, paraformaldehyde, chloroform, sodium chloride, xylene
BDH Laboratory Supplies; Poole, Dorset	Powdered gelatin, chromium potassium sulphate, ethylenediaminetetraacetic acid disodium salt, sodium phosphate, sodium orthophosphate, sodium pyrophosphate, glass slides, cover slips
Vector Laboratories Inc. Burlingame, California, USA	ABC kit, DAB substrate kit

2.5.2. Drugs

The drugs used (with supplier) used in ISH and ICC experiments are tabulated below.

Table 2.2. Drug sources for ISH and ICC experiments.

Supplier	Drug
Tocris Cookson Ltd; Bristol, UK	CGP 46381, CGP 55845, GBR 12909, maprotiline, tranylcypromine, THIP, flunitrazepam, methylphenidate
Sigma; Poole, Dorset, UK	Baclofen, desipramine
Lundbeck Pharmaceticals; Hartlepool, UK	Citalopram
Glaxo Smith-Kline	Paroxetine
Lilly	Fluoxetine

Chapter Three

The Effect of Acute Antidepressant Drug Administration on BDNF mRNA and BDNF exon mRNA in the Rat Hippocampus

3.1. Introduction

The present chapter relates to the effect of acute administration of various classes of antidepressant drugs on the expression of total BDNF mRNA (exon V), as well as variable transcript forms which are generated from the gene (containing exons I-IV), in the adult rat hippocampus. A series of *in-situ* hybridisation experiments were performed examining the effect of a single injection of six different antidepressant drugs on BDNF mRNA, as well as the transcript forms containing BDNF exon I and exon IV mRNA. Attempts were made to measure exon II and exon III mRNA, however basal levels were deemed insufficient to ensure reliable measurements.

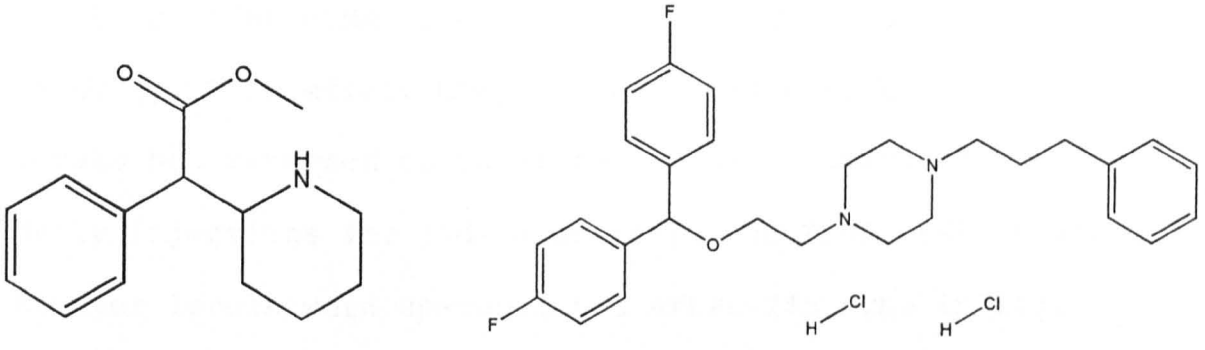
The hippocampus was chosen as the region of focus for the series of experiments. Initial experiments indicated the highest level of basal expression of BDNF mRNA was present in this region in comparison with other areas measured, where low levels of basal expression were found *(See Section 3.3.1)*. In addition, as explained in *Section 1.7.3*, alterations in hippocampal function have long been implicated in the pathophysiology of depression. Furthermore, a high degree of neuronal plasticity is known

to take place in the hippocampus and BDNF has been shown to influence synaptic function and induce neuronal sprouting in this region *(See Section 1.6.2.3)*.

The antidepressants used comprised of the SSRIs fluoxetine, paroxetine and citalopram; and the selective NARIs desipramine and maprotiline. The antidepressants were chosen because of their varying affinities and potency for the serotonin and noradrenaline reuptake sites (Hytel, 1994). The non-selective MAOI tranylcypromine (TCP) was also tested.

In addition for comparison, the effect of the non-selective noradrenaline (NA) / dopamine (DA) reuptake blocker methylphenidate and the selective DA transporter blocker GBR 12909 were assessed. Methylphenidate is a psycho-stimulant that binds with a similar affinity to the DA and NA transporters that produces an increase in extracellular brain concentrations of both DA and NA in rodents (Gatley et al, 1996; Kuczenski and Segal, 1997). GBR 12909 has been shown to show bind with a high affinity to the DA transporter (Rothman et al, 2003), and has been shown to bind with a substantially less (over 100 times) affinity to the 5-HT transporter (Andersen, 1989). It has also been

shown to have no effect on NA metabolism or LC firing rate (Nissbrandt et al, 1991).



Methylphenidate GBR 12909

Figure 3.1. The structure of methylphenidate and GBR 12909

3.1.1. The effect of acute administration of antidepressant drugs on BDNF mRNA

As explained earlier *(See Section 1.6.2.4)*, several groups have shown an enhancement in BDNF mRNA after chronic administration of antidepressant drugs (Nibuya et al, 1995; Russo-Neustadt et al, 2000; Coppell et al, 2003). However, considerable variations exist in the magnitude of the increase between these studies. It has been suggested that such anomalies may be due to the time period after the last injection. Coppell et al (2003) found that administration of

antidepressant drugs produced a "bi-phasic effect" on BDNF mRNA within the rat hippocampus. Thus, a single injection of antidepressant (paroxetine, fluoxetine, sertraline) inhibited BDNF mRNA levels in the CA1, CA3 and DG regions after 4h. This effect however was transient, as after 24h levels had returned to baseline. Chronic administration (2 daily injections for 14d) also inhibited BDNF mRNA at 4h, however levels were up-regulated after 24h. The initial down-regulatory response was attributed to the enhancement of local extracellular 5-HT levels, as a result of acute 5-HT reuptake blockade (Coppell et al, 2003). Zetterström et al (1999) demonstrated that acute elevation of brain 5-HT levels either by administration of the 5-HT release agent *p*-chloroamphetamine (*p*CA), the SSRI paroxetine, or a combination of the 5-HT precursor l-tryptophan and the non-selective MAOI TCP, significantly reduced BDNF mRNA in the DG at 4h. In contrast, rapid depletion of 5-HT levels with multiple injections of the tryptophan hydroxylase-selective inhibitor *p*-chlorophenylalanine (*p*CPA) significantly increased BDNF mRNA in the DG (Zetterström et al, 1999). The results suggest that acute enhancements in 5-HT levels have a transient inhibitory effect on BDNF mRNA expression in the hippocampus. The role of 5-HT receptors in the acute regulation of BDNF mRNA was demonstrated by Vaidya et al

(1997). Acute administration of the 5-HT_2 receptor agonist DOI reduced BDNF mRNA in the DG at 4h. This effect was attenuated by the 5-HT_{2A} receptor antagonist MDL 100907, but not by the 5-HT_{2C} antagonist SB 206553, indicating a central role for the 5-HT_{2A} receptor in the inhibitory BDNF mRNA response (Vaidya et al, 1997).

It is not clear however whether the acute down-regulatory response is due specifically to acute manipulations in 5-HT, or occurs due to generalised changes in monoamine concentrations. For this reason the present study examined various antidepressant compounds with varying affinities to 5-HT and NA reuptake sites, as well as the non-selective MAOI TCP. Additionally, the role of acute DA manipulation was studied with the selective DA transporter blocker GBR 12909 and the non-selective NA/DA reuptake blocker methylphenidate.

The BDNF gene has been suggested as a possible target in antidepressant treatment (Condorelli et al, 1994; Nibuya et al, 1995; Siuciak et al, 1996; Duman, 1998; Duman et al, 1999), thus further examination of the initial down-regulatory response (at 4h) is vital in order to elucidate the mechanism of action of antidepressant action on BDNF.

The unique complexity of the BDNF gene among neurotrophins allows the opportunity to study the effect of antidepressants, when given acutely, on specific exon-containing transcripts. During the course of this series of experiments, a similar study was published demonstrating evidence of differential BDNF mRNA exon expression (exons I-IV), following the acute administration of antidepressants (Dias et al, 2003). However, a full appraisal of the effects in the previous study was hampered by the lack of inclusion of data for the full BDNF gene (exon V), which codes for BDNF protein. In the present study we have simultaneously included total BDNF mRNA data (exon V), as well as a representative form of protein-dependent exon mRNA (exon I) and a non-protein synthesis-dependent 'IEG-like' exon mRNA (exon IV; Lauterborn et al, 1996; Russo-Neustadt et al, 2001; *See Section 1.6.3*).

The aim of this chapter therefore is to examine the effect of acute (single injection) of numerous antidepressant compounds, as well as methylphenidate and GBR 12909 on BDNF mRNA, as well as exon I and exon IV mRNA expression in the hippocampus at 4h.

3.2. Methods

Male Sprague-Dawley rats (225-250g) were injected intra-peritoneally (i.p) once with either: saline (1ml/kg), or fluoxetine (10mg/kg), paroxetine (5mg/kg), citalopram (10mg/kg), desipramine (10mg/kg), maprotiline (4mg/kg), TCP (5mg/kg), methylphenidate (4mg/kg) or GBR 12909 (10mg/kg) in 0.9% saline, before being sacrificed 4h later. Brains were isolated, then flash-frozen in cooled isopentane and then stored at -70°C until further use. *In-situ* hybridisation and densitometric analysis procedures are described in *Chapter 2*.

3.3. Results

3.3.1. Basal distribution of total BDNF mRNA and individual BDNF exon mRNA in the rat brain

3.3.1.1. Basal total BDNF mRNA expression

Coronal sections through frontal cortex, striatum and hippocampal areas were assessed for total (exon V) BDNF mRNA distribution. Signals were detected in the cingulate *(Figure 3.2, A and B)* and piriform *(Figure 3.2, B and C)* cortices, however they were generally low and difficult to quantify by densitometry. A signal was also detected in the parietal cortex *(Figure 3.2, C)*, this signal was very weak and there were no differences in expression through the different layers of this region.

Basal levels were found to exist in a measurable quantity the hippocampal region only *(Figure 3.2, C and D)*. Within the hippocampus, the highest levels were present in the CA3 and DG regions, with lower levels present in the CA1 *(Figures 3.2, C and D)*.

Figure 3.2, D displays total BDNF mRNA expression in a saggital section through the adult rat brain. Higher levels of expression are seen the granular cell layer of the cerebellum, as well as the hippocampus.

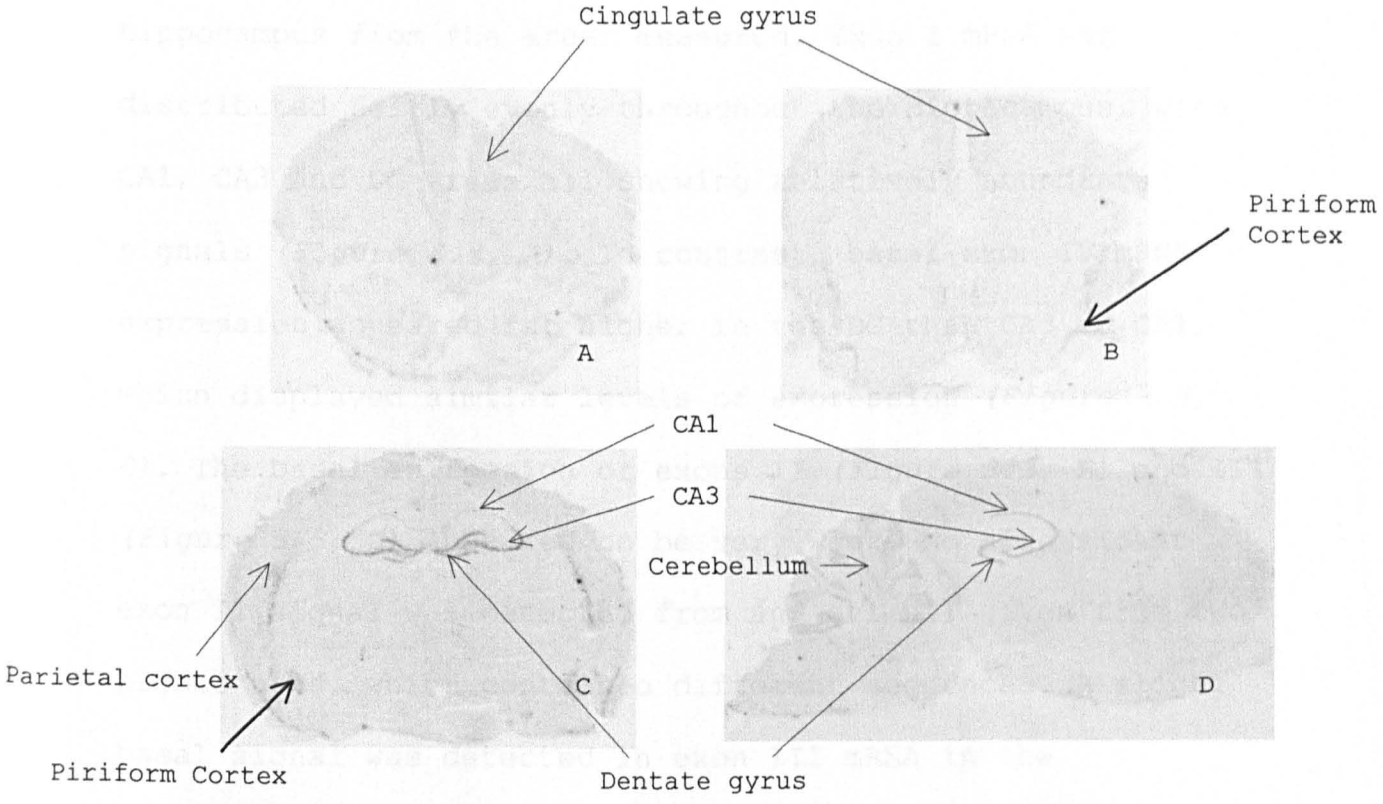


Figure 3.2. Basal total BDNF mRNA expression. A, coronal section through the frontal cortex region; B, coronal section through the striatum region; C, coronal section through the hippocampus region; D, saggital section through the rat brain.

3.3.1.2. Basal individual BDNF exon transcript mRNA expression

The oligonucleotide probes used corresponding to mRNAs encoding for each individual exon yielded varying levels of expression among exons I, II, III and IV. However, any significant expression of each exon was confined to the hippocampus from the areas measured. Exon I mRNA was distributed fairly evenly throughout the hippocampus, with CA1, CA3 and DG areas all showing relatively abundant signals *(Figure 3.3, A)*. In contrast, basal exon IV mRNA expression appeared far higher in the DG than CA3 or CA1, which displayed similar levels of expression *(Figure 3.3, D)*. The basal expression of exons II *(Figure 3.3, B)* and III *(Figure 3.3, C)* appeared to be very weak. No significant exon II signal was detected from any stimuli given from two probes used, which contained different sequences. A slight basal signal was detected in exon III mRNA in the hippocampus and a significant response was detected after strong stimuli (acute ECS), suggesting that the probe used was able to hybridise with exon III. However, as the basal level was so weak, the accuracy of analysis could be questioned. It was therefore decided that the series of experiments in this and proceeding chapters would focus on

the differential effects on exon I and IV mRNAs, in addition to total BDNF mRNA (exon V).

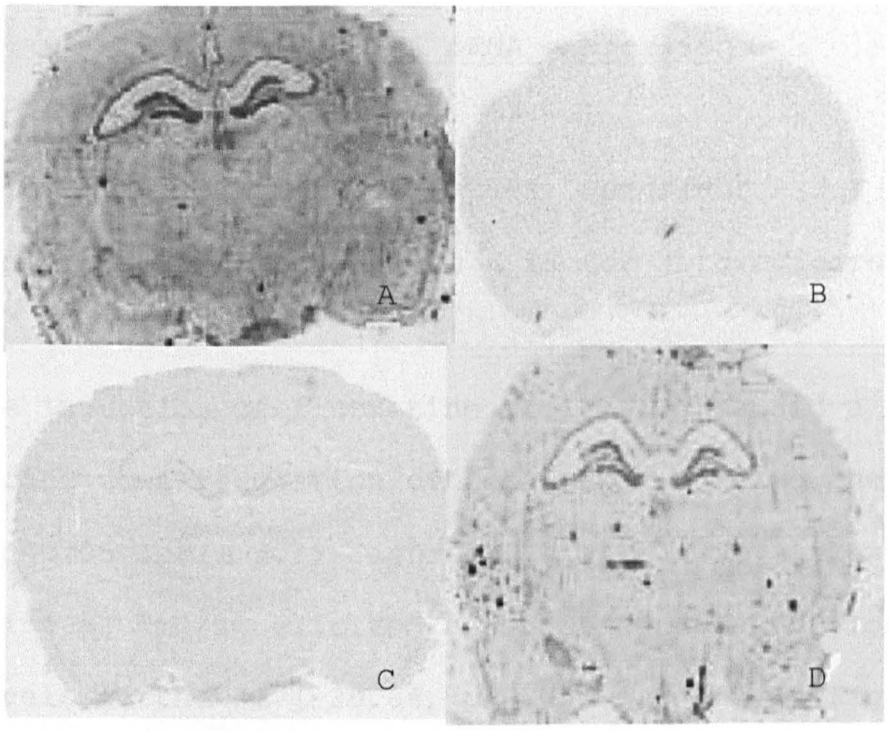


Figure 3.3. Basal expression of BDNF exon transcript mRNA: A, exon I; B, exon II (PROBE B); C, exon III; D, exon IV.

3.3.2. Effects of acute admnistration of antidepressant drugs on total BDNF mRNA and individual BDNF exon mRNA

3.3.2.1. Effect on total BDNF mRNA expression

See Tables 3.1 for numerical values. See Figures 3.4 to 3.11 for graphs. See Figures 3.12 to 3.19 for autoradiographs.

A single injection of fluoxetine at 10mg/kg caused a significant down-regulation of total BDNF mRNA in the DG (-27.5%; $p<0.05$ *Table 3.1; Figures 3.4 and 3.12*). Likewise, paroxetine at 5mg/kg elicited a significant inhibition in mRNA levels in the DG (-20.6%, $p<0.05$; *Table 3.1; Figures 3.5 and 3.13*), as well as the CA3 (-18.6%; $p<0.05$). Citalopram administration inhibited DG levels significantly (-37.8%, $p<0.01$; *Table 3.1; Figures 3.6 and 3.14*). Among the noradrenargic-selective antidepressants tested, desipramine (-39.6%, $p<0.05$; *Table 3.1 Figures 3.7 and 3.15*) and maprotiline (-33.4%, $p<0.01$; *Table 3.1. Figures 3.8 and 3.16*) significantly inhibited DG BDNF mRNA levels. The MAOI TCP (5mg/kg) caused the largest inhibition in BDNF levels in the DG among antidepressant drugs tested (-42.6%, $p<0.001$; *Table 3.1, Figures 3.9 and 3.17).*

Acute administration of either the non-selective NA/DA transporter blocker methylphenidate (-33.4%, $p<0.05$; *Table 3.1; Figures 3.10 and 3.18*) or the DA transporter blocker GBR 12909 (-39.9%, $p<0.001$; *Table 3.1; Figures 3.11 and 3.19*) significantly inhibited BDNF mRNA expression in the DG region.

3.3.2.2. Effect on BDNF exon I mRNA expression

See Table 3.2 for numerical values. See Figures 3.4 to 3.11 for graphs. See Figures 3.12 to 3.19 for autoradiographs.

The acute injection of any antidepressant tested had no significant effect on BDNF exon I mRNA in any region of the hippocampus at 4h. However, there was a small, but not significant fall in exon I mRNA in CA1, CA3 and DG subfields after desipramine and fluoxetine administration. In addition, modest, non-significant reductions were seen after TCP administration in the CA1 and DG and in the CA3 subfield after citalopram administration.

3.3.2.3. Effect on BDNF exon IV mRNA expression

See Table 3.2 for numerical values. See Figures 3.4 to 3.11 for graphs. See Figures 3.12 to 3.19 for autoradiographs.

In accordance with BDNF mRNA, acute injection of all antidepressants tested produced an inhibitory effect on BDNF exon IV mRNA in the DG. Fluoxetine elicited a significant 36.5% ($p<0.01$) inhibition in the DG (*Table 3.3; Figures 3.4 and 3.12*). Paroxetine administration caused a significant 24.7% ($p<0.05$) reduction in the DG (*Table 3.3; Figures 3.5 and 3.13*). Acute citalopram caused significant inhibition in both DG (34.4%; $p<0.05$) and CA3 (-35.1% $p<0.05$; *Table 3.3; Figures 3.6 and 3.14*). Desipramine administration produced a significant inhibitory effect in the DG (-32.1%, $p<0.05$); *Table 3.3; Figures 3.7 and 3.15*). Maprotiline also produced a significant reduction within the DG (-37.9%, $p<0.05$) and CA3 (-34.7%; $p<0.05$; *Table 3.3 Figures 3.8 and 3.16*). Acute TCP administration also resulted in a significant reduction in exon IV mRNA expression in the DG (-30.2%, $p<0.05$; *Table 3.3; Figures 3.9 and 3.17*). Additionally, the DA-/NA-selective reuptake inhibitor methylphenidate also produced a significant inhibitory effect in the DG (-37.7%; $p<0.05$) and CA3 (-34.7%; $p<0.05$; *Table 3.3; Figures 3.10 and 3.18*). The

DA-selective compound GBR 12909 resulted in the largest inhibition in DG levels (-50.0%; $p<0.01$; *Table 3.3; Figures 3.11 and 3.19*). CA3 levels were also significantly reduced by 34.1% ($p<0.05$). CA1 levels were not significantly affected by acute treatment of any compound tested at 4h.

The data presented in the proceeding tables (chapters 3-6) is presented in indexed form. This enabled figures from different experiments to be standardised for comparison (Zetterström et al, 1999; Coppell et al, 2003).

***Table 3.1.* Effect of acute drug treatment on BDNF mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

	CA1	CA3	DG
Vehicle n=6	100±5.3	100±4.8	100±11.3
Fluoxetine n=6	82.3±18.1	82.7±7.8	72.5±6.0*
Saline n=6	100±2.3	100±1.9	100±7.1
Paroxetine n=6	100±4.9	81.4±3.4*	79.4±5.6*
Saline n=5	100±10.1	100±13.2	100±11.2
Citalopram n=5	90.0±8.9	75.6±3.0*	62.2±6.3**
Vehicle n=6	100±5.3	100±4.8	100±11.3
Desipramine n=6	76.0±23.4	78.7±7.8	60.4±12.8*
Saline n=5	100±10.1	100±13.2	100±11.2
Maprotiline n=5	96.8±12.5	86.0±8.8	66.6±8.4**
Saline n=6	100±2.3	100±1.9	100±7.1
Tranylcypromine n=5	94.0±3.3	79.1±3.3*	57.4±4.0***
Saline n=6	100±6.0	100±7.1	100±5.3
Methylphenidate n=5	109.3±8.1	85.2±3.8	66.6±10.0*
Saline n=5	100±10.1	100±13.2	100±11.2
GBR 12909 n=5	94.1±9.3	81.4±9.3	60.1±9.1***

***Table 3.2.* Effect of acute drug treatment on BDNF exon I mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

	CA1	CA3	DG
Vehicle n=6	100±6.1	100±7.0	100±12.9
Fluoxetine n=6	87.6±18.1	85.3±4.7	84.4±7.3
Saline n=6	100±5.4	100±5.8	100±3.9
Paroxetine n=6	100±7.0	105±2.0	101.1±3.1
Saline n=4	100±8.9	100±13.2	100±13.0
Citalopram n=4	98.4±8.3	84.8±19.0	99.1±9.9
Vehicle n=6	100±6.1	100±7.0	100±12.9
Desipramine n=6	84.3±13.4	84.3±13.3	84.4±7.3
Saline n=4	100±8.9	100±13.2	100±13.0
Maprotiline n=4	112.9±11.0	80.0±7.0	86.1±4.4
Saline n=6	100±4.4	100±2.8	100±0.9
Tranylcypromine n=6	90.6±2.8	101.1±3.7	87.0±3.7
Saline n=4	100±8.9	100±13.2	100±13.0
Methylphenidate n=4	111.1±11.1	88.3±10.9	112.3±6.0
Saline n=4	100±8.9	100±13.2	100±13.0
GBR 12909 n=4	103.6±11.0	109±9.6	103.5± 19.9

***Table 3.3.* Effect of acute drug treatment on BDNF exon IV mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

	CA1	CA3	DG
Vehicle n=6	100±16.7	100±10.7	100±16.4
Fluoxetine n=6	89.4±8.5	87.8±7.3	63.5±6.5**
Saline n=6	100±2.8	100±1.7	100±3.2
Paroxetine n=6	91.9±4.1	85.4±3.4	75.3±3.1*
Saline n=5	100±5.8	100±8.8	100±6.3
Citalopram n=5	72.1±20.6	64.9±16.1*	65.6±18.2*
Vehicle n=6	100±16.7	100±10.7	100±16.4
Desipramine n=6	85.3±6.5	76.9±8.1	67.9±5.8*
Saline n=5	100±5.8	100±8.8	100±6.3
Maprotiline n=4	71.5±7.8	65.3±11.3*	62.1±15.4*
Saline n=6	100±2.8	100±1.7	100±3.2
Tranylcypromine n=6	85.4±2.8	83.8±3.9	69.8±5.0**
Saline n=4	100±8.9	100±13.2	100±13.0
Methylphenidate n=4	71.5±9.3	65.3±8.1*	62.3±6.8*
Saline n=4	100±5.8	100±9.4	100±6.5
GBR 12909 n=4	86.1±9.3	65.9±9.6*	50.0± 19.9**

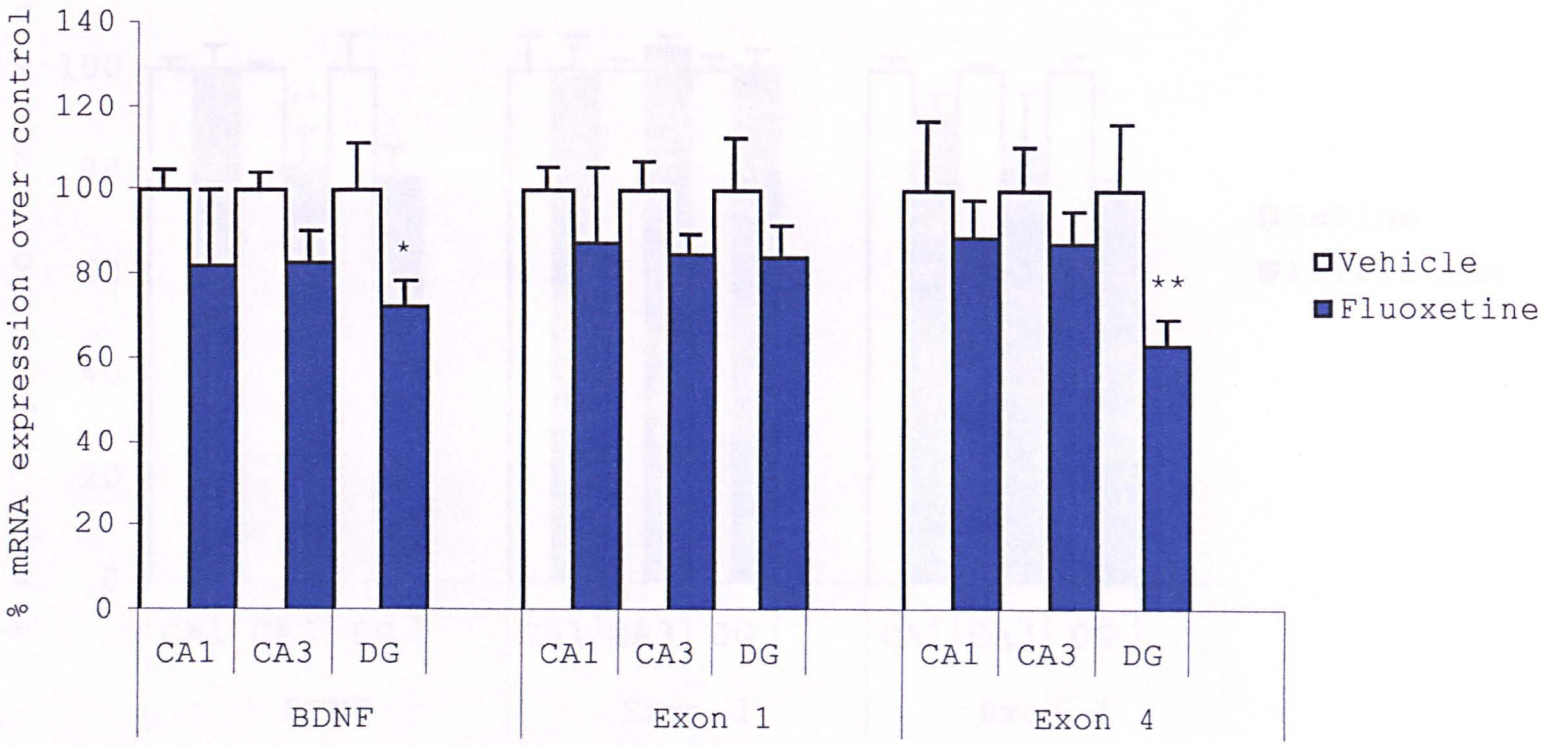


Figure 3.4. **Effect of acute fluoxetine (10mg/kg i.p) treatment on total BDNF mRNA, BDNF exon I and BDNF exon IV mRNA expression in the rat hippocampus, 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

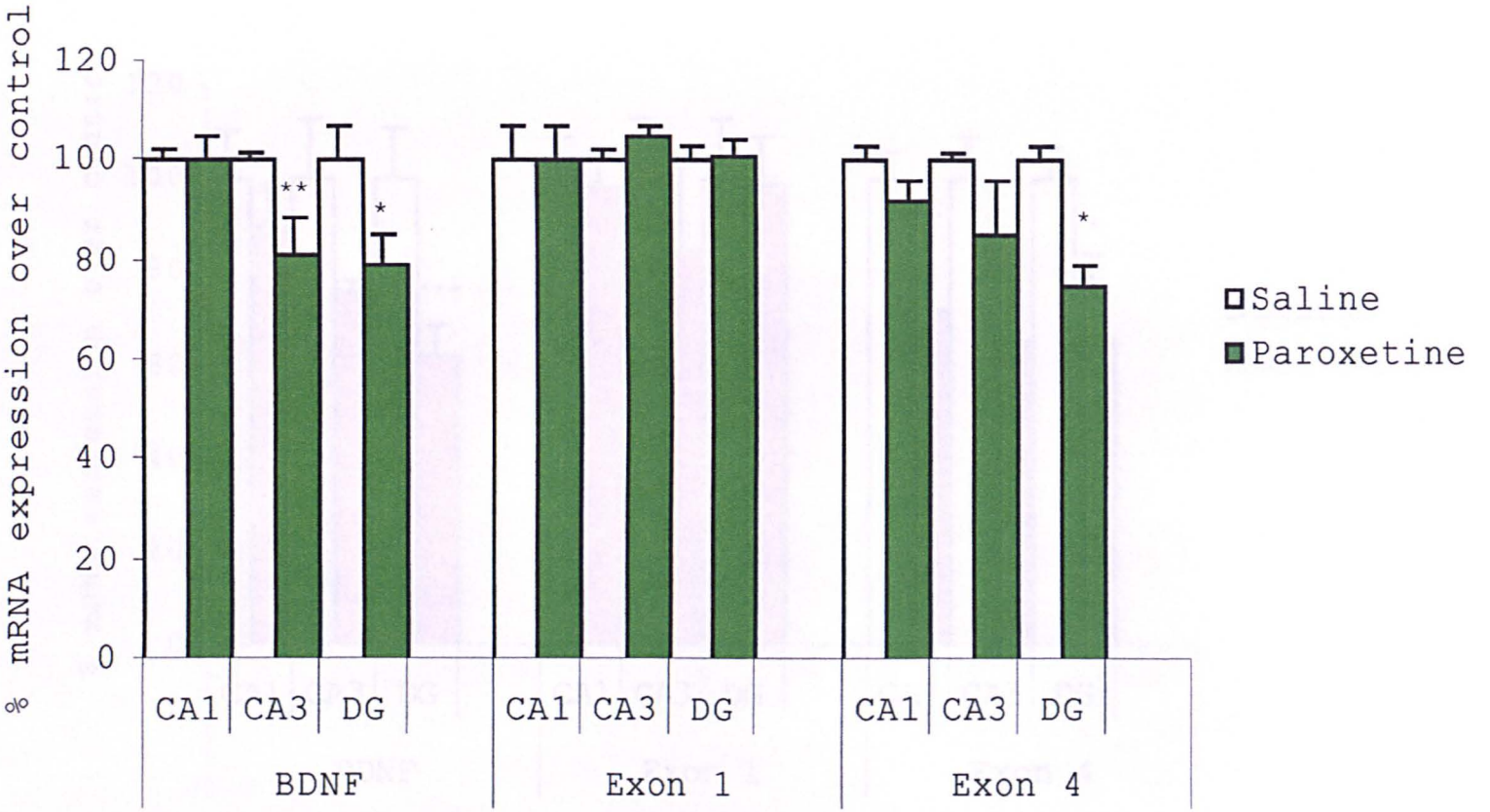


Figure 3.5. **Effect of acute paroxetine (5mg/kg i.p) treatment on total BDNF mRNA, BDNF exon I and BDNF exon IV mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

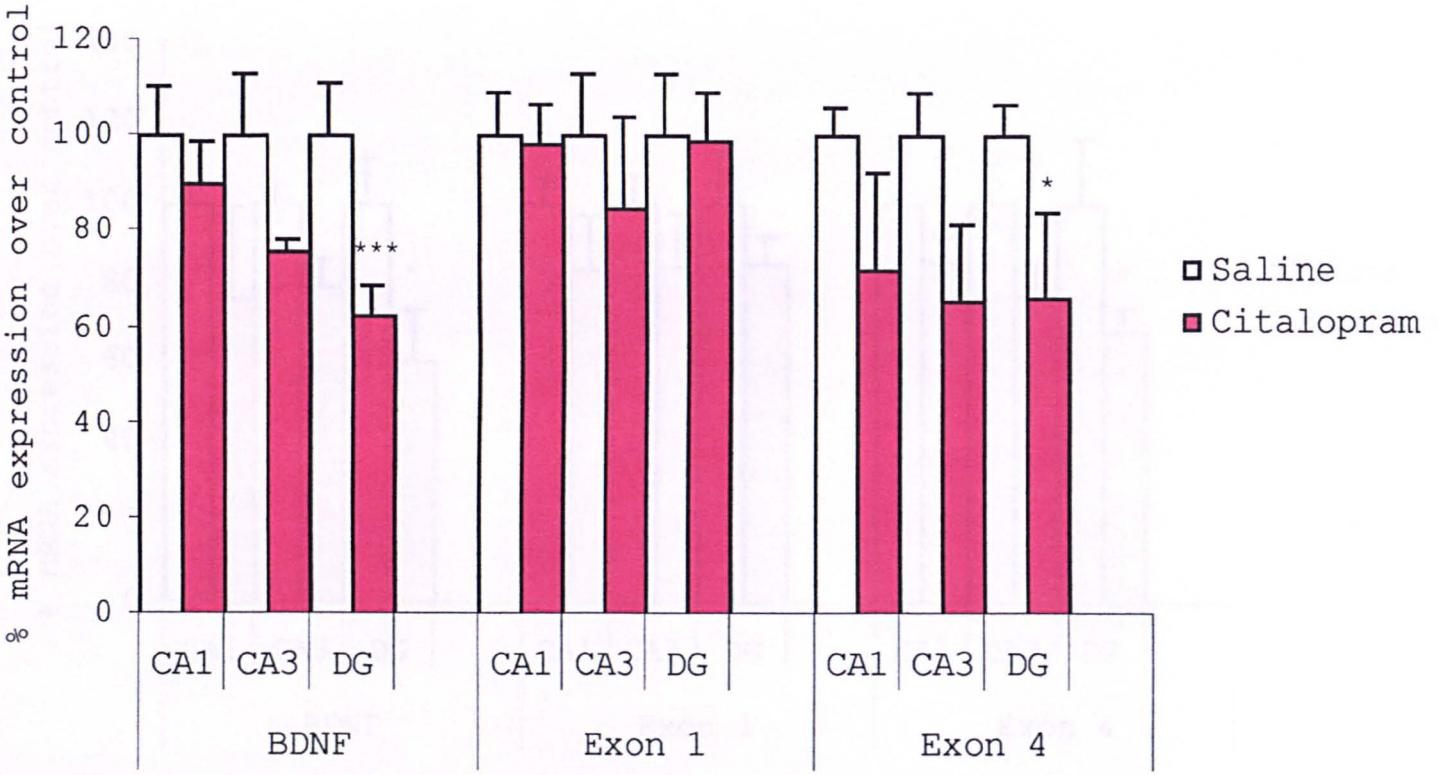


Figure 3.6. **Effect of acute citalopram (10mg/kg i.p) treatment on total BDNF mRNA, BDNF exon I mRNA and BDNF exon IV mRNA expression in the rat hippocampus, 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

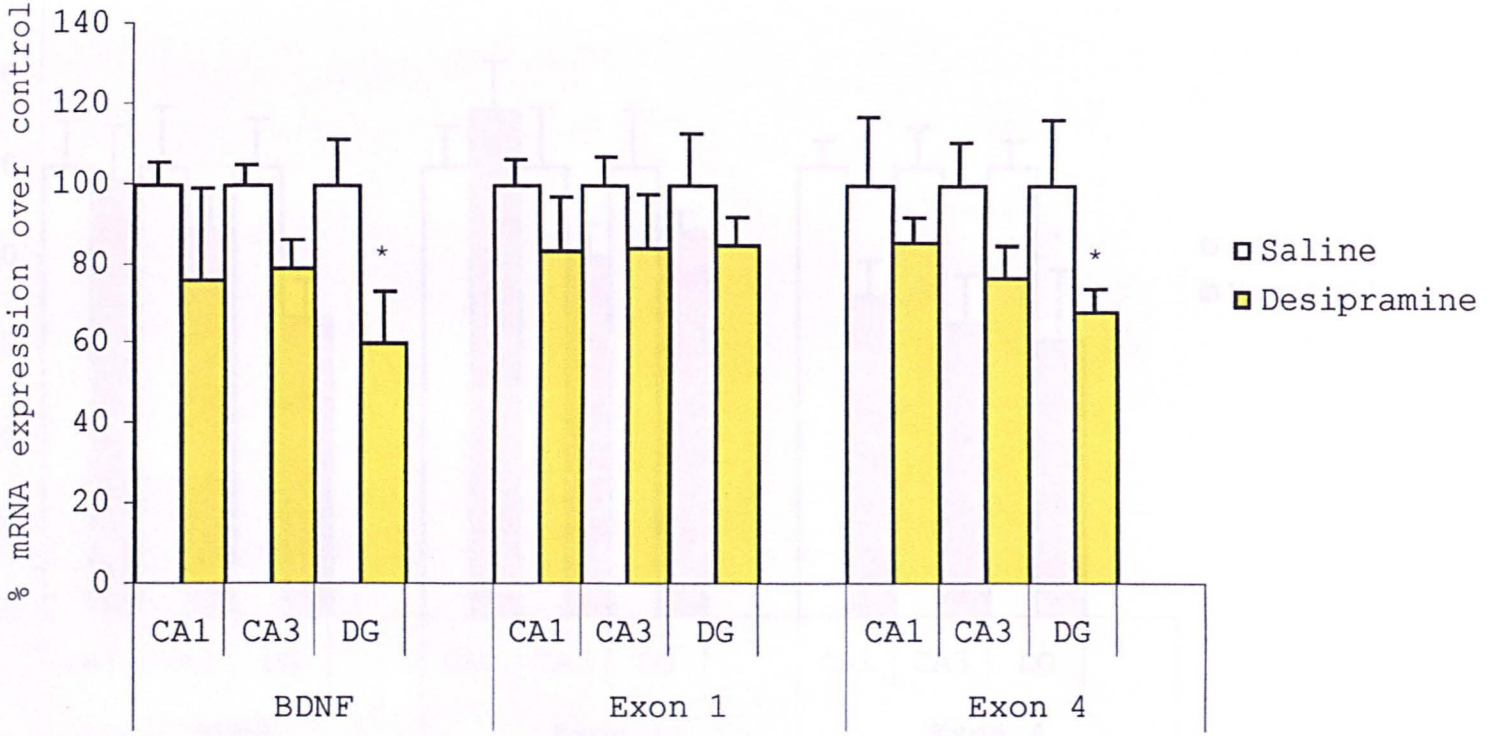


Figure 3.7. **Effect of acute desipramine (10mg/kg i.p) treatment on total BDNF mRNA, exon I mRNA and exon IV mRNA expression in the rat hippocampus, 4h after injection.** Data presented as percentage of control **p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

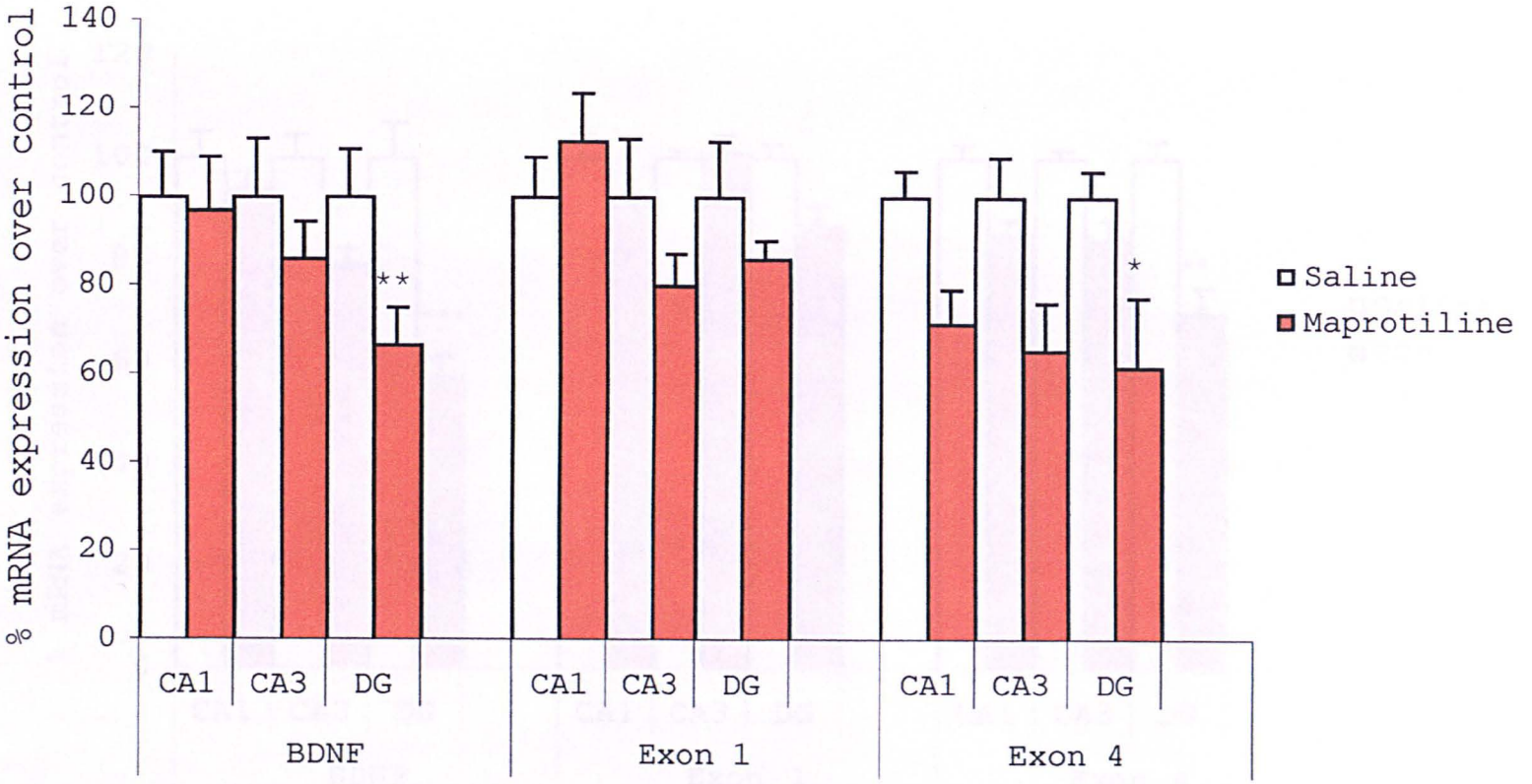


Figure 3.8. **Effect of acute maprotiline (10mg/kg i.p) treatment on total BDNF mRNA, BDNF exon I and BDNF exon IV mRNA expression in the rat hippocampus, 4h after injection.** Data presented as percentage of control **p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

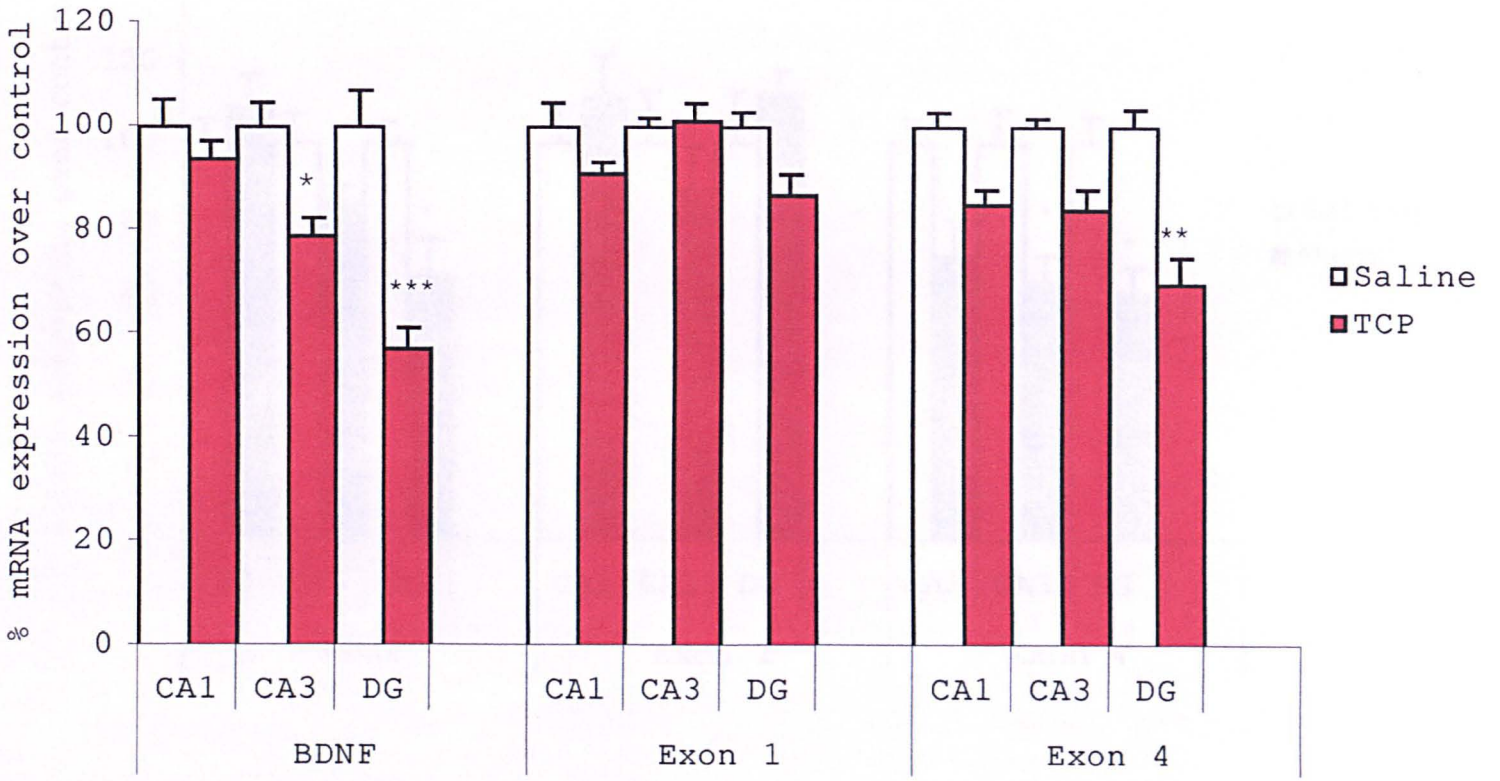


Figure 3.9. **Effect of acute tranylcypromine (5mg/kg i.p) treatment on total BDNF mRNA, exon I mRNA and exon IV mRNA expression in the rat hippocampus, 4h after injection.** Data presented as percentage of control **p<0.001, p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

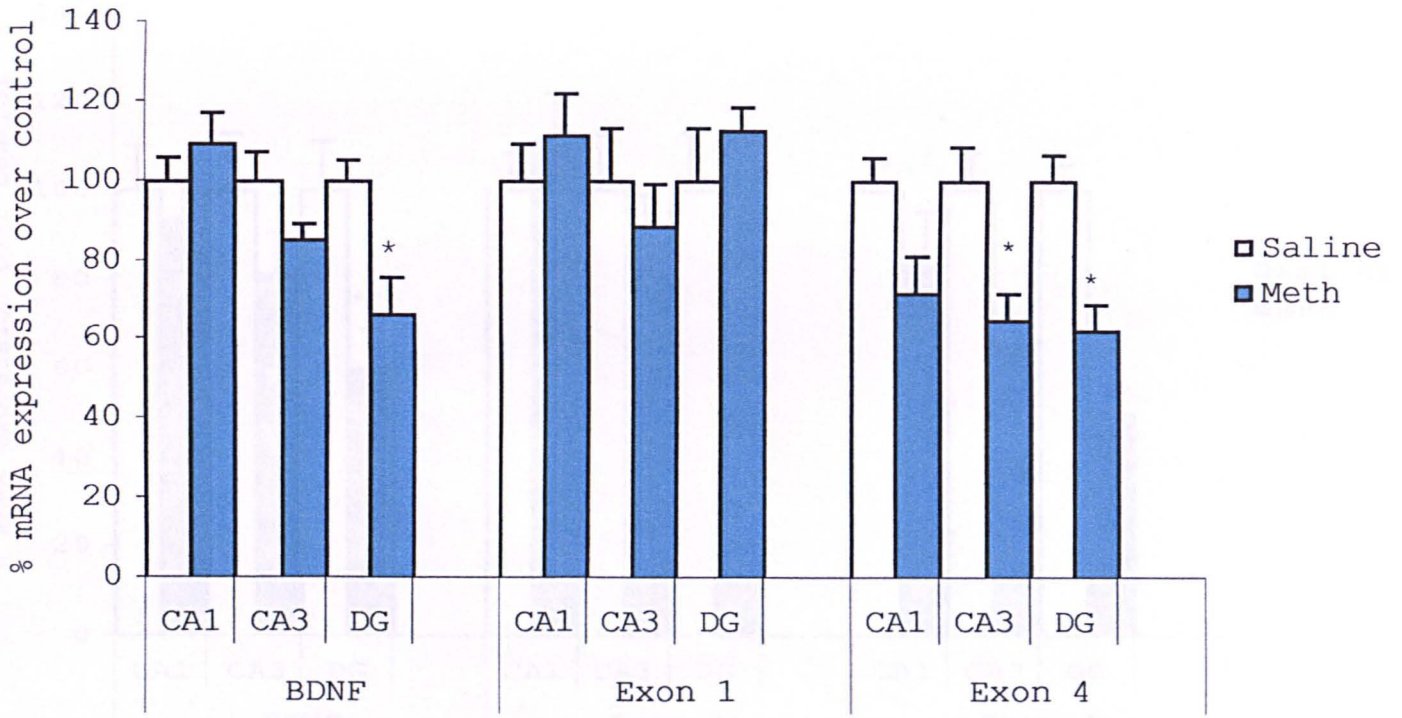


Figure 3.10. **Effect of acute methylphenidate (4mg/kg i.p) treatment on total BDNF mRNA, exon I mRNA and exon IV mRNA expression in the rat hippocampus, 4h after injection.** Data presented as percentage of control **p<0.001, p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

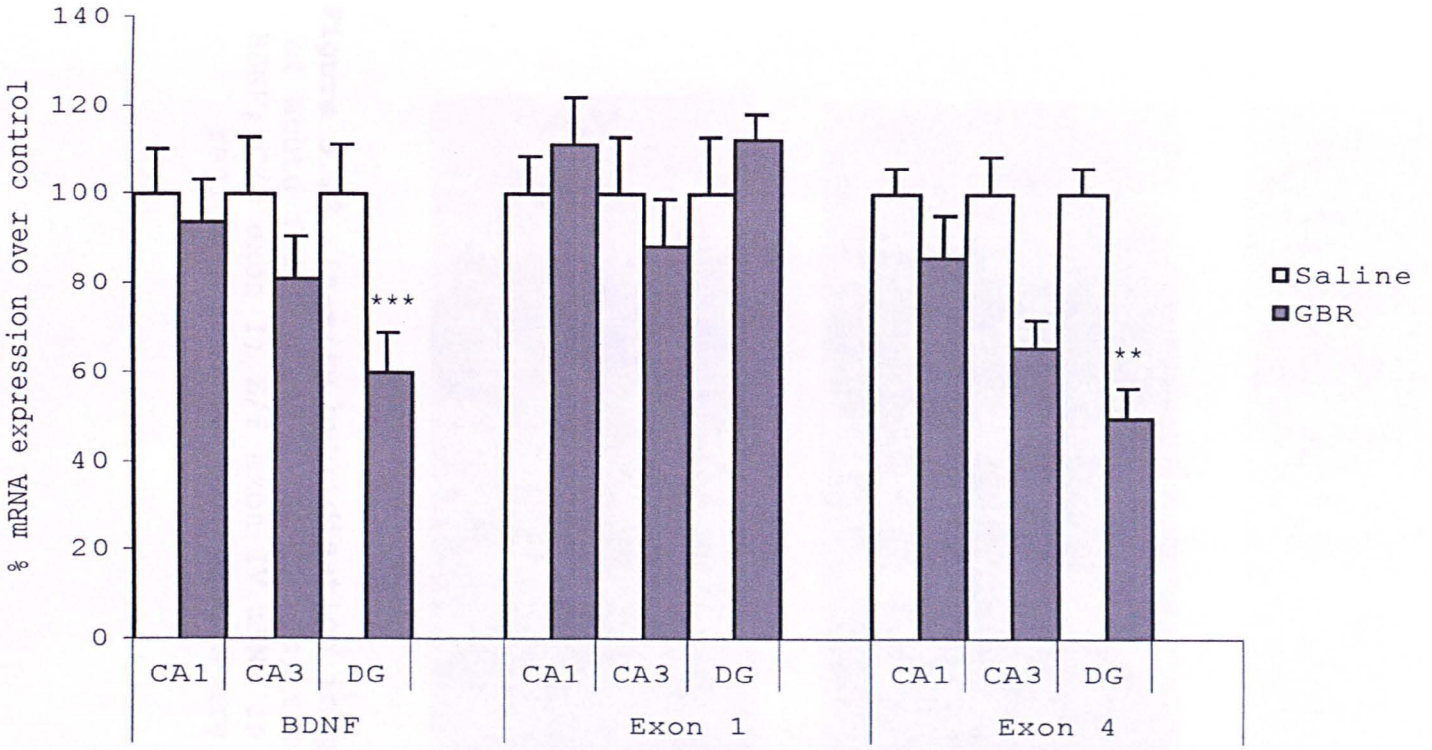


Figure 3.11. **Effect of acute GBR 12909 (10mg/kg i.p) treatment on total BDNF mRNA, BDNF exon I mRNA and BDNF exon IV mRNA expression in the rat hippocampus, 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test

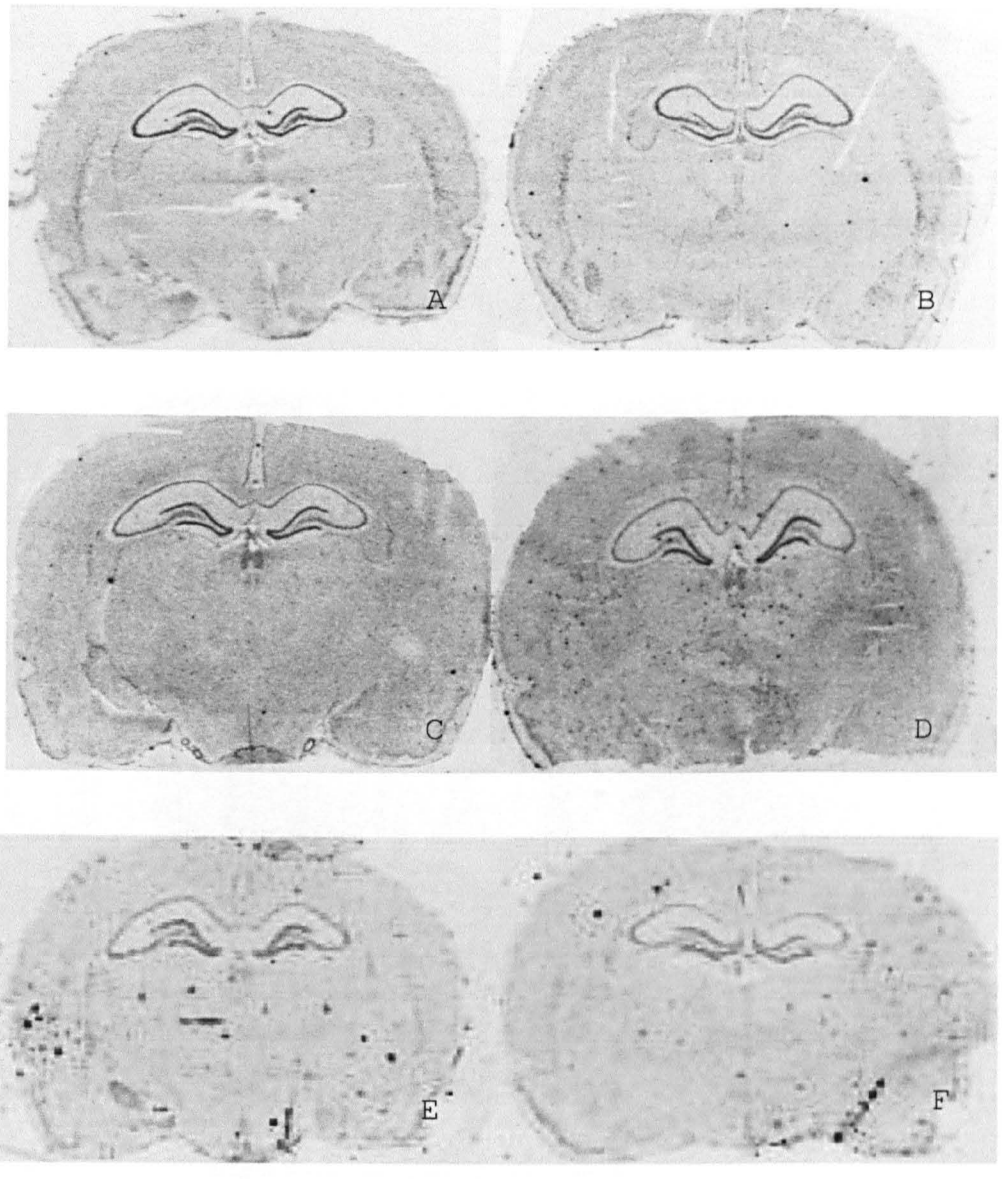


Figure 3.12. *In-situ* hybridisation images showing the effect of acute fluoxetine (10mg/kg i.p) treatment on A/B total BDNF; C/D exon I; E/F exon IV mRNA in the rat hippocampal region at 4h. Control groups are situated left.

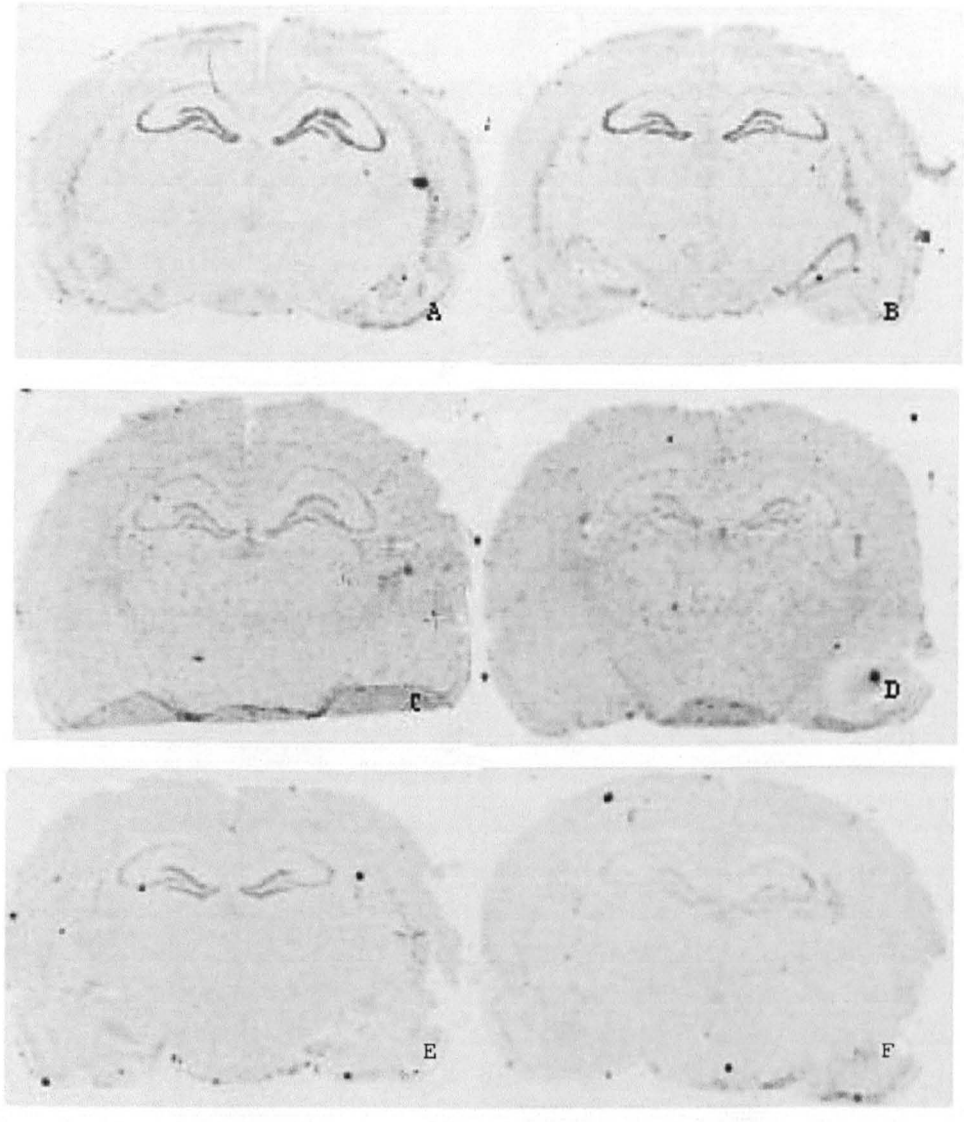


Figure 3.13. *In-situ* hybridisation images showing the effect of acute paroxetine (5mg/kg i.p) treatment on A/B total BDNF; C/D exon I; E/F exon IV mRNA in the rat hippocampal region at 4h. Control groups are situated left.

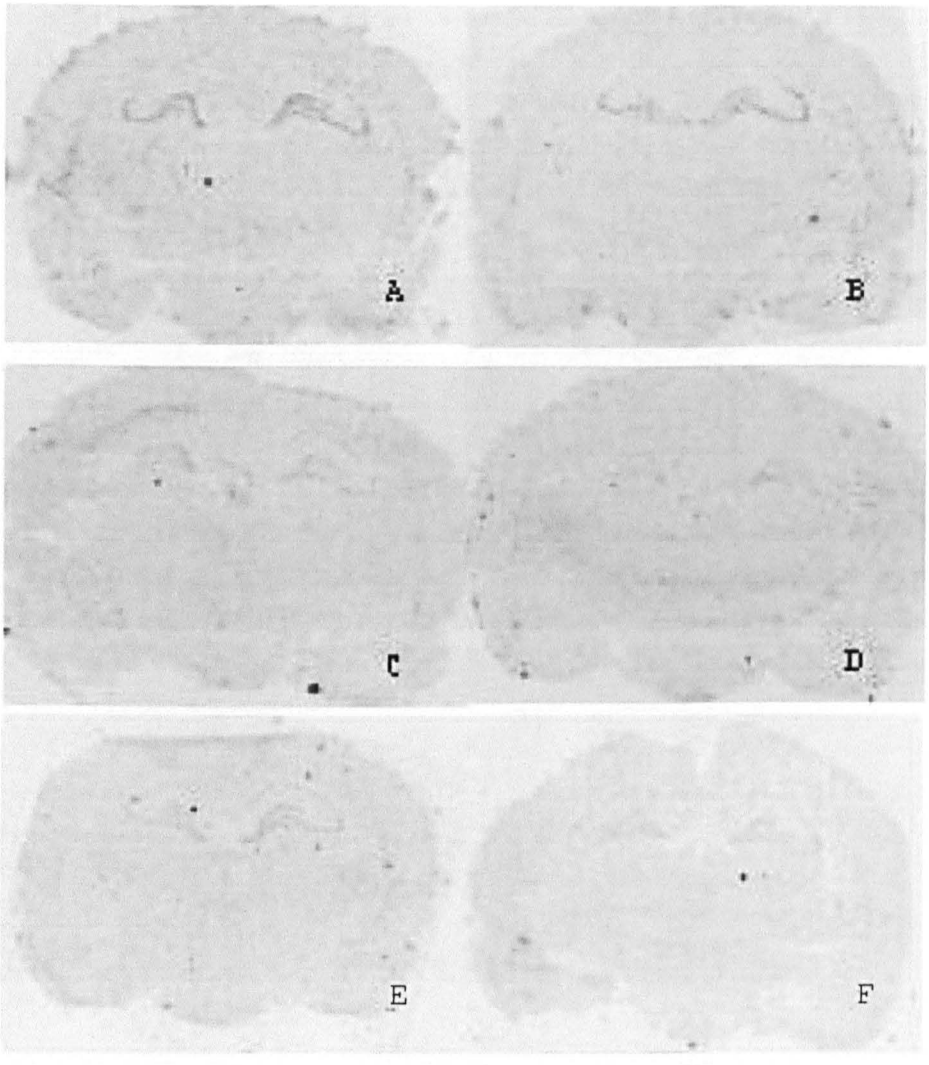


Figure 3.14. *In-situ* hybridisation images showing the effect of acute citalopram (10mg/kg i.p) treatment on A/B total BDNF; C/D exon I; E/F exon IV mRNA in the rat hippocampal region at 4h. Control groups are situated left.

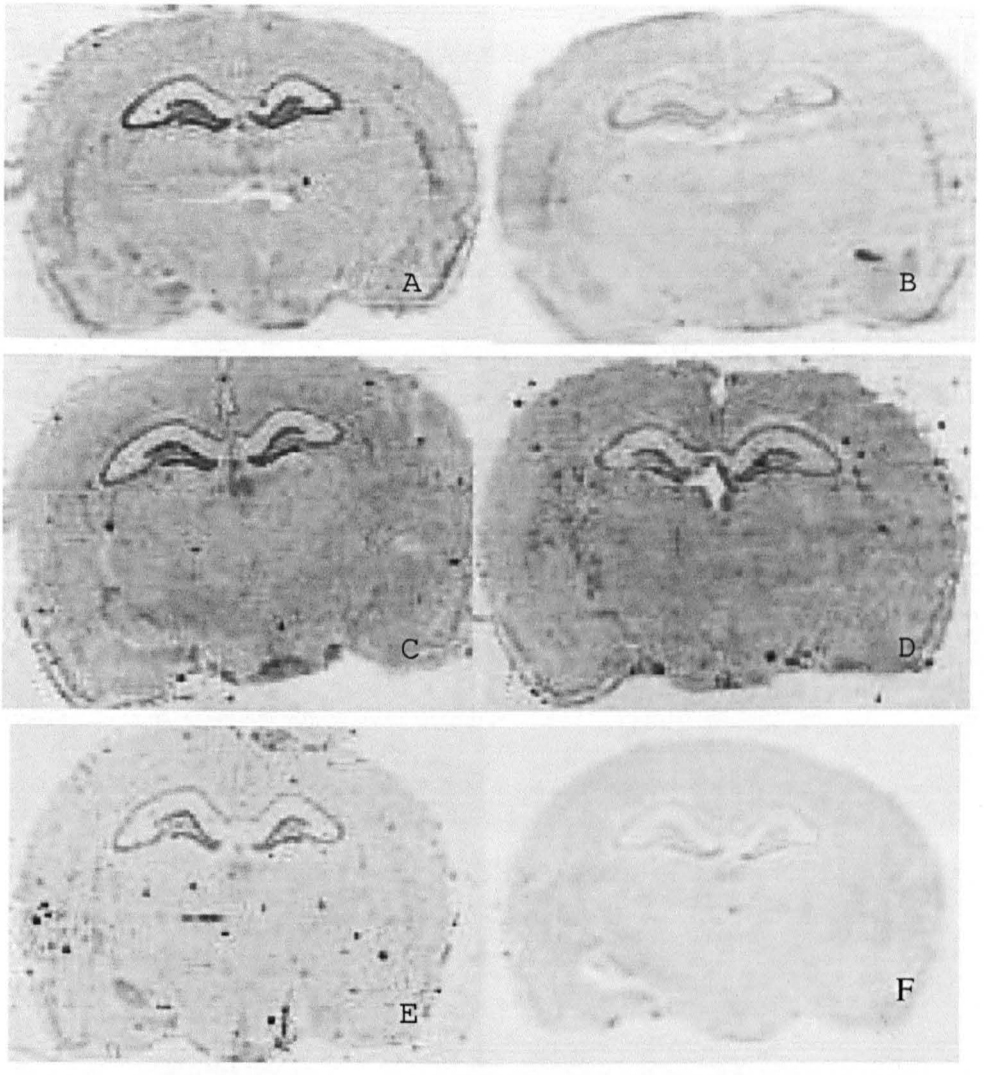


Figure 3.15. *In-situ* hybridisation images showing the effect of acute desipramine (10mg/kg i.p) treatment on A/B total BDNF; C/D exon I; E/F exon IV mRNA in the rat hippocampal region at 4h. Control groups are situated left.

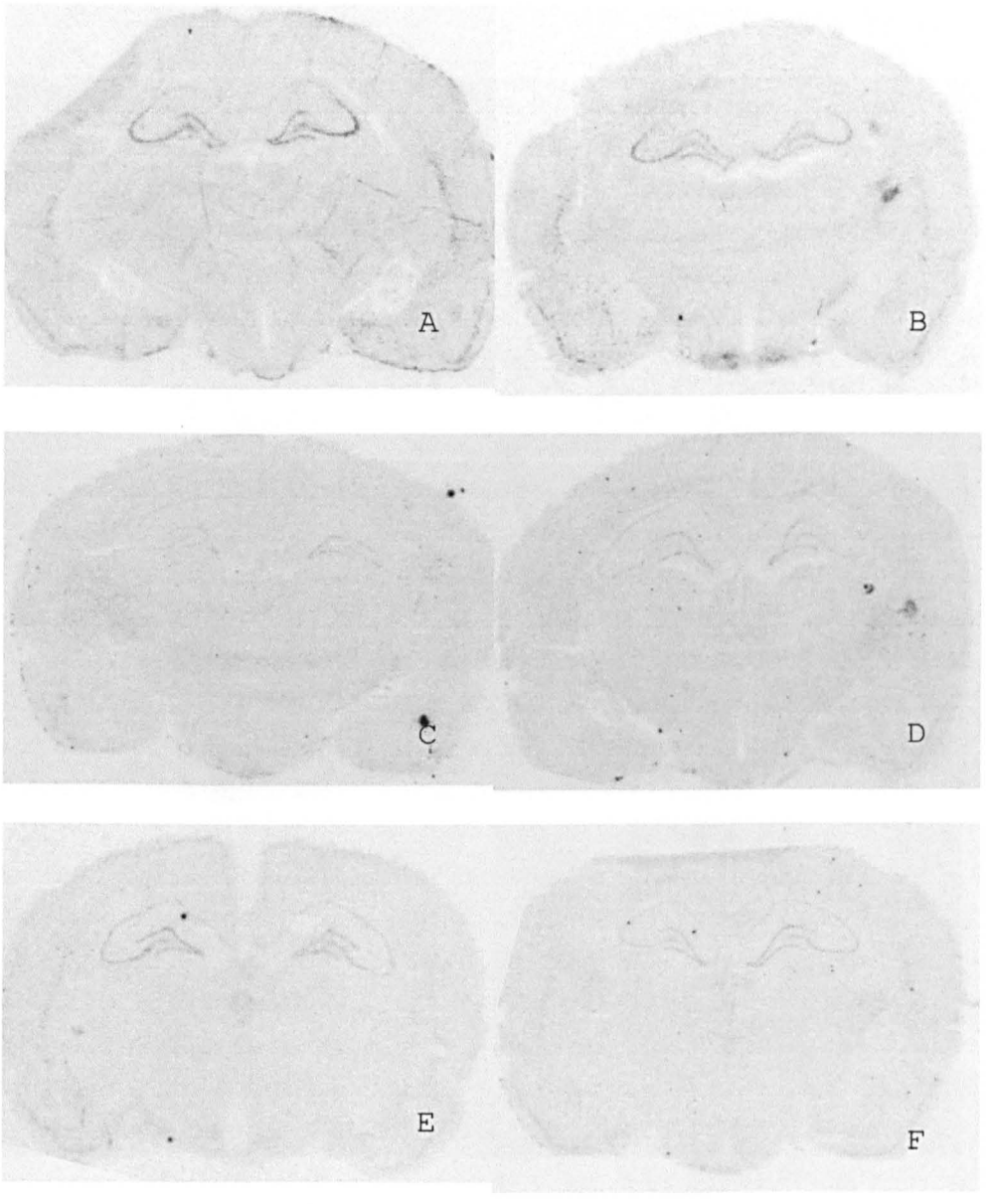


Figure 3.16. In situ hybridisation images showing the effect of acute maprotiline (10mg/kg i.p) treatment on A/B total BDNF; C/D BDNF exon I mRNA and E/F exon IV mRNA in the rat hippocampal region at 4h. Control groups are situated left.

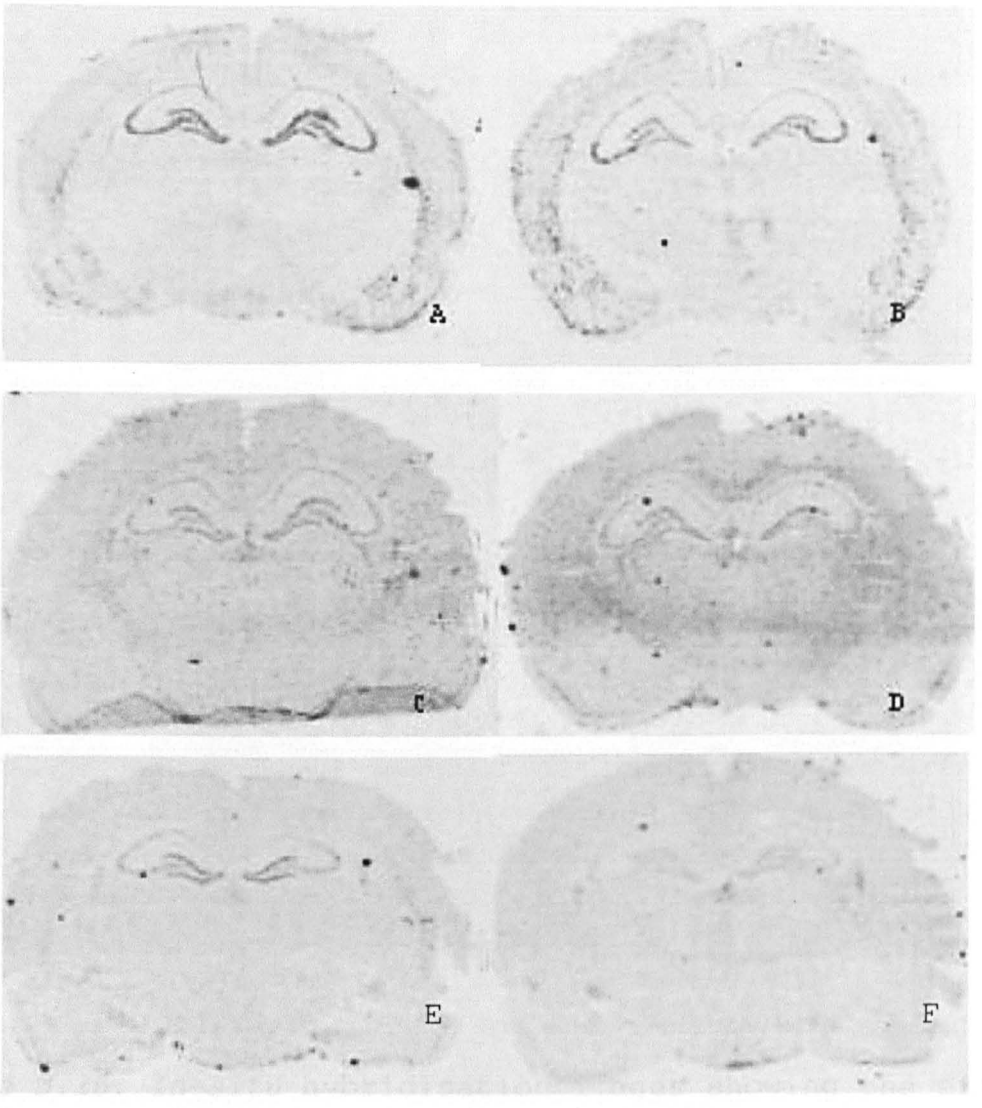


Figure 3.17. *In-situ* hybridisation images showing the effect of acute tranylcypromine (5mg/kg i.p) treatment on A/B total BDNF; C/D exon I and E/F exon IV mRNA in the rat hippocampal region at 4h. Control groups are situated left.

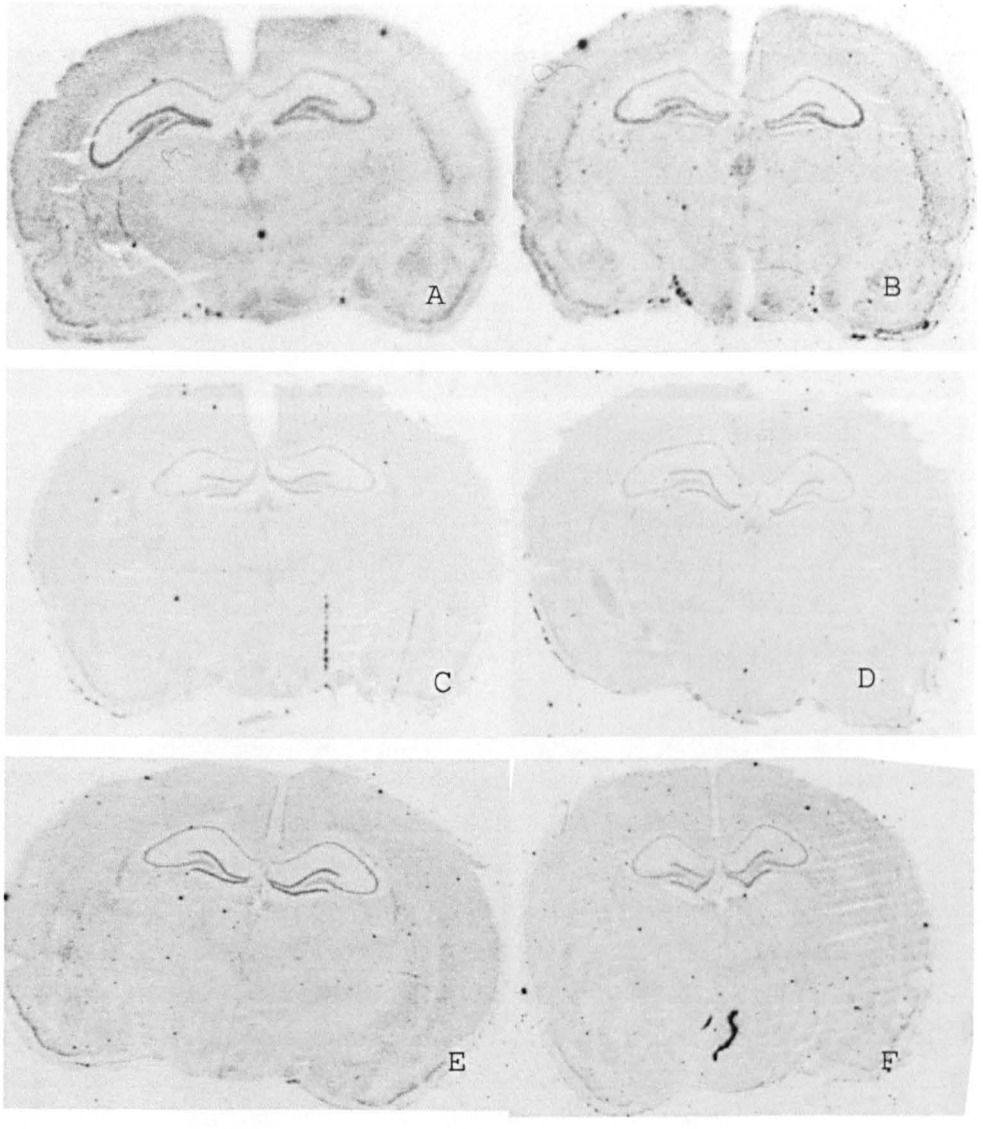


Figure 3.18. *In-situ* hybridisation images showing the effect of acute methylphenidate (4mg/kg i.p) treatment on A/B total BDNF; C/D exon I mRNA and E/F exon IV mRNA in the rat hippocampal region at 4h. Control groups are situated left.

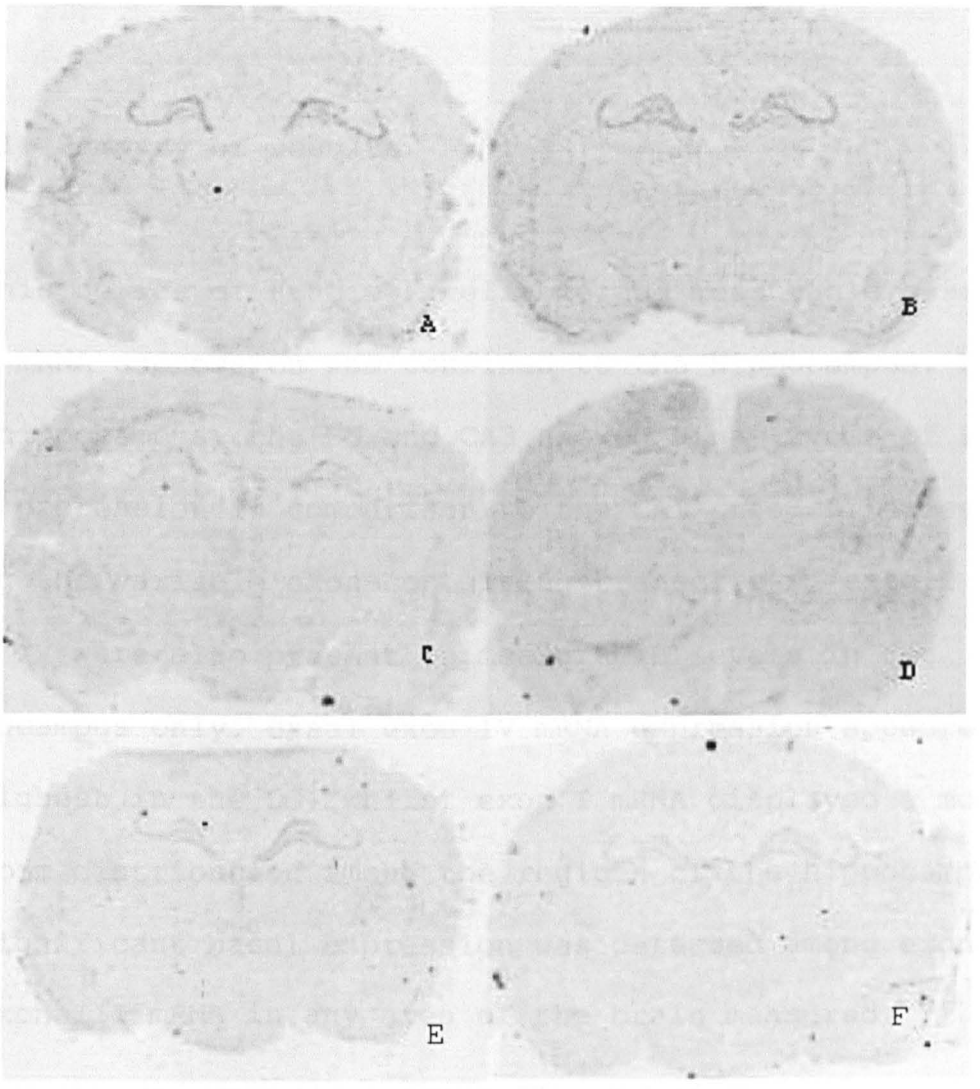


Figure 3.19. *In-situ* hybridisation images showing the effect of acute GBR 12909 (10mg/kg i.p) treatment on A/B total BDNF; C/D exon I mRNA and E/F exon IV mRNA in the rat hippocampal region at 4h. Control groups are situated left.

3.4. Discussion

3.4.1. Summary of results

In this series of studies, reliable and measurable basal BDNF mRNA expression was confined to the hippocampus. Within the hippocampus, the DG and CA3 showed high levels of BDNF mRNA expression in comparison to the CA1 area. Likewise, among the variable exon-containing transcripts, exon I and exon IV were also present in measurable levels in the hippocampus only. Basal exon IV mRNA expression appeared to be highest in the DG, whilst exon I mRNA displayed a more uniform distribution among the regions of the hippocampus. No significant basal expression was detected among exon II or exon III mRNA in any area of the brain measured.

This chapter aimed to examine the effect of a number of antidepressant drugs, when given acutely, on total BDNF mRNA, as well as mRNAs corresponding to the variable BDNF exons I and IV expression in the rat hippocampus at 4h. The antidepressant drugs used comprised of the SSRIs fluoxetine, paroxetine and sertraline; the noradrenergic TCA desipramine; the noradrenergic tetracyclic compound maprotiline and the non-selective MAOI tranylcypromine. In

addition for comparison, two compounds with varying affinities for dopamine reuptake blockade, the non-selective NA/DA reuptake blocker methylphenidate and the selective DA transporter blocker GBR 12909 were assessed. All compounds assessed resulted in a significant inhibition in total BDNF mRNA levels in the DG region of the hippocampus, paroxetine and TCP also down-regulated BDNF mRNA levels in the CA3. Additionally, all compounds assessed significantly inhibited BDNF exon IV mRNA in the DG acute citalopram, maprotiline, methylphenidate and GBR 12909 also down-regulated CA3 levels. However, all compounds assessed had no significant effect on exon I mRNA expression in any area of the hippocampus.

3.4.2. Basal distribution of total BDNF mRNA

The present study showed that basal levels were found to exist in a measurable quantity in the hippocampal region only. Within the hippocampus, the highest levels were present in the CA3 and DG regions, with lower levels present in the CA1. Signals were detected in the piriform and cingulate cortices, however they were generally low and immeasurable. A signal was also detected in the parietal cortex, this signal was very weak and there were no

difference in expression through the different layers of this region. No labelling was present in the striatum. The lack of mRNA expression against background levels in areas other than the hippocampus may have led to inaccurate analysis, thus the study focussed on hippocampal areas.

Previous studies have also demonstrated the highest basal incidence of total BDNF mRNA in hippocampal areas (Ernfors et al, 1990; Hofer et al, 1990; Phillips et al, 1990; Schmidt-Kastner et al, 1996). Additionally, in common with this study, differential BDNF mRNA expression has been found within the hippocampus. High levels were recorded in the CA3 band as well as the DG, with low to moderate levels in the CA1 (Hofer et al, 1990; Phillips et al, 1990; Timmusk et al, 1993; Schmidt-Kastner et al, 1996). However, in contrast to our study, significant, measurable levels have also been found in cerebral and piriform cortices in other *in-situ* hybridisation studies (Hofer et al, 1990; Timmusk et al, 1993).

A possible reason for the differences in density between the current and previous studies may have been due to the type of radioactive probes used in the *in-situ* hybridisation experiments. The present study used S^{35}-labelled

oligonucleotides, while other studies (Timmusk et al, 1993; Schmidt-Kastner et al, 1996) have used P^{32} riboprobes, as opposed to DNA probes used in the present study. Riboprobes are generally more selective and therefore more sensitive, and possess the ability to detect very low levels of basal mRNA (Braissant and Wahli, 1998).

In accordance with previous studies a significant level of basal exon I transcript expression was detected in the hippocampus (Bishop et al, 1994; Berchtold et al, 1999; Russo-Neustadt et al, 2000). However, whilst the present study found relatively little difference between the various hippocampal sub-regions a previous study (Russo-Neustadt et al, 2000) reported a somewhat lower density in the CA1 compared to the CA3 or DG. Generally, basal exon IV mRNA expression appeared to be less abundant than basal exon I mRNA and was mainly restricted to the DG region of the hippocampus. Exon IV has been shown to be present in the brain, with high levels present in the hippocampus (Timmusk et al, 1993; Bishop et al, 1994). Previous studies have found a similar pattern of expression of exon IV mRNA to this study within the hippocampus, with expression being predominant in the DG granular cell layer (Timmusk et al, 1993; Bishop et al, 1994).

In contrast to some previous studies (Timmusk et al, 1993; Lauterborn et al, 1998; Russo-Neustadt et al, 2000), the oligonucleotide probes used in our experiments failed to detect significant basal exon II or exon III mRNA signal in any area of the brain. No signal was detected in any experiment using two different probes with sequences corresponding to exon II mRNA. In contrast to exon II mRNA, a strong exon III mRNA signal was detected in hippocampal areas after strong stimuli (acute ECS), indicating that the exon III probe could hybridise. However, the basal expression was deemed too weak against background levels to ensure accuracy. The reasons behind the lack of basal exon II and exon III signal are unclear, however earlier studies mainly used more sensitive riboprobes in favour of DNA probes used here. Therefore a weaker signal, such as that found with exon III mRNA in the present study, may have been more easily detected in earlier studies.

3.4.3. The effect of acute administration of antidepressant drugs on BDNF mRNA in the hippocampus at 4h

The individual acute administration of a variety of antidepressant drugs, as well as two centrally-acting

stimulants: the selective DA transporter GBR 12909 and the non-selective NA/DA reuptake blocker methylphenidate, all elicited a significant inhibition of BDNF mRNA in the DG area of the hippocampus at 4h. In addition, acute paroxetine or TCP treatment also caused a significant inhibition in BDNF mRNA levels in the CA3 region. No significant changes were seen in the CA1, after acute administration of any of the compounds tested.

A previous study using similar ISH techniques, doses and experimental procedure (Coppell et al, 2003), found similar reductions in BDNF mRNA from acute SSRI (fluoxetine at 10mg/kg, paroxetine at 5mg/kg and sertraline at 10mg/kg) and MAOI (TCP; 5mg/kg) treatment in the DG at 4h. However, in contrast to the present study, no changes in BDNF mRNA were seen after acute administration of the selective NARI desipramine (Coppell et al, 2003). As acute desipramine elicited a significant response in BDNF mRNA in the present study, a further NA-selective compound was tested. Acute maprotiline administration also resulted in a reduction in BDNF mRNA at 4h in the DG region. The results in the present study therefore appear to contradict the assertion that acute inhibitory response is predominantly a 5-HT-mediated

mechanism (Coppell et al, 2003) and imply an equivocal role for NA.

Acute administration of the MAOI TCP resulted in the acute down-regulation of BDNF mRNA in the DG and CA3 in both studies (the largest reduction in DG levels of any antidepressant tested in our study). TCP is non-selective among MAO isoenzymes, therefore it has effects on type B MAO, which deaminates DA (Murphy et al, 1987). TCP has been demonstrated to cause a significant decrease in DA metabolism (Dyck et al, 1993; Martin et al, 1995). This suggested a possible role for DA-related mechanisms in the acute inhibition in BDNF mRNA. Thus, two centrally-acting DA stimulants, the mixed DA/NA reuptake blocker methylphenidate and the selective DA transporter blocker GBR 12909, were administered acutely. A single treatment with either drug resulted in the inhibition of BDNF mRNA levels of a similar magnitude to the antidepressants tested in the DG at 4h. This implicates a role for DA in the acute regulation of BDNF mRNA expression in the DG region of the hippocampus. In addition, as both methylphenidate and GBR 12909 have a negligible effect on serotonergic transmission, suggesting that the acute inhibitory BDNF response may occur independently of 5-HT.

Studies relating to the effect of acute DA receptor blockade on BDNF mRNA have revealed interesting findings. Acute administration of the unselective DA antagonist haloperidol has been shown to lead to a reduction in hippocampal BDNF mRNA (Lipska et al, 2001; Meredith et al, 2004). D_2 receptors are linked to cAMP via G_i proteins and are inhibitory on consequential cAMP-mediated signalling. Therefore, if located on DA neurones, they will cause a reduction in DA release (Missale et al, 1998; Meredith et al, 2004). Thus blockade of hippocampal D_2 autoreceptors by haloperidol may result in a similar effect on extracellular DA concentration as a blockade on the DA transporter by GBR 12909 and methylphenidate.

Overall, the results in the present study indicate that acute inhibition in BDNF mRNA in the DG is a non-selective phenomenon that may be induced by general facilitation of monoamine NA, 5-HT or possibly DA transmission.

3.4.4. The effect of acute administration of antidepressant drugs on BDNF exon transcript mRNA in the hippocampus at 4h

Acute administration of various types of antidepressant drug, as well as the NA/DA reuptake blocker methylphenidate

and the selective DA transporter blocker GBR 12909 had a variable effect on mRNA for specific BDNF exon transcripts at 4h. In accordance with the effect on total (exon V) BDNF mRNA, exon IV mRNA was inhibited after all antidepressants tested acutely, as well as methylphenidate and GBR 12909. Additionally, acute methylphenidate or GBR 12909, or citalopram or maprotiline, also significantly reduced CA3 exon IV mRNA levels at 4h.

As mentioned earlier, exons III and IV have been shown to share properties with IEGs (Lauterborn et al, 1996; 1998). This implies that acute alterations in expression may occur in exon IV mRNA without intervening protein synthesis-dependent mechanisms. It is thus feasible that changes could occur in the expression within the short space of time post-injection (4h) in this series of experiments. In contrast, the acute administration of the various antidepressants, as well as methylphenidate and GBR 12909, had no effect on exon I mRNA at 4h in any area of the hippocampus. Changes in exon I and II transcription have been demonstrated to protein synthesis-dependent (Lauterborn et al, 1996; 1998) and display a delayed induction process (Kokaia et al, 1994). This implies that the time period post-injection may have

been insufficient for significant changes in exon I mRNA to occur.

Net BDNF expression in the brain depends on the cumulative activities of its multiple alternative exon-containing transcripts (Timmusk et al, 1993; Nakayama et al, 1994; Bishop et al, 1997). Therefore, the inhibition in exon IV-containing transcripts may account in part for the net inhibition in BDNF mRNA in the DG. However, the influence of exon IV mRNA inhibition in the CA3 area appears more complex. Whilst total BDNF mRNA was significantly inhibited in the CA3 after acute paroxetine and TCP, exon IV mRNA was significantly inhibited in the CA3 by citalopram, maprotiline as well as methylphenidate and GBR 12909 at 4h. However, there was a general inhibitory trend on both total BDNF and exon IV transcripts in the CA3 and DG. The mixed pharmacological nature of the compounds which inhibited exon IV as well as total BDNF transcription, suggests that the inhibitory response in the CA3 and DG areas is not solely specific to either 5-HT or NA manipulations, but a more general response, possibly also involving DA.

A recently published BDNF exon study (Dias et al, 2003), has found fairly conflicting findings to the results presented

here. A reduction in exon IV mRNA was recorded in the CA1 area after acute fluoxetine treatment, however acute desipramine and TCP treatment did not influence exon IV mRNA, no drug affected CA3 or DG mRNA expression. Variation in drug doses between the experiments could have accounted for this lack of effect. The post-injection time period (2h) was also half the length of that in our experiment. It may be possible that the time period in this experiment is too short for the threshold for a change in exon IV mRNA expression to occur. A further finding in the Dias et al (2003) study appears to contradict earlier reports (Lauterborn et al, 1996; 1998). Dias et al (2003), found that exon II mRNA was inhibited after acute desipramine and TCP. Changes in exon II mRNA have been shown to be protein synthesis-dependent. This would imply that a prolonged period of time would be required for proceeding activation of further responsive genes (Clayton, 2000). As our study failed to find any significant basal signal using exon II mRNA-specific probes this inhibitory effect cannot be confirmed, though the lack of effect on exon I mRNA from acute treatment of the various compounds tested appears to support earlier findings (Lauterborn et al, 1996; 1998).

A further recently published study RT-PCR study (Altieri et al, 2004) however appears to support the inhibitory effect seen on exon IV mRNA in the hippocampus after acute antidepressant treatment as seen in the present study. The group found similar significant reductions in overall hippocampal exon IV, but not exon I, II or III mRNA after acute fluoxetine (5mg/kg) administration at 8h.

In conclusion, it was found in the present study that the acute administration of the antidepressants fluoxetine, paroxetine, citalopram, desipramine, maprotiline or TCP, or the mixed NA/DA reuptake blocker methylphenidate or the selective DA transporter blocker GBR 12909, all resulted in the significant inhibition in total BDNF mRNA in the DG area of the hippocampus. The inhibitory effect on total BDNF mRNA in the DG appeared to be due in part to the inhibition of BDNF exon IV mRNA, which was significantly down-regulated 4h in the same area after acute fluoxetine, paroxetine, citalopram, desipramine, maprotiline or TCP administration. In addition, acute paroxetine or acute TCP significantly reduced CA3 total BDNF mRNA levels. Exon IV mRNA expression was significantly inhibited in the CA3 by acute citalopram, methylphenidate or GBR 12909. In contrast, no change was seen in exon I mRNA after acute administration of the

various antidepressants tested in any area of the hippocampus at 4h.

The next chapter aims to elucidate the mechanisms behind the apparent acute inhibition in BDNF mRNA in the adult rat hippocampus.

Chapter Four

Involvement of GABA Receptor Activation in the Acute Down-Regulation of BDNF mRNA Expression in the Rat Hippocampus

4.1. Introduction

Chapter three outlined the acute effects of several antidepressant compounds on the BDNF gene in the rat hippocampus. It was discovered that acute administration of several classes of antidepressants, as well as the selective DA transporter blocker GBR 12909 or the NA/DA reuptake blocker methylphenidate, inhibited BDNF mRNA in the DG (as well as in the CA3 after paroxetine or TCP treatment) at 4h. This net reduction in the expression for the whole gene appeared to be due in part to selective inhibition in variable exon IV-containing transcript expression. The reasons behind the acute inhibition in total BDNF mRNA and corresponding individual exon mRNA expression in the hippocampus remain erroneous.

It has been suggested that the initial transient inhibitory effect on BDNF mRNA as a result of acute antidepressant administration may be due to the presence of GABA interneurone architecture in the polymorphic layers of the hippocampus (Vaidya et al, 1997; Zetterström et al, 1999; Vaidya and Duman, 2001). Electrophysiological studies have revealed the presence of excitatory 5-HT receptors (i.e. 5-HT_2, 5-HT_4, 5-HT_6, 5-HT_7), in high levels on hippocampal

interneurones (Freund et al, 1990; Piguet and Galvan, 1994). The acute activation of these receptors is thought to facilitate spontaneous GABA release and increase inhibitory control over the hippocampal cells (Piguet and Galvan, 1994). GABA has been shown to inhibit BDNF mRNA levels (Zafra et al, 1991; Marmigere et al, 2003). Thus, an increase in GABA release as a result of activation of 5-HT receptors present on interneurones may underlie the acute inhibitory effect of antidepressant drugs on BDNF mRNA.

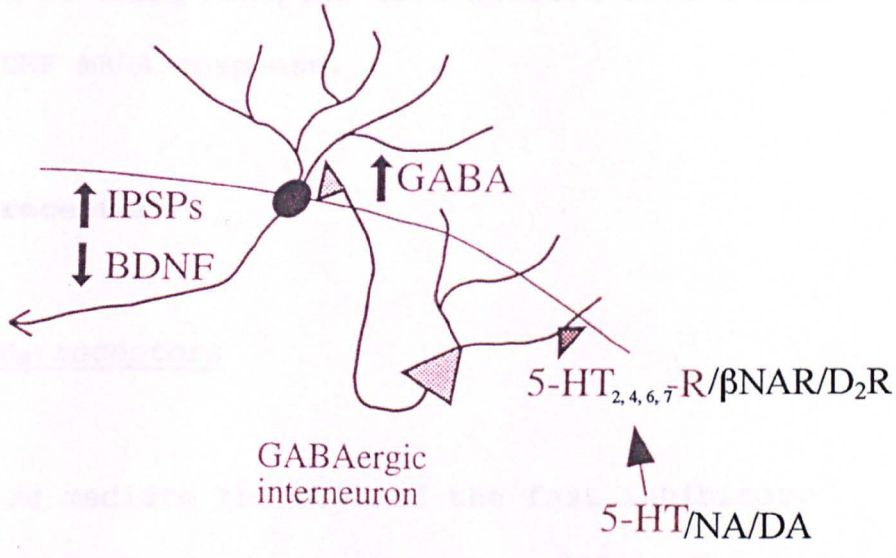


Figure 4.1. Possible mechanism of monoamine-mediated inhibition of BDNF MRNA in the DG region of the hippocampus. Activation of excitatory monoamine receptors increases the firing rate of local GABAergic inteneurones and thereby increases IPSPs in the granule cells. Thus increased inhibitory control over BDNF mRNA (Adapted from Vaidya et al, 1997).

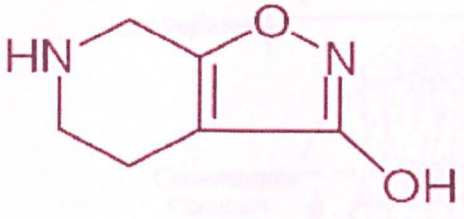
In order to elaborate on the inhibitory effect of antidepressant drugs on BDNF mRNA in the hippocampus, the effect of selective GABA receptor stimulation was studied. In this chapter the effect of three GABAergic compounds: the $GABA_A$ receptor agonist 4, 5, 6, 7-tetrahydroisoxazolo{5-4-C]pyridin-3-0 (THIP), the benzodiazepine flunitrazepam and the $GABA_B$ agonist baclofen were assessed. In addition, the actions of two $GABA_B$ antagonists: CGP 55845 and CGP 46381 was studied, both independently and in conjunction with the SSRI paroxetine or baclofen in order to establish the influence of $GABA_B$ receptor transmission in the acute inhibitory BDNF mRNA response.

4.1.1. GABA receptors

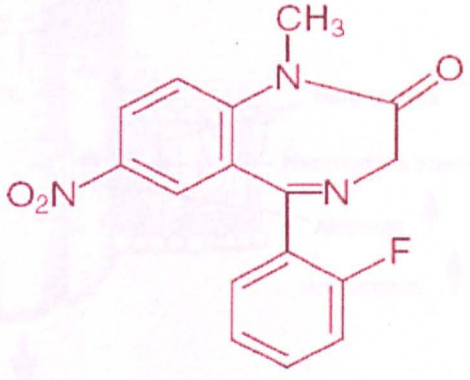
4.1.1.1. $GABA_A$ receptors

$GABA_A$ receptors mediate the bulk of the fast inhibitory neurotransmission in the brain (Bormann, 2000). They are classified $GABA_{A1}$ to $GABA_{A6}$ based on subunit structure and receptor function (Barnard et al, 1998). The receptor subtypes are formed from different combinations of subunits and subtype which vary in different brain cells and areas. Changes in subunit expression may occur in response to drug

treatment, such as with alcohol (Mhatre and Ticku, 1993; Grobin et al, 1998) or benzodiazepines (Holt et al, 1996; Impagnatiello et al, 1996). The receptor is ligand-gated with a heteropentameric structure and integral anion channel. THIP is a potent $GABA_A$ activator and binds tightly to the $GABA_A$ receptor (Krogsgaard-Larsen et al, 1977; 1979) It is capable of activating (with differing potencies) a broad range of $GABA_A$ receptor subunit configurations (Ebert et al, 1994).



THIP



Flunitrazepam

Figure 4.2. Structures of selected $GABA_A$-related compounds: the $GABA_A$ agonist THIP and the benzodiazepine agonist flunitrazepam

The $GABA_A$ receptor also contains a benzodiazepine-binding site, which when activated increases the affinity for GABA to bind with the $GABA_A$ receptor (Korpi et al, 2002). Flunitrazepam is a full benzodiazepine agonist, which

induces its effects by increasing the opening frequency of the integral $GABA_A$ Cl^- receptor channel (Korpi et al, 2002; Davies and Alkana, 2003)

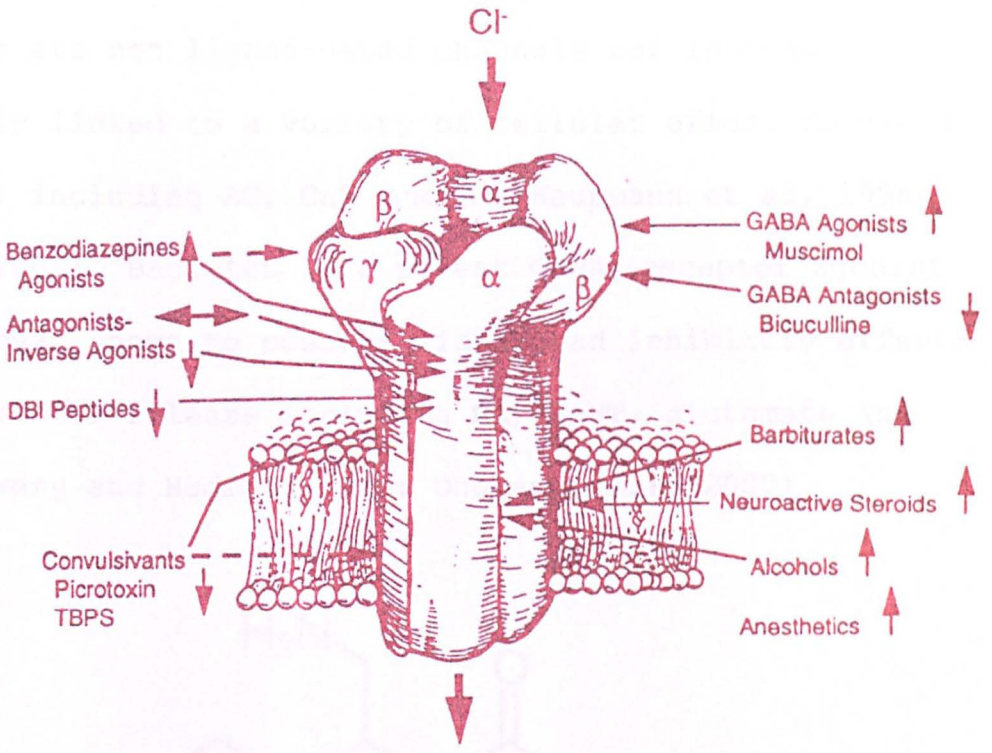


Figure 4.3. Schematic illustration of the $GABA_A$ receptor structure containing two α and β subunits and a single γ subunit to form an intrinsic Cl- ion channel. Putative ligands and drugs are known to interact at one of the major sites associated with the $GABA_A$ receptor and to either positively or negatively modulate GABA-gated Cl- ion conductance are also illustrated (Adapted from Cooper et al, 1996)

4.1.1.2. GABA$_B$ receptors

The GABA$_B$ receptor is distributed widely throughout the rodent and human CNS, including the hippocampus (Bowery et al, 1987; Chu et al, 1990). In contrast to GABA$_A$, GABA$_B$ receptors are not ligand-gated channels but instead are negatively linked to a variety of cellular effectors via G_i proteins, including AC, Ca^{2+} and K^+; Kaupmann et al, 1998; Bowery, 2002). Baclofen is a potent GABA$_B$ receptor agonist and has been shown to possess widespread inhibitory effects on transmitter release including NA, 5-HT, glutamate and GABA (Bowery and Hudson, 1979; Ong and Kerr, 2000).

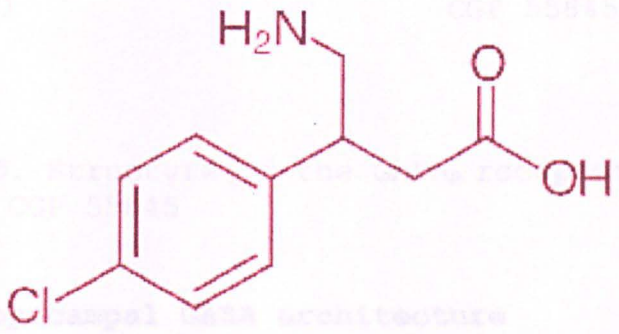
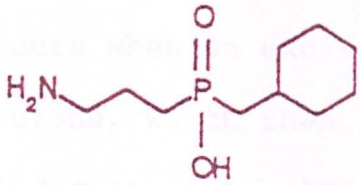
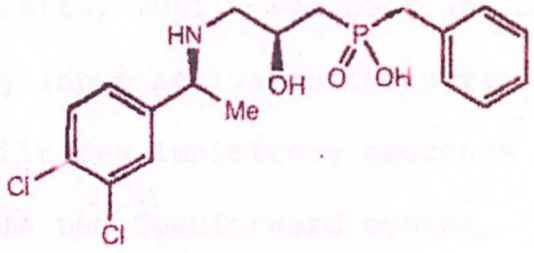


Figure 4.4. Structure of the GABA$_B$ receptor agonist baclofen

The two antagonist compounds used in this study, CGP 55845 and CGP 46381, are both potent to the $GABA_B$ receptor. CGP 46381 is highly brain penetrant and has been demonstrated to block late inhibitory postsynaptic potentials (IPSPs) *in vivo* and enhance cAMP production (Lingenhoehl and Olpe, 1993). CGP 55845 is less penetrant than CGP 46381, but has been demonstrated to be a potent antagonist of hippocampal $GABA_B$ receptors in the hippocampus (Davies et al, 1993).



CGP 46381



CGP 55845

Figure 4.5. Structure of the $GABA_B$ receptor antagonists CGP 46381 and CGP 55845

4.1.2. Hippocampal GABA architecture

GABA interneurones form a core component to the cortico-limbic circuitry and have been identified using Golgi staining and more recently through cytochemical and electrophysiological techniques (Freund and Buzsaki, 1996). These cells provide both inhibitory and dis-inhibitory

modulation of cortical and hippocampal circuits and discriminate sensory information within cortico-limbic areas (Benes and Beretta, 2001).

4.1.2.1. Function of hippocampal GABAergic neurones

Hippocampal electrophysiological studies have revealed that the action of GABA is typically inhibitory (Krnjevic, 1981; Ben-Ari, 1981) and involved in feedback or feedforward inhibition (Benes and Beretta, 2001). Feedback inhibition occurs when an excitatory input activates the pyramidal neurone, which then facilitates inhibitory neurones (Andersen et al, 1964). In the feedforward system, pyramidal neurones of the CA3 project to other pyramidal cells in the CA1. These excitatory neurones then feed into the inhibitory neurones of the CA1 causing a reduction in the excitability of pyramidal neurones in the CA1 (Buzsaki, 1984).

The present chapter focuses on the effect of acute administration of three compounds with varying GABA receptor agonistic actions on hippocampal BDNF mRNA, as well as BDNF exon I and exon IV mRNA at 4h. Additionally, in order to confirm the role of the $GABA_B$ receptor

activation in the inhibitory BDNF response after acute antidepressant drug treatment, two $GABA_B$ receptor antagonists were tested, given before acute paroxetine treatment.

4.2. Methods

4.2.1. Administration of acute GABA receptor agonists

Male Sprague-Dawley rats (225-250g) were injected (i.p) once with either saline (1ml/kg), the $GABA_A$ receptor agonist THIP (10mg/kg), the benzodiazepine flunitrazepam (10mg/kg) or the $GABA_B$ receptor agonist baclofen (10mg/kg) in 0.9% saline and observed for behavioural changes for 4h before being sacrificed. Brains were isolated before being placed in cooled isopentane and stored at -70°C until further use. BDNF and various BDNF exon mRNA expression was measured by densitometric analysis as described in *Chapter 2*.

4.2.2. $GABA_B$ antagonist pre-treatment

Male Sprague-Dawley rats (225-250g) were injected (i.p) once with either saline (1ml/kg), CGP 55845 (10mg/kg) or CGP 46381 (10mg/kg) in 0.9% saline and observed for behavioural changes for 30 mins, before being injected (i.p) with either saline (1ml/kg), baclofen (10mg/kg) or paroxetine (5mg/kg). Animals were observed for a further 4h before being sacrificed. Brains were isolated before being frozen in cooled isopentane and stored at -70°C until

further use. BDNF and various BDNF exon mRNAs were measured by densitometric analysis as described in *Chapter 2*.

4.3. Results

4.3.1. The effect of acute administration of GABA receptor compounds on BDNF mRNA and BDNF exon mRNA expression

4.3.1.1. Effect on total BDNF mRNA

The $GABA_A$ receptor agonist THIP failed to produce any significant effect on BDNF mRNA in any area of the hippocampus at 4h. Although CA3 levels were inhibited slightly, mRNA increased in the CA1 and the DG *(Table 4.1; Figures 4.6, 4.9)*. The full benzodiazepine receptor agonist flunitrazepam failed to significantly alter BDNF mRNA, though levels were inhibited slightly in the CA1, CA3 and DG *(Table 4.1; Figures 4.7, 4.10)*. The $GABA_B$ receptor agonist baclofen significantly inhibited BDNF mRNA in the CA3 (-20.9%; $p<0.05$) and DG (-42.6%; $p<0.001$), though not in the CA1 *(Table 4.1; Figures 4.8, 4.11)*.

4.3.1.2. Effect on BDNF exon I mRNA expression

None of the GABA-ergic agonist compounds used had any significant effect on BDNF exon I mRNA in any area of the hippocampus *(Table 4.2; Figures 4.6 to 4.11)*.

4.3.1.3. Effect on BDNF exon IV mRNA expression

The three compounds tested had varying effects on BDNF exon IV mRNA in the rat hippocampus. THIP *(Table 4.3; Figures 4.6, 4.9)* and flunitrazepam *(Table 4.3; Figures 4.7, 4.10)* had no significant effect on exon IV mRNA in any area of the hippocampus. Baclofen however significantly inhibited levels in the DG (-40.1%; $p<0.001$), but not the CA3 or CA1 (*Table 4.3; Figures 4.8, 4.11*).

4.3.2. The effect of $GABA_B$ antagonist pre-treatment on baclofen/paroxetine-mediated down-regulation of BDNF mRNA

4.3.2.1. Effect of acute baclofen and paroxetine administration

When administered without either CGP compound (30 minutes after saline), baclofen produced a significant down-regulation in BDNF mRNA levels in the DG (-48.8%; $p<0.001$) and the CA3 (-35.4%; $p<0.01$) regions of the hippocampus, no significant effect was seen in the CA1 *(Table 4.4; Figures 4.12 and 4.13)*. Acute paroxetine administration also significantly inhibited DG (-25.4%; $p<0.05$) and CA3 (-

27.8%; $p<0.05$) regions, no significant effect was seen in the CA1 *(Table 4.4; Figures 4.12 and 4.13)*.

4.3.2.2. Effect of CGP 55845

CGP 55845 alone (30 minutes after saline) produced a small, non-significant reduction in BDNF mRNA in all areas of the hippocampus. Pre-administration with CGP 55845 significantly attenuated the effect of acute baclofen treatment in the DG region (CGP 55845/baclofen vs. saline/baclofen, 30.7%; $p<0.05$). CA3 levels were also attenuated, though not significantly. However, 55845 failed to attenuate the effect of acute paroxetine treatment in the CA3 and DG *(Table 4.4; Figures 4.12 and 4.13)*.

4.3.2.3. Effect of CGP 46381

CGP 46381 alone (30 minutes after saline) significantly increased BDNF mRNA in the DG (35.8%; $p<0.05$), a smaller non-significant increase was also seen in the CA1. In addition CGP 48381 pre-treatment caused a significant attenuation from baclofen administration (CGP 46381/baclofen vs. saline/baclofen, 31.3%; $p<0.05$) in the DG, with a smaller non-significant attenuation in the CA3.

The acute paroxetine-mediated inhibition of BDNF mRNA was also significantly attenuated by pre-treatment with CGP 46381 in the DG (saline/paroxetine vs. CGP 46381/paroxetine, 25.6%; $p<0.05$) and CA3 (31.0%; $p<0.05$; *Table 4.4; Figures 4.12 and 4.14)*.

***Table 4.1.* Effect of GABA-ergic compounds on BDNF mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

	CA1	CA3	DG
Saline n=6	100±5.3	100±6.2	100±9.1
Flunitrazepam n=5	86.2±8.0	89.2±6.3	92.5±4.3
Saline n=5	100±7.8	100±7.8	100±8.8
THIP n=5	112.2±8.4	84.4±15.5	106.7±9.8
Saline n=6	100±1.8	100±2.4	100±4.6
Baclofen n=6	94.0±3.4	79.1±3.3*	57.4±4.0***

***Table 4.2.* Effect of GABA-ergic compounds on BDNF exon I mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

	CA1	CA3	DG
Vehicle n=6	100±8.8	100±7.6	100±11.0
Flunitrazepam n=5	93.2±8.7	84.0±4.6	81.5±4.8
Saline n=5	100±4.6	100±5.1	100±5.3
THIP n=5	97.3±10.6	101.2±13.0	93.4±6.1
Saline n=6	100±9.8	100±11.4	100±6.8
Baclofen n=6	101.5±4.7	88.3±16.4	90.4±12.7

***Table 4.3.* Effect of GABA-ergic compounds on BDNF exon IV mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

	CA1	CA3	DG
Vehicle n=6	100±5.3	100±6.2	100±9.1
Flunitrazepam n=5	86.2±8.0	89.2±6.3	92.5±4.3
Saline n=5	100±7.8	100±7.8	100±8.8
THIP n=5	112.2±8.4	84.4±15.5	106.7±9.8
Saline n=6	100±3.3	100±4.0	100±2.9
Baclofen n=6	96.0±7.7	80.0±6.3	59.1±3.0**

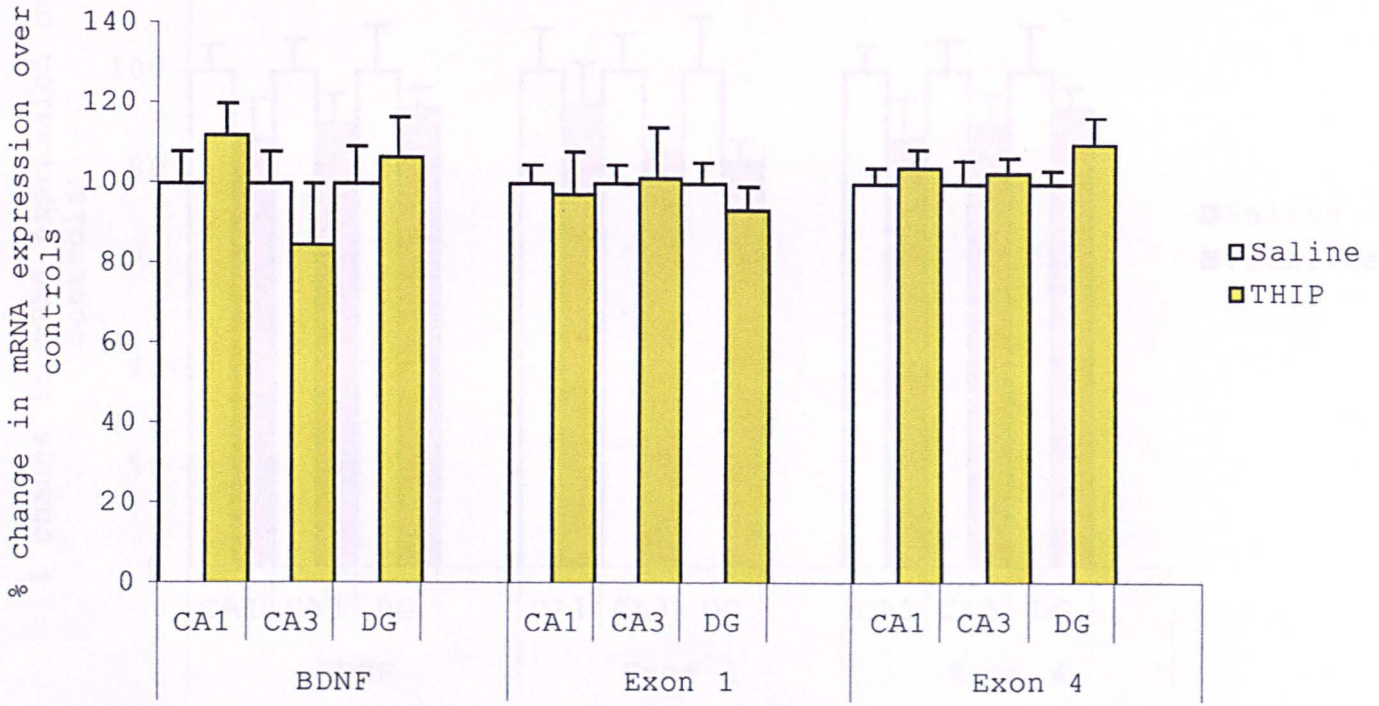


Figure 4.6. **Effect of acute THIP treatment on total BDNF mRNA, BDNF exon I and BDNF exon IV mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

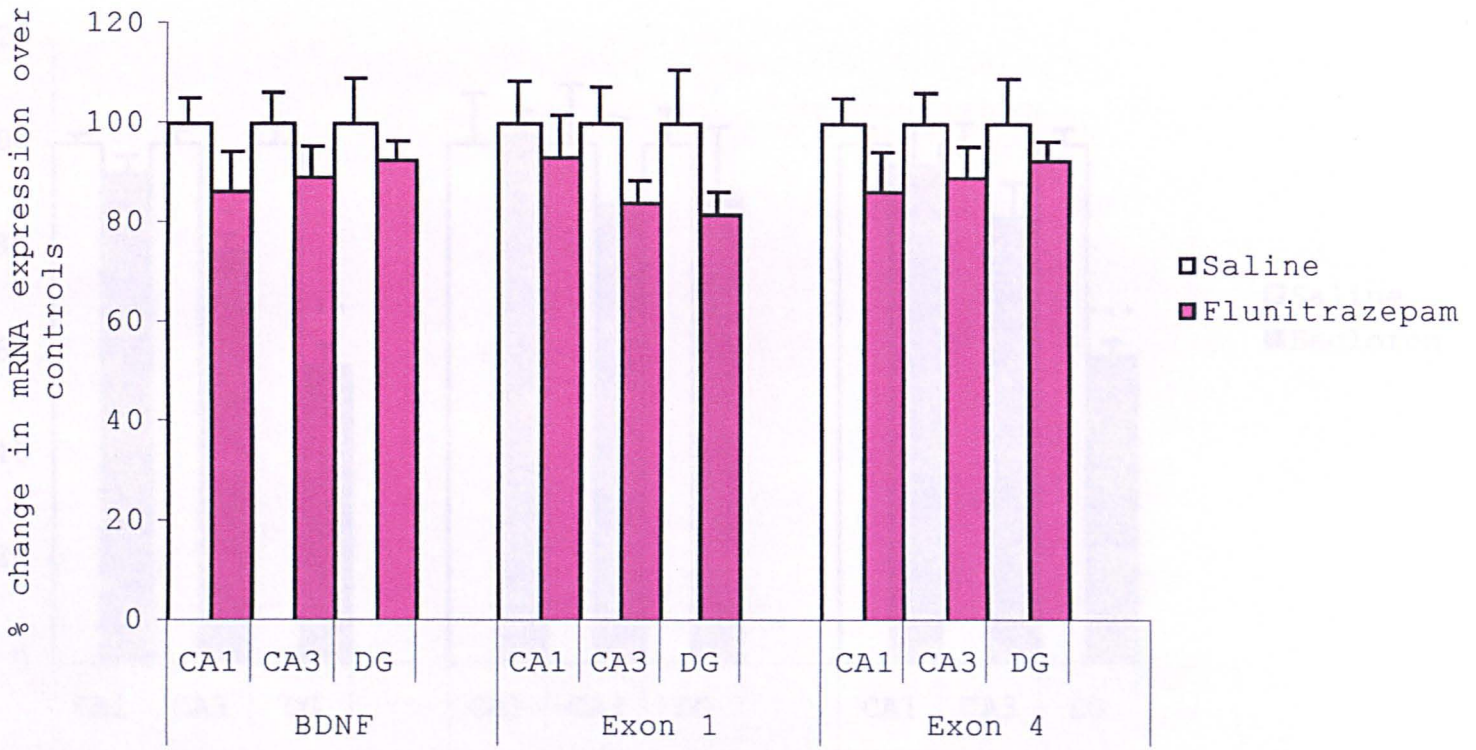


Figure 4.7. **Effect of acute flunitrazepam treatment on total BDNF mRNA, BDNF exon I and BDNF exon IV mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control **p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

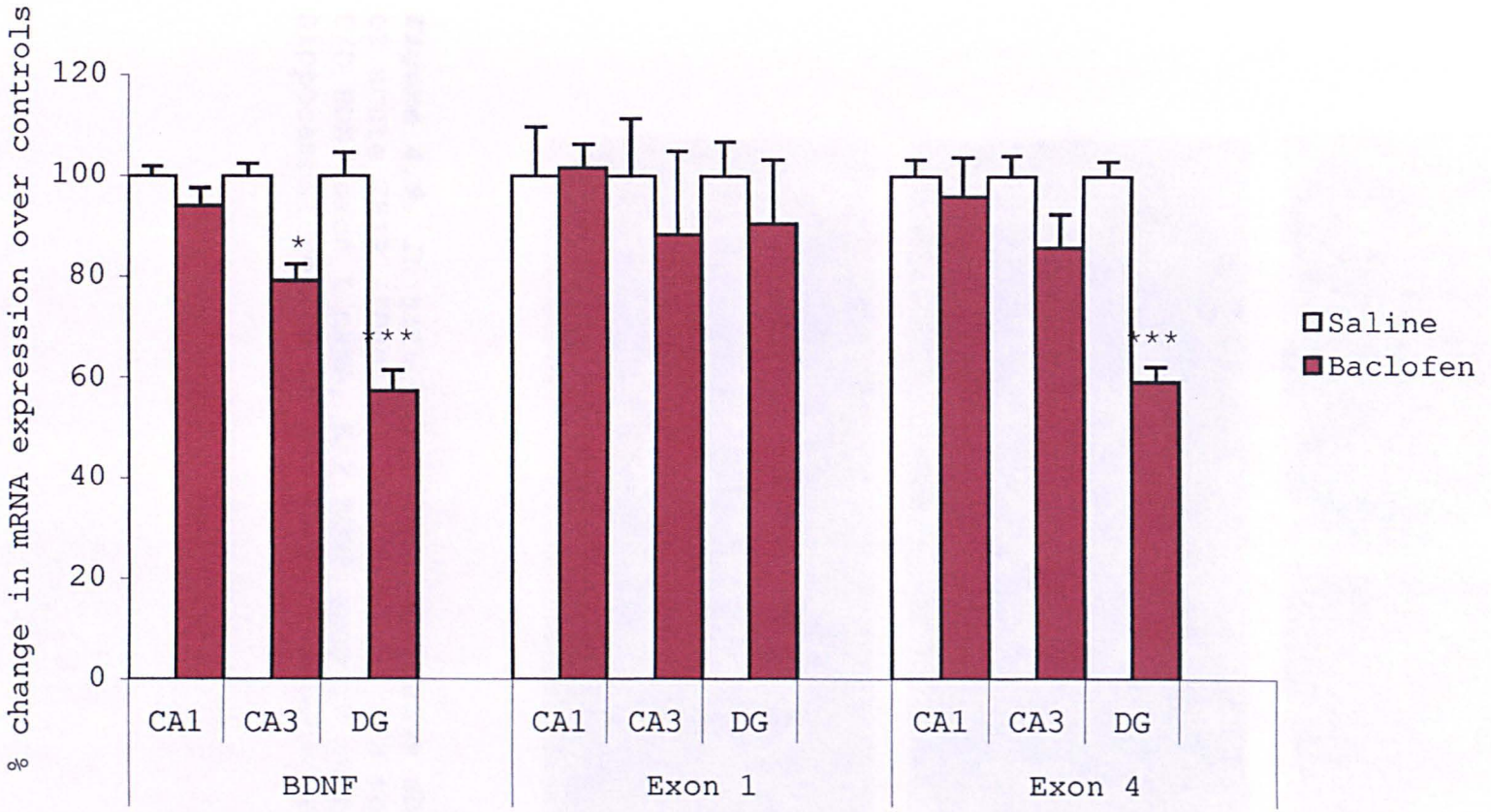


Figure 4.8. **Effect of acute baclofen treatment on total BDNF mRNA, BDNF exon I and BDNF exon IV mRNA expression in the rat hippocampus 4h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

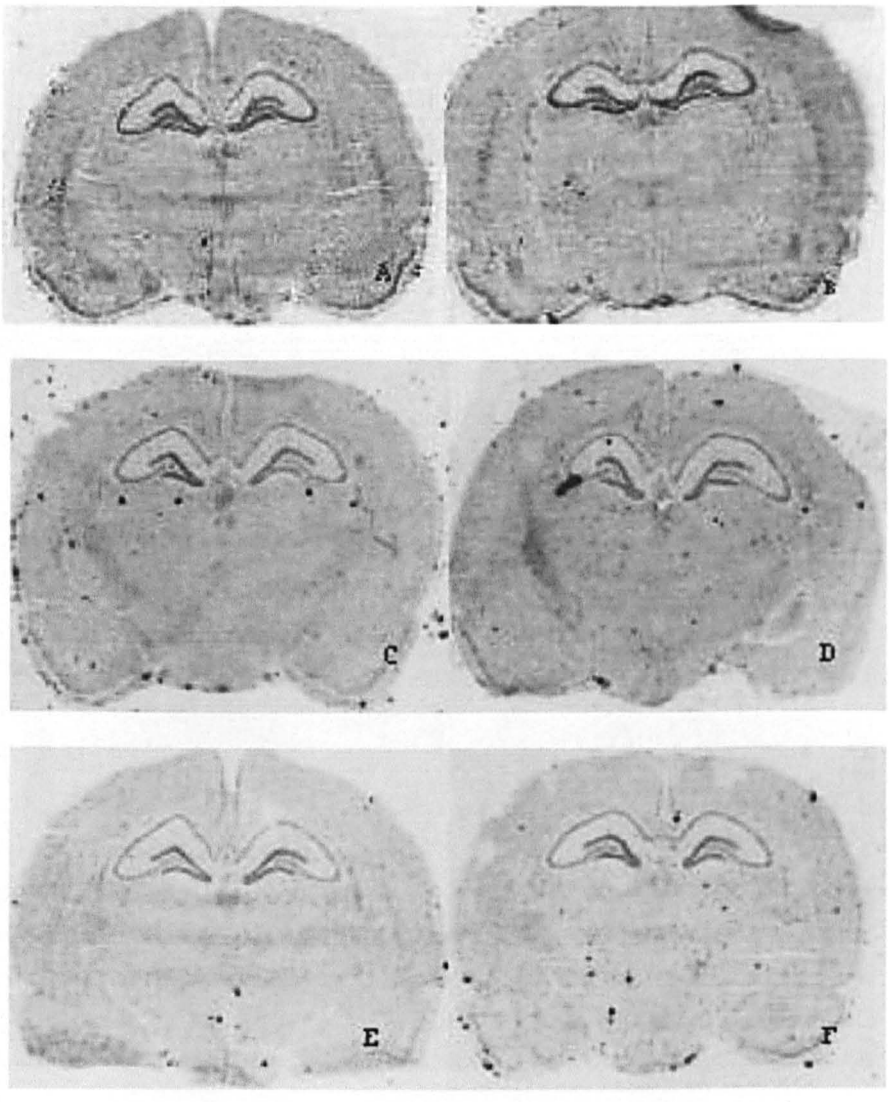


Figure 4.9. *In situ* hybridisation images showing the effect of acute THIP (10mg/kg) treatment on A/B total BDNF mRNA, C/D BDNF exon I mRNA, E/F BDNF exon IV in the rat hippocampal region at 4h. Control groups are situated left.

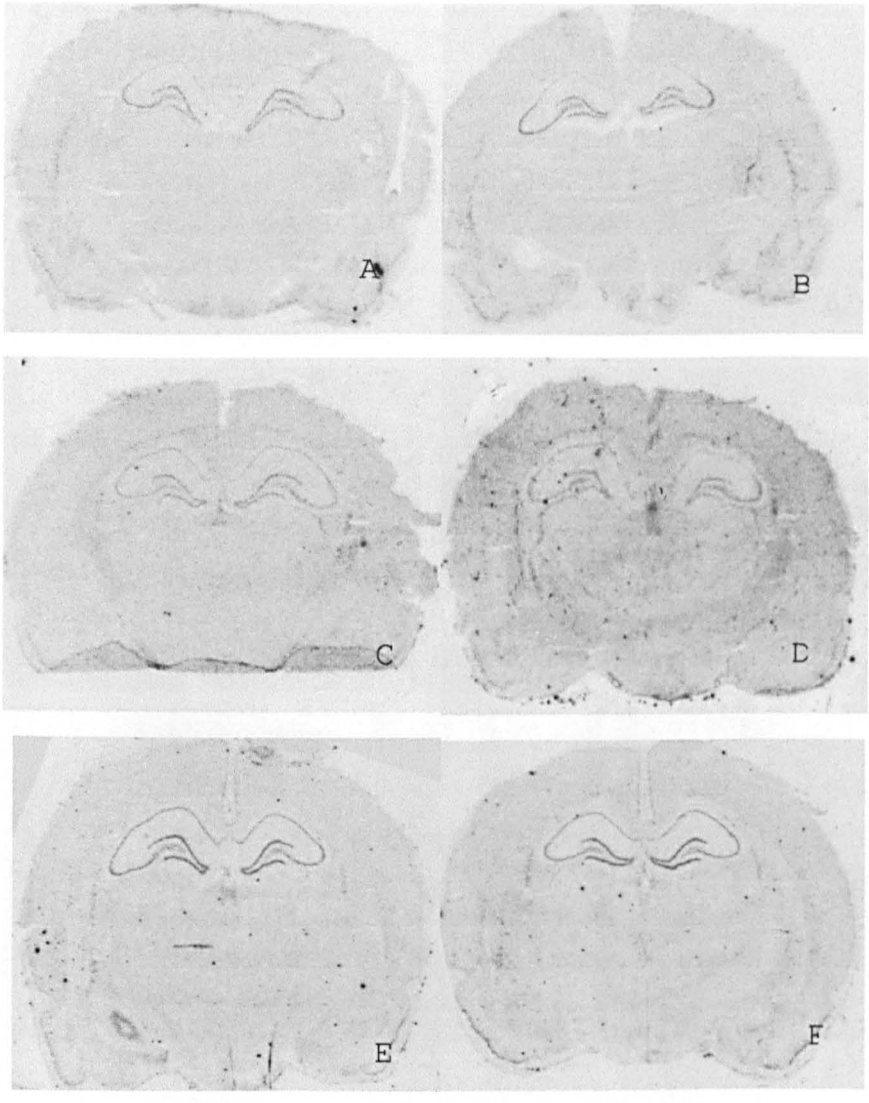


Figure 4.10. *In-situ* hybridisation images showing the effect of acute flunitrazepam (10mg/kg) treatment on A/B total BDNF mRNA, C/D BDNF exon I mRNA, E/F BDNF exon IV in the rat hippocampal region at 4h. Control groups are situated left.

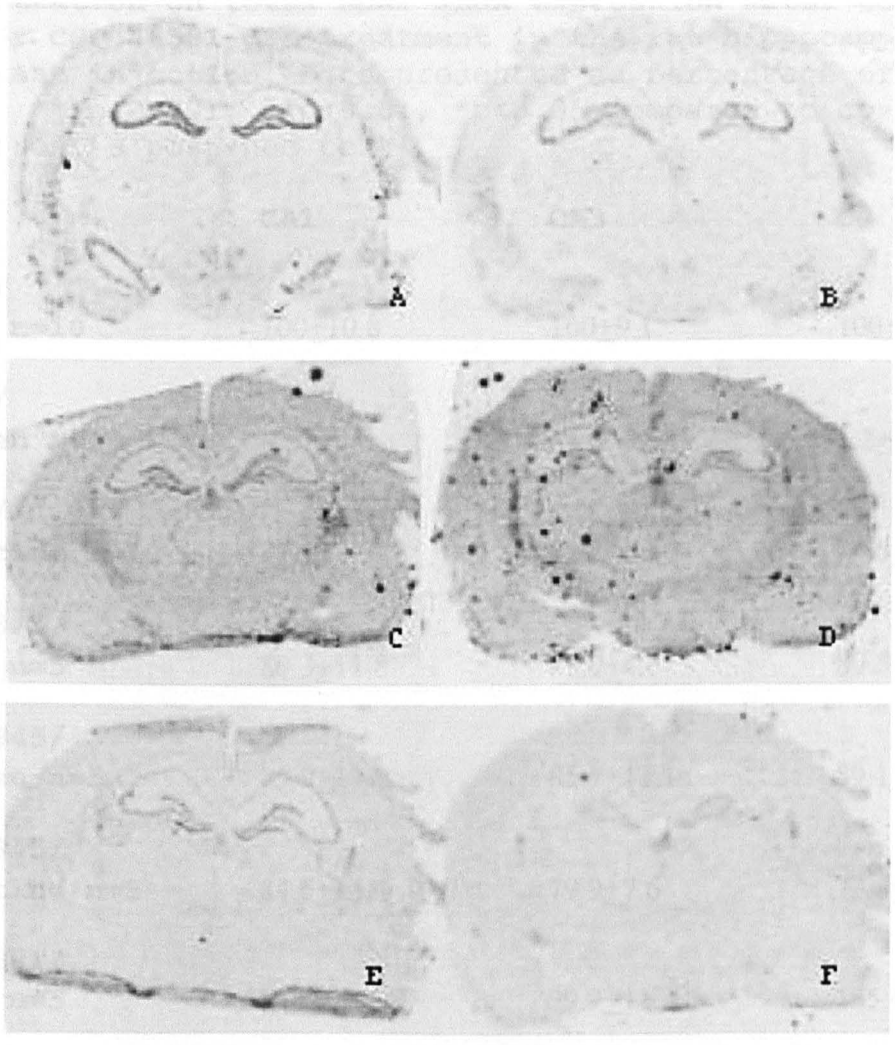


Figure 4.11. *In-situ* hybridisation images showing the effect of acute baclofen (10mg/kg) treatment on A/B total BDNF mRNA, C/D BDNF exon I mRNA, E/F BDNF exon IV in the rat hippocampal region at 4h. Control groups are situated left.

***Table 4.4.* Effect of acute paroxetine and baclofen administration on total BDNF mRNA expression after CGP 55845 or CGP 46381 pre-treatment in the rat hippocampus 4h after last injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (Bonferroni's post-hoc test)

	CA1	CA3	DG
Saline/ Saline n=10	100±10.8	100±9.1	100±11.8
Saline/ Baclofen n=10	81.7±9.6	64.6±8.5**	51.2±14.4***
Saline/ Paroxetine n=10	91.9±7.6	72.2±6.4*	74.6±12.0*
CGP 55845 Saline n=5	80.3±11.8	85.0±4.6	80.5±9.4
CGP 55845/ Baclofen n=5	85.9±14.1	85.7±11.1	89.1±15.1
CGP 55845/ Paroxetine n=5	84.6±14.4	79.9±7.6	73.8±11.4*
CGP 46381/ Saline n=5	118.9±11.8	99.9±11.4	135.4±12.9*
CGP 46381/ Baclofen n=5	109.5±12.1	86.4±2.5	82.9±17.8
CGP 46381/ Paroxetine n=5	123.9±13.7	104.7±7.0	100.3±12.3

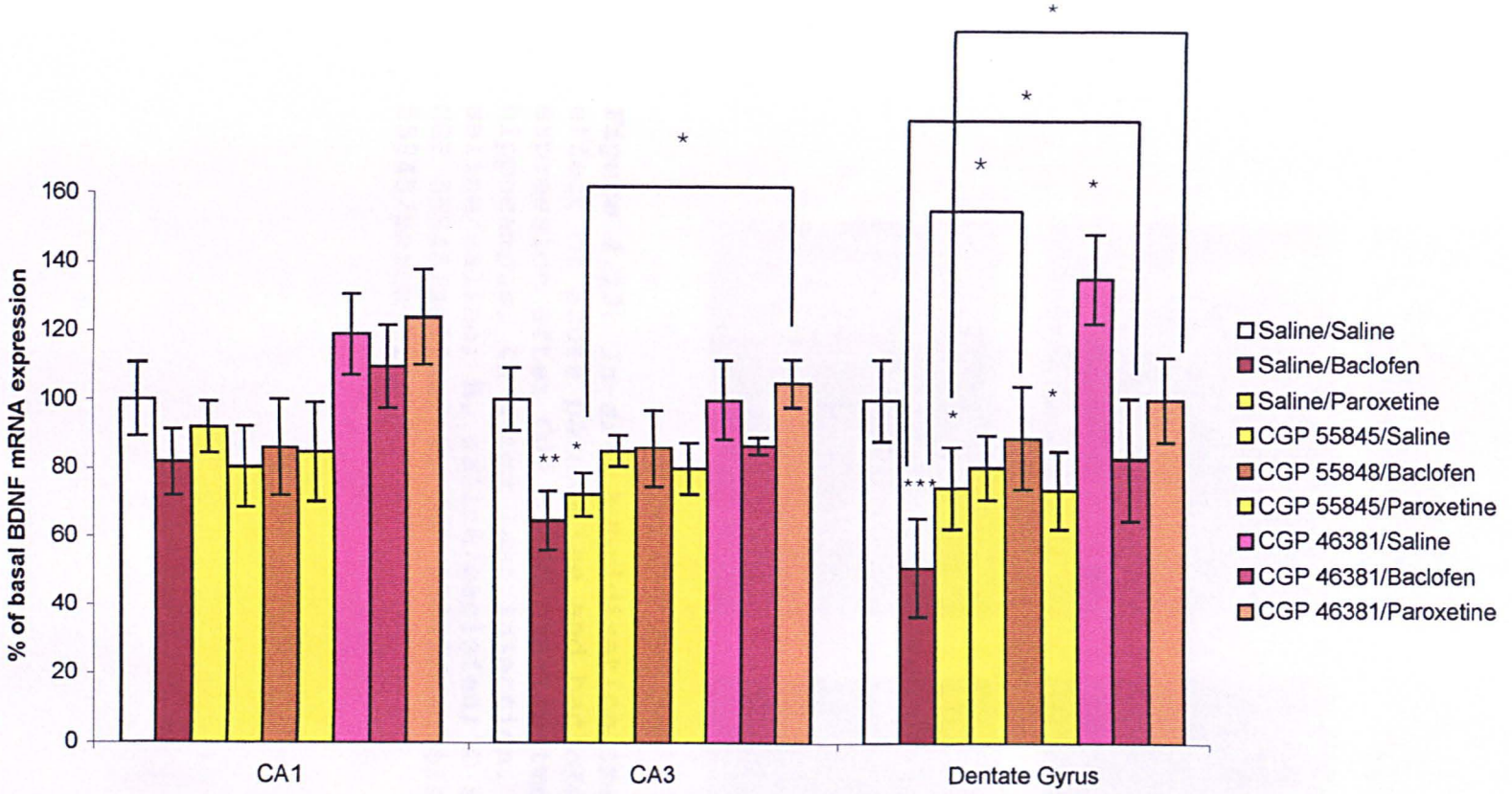


Figure 4.12. **Effect of acute paroxetine and baclofen administration on total BDNF mRNA expression after CGP 55845 and CGP 46381 pre-treatment in the rat hippocampus at 4h after last injection.** Data presented as percentage of control; ***p<0.001, **p<0.01, *p<0.05 compared with control or saline pre-treated control animals (vs. CGP treated animals).

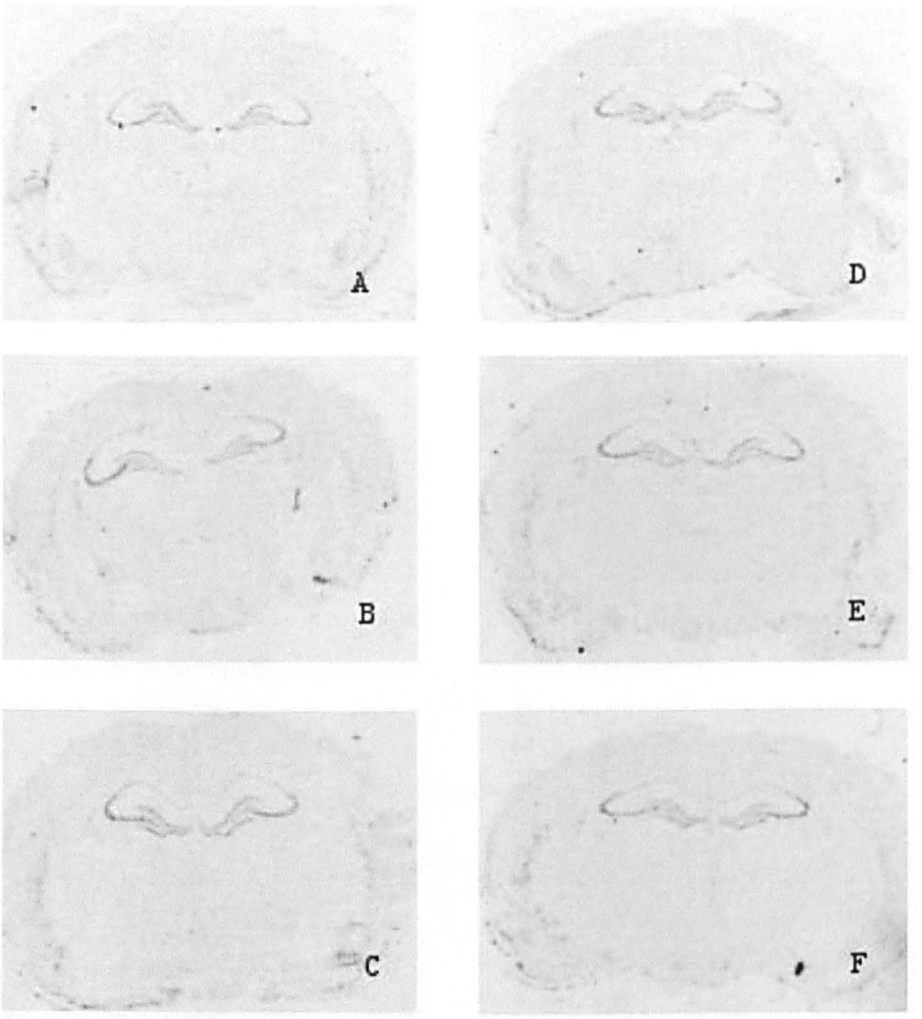


Figure 4.13. *In-situ* hybridisation images showing the effect of acute paroxetine and baclofen on total BDNF mRNA expression after CGP 55845 pre-treatment in the rat hippocampus, 4h after last injection. KEY: A, saline/saline; B, saline/baclofen; C saline/paroxetine; D, CGP 55845/saline; E, CGP 55845/baclofen; F, CGP 55845/paroxetine.

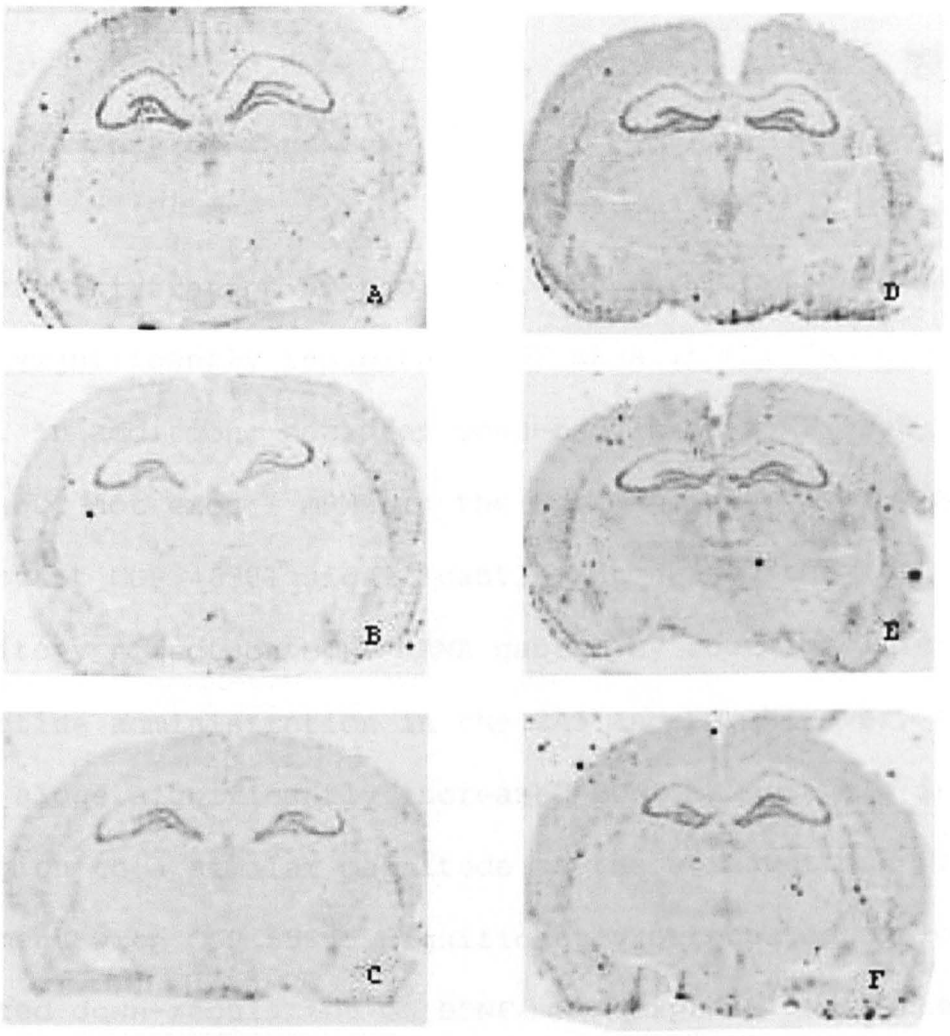


Figure 4.14. *In-situ* hybridisation images showing the effect of acute paroxetine and baclofen on total BDNF mRNA expression after CGP 46381 pre-treatment in the rat hippocampus, 4h after last injection. KEY: A, saline/saline; B, saline/baclofen; C saline/paroxetine; D, CGP 46381/saline; E, CGP 46381/baclofen; F, CGP 46381/paroxetine.

4.4. Discussion

4.4.1. Summary of findings

Acute administration of baclofen, but not flunitrazepam or THIP, significantly inhibited BDNF mRNA in the CA3 and DG at 4h. In addition, baclofen down-regulated BDNF exon IV mRNA, but not exon I mRNA in the DG. The selective $GABA_B$ antagonist CGP 46381 significantly attenuated the inhibitory effect on BDNF mRNA caused by acute baclofen or paroxetine administration in the CA3 and DG. However, CGP 46381 alone significantly increased BDNF mRNA levels in the DG region to a similar magnitude as the attenuation. Pre-treatment with CGP 55845 significantly attenuated baclofen-mediated down-regulation of BDNF mRNA expression in the DG, however it failed to attenuate paroxetine-mediated down-regulation in the CA3 and DG region of the hippocampus.

4.4.2. The effect of acute GABA-ergic receptor agonist administration on BDNF mRNA and BDNF exon mRNA at 4h

The main aim of this chapter was to elaborate on the role of GABAergic transmission in the acute regulation of BDNF mRNA in the rat hippocampus, in an attempt to link

facilitation in GABA transmission to antidepressant drug-induced reductions in BDNF mRNA levels. Three GABA receptor agonists were tested which had varying effects on BDNF mRNA levels. The two agonists working on the $GABA_A$ receptor, the $GABA_A$ receptor agonist THIP and the benzodiazepine receptor agonist flunitrazepam, both failed to produce any significant effect on BDNF mRNA in any area of the hippocampus at 4h. This was in direct contrast to baclofen, a $GABA_B$ receptor agonist, which significantly inhibited levels in the CA3 and DG. GABAergic mechanisms have been suggested to be involved in the acute inhibitory effect of antidepressants (Vaidya et al, 1997; Zetterström et al, 1999; Vaidya and Duman, 2001; *Section 4.1*). The present results therefore indicate a preferential role for $GABA_B$ receptor-mediated mechanisms in the acute inhibition of BDNF mRNA in the CA3 and DG of the hippocampus. $GABA_B$ receptors have been observed in high quantities in the CA3 and DG in comparison to the CA1 (Knott et al, 1993).

In correspondence with the effect of acute antidepressant treatment, acute baclofen administration also significantly reduced BDNF exon IV (IEG-like), but not BDNF exon I (protein synthesis-dependent) mRNA in the DG region.

4.4.3. The effect of $GABA_B$ receptor antagonists on baclofen/paroxetine-mediated inhibition of BDNF mRNA at 4h

In order to confirm the influence of $GABA_B$ receptor activation in the paroxetine/baclofen-mediated down-regulation of BDNF mRNA, two selective $GABA_B$ receptor antagonists were employed at non-convulsive doses (10mg/kg), 30 minutes prior to injection with either baclofen or the SSRI paroxetine. Baclofen, when administered with saline, inhibited BDNF mRNA to a similar magnitude as the previous experiment. Likewise, acute paroxetine also significantly inhibited BDNF mRNA levels to similar levels as previous experiments *(See Section 3.3.1.1)*.

Non-convulsive doses of CGP 55845 did not significantly enhance (or reduce) BDNF mRNA in any area of the hippocampus. Thus, the apparent attenuation of the acute baclofen-mediated inhibitory response on DG BDNF mRNA by CGP 55845 pre-treatment appears to be as a result of true $GABA_B$ receptor blockade, as opposed to the any overriding effect of the antagonist. However, the compound failed to attenuate the paroxetine-mediated BDNF mRNA down-regulation in the DG. Therefore, if it had successfully blocked local

$GABA_B$ receptor transmission, one may conclude that CGP 55845 blocks acute baclofen-, but not paroxetine-mediated down-regulation in the DG region.

In comparison, when administered with saline at non-convulsive doses, CGP 46381 significantly up-regulated BDNF mRNA in the DG. Similar, transient increases in BDNF mRNA have been reported following acute administration of the $GABA_B$ receptor antagonists (CGP 56999 and CGP 56433) in the hippocampus and cortex at 6h, with levels returning back to baseline at 24h (Heese et al, 2000). Such increases may be anticipated due to the reduction in $GABA_B$-mediated inhibition of BDNF mRNA in the DG, due to local receptor blockade. Thus, whilst pre-treatment with CGP 46381 significantly attenuated the acute down-regulatory response of BDNF mRNA in the DG after acute baclofen or paroxetine, when the up-regulatory effect CGP 46381 (+35.4%) is considered, the attenuation is minimal. Therefore, it may be debated whether the lack of reduction of BDNF in the DG region was due primarily to an additive effect of CGP 46381 via glutamatergic induction, or true antagonism of the effects of baclofen or paroxetine.

However in the CA3 area, CGP 46381 alone had little effect on BDNF mRNA levels. Thus, the significant attenuating effect of CGP 46381 pre-treatment before paroxetine treatment was not influenced by any enhancing effect from the compound. Thus, it appears that the attenuation in CA3 levels may be genuine and may implicate the activation of the $GABA_B$ receptor in the acute inhibition of BDNF after acute paroxetine treatment in the CA3.

Returning to the supposition that forms the basis behind these experiments, which suggested a role for localised GABAergic interneurone activation via excitatory 5-HT receptor activation in the down-regulation of BDNF mRNA in the hippocampus (Vaidya et al, 1997; Zetterström et al, 1999; Vaidya and Duman, 2001). It appears that the results from this chapter partially confirm this hypothesis. Firstly, acute baclofen, but not THIP or flunitrazepam administration significantly down-regulated BDNF mRNA in the DG region, this effect was blocked by both CGP 55845 and CGP 46381 pre-treatment. This suggests a predominant role for $GABA_B$ receptor-mediated inhibition in BDNF mRNA in the DG. In addition, administration of the $GABA_B$ antagonist CGP 46381, but not CGP 55845 significantly attenuated paroxetine-mediated down-regulation in the DG. However, the

additive effect of CGP 46381 may have influenced the attenuating effect in the DG. Nevertheless, the results suggest activation in $GABA_B$ receptor-mediated transmission in the down-regulatory effect of paroxetine in the CA3 region of the hippocampus.

The results in this chapter appear to confirm the hypothesis that suggests a role for excitatory 5-HT receptors on GABAergic interneurones within the hippocampus. However, it was demonstrated in *Chapter 3* that acute administration of antidepressant drugs with varying affinities to NA/5-HT reuptake or metabolism (as well as two compounds with no potency to 5-HT) had an equivocal effect on BDNF mRNA inhibition. Thus, it was concluded that the inhibition of BDNF mRNA at 4h after acute antidepressant treatment was not exclusively 5-HT-mediated and may be induced by a general facilitation of 5-HT, NA (or possibly DA) transmission in the hippocampus. Excitatory β-adrenoceptor stimulation has been shown to depolarise groups of interneurones within the hippocampus and increase IPSP amplitude in CA1 and CA3 pyramidal cells (Bergles et al, 1996). In addition, both $GABA_A$ and $GABA_B$-receptor-mediated inhibition in DG granule cells is enhanced by β-adrenoceptor activation (Bijak and Misgeld,

1995). Electrophysiological data also suggests that GABAergic interneurones can be excited via α_1 adrenoceptors resulting in pyramidal and granule cell inhibition (Bergles et al, 1996). This therefore suggests the presence of excitatory adrenoceptors on hippocampal interneurones, activation of which may result in the inhibition of BDNF mRNA in the DG and CA3 areas of the hippocampus. The role of DA in BDNF mRNA inhibition within the hippocampus is unclear. Little evidence exists for the presence of excitatory DA receptors on GABAeregic interneurones. However, inhibitory D_2 receptors have been shown to be present in the DG and at the layer of the mossy fibre zone (Khan et al, 1998). Facilitation in extracellular hippocampal DA levels therefore may result directly in the activation of inhibitory control over DG granule cells, thereby inhibiting local BDNF mRNA levels.

In conclusion, it appears that BDNF mRNA is regulated acutely via $GABA_B$-mediated mechanisms in the DG and CA3 regions of the hippocampus. Moreover, the inhibitory effect of the SSRI paroxetine on BDNF mRNA appears to be significantly influenced by $GABA_B$ receptor blockade in the CA3, possibly implicating $GABA_B$ activation in the inhibitory effect of paroxetine on BDNF mRNA. Overall, this suggests

that $GABA_B$ transmission participates in the early effects of antidepressant drug action on BDNF mRNA in the hippocampus.

The next chapter examines the effect of chronic antidepressant administration on total BDNF mRNA, as well exon I and exon IV mRNA in the rat hippocampus.

Chapter Five

Effect of Chronic Administration of Antidepressant Drugs on BDNF mRNA and BDNF exon mRNA in the Rat Hippocampus

5.1. Introduction

As explained earlier *(See Section 1.5)*, a central flaw in monoamine hypothesis is the failure to explain the need for chronic treatment of antidepressant drugs, despite their acute effects on monoamine transmission. This has led to research focusing on post-synaptic events beyond the monoamine receptor. The actions of 5-HT and NA receptors at both pre- and post-synaptic sites are mediated by their coupling to respective intracellular signal transduction pathways (Duman, 1998; Shelton, 2000). These monoamine-linked cellular mechanisms therefore would be expected to mediate the actions of antidepressants, via increased extracellular 5-HT/NA and the subsequent stimulation of monoamine receptors. Furthermore, the long-term activation of such mechanisms has been postulated to result in adaptation that may result in the eventual therapeutic efficacy of antidepressant drugs (Nestler et al, 1989; Manji et al, 1995).

5.1.1. The effect of chronic antidepressant drug treatment on receptor-coupled signalling cascades

Several studies have demonstrated an up-regulation in the components comprising the cAMP signalling cascade after the chronic administration of antidepressants via noradrenergic β-AR; or serotonergic 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7 receptor activation (Nestler et al, 1989; Perez et al, 1991; Ozawa and Rasenick, 1991; Thome et al, 2000). Additionally, the phosphoinositide (PI) pathway, which is linked via G_q proteins to noradrenergic α_1 and 5-HT_2 receptors, has also been shown to be affected by chronic antidepressant treatment. However, its precise role is more complex. Some studies have reported increased PI turnover (Newman et al, 1989; Pandey et al, 1991), while others have reported decreases (Li et al, 1988; Dwivedi et al, 2002) or no effects (Coull et al, 2000) after chronic antidepressant drug treatment.

A downstream target of both the cAMP at PI pathways that has been suggested to mediate the action of antidepressants is the cAMP response element binding protein (CREB; Meyer and Habener, 1993; Thome et al, 2000). CREB is a transcription factor, which is activated by increased cAMP

production as a consequence of stimulation by G_s-coupled receptors. CREB mediates the actions of cAMP on gene expression and could thereby underlie some of the effects of chronic antidepressant treatment. Nibuya et al (1996) demonstrated that chronic, but not acute administration of several different types of antidepressants (SSRIs, NA-selective reuptake inhibitor, MAOI, atypical) enhanced CREB mRNA and its corresponding protein in the rat brain. Phosphorylation of CREB is increased after chronic, but not acute antidepressant treatment (Duman et al, 1997; 1999; Thome et al, 2000) and the function of CREB is regulated by its state of phosphorylation at serine residue 133 (Mayr et al, 2001). Phosphorylation of CREB by kinases (PKA, PKC) leads to increased binding to the CREB response element (CRE) sequence sites in promoters (Nichols et al, 1992; Lonze and Ginty, 2002).

Deletion and mutational analysis of the promoter directly upstream of the BDNF gene has revealed that gene activation is dependent on an element 5' to the mRNA start site. The element (located between 40 and 30 bp upstream of the mRNA start site) within the BDNF gene matches the consensus sequence of the CRE and is required for activation of the promoter (Shieh et al, 1998; Tao et al, 1998). The CRE-

dependent component of the response appears to be mediated by CREB as it is part of the complex that binds to this CRE and since negative mutants of CREB attenuate activation of the promoter (Shieh et al, 1998).

An additional area within the BDNF gene, upstream from the CRE promoter region (between 47 and 72bp upstream of the mRNA start site) has been identified. This site is a novel calcium response element site and is required for calcium-dependent BDNF expression (Shieh et al, 1998; Tao et al, 1998). BDNF has been suggested to be a likely target gene of calcium signalling (Shieh and Ghosh, 1999). Activation of voltage-sensitive Ca^{2+} channels or NMDA type glutamate receptors leads to the enhancement of BDNF mRNA and stimulates the release of BDNF protein (Zafra et al, 1990; 1991; Ghosh et al, 1994). Ca^{2+} influx also triggers phosphorylation of CREB and interfering with CREB inhibits calcium-dependent BDNF transcription (Finkbeiner, 2000), thus indicating a common reciprocal pathway.

The possibility that induction of BDNF is mediated by CREB is further supported by the apparent anatomical co-localisation after chronic antidepressant treatment. Both CREB and BDNF mRNA have been shown to be significantly up-

regulated in hippocampal, as well as cortical areas (Nibuya et al, 1995; 1996; Duman et al, 1997; Coppell et al, 2003). A role for the cAMP pathway in regulating this response in these areas is supported by studies using phosphodiesterase (PDE) inhibitors, which inhibit the breakdown of cAMP molecules. Chronic administration of the PDE inhibitor rolipram enhances CREB and BDNF mRNA expression (Nibuya et al, 1996; Fujimaki et al, 2000).

Many previous studies have demonstrated an increase in BDNF mRNA levels after chronic, but not acute antidepressant drug treatment (Nibuya et al, 1996; Zetterström et al, 1998; Russo-Neustadt et al, 1999; Coppell et al, 2003). However, significant differences exist in the magnitude of the BDNF mRNA response to chronic antidepressant intervention. Using ISH and Northern blot techniques, Nibuya et al (1996) found a robust (2-3 fold) increase in BDNF mRNA in the CA1 area hippocampus, after administration of a wide range of drugs (SSRIs, TCAs, MAOIs, atypical). Subsequent studies have found less pronounced changes also localised to the hippocampus and not common to all antidepressants (Zetterström et al, 1998; Russo-Neustadt et al, 1999). For instance, a recent study (Coppell et al, 2003) found significant elevation in BDNF mRNA after

chronic administration of the SSRIs paroxetine, fluoxetine and sertraline, but not with the non-selective MAOI TCP or the noradrenergic TCA desipramine.

As explained earlier *(See Section 1.6.3)*, the BDNF gene has multiple transcripts, each with unique promoters. Transcripts consist of a variable 5′ region (exons I-IV) and a common 3′ segment (exon V), which codes for mature BDNF protein. Previous studies have revealed differential use of the individual BDNF exon mRNA transcripts, which vary in response to chronic antidepressant. Russo-Neustadt et al (2000) found chronic administration of the non-selective MAOI TCP, but not the TCA imipramine significantly up-regulated exon I mRNA in the CA3 and DG region of the hippocampus, but no significant change was seen in exon II mRNA. The study however did not examine the corresponding effect on exon III or IV. In contrast, a more recent study (Dias et al, 2003) found chronic administration of TCP or the noradrenergic TCA desipramine significantly up-regulated exon II and exon III mRNA, but not exon I or exon IV mRNA.
Both studies however lacked data on total BDNF mRNA making conclusions regarding predicted net effects on the translation of the corresponding protein difficult.

In *Chapter 3,* it was found that that acute (single injection) administration of several antidepressant compounds had varying effects on individual BDNF exon transcripts in the rat hippocampus at 4h. Acute antidepressant treatment resulted in an overall inhibition in total BDNF mRNA expression, which seemed to be due in part to exon IV, but not exon I mRNA down-regulation. Therefore as a continuation of this series of experiments, this chapter aims to investigate which of the exon transcripts studied is influenced by chronic administration of antidepressant drugs.

In order to clarify the influence of chronic antidepressant drug treatment on varying individual exon mRNA, as well as total BDNF mRNA regulation, this series of experiments investigates the effect of chronic administration of three antidepressants. Two antidepressants with varying affinities to the NA/5-HT reuptake sites (the noradrenergic TCA desipramine and the SSRI fluoxetine), together with the non-selective MAOI TCP were administered for a 3-week period. In the following series of ISH experiments, we utilised oligonucleotide probes selective to total BDNF mRNA (exon V), as well as a representative form of protein-

dependent exon mRNA (exon I) and a non-protein synthesis-dependent 'IEG-like' exon mRNA (exon IV; Lauterborn et al, 1996; 1998; *See Section 1.6.3*).

5.2. Methods

Male Sprague-Dawley rats (225-250g) were injected (i.p) once daily for 21d with either: saline (1ml/kg) or fluoxetine (10mg/kg), desipramine (10mg/kg) or TCP (5mg/kg) in 0.9% saline, and sacrificed 24h after the last injection. Brains were isolated and flash-frozen in cooled isopentane, before being stored at -70°C until further use. *In-situ* hybridisation and densitometric analysis procedures are described in *Chapter 2.*

5.3. Results

5.3.1. Effect of chronic administration of antidepressant drugs on BDNF mRNA and BDNF exon mRNA at 24h

5.2.1.1. Effect on total BDNF mRNA

Three different antidepressant drugs were assessed for their effect on BDNF mRNA at 24h, the SSRI fluoxetine, the TCA desipramine and the non-selective MAOI TCP. Daily injections were administered over a three-week period. Fluoxetine (10mg/kg) caused a significant up-regulation of BDNF mRNA in the DG area (36.4%; $p<0.05$) of the hippocampus. No significant changes were observed in the CA1 or CA3 regions *(Table 5.1; Figures 5.1 and 5.4)*. Repeated administration of desipramine (10mg/kg) also caused a significant up-regulation in the DG (36%; $p<0.05$), no significant changes were observed in the CA1 or CA3 areas *(Table 5.1; Figures 5.2 and 5.5)*. Likewise, chronic TCP (5mg/kg) administration caused a significant up-regulation in BDNF mRNA in the DG (48%; $p<0.01$) at 24h. In addition, chronic TCP administration caused a significant up-regulation in the CA3 (37.5%; $p<0.05$) field of the

hippocampus, but not the CA1 *(Table 5.1; Figures 5.3 and 5.6)*.

5.3.1.2. Effect on BDNF exon I mRNA

Chronic treatment with fluoxetine caused a significant up-regulation in BDNF exon I mRNA at 24h in the DG (64.4%; $p<0.001$) and also in the CA3 (37.5%; $p<0.05$), but not in the CA1 *(Table 5.2; Figures 5.1 and 5.4)*. Unlike the effect on whole BDNF mRNA, desipramine had no significant effect on BDNF exon I mRNA in any area of the hippocampus *(Table 5.2; Figures 5.2 and 5.5)*. Chronic TCP treatment resulted in a significant up-regulatory effect in the DG (51.2%; $p<0.01$) and the CA3 (47.5%; $p<0.05$; *Table 5.2; Figures 5.3 and 5.6*). No significant effect was seen in the CA1.

5.3.1.3. Effect on BDNF exon IV mRNA

Chronic treatment with either fluoxetine, TCP or desipramine had no significant effects on BDNF exon IV mRNA in any area of the rat brain tested *(Table 5.3; Figures 5.1 to 5.6)*.

***Table 5.1.* Effect of chronic drug treatment on BDNF mRNA expression in the rat hippocampus 24h after the last injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

	CA1	CA3	DG
Saline n=6	100±3.8	100±5.9	100±4.9
Fluoxetine n=6	105.5±6.4	113.4±6.1	136.4±6.3*
Saline n=6	100±3.8	100±5.9	100±4.9
Desipramine n=6	118.4±4.6	123.6±5.1	136.0±6.0*
Saline n=6	100±3.8	100±5.9	100±4.9
Tranylcypromine n=6	123.6±11.1	137.5±3.9*	148.0±11.4**

***Table 5.2.* Effect of chronic drug treatment on BDNF exon I mRNA expression in the rat hippocampus 24h after the last injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

	CA1	CA3	DG
Saline n=6	100±8.8	100±14.5	100±10.5
Fluoxetine n=6	113.4±6.1	137.8±7.7	164.4±5.2**
Saline n=6	100±8.8	100±14.5	100±10.5
Desipramine n=6	81.4±9.7	101.9±18.0	119.0±18.3
Saline n=6	100±8.8	100±14.5	100±10.5
Tranylcypromine n=6	113.6±7.6	147.5±11.9*	151.2±11.0**

***Table 5.3.* Effect of chronic drug treatment on BDNF exon IV mRNA expression in the rat hippocampus 24h after the last injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

	CA1	CA3	DG
Saline n=6	100±10.1	100±8.6	100±7.5
Fluoxetine n=6	115.0±6.4	121.0±5.6	100.7±7.3
Saline n=6	100±10.1	100±8.6	100±7.5
Desipramine n=6	89.4±4.6	93.3±14.1	84.3±7.6
Saline n=6	100±10.1	100±8.6	100±7.5
Tranylcypromine n=6	80.4 ± 13.4	103.3±9.8	81.8±6.7

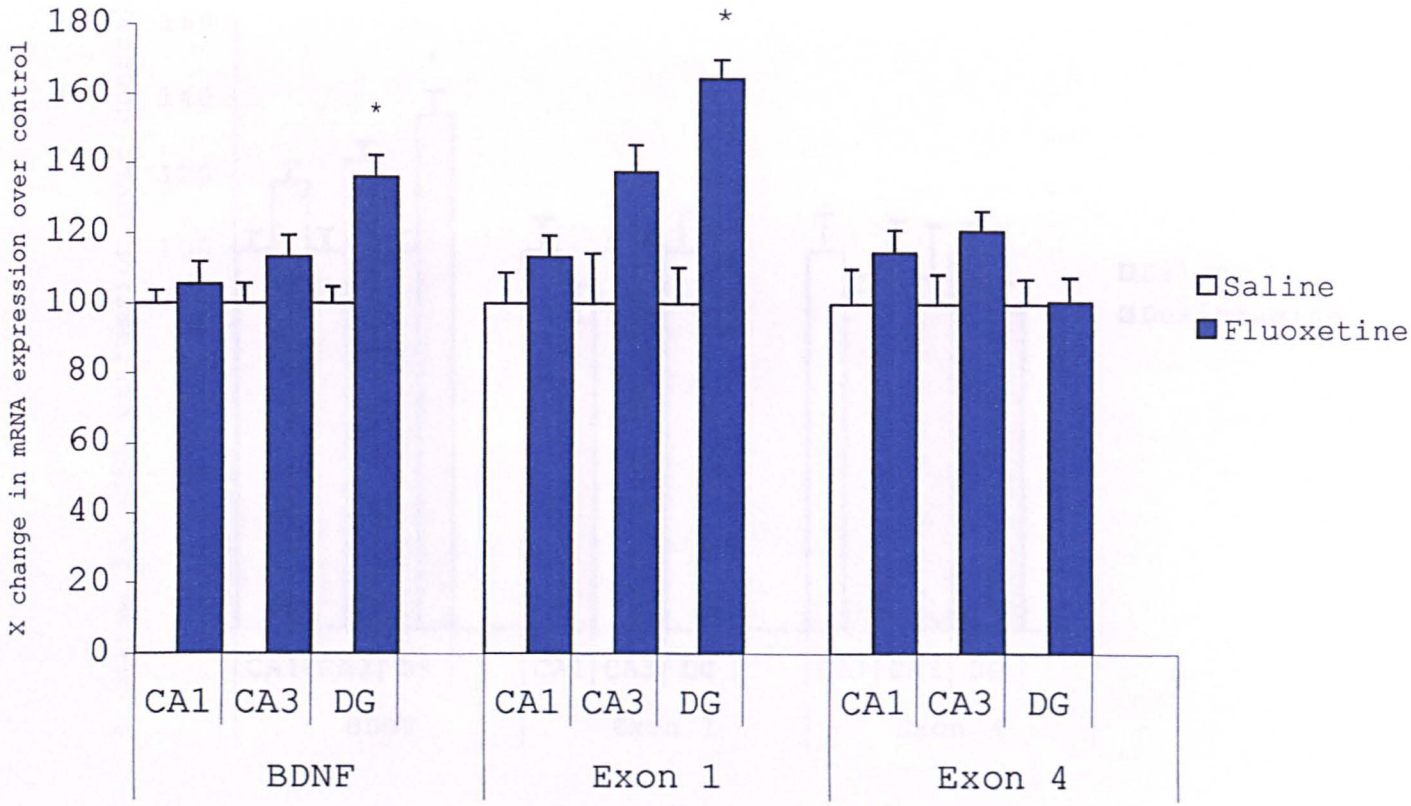


Figure 5.1. The effect of chronic fluoxetine (21 days at 10mg/kg) administration on total BDNF mRNA, exon I mRNA and exon IV mRNA at 24h in the CA1, CA3 and dentate gyrus (DG) regions of the hippocampus. Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (Bonferroni's post-hoc test).

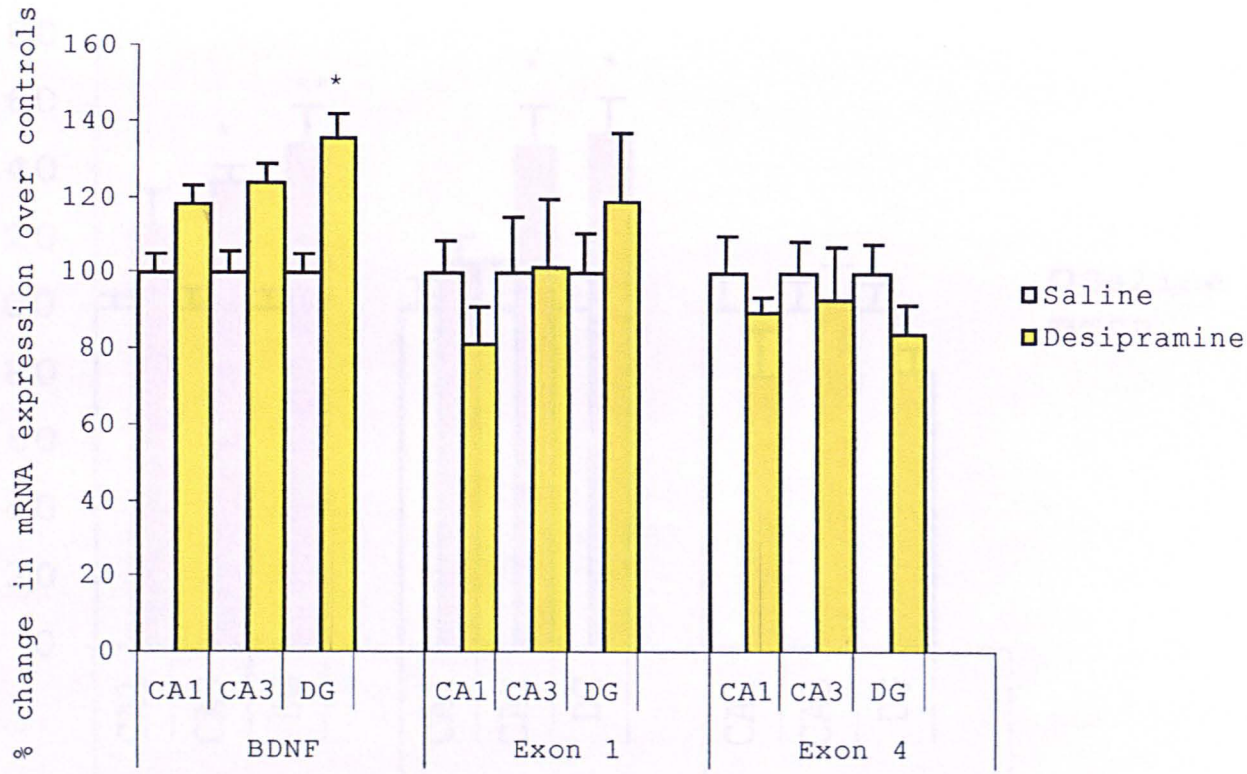


Figure 5.2. The effect of chronic desipramine (21 days at 10mg/kg) administration on total BDNF exon mRNA, exon I mRNA and exon IV mRNA at 24h in the CA1, CA3 and dentate gyrus (DG) regions of the hippocampus. Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (ANOVA with Bonferroni's post-hoc test)

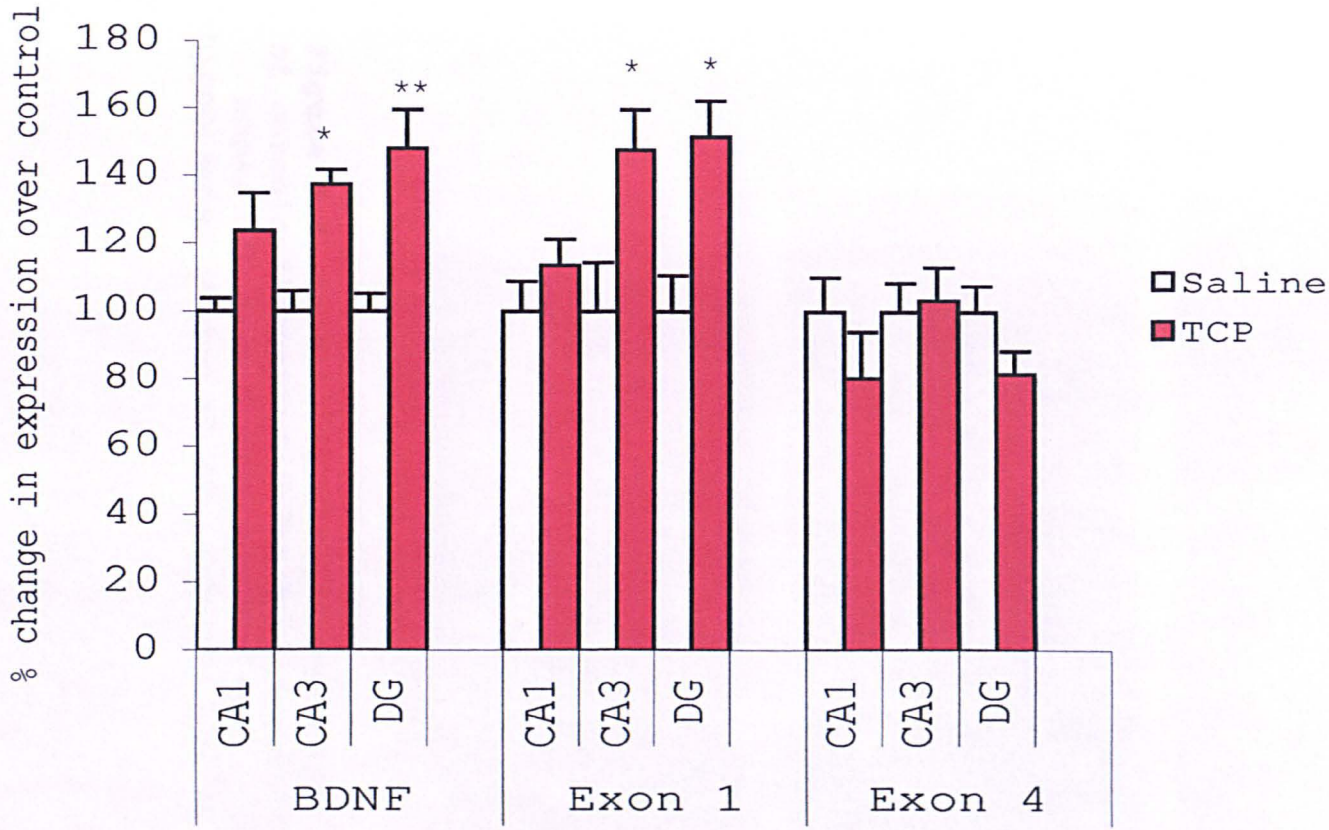


Figure 5.3. The effect of chronic tranylcypromine (TCP; 21 days at 5mg/kg) administration on total BDNF mRNA, exon I mRNA and exon IV mRNA at 24h in the CA1, CA3 and dentate gyrus (DG) regions of the hippocampus. Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (Bonferroni's post-hoc test).

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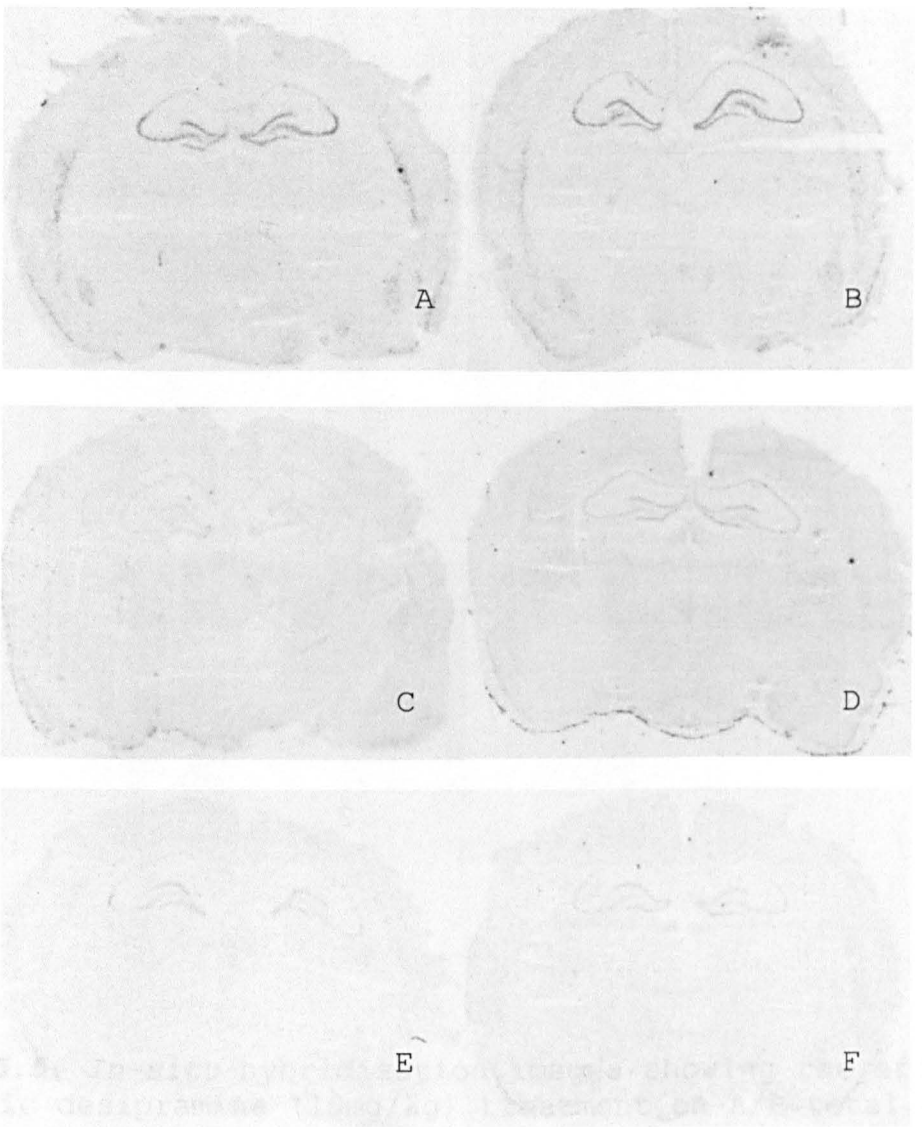

Figure 5.4. In-situ hybridisation images showing the effect of chronic fluoxetine (10mg/kg) treatment on A/B total BDNF mRNA; C/D exon I mRNA and E/F exon IV mRNA in the rat hippocampal region at 24h. Control groups are situated left.

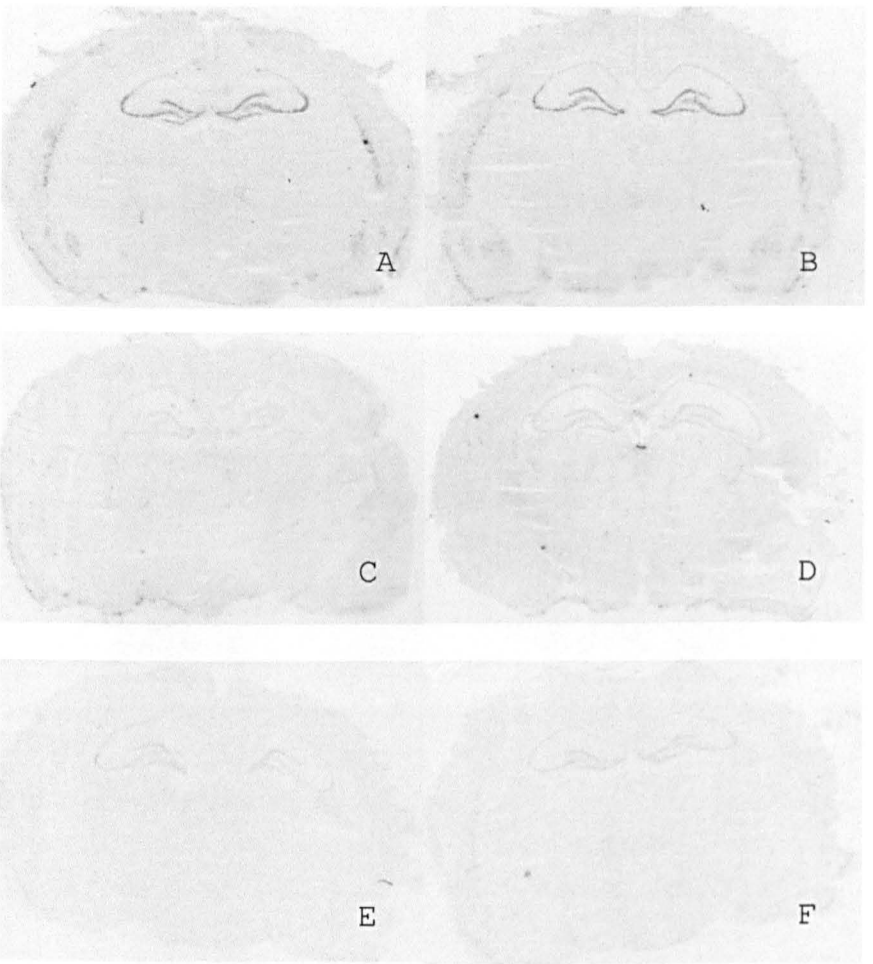

Figure 5.5. *In-situ* hybridisation images showing the effect of chronic desipramine (10mg/kg) treatment on A/B total BDNF mRNA; C/D exon I mRNA and E/F exon IV mRNA in the rat hippocampal region at 24h. Control groups are situated left.

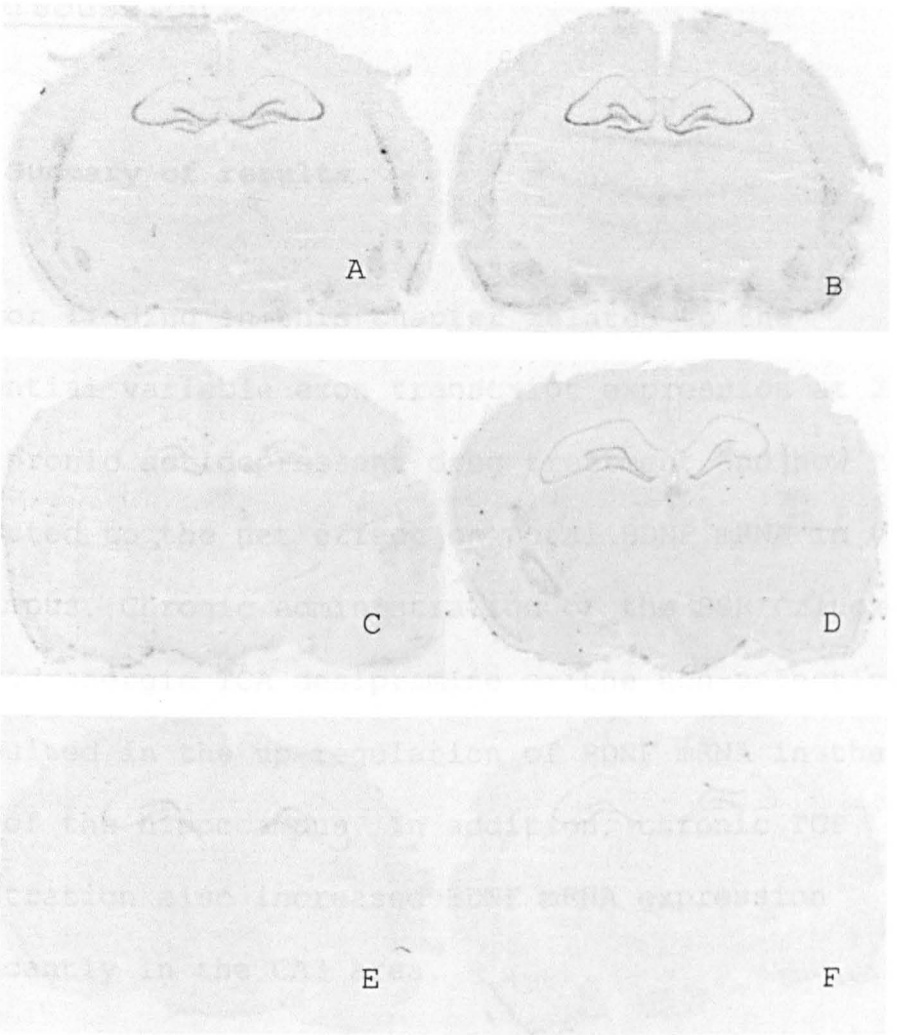

Figure 5.6. In situ hybridisation images showing the effect of chronic tranylcypromine (5mg/kg) treatment on A/B total BDNF mRNA; C/D exon I mRNA; E/F exon IV mRNA in the rat hippocampal region at 24h. Control groups are situated left.

5.4. Discussion

5.4.1. Summary of results

The major finding in this chapter related to the differential variable exon transcript expression at 24h after chronic antidepressant drug treatment and how this contributed to the net effect on total BDNF mRNA in the rat hippocampus. Chronic administration of the SSRI fluoxetine, the noradrenergic TCA desipramine or the non-selective MAOI TCP resulted in the up-regulation of BDNF mRNA in the DG region of the hippocampus. In addition, chronic TCP administration also increased BDNF mRNA expression significantly in the CA3 area.

Among the variable exon transcripts, exon I mRNA was significantly up-regulated after chronic fluoxetine and TCP, but not desipramine administration. In contrast, chronic treatment of fluoxetine or TCP (as well as desipramine) had no significant effect on exon IV mRNA expression in any area of the hippocampus.

5.4.2. The effect of chronic antidepressant drug administration on total BDNF mRNA

As with earlier experiments (Nibuya et al, 1996; Russo-Neustadt, 1999; Coppell et al, 2003), different antidepressants, when given chronically, up-regulated total BDNF mRNA in areas of the hippocampus. However, there are some differences between the present findings and previous experiments.

Nibuya et al (1996) chronically administered two antidepressants the SSRI fluoxetine or MAOI TCP. Unlike the present study, they found the largest up-regulatory effect on BDNF mRNA in the CA1 after chronic fluoxetine (161%) administration. Chronic TCP administration also significantly increased CA1 levels (75%), though not to the extent of fluoxetine. However, similar significant increases in BDNF mRNA expression to the present study were recorded in the CA3 and DG after chronic fluoxetine and TCP administration. The reason for the apparent differences between the present and the previous studies are unclear. However, the period after the last injection however was shorter in the Nibuya et al (1996) study (18h). Thus, it may be that such increases may have subsided in the subsequent

period (6h) from where the current study was measured. Russo-Neustadt et al (1999) found similar significant increases in the DG (57%) to the present study after chronic TCP treatment from a similar treatment regimen (20d, once daily). CA1 levels were also significantly increased (25%), though to a less extent than the Nibuya study. No significant change was seen in CA3 levels.

Coppell et al (2003) found repeated injection of fluoxetine, paroxetine, sertraline or TCP (14d, twice daily) increased BDNF expression in the hippocampus at 24h. This effect however was confined to the DG, with the exception of chronic TCP, which also caused an increase in the CA1 region. In comparison to the present study, which found a significant increase in total BDNF mRNA levels after chronic desipramine treatment, Coppell et al (2003) failed to find any change in BDNF mRNA after chronic administration of the noradrenergic TCAs desipramine and maprotiline at 24h. As explained in *Section 3.4.3,* the Coppell study also failed to find any significant change (inhibition) after acute administration of desipramine, whilst other SSRI compounds inhibited BDNF mRNA in the DG, this indicated a predominant role for 5-HT-mediated mechanisms in acute (at 4h) and chronic (at 24h) regulation of BDNF mRNA in the hippocampus.

The present study found changes in BDNF mRNA after desipramine treatment at both these time points therefore counteracting this assertion. It is unclear why these anomalies exist, however it may be speculated that a significant inhibitory response is required acutely in order to gain a significant increase after chronic treatment.

The results from this chapter appear to correspond with previous studies, which have identified the BDNF as a target gene for chronic antidepressant drug treatment (Nibuya et al, 1996; Russo-Neustadt et al, 1999; Coppell et al, 2003). Additionally, the up-regulation in BDNF mRNA levels appears to follow the time-course for the commencement of therapeutic effect from antidepressants (i.e. 10-21d; Heninger and Charney, 1987; Vaidya and Duman, 2001). Previous studies (Nibuya et al, 1996; Ying et al, 2002) have revealed significantly similar up-regulatory effects of the transcription factor CREB in proximity to that of BDNF mRNA (i.e. the DG region of the hippocampus). CREB function is augmented by the up-regulation of the cAMP system, which results in the phosphorylation of CREB by PKA (Meyer and Haebner, 1993; Ghosh and Greenberg, 1995). Activation of the cAMP cascade may occur as a result of increased excitatory monoamine receptor (i.e. 5-HT_4, 5-HT_{5A}, 5-HT_6, 5-HT_7, βNA)

activation due to prolonged increases in extracellular monoamine concentration (Duman, 1998; Russo-Neustadt et al, 2003). This has suggested a common series of signal transduction events, resulting in the increase in BDNF mRNA expression in the hippocampus. Indeed, culture studies have indicated that activation of cAMP increases BDNF mRNA expression (Tao et al, 1998; Shieh et al, 1998) and identified a CRE binding element within the promoter region of the BDNF gene *(See Section 1.5.2)*. The PI pathway also may be activated by prolonged 5-HT_2, α_1 NA receptor stimulation, which also has been shown to target CREB *(See Section 1.5.3)*. CREB may therefore represent a common target for various antidepressant drug types (Duman et al, 1997; 1999; Shelton, 2000).

5.4.3. The effect of chronic antidepressant administration on BDNF exon mRNA

Chronic antidepressant administration produced differential effects among the various BDNF transcripts tested in the DG area of the hippocampus at 24h. Chronic administration of the SSRI fluoxetine and the MAOI TCP resulted in significant increases in exon I mRNA expression, however no significant increase was seen after chronic desipramine treatment. In

contrast, no effect was seen in exon IV mRNA in any area of the hippocampus after any drug tested. These findings contrast those found after acute administration of various antidepressants at 4h, where exon IV mRNA was significantly altered (inhibited) and exon I mRNA was unchanged *(See Chapter 3)*. This may therefore suggest the differential usage of the variable exon transcripts in response to differing antidepressant interventions at different time points, which contribute to the overall change in total BDNF mRNA.

It has been suggested that altered transcription of exon I and II mRNA is regulated by intervening protein synthesis mechanisms (Lauterborn et al, 1996) and influenced by calcium/calmodulin-dependent protein kinase activation (Murray et al, 1998). Facilitation of such mechanisms has been implicated in the function of chronic antidepressant treatment. Indeed, a cAMP-response element has been identified in the promoter region of exon I sequence (Sheih et al, 1998; Tabuchi et al, 2002). Using a similar treatment regimen to the current study (7.5mg/kg; 20d, once daily), Russo-Neustadt et al (2000) also found a similar up-regulatory effect in exon I mRNA after chronic TCP treatment in the DG region at 24h. This may suggest a pivotal role for

exon I mRNA transcription in the up-regulatory response of antidepressants on the BDNF gene and may reflect the initiation of a stable long-term change in neurotransmission. Exon IV-containing transcripts, which were predominantly affected by acute antidepressant administration at 4h, were unaffected by chronic antidepressant administration at 24h. Regulation of exon III and IV mRNA is not protein synthesis-dependent, they are thought to share properties with IEGs. Thus, time course studies indicate that exon III and IV mRNAs are changed more rapidly and maximal or minimal levels are attained earlier than exon I and II mRNAs (Lauterborn et al, 1996). As the chronically-treated groups were assessed 24h after the last injection, any acute alteration in exon IV mRNA transcription may have elapsed, with levels returning to normal.

A difference in the effects of chronic SSRI fluoxetine or the non-selective MAOI TCP occurred with the noradrenergic TCA desipramine. Though chronic desipramine administration significantly up-regulated total BDNF mRNA levels in the DG, a corresponding significant up-regulation in exon I transcript mRNA was not seen (though an upward trend was recorded). The net BDNF expression depends on the cumulative

effect of the multiple alternative exon-containing transcripts (Timmusk et al, 1993; Nakayama et al, 1994; Bishop et al, 1997). This may therefore implicate the up-regulation of either exon II and/or exon III in the DG after chronic desipramine treatment, which contributes to the overall increase in total BDNF mRNA. As the present study was unable to reliably measure exon II or exon III-containing transcripts, it was not possible to confirm which exon primarily contributed to the overall effect. However, the findings may represent the differential use of BDNF exon transcripts in response to the chronic administration of varying types of antidepressant drugs. In addition, there was no induction of any BDNF exon transcript in frontal cortex and striatal areas (data not shown).

In conclusion, the chronic administration of three antidepressant drugs each resulted in the up-regulation of BDNF mRNA in the DG region of the hippocampus at 24h. Chronic TCP administration also up-regulated BDNF mRNA in the CA3 region. Chronic treatment with fluoxetine or TCP also significantly up-regulated exon I mRNA levels. Chronic fluoxetine, TCP or desipramine administration had no effect on exon IV mRNA in any area of the hippocampus at 24h.

There was no induction in BDNF, BDNF exon I or BDNF exon IV in either striatal or frontal cortex areas (data not shown).

Chapter Six

The Effect of Electroconvulsive Shock on BDNF mRNA in the Rat Hippocampus

6.1. Introduction

Chapters three and five outlined the bi-phasic effect of antidepressant drugs on total BDNF mRNA in the rat hippocampus, whereby acute antidepressant drug treatment inhibited BDNF mRNA in the CA3 and DG of the hippocampus at 4h. Whereas chronic, but not acute treatment, up-regulated BDNF mRNA in the same areas at 24h. Additionally, it was shown that these changes are achieved by the differential use of the variable exon-containing transcripts. The acute down-regulatory response appeared to be mainly due to the inhibition in the immediate early gene-like (Lauterborn et al, 1996; 1998) exon IV mRNA transcription. The chronic up-regulatory response however, appeared to be due to the comparable increase in the protein synthesis dependent exon I transcription, changes in expression of which has been shown to be protein synthesis dependent (Lauterborn et al, 1996; 1998).

Electroconvulsive therapy (ECT) is considered to be one of the most effective forms of therapy available for severe types of depression (Greenberg et al, 1988; Fink, 1990; Mann, 1998). Like antidepressant drug treatment however, ECT requires the induction of a series of convulsions over

a period of weeks and the mechanisms behind its efficacy remain ambiguous (Greenberg et al, 1988; Fink, 1990).

6.1.1. The effect of ECS treatment on central monoamine function

Several studies have reported marked effects on both 5-HT and NA function after chronic application of ECS. Repeated ECS in rodents was found to produce a significant increase in 5-HT_2 receptor binding in the cortex and hippocampus. These changes corresponded with behavioural alterations indicative of such a change ('head twitch behaviour'; Green et al, 1983; Goodwin, 1984). Repeated ECS application has also been shown to increase 5-HT_{1A} receptor mRNA and binding site densities in the DG (Hayakawa et al, 1994; Burnet et al, 1995). An increase in interstitial 5-HT concentration in the hippocampus has been recorded in microdialysis studies after chronic ECS application (Zis et al, 1992). This facilitation in 5-HT release appears to be as a result of neuronal activation as such an increase is blocked by the sodium channel blocker tetrodoxin (Zis et al, 1992).

It has been demonstrated that locus coeruleus (LC) electrophysiological activity is decreased after chronic ECS

treatment (Grant and Weiss, 2001), despite elevation in tyrosine hydroxylase (TH) activity in the LC area (Weiner et al, 1991; Kapur et al, 1993; Brady et al, 1994), though decreases in TH immunoreactivity activity have also been recorded in the LC area (Nestler et al, 1990). It has been suggested that this inhibition in activity may be due to stimulation of somatodendritic α_2 adrenoceptors on LC neurones (Cedarbaum and Aghajanian, 1976; Aghajanian and Vandermaelen, 1982; Simson and Weiss, 1987). Thus, the elevation in interstitial NA levels may result in an increase in stimulation of such receptors, which will inhibit depolarisation of LC neurones. 5-HT receptors have also been found on LC cell bodies (Pickel et al, 1997). These are also thought to be inhibitory in nature (Segal, 1979), thus increases in interstitial 5-HT levels arising from ECS application may also inhibit LC neuronal depolarisation. Interestingly, Teppet et al (1992) found a decrease in responsiveness of somatodenritic α_2 adrenoceptor of LC NA neurones to the inhibitory effect of intravenous clonidine after repeated ECS, which may suggest an adaptive response to ECS. However, the desensitising effect was also seen after acute ECS, which does not produce a therapeutic response in humans.

6.1.2. The effect of ECS treatment on receptor-coupled signalling cascades

Seizure is known to produce dramatic and diverse effects on a variety of signalling components including neurotransmitters (Nomikos et al, 1991; Zis et al, 1991; 1992; McGarvey et al, 1993; Stenfors et al, 1995; Zetterström et al, 1998; Gur et al, 2002), neurotransmitter receptors (Burnet et al, 1999; Ishihara et al, 2001; Gur et al, 2002; Dremencov et al, 2003), G-proteins (Ozawa and Rasenick, 1991), protein kinases (Nestler et al, 1989) and transcription factors (Nibuya et al, 1995; Jeon et al, 1997), potassium channels (Pei et al, 1997) and structural proteins (Pei et al, 1998). The similarities in many key components activated by both chronic antidepressant treatment and ECS has suggested that prolonged ECS and antidepressant application may activate similar cascades, leading to a common therapeutic response. Chronic ECS has been demonstrated to augment the cAMP signalling system at various levels. The coupling of stimulatory G-proteins to adenylate cyclase is increased (Ozawa and Rasenick, 1991), as well as the expression of cAMP phosphodiesterase (Sattin, 1971; Lust et al, 1976; Clarenbach et al, 1978). ECT has been demonstrated to result in an increase in

plasma and urine cAMP concentration in depressed patients (Lykouras et al, 1990). The expression of downstream targets of cAMP, including PKA (Nestler et al 1989) and CREB (Nibuya et al, 1995; 1996) are enhanced by ECS application. Seizure induction is known to have widespread effects on the regulation of neurotrophic factors. Chemically- and electrically- induced seizures elicit a dramatic up-regulation in BDNF mRNA and its receptor, trkB (Zafra et al, 1991; Ballarin et al, 1991; Gall et al, 1991; Nibuya et al, 1995; Zetterström et al, 1998).

This chapter aims to elaborate on the effect of acute and chronic ECS treatment on the BDNF gene, focussing on the effect on individual BDNF transcript (exon I and exon IV) mRNA as well as on the whole gene.

6.2. Methods

6.2.1. ECS application

The protocol used for ECS application is listed in *Section 2.1.1.3.*

Animals were sacrificed 4h after acute ECS application and 24h after chronic application. The time-points were chosen to correspond with the antidepressant drug treatment protocols *(See Chapters 3 and 5).*

6.3. Results

6.3.1. Effect of acute ECS on total BDNF mRNA

Acute ECS elicited more than a four-fold increase in total BDNF mRNA in the DG (385.4%; $p<0.001$) at 4h, compared with the sham treated animals. However, CA1 and CA3 levels were not significantly altered *(Table 6.1; Figures 6.1, 6.3)*.

Exon I mRNA levels increased three-fold in the DG (221.5%; $p<0.001$) after acute ECS treatment at 4h, no significant changes were seen in the CA3 or CA1 areas *(Table 6.1; Figures 6.1, 6.3)*.

Acute ECS resulted in over a five-fold increase (446.0%; $p<0.001$) in DG exon IV mRNA levels at 4h, no significant changes were seen in CA1 or CA3 levels *(Table 6.1; Figures 6.1, 6.3)*.

6.3.2. Effect of chronic ECS on total BDNF mRNA, exon I mRNA and exon IV mRNA

Chronic ECS treatment resulted in an increase in whole BDNF gene mRNA in the DG at 24h after the last shock (144.3%; $p<0.001$). In comparison, BDNF mRNA levels were not significantly altered in CA1 and CA3 areas *(See Table 6.2; Figures 6.2, 6.4)*.

Exon I mRNA was significantly increased in the DG (77.4%; $p<0.01$), 24h after chronic ECS administration. No change was seen in CA1 or CA3 areas *(See Table 6.2; Figures 6.2, 6.4)*.

Chronic treatment had no effect on exon IV mRNA in the DG at 24h, in addition to CA1 and CA3 *(See Table 6.2; Figures 6.2, 6.4)*.

***Table 6.1.* Effect of acute ECS treatment on total BDNF mRNA, exon I mRNA and exon IV mRNA expression in the rat hippocampus 4h after shock.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (Student's *t*-test)

	CA1	**CA3**	**DG**
BDNF			
Acute Sham n=4	100±11.4	100±10.1	100±17.0
Acute ECS n=4	110.5±4.2	91.9±4.5	485.4±14.2***
Exon I mRNA			
Acute Sham n=4	100±8.1	100±8.1	100±12.4
Acute ECS n=4	123.4±5.6	100.3±6.8	321.5±10.8***
Exon IV mRNA			
Acute Sham n=4	100±14.4	100±17.3	100±21.9
Acute ECS n=4	123.2±7.6	132.4±5.6	546.0±15.0***

Table 6.2. **Effect of chronic ECS treatment on total BDNF mRNA, exon I mRNA, and exon IV mRNA expression in the rat hippocampus 24h after shock.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (Student's *t*-test)

	CA1	**CA3**	**DG**
BDNF			
Chronic Sham n=5	100±14.8	100±11.0	100±15.3
Chronic ECS n=5	79.3±11.4	77.5±17.7	244.3±11.6***
BDNF Exon I			
Chronic Sham n=5	100±6.8	100±5.4	100±12.4
Chronic ECS n=5	82.3±12.5	92.5±9.6	177.4±10.4**
BDNF Exon IV			
Chronic Sham n=5	100±12.7	100±12.1	100±16.4
Chronic ECS n=5	76.5±17.7	92.4±19.8	78.1±8.4

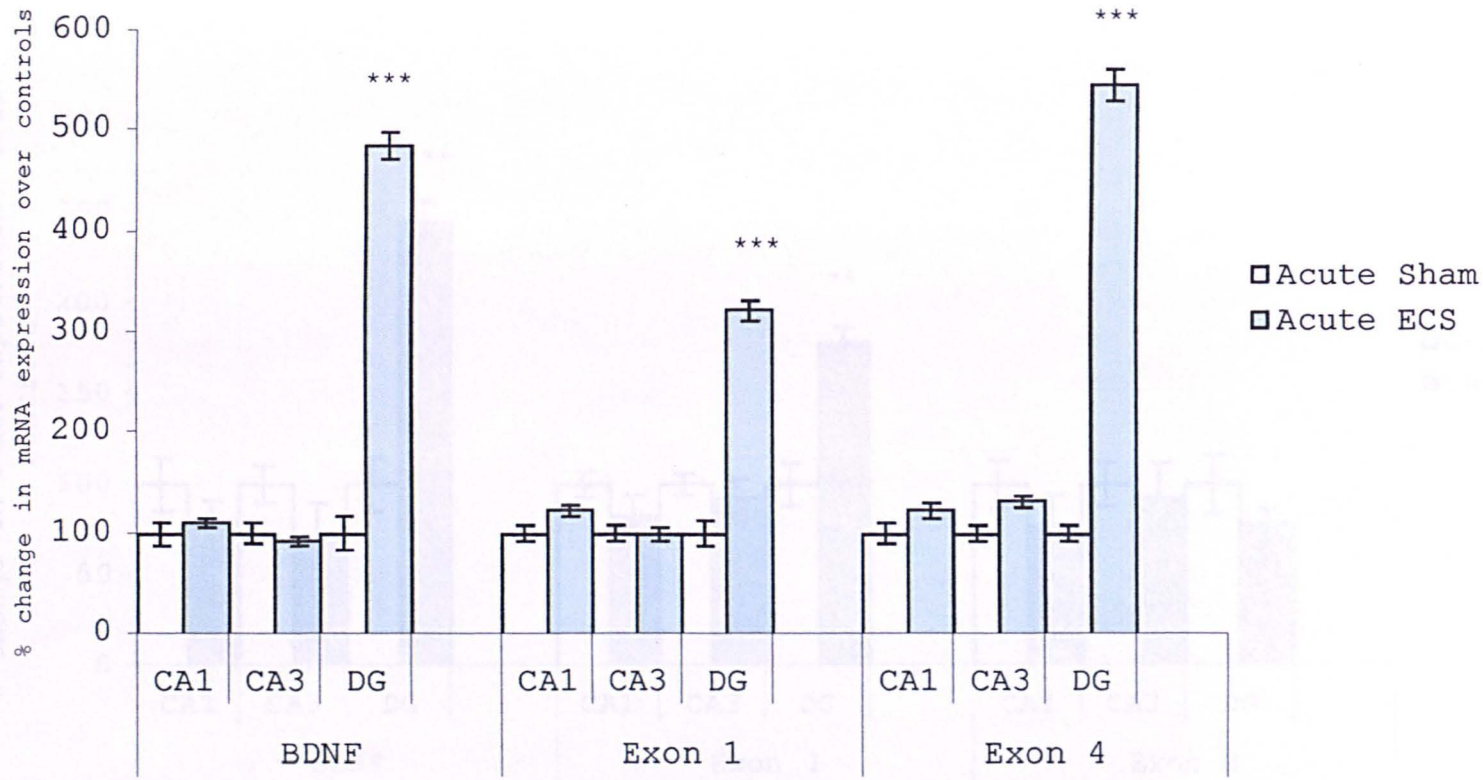

Figure 6.1. **Effect of acute ECS treatment on total BDNF mRNA, exon I mRNA and exon IV mRNA expression in the rat hippocampus 4h after administration.** Data presented as percentage of control **p<0.001, *p<0.05 compared to control (Student's *t*-test)

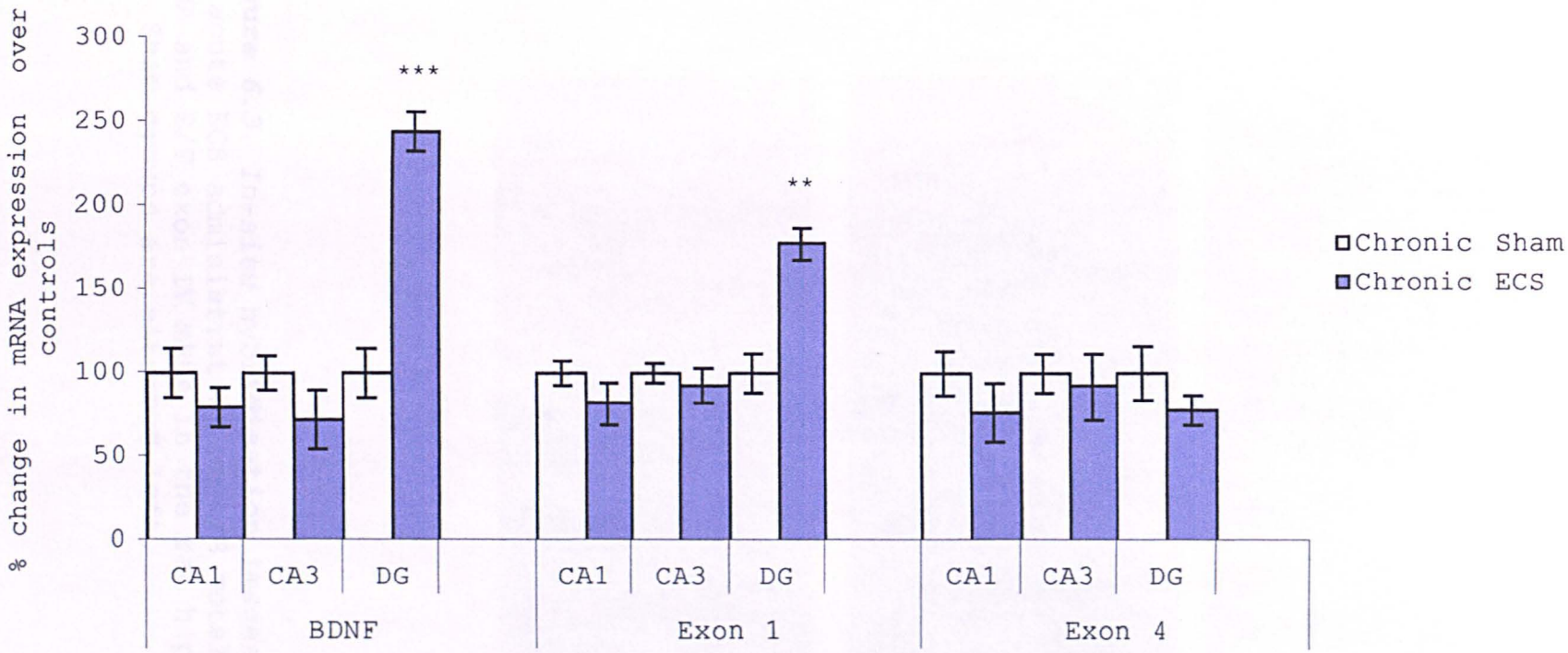

Figure 6.2. **Effect of chronic ECS treatment on total BDNF exon mRNA expression in the rat hippocampus 24h after administration.** Data presented as percentage of control **p<0.001, *p<0.05 compared to control (Student's *t*-test)

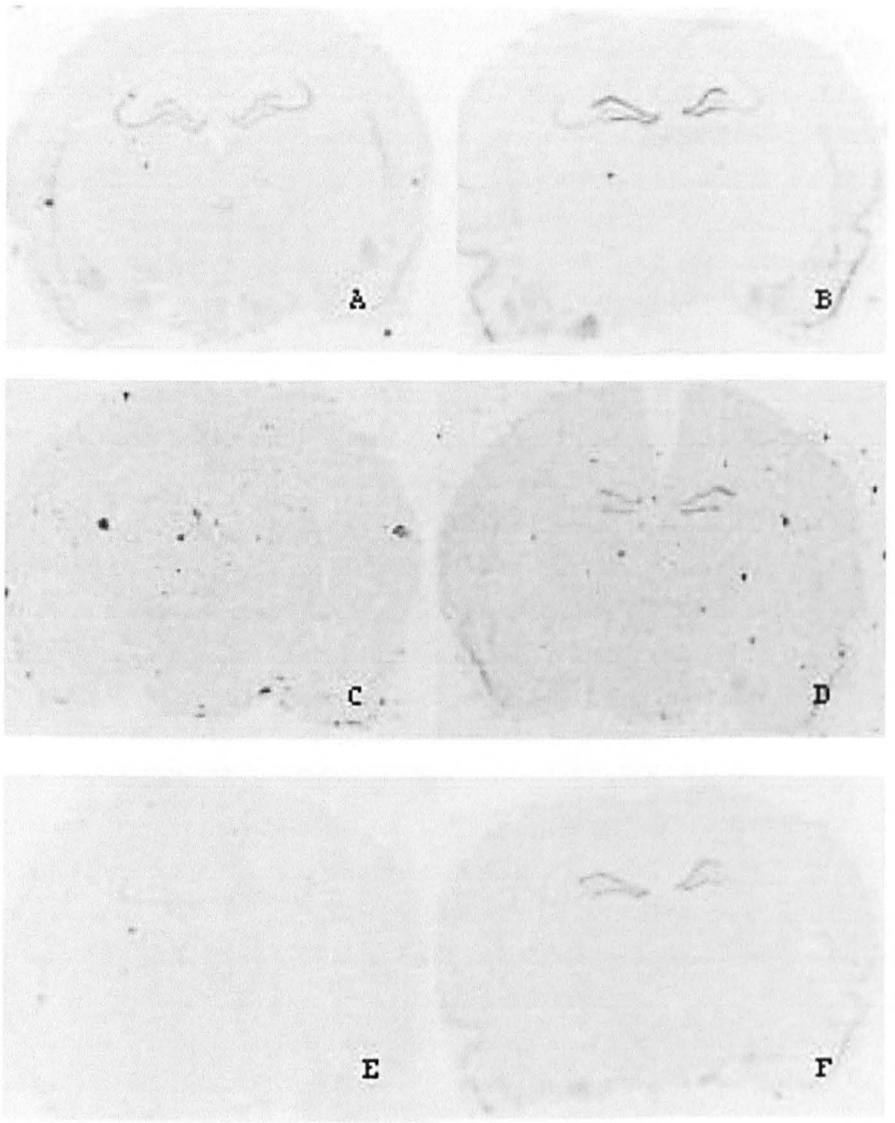

Figure 6.3. *In-situ* hybridisation images showing the effect of acute ECS administration on A/B total BDNF, C/D exon I mRNA and E/F exon IV mRNA in the rat hippocampal region at 4h. Sham groups are situated left.

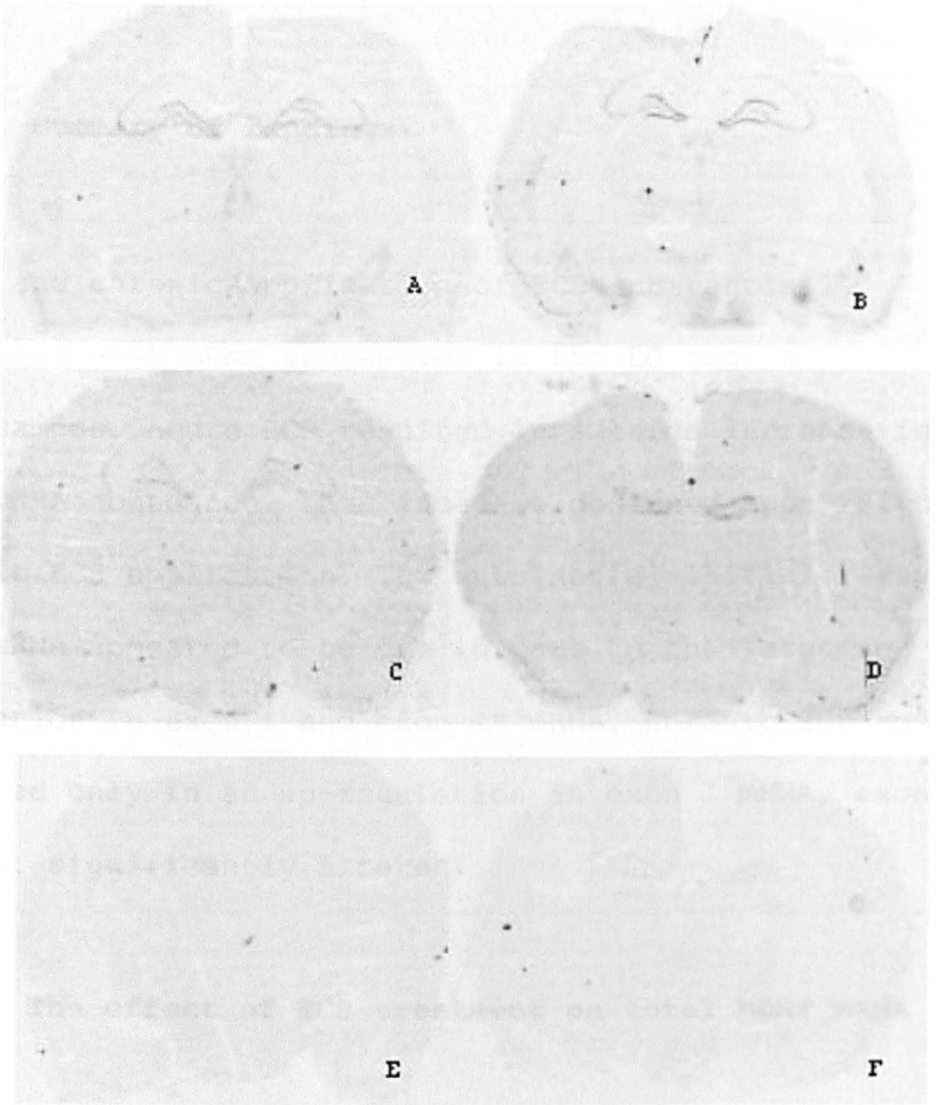

Figure 6.4. *In-situ* hybridisation images showing the effect of chronic ECS administration on A/B total BDNF, C/D exon I and E/F exon IV mRNA in the rat hippocampal region at 24h. Sham groups are situated left.

6.4. Discussion

6.4.1. Summary of findings

Acute and chronic application of ECS substantially increased BDNF mRNA expression in the DG region of the hippocampus. Acute ECS resulted in a large increase in DG BDNF mRNA abundance, this increase declined upon further chronic ECS application. The substantial initial rise in BDNF mRNA appeared to be due in part to the large up-regulation in exon I and exon IV mRNA. Chronic ECS however, resulted only in an up-regulation in exon I mRNA, exon IV was not significantly altered.

6.4.2. The effect of ECS treatment on total BDNF mRNA

In contrast to antidepressant drug treatment, where a bi-phasic BDNF mRNA response was found (an initial acute down-regulatory effect followed by up-regulation after chronic treatment), both acute and chronic ECS up-regulated BDNF mRNA in the DG. Similar rapid facilitation of BDNF mRNA has been shown in response to numerous seizure paradigms including kainic acid-induced seizure (Zafra et al, 1990;

1991), kindling stimulation (Ernfors et al, 1991; Kokaia et al, 1994; 1996; Sato et al, 1996), electrolytic lesions (Isackson et al, 1991), as well as ECS (Nibuya et al, 1995; Zetterström et al, 1998a). However the up-regulation in total BDNF mRNA after acute ECS was transient, with levels returning to controls within 24h (Zetterström et al, 1998a).

The mechanisms behind the rapid induction of BDNF after seizure are unclear. However, as mentioned earlier *(Section 4.4)*, BDNF is acutely regulated by neuronal activity and interplay between glutamate and GABA transmission (Zafra et al, 1991; 1992; Bonanno et al, 1998). ECS has been shown to produce a long-term 'LTP-like' enhancement in neurotransmission in the DG (Stewart et al, 1994; Burnham et al, 1995). This effect has also been demonstrated after kindling stimulation. The kindling phenomenon, where sub-convulsive electrical stimulations become convulsive upon chronic application, has been associated with enhancement in the potency of glutamate at its respective receptor. Thus, kindling seizures have been shown to produce substantial change within NMDA receptor subunits (Kraus et al, 1994; Vezzani et al, 1995; Watkins et al, 1998). Activation of NMDA receptor subtypes leads to the

enhancement of BDNF mRNA levels (Zafra et al 1990; 1991). In addition, stimulation of DG granule cells that evoke LTP has been shown to enhance BDNF mRNA in the same cells (Castren et al, 1993; Dragunow et al, 1993). Such increases appear to be also influenced by non-NMDA glutamate receptors, as the non-NMDA receptor antagonist CNQX blocks the ECS-induced increase in BDNF mRNA in the hippocampus (Zetterström et al, 1998b).

Acute ECS application has been demonstrated to elicit immediate early gene (IEG) induction. Expression of *c-fos* is rapidly increased after acute ECS in the hippocampus and cortex (Cole et al, 1990; Winston et al, 1990). The induction of *c-fos* has been shown to be a reliable marker of neuronal activity (Morgan et al, 1987; Sagar et al, 1988), thereby indicating hippocampal and cortical activity after acute ECS application. Interestingly, acute ECS induction of *c-fos* has been shown to be blocked by the NMDA antagonist MK801 in the hippocampus, but not several other neurotransmitter receptor antagonists (D_1, $5\text{-}HT_{2A/2C}$, α_1NA, βNA; Morinobu et al, 1997), implicating a role for acute glutamatergic activity in the hippocampus in response to acute ECS. The induction of BDNF mRNA after seizure has also been shown to be dependent on Ca^{2+} signalling and

activation of calmodulin-dependent protein kinases (Bading et al, 1993; Lerea and McNamara, 1993; Murray et al, 1998) and BDNF mRNA is regulated in a Ca^{2+}/calmodulin-dependent manner *in vitro* (Murray et al, 1998). In contrast, a fast decrease in hippocampal GABA function has been shown after seizure (kainic acid-induced) at a pre-synaptic level, resulting in a decrease in inhibitory postsynaptic potentials (IPSPs; Zafra et al, 1991). Such an effect could be considered the opposite to the effect demonstrated in *Chapter 4*, where the acute administration of the $GABA_B$ agonist baclofen resulted in the rapid decrease in BDNF mRNA expression in the hippocampus *(See Section 4.3.1)*.

The up-regulatory effect on BDNF mRNA from acute ECS is not consistent with the time-course for the therapeutic action of ECT. Therefore it seems that up-regulation of BDNF mRNA alone is not sufficient to bring therapeutic response in humans, it appears that sustained elevation from chronic treatment may be necessary for eventual efficacy. ECS has been reported to up-regulate the cAMP signalling system at several levels (Ozawa and Rasenick, 1991), suggesting prolonged cAMP cascade activation may underlie the therapeutic mechanism. As explained in *Chapter 5*, various 5-HT and NA receptor subtypes are linked to the cAMP

systems via G-protein activation. Microdialysis studies have revealed enhanced release of both 5-HT (Zis et al, 1992) and NA (Thomas et al, 1991; 1992) after acute and chronic ECS application. Additionally, adaptive changes in monoamine receptor number and sensitivity have been demonstrated. Post-synaptic 5-HT_{1A} receptor mRNA up-regulation has been found in DG granule cells following chronic ECS (Burnett et al, 1999) and decreased sensitivity has been reported among 5-HT_{1A} receptors to agonist compounds (Gur et al, 2002). Down-regulation in β-adrenoreceptor number has also been observed (Nutt et al, 1989; Seo et al, 1999). As with chronic antidepressant drug treatment, persistent increases in monoamine concentration from chronic ECS application may activate similar downstream components. One possible converging target point for both ECS and antidepressant drug treatment may be the transcription factor CREB. CREB levels are increased in the hippocampus by ECS (Nibuya et al, 1996; Vaidya and Duman, 1998) and antidepressant drug treatment (Nibuya et al 1996 Duman et al, 1997, 1999; Thome et al, 2000). CREB has been associated as a transcription factor for the BDNF gene (Nibuya et al, 1996; *See Section 5.1*).

Overall, it appears that glutamate may be a likely mediator of BDNF mRNA induction after acute ECS induction, however the maintenance of BDNF mRNA elevation after chronic ECS appears more complex. The prolonged facilitation of monoamine receptor-linked signalling cascades, resulting in CREB induction may lead to longer-term BDNF augmentation and may possibly underlie ECS therapeutic action.

6.4.3. The effect of ECS on individual BDNF exon mRNA

The induction of acute and chronic ECS produced differential effects on the transcription of exon I and IV mRNA in the DG region of the hippocampus. Acute ECS resulted in an up-regulation in both exon I and IV mRNA. Exon IV-containing mRNAs have been shown to share properties with IEGs (Lauterborn et al, 1996; 1998). Therefore changes in exon IV mRNA may have been expected after 4h, reflecting the overall change in BDNF. However, the induction of exon I mRNA has been shown to be dependent on ongoing Ca^{2+}/calmodulin protein synthesis mechanisms (Lauterborn et al, 1996; 1998) and displays a delayed induction process (Kokaia et al, 1994). It is therefore surprising that the response in exon I mRNA is evident after only 4h post-injection. Acute ECS results in a

massive depolarising effect on granular cells of the DG, resulting in Ca^{2+} influx into neurones. Several studies have indicated that activation of L-type voltage-sensitive Ca^{2+} channels can lead to a transient increase in exon I (as well as exon II and III) containing transcripts (Timmusk et al, 1993; Metsis et al, 1993; Tao et al, 1998), increases may take place as early as 3h after acute ECS application (Tao et al, 1998). Indeed, putative calcium-responsive elements have been detected within the promoter region of exon I, it may be a possibility therefore that fast exon I transcription may occur in response to rapid depolarising stimuli, such as from acute ECS.

As with the effect acute antidepressant drug treatment, alteration in exon IV mRNA transcription mirrored the change to total BDNF mRNA. This suggests a primary role for exon IV transcripts in the BDNF mRNA response to acute stimuli. Like chronic antidepressant drug treatment, chronic ECS application resulted in increases in exon I mRNA, but not exon IV mRNA in the DG region of the hippocampus. This further implicates exon I activation in long-term interventions involving intervening protein synthesis mechanisms.

In conclusion, the effect of acute application of ECS on total BDNF mRNA and two individual exon transcripts differs from that of acute antidepressant drug treatment. Unlike acute drug treatment where total BDNF mRNA as well as exon IV mRNA were inhibited, acute ECS up-regulated total BDNF mRNA as well as both exon I and IV mRNA. As the up-regulatory effect on BDNF mRNA from acute ECS is not consistent with the time-course for the therapeutic action of ECS it appears that that up-regulation of BDNF mRNA alone is not sufficient to bring therapeutic response.

Chapter Seven

The Effect of Chronic Antidepressant Treatment on BDNF Immunoreactivity in the Rat Hippocampus

7.1. Introduction

The majority of studies relating to the regulation of BDNF after chronic antidepressant treatments have been based on the analysis of BDNF mRNA and not on the corresponding protein product. This has been due in part to technical limitations, such as lack of sensitive tissue assays for BDNF protein, as well as difficulties in quantifying (Altar et al, 2003). However, examination of protein expression is vital in order to elucidate the functional properties of BDNF. In addition, its localisation in relation to BDNF mRNA alludes to how the protein is synthesised and transported.

7.1.1. Basal distribution of BDNF protein in the rat brain

Previous animal (Conner et al, 1997; Yan et al, 1997) and human studies (Iritani et al, 2003) using similar immunocytochemistry (ICC) strategies to those in this chapter have revealed distinct BDNF immunoreactivity in several structures of the brain. These included the frontal and parietal cortices, as well as the hippocampus (Yan et al, 1997; Conner et al, 1997). Within the hippocampal formation, relatively dense staining has been found in the

CA3, particularly in the mossy fibre zone (MFZ), which connects granule cells of the DG with the CA3 pyramidal cells (Conner et al, 1997). Less basal staining has been reported in the CA3 area, and DG staining minimal (Conner et al, 1997, Iritani et al, 2003).

7.1.2. The effect of chronic ECS treatment on BDNF immunorectivity in the rat hippocampus

Previous studies examining the effect of chronic ECS on BDNF protein have generally found an up-wards trend in expression in several areas of the brain. A recent enzyme-linked sorbent assay (ELISA) study (Altar et al, 2003) found 10 consecutive days of ECS treatment elicited rapid, large and widespread increases in BDNF protein in the parietal cortex (219%), hippocampus (132%), frontal cortex (94%) and neostriatum (67%). These increases peaked at 15 hours after the last treatment and were sustained for a 3-day period. Another ELISA study (Angelucci et al, 2002) found increased immunoreactivity restricted to the hippocampus and frontal cortex after 8 consecutive days of ECS. However, these findings were not replicated by the same group (Angelucci et al, 2003).

Within the hippocampus, Smith et al (1997) found increased immunoreactivity localised in the hippocampal mossy fibres projecting from the DG granule cells to the CA3 pyramidal cells after chronic ECS application.

The present study used the ICC technique, with specific affinity-purified antibodies selective to BDNF, to assess the effect of chronic application of ECS within the rat hippocampus. In addition, basal levels of BDNF protein in coronal sections through three major areas of the rat brain: the frontal cortex, striatum and hippocampus were established.

7.2. Methods

Immunocytochemistry and densitometric analysis procedures are described in *Chapter 2*.

7.3. Results

7.3.1. Basal distribution of BDNF protein in the rat brain

Basal BDNF immunoreactivity was found in the hippocampus, as well as cortical and striatal areas. Significant labelling was established in the parietal *(See Figure 7.4)* and frontal cortices *(See Figure 7.5)*. Within the hippocampus, basal BDNF immunoreactivity was found in the pyramidal cells of the CA1 and CA3. However, the most intense staining appeared to be within the MFZ. No significant basal expression was seen in the granule cells of the DG *(See Figure 7.3)*. As the DG basal level was so weak the accuracy of analysis could be questioned, therefore the effect of antidepressant treatments was focussed on the CA1, CA3 and MFZ within the hippocampus.

A high level of magnification appeared to highlight BDNF protein labelling in somatic and axonal regions of cortical pyramidal neurones. Labelling appeared to be concentrated in somatic areas, which occasionally extended along axonal areas. However, under close scrutiny considerably less staining appeared to be present in central areas of the soma in many cells *(See Figure 7.4B)*.

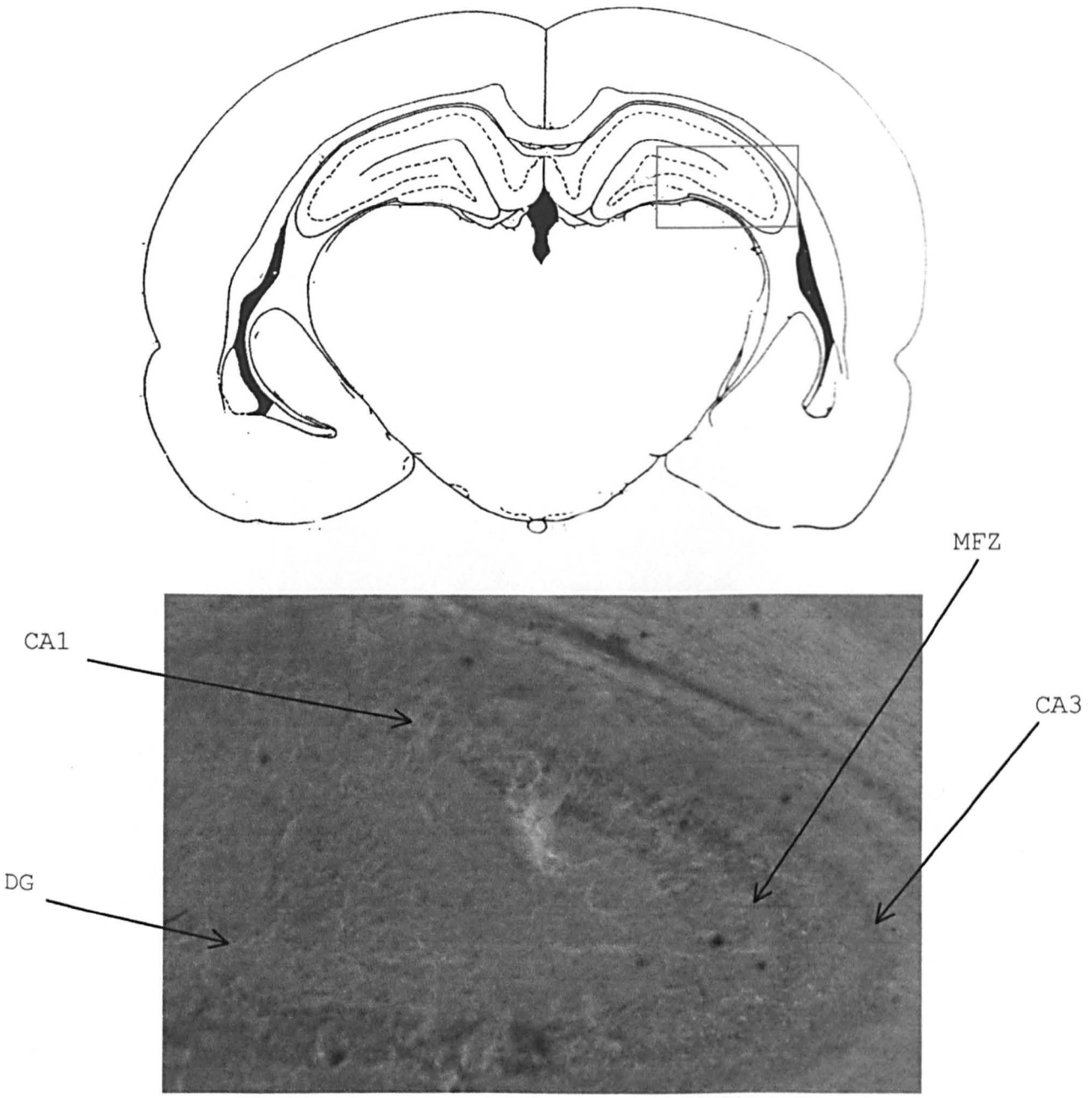

Figure 7.1. Photomicrograph showing basal expression of BDNF protein in the rat hippocampus. The anatomical location is highlighted on a schematic diagram above (Adapted from Paxinos and Watson, 1986; Plate 34).

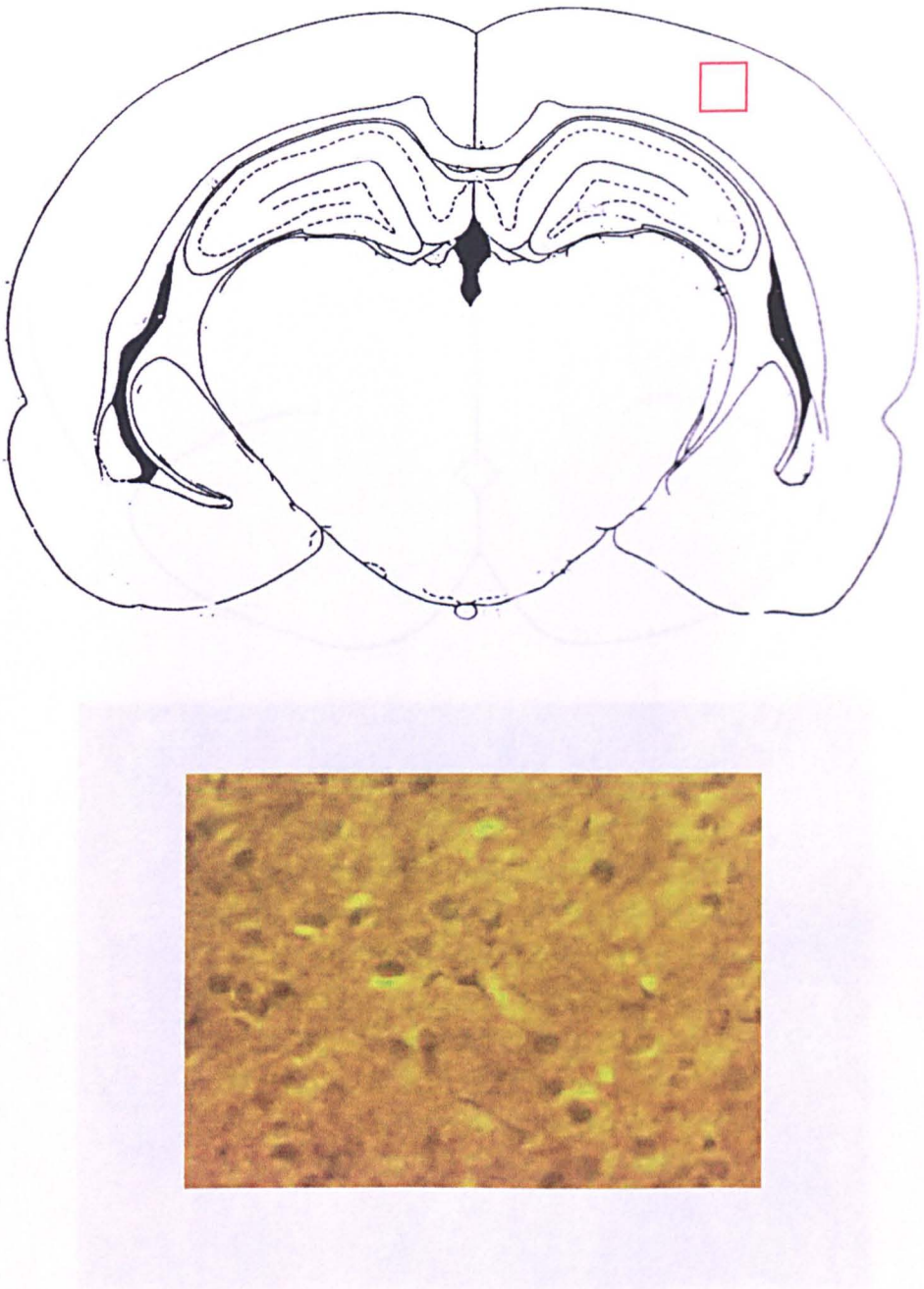

Figure 7.2. Photomicrograph showing the basal expression of BDNF protein in the rat parietal cortex. The anatomical location is highlighted on a schematic diagram above (Adapted from Paxinos and Watson, 1986; Plate 34).

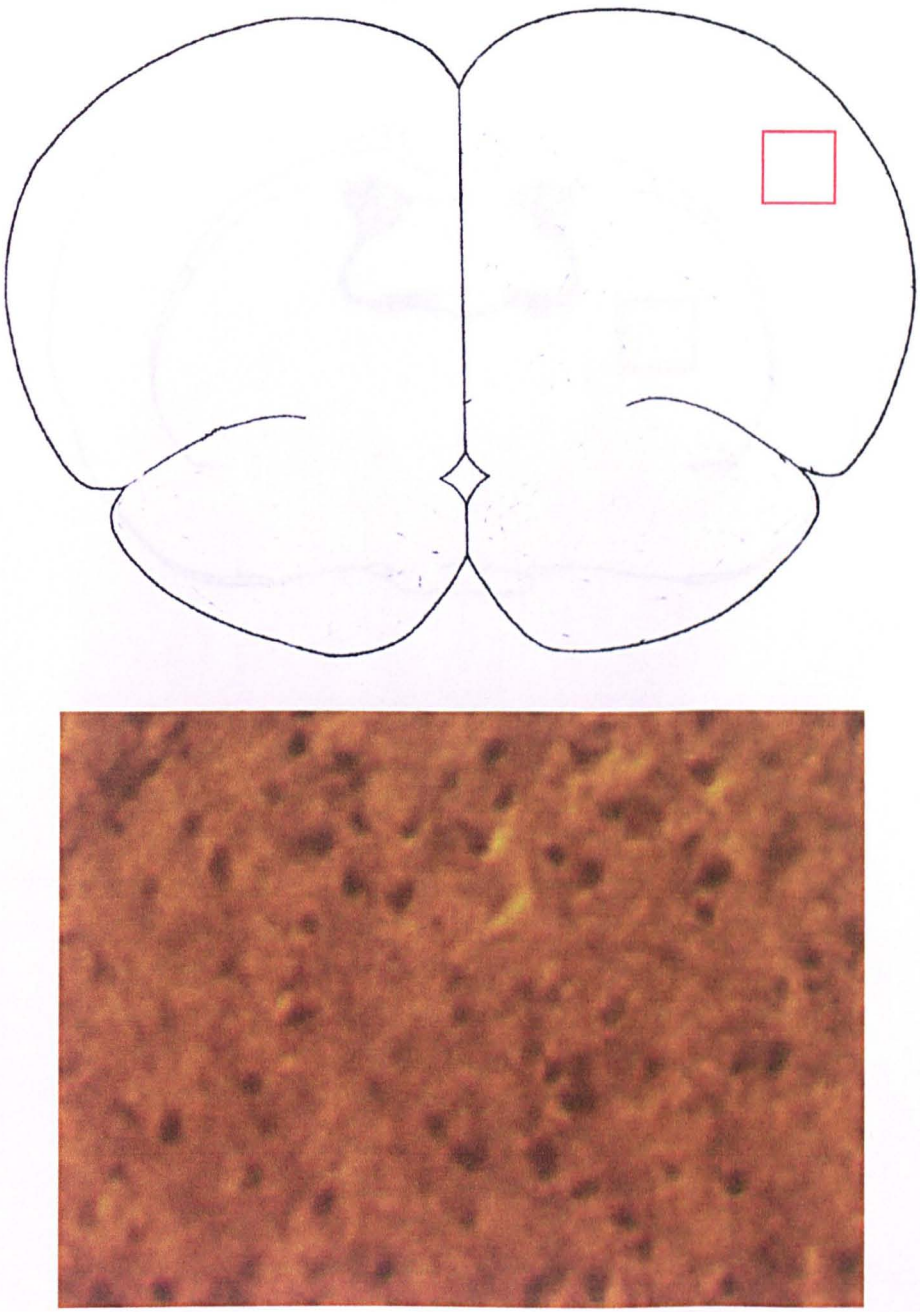

Figure 7.3. Photomicrograph showing basal expression of BDNF protein in the rat frontal cortex. The anatomical location is highlighted on a schematic diagram above (Adapted from Paxinos and Watson, 1986; Plate 8).

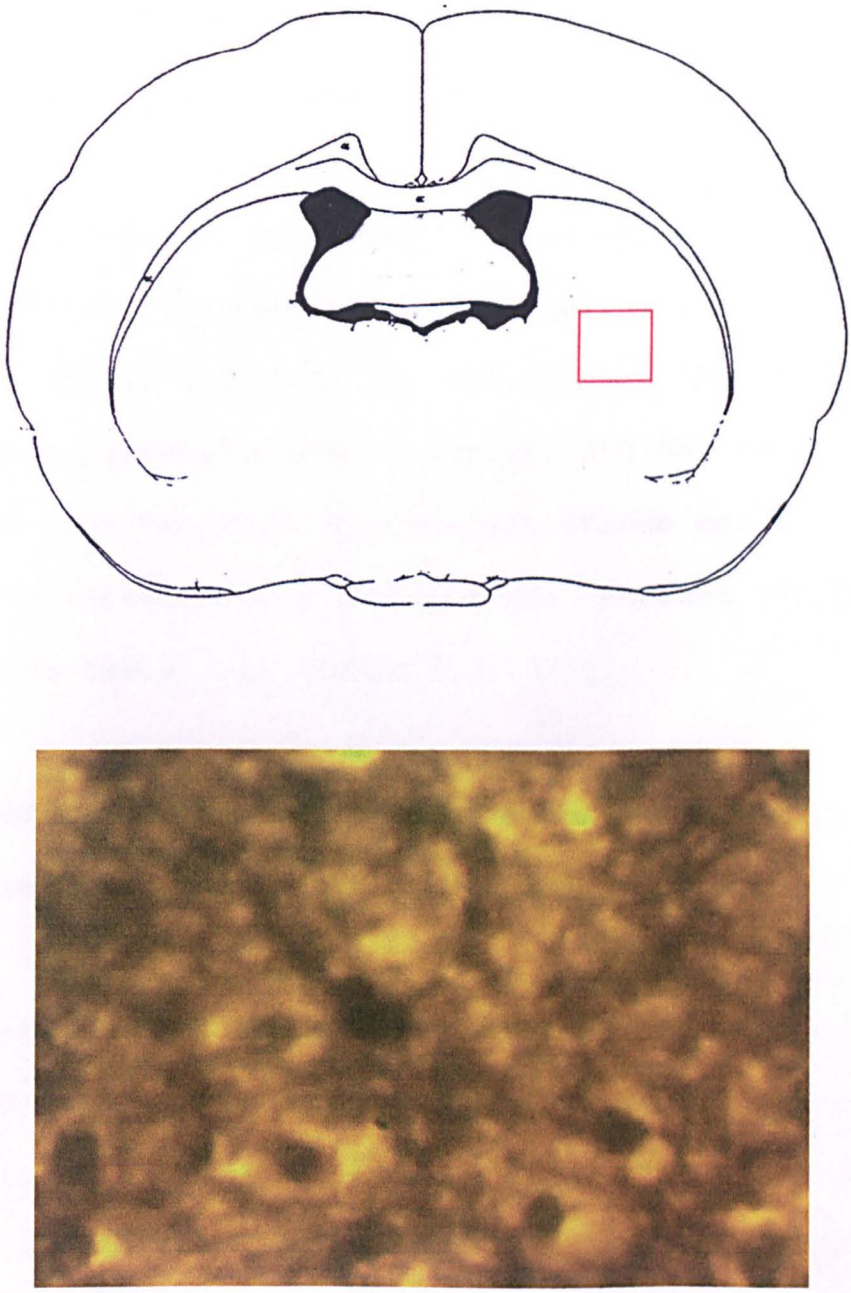

Figure 7.4. Photomicrograph showing basal expression of BDNF protein in the rat striatum (caudate putamen). The anatomical location is highlighted on a schematic diagram above (Adapted from Paxinos and Watson, 1986; Plate 22).

7.3.2. Effect of chronic ECS treatment on BDNF immunoreactivity in the rat hippocampus at 24h

Chronic application of ECS elicited significant increases in BDNF protein expression in all areas assessed over sham-treated animals. CA1 (+143.3%, $p<0.01$) and CA3 (+144.6, $p<0.05$) and parietal cortex (+166.0%, $p<0.05$) levels increased over two-fold. The largest change was in the MFZ, where over a twelve-fold increase was recorded (+1159.3%, $p<0.01$; *See Table 7.1; Figure 7.5; 7.6*).

7.3.3. Effect of chronic antidepressant treatment on BDNF immunoreactivity in the rat hippocampus at 24h

An attempt was made to measure the effect of chronic antidepressant (TCP) treatment on BDNF immunoreactivity in the rat brain. However, only a cursory examination was possible due to low sample numbers (n=2; data not shown).

Table 7.1. **Effect of chronic ECS treatment on BDNF protein expression in the rat hippocampus 24h after injection.** Raw data presented ***p<0.001, **p<0.01, *p<0.05 compared to control (Unpaired two-tailed Student's *t*-test). Units corrected for non-specific signalling over white matter (corpus callosum)

	CA1	CA3	MF	Par Ctx
Sham n=3	0.1007±0.015	0.1073±0.028	0.0133±0.0133	0.047+0.006
ECS n=4	0.245±0.027**	0.2625±0.042*	0.1675±0.032**	0.125+0.017*

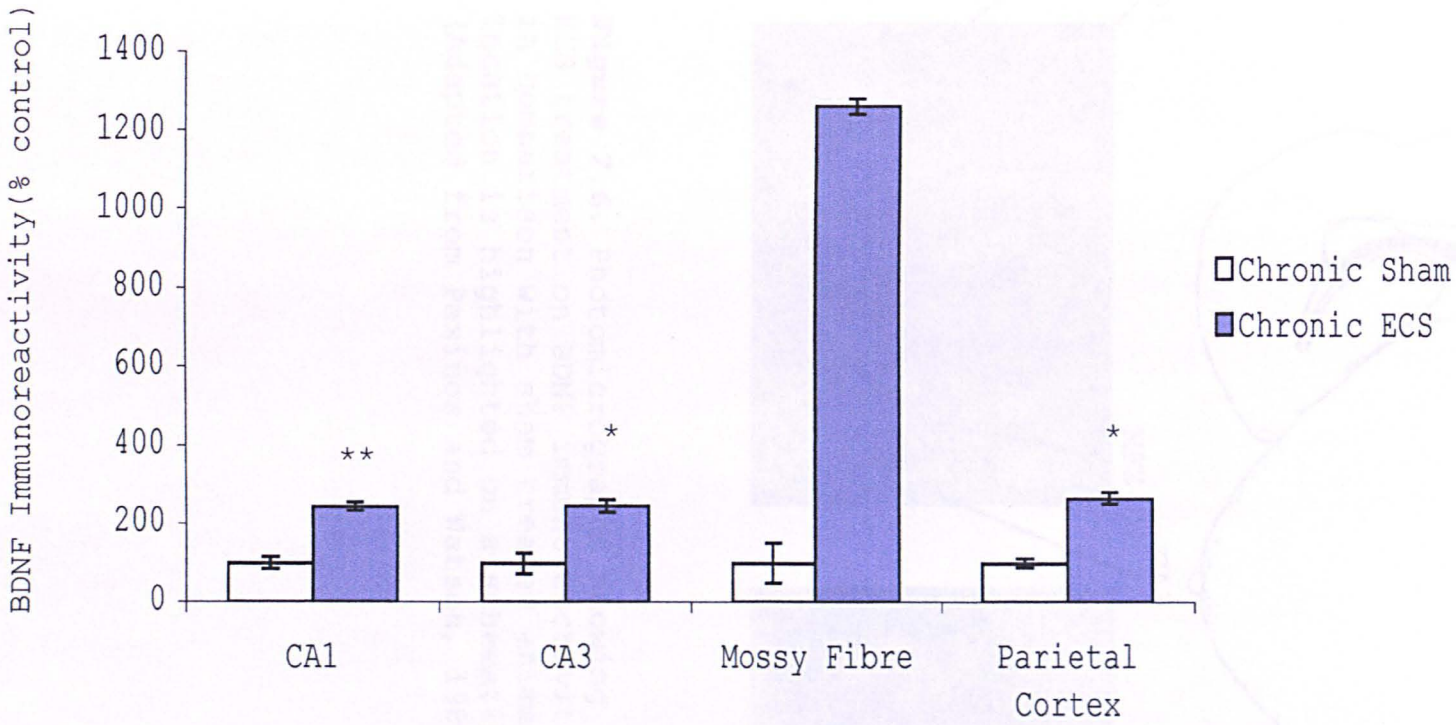

***Figure 7.5.* Effect of chronic ECS treatment on BDNF protein expression in the rat hippocampus 24h after injection.** Data presented as percentage of control ***p<0.001, **p<0.01, *p<0.05 compared to control (Unpaired two-tailed Student's *t*-test). Units corrected for non-specific signalling over white matter (corpus callosum).

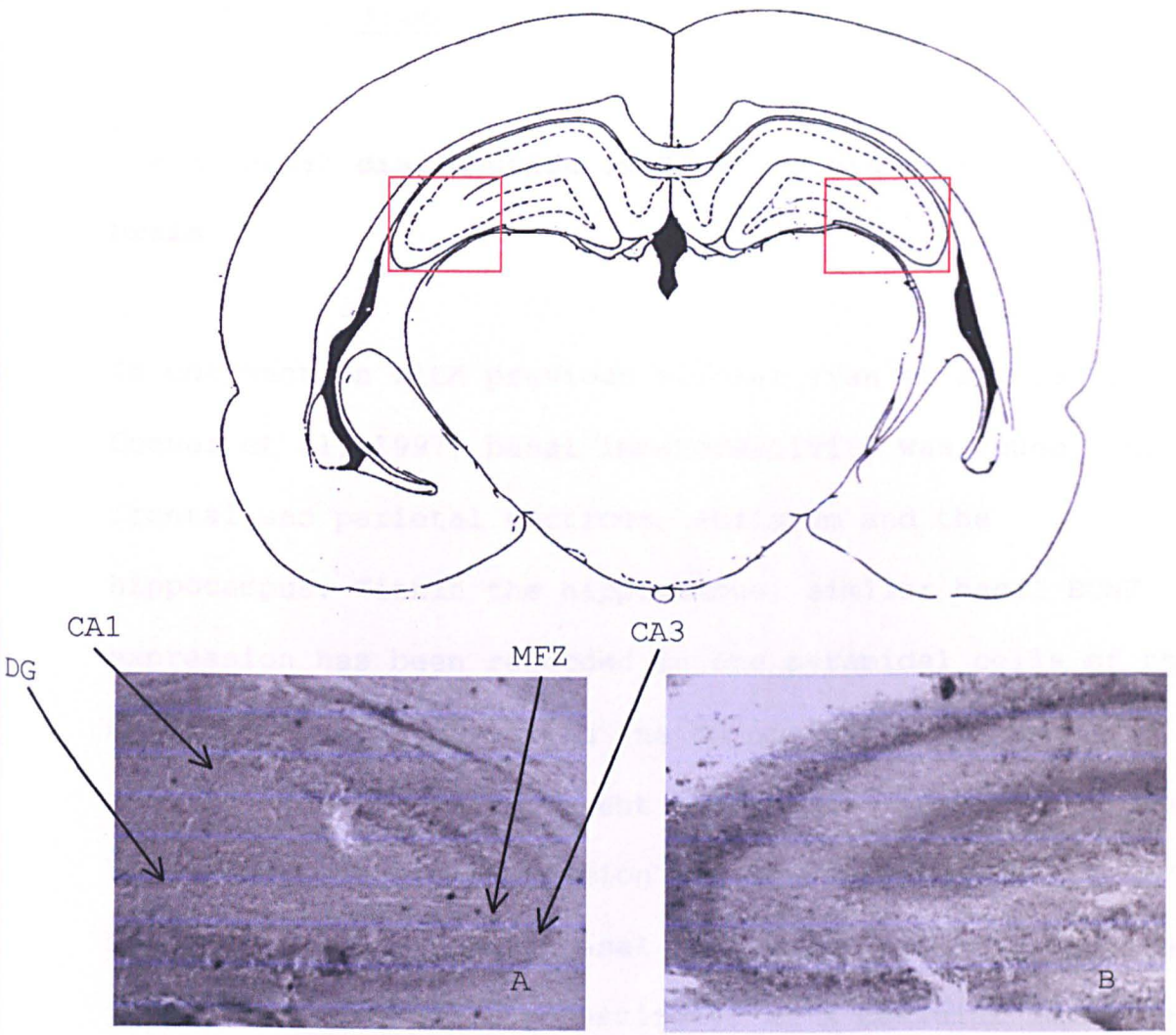

Figure 7.6. Photomicrographs showing the effect of chronic ECS treatment on BDNF immunoreactivity in the hippocampus in comparison with sham treated animal (A). The anatomical location is highlighted on a schematic diagram above (Adapted from Paxinos and Watson, 1986; Plate 33).

7.4. Discussion

7.4.1. Basal distribution of BDNF protein in the adult rat brain

In conjunction with previous studies (Yan et al, 1997; Conner et al, 1997) basal immunoreativity was found with frontal and parietal cortices, striatum and the hippocampus. Within the hippocampus, similar basal BDNF expression has been recorded in the pyramidal cells of the CA3 and the MFZ connecting the DG granule cells and the CA3. In addition, the present study also found significant basal BDNF protein expression was found in the CA1. However, no significant basal BDNF expression was detected throughout the DG. Interestingly, at a cellular level, a distinct pattern of BDNF distribution appeared to exist. A high level of immunoreactivity was present in the periphery of the soma, sometimes extending along axons, however a lack of staining was observed in the centre of the soma. This appears to confirm previous findings (Conner et al, 1997), that unlike BDNF mRNA, BDNF protein is not present within the nucleus and is localised to the cytoplasm, extending into proximal processes.

7.4.2. The effect of chronic ECS treatment on BDNF immunoreactivity

Chronic ECS treatment produced widespread, significant increase in BDNF protein in all areas assessed. Within the hippocampus, the largest increase in protein levels was seen in the MFZ. Such large increases in MFZ levels have also been recorded in a previous ICC study (Smith et al, 1997). Previous ELISA studies (Altar et al, 2003; Angelucci et al, 2002) have found significant increases in overall hippocampal BDNF protein levels.

From the data gained in this chapter, as well as the data concerning BDNF gene alterations after chronic ECS treatment, it is seems that chronic ECS administration elicits a profound effect on both BDNF mRNA and it protein product in the hippocampus. It must be stressed that considerable limitations of ICC exist from the inherent inability to quantify changes in immunoreactivity. Technique such as western immunobotting or immunoautoradiography may have provided more accurate readings (Xu et al, 2002; Altar et al, 2003).

In addition, variability exists in the localisation of alterations in BDNF gene and protein expression within the hippocampus. Basal expression of BDNF mRNA was high in granule cells of the DG, with lower expression in the CA1 *(See Section 3.3.1)*. However, basal BDNF protein level in DG granule cells was minimal, with expression present in the pyramidal cells of the CA1 and CA3, as well as the MFZ. As explained in *Section 1.7.3,* soma arising from the DG area project mossy fibres axons through the MFZ to the CA3 pyramidal layer cells. ICC visualisation studies on BDNF at a cellular level have confirmed that neurones expressing BDNF mRNA also synthesise their corresponding protein (Wetmore et al, 1991; Wetmore et al, 1994; Dugich-Djordjevic et al, 1995). The soma of such neurones have been shown to be rich in BDNF mRNA (Altar and DiStefano, 1998). BDNF protein has been shown to be mainly expressed in axonal and terminal areas (Altar et al, 1997). Thus, it has been suggested that the predominant direction for transport of BDNF is anterograde (Altar and DiStefano, 1998). Indeed, transection of the dorsal root ganglion axon has revealed accumulation of BDNF protein on the proximal side of the ligature (Tonra et al, 1998). This phenomenon may also explain the apparent lack of BDNF mRNA signal in

striatal areas, compared with rich BDNF protein labelling in the same area.

The apparent co-localisation of increases in BDNF protein after ECS application with the sprouting of the mossy fibre pathway offers a tempting hypothesis that BDNF enhancement may act as a precursor to impending structural change in areas associated with depression after antidepressant treatment. Furthermore, this analogy appears to suggest a central role for granule cells in the DG and their axons in the mossy fibre tract in the aetiopathology of depression. Indeed, stress models in rodents have been shown to induce atrophy in mossy fibres and dendritic trees of the CA3 pyramidal neurones (Popoli et al, 2002; McEwen, 1999), indicating decreases in synaptic transmission in this critical neuronal pathway (Castren, 2004). In addition, hippocampal neurogenesis has been suggested in areas similar to those where BDNF mRNA is enhanced after antidepressant treatment. Repeated administration of a variety of antidepressant drugs and ECS causes an increase in the number of Brd-U labelled cells in the hippocampus (Malberg et al, 2000; Jacobs and Fornal, 1999; Manev et al, 2001). The new cells have been shown to migrate into the granule cell layer, before extending axons along the mossy

fibre pathway to the CA3 pyramidal layer (van Praag et al, 1999). It appears that new cells exhibit similar characteristics as mature granule cells and integrate into hippocampal circuitry (Duman et al, 2001).

In conclusion, the present study found that chronic application of ECS significantly increased BDNF immunoreactivity in the CA1, CA3 and the MFZ of the hippocampus, as well as in the parietal cortex. Chronic administration of the non-selective MAOI antidepressant drug TCP failed to elicit any significant increase in these areas, however these results were very preliminary due to low group sample numbers.

Chapter Eight

General Discussion

8.1 Background

Major depression is one of the most common and devastating psychiatric disorders and is characterised by a number of mental, as well as somatic symptoms (DSM-IV™, 1997). The serendipitous discovery of antidepressant treatments in the 1950s led to the establishment of numerous theories into the pathophysiology of depression and the mechanism of action of antidepressant drugs. The most prominent of these the "monoamine hypothesis" implicated a role for noradrenaline (NA) and serotonin (5-HT) transmitter systems (Schildkraut, 1965). Most antidepressant drugs facilitate the signalling of 5-HT or NA either by inhibiting their reuptake back into pre-synaptic terminals (e.g. SSRIs, TCAs) or by inhibiting their catabolism (e.g. MAOIs). However, alterations in monoamine turnover take place quickly, within hours of drug administration, but the therapeutic antidepressant effect may take several weeks of chronic drug treatment to manifest (Nestler et al, 2002). In addition, one of the most effective forms of antidepressant treatment electroconvulsive therapy (ECT; animal model, ECS) requires several applications in order for full efficacy to occur (Fink, 1990). This suggests that alterations in monoamine transmission alone cannot explain

the efficacy of antidepressants and has led to research focusing on events beyond the monoamine receptor. The cAMP signalling cascade is regulated by several 5-HT and NA receptor subtypes. Increased levels of cAMP induces protein kinase A (PKA) activation, which in turn catalyses a variety of regulatory proteins including transcription factors such as cAMP response element binding protein (CREB). CREB mRNA and protein has been shown to be increased after chronic antidepressant treatment (Nibuya et al, 1995; 1996). CREB regulates gene transcription by binding to a cAMP response element on the regulatory region of various genes. One such target gene identified as containing such a region is brain-derived neurotrophic factor (BDNF).

The BDNF gene has multiple transcripts with unique promoters. The BDNF transcripts consist of a variable 5′ region (exons I-IV) and an invariable 3′ segment (exon V). The common 3′ exon codes for the mature BDNF protein, whereas exons I to IV arise from the differential use of four different promoters (Russo-Neustadt et al, 2000). The transcripts are differentially expressed across areas of the brain (Bishop et al, 1994) and differing stimuli has been shown to regulate the expression of distinct BDNF exon

transcripts in the brain. It has been demonstrated that exon III- and exon IV-containing transcripts share properties with immediate early genes, whereas changes exon I- and exon II-containing transcript expression is dependent on ongoing protein synthesis mechanisms (Lauterborn et al, 1996; 1998).

Several studies have demonstrated an up-regulatory effect on BDNF after chronic, but not acute antidepressant drug (Nibuya et al, 1995; Zetterström et al, 1998; Russo-Neustadt, 1999; 2000; Coppell et al, 2003) and ECS (Nibuya et al, 1995; Schmidt-Kastner et al, 1996; Zetterström et al, 1998b) treatment. However, a recent study (Coppell et al, 2003) demonstrated an apparent bi-phasic effect of antidepressant drug treatment on BDNF mRNA, involving an initial down-regulation of the BDNF gene in the hippocampus at 4h after acute and chronic treatment, and an up-regulatory effect at 24h following chronic treatment.

Against this background, this thesis aimed to study the effect of numerous antidepressant drugs as well as ECS, when given chronically or acutely, on the total BDNF gene mRNA as well as as a representative form of protein-dependent exon mRNA (exon I) and a non-protein synthesis-

dependent 'IEG-like' exon mRNA (exon IV; Lauterborn et al, 1996; Russo-Neustadt et al, 2001) in the rat hippocampus. Further research into the apparent acute inhibitory effect on BDNF mRNA at 4h was also conducted. In addition, the effect of antidepressant drug and ECS treatment on BDNF immunoreactivity was examined. A summary of the findings, ranked in perceived order of importance is listed below.

8.2. Summary of main findings

8.2.1. Differential effect of acute and chronic antidepressant drug administration on total BDNF mRNA and BDNF exon mRNA distribution in the adult rat hippocampus

One of the key findings within this body of research related to the differential effect of acute (at 4h) and chronic (at 24h) antidepressant drug treatment on BDNF mRNA within the hippocampus and the corresponding effect on the variable exon-containing transcript expression within the hippocampus. A previous study had revealed an apparent "bi-phasic effect" (Coppell et al, 2003), whereby acute antidepressant administration inhibited BDNF mRNA within the DG region of the hippocampus at 4h, whereas chronic antidepressant administration increased BDNF mRNA at 24h.

This effect was confirmed in this thesis, not only after compounds potent to 5-HT reuptake inhibition, but also from drugs with high affinity to noradrenergic/dopaminergic transporters. Furthermore, an apparent differential use of the variable exon-containing transcripts was discovered in response to acute and chronic antidepressant administration. Thus, acute administration of a variety of antidepressant drugs (as well as the non-antidepressant compounds with high affinity for the DA transporter: methylphenidate and GBR 12909), down-regulated BDNF mRNA expression within the DG region of the hippocampus at 4h, an effect which appeared to be mediated in part by an inhibition in exon IV (IEG-like) mRNA transcription, rather than exon I mRNA (protein synthesis-dependent), which was unchanged *(See Section 3.3.2)*. In contrast, chronic administration of the SSRI fluoxetine or the MAOI TCP significantly up-regulated BDNF mRNA in the DG at 24h, an effect which appeared to be mediated in part by an increase in exon I mRNA transcription, rather than exon IV, which was unchanged *(See 5.3.1)*. However, chronic administration of noradrenergic TCA desipramine significantly increased total BDNF mRNA, without a corresponding increase in exon I mRNA expression. This suggested a predominant increase in

an exon transcription in those not tested (exon II or exon III) in NA-mediated up-regulation of BDNF in the DG.

8.2.2. The acute inhibitory effect of antidepressant drugs on BDNF mRNA in the DG is influenced by $GABA_B$-mediated mechanisms

The acute administration of baclofen, but not flunitrazepam or THIP, significantly inhibited BDNF mRNA in the CA3 and DG at 4h, thus suggesting involvement of $GABA_B$, rather than $GABA_A$ receptor-mediated mechanisms. The $GABA_B$-selective antagonist CGP 46381 significantly attenuated the inhibitory effect on BDNF mRNA caused by acute baclofen or paroxetine administration in the CA3 and DG. However, CGP 46381 alone significantly increased BDNF mRNA levels in the DG region to a similar magnitude as the attenuation, suggesting the existence of an endogenous inhibitory tone by GABA on BDNF mRNA in this region. Pre-treatment with an additional $GABA_B$ receptor antagonist, CGP 55845, significantly attenuated baclofen-mediated down-regulation of BDNF mRNA expression in the DG, however it failed to attenuate paroxetine-mediated down-regulation in the CA3 and DG region of the hippocampus.

8.2.3. The effect of acute and chronic ECS application on total BDNF mRNA and BDNF exon mRNA

Both acute ECS at 4h and chronic ECS at 24h significantly up-regulated total BDNF mRNA in the DG region of the hippocampus. The magnitude of the increase however, was larger in acutely treated animals *(See Section 6.3.1 and 6.3.4)*. In addition, the induction of acute ECS at 4h and chronic ECS at 24h resulted in a variable use of BDNF exon transcripts measured in the DG region of the hippocampus. Acute ECS resulted in the up-regulation of both exon I and IV mRNA at 4h, whereas chronic ECS resulted in an increase in exon I mRNA only at 24h, exon IV mRNA expression was unchanged *(See Sections 6.3.2, 6.3.3, 6.3.5 and 6.3.6)*

8.2.4. BDNF protein levels are increased after chronic ECS, but not chronic antidepressant drug treatment

Chronic administration of ECS resulted in a significant increase in immunoreactivity in the CA1, CA3 and mossy fibre zone of the hippocampus, as well as the parietal cortex. Chronic administration of the MAOI antidepressant drug TCP failed to increase BDNF protein in the same areas, however these results were preliminary in nature and

significance may not have been achieved due to low sample numbers (n=2).

8.3. Discussion

8.3.1. Implications of the main findings

8.3.1.1. Differential use of variable BDNF exon transcripts in response to antidepressant drug treatment

As explained in *Chapter 3* and *Chapter 5*, acute and chronic antidepressant drug treatment had a differing effect on the two exon-containing transcripts measured. Whilst like total BDNF mRNA, exon IV-containing transcripts were down-regulated by acute antidepressant drug treatment, exon I-containing transcripts were unchanged. In contrast, following chronic administration, exon I mRNA expression was up-regulated in conjunction with total BDNF mRNA, while exon IV mRNA remained unchanged. The physiological consequences of this phenomenon are unclear. However, it has been shown previously that exon IV- (as well as exon III-) containing mRNAs share properties with IEGs, whereas transcription of exon I (and exon II) mRNA is regulated via intervening protein synthesis mechanisms (Lauterborn et al,

1996; 1998). As each variable exon (exon I-IV) has a unique promoter, it appears that the differential effect on total BDNF mRNA after acute and chronic antidepressant treatment may be as a result of the recruitment of different promoters, which result in the corresponding change in exon transcription and total BDNF mRNA expression. Thus, the presence of numerous transcripts, each with unique promoter regulatory mechanisms might act as fine-tuning mechanism, mediating control over BDNF transcription, and translation into BDNF protein.

8.3.1.2. $GABA_B$-mediated inhibition of BDNF mRNA after acute antidepressant drug treatment

Chapter 4 outlined a hypothetical mechanism for the regulation of BDNF mRNA after acute antidepressant treatment. This involved the activation of excitatory 5-HT receptors present on GABA-ergic interneurone architecture within the hippocampus. This would, in theory, result in increased GABA release and subsequent inhibitory control over hippocampal cells, thereby inhibiting BDNF mRNA. Acute administration of the $GABA_B$ agonist baclofen, but not the $GABA_A$ agonist THIP or the benzodiazepine flunitrazepam significantly inhibited BDNF mRNA in the CA3 and DG.

Furthermore, $GABA_B$ receptor antagonist pre-treatment prior to acute baclofen treatment appeared to attenuate the inhibitory response. This therefore appears to confirm a role for $GABA_B$, but not $GABA_A$ mechanisms in the acute regulation of BDNF in the DG. However, the role of $GABA_B$-mediated mechanisms in acute antidepressant (paroxetine) inhibition of BDNF mRNA in the hippocampus was not fully established. Further research incorporating the acute administration of antidepressants with varying affinities to NA and/or 5-HT reuptake sites after $GABA_B$ receptor antagonist administration is required to confirm this effect.

Several studies, including the present study, have demonstrated an up-regulatory effect on BDNF after chronic, but not acute antidepressant drug treatment (Nibuya et al, 1995; Zetterström et al, 1998; Russo-Neustadt, 1999; 2000; Coppell et al, 2003). These findings have suggested a significant role for BDNF in the mechanism of action of antidepressant drugs, thereby implying an up-regulatory effect on BDNF gene transcription may be beneficial in the treatment of depression. It may be speculated that the initial inhibitory effect on BDNF mRNA found after acute antidepressant administration could be seen as a

detrimental phenomenon which may contribute to the apparent "therapeutic latency period" *(See Section 1.4.3.1)* of antidepressant drugs. Therefore, the elimination of such an effect may enhance the action of antidepressants on BDNF and possibly reduce the latency period. Possible $GABA_B$ receptor-mediated involvement in the acute down-regulatory response in BDNF after acute antidepressant treatment perhaps suggest that $GABA_B$ receptor blockade may be beneficial in the initial phase of antidepressant treatment. This could have important clinical implications for the shortening of the latency period of therapeutic onset of antidepressant drugs.

8.3.1.3. Chronic and acute ECS up-regulate BDNF mRNA in the rat hippocampus

Unlike antidepressant drug treatment where chronic, but not acute administration, resulted in the up-regulation in BDNF, ECS up-regulated BDNF mRNA in the DG, when given both acutely and chronically. BDNF mRNA has been shown to be up-regulated acutely by enhanced neuronal activity and glutamatergic transmission (Zafra et al, 1991; 1992). The up-regulatory effect on BDNF mRNA from acute ECS however is not consistent with the time-course for the therapeutic

action of ECS. Therefore it was suggested in *Chapter 6,* that up-regulation of BDNF mRNA alone is not sufficient to bring therapeutic response. Interestingly, Zetterström et al (1998a) found that though acute ECS produced an up-regulation in BDNF mRNA at 4h, the effect was transient and levels returned to normal after 24h. However, chronic ECS application produced far longer lasting increases in BDNF mRNA, perhaps suggesting chronic adaptation in components mediating BDNF signalling. Both ECS and antidepressant drug treatments potentiate the cAMP signalling cascade at several levels (Ozawa and Rasenick, 1991; Nibuya et al, 1996). An important target for this system is CREB, which has been shown to regulate BDNF transcription (Shieh et al, 1998; Tao et al, 1998). Moreover, chronic administration of antidepressant drugs or ECS increases CREB in the rat hippocampus (Nibuya et al, 1996).

8.3.1.4. The implications of enhanced BDNF signalling

BDNF has been identified as an important mediator in several vital functions within the brain. In addition to promoting the survival of both 5-HT and NA neurones (Altar et al, 1994), direct BDNF infusion has also been shown to produce neurotrophic effects of 5-HT neurones in the

neocortex (Mamounas et al, 1995; 2000). This may represent a reciprocal process between monoamine function and BDNF production, whereby persistent increases in monoamine levels, such as from chronic antidepressant drug treatment, potentiate BDNF levels which in turn produce neurotrophic events, altering morphology and increasing neuronal system efficiency. Conversely, chronic stress, through enhanced CRF and/or glucocorticoid levels may promote morphological deficits in such neuronal systems associated with depression, chronic stress has been demonstrated to result in neuronal atrophy in CA3 neurones (Sapolsky et al, 1990; Watanabe et al, 1992; Magarinos et al, 1996), possibly through deleterious effects on BDNF turnover (Smith et al, 1995). It is therefore tempting to suggest that elevations in BDNF protein may be predictive response for the mechanistic actions of antidepressant interventions. However, despite the apparent up-regulatory effect of chronic ECS, the effect antidepressant drug administration on BDNF protein remains somewhat inconclusive. It is therefore apparent that more studies are needed in this area in order to fully establish the role of BDNF in the mechanistic action of antidepressant drugs.

8.3.2. Future studies and areas of investigation

8.3.2.1. Impact of stress on BDNF exon transcripts

Chronic exposure to stress has long been implicated in the pathophysiology of depression. Additionally, chronic stress models in rats have been shown to lower BDNF mRNA in the hippocampus (Smith et al, 1995; Ueyama et al, 1997), as does elevation of exogenous corticosterone levels (Schaaf et al, 1998), an effect that is blocked by subsequent antidepressant treatment (Nibuya et al, 1995; *See Section 1.6.2.4*). Using exon-specific oligonucleotide probes, the effect of stress paradigms upon variable BDNF transcripts could be assessed. In addition, the impact of subsequent antidepressant treatment after stress on BDNF exon transcripts could be examined. Such studies would help address which BDNF exons are affected by stress and allude to the mechanism by which chronic antidepressant treatment may reverse this effect.

8.3.2.2. Double-labelling strategies

Double ISH and ICC labelling techniques may be employed to help assess the changes in total BDNF and individual BDNF exon transcript mRNA, in relation to the localisation subsets of neurones. For example, this series of experiments may be important in identifying the localisation of GABAergic interneurones in relation to changes in BDNF mRNA after acute antidepressant drug treatment *(See Chapter 4)*.

8.3.3.3. Transgenic animals

Transgenic mice contain additional foreign DNA in every cell, allowing them to be used to study gene function or regulation and to model human diseases. Thus, targeted gene insertion techniques could be used to produce transgenic mice displaying knockout genes to further investigate the role of BDNF in antidepressant function. For instance, to further investigate the role of specific monoamine receptors in BDNF mRNA transcription, specific receptor protein knockout mice could be bred. The effect of antidepressant treatment on BDNF mRNA or individual BDNF

exon transcript mRNA in the receptor knockout mice could then be assessed.

8.4. Concluding remarks

BDNF is a versatile molecule, capable of variable responses to antidepressant challenge. The results presented in this thesis have outlined some novel findings with regard to the regulation of the BDNF gene after antidepressant treatment. Continued study in this area will hopefully facilitate the development of faster, more efficacious and specific treatments for those suffering this devastating affliction.

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The citation of publications is according to the 'Harvard' style of referencing.

Where there are several citations sharing the same principal author, publications are listed in chronological order.

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