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MOLECULAR ABNORMALITIES IN POSTMORTEM BRAINS OF SUBJECTS WITH MOOD DISORDERS

By

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Abstract

Biochemical and structural abnormalities have been reported in postmortem brain tissue from patients with mood disorders. Studies of the molecular pharmacology of drugs used in the treatment of mood disorders have led to a reinterpretation of earlier models of neuropathology in these diseases. Noradrenergic and serotonergic hypotheses have been expanded to include postsynaptic intracellular signal transduction pathways, regulation of gene expression, and synaptic plasticity. Because much of this evidence was obtained from postmortem brain, the experiments in this study also used postmortem tissue to examine the neuropathology of mood disorders. Human postmortem brain tissue was obtained from the Stanley Consortium Neuropathology Foundation and consisted of pieces of temporal and occipital cortex, and slices of prefrontal cortex and hippocampus from patients with bipolar affective disorder (BD), major depressive disorder (MDD), schizophrenia (SCZ) and controls (N = 15 per group). Components of the cAMP system were examined, as were potential target transcription and neurotrophic factors. Morphological consequences arising from altered cAMP signalling such as sprouting and DNA fragmentation were also investigated.

There was a trend towards blunted temporal cortex adenylyl cyclase activity in subjects with mood disorders, and decreased occipital cortex $G\alpha_s$ in lithium-treated BD subjects. Temporal cortex CREB levels were decreased in antidepressant-untreated MDD subjects compared to controls, and normal in those subjects treated by

antidepressants at the time of death. Temporal cortex CREB levels were decreased in BD patients treated with anticonvulsants relative to those not treated by anticonvulsants at the time of death. When assessing the effect of suicide on cAMP signalling, subjects who died as a result of suicide had lower temporal cortex CREB levels and CRE-binding than subjects who died of other causes. This effect was most evident in the MDD group. In hippocampus, BDNF levels were increased in subjects treated by antidepressants compared to subjects not treated by these medications at the time of death. This finding was more pronounced in the MDD group. Hippocampal studies also showed that BD subjects had increased mossy fibre staining relative to controls and other diagnoses, suggesting increased sprouting of the dentate gyrus axons.

These postmortem findings are consistent with recent animal and cell models of antidepressant and mood stabilizer pharmacology. The changes in post-receptor signalling and hippocampal sprouting are also consistent with current conceptualizations of the neurobiology of mood disorders. These studies support the use of postmortem brain tissue as a clinically relevant research model, and add to the growing literature elucidating the pathophysiology of mood disorders and the molecular pharmacology of their treatments.

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To my committee members Drs. *Mishra*, *MacQueen* and *Joffe*, for your input, your feedback, and for simplifying the administrative matters, which allowed me to concentrate on the research. I am further indebted to Dr. MacQueen for her support, advice and numerous discussions about long-term goals;

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Dar Dowlatshahi

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Preface

This thesis consists of previously published manuscripts submitted in the format of a "Sandwich Thesis". It is consistent with the requirements for Sandwich Theses as per the *McMaster University School of Graduate Studies Guide for the Preparation of Theses*, as well as the new requirements from the Graduate Programs in Health Sciences announced by Dr. Haslam in April 1998. Moreover, in accordance with the *McMaster University School of Graduate Studies Guide for the Preparation of Theses* section 3c, this preface serves to disclose specific contributions of the author to the published manuscripts included herein.

Note: In the following paragraphs, "*the author*" refers to the author of this thesis, Mr. D. Dowlatshahi.

1) Dowlatshahi D & Young LT. (2000) Molecular abnormalities in brains of depressed patients. *The Neuroscientist* (6): 401-410.

This publication reviews the molecular pathology and pathophysiology of major depressive disorder. As such, its modified form is included as an introduction to this thesis. The manuscript was researched and written by the author, with direction from Dr. Young. Each draft was prepared by the author and edited by Dr. Young. Following Dr. Young's approval, the final draft was written and submitted by the author.

2) Dowlatshahi D, MacQueen GM, Wang JF & Young LT. (1998) Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. *The Lancet* (352): 1754-55.

This experiment measured CREB levels and was the first in a series of experiments by the author measuring components of the cAMP signalling pathway in postmortem brain samples. The author completed the experiment, data acquisition and statistical analyses. The author was also responsible for preparation of the manuscript. Due to her extensive experience with statistics, Dr. MacQueen independently verified statistical analyses for this and all subsequent experiments. Dr. MacQueen was also intimately involved in the writing of this manuscript. Dr. Wang provided troubleshooting advice, and reviewed final manuscripts for this and subsequent publications. Dr. Young was involved in the conceptual design of experiments and editing of manuscripts in this and subsequent publications.

3) Dowlatshahi D, MacQueen GM, Wang JF, Reiach JS & Young LT. (1999) G-protein coupled cyclic AMP signaling in postmortem brain of subjects with mood disorders: effects of diagnosis, suicide, and treatment at the time of death. *Journal of Neurochemistry* (73): 1121-26.

This publication describes several experiments where the author measured components of the cAMP cascade. Mr. Reiach optimised conditions for, and assisted with Gαi and Gαs immunoblotting procedures. The author conducted all other experimental procedures alone (cAMP levels, AC activity, CREB levels). The author

was also responsible for data acquisition, statistical analysis and manuscript preparation. Dr. MacQueen reviewed and edited the final manuscript. Drs. Wang, and Young were involved as previously described.

4) Stewart RJ, Chen B, Dowlatshahi D, MacQueen GM & Young LT. (2001) Abnormalities in the cAMP signaling pathway in post-mortem brain tissue from the Stanley Neuropathology Consortium. *Brain Research Bulletin* (55): 625-29.

This publication reviews cAMP signalling abnormalities in postmortem brains of patients with major depressive disorder and bipolar affective disorder, with some original CaM kinase data. It also reviews the Stanley Foundation Neuropathology Consortium brain tissue used for all experiments in this thesis.

Dr. Stewart prepared the first draft of this manuscript. Dr. Chen completed a CaM Kinase II/IV immunoblotting assay, which was included in this review as an unpublished experiment. The author assisted Dr. Stewart's literature review of molecular pathology in mood disorders. Furthermore, the author was responsible for second and subsequent drafts of the manuscript following Dr. Stewart's departure from McMaster University. Drs. Young and MacQueen edited all drafts.

5) Chen B, Dowlatshahi D, MacQueen GM, Wang JF & Young LT. (2001) Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. *Biological Psychiatry* (50): 260-5.

BDNF is a downstream target of cAMP-dependant gene expression and was measured to add to the author's previous findings. Dr. Chen optimised the BDNF immunohistochemistry procedure. Dr. Chen and the author shared the experiment and data gathering. The author was responsible for statistical analysis and manuscript preparation. Drs. MacQueen, Wang and Young were involved as previously described.

6) Dowlatshahi D, MacQueen GM, Wang JF, Chen B & Young LT. (2000) Increased hippocampal supragranular Timm staining in subjects with bipolar disorder. *Neuroreport* (11): 3775-78.

Following the cAMP pathway experiments, the author measured morphological changes in brain regions with altered BDNF levels. The author was responsible for completing the experiment, data gathering, statistical analyses and manuscript preparation. Dr. Chen's histology expertise was consulted during the modification of the Timm assay for optimisation with our postmortem brain samples. Drs. MacQueen, Wang and Young were involved as previously described.

Also in accordance with the Graduate Programs in Health Sciences Sandwich Theses guidelines, detailed methods for the above experiments, as well as those from unpublished experiments, are included in Appendix A. Appendix B and C contain subject data and unpublished works, respectively.

Table of Contents

Abbreviations

AC	adenylyl cyclase
AD	antidepressant
ANOVA	analysis of variance
ATF	activating transcription factor
ATP	adenosine triphosphate
BA	Brodmann's area
BD	bipolar affective disorder
BDNF	brain derived neurotrophic factor
CaMK	calcium/calmodulin-dependent kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CONT	control subjects
CRE	cAMP response element
CREB	cAMP response element binding protein
CSF	cerebrospinal fluid
DG	dentate gyrus
DOPAC	dihydroxyphenlyacetic acid
DSM-IV	Diagnostic and Statistical Manual, 4th edition
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECT	electroconvulsive therapy
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol tetra acetic acid
GAP-43	growth associated protein-43
GTP	guanosine triphosphate
5-HIAA	5-hydroxyindoleacetic acid
HPC	hippocampus
5-HT	5-hydroxy tryptophan a.k.a. serotonin
HVA	homovanillic acid
IML	inner molecular layer
IMPase	inositol monophosphatase
kDa	kiloDaltons
Li	lithium
LV	lateral ventricle
MAOI	monoamine oxidase inhibitor
MARCKS	myristolated alanine-rich C kinase substrate
MBP	myelin basic protein
MDD	major depressive disorder
MHPG	3-methoxy-4-hydroxyphenylethylene glycol
mRNA	messenger ribonucleic acid

pCREB	phosphorylated CREB
PBS	phosphate-buffered saline
PI	phosphoinositol
PIP_2	phosphatidyl inositol 4,5-bisphosphate
PKA	cAMP-dependant protein kinase
PKC	calcium-dependent protein kinase
PMI	postmortem delay interval
PVDF	polyvinylidene fluoride
ROD	relative optical density
SCZ	schizophrenia
SDS-PAGE	sodium dodecyl sulfate polyacrilamide gel electrophoresis
SEM	standard error of the mean
SG	supragranular
SNAP-25	synaptisome associated protein-25
SSRI	selective serotonin reuptake inhibitors
TCA	tricyclic antidepressants
TH	tyrosine hydroxylase
TUNEL	tranferase dUTP nick end labeling
VPA	valproic acid

1. Introduction

This introduction is a modified version of the original publication by Dar Dowlatshahi and L. Trevor Young; Molecular Abnormalities in Brains of Depressed Patients, *The Neuroscientist* (2000) Volume 6, Number 5: pp401-410. Sections reviewing bipolar affective disorder were added, and references were reformatted and appended to the end of this thesis.

Mood disorders are a group of psychiatric illnesses characterized by episodes of disturbed mood, which cause significant distress and social, occupational or functional impairment. This group of disorders include major depressive disorder, bipolar disorder, dysthymic disorder and cyclothymic disorder. Major depressive disorder (MDD) is among the most common psychiatric disorder with a lifetime prevalence of up to 15% (Kessler et al., 1994). It has a recurrent course in at least 50% of patients, and in a smaller percentage, is refractory to the usual treatments of antidepressants and psychotherapy (Potter WZ et al., 1995). There is substantial mortality, with up to 10% of patients committing suicide. Bipolar affective disorder (BD), characterized by episodes of depression and mania, affects approximately 1.5% of the population (Goodwin & Jamison, 1990). Without treatment, the frequency of manic episodes increases as the patient becomes sensitized to exacerbations of the disease (Winokur et al., 1993). The degree of morbidity with these illnesses is high, and may persist after the mood has returned to normal. Indeed, functional impairment may be greater than that seen with illnesses such as diabetes and heart disease (Wells et al., 1988).

The most commonly used treatment in mood disorders is pharmacotherapy. Nevertheless, other treatment modalities such as electroconvulsive therapy (ECT) and psychotherapy are also used. ECT is generally reserved for depressed patients who are refractory to drug treatment, and for urgent use in severely ill patients at high risk for suicide. Psychotherapy, particularly cognitive behavioural therapy, is efficacious in MDD and is often used in conjunction with antidepressants (Thase et al., 1997).

A variety of antidepressants are available for the treatment of MDD, including selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), and

monoamine oxidase inhibitors (MAOIs). Although these drug classes have different side effect profiles, they are equally effective (Mulrow et al., 2000). For the treatment of BD, the mood stabilizer lithium is considered first line treatment (APA, 2002). Valproic acid (VPA), originally used as an anticonvulsant, is also available as a first line agent, but is more commonly used for mixed states (APA, 2002). Alternate drugs for BD are the anticonvulsants carbamazepine, oxcarbamazepine and lamotrogine; the latter having been recently approved as first line for depressive episodes (APA, 2002). 60-70 % of patients with MDD respond to antidepressant drug treatments (Ebert MH et al., 2000) and 50-80 % of BD patients respond to lithium alone (Calabrese et al., 1996). The most common reasons for treatment failure are low serum drug levels, inadequate duration of treatment and prominent side effects (Ebert MH et al., 2000), although pharmacogenetic factors may play a role in determining drug response in individual patients. However, despite decades of experience with these therapeutic agents, their precise molecular pharmacology is unknown.

Mood disorders are heterogeneous syndromes with multiple causes including genetic predisposition, early life trauma, and reaction to stress (Kalpana IN et al., 1995). They may be precipitated by a variety of medical illnesses and may be associated with abnormalities in the temporal-limbic-frontal-caudate network with the possibility that the orbitofrontal/amygdalar network and the hippocampal/cingulate system regulate mood and cognitive symptoms of the disorders, respectively (Drevets, 1999). A number of specific biochemical pathways have been implicated in mood disorders with a longstanding focus on monoaminergic neurotransmitter systems. These latter pathways

may be particularly important since many, if not all, antidepressant drugs have effects on these pathways.

As early as 1965, Schildkraut (1965) proposed that depression might be due to a deficiency of norepinephrine at synapses in cerebral cortical and limbic regions, and that antidepressant drugs might act to increase the neurotransmission through this pathway. Several years later, the ability for norepinephrine-modulation by serotonin was recognized in the serotonin-permissive hypotheses of Prange (1974). Based on these models, studies of the mechanism of antidepressants have accumulated, which have become increasingly more sophisticated and plausible. Monoaminergic hypotheses of mood disorders have been expanded to include post receptor components of the signal transduction pathways and regulation of gene expression (Duman et al., 1997). Animal studies have shown that treatment with antidepressants increases intracellular cAMP signalling. Studies have also shown increases in the transcription factor CREB in rat hippocampus following chronic treatment with antidepressants. Postmortem brain studies have examined these systems in tissue obtained from subjects with depression, bipolar disorder, and those who died by suicide. These postmortem brain studies are surprisingly consistent with animal studies.

In the following pages we will review the postmortem brain studies in MDD and BD individuals, which have focused on the monoaminergic pathways. Earlier studies examined neurotransmitter levels, their metabolites and receptors. More recent studies measured signal transduction intermediates, whereas current studies are examining transcription factors and the regulation of gene expression.

Postmortem Brain Tissue in Depression & Suicide

Although many advances have been made with brain imaging, biochemical studies of human brain tissue can only be accomplished in tissue slices or homogenates obtained from subjects at the time of death. With the establishment of several large brain tissue banks around the world, there is currently great interest in the use of postmortem brain tissue to study the pathophysiology of mood disorders and the targets of drugs. Surprisingly, many assays work very well on postmortem tissue as long as age and the time interval from death to autopsy (i.e. postmortem delay) are controlled and accounted for in data analysis. Earlier studies used a coroner's diagnosis of psychiatric disease, but it is now considered essential that a patient's medical records are reviewed by experts and diagnoses made with standardized methods. The current standard is the DSM-IV (Diagnostic and Statistical Manual, Fourth Edition, American Psychiatric Association) criteria. Past treatments must be detailed and accounted for and factors such as substance abuse and other psychiatric or medical illnesses detailed. Another approach has been to obtain tissue from subjects who died by suicide. Since most patients who die from suicide suffered from a psychiatric disorder, and depression is strongly associated with suicide, this has been a useful strategy. It has been difficult, however, to ascertain whether any effects are due to depression, suicide or the combination of both. Depression associated with suicide has often been considered a more severe form of the disorder. Others make the case that suicide itself may have associated with clear biochemical markers and may be inherited or transmitted from one generation to the next, independent of factors associated with depression. It is therefore important to

differentiate between three subject groups when reviewing postmortem brain studies: suicide subjects, depressed subjects and depressed-suicide subjects. Literature reviewed in this article includes data from all three groups.

Neurotransmitters and Receptors

There is a substantial body of evidence implicating the monoamine neurotransmitter systems in mood disorders. The noradrenergic and serotonergic pathways have been well studied in depression, whereas dopamine has been implicated to a lesser extent. Noradrenergic cell bodies originate in the brainstem locus coeruleus and project to many brain regions including cerebral cortex and hippocampus. These projections from the locus coeruleus are responsible for modulating overall arousal levels and may be important in psychiatric disorders, specifically depression, mania and anxiety. Serotonin, dopamine, GABA and a variety of other neurotransmitters can modulate the noradrenergic neurons of the locus coeruleus. Furthermore, these neurons can also undergo autoregulation by presynaptic noradrenergic receptors. The presynaptic α_2 receptors are part of a negative feedback system, inhibiting noradrenaline release.

Evidence of abnormalities in noradrenergic activity has been reported in postmortem brain experiments. The results from initial studies of noradrenergic metabolites in postmortem brain in a number of regions including brainstem, cortex, caudate and hypothalamus have been inconsistent (Klimek et al., 1997; Kalpana IN et al., 1995; Crow et al., 1984; Beskow et al., 1976). Since tyrosine hydroxylase (TH) is a rate-

limiting enzyme in the formation of norepinephrine, several studies have measured TH activity and levels in locus coeruleus of suicide victims (Kalpana IN et al., 1995). Increased TH levels were found in subjects who committed suicide, including those who were free of antidepressants (Ordway, 1997; Ordway et al., 1994). Studies measuring β-adrenergic receptor binding have also shown varied results; although some revealed no association between β-adrenergic receptor binding and MDD (Crow et al., 1984; Meyerson et al., 1982), studies using postmortem brain from drug-free suicide subjects have identified increases in β-receptor density (Arango et al., 1990; Biegon & Israeli, 1988; Mann et al., 1986). Subsequent to these findings, there has been much interest in the α_2-adrenergic receptor, as this family of receptors can modulate both serotonergic as well as noradrenergic activity in the brain (Esteban et al., 1996; Trendelenburg et al., 1994).

Several researchers have measured α_2-adrenergic receptor binding in postmortem brains of subjects with MDD. These investigators consistently report increased α_2-adrenergic receptor densities in the frontal cortex, prefrontal cortex, hippocampus and locus coeruleus in MDD subjects (Gonzalez et al., 1994; Ordway et al., 1994; Meana et al., 1992; Meana & Garcia-Sevilla, 1987). Though compelling, these studies included subjects both on and off antidepressant treatment at the time of death. Therefore, it is difficult to differentiate changes in α_2-adrenergic receptors due to MDD diagnosis from potential changes caused by long-term antidepressant therapy. Accordingly, De Paermentier et al. (1997) showed significant increases in temporal cortex α_2-adrenergic

receptor binding in MDD suicide patients that had not received antidepressant treatment for at least three months prior to death, relative to both treated MDD suicides and controls. The increase was specific to receptor subtype as α_1-adrenergic receptor binding was not significantly different between the groups. Subsequently, Garcia-Sevilla et al. (1999) report significant increases in α_2-adrenergic receptor protein levels in prefrontal cortex of untreated MDD suicide subjects relative to controls. These studies support the notion of α_2-adrenergic receptor upregulation in MDD as they control for potential antidepressant effects.

In addition to studies that found noradrenergic abnormalities in depression and suicide, it is important to consider the interactions between the serotonergic and noradrenergic systems in the brain. This interaction has formed the serotonin permissive hypothesis (Prange et al., 1974) and is consistent with the large body of evidence on the regulation of serotonin by antidepressant drugs. Stimulation of the α_2-receptor has been shown to inhibit the synthesis and release of noradrenaline and serotonin (Esteban et al., 1996; Trendelenburg et al., 1994). Therefore, based on the postmortem brain studies, the upregulation of α_2-adrenergic functionality may lead to decreases in both noradrenergic and serotonergic activity in MDD. It would follow that increasing serotonergic and noradrenergic function may result in clinical improvements.

Abnormalities in serotonin transmission have been hypothesized to contribute to the pathophysiology of MDD. Patients with MDD show blunted hormonal release in response to neuroendocrine challenges following serotonin stimulation, relative to non-

psychiatric controls (Cowen & Charig, 1987; Heninger et al., 1984). In addition, cerebrospinal fluid from MDD patients has lower levels of the serotonin metabolite 5-HIAA, relative to control subjects (Risch & Nemeroff, 1992). This suggests that low serotonin turnover is associated with MDD. Such findings have contributed to the development of serotonergic antidepressants. These drugs selectively inhibit serotonin reuptake and show clinical efficacy (Kalpana et al., 1995).

Despite the evidence from antidepressant studies, postmortem brain analysis of serotonergic pathology has been inconsistent. Contrary to the hypothesis of decreased serotonergic activity in MDD, evidence supports a possible upregulation of serotonin receptors. Arango et al. (1995) showed a significant increase in 5-HT_{1A} binding in prefrontal cortex of subjects that died by suicide. This increase was negatively correlated with presynaptic 5-HT transporter binding in these subjects, suggesting an overall increase in synaptic sensitivity. However, these suicide subjects lacked a firm diagnosis of MDD. A recent study showed increased 5-HT_{1A} binding in midbrain dorsal raphe nucleus of MDD suicide subjects (Stockmeier et al., 1998). Lowther et al. (1997) also measured 5-HT_{1A} binding in a sample of suicide subjects with a diagnosis of MDD. Conversely, this study showed no differences between depressed suicides and controls in 5-HT_{1A} binding sites in frontal cortex, occipital cortex, hippocampus and amygdala. This study also showed no changes in 5-HT_{1A} binding associated with antidepressant treatment. Measures of 5-HT_{1B} receptor binding also show no differences between MDD and controls in prefrontal cortex (Huang et al., 1999).

Several groups have found increases in postsynaptic 5-HT_2 receptor binding in the frontal and prefrontal cortex of suicide subjects relative to non-suicide subjects (Turecki et al., 1999; Arango et al., 1990; Mann et al., 1986; Stanley & Mann, 1983). This change was not seen in the temporal cortex (Arango et al., 1990) nor was any change in 5-HT_1 binding detected (Mann et al., 1986). Using a sample of suicide subjects diagnosed with MDD, Hrdina et al. (1993) reported a significant increase in $5HT_2$ receptor binding in prefrontal cortex and amygdala relative to controls. Furthermore, this study showed that the ratio between presynaptic uptake sites and postsynaptic receptors in the amygdala was lower in MDD suicides relative to controls, suggesting synaptic sensitization.

Studies reporting increases in postsynaptic serotonin binding appear at first to be inconsistent with the serotonergic hypothesis of MDD. However, this increased postsynaptic sensitivity may be a compensatory response to overall decreases in serotonin transmission. This theory predicts that increases in post-synaptic receptor binding should also be evident in adrenergic receptors, as noradrenergic activity should also be blunted in MDD. Indeed, Arango et al. (1990) & Mann et al. (1986) showed that increases in postsynaptic 5-HT_2 binding in prefrontal cortex of suicide subjects was accompanied by increases in β-adrenergic binding in both prefrontal and temporal cortex. It is also difficult to completely separate diagnostic-dependent changes in serotonin receptors from any changes that may be due to long-term antidepressant treatment. Chronic antidepressant treatment may result in the sensitization of postsynaptic neurotransmitter receptors, thereby compensating for the clinical manifestations of MDD.

Dopamine has also been implicated in the pathophysiology of depression. Levels of DOPAC and HVA, two major metabolites of dopamine, are decreased in subjects with MDD, relative to controls (Roy et al., 1985; Mendels et al., 1972). However, there was no difference in D_1 and D_2 receptor binding between antidepressant-free suicide MDD subjects and controls in the caudate, putamen or nucleus accumbens (Bowden et al., 1997). Antidepressant-treated MDD suicide subjects had increased D_2 receptor binding in all three brain regions, and D_1 binding was increased in the nucleus accumbens. Although the striatum is anatomically part of the basal ganglia and involved in motor behaviours, the nucleus accumbens, where both D_1 and D_2 binding was upregulated following antidepressant treatment, is functionally part of the limbic system (England MA & Wakely J, 1991). Interestingly, a postmortem study measuring morphometry of whole brain sections revealed significant decreases in volumes of nucleus accumbens, pallidum and putamen in subjects with mood disorders relative to controls (Baumann et al., 1999). These studies show a possible degeneration of dopaminergic brain regions in MDD.

In contrast to MDD, noradrenergic activity is thought to be upregulated in BD. Early studies measured peripheral plasma norepinephrine levels in sympathetic challenge paradigms. Plasma norepinephrine levels were increased in BD subjects following orthostatic challenge, as compared to controls (Rudorfer et al., 1985). Although this finding is consistent with increased noradrenergic activity, peripheral norepinephrine levels do not necessarily correlate with the central nervous system. CSF measurements showed higher norepinephrine levels in patients with mania compared to depressed

patients (Post et al., 1978). CSF MHPG levels, a metabolite of norepinephrine, was also elevated in BD subjects (Swann et al., 1987). However, CSF norepinephrine and MHPG levels are dependent on their plasma levels, thereby reflecting peripheral rather than central noradrenergic activity.

More recent studies have been able to measure monoamine levels in postmortem brain of subjects with BD. One study found increased norepinephrine turnover in thalamus and frontal, temporal and occipital cortex (Young et al., 1994a). There were no changes in the levels of norepinephrine, serotonin or dopamine, nor were there any differences in β-receptor binding. However, decreased brain 5HIAA, a metabolite of serotonin, was found in frontal and parietal cortex (Young et al., 1994a), as was a decrease in serotonin uptake receptor sites (Leake et al., 1991).

One of the major difficulties with neurotransmitter hypotheses of mood disorders is the inability to account for the temporal discrepancy between pharmacological and clinical effects of medications. Although antidepressants and mood stabilizers achieve their pharmacological effects shortly after ingestion, clinical improvements often take several weeks (Kalpana et al., 1995). Therefore the mechanism of action of these drugs has to take into account cellular and molecular processes that occur over time. Post-receptor signalling systems link immediate synaptic changes with long-term changes in gene expression, and such long-term effects on gene expression may result in morphological changes that are beneficial to the patient.

Intracellular Signalling Pathways

The cAMP Signaling System

Of the known intracellular signal transduction pathways, the G-protein coupled cAMP-signaling pathway is one of the best understood (figure 1). G-proteins are GTP binding proteins consisting of three subunits: α, β and γ. The β and γ subunits are believed to anchor the G-protein to the cell membrane, whereas α is the catalytic subunit. The α subunit can exist as α_s or α_i, having stimulatory or inhibitory actions respectively, or as αq, which is linked to the phosphoinositol pathway. Following the binding of a ligand to a G-protein coupled receptor, the G-protein will bind GTP, releasing the functional subunit $G\alpha_s$ or $G\alpha_i$. These subunits will activate or inhibit, respectively, the membrane bound enzyme adenylyl cyclase (AC). An activated AC will catalyze the formation of cAMP from ATP, which will subsequently bind to the cAMP-dependent protein kinase (PKA). PKA consists of both cAMP-binding regulatory subunits and catalytic subunits. Upon binding cAMP, the regulatory subunits release the catalytic units, which can subsequently catalyze a variety of intracellular proteins, including ion channels, enzymes and transcription factors.

Among the many known transcription factors, the cAMP responsive element binding protein (CREB) is one of the most studied and perhaps most relevant to the pathophysiology of mood disorders. The CREB family of transcription factors includes

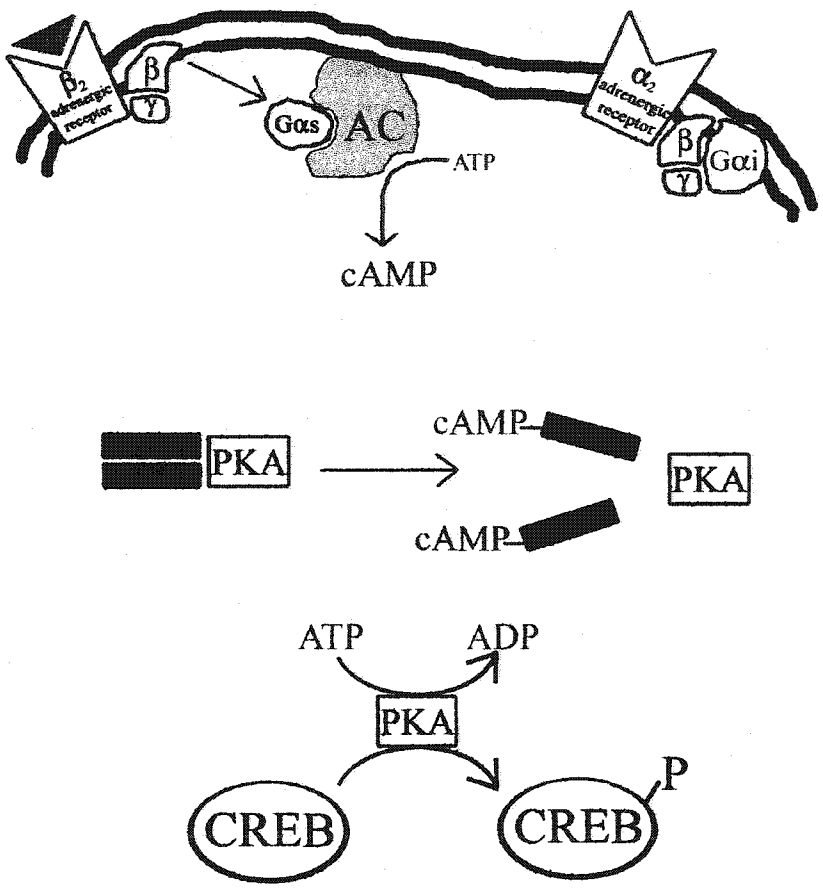

Figure 1: The cAMP intracellular signaling pathway.

many CREB isoforms, activating transcription factors (ATFs), the CRE modulator (CREM) and the inducible cAMP early repressor (ICER). CREB contains a phosphoacceptor site on Ser133, a substrate for phosphorylation by PKA as well as Ca^{2+}-calmodulin dependent kinases (CaMKs). Phosphorylation at this site, producing phosphoCREB (pCREB), is required for the transcriptional activation of CREB. Genes whose expression is dependent on cAMP contain a cAMP-response element (CRE), which interacts with CREB. Although, CREB can bind to the CRE in its unphosphorylated state, only pCREB can initiate transcription. Evidence indicates that a CREB binding protein (CBP) may be recruited by the phosphorylated Ser133, mediating subsequent interactions with transcriptional machinery.

Abnormalities in signal transduction pathways have been reported to be associated with a variety of neuropsychiatric disorders (Wang et al., 1997; Kalpana IN et al., 1995). Using both *in vitro* and animal models, several groups have shown antidepressant-induced changes in a variety of intracellular processes (Duman et al., 1997), particularly with respect to the cAMP-signalling pathway. Treatment with antidepressant medications results in the upregulation of components of the cAMP pathway in both rats and cell lines; antidepressants increase levels of CREB, phosphorylation of CREB, and its subsequent biding to the CRE region. Brain derived neurotrophic factor (BDNF) and its receptor trkB, both genes regulated by CRE-binding, are also upregulated by antidepressant treatment (Duman et al., 1997). Based on this work, it has been hypothesized that MDD may be associated with the blunting of the

cAMP system. Antidepressants, therefore, may exert their clinical effects by inducing a compensatory increase in cAMP signalling.

Garcia-Sevilla et al. (1999) reported that the increases in α_2-receptor levels in antidepressant free MDD suicide subjects was associated with a significant increase in $G\alpha_i$ and $GRK_{2/3}$ protein levels. $GRK_{2/3}$ desensitizes β-adrenergic receptors via phosphorylation, whereas $G\alpha_i$ inhibits AC. Since β-adrenergic receptors are linked to stimulatory G-proteins, increases in both $G\alpha_{i1/2}$ and $GRK_{2/3}$ may lead to a synergistic blunting of the cAMP cascade. Conversely, Pacheco et al. (1996) found decreases in $G\alpha_{i2}$ levels and increases in $G\alpha_s$ (short form) in frontal cortex of MDD suicide subjects relative to controls. This study did not separate subjects based on drug therapy, as such, the apparent upregulation in cAMP signalling may be partially due to antidepressants effects. Accordingly, Garcia-Sevilla et al. (1999) showed that antemortem antidepressant treatment was associated with a significant reduction of $G\alpha_{i1/2}$ protein (32%).

Several studies report blunting of AC activity in subjects with MDD. Decreased stimulated-AC activity was found in frontal cortex of suicide subjects relative to non-suicide subjects (Lowther et al., 1996; Cowburn et al., 1994). A similar trend towards decreased forskolin-stimulated AC activity was also found in subjects with mood disorders (Dowlatshahi et al., 1999). Reiach et al. (1999) reported a significant decrease in forskolin-stimulated AC activity in MDD suicide subjects compared to controls. This study also showed a concordant decrease in AC type IV protein levels in MDD subjects who committed suicide. In general, studies show normal basal AC activity, with blunted

responses to stimulation. These findings may indicate that changes in upstream components of this signalling pathway are important in depression.

Postmortem brain studies in BD also reveal abnormal cAMP signalling. Several laboratories have reported increased levels of Gαs protein in frontal, temporal and occipital cortex brain tissue from subjects with BD, as compared to controls (Young et al., 1993; Young et al., 1991). Other experiments demonstrate increased forskolin-stimulated AC activity in BD brain tissue (Young et al., 1993), without any increase in AC levels (Reiach et al., 1999). Moreover, the binding of cAMP to PKA is increased in BD brain tissue (Rahman et al., 1997).

In support of the postmortem brain studies, increased Gαs protein was found in mononuclear leukocytes (MNL) obtained from patients with BD (Young et al., 1994b; Schreiber et al., 1991). These findings were not present in patients with MDD, and appear to be independent of mood state (Manji et al., 1995). Furthermore, cAMP-dependent phosphorylation was also increased in platelets obtained from patients with BD (Perez et al., 1995). These observations suggest that the cAMP-dependent signal transduction pathway is upregulated in BD. Consistent with this hypothesis, lithium has been shown to blunt cAMP signalling in animal and *in vitro* models. Chronic lithium treatment attenuates norepinephrine stimulated cAMP production (Forn & Valdecasas, 1971), blunts agonist medicated GTP binding (Avissar et al., 1988), and decreases $G\alpha_s$ and $G\alpha_i$ mRNA in rat brain (Li et al., 1991; Colin et al., 1991).

It has been established that cross-talk exists between the cAMP signalling system and other second messenger systems, most notably the phosphoinositol (PI) pathway. Research in bipolar disorder has revealed abnormalities in both of these signalling pathways (Wang et al., 1997). Increased PIP_2 levels have been reported in platelets obtained from untreated BD patients (Brown et al., 1993). Platelet protein kinase C activity was also increased in BD patients (Friedman et al., 1993). Furthermore, basal and agonist-stimulated intracellular Ca^{2+} levels were increased in lymphocytes and platelets obtained from subjects with BD (Dubovsky et al., 1992; Tan et al., 1990; Dubovsky et al., 1989), although this was not consistently replicated (Bothwell et al., 1994; Eckert et al., 1994). Interestingly, lithium is known to bind and inhibit IMPase, an enzyme involved in the regeneration of inositol. Accordingly, the upregulated protein kinase C activity in BD platelets decreased following 1-2 weeks of lithium treatment (Friedman et al., 1993).

Similarly, the PI-pathway is also affected in MDD. A significant decrease in GTP-γ-S stimulated PI hydrolysis was found in MDD suicide subjects relative to controls (Pacheco et al., 1996). Such decreases in PI hydrolysis may result in decreased activity of protein kinase C. Hrdina et al. (1998) showed that protein kinase C levels in prefrontal cortex of MDD suicide subjects were not different than controls. Furthermore, measurements of MARCKS expression, a protein kinase C substrate, showed no difference between suicide subjects and controls in hippocampus or prefrontal cortex (McNamara et al., 1999). It is difficult at this time, however, to formulate a role of PI-signalling in MDD due to the lack of available information.

The observed changes in upstream cAMP signalling should have downstream consequences on cAMP-dependent gene regulation. Accordingly, temporal cortex protein levels of the transcriptional regulator CREB are decreased in untreated MDD subjects relative to both antidepressant-treated MDD subjects and controls (Dowlatshahi et al., 1998). Furthermore, suicide subjects have lower temporal cortex CREB levels relative to non-suicide subjects (Dowlatshahi et al., 1999). Antidepressant-treated MDD subject CREB levels are not different than controls (Dowlatshahi et al., 1998). This suggests that antidepressants may restore CREB activity to control levels by upregulating the cAMP signalling system. In keeping with the model of antidepressant action in which CREB levels are increased, the expression of a possible target gene for this pathway, BDNF, is also increased by antidepressants. Although BDNF levels are difficult to measure in postmortem brain, increased levels of its receptor trkB have been found in antidepressant treated MDD subjects compared to untreated subjects, lending further support to this hypothesis (Bayer et al., 2000).

Numerous studies have therefore shown evidence of altered cAMP signalling in postmortem brain from mood disorder subjects at multiple sites in this transduction pathway. In general, the literature suggests that the function or levels of components of this pathway are downregulated in MDD and upregulated in BD. Although this signalling pathway is shared by a variety of neurotransmitter systems, the changes are consistent with the hypothesis of decreased noradrenergic signalling in depression, and increased noradrenergic signalling in BD. The expression of several genes linked to this signalling pathway have been shown to be increased by antidepressants and possibly

decreased in depression. One target of interest has been BDNF and its receptor TrkB. Since this target and other genes are involved in neuronal plasticity, researchers have begun to focus on downstream targets of these pathways, specifically structural changes in discrete brain regions. These studies are reviewed next.

Morphological Abnormalities

Abnormalities in the regulation of gene expression can result in biochemical and structural changes at the cellular level. Several genes, downstream to signalling systems, are important for regulating morphological characteristics of neurons. Neurotrophin expression, for example, is implicated in neuronal sprouting, and may be important in the maintenance of cell viability (Duman et al., 1997). Changes in cAMP signalling can lead to abnormal BDNF expression, which may have consequences for neuronal growth and survival. Antidepressant treatment in animal models reportedly upregulates neurotrophin expression and induces morphological changes, including neuronal sprouting (Duman et al., 1997).

Imaging studies have demonstrated structural changes in brains of MDD subjects. Studies consistently show that abnormal volume and activity levels in prefrontal cortex and limbic regions are associated with MDD (Kishimoto et al., 1998). A series of recent molecular studies using postmortem brain from MDD subjects also revealed structural abnormalities in the prefrontal cortex.

Ongur et al. (1998) found structural abnormalities in prefrontal cortex (Brodmann's area 24) of subjects with familial mood disorders using unbiased-stereological techniques. Subjects had significantly decreased number of glia relative to controls. Cortical volume

was also decreased in the subjects with familial mood disorders (Ongur et al., 1998). A trend towards decreased glial number was seen in non-familial forms of mood disorders.

Consistent with these findings, Rajkowska et al. (1999) reported decreased cortical thickness, neuronal size and neuronal and glial density in layers II-IV of the rostral orbitofrontal region of subjects with MDD. Neuronal size and glial density were also reduced in layers V-VI of caudal orbitofrontal cortex in subjects with MDD. Furthermore, MDD subjects had reductions in density and sizes of neuronal and glial cells in both supra- and infragranular layers of the dorsolateral prefrontal cortex (Brodmann's area 9) (Rajkowska et al., 1999); glial density was decreased in layer V, and neuronal sizes were decreased in layer VI (Cotter et al., 2002). Decreased glial density and neuronal sizes were also found in layer VI of anterior cingulate cortex of MDD subjects (Cotter et al., 2001).

Similar cellular changes were reported in brains of subjects with BD. In dorsolateral prefrontal cortex, there is a decrease in neuronal and glial density in layer III (Rajkowska et al., 2001), a decrease in pyramidal cell density in layers III and V (Rajkowska et al., 2001), and decreased neuronal sizes in layers V and VI (Cotter et al., 2002). In anterior cingulate cortex (BA 24) of subjects with BD, there was a 43% increase in layer II dendritic spine synaptic density (Aganova & Uranova, 1992).

Markers for neuronal plasticity and oligodendrocytes have also been examined in brains of MDD suicide subjects (Honer et al., 1999). Myelin basic protein (MBP), a marker for oligodendrocytes, was decreased in anterior frontal cortex of MDD suicide subjects, relative to controls. Furthermore, changes in growth-associated protein-43 (GAP43), a protein associated with synaptic plasticity, were reported in these subjects. Expression of

these proteins varies during development, and appears to vary with neuronal growth and injury. Hrdina et al. (1998) have also reported changes in GAP43 in postmortem brain. They found that antidepressant-free MDD suicide subjects had decreased prefrontal cortex (BA 9) GAP43 protein and mRNA levels as compared to controls. This change was not seen in frontal cortex (BA 10), thereby suggesting a regional specificity. In hippocampus stratum moleculare and presubiculum, MDD subjects had increased levels of SNAP-25 (Fatemi et al., 2001), a synaptosome associated protein involved in plasticity.

In BD, another marker for synaptic function, synapsin, was decreased in hippocampal tissue from BD subjects as compared to controls (Vawter et al., 2000). SNAP-25 levels were also decreased in hippocampul stratum oriens, alveous and presubiculum of BD subjects (Fatemi et al., 2001). Furthermore, expression of the synaptic protein complexin was decreased in CA4, subiculum and parahippocampal gyrus of BD subjects (Eastwood & Harrison, 2000). A 140kd variable alternatively spliced exon of a neural cell adhesion molecule was also increased in hippocampus of BD subjects (Vawter et al., 1998). Although the functional implications of these changes are not yet understood, they suggest altered synaptic organisation in cortical and limbic structures of subjects with mood disorders.

Summary

Recent studies on the molecular pharmacology of antidepressants and mood stabilizers have lead to a reinterpretation of earlier models of the pathophysiology of mood disorders. In particular it has extended the monoaminergic hypotheses to include

signal transduction pathways, transcription factors and structural changes in specific brain regions. This understanding of the mechanism of action of antidepressants and mood stabilizers is consistent with a delayed onset of action of these drugs, and also with their effects over the long term including prevention of future episodes.

Concurrently, there has been increased interest in the use of postmortem brain tissue to study specific psychiatric disorders. Subject selection has become more sophisticated, as have the specific molecular mechanisms to be studied. Earlier findings suggestive of altered noradrenergic neurotransmission in these illnesses are supported by studies on components of the cAMP signal transduction pathway. A recent interest in structural changes, particularly in frontal and orbitofrontal regions of MDD subjects, is consistent with long lasting alterations in such specific neurotransmitter pathways. Furthermore, findings from postmortem brain are remarkably consistent with animal models of psychiatric drug actions.

2. Hypotheses

2.1 The cAMP signalling cascade

Several lines of evidence suggest abnormal cAMP signalling may be important in the pathophysiology of mood disorders. The objective was to determine if components of the cAMP signalling system were differentially active in the Stanley Foundation Neuropathology Consortium brain tissue obtained from subjects with MDD and BD as compared to age and sex matched non-psychiatric controls. Furthermore, we wanted to assess the effects of drug treatment at the time of death in the same subjects. We chose to measure Gαi, Gαs, AC activity and cAMP levels as surrogates of cAMP signalling activity.

At the time of these experiments, we had access to occipital and temporal cortex tissue. Cyclic AMP signalling abnormalities had previously been reported in these brain regions from subjects with mood disorder (Young et al., 1993) thereby providing a rationale for their use.

Specific hypotheses:

1. Subjects with a diagnosis of BD have increased temporal and occipital cortex Gαs levels, AC activity, cAMP levels and decreased cortical Gαi levels.
2. Gαs levels, Gαi levels, AC activity and cAMP levels, are restored to control levels in temporal and occipital cortex of BD subjects treated with mood stabilizers at the time of death.

3. MDD subjects have decreased temporal and occipital cortex Gαs levels, AC activity, cAMP levels and increased cortical Gαi levels.
4. Gαs levels, Gαi levels, AC activity and cAMP levels are restored to control levels in temporal and occipital cortex of MDD subjects treated with antidepressants at the time of death.

2.2 CREB transcription factor

CREB-mediated transcription is dependent on several factors, including the binding of CREB to the CRE region, the phosphorylation of CREB and the total amount of CREB protein. As CREB activity is regulated by cAMP signalling, our objective was to measure CREB levels, phosphorylation and binding to the CRE-region. Occipital and temporal cortex brain tissues were used to maintain consistency with the cAMP signalling experiments above.

It was not possible to measure temporal cortex phosphorylated CREB levels using the Western immunoblotting procedure. One year following the completion of the CREB and CRE-binding experiments, a sensitive immunohistochemistry assay to measure phosphorylated CREB was developed. Prefrontal cortex slices were obtained from the Stanley Foundation for use in this experiment; temporal and occipital cortex slices were not available. As reviewed in the introduction, several MDD studies have implicated the prefrontal and frontal cortices as sites of cAMP signalling abnormalities, justifying their use in this assay.

Specific hypotheses:

5. CREB levels, CREB phosphorylation and CRE-binding are increased in cerebral cortex of subjects with BD compared to age and sex matched controls, and are restored to control levels BD subjects treated with mood stabilisers at the time of death.
6. CREB levels, CREB phosphorylation and CRE-binding are decreased in cerebral cortex of subjects with MDD compared to age and sex matched controls, and are restored to control levels in MDD subjects treated with antidepressants at the time of death

2.3 CaMK II and IV levels

In addition to phosphorylation by PKA, CREB can be phosphorylated and activated by CaMK IV. CaM Kinases are enzymes regulated by intracellular Ca^{2+} levels. Increased intracellular Ca2+ levels have consistently been reported in cells obtained from BD patients (Emamghoreishi et al., 1997), which may have downstream effects on CaMK activity. The mood stabiliser lithium is known to inhibit IMPase, which ultimately decreases intracellular Ca^{2+} levels and potentially downregulates CaM Kinases. Since CREB levels and activity were measured, CaM Kinases II and IV were also measured to determine the extent of their influence on CREB. Although a reliable method for measuring CaMK activities in postmortem brain was unavailable, it was possible to measure CaMK II and IV protein levels in temporal cortex.

Specific hypotheses:

7. CaMK II and IV levels are increased in temporal cortex of subjects with BD compared to age and sex-matched controls, and restored to control levels in BD subjects treated with lithium at the time of death.

2.4 BDNF levels

BDNF is a CREB-regulated neurotrophic factor that has been proposed as a target for antidepressant treatments (as previously discussed). The objective was to measure BDNF protein levels in subjects receiving antidepressant treatments at the time of death. Most BDNF findings were derived from animal experiments using hippocampus sections. We acquired hippocampal slices from the Stanley Foundation and developed a sensitive BDNF immunohistochemistry assay. Although our CREB and cAMP findings were restricted to the temporal and occipital cortices, slices from these regions were not available.

Specific hypotheses:

8. Hippocampal BDNF protein levels are decreased in MDD subjects compared to age and sex-matched controls, and are restored to control levels in antidepressant-treated MDD subjects.

2.5 Mossy Fibre Sprouting

Neurotrophic factors are thought to be critical for neuronal survival and synaptic plasticity. As such, altered BDNF levels in the hippocampus of MDD subjects may lead to morphological changes in this brain region. Recent studies have shown an increase in

mossy fibre sprouting in the dentate gyrus following electroconvulsive stimulation, a model for ECT-treatment of mood disorders (Vaidya et al., 1999). We wondered if similar changes occur following antidepressant treatments in subjects with MDD. Furthermore, animal kindling experiments, which have been proposed as a model for BD, also reveal increases in dentate gyrus mossy fibre sprouting. The objective was to measure mossy fibre sprouting in the dentate gyrus of the Stanley Foundation hippocampus tissue using Timm staining as a surrogate.

Specific hypotheses:

9. Dentate gyrus Timm staining is increased in MDD subjects receiving antidepressant treatment at the time of death compared to untreated MDD subjects.
10. Dentate gyrus Timm staining is increased in subjects with BD compared to age and sex matched controls, and is restored to control levels in BD subjects treated with mood stabilisers at the time of death.

2.6 DNA fragmentation

BDNF is critical for neuronal survival and plays an important role in regulating the equilibrium between hippocampal neurogenesis and apoptosis (see Appendix C). Antidepressant treatments in animal models increase dentate gyrus neurogenesis, an effect that may be mediated by BDNF. These studies suggest that MDD may be associated with a decrease in dentate gyrus neurogenesis, which results in an equilibrium shift towards apoptosis. Although it was not possible to measure neurogenesis in the Stanley Foundation

brains tissue, we were able to employ the TUNEL technique to measure DNA fragmentation, a method frequently used in postmortem brain to measure apoptosis. The objective was to measure TUNEL staining as a surrogate for apoptosis in hippocampus of the Stanley Foundation brain tissue.

Specific hypotheses:

11. Dentate gyrus TUNEL staining is increased in subjects with MDD compared to age and sex matched controls, and restored to control levels in MDD subjects treated with antidepressant sat the time of death.

3.1 Increased temporal cortex CREB concentrations and antidepressant treatment in major depression

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Blunted brain cAMP signalling is purported to occur in major depressive disorder (MDD)[1]. Furthermore, antidepressants increase concentrations of the critical downstream target of cAMP signalling, cAMP regulatory element binding protein (CREB)[2]. CREB is a transcription factor involved in regulation of genes important for neuronal regulation and survival of proteins such as brain derived neurotrophic factor. This increase in CREB may underlie the long-term changes thought to be responsible for the delayed therapeutic effect of antidepressants. These hypotheses are intriguing but are derived solely from animal studies and remain to be evaluated in a clinical sample. We investigated CREB concentrations in untreated patients and those treated with antidepressants.

We used postmortem brain tissue[3] from sixty patients (N=15 each MDD, bipolar disorder (BD), schizophrenia (SCZ) and nonpsychiatric controls). The medical records and family interviews were reviewed by two senior psychiatrists to ensure that all patients met DSM-IV criteria. Groups were matched on age (overall sample mean = 45.4yr, SD=11.2), sex (overall 24 female, 36 male), and postmortem interval (overall sample mean = 29.4h, SD = 13.4). Brain extracts were prepared from temporal (Brodmann Areas 20 and 21) and occipital cortex (Brodmann Areas 18 and 17)[4] and we analysed 30 μg per patient by Western blotting [5] with anti-CREB antisera (Upstate Biochemicals Inc., 1:2000 dilution) and the ECL detection system (Amersham). Each gel contained one subject per group and five samples (10-50μg) derived from a single control subject obtained from the Canadian Brain Tissue Bank (CBTB) which were run as a standard. Densitometric values were determined and normalised to the CREB

concentrations obtained from the linear range of the control standard sample. All assays were performed blind to diagnosis and repeated.

There was no correlation between temporal cortex CREB levels and age ($r=0.046$, $p=0.73$) or postmortem interval ($r=-0.039$, $p=0.77$). In the MDD group, higher temporal cortex CREB concentrations were found in patients treated with antidepressants at the time of death (see Figure 1) compared with those who were not ($df\ [1, 14]$, $F=8.48$; $p=0.01$). Furthermore, MDD patients not on antidepressants at the time of death had lower CREB concentrations than in controls ($df\ [1, 19]$, $F=6.27$; $p=0.02$), whereas treated patients did not differ from the control group ($df\ [1, 24]$, $F=1.97$; $p=0.17$). There were no differences in the occipital cortex, which suggests that the effects of antidepressants may be regional. There were no differences in CREB concentrations comparing BD or SCZ subjects to controls or comparing antidepressant treated and non-antidepressant treated BD or SCZ subjects, respectively, suggesting this drug effect may be specific to MDD.

Downstream changes in the cAMP pathway may occur in patients with MDD and that antidepressant treatment may be associated with a return to normal temporal cortex levels of CREB in patients with MDD. The cAMP pathway might ultimately be one of many intracellular pathways contributing to the pathophysiology of depression and its treatment. Studies of this system are providing new insights into the plasticity of the brain at the level of gene expression in response to environmental and pharmacological stimulation.

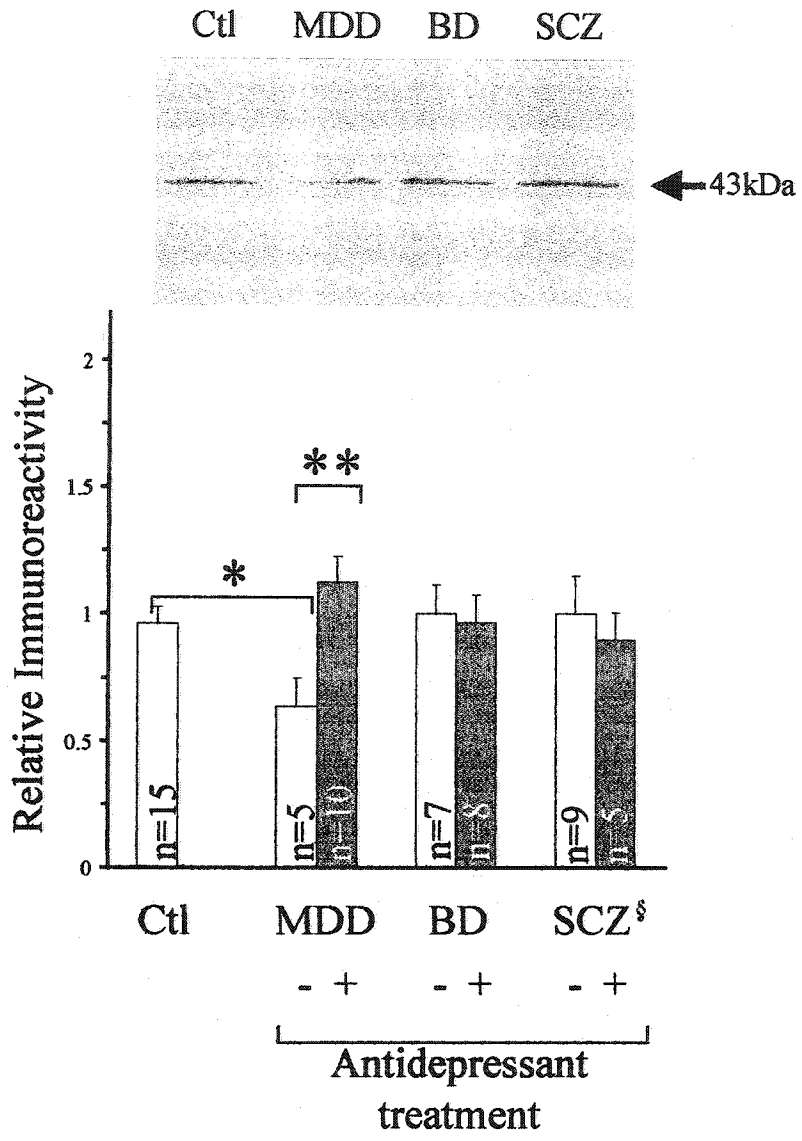

Figure 1: CREB levels in temporal cortex of antidepressant treated and non-antidepressant treated psychiatric patients and controls

Inset depicts a representative immunoblot of the 43 kDa CREB band from three untreated patients and one control. In the graph, * indicates $p<0.05$ for untreated MDD patients compared to controls whereas ** indicates $p=0.01$ for antidepressant treated MDD patients compared to untreated MDD patients. § indicates $n=14$ for SCZ group as one non-antidepressant treated subject was omitted due to almost undetectable CREB levels.

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3.2 G-protein coupled cAMP signaling in postmortem brain of subjects with mood disorders: effects of diagnosis, suicide and treatment at the time of death

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Abstract

Components of cAMP signaling were examined in postmortem cerebral cortex of a well characterized group of patients with mood disorders and non-psychiatric controls. We measured G-protein levels, adenylyl cyclase activity and CREB levels in cerebral cortex of the subjects with respect to diagnoses, treatment and suicide. There was no effect of diagnosis on any measure, except for a trend towards decreased stimulated adenylyl cyclase activity in subjects with mood disorders relative to controls. We also detected a significant effect of suicide on temporal cortex CREB levels in subjects that died as a result of suicide, relative to those that did not, which was more evident in patients with MDD. Bipolar disorder (BD) subjects treated with anticonvulsants at the time of death have decreased temporal cortex CREB levels relative to those not receiving anticonvulsants. Furthermore, we found a trend towards decreased occipital cortex $G\alpha_s$ (short) levels in BD subjects treated with lithium. These results support the hypothesis of altered cAMP signaling in mood disorders and raise the possibility that factors other than diagnosis, such as treatment and suicide, may be relevant to cell-signaling abnormalities reported in the literature.

Numerous studies have revealed abnormalities in brain intracellular signal transduction systems in patients with mood disorders (reviewed in Wang et al., 1997). Specifically, altered cAMP signaling has been implicated in bipolar disorder (BD), major depressive disorder (MDD) and suicide (Young et al., 1993; Cowburn et al., 1994; Pacheco et al., 1996), and as a potential target for antidepressant and mood stabilizer treatments (Ozaki and Chuang, 1997; Dowlatshahi et al., 1998; Wang et al., in press). The cAMP signaling pathway is of particular interest as its downstream target, the cAMP response element binding protein (CREB), regulates transcriptional activity thereby linking immediate post-synaptic events with longer term neuronal changes thought to occur in these disorders (Duman et al., 1997).

Increased levels of the stimulatory G-protein alpha subunit, $G\alpha_s$, have been found in temporal and occipital cerebral cortex of subjects with BD by two laboratories (Young et al., 1993; Friedman & Wang, 1996). Increased $G\alpha_s$ levels were found also in cerebral cortex of subjects with MDD (Pacheco et al., 1996), suggesting that these changes may be related to the diagnosis of a mood disorder rather than specific to BD. In addition, there is evidence that factors such as mood state or treatment with mood stabilizers may be relevant to altered levels of this protein (Schreiber et al., 1991; Avissar et al., 1997). The effects of mood stabilizer treatment or diagnosis of BD on downstream targets of cAMP signaling such as CREB in postmortem temporal and occipital cerebral cortex remains to be determined.

Recent evidence also suggests that decreased cAMP signaling, which may result in decreased CREB-dependent gene transcription, may be associated with the clinical

manifestations of MDD. Several studies have demonstrated altered adenylyl cyclase (AC) activity in cerebral cortex of subjects with MDD (Cowburn et al., 1994; Lowther et al., 1996). Decreased GTPγS and forskolin stimulated AC activity have also been reported in frontal cortex of subjects who died by suicide (Cowburn et al., 1994). Furthermore, animal studies have shown that antidepressant treatment could increase AC and cAMP-dependent protein kinase (PKA) activities (Menkes et al., 1983; Nestler et al., 1989; Perez et al., 1989). Nibuya et al (1996) demonstrated that chronic antidepressant treatment increased rat hippocampal CREB protein, mRNA levels and its binding to the consensus cAMP response element (CRE). We have recently reported that temporal cortex CREB levels were lower in MDD patients not treated with antidepressants at the time of death relative to both treated subjects with MDD and non-psychiatric control subjects (Dowlatshahi et al., 1998). Since CREB levels may be increased by antidepressant treatment, lower levels of this transcription factor would be expected to be associated with the clinical features of MDD.

With the availability of brain tissue through the Stanley Neuropathology Consortium, we decided to reexamine the issue of altered $G\alpha_s$ levels and downstream cAMP-signaling in cerebral cortex obtained from a larger and well characterized sample of subjects with mood disorders. In the present study, we measured $G\alpha_s$ and $G\alpha_i$ levels in temporal and occipital cortex, brain regions in which differences were initially detected, together with basal and forskolin-stimulated AC activity and the downstream

target, CREB, in temporal cortex obtained from a group of age- and sex-matched subjects with BD, MDD and non-psychiatric controls.

Materials and Methods

Postmortem tissue sample and preparation of membrane and cellular extracts

Postmortem brain tissue was generously donated by the Stanley Foundation Neuropathology Consortium (Johnston et al., 1997) and consisted of subjects diagnosed with MDD, BD and age and sex matched non-psychiatric controls (n=15 per group). Temporal and occipital cortical areas were dissected according to the gyral and sulcal landmarks as defined by Brodmann (1909), and the groups were matched for brain hemispheres used. Microscopic examinations were made by two neuropathologists on all cases with toxicological examinations on the majority. Extensive medical records were available for all subjects and were reviewed by two psychiatrists. Where clarification of medical history was required, non-structured interviews were conducted with family members and the treating mental health professional by one psychiatrist to clarify clinical information. Based on the data gathered in this manner, diagnoses were established by two psychiatrists using DSM-IV criteria (demographics shown in tables 1 and 2). Control subjects were confirmed to be free of both psychiatric illness and substance abuse by this same method. Cause of death, substance abuse history, medications at time of death and lifetime intake of antipsychotics was available for all subjects (table 2).

Membrane preparations for cAMP assays and G-protein immunoblotting were isolated from 50 mg of temporal and occipital postmortem cerebral cortex (B.A. 20/21 and 18, respectively) as previously described (Young et al., 1993). Whole cell protein

Table 1: Subject demographics

	MDD (n = 15)	BD (n = 15)	Control (n = 15)
Age (mean ± SEM, yrs)	46.5 ± 2.4 [range 30-65]	42.3 ± 3.0 [25-61]	48.1± 2.8 [29-68]
Sex (M / F)	9 / 6	9 / 6	9 / 6
PMI (mean ± SEM, hrs)	27.5 ± 2.8 [range 7-47]	32.5 ± 4.2 [13-62]	23.7 ± 2.6 [8-42]
Suicide (Y / N)	7 / 8	9 / 6	0 / 15

PMI = postmortem interval

Table 2: Cause of death and treatment information of subjects with BD or MDD

BD subjects

Age / Sex	Postmortem interval (hours)	Antidepressant / mood stabilizer medications at time of death	Other medications	Cause of death	Alcohol / substance abuse
25 / F	24	Li, trazadone CBMZ	NL	Suicide	Current
37 / F	29	Li, bupropion	BDZ	Suicide	Past
30 / M	56	None [a, b, c]	None	Suicide	None
34 / M	23	VPA, venlaflaxine [b, c]	NL	Suicide	Past
48 / M	13	None [b, c]	None	Suicide	None
31 / M	28	Trazadone	Trihexphenidyl, NL	Suicide	Current
30 / M	45	VPA, bupropion	None	Suicide	Past
61 / F	60	VPA, SSRI [a, b]	None	Suicide	None
50 / M	19	VPA	BDZ, benzotropine, clozapine	Suicide	None
48 / F	22	VPA [b], SSRI, [b, c] CBMZ [b]	NL	Pneumonia	Current
54 / M	39	Li, CBMZ	None	Hematoma	Past
30 / M	31	Li	Clozapine	Pneumonia	None
57 / M	19	None [a, b, c]	NL, Diphenhydramine	Cardiac	Past
50 / F	18	None [a]	None	Malnutrition	Current
50 / F	62	VPA, TCA	None	Pulmonary	None

MDD subjects

Age / Sex	Postmortem interval (hours)	Antidepressant / mood stabilizer medications at time of death	Other medications	Cause of death	Alcohol / substance abuse
32 / F	47	TCA [b, c]	BDZ	Suicide	None
44 / F	32	TCA [b, c], SSRI [c]	BDZ	Suicide	None
46 / M	26	None [a, b]	BDZ, diphenhydramine	Suicide	None
51 / M	26	Nefazadone [a]	Hydroxyzine	Suicide	Past
39 / M	23	None [a, b]	None	Suicide	Current
42 / M	7	None [a, b]	None	Suicide	None
30 / F	33	TCA [a, b], SSRI	BDZ	Suicide	None
53 / F	40	Li, trazadone	None	Acute alcohol intoxication	Current
65 / M	19	None [b, c]	Phenytoin	Cardiac	None
52 / M	12	None	None	Cardiac	None
42 / F	25	SSRI, Li	None	Cardiac	None
56 / M	23	SSRI	None	Cardiac	None
56 / F	28	SSRI	BDZ, buspirone	Pulmonary	None
43 / M	43	TCA	None	Cardiac	Current
47 / M	28	Nefazadone, SSRI	None	Cardiac	None

TCA = tricyclic antidepressant, SSRI = selective serotonin reuptake inhibitor, Li = lithium, BDZ = benzodiazepine, NL = typical neuroleptic, VPA = sodium valproate, CBMZ = carbamazepine; Where possible, antidepressant / mood stabilizer treatment was confirmed with toxicological data obtained from either a) urine, b) blood or c) brain.

homogenates for CREB immunoblotting were isolated as described (Widnell et al., 1994). Protein concentrations were quantified as per the method of Bradford (1976).

Basal and stimulated adenylyl cyclase activity

Basal and forskolin-stimulated cAMP was measured as previously described (Young et al., 1993). Briefly, temporal cortex membrane preparations (5 μg protein) were incubated in 200 μl of reaction mixture consisting of 50 mM Tris-HCl (pH 7.4), 1.5 mg/ml of bovine serum albumin, 2mM EGTA, 25 mM phosphocreatine, 250 U/ml creatine phosphokinase, 5 mM $MgCl_2$ and 100 μM isobutylmethylxanthine for 10 minutes at 4°C. After preincubation, reactions were initiated by the addition of 0.5 mM ATP together with either 100 μM forskolin or 50 mM Tri-HCl (pH 7.4) as control for 10 minutes at 37°C. The reaction was stopped by boiling samples for 5 minutes and centrifuging at 13,000xg for 5 minutes at 4°C. The supernatants were assayed for cAMP using a radioimmunoassay kit (Amersham Pharmacia Biotech, cat#RPA 509). All subjects were analyzed in duplicate and assays were performed blind to diagnoses. Data were expressed as pmol of cAMP produced per mg protein per minute.

Immunoblotting

Temporal and occipital cortex preparations were immunoblotted for G-proteins (10 μg used) and CREB (30 μg used) as previously described (Young et al., 1993; Wang et al., in press). Briefly, protein extracts were subjected to SDS-PAGE with a 12% acrylamide gel at 120V for 1.5 hours. Proteins were transferred to PVDF membranes for 1 hours at 100V and 4°C. Blots were blocked in 5% milk-PBS for 45 minutes and

incubated overnight with either $G\alpha_s$ antisera (1:10000 dilution, NEN Life Science), $G\alpha_{i(1\&2)}$ antisera (1:5000 dilution, NEN Life Science) or CREB antisera (1:2000 dilution, Upstate Biologicals Inc). Blots were washed and incubated with secondary antibody goat anti-rabbit IgG conjugated to horseradish peroxidase diluted to 1:2000 in blocking buffer for 1 hour at room temperature, and immunoreactive bands detected with the enhanced chemiluminescence (ECL) system (Amersham). Each gel contained a prestained broad range protein ladder to measure molecular weights of individual bands. Immunoblotting with $G\alpha_s$ antisera resulted in two bands corresponding to the long and short forms of $G\alpha_s$ protein (figure 1). Immunoblotting with $G\alpha_i$ antisera resulted in a single band, whereas immunoblotting with CREB antisera yielded a 43 kDa CREB band. A non-specific band migrating at 55 kDa was also found using the CREB antiserum (data not shown). The 43 kDa CREB band was confirmed using control HeLa cell extract (Upstate Biologicals, inc). A linear range (10 to 40 μg for CREB, 2.5 to 20 μg for G-proteins) of postmortem brain protein from a separate control human subject was run on each gel (figure 1) thereby allowing standardization of values as previously described (Dowlatshahi et al., 1998). Again, samples were run in duplicate and blind to diagnosis.

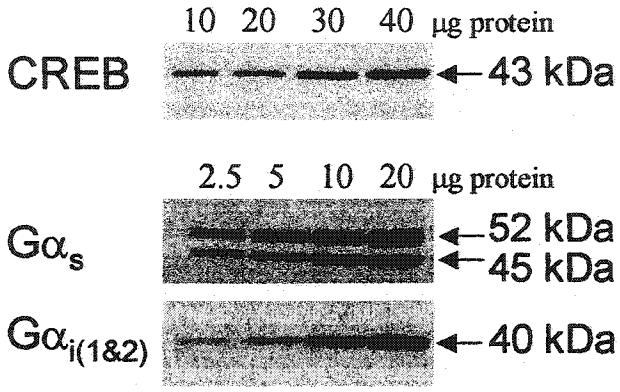

Figure 1. Representative temporal cortex immunoblots depicting linear ranges of protein used for standardization (10 – 40 µg for CREB, 2.5 to 20 µg for G-proteins).

Results

There were no significant differences in age ($F = 1.17$, df [2,44], $p = 0.32$), sex ($F < 0.01$, df [2,44], $P > 0.999$) or postmortem delay ($F = 1.85$, df [2, 44], $p = 0.17$) between control, MDD or BD subjects. There was no overall effect of age, sex or postmortem interval on temporal and occipital cortex $G\alpha_s$, $G\alpha_{i(1\&2)}$, and CREB levels, or on temporal cortex basal or forskolin stimulated AC activity ($p > 0.16$ in all cases). All measures were stable with respect to the age and postmortem intervals of the tissue samples used in this study (figure 2).

As abnormalities were previously detected in these measures in patients with mood disorders, we first looked for differences across diagnostic groups. In contrast to previous studies, no significant differences were detected in the levels of $G\alpha_s$ (long or short form) or $G\alpha_i$ across diagnostic groups in either temporal or occipital cortex (figure 3). Although no differences were evident comparing basal AC activity across patient groups, there was trend towards decreased forskolin-stimulated AC activity in both groups of patients compared to non-psychiatric control subjects ($F = 2.61$, df [2,44], $p = 0.085$).

Comparing subjects who died as a result of suicide to those who did not, there were no differences in $G\alpha_s$ (long or short) levels, $G\alpha_i$ levels, or in basal or forskolin-stimulated cAMP levels (data not shown). As we had previously reported an effect of treatment with antidepressants at the time of death to increase CREB levels (Dowlatshahi et al., 1998) and since there was overlap between antidepressant use at the time of death

and suicide, we took this variable into account in subsequent analyses of CREB levels. When we covaried for antidepressant treatment at the time of death, we found a significant main effect for suicide on temporal cortex CREB levels ($F = 4.63$, df [1,44], $p = 0.037$), with lower levels in the group that died by suicide (figure 4). We then examined whether the effect of suicide associated with lower temporal cortex CREB levels was restricted to either diagnosis. There was no significant difference in the temporal cortex CREB levels comparing BD subjects who died by suicide with those who did not ($T = -0.31$, df [9], $p = 0.77$). In contrast, there was a trend towards lower CREB levels in this same region in MDD suicide victims relative to MDD patients who died from other means ($T = -1.95$, df [10], $p = 0.08$; data not shown). As can be seen in table 2, there is an overlap between subjects on antidepressants at the time of death and those who died by suicide (4/7 in the suicide group and 6/8 in the non-suicide group).

We previously reported on the effects of antidepressants on downstream cAMP signaling in this sample (Dowlatshahi et al., 1998). In this study, we examined the effects of treatment with mood stabilizers at the time of death. Patients with BD were classified based on whether they were treated with either lithium or anticonvulsants (carbamazepine or divalproex sodium) at the time of death. We found a trend toward lower $G\alpha_s$ (short form) levels in occipital cortex of BD patients on lithium at the time of death ($T = -2.09$, df [10], $p = 0.063$; figure 5A). No other lithium effects were evident. Treatment with anticonvulsants at the time of death had no apparent effect on either G-protein, basal cAMP or forskolin-stimulated cAMP levels. However, temporal cortex

CREB levels were significantly lower in BD subjects on anticonvulsants at the time of death compared with those who were not ($T = -2.27$, df [11], $p = 0.044$; figure 5B).

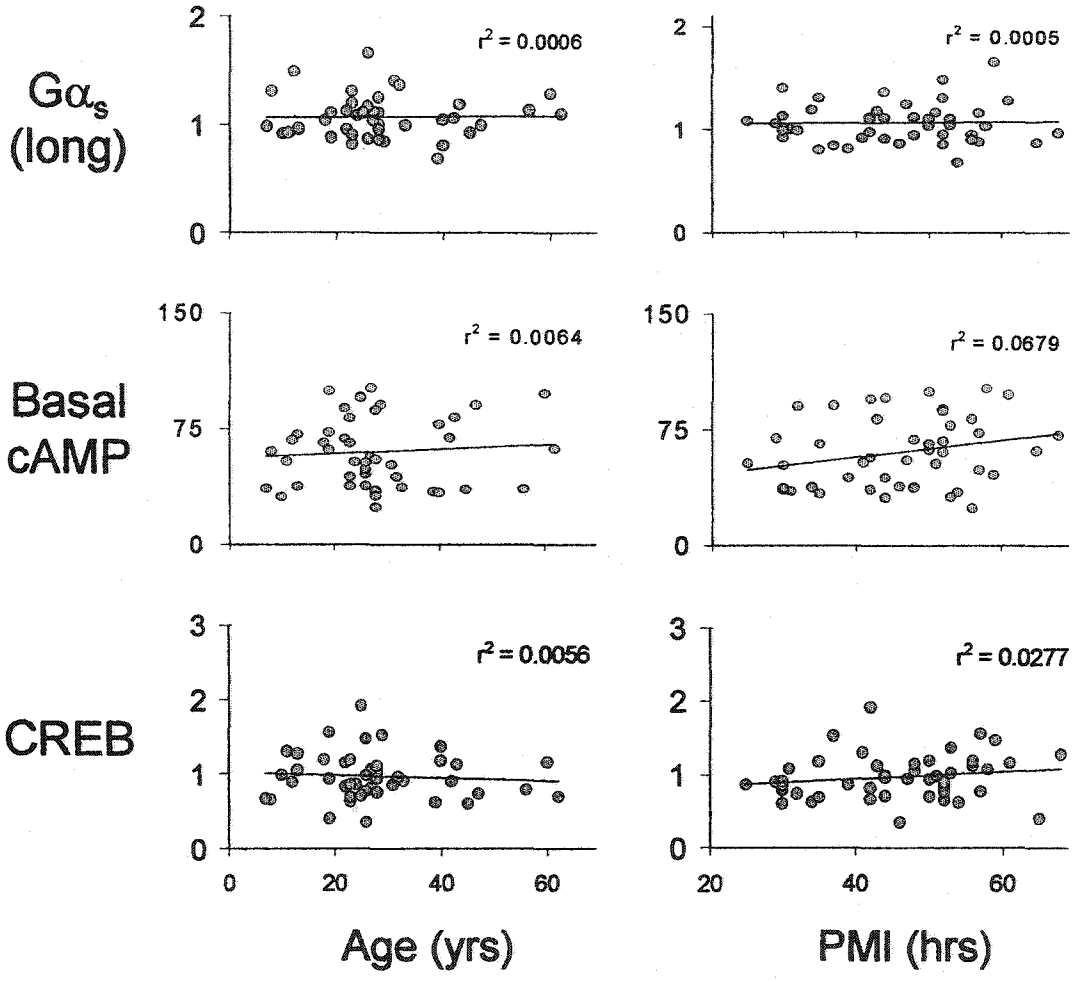

Figure 2. Temporal cortex $G\alpha_s$ (long form) immunoreativity, basal adenylyl cyclase activity and CREB immunoreactivity of all subjects (n = 45) with respect to postmortem intervals and age.

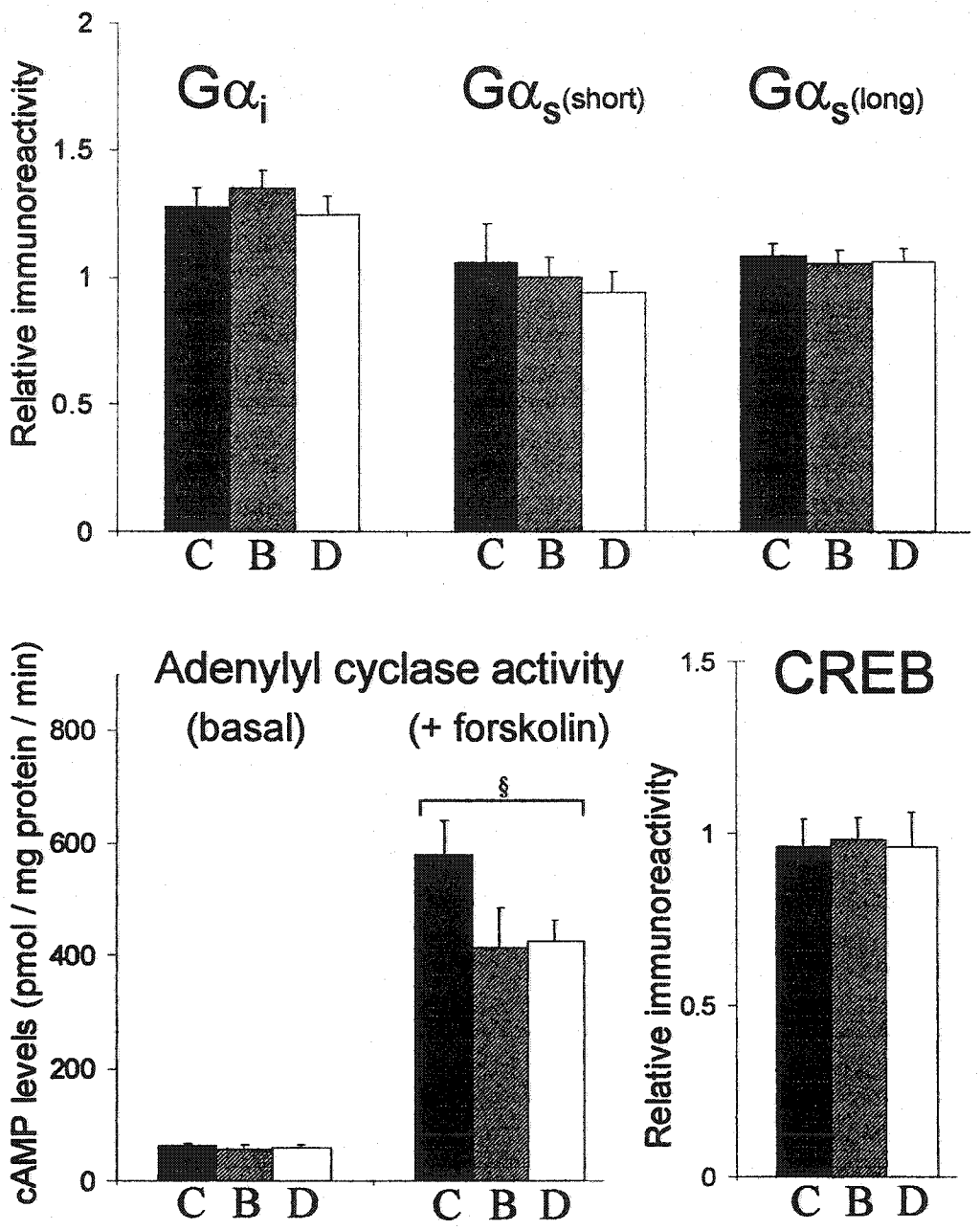

Figure 3. Temporal cortex immunoreactivities and adenylyl cyclase activities of control subjects (C), bipolar disorder subjects (B) and major depressive disorder subjects (D). Values are expressed as mean ± SEM, and n = 15 for each group. § p=0.085.

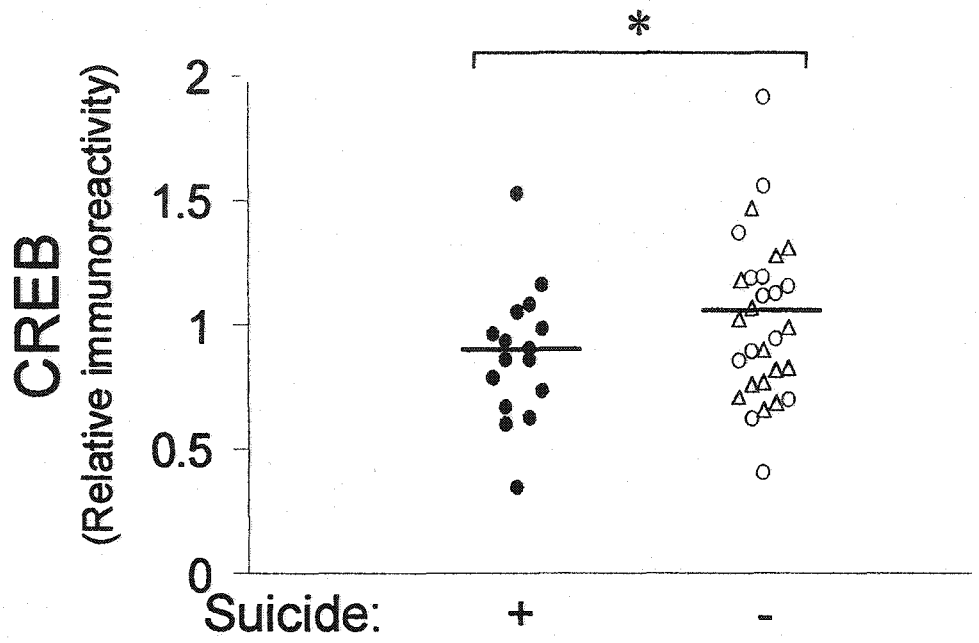

Figure 4. Temporal cortex CREB immunoreactivities (mean ± SEM) of suicide (+) and non-suicide (-) subjects. Δ = non-psychiatric control subjects. * p = 0.037.

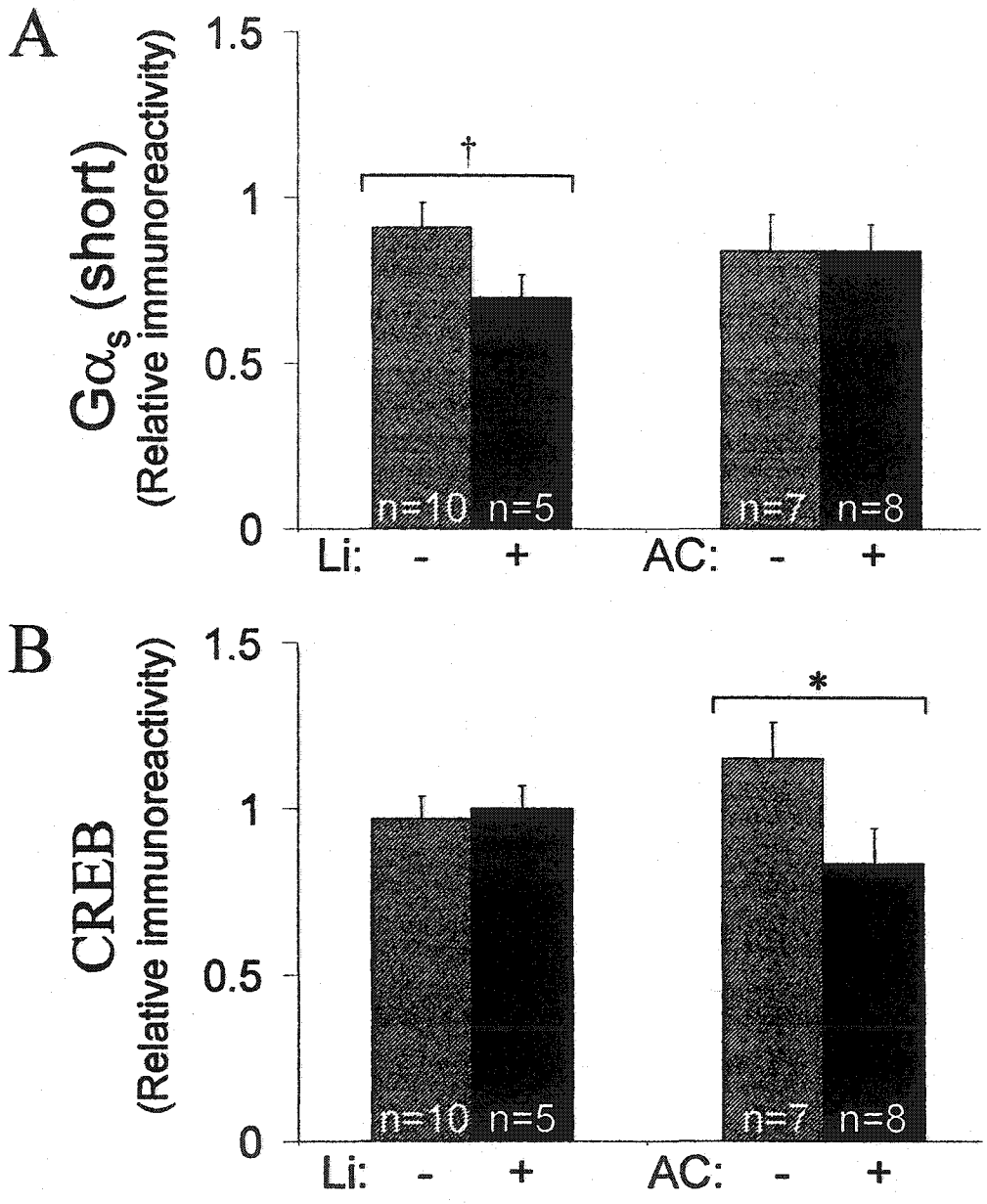

Figure 5. Effects of lithium (Li) and anticonvulsant (AC) treatment at the time of death on **A)** occipital cortex $G\alpha_s$ (short form) immunoreactivity and **B)** temporal cortex CREB immunoreactivity in subjects with bipolar disorder. † $p = 0.063$, * $p = 0.044$.

Discussion

In contrast to studies using smaller numbers of subjects, we found no evidence of increased $G\alpha_s$ levels in temporal or occipital cortex of subjects with BD. Increased cerebral cortex $G\alpha_s$ levels may therefore be the result of different clinical and demographic variables between subject groups. For example, treatment with mood stabilizing medications, which were commonly used in our BD patients, may account in part for differences between the present study and previous reports. We found a trend toward lower occipital cortex $G\alpha_s$ levels (short form) in subjects treated with lithium at the time of death compared to those not on this drug. Lithium decreases $G\alpha_s$ mRNA and protein levels in rat brain and cultured PC12 cells (Li & Jope, 1995); increased levels of this protein may thus occur in untreated BD, with lithium treatment near the time of death causing decreased $G\alpha_s$ levels and possibly accounting for the differences between our results and previous reports. Furthermore, studies in lymphocytes obtained from subjects with BD suggested that mood state may also affect levels of $G\alpha_s$ (Schreiber et al., 1991; Avissar et al., 1997). Since mood state at the time of death is difficult to infer, the discrepancy between studies may reflect heterogeneity of mood states across the subject groups.

In accordance with the $G\alpha_s$ data , but in contrast to a previous study (Young et al., 1993), there were no significant differences in basal AC activity and a trend towards decreased AC activity in the BD subjects. The factors that may have accounted for the observed discrepancy in $G\alpha_s$ levels may also be relevant to AC activity. In addition,

antidepressants are known to increase cerebral cortex AC activity in rat brain (Menkes et al., 1983) and in brain from treated depressed subjects (Reiach et al., in press). Since subjects in earlier and present studies were variably treated with antidepressants, these medications may have contributed to the AC findings.

When cerebral cortex CREB levels were examined, the most striking finding was that CREB levels were significantly lower in BD subjects on anticonvulsants at the time of death, an effect opposite to that of antidepressants (Dowlatashi et al, 1998). This suggests that CREB may be affected by treatment with anticonvulsants and supports studies in rat brain and cultured cells demonstrating that mood stabilizers regulate the levels and function of specific transcription factors (Ozaki and Chuang, 1997; Chen et al., 1999; Wang et al., in press). The fact that anticonvulsants and antidepressants have opposite effects on CREB levels is concordant with the apparently opposite clinical effects that these medications exert in conditions such as mania.

Previously, we reported a diagnostically specific decrease of temporal cortex CREB levels in the antidepressant untreated MDD subjects (Dowlatshahi et al., 1998). Here, we looked for corresponding alterations in upstream components of the cAMP signaling pathway. There were no differences in $G\alpha_s$ (long or short) or $G\alpha_i$ levels in temporal or occipital cortex when control and MDD subjects were compared. Furthermore, no changes were apparent in basal cAMP levels in MDD subjects, although there was a trend toward blunted forskolin-stimulated cAMP levels in temporal cortex compared to control subjects. In contrast to the reported changes in temporal cortex CREB levels, we found that antidepressant treatment had no effect on G-protein levels

and AC activity. These results suggest that the previously reported antidepressant effect may be specific to CREB or other downstream components of the cAMP pathway. When we compared subjects with MDD who died by suicide versus those who died by other means, however, there was a trend towards decreased temporal cortex CREB protein levels in subjects with MDD who died from suicide. When all groups were compared in this way, CREB levels were lower in temporal, but not occipital, cortex of patients who suicided. While this relationship between suicide and temporal lobe CREB levels was apparent across diagnostic groups, it appeared maximal in subjects with MDD. Furthermore, the relationship between suicide and CREB levels appears to be independent of the previously reported effect of antidepressant treatment on temporal cortex CREB levels.

These findings support the growing literature suggesting that downstream targets of the cAMP signaling pathway are important in mood disorders, but they must be interpreted with caution. First, the levels of phosphorylated CREB or its binding to the CRE consensus sequence could not be reliably measured in postmortem brain tissue, making it difficult to determine the functional relevance of decreased CREB levels with respect to neuronal gene expression. Second, it is not possible to know the mood state of the subjects at the time of death; although suicide is likely to be associated with multiple depressive symptoms, it is not known whether the changes in CREB levels are directly associated with severity of depression. Third, to clearly differentiate the relations between suicide and antidepressant treatment on cortical CREB levels, a larger sample of depressed subjects who were not exposed to antidepressant treatment but died by suicide

would be required. Fourth, medication effects are based on drugs prescribed at the time of death. Knowledge about patient compliance, length of time of treatment and past treatment would help to further clarify the relationship between drug treatment and these measures. Finally, although the trend towards decreased forskolin-stimulated AC activity is consistent with an earlier study in frontal cortex of depressed suicide victims (Cowburn et al., 1994), the lack of changes in $G\alpha_s$ or $G\alpha_i$ levels in temporal cortex of MDD subjects are not concordant with published findings. Cowburn et al. (1994) found no difference in the levels of these G-proteins in frontal cortex of depressed subjects who died by suicide, but a more recent study on a well characterized group of depressed subjects found a significant increase in $G\alpha_s$ levels in frontal cortex (Pacheco et al., 1996). It is difficult to know what factors contribute to this variability in results.

In the present study, we analyzed components of the cAMP signaling pathway in postmortem tissue from a well characterized group of psychiatric subjects. Overall, patients with BD did not differ from controls on any measure, but BD subjects treated with anticonvulsants at the time of death had significantly lower levels of CREB in temporal cortex. As well, there was a trend towards decreased occipital cortex $G\alpha_s$ in BD patients on lithium at the time of death. In patients with MDD, we report an apparent relationship between decreased temporal cortex CREB levels and death by suicide; this relationship was present in the larger population of subjects, but was most evident in subjects with MDD. In summary, these data suggest the presence of abnormalities in components of the cAMP signaling pathway in subjects with mood disorders.

Furthermore, they emphasize the importance assessing clinical factors such as suicide and drug treatment in interpreting results of human postmortem brain studies.

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3.3 Abnormalities in the cAMP signaling pathway in post mortem brain tissue from the Stanley Neuropathology Consortium

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Abstract

There is an established relationship between the monoaminergic neurotransmitter system and mood disorders. In an attempt to define further the pathophysiology of mood disorders, research is focussing on intracellular second messenger systems including cyclic adenosine 3', 5'-monophosphate (cAMP) and the polyphosphoinositol (PI) generated second messengers. The availability of tissue from the Stanley Foundation Neuropathology Consortium has offered us the opportunity to make a number of observations with respect to these second messenger systems in tissue from patients with major depressive disorder and bipolar affective disorder. There is evidence that antidepressants stimulate components of the cAMP pathway in patients with depression while mood stabilizers blunt the same pathway in patients with bipolar disorder; furthermore, downstream targets of this pathway appear to be altered in patients with mood disorders. The relations between changes in second messenger systems, gene transcription and clinical effects of current therapeutic regimens has implications for development of novel treatments of mood disorders.

Introduction

Major depressive disorder and bipolar affective disorder are amongst the most common and debilitating psychiatric disorders, but the pathophysiology of these disorders remains unknown. It is widely thought that patients with mood disorders suffer from a dysfunction of monoaminergic neurotransmitter systems, likely resulting from a complex interaction of genetic predisposition, adverse early life experience and environmental stress [18, 26]. In an attempt to elucidate the pathophysiology of mood disorders and explain the long observed relation between monoaminergic systems and antidepressant and mood stabilizing medications [17, 25, 26], studies have increasingly focussed on intracellular second messenger systems. Recent evidence suggests that signal transduction abnormalities occur in mood disorders, and may also be relevant in pharmacologic treatments for major depressive disorder and bipolar disorder [7, 22].

Neurotransmitters and neuropeptides regulate receptor-coupled second messengers including cyclic adenosine 3', 5'-monophosphate (cAMP) and the polyphosphoinositol (PI) generated second messengers among others. Of these, the cAMP pathway is currently among the best studied in the pathophysiology of mood disorders [7, 22]. In this pathway, receptor-binding is coupled to cAMP production via G-proteins, which can either stimulate (Gαs) or inhibit (Gαi) adenylyl cyclase (AC) to convert ATP to cAMP. Cyclic AMP is also coupled to downstream transcriptional

regulation by activating protein kinase A (PKA), which can phosphorylate the transcription factor CREB.

Studies using cell lines, animal models and human *post mortem* brain tissue have demonstrated changes in the cAMP signaling pathway that may be relevant to the pathophysiology and treatment of mood disorders. Antidepressant (AD) treatment in rodents results in increased forskolin-stimulated AC activity, increased Gαs levels and increased coupling between Gαs and AC in rat brain[3]. Chronic AD administration also increases CREB mRNA, and protein levels as well as CREB phosphorylation [19]. Interestingly, long-term, but not short-term administration of antidepressants also increased expression of the cAMP regulated gene BDNF, and its receptor trk B, in rat hippocampus [20]. Since these changes occur after 10-21 days of treatment, this time course is consistent with the observed lag between AD initiation and improvement of symptoms in major depressive disorder patients [13].

Post mortem brain studies have shown that antidepressant-free major depressive disorder subjects have increased Gαi and GRK 2/3 protein levels, both of which can desensitize the cAMP pathway [10]. These studies also found that decreased Gαi levels are present in subjects treated with antidepressants. Other studies have shown decreased basal, forskolin- and GTP-stimulated AC activity in the frontal cortex of major depressive disorder subjects, and decreased stimulated AC activity in the frontal cortex of suicide victims compared to controls [4, 15]. These studies suggest that major depressive

disorder is associated with blunted cAMP signaling, while antidepressant treatment increases signaling at a number of points along the cAMP pathway.

Studies of bipolar disorder have also found cAMP signaling abnormalities [16]. Lithium administration affects G-protein coupled cAMP signaling by blunting agonist- and GTP-stimulated cyclic AMP production, regulating AC and Gαs expression, and increasing levels of PKA substrate DARPP-32[12] in rat brain. Lithium also inhibits CREB phosphorylation in rat cortex and hippocampus, and decreases forskolin-stimulated CRE binding to DNA in cultured cells, with no observed effect on CREB expression [2, 27]. *Post mortem* brain tissue and blood cells from bipolar disorder patients reveal increased levels of Gαs, which decrease following mood stabilizer treatment [9, 18, 28]. *Post mortem* brain studies also reveal increased cAMP-binding and PKA activity in patients with bipolar disorder [8, 23]. These studies suggest that mood stabilizer drugs affect the cAMP-signaling pathway at various sites.

Given the recent availability of tissue from the Stanley Foundation Neuropathology Consortium, we proposed to test components of these second messenger systems in *post mortem* brains from a well-characterized group of patients with major depressive disorder, bipolar disorder and schizophrenia and control subjects.

MATERIALS AND METHODS

Stanley foundation tissue sample

Post mortem brain tissue was obtained from 15 subjects in each of the bipolar disorder, major depressive disorder, schizophrenia and non-psychiatric, non-neurologic control groups [5]. Detailed information was available on all subjects through medical records that included demographic data, medical history, substance abuse history, psychotropic drug treatment history, cause of death, and medication at the time of death. Diagnoses were established according to DSM-IV criteria by two senior psychiatrists after reviewing the medical records and interviewing family members. Similar review of control subjects confirmed lack of psychiatric illness and substance abuse. All groups were matched for age, sex and *post mortem* delay interval. In our studies we used cerebral cortex (frontal, temporal and occipital) and hippocampus, regions in which abnormalities have been demonstrated in subjects with mood disorders.

Basal and stimulated adenylyl cyclase activity

Temporal cortex membrane preparations (5 μg protein) were incubated in 200 μl of reaction mixture consisting of 50 mM Tris-HCl (pH 7.4), 1.5 mg/ml of bovine serum albumin, 2mM EGTA, 25 mM phosphocreatine, 250 U/ml creatine phosphokinase, 5 mM $MgCl_2$ and 100 μM isobutylmethylxanthine for 10 minutes at 4°C. After preincubation, reactions were initiated by the addition of 0.5 mM ATP together with either 100 μM forskolin or 50 mM Tri-HCl (pH 7.4) as control for 10 minutes at 37°C. The reaction

was stopped by boiling samples for 5 minutes and centrifuging at 13,000xg for 5 minutes at 4°C. The supernatants were assayed for cAMP using a radioimmunoassay kit (Amersham Pharmacia Biotech, NJ).

Immunoblotting Analysis

Temporal and occipital cortex preparations were immunoblotted for G-proteins (10 μg used), CREB (30 μg used) and CaM Kinase II and IV (30 μg used) as previously described [27, 28]. Briefly, protein extracts were subjected to SDS-PAGE with a 10% acrylamide gel at 120V for 1.5 hours. Proteins were transferred to PVDF membranes for 1 hours at 100V and 4°C. Blots were blocked in 5% milk-PBS for 45 minutes and incubated overnight with either $G\alpha_s$ antisera (1:10000 dilution, NEN Life Science), $G\alpha_{i(1\&2)}$ antisera (1:5000 dilution, NEN Life Science), CREB antisera (1:2000 dilution, Upstate Biologicals Inc), CaMKII or CaMKIV mouse monoclonal antibody (1:1000 dilution, Transduction Laboratories). Blots were washed and incubated with secondary antibody goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase diluted to 1:2000 in blocking buffer for 1 hour at room temperature, and immunoreactive bands detected with the enhanced chemiluminescence (ECL) system (Amersham). Each gel contained a prestained broad range protein ladder to measure molecular weights of individual bands. Samples were run in duplicate and blind to diagnosis.

Summary of Results

In this section, we will summarize results from our recent experiments that examined the cAMP signaling pathway in this tissue sample. These data are summarized in figure 1. We will also present previously unpublished results on another important factor, calmodulin dependent kinase study (CaMK) which may also regulate CREB phosphorylation and therefore be relevant to our understanding of the pathophysiology of major depressive disorder and bipolar disorder.

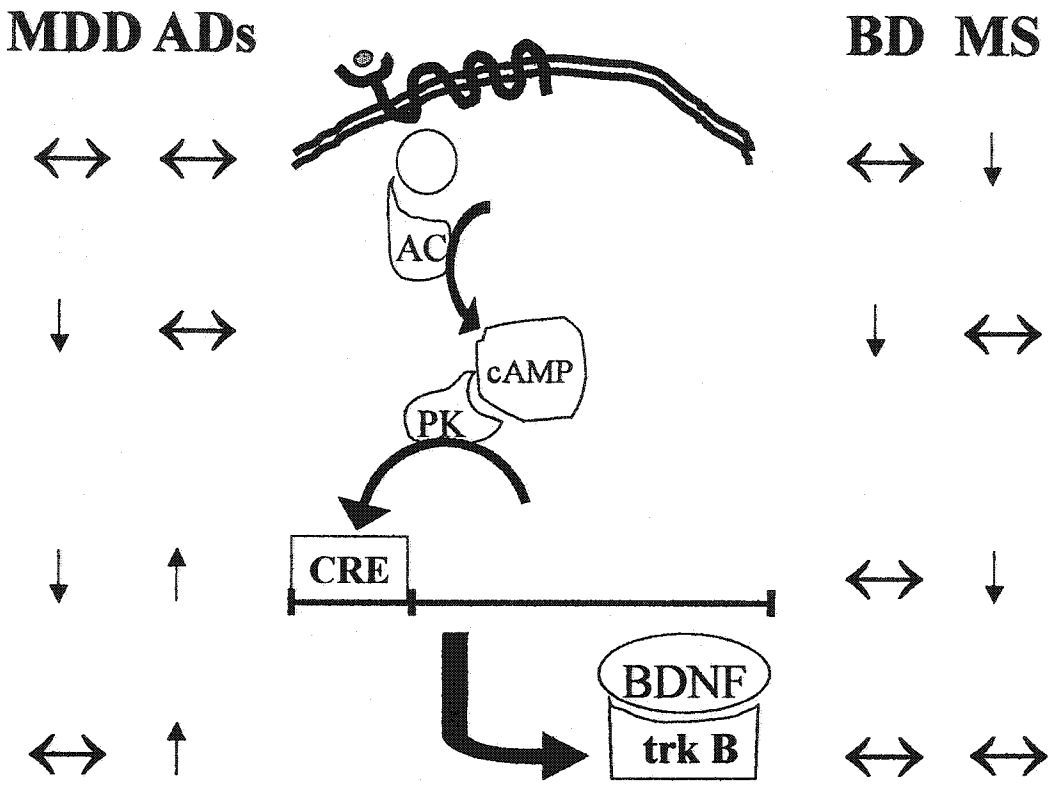

Figure 1 Cyclic AMP signaling pathway abnormalities in Stanley Foundation postmortem brain tissue.

Bipolar Disorder and the Effects of Treatment with Mood Stabilizing Drugs

We measured components of the cAMP pathway in bipolar disorder subjects. No differences between diagnostic groups were found in the levels of Gαs and Gαi in either temporal or occipital cortex [5], regions in which we and others have previously found changes in levels of these proteins. There was, nonetheless, a trend towards lower levels of Gαs in bipolar disorder subjects on lithium at the time of death compared to those subjects without lithium treatment, suggesting that treatment with this drug may be a critical factor when examining results of *post mortem* brain studies. There was also a trend towards lower forskolin-stimulated AC activity in this group compared with control subjects, providing further evidence of abnormalities in this pathway in bipolar disorder. CREB was also studied, with evidence of lower CREB levels in temporal cortex in subjects treated with mood stabilizing anticonvulsants at the time of death although there were no overall diagnostic group differences.

Major depressive disorder and the effect of antidepressants

As in bipolar disorder subjects, there were no differences in levels of Gαs and Gαi in the major depressive disorder subject group compared to the control group [5]. Basal AC activity was not different in the major depressive disorder group compared to controls, but there was a trend towards decreased forskolin-stimulated AC activity in this group. Further downstream, we found temporal cortex CREB levels to be lower in major depressive disorder subjects not treated with antidepressants at the time of death

compared to those receiving antidepressants [6]. Moreover, CREB levels in antidepressant treated patients with major depressive disorder were equal to those of control subjects.

To further evaluate the significance of changes in CREB levels in these subjects, we wished to study CREB phosphorylation as phosphorylation is critical to CREB function. However, we were unable to directly measure phosphorylated CREB levels in *post mortem* brain tissue. An indirect approach was therefore used, focussing on specific kinases which are known to phosphorylate CREB. Since intracellular calcium levels have been consistently shown to be altered in subjects with mood disorders, we next focussed on measuring both CaM kinase II and IV levels in homogenate and brain tissue slices. Using previously described techniques [5] and specific antisera from commercial sources, we were able to detect distinct immunoreactive bands for each kinase and intracellular staining in neurons in cerebral cortical sections (figure 2). Thus far, we have found no significant differences between diagnostic groups in CaMKinase II and IV levels. Furthermore, there were no significant effects of treatment with either antidepressants or mood stabilizers on these measures. When we examined the contribution of other clinical factors in these subjects, we did find significantly lower ($T=2.0$, $df(48,1)$, $p<0.05$) CaMKinase II levels in frontal cortex of subjects with a history of substance abuse (1.40 ± 0.22 ROD, $N=9$) compared to subjects with no such history (2.1 ± 0.15 ROD, $N=40$). No similar effect was evident in CaMKinase IV levels. The relevance of the CaMKinase II data remains to be established.

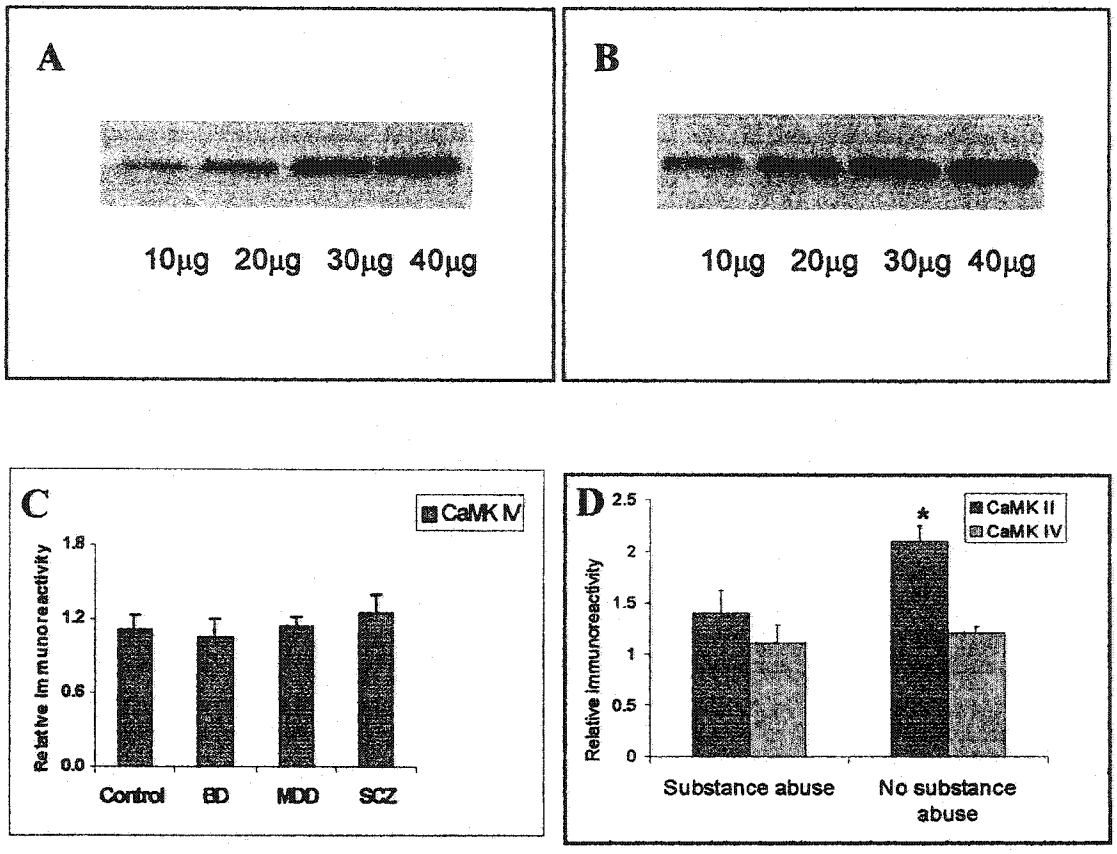

Figure 2 Immunoblotting with CaMKIV antisera resulted in a single band at 60KDa for **A** CaMKII, and **B** CaMKIV. A linear range (10, 20, 30 and 40 µg) of postmortem brain protein from a separate control human subject was run on each gel. **C.** Temporal cortex CaMK immunoreactivities (mean ±SEM) of subjects with BD, MDD, SCZ and matched controls. **D.** Temporal cortex CaMK immunoreactivities of subjects meeting criteria of substance abuse relative to subjects not meeting the criteria. * $p = 0.05$ compared to CaMKII levels of subjects meeting substance abuse criteria.

DISCUSSION

The availability of Stanley Foundation tissue has offered a unique opportunity to study the relation between mood disorders and intracellular signal transduction pathways (figure 1) in a large well-characterized *post mortem* sample. To date, several associations have emerged. First, both major depressive disorder and bipolar disorder subjects appear to have decreased stimulated-AC activity compared to controls. Second, antidepressant and mood stabilizing agents exert multiple effects on the cAMP pathway. Lithium treatment may decrease cortical $G\alpha_s$ levels. Antidepressant treatment may increase CREB levels in temporal cortex of subjects with major depressive disorder. Anticonvulsant mood stabilizing drugs may have opposite effects on cortical CREB levels in patients with bipolar disorder. Third, there are components of the cAMP pathway that are not influenced by treatment modality or diagnosis. These included levels of $G\alpha i$, basal AC activity and CaM Kinase II and IV levels. Fourth, other clinical variables may also be important in the regulation of this pathway as evidenced by the finding of lower cortical CaM Kinase II levels in subjects with a history of comorbid substance abuse. In the aggregate, these results reinforce the importance of the cAMP pathway in the diagnosis and treatment of mood disorders. Furthermore, the observed changes in downstream targets such as CREB suggest the involvement of gene regulation in mood disorders and their treatment.

Results of other studies on intracellular second messenger systems in *post mortem* brains of major depressive disorder and bipolar disorder subjects are generally consistent

with those from our lab. However, one study reported increases in Gαi levels in antidepressant-free major depressive disorder subjects [10] and another found decreased $G\alpha i_2$ and increased Gαs in major depressive disorder suicide patients [21]. The latter study did not separate patient groups based on treatment, which may account for the discrepancy in results. In contrast to our present findings, increased Gαs and increased stimulated-AC activity have been previously reported [9, 28, 29]. These differences may be related to mood state at the time of death (see below), or may reflect a differential intensity of psychotropic drug treatment prior to death. In support of the latter possibility is the fact that mood stabilizer drug treatments altered levels of G-proteins in bipolar disorder subjects. The findings of altered CREB levels in major depressive disorder and bipolar disorder patients related to drug treatment are supported by previous studies in rat brain and cultured cells which showed mood stabilizing and antidepressant effects on transcription factors [19, 20, 23, 27]. The increased BDNF expression in response to antidepressant treatment is also in accordance with the CREB data and previous studies measuring BDNF levels in rat brain exposed to antidepressant medication. The association of lower cortical CaM Kinase II levels in subjects with mood disorders and comorbid substance abuse requires further study. In the current subject group, this rate of comorbid substance abuse was low due to case selection, further limiting any conclusions about these results.

Post mortem brain studies must be interpreted with caution because of the limitations inherent in working with such tissue sample. First, mood state cannot be

determined in *post mortem* samples, so the extent to which biochemical changes are state dependent cannot be evaluated in this model. Second, although treatment histories were obtained from medical records, some information that could help to clarify the relation between drug treatment and biochemical abnormalities was not available. Important factors might include treatment intensity, duration, and effectiveness as well as compliance estimates. Third, variability in tissue quality between subjects makes unstable markers such as phosphoCREB difficult to measure in *post mortem* brain. Fourth, findings from Stanley Foundation tissue may be representative of a specific subset of patients, possibly those with a more severe illness or specialized treatment, and may be difficult to generalize to other patients with these disorders. Finally, definitive evaluation of the effect of multiple clinical variables on several biochemical variables would benefit from even larger samples sizes.

In summary, the cAMP second messenger pathway may be important in the pathophysiology and treatment of major depressive disorder and bipolar disorder. We now have evidence that antidepressants increase CREB, and based on the work of others, the BDNF receptor (trkB) in major depressive disorder patients [1], and mood stabilizers decrease CREB levels in bipolar disorder patients. The opposite effects of these drugs on the cAMP pathway parallel their clinical effects in depressed and manic patients. Furthermore, the observation that gene expression changes occur in response to these agents fits temporally with the observed delay seen between institution of antidepressant and mood stabilizer treatment and clinical effect. These findings may contribute to novel approaches in the design of therapeutic agents for these disorders.

ACKNOWLEDGEMENTS

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3.4 Increased Hippocampal BDNF Immunoreactivity in Subjects Treated with Antidepressant Medication

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Abstract

Background: The cAMP signaling pathway, and its downstream neurotrophic factor BDNF, are major targets of antidepressant medications. Abnormalities in this pathway have previously been reported in postmortem brain of subjects with mood disorders. This study was designed to test whether the diagnosis of a mood disorder, or treatment with an antidepressant or mood stabilizer was associated with changes in hippocampal BDNF in postmortem brain.

Methods: Frozen postmortem anterior hippocampus sections were obtained from the Stanley Foundation Neuropathology Consortium. Tissue from subjects with major depression, bipolar disorder, schizophrenia and non-psychiatric controls were stained for BDNF using immunohistochemistry.

Results: Increased BDNF expression was found in dentate gyrus, hilus and supragranular regions in subjects treated with antidepressant medications at the time of death, compared with antidepressant-untreated subjects. Furthermore, there was a trend towards increased BDNF expression in hilar and supragranular regions in depressed subjects treated with antidepressants, compared to the subjects not on these medications at the time of death.

Conclusions: These findings are consistent with recent studies measuring CREB levels in this same subject sample, and support current animal and cellular models of antidepressant function.

Introduction

Cell signaling abnormalities may be critical to the pathophysiology and treatment of major depressive disorder (MDD) (Duman et al 1997). Postmortem brain studies report changes at multiple sites of the cAMP pathway in MDD (Cowburn et al 1994; Dowlatshahi et al 1999, 1998; Lowther et al 1997; Reiach et al 1999). Antidepressant (AD) treatment and electroconvulsive shock (ECS) consistently upregulate several components of this pathway in rat and mouse brain, including CREB and phosphorylated CREB levels, and these effects may be important in their mechanism of action (Fitzgerald et al 1996; Jensen et al 2000; Jeon et al 1997; Mori et al 1998; Nibuya et al 1996; Pilc and Legutko 1995a, 1995b; Shimizu et al 1995; Takahashi et al 1999; Thome et al 2000). Studies of downstream targets of the cAMP pathway revealed that brain derived neurotrophic factor (BDNF), which regulates neuronal survival and synaptic plasticity, is also increased by AD and ECS in cerebral cortex and hippocampus (Fujimaki et al 2000; Nibuya et al 1995).

We previously reported a decrease in the transcription factor CREB in cerebral cortex of subjects with MDD, that is restored in MDD subjects treated with AD at the time of death (Dowlatshahi et al 1998). Using the same tissue sample, another laboratory found increased levels of the mRNA for the BDNF receptor, trkB, associated with AD treatment (Bayer et al, 2000). With the recent availability of hippocampal sections from subjects with MDD, bipolar disorder (BD) and schizophrenia (SCZ), we were able to measure BDNF using immunohistochemistry, as this may be a critical target in MDD and its treatment with antidepressant medications.

Materials & Methods

Postmortem brain samples

Frozen postmortem anterior hippocampal sections were obtained from the Stanley Foundation Neuropathology Consortium consisting of subjects with MDD, BD, SCZ and age and sex matched controls (n = 15 per group). Diagnoses were retrospectively established by two senior psychiatrists using DSM-IV criteria. Detailed clinical information and diagnostic procedures are provided elsewhere (Dowlatshahi et al 1999). As previously described, of the sections obtained from the 60 subjects, we were able to identify anterior hippocampus with good structural integrity in 50 subjects. The final sample consisted of 11 BD subjects, 12 MDD subjects, 12 SCZ subjects and 15 controls. Sections from these subjects included a clearly recognizable hilar region, dentate gyrus, and inner molecular layer. In addition these regions, the lateral ventricle, Ammon's horn and hippocampal sulcus were also present in the majority of the slides.

BDNF Immunohistochemistry

Sections were warmed to room temperature and fixed in 10% PBS buffered formalin (Sigma). Sections were subsequently treated with 0.3% Triton X-100/PBS for 10 min. Next, sections were covered by 10% normal goat serum for 30 min, followed by incubation with 2μg/ml Chicken anti-human BDNF bAb (Promega, MADISON, USA) in antibody dilutant (Zymed, ON) at 4 ° C overnight. After extensive washing with PBS, sections were incubated with 1:500 Goat anti-chicken IgY, conjugated with alkaline

phosphatase (Promega, MADISON, USA) in antibody dilutant (Zymed, ON) for 60 min. Sections were then incubated with BCIP/NBT colour solution (Boehringer Manhnem, Wilmington) for 2 h. Endogenous phosphatase activity was inhibited by the addition of 1mM levamisole. Duplicate samples for each subject were stained by BDNF immunohistochemistry. To control for nonspecific binding, control sections were incubated as described above but without the primary antibody. Specificity of the antibody for human BDNF was also tested using Western immunoblotting.

Immunoblotting Analysis.

SH-SY5Y cell extract and recombinant BDNF (Amgen, CA) were used to determine the specificity of anti-human BDNF antidbody. Total protein was isolated from SH-SY5Y cell as previously described (Wang et al 1999). Ten μg of protein were subjected to SDS-PAGE with a 15 % acrylamide gel for 1 h at 120 V. Proteins were transferred to PVDF membranes at 100 V for 1 hr, blocked in 3% milk-phosphate buffered saline (PBS) (30 min, 22° C), and incubated overnight at 22° C with a polyclonal anti-human BDNF antidbody (Promega, WI) at a 1:1000 dilution. Blots were then incubated with secondary antibody, goat anti-chicken IgY conjugated to alkaline phosphatase diluted 1:5000 in blocking buffer for 1 hr at 22° C. Immunoreactive bands were detected with the standard NBT/BCIP (Promega, WI).

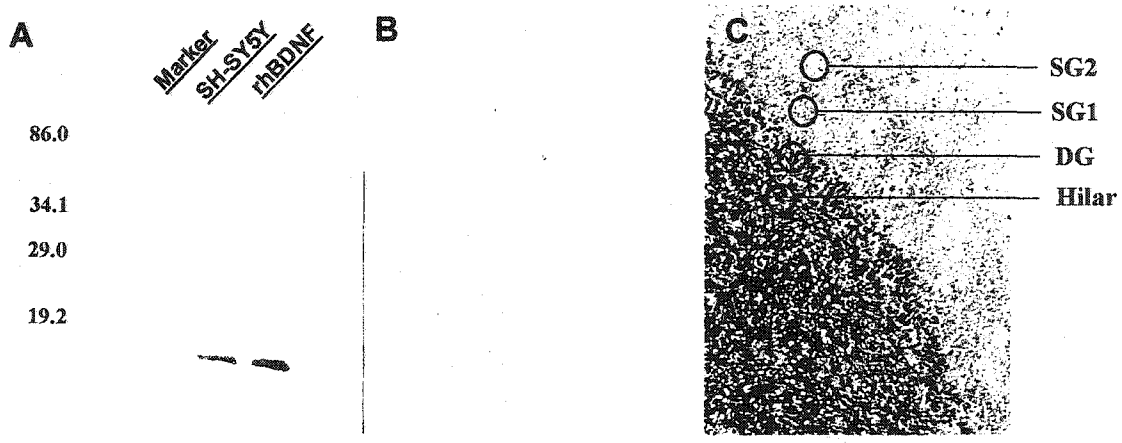

Figure 1. The BDNF level in human neuroblastoma SH-SY5Y cell extract and hippocampus of human brain. (A) BDNF immunoreactivity in human neuroblastoma SH-SY5Y cell extract. (B) Background staining in control slide incubated without the specific BDNF antisera. (C) BDNF immunoreactivity was determined by measuring optical density of ten 50 μm radius circles from four regions (Hilar, DG, SG1, SG2; 200 X magnification). SG1 and SG2 are immunoreactivity measurements from 0-100 μm and 100-200 μm into the inner molecular region. DG = dentate gyrus; SG = supragranular region.

Quantitative analyses

Immunohistochemical stained sections were examined at 50X magnification by creating a digitized image with the Bioquant Pure Color Windows98 imaging system (R&M Biometrics, Inc., U.S.A.) attached to a light microscope with an electronic stage encoder (Zeiss Axioskop, Oberkochen, Germany). Using the imaging system, the average integrated optical density (IOD) of BDNF immunoreactivity in the hilar region, the dentate gyrus, and the supragranular (SG) zone adjacent to the dentate gyrus were measured by averaging measurements obtained from ten adjacent circles with 50 μm radii (Figure 1). Two measurements were obtained from the supragranular region: the first from a 100μm band outside the dentate gyrus (SG1), and the second starting at 100μm from the dentate gyrus and extending a further 100μm (SG2). The density of immunostaining in different regions was expressed as relative optical density (ROD). Since glass slides absorb pigment during immunostaining, the Bioquant imaging system included an automatic background correction algorithm. This correction calibrates analysis to a baseline image obtained from a tissue-free region of the slide. These background corrections were made for each section, as per the Bioquant protocols (R & M Biometrics Inc., USA). All assays were performed blind to diagnosis and treatment history.

Statistical analysis

Group differences in age, sex and postmortem delay were tested using ANOVAs. Effects of age and postmortem delay on BDNF immunoreactivity were determined by Pearson's product moment tests. ANOVAs were also used to examine overall effects of diagnosis and drug treatments. Drug treatment effects within groups were examined using t-tests. All statistics were done using SPSS for Windows.

Results

The specificity of the BDNF antisera was confirmed with SH-SY5Y cell extract and recombinant BDNF (figure 1A). As expected, this antisera detected a 14 Kd band in cellular extract (Murer et al, 1999). In frozen postmortem sections, we were able to detect BDNF immunoreactivity in several regions of anterior hippocampus. High levels of immunoreactivity were detected in hilus, dentate gyrus as well as supragranular regions adjacent to the dentate gyrus. Lower levels were detected in other regions, with the lowest found in white matter. BDNF immunoreactivity was found principally in cytoplasm (figure1C). There is virtually no signal in control slides incubated without the specific BDNF antisera (figure 1B)

There were no significant differences in age [F=0.77, df (3,49), p = 0.52], sex [F=0.12, df (3,49), p = 0.95], or postmortem delay [F = 2.09, df (3,49), p = 0.115] between the four groups, nor was there a correlation between BDNF levels and age, sex or postmortem delay (figure 2).

There was no overall effect of diagnosis on BDNF levels in any of the hippocampal regions. BDNF levels were, however, higher in subjects treated with ADs relative to untreated subjects in hilus [t= 2.71, p = 0.009], dentate gyrus [t = 2.22, p = 0.031], SG1 [t = 2.88, p = 0.006], and SG2 [t = 2.80, p = 0.007] (figure 3A). Following corrections for repeated comparisons (using p = 0.0125 as significant), we determined that BDNF levels were significantly increased in subjects treated with ADs in hilus, SG1 and SG2 regions, with a non-significant trend towards increased BDNF in dentate gyrus. When we examined other illness variables such as suicide, length of illness or substance

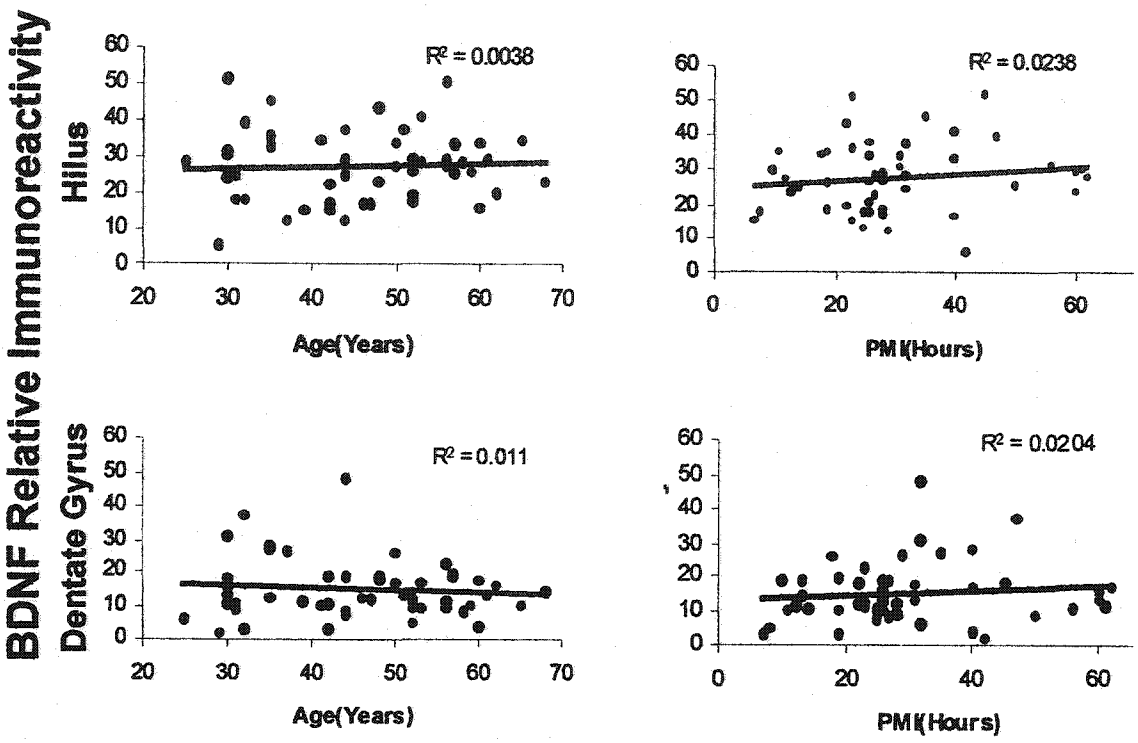

Figure 2: Hilus and dentate gyrus BDNF immunoreactivity in all subjects (n = 50) with respect to postmortem intervals and age.

abuse, we found no effect of any of these variables on BDNF immunoreactivity in any region.

As earlier studies in this patient sample found antidepressant effects on components of the cAMP signalling pathways to be most evident in subjects with MDD (Bayer et al, 2000; Dowlatshahi et al 1998), we made further comparisons within this group. There were trends toward increased BDNF levels in antidepressant treated MDD subjects ($n = 8$) relative to MDD subjects not treated with AD ($n = 4$) in hilus [$t = 1.90$, $p = 0.09$], dentate gyrus [$t = 1.66$, $p = 0.13$], SG1 [$t = 2.09$, $p = 0.06$], and SG2 [$t = 1.77$, $p = 0.11$] (figure 3B). There was no effect of either suicide or length of illness on BDNF levels in subjects with MDD.

Examining the BD group, we found no effect of AD treatment on BDNF levels in subjects with BD in any region (figure 3B). Furthermore, there was no effect of lithium treatment, anticonvulsant treatment, length of illness or suicide on BDNF levels in subjects with BD. There were no significant effects of any illness or treatment related variables on BDNF levels in the samples from patients with schizophrenia.

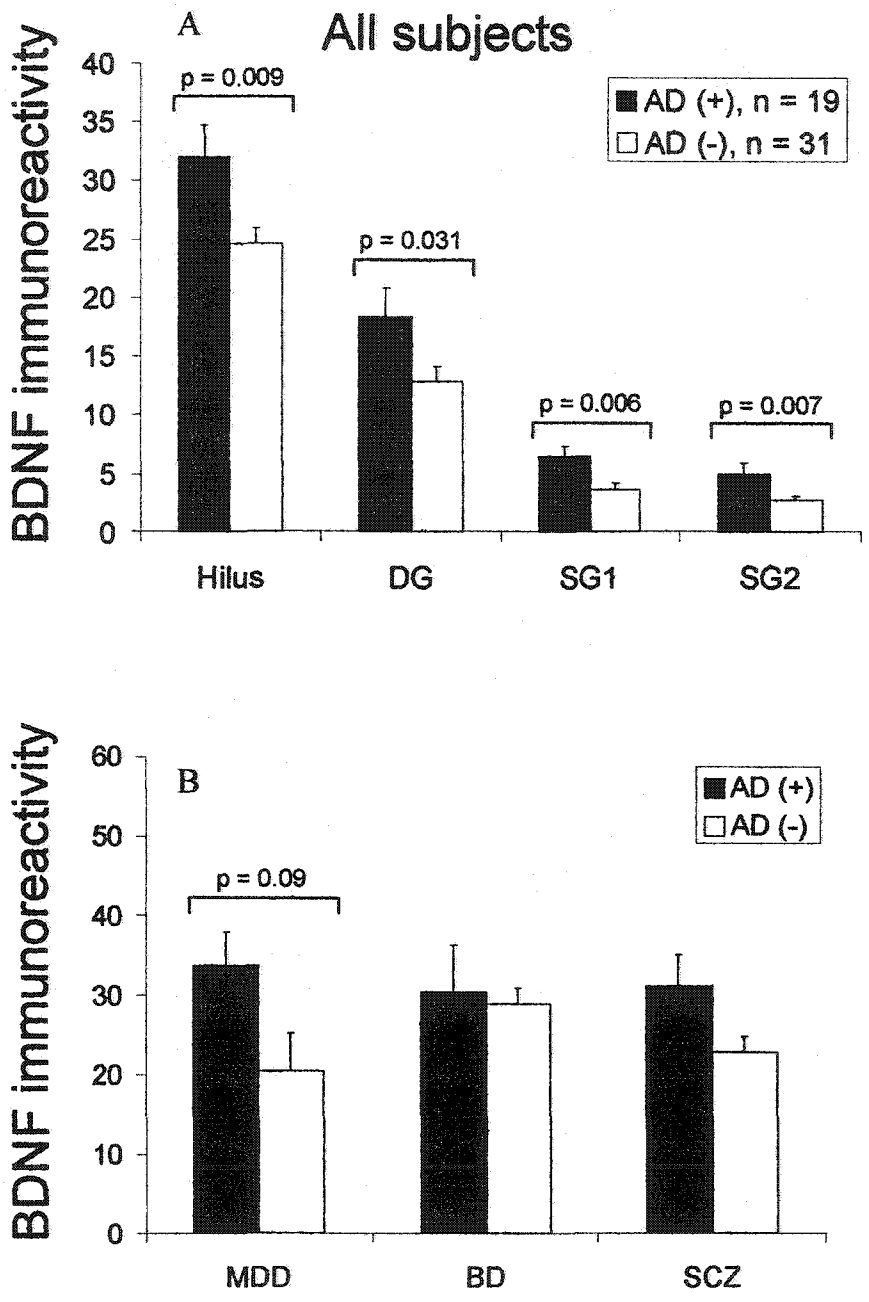

Figure 3: A) Hippocampal BDNF immunoreactivity is increased in subjects treated with ADs in all four regions of interest (mean ± SEM). B) Hilar BDNF immunoreactivity is increased in MDD subjects treated with ADs. BDNF immunoreactivity was unchanged in BD and SCZ with respect to AD treatment.

Discussion

These results add further support to the notion that BDNF may be regulated by AD medications, suggesting its possible involvement in the pathophysiology of MDD, and possibly depressive symptoms in BD and schizophrenia. These results provide the first demonstration from patient samples that BDNF may be regulated by AD medications that target the cAMP signaling system, and lend further support to previous animal studies in which similar effects have been consistently shown. Increased hippocampal BDNF levels after AD treatment is consistent with several recent reports that antidepressant medications increase levels of the transcriptional regulator, CREB (Dowlatshahi et al 1998; Nibuya et al 1996), known to regulate BDNF expression, and increase the BDNF receptor, TrkB, in postmortem brain from this same group of subjects (Bayer et al, 2000). The effect of AD treatment on hippocampal BDNF levels appeared most evident in MDD, suggesting an interaction between this illness and AD treatment on BDNF levels. It is also possible that AD treatment may have similar targets in diverse psychiatric disorders, since the effect of AD treatment was robust and found in the entire subject group. These results add to the growing body of data, which suggest that the cAMP signaling system and its downstream targets are important in the mechanism of action of ADs in the pathophysiology of depression.

There are several limitations to this study. First, due to the limited amount of hippocampal tissue available from the subjects, our study was not replicated using ELISA or immunoblotting, nor was a different BDNF antibody used for comparison. However, the specificity of this antibody has previously been demonstrated (Earnest et al 1999) and recent

studies have utilized it in human brain (Hock et al 2000; Kerschensteiner et al 1999). Second, when we limited our analyses to subjects with MDD, we were only able to detect non-significant trends towards higher levels of BDNF in subjects receiving AD treatments. Replication of this study in a larger sample would determine whether changes in BDNF levels are indeed more evident in subjects with this diagnosis, which would help to clarify the relationship between hippocampal BDNF levels and the pathophysiology of depression. Third, drug treatment at the time of death is not necessary reflective of drug treatment history, which limits the evaluation of drug effects on hippocampal BDNF expression. Finally, the clinical significance of these changes in relation to hippocampal function and specific components of antidepressant efficacy, such as relief of symptoms or prevention of relapses, needs to be further clarified.

In summary, these results provide the first demonstration of AD-dependent BDNF changes in human samples, supporting the data from pre-clinical studies suggesting that AD treatments may target biochemical pathways of hippocampal structures. Abnormalities of both hippocampal structure and function have been demonstrated in clinical samples of patients with recurrent MDD (Bremner et al 2000; Sheline et al 1999, 1996); the results from this study suggest that AD treatment may normalize hippocampal levels of an important neuroprotective factor. The clinical significance of this finding, including the possibility that AD agents may prevent or minimize the hippocampal changes that occur in some patients with MDD clearly requires further investigation.

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3.5 Increased Hippocampal Supragranular Timm Staining in Subjects with Bipolar Disorder

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Abstract

Biochemical and structural abnormalities have been reported in hippocampus of subjects with mood disorders. This study examined the organization of mossy fibers in anterior hippocampus of subjects obtained from the Stanley Neuropathology Consortium. Frozen postmortem hippocampal sections from subjects with major depression, bipolar disorder, schizophrenia and non-psychiatric controls were stained using the Neo-Timm procedure, which selectively stains mossy fibers. Increased Timm staining in the supragranular layer was found in subjects with bipolar disorder relative to control subjects. These results are suggestive of neuronal sprouting in hippocampus of subjects with bipolar disorder. There were no significant associations between supragranular Timm staining and suicide, length of illness or drug treatment at the time of death.

Introduction

Abnormalities in signal transduction pathways have been identified in postmortem brain of subjects with mood disorders. Blunted cAMP signaling has consistently been found in tissue from patients with major depressive disorder (MDD) and suicide [1-5], whereas increased cAMP signaling has been found in most but not all studies examining tissue from subjects with bipolar disorder (BD) [2,6-9]. These results are consistent with the known action of antidepressant (AD) and mood-stabilizing agents, with a preponderance of data demonstrating up-regulation of components of the cAMP pathway by chronic AD treatment [10,11] and an opposite effect of lithium [12,13] in animal models and cell cultures. We have recently confirmed a number of these drug effects in postmortem tissue from patients with MDD or BD [1,2].

One possible consequence of chronic changes in cAMP signaling is altered function and/or levels of cAMP regulated transcription factors such as CREB, with consequential changes in expression of cAMP regulated target genes. For instance, chronic AD treatment has been shown by several groups to increase CREB levels and phosphorylation [10], which was associated with increased expression of brain derived neurotrophic factor (BDNF) [10], a cAMP-regulated target gene, in rat cerebral cortex and hippocampus. Our lab and others have found similar effects of AD treatment in postmortem samples from subjects with mood disorders [1,14].

The possible neuroanatomical consequences of up-regulated cAMP signaling and BDNF levels have not previously been examined in postmortem brain tissue from subjects with mood disorders. However, converging lines of evidence suggest that

neuroanatomical changes are detectable in these disorders. Postmortem brain studies have demonstrated decreased non-pyramidal cell density [15] and increased levels of N-CAM [16], a cellular adhesion molecule, in hippocampus from patients with BD. Furthermore, several imaging studies of patients with mood disorders have found volumetric abnormalities in temporo-limbic structures [17]. The factors leading to such structural changes are unclear, but may be associated with alterations in cAMP signaling and neurotrophic factors. A recent study reported that electroconvulsive shock, a model for electroconvulsive therapy used in mood disorders, results in neuronal sprouting as assessed by increased Timm staining in rat hippocampus [18]. This neuronal sprouting occurs as a result of proliferation of axonal collaterals from dentate gyrus granular cells, known as mossy fibres, and is also seen in the animal kindling model [19] which as been proposed as a non-homologous model of BD [25]. At this time, human postmortem brain studies using Timm staining have been restricted to subjects with temporal lobe epilepsy [20 - 23].

To determine whether there is evidence for changes in hippocampal synaptic organization in patients with MDD or BD, we used Timm staining to measure mossy fibre sprouting. These studies were carried out in the well-characterized clinical sample in which our lab and others have previously found changes in cAMP signaling.

Materials and Methods

Frozen unfixed postmortem anterior hippocampal sections from the Stanley Foundation Neuropathology Consortium were obtained from subjects with BD, MDD, schizophrenia (SCZ) and non-psychiatric controls (15 per group). Diagnoses were retrospectively established using DSM-IV criteria by two independent senior psychiatrists. Subjects were matched for age, sex, race, postmortem delay interval and hemisphere. We have previously described this sample in detail [2]. All sections examined in this study included a clearly recognizable hilar region, dentate gyrus, and inner molecular layer. Subjects with hippocampal sections not including these regions were excluded, resulting in a final sample size of 15 controls, 13 BD, 14 MDD and 13 SCZ. In addition to the dentate gyrus, hilus and inner molecular layer, the lateral ventricle, Ammon's horn and hippocampal sulcus were also present in the majority of slides (figure 1). Demographic information for this sample is provided in table 1.

Two consecutive frozen anterior hippocampus sections (14 μm) for each subject were thawed for two hours at 21°C. Silver sulfide staining was performed with the Neo-Timm procedure developed for human hippocampal sections [20 - 23]. Briefly, slides were fixed in 0.12M Millonig's buffer (pH 7.3) with 0.1% Na_2S, 0.002% $CaCl_2$ and 4% gluteraldehyde for 10 minutes and air-dried. Slides were then incubated for 60 minutes in the dark in 100ml of Timm developer, consisting of a 60:30:10 ml mixture of gum arabic, hydroquinone, and citric acid-sodium citrate, containing 0.5 ml of silver nitrate solution. Incubation periods were optimized first in rat, and subsequently in human hippocampal tissue. Incubations times during optimization ranged from 30 minutes to 90

minutes, with 60 minutes yielding the best signal to noise ratio. Silver staining specificity was confirmed by comparing light (50 min) and dark (70 min) exposures as previously described [20, 22]. Following Timm staining, slides were rinsed, dried, incubated in xylene, hydrated with ethanol, and counterstained in 0.1% thionin/0.1 M sodium acetate. Cell bodies were counterstained with thionin to facilitate identification of the dentate gyrus granule cells, as well as other hippocampal neuroanatomical landmarks (figure 1). Experiments were conducted blind to group assignment and were replicated.

Distribution of silver staining in the supragranular region of the dentate gyrus was analyzed blind to diagnosis using the Bioquant TCW 98 imaging system with a topography module and a Zeiss Axioskop light microscope with stage encoder. The supragranular region was defined as the region of the inner molecular layer located directly adjacent to the dentate gyrus (figure 1). Supragranular silver staining was quantified using a Timm index[19, 24]. A Timm index provides a computer-assisted non-biased measurement representing the pattern of supragranular silver staining. Using computer topography, the total area of supragranular silver stain in the inner molecular layer was measured and divided by the perimeter of the dentate gyrus. This Timm index was subsequently averaged from duplicate slides for each subject.

Age, postmortem delay and sex effects were determined using correlational analysis. Subject group differences and medication effects were examined using analyses of variance (ANOVA) followed by post hoc Duncan's tests to identify specific group differences. All statistical analyses were performed using the SPSS statistical package.

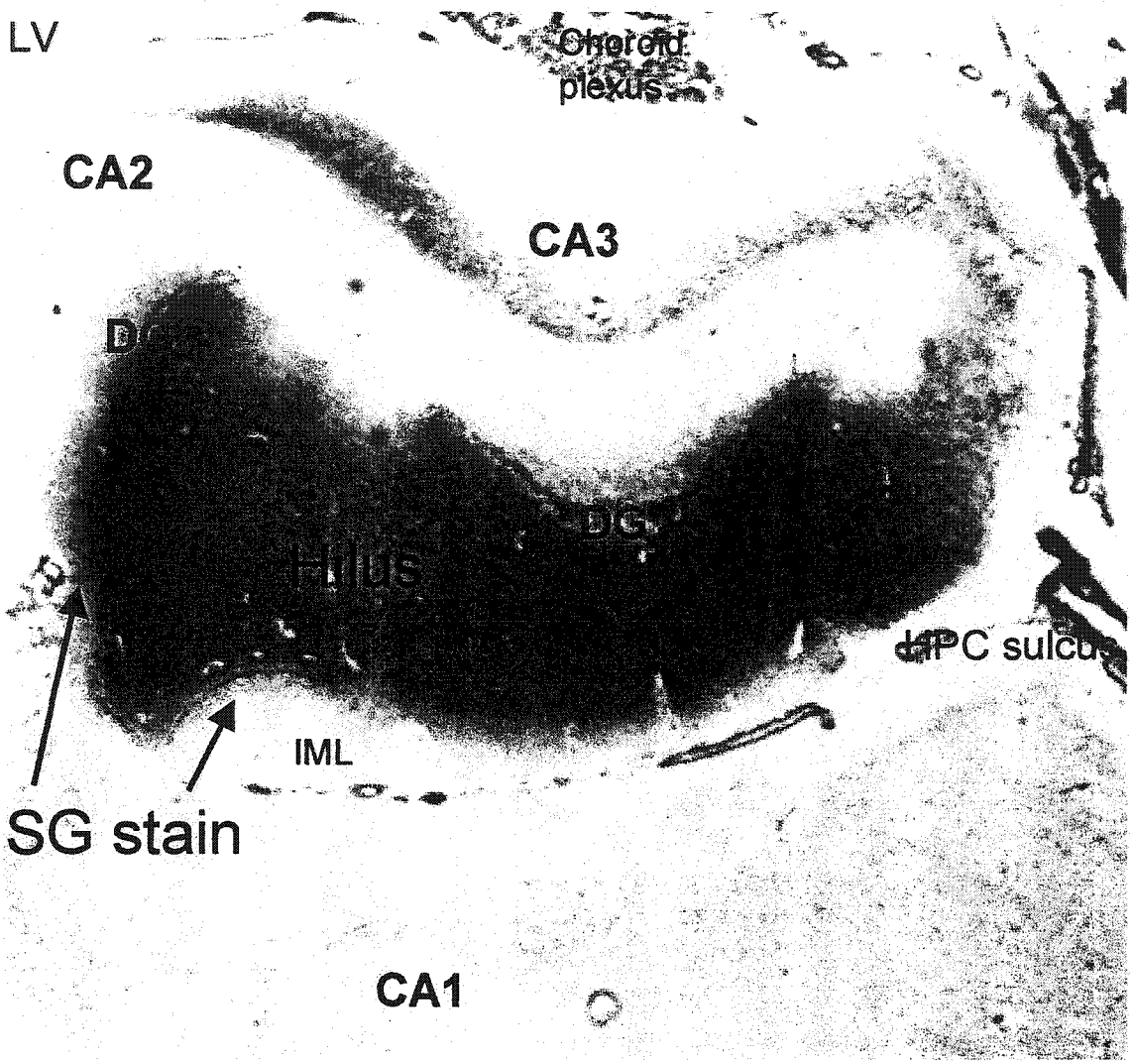

Figure 1: Representative hippocampal section (5X magnification) showing Timm staining in hilar and supragranular regions (SG stain). Timm index for the SG staining was obtained by measuring the area of Timm stain in the inner molecular region (IML) directly adjacent to the dentate gyrus (DG), and dividing it by the total length of the DG. LV = lateral ventricle; HPC sulcus = hippocampal sulcus.

Table 1: Subject demographics

	Control (n = 15)	BD (n = 13)	MDD (n = 14)	SCZ (n = 13)
Age (mean ± SEM, yrs)	48.1± 2.8 [range 29-68]	42.4 ± 3.4 [25-61]	46.1 ± 2.5 [30-65]	42.9 ± 3.7 [25-62]
Sex (M / F)	9 / 6	7 / 6	8 / 6	8 / 6
PMI (mean ± SEM, hrs)	23.7 ± 2.6 [range 8-42]	34.3 ± 4.6 [13-62]	28.6 ± 2.7 [7-47]	32.2 ± 3.7 [12-60]
Suicide (Y / N)	0 / 15	7 / 6	7 / 7	5 / 8

PMI = postmortem interval

Results

As previously reported, silver staining was darkest in the hilar regions, with varying degrees of staining in the supragranular regions (figure 1). There were no statistical differences in age [$F = 0.77$, df (3,54), $p = 0.515$], sex [$F = 0.06$, df (3,54), $p = 0.981$], or postmortem delay [$F = 1.87$, df (3,54), $p = 0.147$] between the four groups. There were no significant correlations between Timm index and age [$r^2 = 0.022$, df (1,54), $p = 0.283$], sex [$F = 0.20$, df (1, 54), $p = 0.657$] or postmortem delay [$r^2 = 0.036$, df (1,54), $p = 0.164$]. There were no significant effects of age of onset, length of illness, or suicide on Timm index.

A one-way ANOVA examining the effect of diagnosis on Timm index was conducted including treatment status (AD, lithium, anticonvulsant and antipsychotic medication) as co-varying factors because of previous findings demonstrating the effect of ADs and mood stabilizers in these patients [1, 2, 14]. There was a main effect for diagnosis [$F = 2.96$, df (3,50), $p = 0.043$] and AD treatment was a significant co-variant [$F = 4.27$, df (1,50), $p = 0.045$]. Post hoc analyses using Duncan's test revealed significantly higher Timm index in subjects with BD compared to control subjects (figure 2).

Subjects treated with AD medication at the time of death had a non-significant trend towards decreased Timm index (mean ± SEM = 101 ± 19) compared to patients not on these drugs (mean ± SEM = 170 ± 35) [$T = -1.83$, $p = 0.075$]. There were no significant effects of treatment with lithium, anticonvulsant mood stabilizers or neuroleptics on Timm index these subjects.

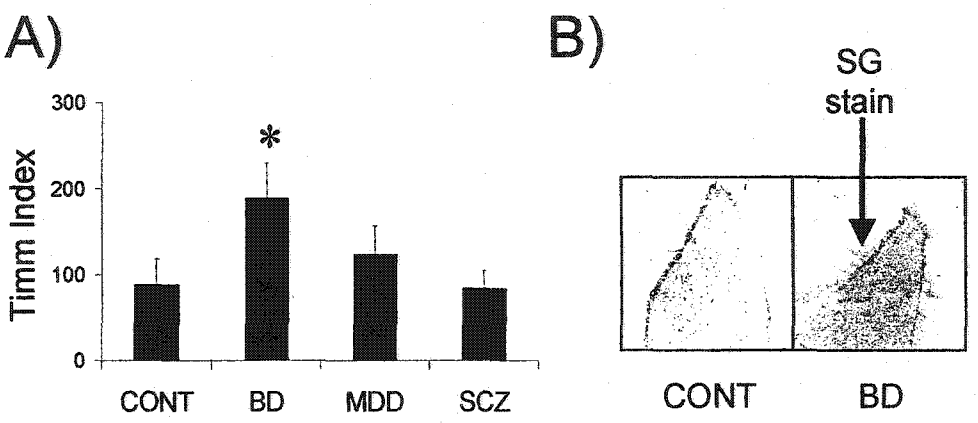

Figure 2: **A)** Subjects with bipolar disorder have significantly higher Timm index compared to controls subjects. * One-way ANOVA $p = 0.043$. **B)** Representative hippocampal sections (50X magnification) from a subject with bipolar disorder (BD) and a non-psychiatric control (CONT) showing supragranular Timm staining (SG stain).

Discussion

The present study adds to the growing body of data on hippocampal abnormalities in BD [15, 18, 16], providing evidence that neuronal sprouting may occur in the dentate gyrus of patients with this disorder. These findings appear to be specific to patients with BD, with no evidence for similar changes in patients with MDD or SCZ, and are consistent with the hypothesis that altered limbic neuronal excitability occurs in BD [25].

We found that AD treatment was associated with a trend towards lower Timm index. The observed effects were opposite to effects of these drugs in rat hippocampus [19]. It is possible that these drugs may regulate this process differentially in healthy vs. pathological tissue, or that treatment over longer periods than those utilized in animal models is required to observe the final effect of these agents.

The present findings must be interpreted with caution as they are preliminary and have a number of limitations. First, although the number of subjects is relatively large for a postmortem brain study and the clinical data is extensive, these findings may only reflect a subset of patients with BD, possibly those with a more severe course. Furthermore, treatment at the time of death may not adequately reflect treatment history over the course of illness, thus limiting evaluation of drug treatment effects in these subjects. Finally, the increased Timm staining may not reflect neuronal sprouting of mossy fiber collaterals, but may indicate a reorganization of granule cell axons or simply alterations in endogenous zinc distribution due to other causes. It is possible that one or more of these factors may have contributed to the lack of differences comparing MDD and control groups.

Conclusion

Structural changes in hippocampus of subjects with BD may be related either to symptoms or course of illness, and are consistent with the hypothesis that increased limbic excitability occurs in this disease [25]. In this study, we have reported an increase in Timm staining in the SG layer of the hippocampus of subjects with BD as compared to controls. This may reflect mossy fiber sprouting, but further studies are required to determine the functional significance of this increased staining and its relation to the course of illness and treatment of BD.

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4. General Discussion

In order to assess potential cAMP signalling abnormalities in mood disorders, postmortem brain tissue was obtained from the Stanley Foundation Neuropathology Consortium. Because previous studies have reported altered cAMP signalling in temporal and occipital cortex of subjects with BD (Young et al., 1993; Young et al., 1991), it was thought that these brain regions would be appropriate for use in replicating earlier G-protein studies, and for examining downstream components of the cAMP system. Prefrontal cortex sections obtained from the same subject sample became available following the completion of temporal and occipital cortex studies. Although we were unable to complete phosphorylated CREB immunoblotting in the temporal cortex due to poor signal, prefrontal cortex sections became available and allowed for immunohistochemical analysis of phosphorylated CREB levels, a technique superior in sensitivity. Moreover, altered cAMP signalling has also been reported in this brain region (Lowther et al., 1996; Cowburn et al., 1994), emphasizing its relevance to the objectives of this study.

Previous molecular studies of antidepressant and mood stabilizer pharmacology using animal models show alterations in cAMP signalling, CREB and BDNF expression in rat hippocampus (Chen et al., 1999; Duman et al., 1997). Human imaging studies reveal a reduction in hippocampal volume in patients with MDD (Bremner et al., 2000; Sheline et al., 1999; Sheline et al., 1996). Furthermore, the dentate gyrus of the hippocampus receives

afferent fibres from the temporal cortex, supporting its use in our studies. With the subsequent acquisition of hippocampal sections, remaining experiments focused on this highly relevant brain region.

Using temporal and occipital cortex tissue, we were not able to replicate previous findings of increased $G\alpha_s$ or forskolin-stimulated AC activity in subjects with BD. Correspondingly, there were no downstream changes in cAMP signalling; CREB levels, phosphorylated CREB levels and CRE-binding were also unchanged. The CREB-regulated BDNF was unchanged in this group, as was DNA-fragmentation (see appendix C). Interestingly, a previous study conducted by the Young lab failed to show increased $G\alpha_s$ mRNA in cerebral cortex of patients with BD (Young et al., 1996). These inconsistencies may be due to small sample sizes resulting in insufficient statistical power to detect subtle changes in cAMP signalling. Alternately, there may be heterogeneous subgroups of BD patients, with slightly differing pathologies. This notion is supported by the differing clinical presentations and responsiveness to therapy in patients meeting criteria for BD. To address these questions, larger groups of BD subjects are required to provide sufficient statistical power for a detailed subgroup analysis based on clinical variables. These studies could deal with more complicated issues that may be correlated with neuropathological findings, such as treatment response, severity of illness, and length of illness.

Subgroup analysis of subjects with BD revealed a trend towards decreased $G\alpha_s$ in occipital cortex of lithium-treated patients. This finding is consistent with animal studies showing blunted cAMP signalling following lithium treatment (Carli et al., 1994; Li et al.,

1993; Masana et al., 1991; Avissar et al., 1988; Forn & Valdecasas, 1971). Interestingly, well-documented side effects of lithium use may also be attributed to blunting of intracellular cAMP signalling. Hypothyroidism seen in lithium use is associated with a disruption of iodide transport into thyroid cells (Hardman & Limbird, 2001). Iodide normally enters thyroid cells to be synthesized into thyroid hormone following stimulation of the TSH receptor. These receptors are $G\alpha_s$-coupled transmembrane proteins, and activation of AC with cAMP production is necessary for the iodide transport. Another side effect of lithium toxicity is diabetes insipidus, caused by a decreased responsiveness to anti-diuretic hormone (ADH) by ADH receptors in the kidneys (Hardman & Limbird, 2001). The ADH receptor is also a $G\alpha_s$-coupled transmembrane protein, capable of activating the cAMP system for fluid homeostasis. Both these receptor systems, whose only common biochemical pathway is cAMP production, become desensitized to their respective ligands following lithium treatment. Although these scenarios may be unrelated effects of lithium, they provide a separate line of evidence to support the notion that lithium blunts cAMP signalling. Overall, the results do not support an abnormal increase in cAMP signalling in BD; nevertheless they show the importance of its regulation for the treatment of BD. Accordingly, mood-stabilizing anticonvulsant treatment of BD subjects was shown to decrease temporal cortex CREB levels, thereby interfering with the downstream target genes of the cAMP system.

As predicted by the kindling model of altered limbic excitability in BD (Post & Weiss, 1989), Timm staining was increased in the hippocampus of subjects with BD. Based on the increased mossy fibre sprouting seen in rat hippocampus following electroconvulsive

stimulation (Vaidya et al., 1999), it was also hypothesised that Timm staining would be increased following antidepressant treatment. However, this postmortem study did not find any change in Timm staining in subjects treated with antidepressants at the time of death. This finding is consistent with a study published in 2001, the year following this study. The 2001 study reported an increase in mossy fibre sprouting in rat hippocampus with electroconvulsive stimulation, but not with antidepressant treatment (Lamont et al., 2001).

When measuring cAMP signalling in subjects with MDD, this study found no changes in G-protein levels. However, there was a trend towards decreased forskolin-stimulated cAMP production in temporal cortex of patients with MDD. This effect was not specific to the MDD group, but was also seen in BD subjects. As this effect did not meet the criteria for statistical significance, it is difficult to comment on its importance. Nevertheless, it is possible that there is a blunting of AC activity in mood disorders, as previously reported in depressed suicide subjects (Reiach et al., 1999).

The finding of decreased CREB levels in temporal cortex of untreated MDD subjects, which appear to be restored to control levels in subjects treated with antidepressants, are consistent with animal and cell models of depression and antidepressants pharmacology (Duman et al., 1997). Phosphorylated CREB levels were not elevated, which may be related to the use of a different brain region. Ideally, an immunohistochemistry study measuring phosphorylated CREB in temporal cortex should be conducted, once sections from this region are available. CRE-binding was not decreased in temporal cortex of untreated MDD patients either. However, temporal cortex CRE-binding was decreased in MDD suicide subjects, relative to MDD non-suicide subjects. This finding

may represent a subset of MDD subjects with a more severe course of illness; although suicide may be a pathological entity of its own as demonstrated by an overall decrease in CREB levels in subjects with suicide, this CRE-binding decrease was not seen in any of the other groups, potentially suggesting a diagnostic specificity.

Increased hippocampal BDNF levels found in subjects treated with antidepressants are also consistent with animal and cell models (Duman et al., 1997). These models show antidepressant treatment increases hippocampal BDNF levels, as well as hippocampal CREB transcriptional activity and cAMP-dependent gene expression (Thome et al., 2000). There was also a trend towards decreased BDNF levels in untreated MDD subjects relative to antidepressant-treated MDD subjects, an effect not seen in other diagnostic groups. A larger sample size may provide the necessary statistical power to detect a significant effect of diagnosis on BDNF levels.

In patients with MDD, blunted cAMP signalling may decrease expression of BDNF and other neuroprotective factors in dentate gyrus and hippocampus, and potentially affect cell viability and neurodegeneration of these brain regions. A recent study demonstrated that neurogenesis is dependent on cAMP signalling (Nakagawa et al., 2002). Neurogenesis is a process where granular cells are generated throughout the life cycle in a proliferative region of the dentate gyrus (McEwen, 1999). These cells migrate to the granular layer of the dentate gyrus and ultimately undergo apoptosis, resulting in a constant turnover of dentate gyrus neurons. In mouse dentate gyrus, increased neurogenesis is associated with an increase in CREB levels and CREB phosphorylation (Nakagawa et al., 2002). CREB knockout mice have decreased cell proliferation in the dentate gyrus (Nakagawa et al.,

2002). Similarly, the decreased cAMP signalling seen in MDD patients may cause a shift in the equilibrium between neurogenesis and apoptosis in the dentate gyrus, resulting in a relative increase in granular cell apoptosis. In this study, TUNEL staining did not show an increase in DNA-fragmentation in dentate gyrus of MDD subjects (Appendix C). However, due to lack of hippocampal slices, tissue from only 5 MDD subjects were available for use in this study. When single slides from other MDD subjects were added to the experiment, there was an increase in DNA-fragmentation in the MDD group relative to controls. Since the *a priori* protocol for this experiment called for duplicate slides per patient, this data was not used. Nevertheless, replication of the TUNEL experiment using a larger MDD sample is warranted.

The postmortem brain findings in subjects with MDD can be interpreted within the context of two models. First, degeneration of dentate gyrus cells secondary to abnormal cAMP signalling may confer a vulnerability to the hippocampus for neurodegenerative events. Animal models consistently show that chronic environmental stress causes atrophy of the hippocampal CA3 pyramidal cell dendrites, possibly due to hypothalamus-pituitary-adrenal axis dependent increases in circulating glucocorticoids (McKittrick et al., 2000; Magarinos et al., 1997; Magarinos et al., 1996; Watanabe et al., 1992). Assuming patients with MDD have decreased cell proliferation in dentate gyrus, they may be more vulnerable to dendritic atrophy following exposure to highly stressful stimuli. Furthermore, this combination of CA3 pyramidal and dentate gyrus atrophy may contribute to the decreased hippocampal volumes seen in imaging studies on MDD patients (Bremner et al., 2000; Sheline, 2000; Sheline et al., 1999). Conversely, antidepressant treatment may increase

neurogenesis in these patients, providing greater resistance against CA3 dendritic atrophy caused by chronic stress.

A second interpretation of the postmortem findings is that decreased cAMP signalling is part of the biochemical pathway leading to CA3 dendritic atrophy in response to chronic stress. Since dentate gyrus axons project to CA3 pyramidal cells, dentate gyrus BDNF may play an important role in the synaptic organization of the pyramidal cells. In this model, stress-induced increases in circulating glucocorticoids in patients with MDD may cause decreased cAMP signalling leading to dendritic atrophy. Indeed, glucocorticoids have been shown to blunt cAMP signalling in rat hippocampus (Dwivedi & Pandey, 2000; Gannon & McEwen, 1990). Furthermore, animal models have also shown that immobilization stress decreases BDNF expression in rat dentate gyrus (Smith et al., 1995). It is then possible to predict that antidepressant treatment may interfere with CA3 atrophy following exposure to stress by upregulating the cAMP system.

These interpretations of the postmortem findings illustrate one of the limitations of postmortem brain models: the inability to establish causation. In one model, the signalling abnormalities may represent an underlying pathology *causing* a vulnerability to stress and, therefore, a predisposition to hippocampal atrophy. In the second model, the signalling abnormalities may themselves represent a pathophysiology of hippocampal atrophy *caused* by stress. Postmortem brain data requires interpretation within the context of animal and cell studies, where cause and effect have been established. Other limitations of postmortem brain tissue will be discussed in detail below.

While measuring phosphorylated CREB levels in prefrontal cortex, there was a serendipitous finding of increased phosphorylated CREB levels in patients with SCZ (Appendix C). Furthermore, subgroup analysis revealed a lowering of phosphorylated CREB levels in SCZ treated with neuroleptic medications, as compared to SCZ not treated with neuroleptics at the time of death. Although previous postmortem brain studies have reported altered cAMP signalling in SCZ (Nishino et al., 1993) and genetic analysis has found two CREB variants expressed only in patients with SCZ (Kawanishi et al., 1999), this diagnosis was beyond the scope of the thesis, and the results were not investigated further.

The findings in this thesis support the use of human postmortem brain as a valid strategy for psychiatric research. The advantage of the postmortem approach is that it provides a model where the pathological condition of interest is present. Many treatments used for mood disorders have no clinical effect on healthy people. This phenomenon reinforces the need to conduct pharmacology studies in relevant human tissues. Nevertheless, postmortem brain studies are one component of a multi-model approach for mood disorder research. *In vitro* cell models provide a strictly controlled environment where biochemical mechanisms can be examined in detail, but are limited by the lack of physiological interactions that take place in *in vivo* systems. Animal models provide these interactions, but can only approximate the clinical condition for which they are designed. Human postmortem brain tissue provides a relevant clinical model, and can be used to complement *in vitro* and animal models.

The postmortem brain approach is subject to several limitations. Assays using postmortem brain are technically difficult. Tissue samples are often quite delicate due to the

postmortem interval, which, in the Stanley Foundation tissue, averaged at almost 30 hours. Many assays are affected by postmortem delay, such as the TUNEL stain and phosphorylated CREB levels. These effects can be measured using covariate analysis, but they nevertheless increase variability in the samples and decrease overall statistical power.

Although most clinical variables were included with each subject, relevant factors such as length of treatment or medication compliance were not. Nor can mental state at the time of death be determined. Several other factors, such as education, income and functional status, can affect prognosis of mood disorders (Chandarana et al., 1997) and may therefore be relevant to the underlying pathology.

Finally, it is difficult to obtain large samples of postmortem brain tissue. The tissue obtained from the Stanley Foundation Neuropathology Consortium represents one of the largest collections of mood disorder brains. Nevertheless, sample sizes were frequently too small to detect subtle changes between groups.

Despite the limitations of postmortem brain tissue, when used in conjunction with cell and animal studies, it provides a relevant clinical approach for the study of mood disorders. To continue this line of experimentation, it would be important to secure more brain tissue samples, particularly hippocampal slices. Collaboration with other research groups using the postmortem approach may facilitate this goal.

To build on the current findings, future research directions could include measuring phosphorylated CREB levels in temporal cortex and hippocampal slices, and CREB levels in hippocampus. The TUNEL study could be replicated with a larger group of MDD subjects, and complimented with assays measuring other markers of apoptosis such as

caspase-3 or p53 induction. These hippocampal studies could take advantage of confocal microscopy to differentiate the relative involvement of neurons and glia. In BD, Timm assays could be conducted using electron microscopy to assess microstructural changes in synaptic organization. These studies could be complemented with assays for synaptic marker proteins. Finally, studies can shift focus towards the amygdala, a target organ for hippocampal efferents and a region known to be affected by mood disorders (Strakowski et al., 1999; Sheline et al., 1998).

This postmortem brain study has added to the growing literature into the pathophysiology of mood disorders and their treatments. In summary, there was blunted temporal cortex adenylyl cyclase activity in subjects with mood disorders and decreased occipital cortex $G\alpha_s$ in lithium-treated BD subjects. Temporal cortex CREB levels were decreased in antidepressant-untreated MDD subjects, and restored in those subjects treated by antidepressants at the time of death. Temporal cortex CREB levels were decreased in BD patients treated with anticonvulsants relative to those not treated by anticonvulsants at the time of death. Subjects who died from suicide had lower temporal cortex CREB levels and CRE-binding than subjects who died of other causes. Hippocampal BDNF levels were increased in subjects treated by antidepressants compared to subjects not treated by these medications. BD subjects had increased mossy fibre staining relative to controls and other diagnoses. These results complement recent animal models and help to validate the use of postmortem brain tissue for molecular psychiatry research. Although the full implications of the findings in this study are not yet

known, it provides a basis to conduct further experiments with the goal of determining an aetiology and treatment for mood disorders.

Appendix A: Detailed Methods

1. Postmortem brain tissue

Postmortem brain tissue was obtained from the Stanley Foundation Neuropathology Consortium. Samples included subjects with bipolar disorder, schizophrenia, major depressive disorder and controls (n=15 for each), all of which have been matched for age, sex and postmortem delay. DSM-IV diagnoses have been rigorously applied to all psychiatric patients and a detailed treatment history is available (See Appendix B for clinical information). Tissue chunks from temporal cortex (B.A. 20 and 21) and occipital cortex (B.A. 17 and 18), and fresh-frozen 14 μm slices from anterior hippocampus and prefrontal cortex were received on dry ice and stored at –80°C until use. All samples were number coded and tested blind to diagnosis (codes were known only to GM who was not involved in experimental assays or data acquisition).

2. Mononuclear lymphocytes

Mononuclear lymphocytes (MNL) samples were obtained from outpatients in the Mood Disorders Program at McMaster University. Subjects met DSM-IV criteria for bipolar disorder and major depressive disorder and gave informed written consent. Drug free patients were unmedicated (with the exception of acetaminophen, aspirin, oral contraceptives, or clonazepam/lorazepam) for at least two weeks prior to obtaining a

sample. Drug free patients had not received mood stabilizers or antidepressants for at least three months and were free of acute medical illness or recent drug abuse. A group of healthy comparison subjects without past or family history of psychiatric illness was matched for age and sex. A 30ml blood sample was obtained from each subject in acid-citrate-dextrose vacutainers and processed within 2 hours. Samples were centrifuged at 100 x *g* for 20 minutes at room temperature and plasma was removed. Samples were then centrifuged at 1000 x *g* for 20 minutes at room temperature, mixed with RPMI-1640, layered with 60% percoll solution and re-centrifuged at 850 x *g* for 20 minutes. The MNL pellet was then rinsed with RPMI-1640 and stored at –80°C until use.

3. Chemicals

Acrylamide, forskolin, ATP, bovine serum albumin, phosphocreatine, creatine phosphokinase, aprotinin, EGTA, EDTA, paraformaldehyde, Triton X-100, gum arabic, hydroquinone, silver nitrate, xylene, thionin, methyl green and gluteraldehyde were purchased from Sigma Chemicals (St. Louis, MO). SDS-PAGE ladder and Bradford assay kit were purchased from Biorad (Mississaugua, Ontario). Carnation instant skim milk powder used for membrane blocking was purchased from Nestle (Don Mills, Ontario). ECL kit, Cyclic AMP assay kit and Hybond-PVDF membrane were purcahsed from Amersham (Oakville, Ontario). Leupeptin and isobutylmethylxanthine were purchased from ICN Biochemicals (Aurora, Ohio). Apoptaq TUNEL-stain kit was purchased from Intergen (Purchase, NY). γ^{32}P-dATP was purchased from Mandel (Guelph, Ontario). Immunohistochemistry kit was purchased from Zymed (San

Francisco, CA). CREB and pCREB polyclonal antibodies were purchased from Upstate Biologicals, Ceaderlane Labs (Hornby, Ontario). BDNF monoclonal antibody, alkaline phosphatase, T4 polynucleotide kinase and levamisole were purchased from Promega (Madison, WI). Recombinant BDNF protein was purchased from Amgen (Thousand Oaks, CA). Gαs long, Gαs short and Gαi antibodies were obtained from NEN Life Sciences (Boston, MA). CaMK II and CaMK IV antibodies were purchased from Transduction Laboratories (San Jose, CA). Millonig's buffer was purchased from Electron Microscopy Sciences (Ft Washington, PA). BCIP/NBT colour solution and G-25 Sephadex columns were purchased from Boehringer Mannheim (Wilmington, NC).

4. Preparation of tissue samples

4.1 Whole cell preparations

Whole cell extracts were prepared by sonicating (Artek Systems Sonic 300 Membrator, 5s, 35% intensity) brain tissue (50mg) in 0.6ml of 20mM Hepes (pH7.9), 0.4M NaCl, 20% glycerol, 5mM $MgCl_2$, 0.5mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 0.1mM PMSF, 5mM dithiothreitol, aprotenin and leupeptin. The solution was then incubated at 4°C for 30 minutes and subsequently centrifuged at 15000 x *g* for 25 minutes at 4°C and the supernatant used. Protein concentrations were determined as per the method of Bradford (1976).

4.2 Membrane preparations

Brain tissue (50mg) was sonicated (Artek Systems Sonic 300 Membrator, 10s, 35% intensity) in 10mM Tris (pH 7.5), 2 mM EGTA, 5mM aprotinin and leupeptin.

The homogenates were centrifuged at 13000 x *g* for 10 minutes at 4°C and pellets were resuspended in 4 volumes of 50 mM Tris (pH 7.5), 2 mM EGTA, 5mM $MgCl_2$, 5mM aprotinin and leupeptin. Protein concentrations were determined as per the method of Bradford (1976).

4.3 Glutaraldehyde-fixing of frozen sections

Anterior hippocampus sections were thawed to room temperature over 2 hours. Air-dried slides were then incubated in 0.1% Na_2S in 4% glutaraldehyde/0.12M Millonig's buffer for 5 minutes. Slides were then rinsed in dH_2O and air-dried prior to Neo-Timm staining procedure.

4.4 Paraformaldehyde-fixing of frozen sections

Anterior hippocampus sections were thawed to room temperature over 32 hours and air dried. Slides were then incubated in 1% paraformaldehyde/PBS for 10 minutes at room temperature. Slides were rinsed twice in PBS for 5 minutes each and incubated in precooled 2:1 ethanol:acetic acid for 5 minutes at –20°C. Slides were then rinsed with PBS twice for 5 minutes each. Slides were air-dried then used for TUNEL staining.

5. Immunoblotting

5.1 Electrophoresis and transfer to PVDF membrane

Thirty μg of whole cell protein or membrane protein samples were incubated in 62.5 mM Tris-HCL buffer (pH 6.8) containing 3% SDS, 10% glycerol and 5% β-mercaptoethanol and boiled for 4 minutes. A broad range pre-stained protein ladder was

used in lane one of the acrylamide gel. Lanes 2 to 5 contained protein corresponding to subjects with BD, MDD, SCZ and control (one of each, blinded with random lane placement). Lanes 6 to 10 contained protein from a standard tissue (see below). The samples were then subjected to SDS-PAGE on a 12% acrylamide gel at 120V for 1.5 hours at room temperature. Gels were incubated in transfer buffer for 10 minutes at 4°C then transferred to PVDF membranes at 100V for 1 hour at 4°C. Prior to immunolabeling, successful transfer was confirmed using 0.2% Ponceau S in 4% acetic acid.

5.2 Primary and secondary immunolabeling

Membranes were blocked in 5% milk-phosphate buffered saline (PBS) for 1.5 hours at room temperature, then incubated overnight at 4°C with either CREB antiserum (1:2000 dilution), pCREB antiserum (1:1000 dilution), Gas antiserum (1:10000 dilution), Gai antiserum (1:5000 dilution), or CaMK II & IV antisera (1:1000 dilution). Blots were then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:2000 in 5% milk-PBS blocking buffer for 1hour at room temperature. Membranes were subsequently washed 3 times in PBS for five minutes each.

5.3 Chemiluminescent detection

Following PBS washes, membranes were blotted dry and evenly covered with ECL detection reagent for 60 seconds. The ECL reagent consisted of an HRP/hydrogen peroxide catalyzed oxidation of luminol, emitting a 428nm wavelength of light that was detected using Kodak X-AR film. Film exposure times varied between 3 seconds and 30 minutes.

5.4 Quantification

Bands visualized on the Kodak X-AR film were quantified using an MCID image analysis system with the Northern Exposure program (ImagExports, Oakville, On). A numerical value for optical density (OD) was obtained for each band.

5.5 Approach to standardization and multiple sample comparison

A protein sample obtained from cerebral cortex of a control subject (not part of the test subjects) was used as a standard on each gel, allowing inter-gel comparisons. Each gel contained 10μg to 50μg of the standard protein sample in lanes 6 to 10. The ODs from these five standards were plotted to obtain a regression line and linear equation for each gel (figure 1). If regression analysis of this linear range failed to show significance (at $p<0.05$) or if the r^2 was smaller than 0.9, the gel was discarded without further analysis and those subjects were run again. If the regression analysis met these quality criteria, the linear equation was used to calculate a reference value for 30μg of protein, which was the amount loaded for each test subject on that gel. ODs obtained from the four test subjects on the gel (lanes 2 to 5) were divided by this reference value, producing a standardized OD. As each subject was run in duplicate, the mean standardized OD was used for statistical analysis.

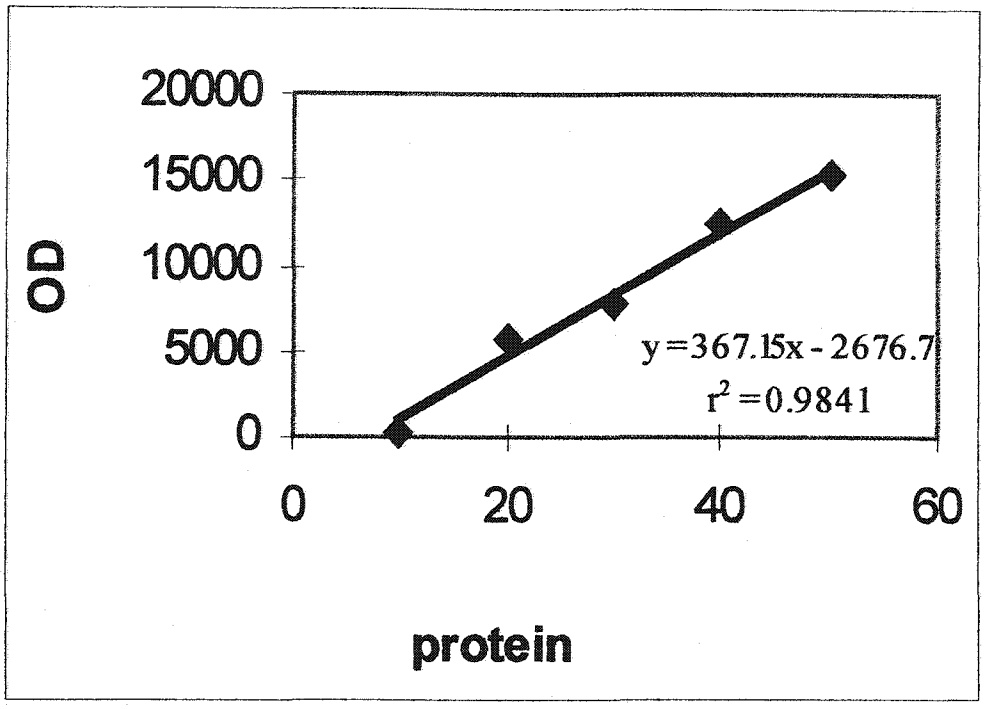

Figure 1: Regression analysis of 10μg to 50μg of reference protein was used to generate a linear range and regression equation for each gel. A reference OD was calculated from this equation for 30μg of protein, and was used to standardize ODs from each test subject on the gel.

5.6 Intra-blot and inter-blot variation

In order to test the reliability of the standardization technique to produce consistent OD values for a subject across gels, we analyzed the inter- and intra-blot variability of this technique. A single subject was run four times on one gel, along with the reference range. This was repeated for four subjects. Each sample was standardized to the regression equation for its gel and the values were compared (figure 2A). Subsequently, four separate subjects were run on a single gel, along with a reference range. A total of four gels containing this group of subjects were run, with randomized lane-placements of the subjects. Samples were standardized and compared (figure 2B). This technique revealed minimal variation of standardized values obtained from a single subject whether run four times on one gel, or on four separate gels.

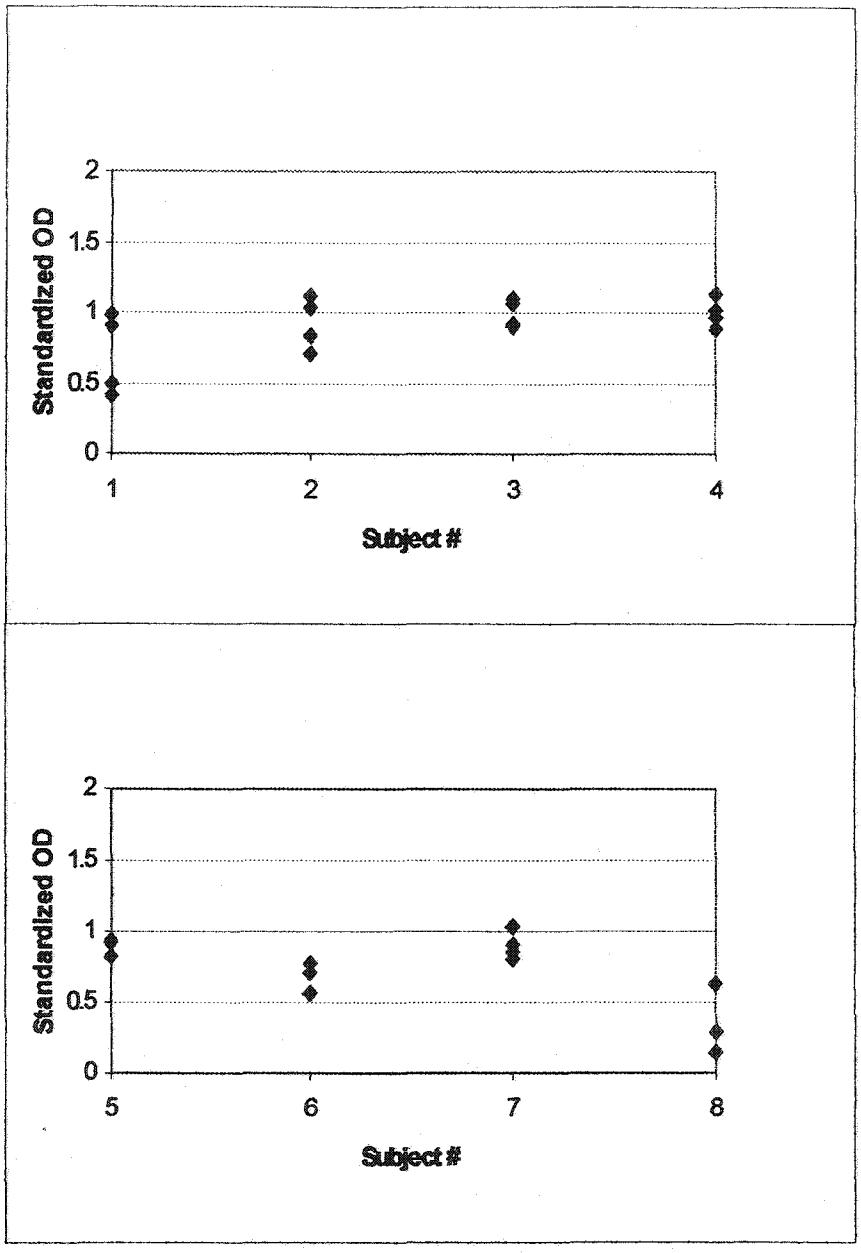

Figure 2: A) *Intra-blot* variability. Each subject was run four times on the same gel and standardized ODs were compared. B) *Inter-blot* variability. Four gels were run, each containing four separate subjects. Standardized ODs were compared.

6. Immunohistochemistry

6.1 Phosphorylated CREB

Frozen prefrontal cortex sections (3 per subject) were thawed to room temperature over 2 hours. The thawed slides were incubated in 0.3% Triton X-100/PBS for 10 minutes at room temperature and rinsed 3 times in PBS. Slides were then incubated in 3% H_2O_2/methanol for 5 minutes at room temperature to quench endogenous peroxidases. Following a PBS rinse, slides were blocked in 10% non-immune goat serum for 10 minutes and drained. Slides were subsequently incubated in polyclonal rabbit-antihuman phosphorylated CREB antibody (1:500) for 26 hours at 4°C. Negative controls used non-immune serum instead of antibody. After washing 3 times in PBS, slides were incubated in biotinylated 2° antibody for 30 minutes and rinsed. Streptavidin congugated with peroxidase was added and slides were incubated for 30 minutes. Following 3 rinses with PBS, slides were covered with DAB for 5 minutes then rinsed with distilled water. Slides were dehydrated in successive 5-minute washes of 20% ethanol, 50% ethanol, 75% ethanol, 95 % ethanol, 100% ethanol, two xylene washes, then xylene-mounted.

6.2 BDNF

Frozen anterior hippocampus sections (2 per subject) were thawed to room temperature and fixed in 10% PBS buffered formalin for 5 minutes. Slides were then incubated in 0.3% Triton X-100/PBS fro 10 minutes at room temperature and rinsed. Endogenous phosphatase activity was quenched using 1M levamisole. Slides were blocked in 10% non-immune goat serum for 30 minutes, and then incubated with

chicken-antihuman BDNF antibody (1:500) overnight at 4°C. Negative controls were incubated in goat serum instead of primary antibody. Slides were washed in PBS then incubated with goat-antichicken IgY conjugated to alkaline phosphatase for 60 minutes at room temperature. Sections were rinsed and incubated in BCIP/NBT solution for two hours, rinsed in water, dehydrated in ethanol and xylene mounted as above.

6.3 Quantification

Immunohistochemical stained sections were examined at 50X magnification by creating a digitized image with the Bioquant Pure Color Windows 98 imaging system (R&M Biometrics, Inc., U.S.A.) attached to a light microscope with an electronic stage encoder (Zeiss Axioskop, Oberkochen, Germany). Using the imaging system, the average integrated optical density (IOD) of BDNF immunoreactivity in the hilar region, the dentate gyrus, and the supragranular (SG) zone adjacent to the dentate gyrus were measured by averaging measurements obtained from ten adjacent circles with 50 μm radii. Two measurements were obtained from the supragranular region: the first from a 100μm band outside the dentate gyrus (SG1), and the second starting at 100μm from the dentate gyrus and extending a further 100μm (SG2). The density of immunostaining in different regions was expressed as relative optical density (ROD). For prefrontal cortex phosphorylated CREB immunohistochemistry, optical density values were obtained from five random fields of view of grey matter (50X magnification) per slide. The five field ODs were averaged for each slide, and a mean OD was calculated from the three slides per subject. Since glass slides absorb pigment during

immunostaining, the Bioquant imaging system included an automatic background correction algorithm. This correction calibrates analysis to a baseline image obtained from a tissue-free region of the slide. These background corrections were made for each section, as per the Bioquant protocols (R & M Biometrics Inc., USA). All assays were performed blind to diagnosis and treatment history.

7. Basal and forskolin-stimulated cAMP levels

7.1 Forskolin-stimulation

Temporal cortex membrane preparations (5 μg protein) were incubated in 200 μl of a pre-incubation mixture consisting of 50 mM Tris-HCl (pH 7.4), 1.5 mg/ml of bovine serum albumin, 2mM EGTA, 25 mM phosphocreatine, 250 U/ml creatine kinase, 5 mM $MgCl_2$ and 100 μM isobutylmethylxanthine for 10 minutes at 4°C. After preincubation, reactions were initiated by the addition of 0.5 mM ATP together with either 100 μM forskolin or 50 mM Tri-HCl (pH 7.4) as control for 10 minutes at 37°C. The reaction was stopped by boiling samples for 5 minutes and centrifuging at 13,000 x *g* for 5 minutes at 4°C. The supernatants were subsequently assayed for cAMP levels.

7.2 Cyclic AMP assay

The principle of this assay is a competition between cAMP in the subject sample and radiolabelled cAMP for a fixed number of cAMP-antibody binding sites. An increase in cAMP results in decreased radiolabelled cAMP binding. Test tubes containing 25, 50, 100, 200, 400, 800 and 1600 fmol of cAMP in 500 μl of 0.05M acetate

buffer (pH 5.8) were used as reference standards. 15 µl of supernatant from each subject was diluted to 500 µl with acetate buffer. 15 µl of each cAMP reference standard was also diluted to 500 µl with acetate buffer. A negative control tube contained 500 µl of acetate buffer. 100 µl of succinyl-cAMP [^{125}I]-tyrosine methyl ester was added to each tube. A total counts tube (TC) consisted of only 100µl of the radiolabelled cAMP ^{125}I. 100 µl of rabbit-antisuccinyl cAMP was added to all tubes except TC. Tubes were vortexed, stoppered and incubated at 4°C for 3 hours. 500µl of secondary antibody (Amerlex-M) was added to all tubes except the TC and vortexed. Tubes were then incubated for 10 minutes at 25°C and centrifuged for 15 minutes at 2000 x *g*. Supernatants were decanted and discarded from all tubes except TC. All test tubes, including subjects, standards and controls were assayed in duplicate. Assay was performed blind to diagnosis.

7.3 Scintillation & quantification

Test tubes were placed in a γ scintillation counter to obtain an average count per minute (CPM) from each sample. Binding was expressed as $\%B/B_0$, where B = reference standard or sample CPM, and B_0 = negative control CPM. Reference standard $\%B/B_0$ was plotted on a graph using Fig P (Biosoft, MO, USA) to derive a logarithmic equation. Mean cAMP levels from the two samples per subject were calculated using this equation, and expressed as pmol of cAMP produced per mg protein per minute.

8. Electrophoretic mobility shift assay (EMSA)

8.1 Labelling of consensus oligonucleotide

A double-stranded synthetic CRE oligonucleotide with the sequence 5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3' was chosen based on the Promega protocol for 5' labelling. 3.5 pmol of the CRE oligonucleotide was incubated in a 10μl mixture containing 1μl [γ-^{32}P]-ATP (3000Ci/mmol at 10mCi/ml), 10U T4 polynucleotide kinase, 5mM DTT, 10mM $MgCl_2$, and 70mM Tris-HCl (pH 7.6) for 10 minutes at 37°C. The reaction was stopped by adding 1μl of 0.5M EDTA. 89μl of TE buffer containing 10mM Tris-HCl (pH 8.0) and 1mM EDTA was added and the mixture was centrifuged through a G-25 Sephadex column for 4 minutes at 1100 x *g*. The eluate contained the CRE-probe for the EMSA procedure.

8.2 Gel shift assay

50μg of temporal cortex whole cell protein extract was incubated in 4% glycerol, 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM DTT, 50mM NaCl, 0.25 mg/ml poly(dI-dC)·poly(dI-dC), and 10mM Tris-HCl (pH 7.5) for 10 minutes at room temperature. A Negative control sample contained Tris-HCl buffer instead of protein. A positive control sample substituted temporal cortex protein with 5μg of HeLa cell extract. Protein from temporal cortex of a reference subject, not part of the test subjects, was included on each gel for standardization and inter-gel comparisons. A supershifted sample contained 1μl of CREB antibody in the incubation mixture. 1μl CRE probe (≈200,000 CPM) was added and samples were incubated at room temperature for 20 minutes. Samples were

combined with 1μl of loading buffer, consisting of 25mM Tris-HCl (pH 7.5), 0.2% bromophenol blue and 40% glycerol, and loaded on a 6% polyacrylamide gel for electrophoresis at 100V at 4°C for 2 hours. Gels were transferred to Whatmann 3MM paper and dried on a gel drier, then exposed to X-ray film overnight at –80°C. All samples were run in duplicate and blind to diagnosis.

8.3 Imaging and quantification

Bands corresponding in size to those supershifted were visualized on Kodak X-AR film and quantified using an MCID image analysis system with the Northern Exposure program. A numerical value OD was obtained for each band. Test subject ODs were divided by the reference sample on each gel to provided a standardized measure for comparison across gels. To limit radiation exposure, only one reference sample was included on each gel. This provided sufficient space to run four subjects in duplicate on each gel. The mean standardized OD for each subject was used for analysis.

9. Timm staining

9.1 The sulfide-silver method

The Timm stain method is used to detect group IIb heavy metals, such as the Zinc in dentate gyrus axons (mossy fibres). The basis of this technique is the conversion of endogenous zincs in mossy fibres into zinc sulphides, which catalyse the reduction of silver into molecular silver. This reaction occurs in a solution of Ag^{+}, a colloid, and hydroquinone, a reducing substance. Two consecutive frozen anterior hippocampus sections for each subject were fixed in glutaraldehyde and sodium sulfide

(see 4.3 above)(Ying et al., 1998; Babb et al., 1996; Mathern et al., 1995a). Timm developer was prepared in the dark and consisted of a 60:30:10ml mixture of 50% gum arabic, 5.67% hydroquinone, and 25.5% citric acid-23% sodium citrate, with 0.5 ml of 17% silver nitrate solution. Slides were incubated for 60 minutes in the dark in 100ml of Timm developer. Incubation periods were optimized first in rat, and subsequently in human hippocampal tissue. Incubations times during optimization ranged from 30 minutes to 90 minutes, with 60 minutes yielding the best signal to noise ratio. Silver staining specificity was confirmed by comparing light (50 min) and dark (70 min) exposures (Mathern et al., 1995a; Mathern et al., 1995b; Babb et al., 1991). Following Timm staining, slides were rinsed with water and air-dried. Slides were then incubated in xylene for 5 minutes, hydrated in 90% and 70% ethanol, and counterstained in 0.1% thionin/0.1 M sodium acetate. Slides were rinsed, dehydrated and xylene-mounted. The assay was conducted blind to diagnosis.

9.2 Quantification

Distribution of silver staining in the supragranular region of the dentate gyrus was analyzed blind to diagnosis using the Bioquant TCW 98 imaging system with a topography module and a Zeiss Axioskop light microscope with stage encoder. The supragranular region was defined as the region of the inner molecular layer located directly adjacent to the dentate gyrus. Supragranular silver staining was quantified using a Timm index (Vaidya et al., 1999; Watanabe et al., 1996). A Timm index provides a computer-assisted non-biased measurement representing the pattern of supragranular silver staining. Using computer topography, the total area of supragranular silver stain in

the inner molecular layer was measured and divided by the perimeter of the dentate gyrus. This Timm index was subsequently averaged from duplicate slides for each subject.

10. Terminal dUTP nick-end labelling (TUNEL)

10.1 Sample size

As most of the hippocampal tissue was used in the previous experiments, the number of subjects available for TUNEL staining was 8 controls, 9 bipolar disorder, 5 major depressive disorder and 6 schizophrenia. These subjects had a minimum of two hippocampal slides available, as the assay was done in duplicate.

10.2 TUNEL procedure

The principle of the TUNEL procedure is to label free 3'OH DNA termini *in situ* with nucleotides labelled with digoxigenin. An anti-digoxigenin antibody conjugated with peroxidase is used to bind these labelled nucleotides. Two consecutive hippocampal slices per subject were fixed in paraformaldehyde (see 4.4 above). To quench endogenous peroxidases, slides were incubated in 3% hydrogen peroxide/PBS for 5 minutes at room temperature. Slides were then placed in an equilibration buffer (Intergen, S7101) for 1 minute at room temperature. Slides were blotted dry and incubated in terminal deoxynucleotidyl transferase (TdT) enzyme and reaction buffer (Intergen, S7105) for 1 hour at 37°C. The reaction was stopped by incubating slides in the Stop/Wash buffer (Intergen, S7101#4) for 10 minutes at room temperature. Slides were washed 3 times in PBS for 1 minute each, then incubated with Anti-Digoxigenin

antibody for 30 minutes in a humid chamber at room temperature. Slides were washed 4 times in PBS for 2 minutes each and stained with DAB for 6 minutes.

10.3 Controls

Hippocampal slides used as positive controls were pre-treated with DNAse I immediately following parafolmaldehyde-fixing. Slides were incubated in DN buffer consisting of 30mM Trizma base (pH 7.2), 4mM $MgCl_2$ and 0.1mM DTT at room temperature for 5 minutes. Slides were then incubated with DNAse I (10U/ml) in DN buffer for 10 minutes at room temperature. Slides were washed 5 times with dH_2O for 3 minutes each. Hippocampal slides used as negative controls substituted TdT enzyme with equilibration buffer.

10.4 Counterstaining

Slides were rinsed with tap water, incubated for 5 minutes in dH_2O then stained with eosin for 1 minute. Slides were subsequently rinsed in dH_2O for 30 seconds and incubated in 0.5% methyl green for 10 minutes at room temperature. Slides were rinsed in 3 changes of dH_2O, 3 changes of 100% n-butanol, and 2 changes of 100% xylene, and then mounted. This procedure stained fragmented nuclei brown, and counterstained all cytoplasm pink, and non-fragmented nuclei green.

10.5 Quantification

TUNEL stained sections were examined at 400X magnification by creating a digitized image with the Bioquant Pure Color Windows 98 imaging system (R&M Biometrics, Inc., U.S.A.) attached to a light microscope with an electronic stage encoder (Zeiss Axioskop, Oberkochen, Germany). All granular cells in the dentate gyrus

were counted and an apoptotic index was calculated by dividing the number of TUNEL positive granular cells by total number of granular cells.

11. Statistical analysis

All statistical analyses were conducted using Minitab 12.1 and SPSS for Windows 98. Group differences in age, sex and postmortem delay were tested using one-way ANOVAs. Regression analysis was used to determine postmortem delay and age effects on all measures. ANOVAs were used to assess diagnostic, treatment and suicide effects on all measures. Unpaired two-way t-tests were used to analyze treatment and suicide effects within groups. An α=0.05 level of statistical significance was used in all tests.

Appendix B: Subject Data

STANLEY FOUNDATION NEUROPATHOLOGY CONSORTIUM

	15 schizophrenia	15 bipolar disorder	15 depression without psychosis	15 normal controls
age	44.2 (25–62)	42.3 (25–61)	46.5 (30–65)	48.1 (29–68)
sex	9 M, 6 F	9 M, 6 F	9 M, 6 F	9 M, 6 F
race	13 W, 2 As	14 W, 1 B	15 W	14 W, 1 B
PMI (hrs)	33.7 (12–61)	32.5 (13–62)	27.5 (7–47)	23.7 (8–42)
mRNA yield	10 A 2 B 3 C	13 A 2 B	11 A 2 B 2 C	12 A 2 B 1 C
pH	6.1 (5.8–6.6)	6.2 (5.8–6.5)	6.2 (5.6–6.5)	6.3 (5.8–6.6)
side of brain frozen	6 R, 9 L	8 R, 7 L	6 R, 9 L	7 R, 8 L

5/22/97 (neuropath\sf cons.)

Schizophrenia

Brain No.	Age, race, sex; occupation	Cause of death	PMI (hrs)	Side of brain	Family history – severe psychiatric disorder	Age of onset	Diagnosis	CNS medications at time of death	Est. lifetime antipsychotics (fluphenazine mg equivalents)	Substance abuse history
S-13	30, white female blue collar worker	Suicide: jumped	60	R	Bipolar in 2° relative	22	Schiz. Disorganized: 295.10	thiothixene, desipramine	6,000	Marihuana abuse
S-18	52, white male lived in public shelter	Cardiac	61	L	Depression in 1° relative	20	Schiz., undifferentiated 295.92	None; untreated for over 20 yrs.	9,000	No use of alcohol or drugs
S-30	30, white male, on disability, lived in group home	Pneumonia	32	L	Psychosis in 2° relative	13	Schiz., undifferentiated: 295.92	risperidone, thioridazine	50,000	Moderate use of alcohol; no use of drugs
S-41	62, Asian female housewife	Motor vehicle accident	26	L	Negative	38	Schiz., paranoid: 295.30	none; untreated for several months	50,000	Light use of alcohol; no use of drugs
S-43	60, white female, lived with family	Cardiac	40	L	Negative	15	Schiz., undifferentiated: 295.92	none; had ECT but probably never treated otherwise	0	No use of alcohol or drugs
S-64	60, white male, on disability, lived in supported housing	Accidental drowning	31	R	Negative	27	Schiz., undifferentiated: 295.92	thioridazine, amitriptyline	80,000	No use of alcohol or drugs
S-66	32, Asian/white male, on disability, lived in group home	Acute alcohol intoxication	19	L	Negative	27	Schiz., undifferentiated: 295.92	clozapine	15,000	Alcohol abuse, amphetamine abuse
S-81	31, white male, on disability, lived in group home	Suicide: jumped	14	L	"mental problems" in 2° relative	18	Schiz., undifferentiated: 295.92	clozapine	4,000	Light use of alcohol; no use of drugs

Brain No.	Age, race, sex, occupation	Cause of death	PMI (hrs)	Side of brain	Family history – severe psychiatric disorder	Age of onset	Diagnosis	CNS medications at time of death	Est. lifetime antipsychotics (fluphenazine mg equivalents)	Substance abuse history
S-82	58, white female clerical worker	Cardiac	26	R	Bipolar in two 1° relatives; schiz. in one 1° relative	42	Schiz.; paranoid: 295.30	haloperidol, diphenhydramine	35,000	Past alcohol abuse, no use of drugs
S-93	25, white male, on disability, lived w/family	Suicide: hanged	32	L	Psychosis in 2° relative	20	Schiz.: undifferentiated: 295.92	risperidone, paroxetine	4,000	Past alcohol abuse and marihuana use
S-100	44, white male, on disability, lived in group home	Cardiac	50	R	Schiz. in three 1° relatives	17	Schiz., undifferentiated: 295.92	haloperidol, carbamazepine, fluoxetine, clonazepam, benzotropine	100,000	Moderate alcohol use, little or no use of drugs
S-116	44, white male, on disability, lived in group home	Pulmonary disease	29	L	"Mental illness" in 2° relative	21	Schiz., paranoid: 295.30	clozapine, chlorpromazine, lithium	130,000	Alcohol dependence; past polysubstance abuse
S-118	56, Asian female, on disability, lived with boyfriend	Suicide: overdose	12	R	Schiz. in 1° relative	24	Schiz.: undifferentiated: 295.92	haloperidol, lithium, diphenhydramine, chloral hydrate	150,000	No alcohol use, occ. past marihuana use
S-120	35, white male, on disability, lived in supported housing	Cardiac	35	R	"Mental illness" in 2° relative	19	Schiz., paranoid: 295.30	clozapine, chlorpromazine, maprotiline, benzotropine, diphenhydramine	50,000	Moderate alcohol use; polysubstance abuse
S-173	49, white female, on disability, lived in group home	Cardiac	38	L	Depression in 1° relative	25	Schiz., undifferentiated: 295.92	haloperidol, clozapine, clonazepam	>200,000	No use of alcohol or drugs

Bipolar Disorder

Brain no.	Age, race, sex, occupation	Cause of death	PMI (hrs)	Side of brain	Family history – severe psychiatric disorder	Age of onset	Diagnosis	CNS Medications at time of death	Est lifetime antipsychotics ((fluphenazine mg equivalents))	Substance abuse
S-33	25, white female blue collar worker	Suicide: hanged	24	R	Bipolar in two 2° relatives	19	Bipolar w/psychotic features: 296.54	thiothixene, carbamazepine, lithium, trazadone ✓	7,500	Alcohol dependence; occ. use of marihuana, mushrooms, nitrous oxide, LSD (x2), PCP (x1)
S-34	48, white female technician	Pneumonia	22	L	Negative	16	Bipolar w/psychotic features: 296.64	valproate, ✓ sertraline, chlorprothixene, carbamazepine	32,000	Alcohol abuse; methadone abuse
S-47	37, white female clerical worker	Suicide: overdose	29	R	Bipolar in a 1° relative and depression in a 1° relative	14	Bipolar, w/psychotic features: 296.64	lithium, ✓ bupropion, clonazepam, lorazepam	1,200	In teens used marihuana, cocaine (x2), LSD (x1) but none thereafter
S-48	54, white male technician	Subdural hematoma	39	R	Depression in a 1° relative	39	Bipolar w/o psychotic features: 296.45	lithium, ✓ carbamazepine ✓	2,500	Some alcohol abuse in 20s but abstinent thereafter; no use of drugs
S-60	30, white male, on disability, lived alone	Pneumonia and myocarditis	31	R	Negative	22	Bipolar w/psychotic features: 296.64	lithium, clozapine	60,000	Moderate use of alcohol; no use of drugs
S-68	30, white male blue collar worker	Suicide: carbon monoxide	56	R	Negative	7	Bipolar w/o psychotic features: 296.53	never treated X	0	Light alcohol use, no use of drugs
S-72	57, white male, on disability, lived in supported housing	Cardiac	19	L	Unknown	30	Bipolar w/psychotic features: 296.44	haloperidal, ✓ diphenhydramine	60,000	In 20s abused alcohol; no use of drugs

Brain no.	Age, race, sex, occupation	Cause of death	PMI (hrs)	Side of brain	Family history – severe psychiatric disorder	Age of onset	Diagnosis	CNS Medications at time of death	Est. lifetime ant.psychotics (fluphenazine mg. equivalents)	Substance abuse
S-75	34, white male, clerical worker	Suicide: jumped	23	R	Depression in two 1° relatives	19	Bipolar w/psychotic features: 296.54	risperidone, valproate, venlafaxine ✓	7,000	In teens abused alcohol, then abstinent until few months prior to death; occ. marihuana use
S-83	48, white male technician	Suicide: immolation	13	R	"Insanity" in a 2° relative	27	Bipolar w/psychotic features: 296.44	untreated for over 20 yrs. ✗	200 max.	Moderate alcohol use; marihuana use in college and again in months prior to death
S-88	31, white male, on disability, lived alone	Suicide: jumped	28	R	"Mental breakdown" in a 2° relative	21	Bipolar w/psychotic features: 296.54	haloperidol, trazadone, trihexphenidyl ✓	30,000	Polysubstance abuse including marihuana, mushrooms, glue, cocaine
S-89	30, white male technician	?Suicide: overdose	45	L	Bipolar in two 1° and one 2° relatives	14	Bipolar II: 296.89	valproate, bupriopion ✓	0	In teens used marihuana; occ. cocaine for 1 yr. prior to death
S-91	50, black female professional	Malnutrition and dehydration	18	L	Schiz. in a 1° relative	34	Bipolar w/psychotic features: 296.44	none, untreated for several months ✗	12,000	Marihuana and cocaine abuse
S-103	61, white female housewife	Suicide: overdose	60	L	Bipolar in a 1° relative	18	(1) bipolar w/psychotic features: 296.54, (2) hypothyroidism	fluoxetine, valproate ✓	40,000	Light alcohol use; no use of drugs
S-128	50, white male, on disability, lived alone	Suicide: jumped	19	L	Negative	17	Bipolar w/psychotic features: 296.44	valproate, clozapine, flurazepam, benzotropine ✓	60,000	Light alcohol use; no use of drugs
S-147	50, white female professional	Pulmonary emboli	62	L	Schiz. in a 2° relative	25	Bipolar w/o psychotic features: 296.53	valproate, clomipramine ✓	0	Light alcohol use; no use of drugs

Depression

	Age, race, sex, occupation	Cause of death	PMI (hrs)	Side of brain	Family history severe psychiatric disorder	Age of initial depression	Diagnosis	CNS Medications at time of death	Substance abuse history
S-16	32, white female administrator	Suicide: overdose	47	L	Unknown (adopted)	32	Major depression w/o psychotic features: 296.23	imipramine, amitriptyline, nortriptyline, clonazepam	Some cocaine use
S-38	53, white female blue collar worker	Acute alcohol intoxication	40	R	Depression in 1° relative	11	Major depression w/o psychotic features: 296.33	lithium, trazadone	Severe alcohol dependence
S-46	44, white female professional	Suicide: overdose	32	L	Depression in 1° and 2° relative	27, postpartum	Major depression w/o psychotic features: 296.33	fluoxetine, imipramine, lorazepam	Alcohol use light; no use of drugs
S-59	65, white male business owner	Cardiac	19	R	"Mental problems" in 1° relative	45	Major depression w/o psychotic features: 296.33	phenytoin for a single seizure; no other meds. for 5 yrs.	Alcohol use light; no use of drugs
S-92	52, white male administrator	Cardiac	12	R	Negative	46	Depressive disorder NOS: 311; no psychotic features	no medication for 6 yrs.	1-2 beers/day; no use of drugs
S-99	46, white male professional	Suicide: carbon monoxide	26	R	Depression in two 1° relatives	28	Major depression w/o psychotic features: 296.33	diphenhydramine, clonazepam	No use of alcohol or drugs
S-101	42, white female clerical worker	Cardiac	25	R	Depression in 1° relative	39	Major depression w/o psychotic features: 296.22	fluoxetine, lithium	Alcohol use light; no use of drugs
S-104	51, white male blue collar worker	Suicide: gunshot	26	R	Depression in 1° relative	50	Major depression w/o psychotic features: 296.23	nefazadone, hydroxyzine	Past alcohol dependence but abstinent for 14 yrs.

	Age, race, sex, occupation	Cause of death	PMI (hrs)	Side of brain	Family history severe psychiatric disorder	Age of initial depression	Diagnosis	CNS Medications at time of death	Substance abuse history
S-135	39, white male blue collar worker	Suicide: carbon monoxide	23	L	Depression in two 1° relatives	17	Major depression w/o psychotic features: 296.33	never treated	Alcohol abuse; amphetamine abuse
S-138	42, white male technician	Suicide; hanged	7	L	Depression in two 1° relatives	32	Major depression w/o psychotic features: 296.31	temazepam but off medications for more than 2 weeks	Alcohol use light; occasional marihuana when young
S-156	56, white male salesman	Cardiac	23	L	Depression in 2° relative	52	Major depression w/o psychotic features: 296.32	sertraline	Alcohol use light; no drug use
S-163	56, white female housewife	Pulmonary emboli	28	L	Negative	54	Major depression w/o psychotic features: 296.33	venlafaxine, buspirone, alprazolam	Alcohol use light; no drug use
S-168	30, white female blue collar worker	Suicide: overdose	33	L	Depression in two 1° relatives	19	Major depression w/o psychotic features: 296.33	nortriptyline, alprazolam, clomipramine	Alcohol use light; no drug use
S-171	43, white male blue collar worker, on disability	Cardiac	43	L	Unknown (adopted)	30	Major depression w/o psychotic features: 296.33	trimipramine	Alcohol dependence; some drug use including amphetamines, benzodiazepines, and opiods.
S-172	47, white male salesman	Cardiac	28	L	Bipolar disorder in 1° relative	27	Major depression w/o psychotic features: 296.33	fluoxetine, nefazadone	Alcohol use light; no drug use

Normal Controls

	Age, Race, Sex, Occupation	Cause of death	PMI (hrs)	Side of brain	Family history severe psychiatric disorder	Consulted w/mental health professional or any other evidence of mental illness	Substance abuse history
S-49	52, white male professional	Cardiac	28	L	Negative	None	Alcohol abuse when younger but abstinent last 8 years; no use of drugs.
S-70	44, white female technician	Cardiac	25	R	Negative	None	Alcohol use light; occasional marihuana in younger years
S-73	59, white male contractor	Cardiac	26	R	Negative	None	2-3 beers each night but caused no problems and liver unremarkable; no use of drugs.
S-85	52, white male administrator	Cardiac	8	L	Negative	None	Alcohol use light; no use of drugs.
S-123	52, white male administrator	Cardiac	22	R	Negative	None	4-6 beers/day but never missed work and liver unremarkable. No use of drugs.
S-124	53, white male contractor	Cardiac	28	L	Negative	None	Alcohol abuse in 20s but abstinent for over 30 years. In 20s tried marihuana, LSD & mescaline a few times but none since.
S-126	44, white male business owner	Cardiac	10	L	Negative	None	Alcohol use light; no use of drugs.
S-136	35, white female administrator	Cardiac	23	R	Negative	None	Alcohol use light; no use of drugs.

	Age, Race, Sex, Occupation	Cause of death	PMI (hrs)	Side of brain	Family history - severe psychiatric disorder	Consulted w/mental health professional or any other evidence of mental illness	Substance abuse history
S-141	41, black male administrator	Pulmonary embolus	11	R	Negative	None	Alcohol use light; used marihuana "a few times" in college.
S-149	42, white male administrator	Cardiac	27	R	Negative	None	Alcohol use light; no use of drugs.
S-158	35, white female technician	Pulmonary embolus	40	L	Negative	None	Alcohol use light; no use of drugs.
S-162	68, white female clerical worker	Pulmonary embolus	13	L	Negative	None	Alcohol use light; no use of drugs.
S-165	58, white male blue collar worker	Cardiac	27	L	Negative	None	2 beers/day; no use of drugs.
S-174	29, white female technician	Motor vehicle accident	42	L	Bipolar in 2° relative	Saw counselor for weight control.	Alcohol use light; no use of drugs.
S-179	57, white female housewife	Motor vehicle accident	26	R	Negative	None	Alcohol use light, no use of drugs.

Appendix C: Unpublished results

1. Phosphorylated CREB and CRE-binding

Among the many known transcription factors, the cAMP responsive element binding protein (CREB) is one of the most studied and perhaps highly relevant in the pathophysiology of mood disorders. CREB contains a phosphoacceptor site on Ser133, a substrate for phosphorylation by PKA (Gonzalez & Montminy, 1989) as well as Ca^{2+}-calmodulin dependent kinases (CaMKs) (Sheng et al., 1991). Phosphorylation at this site, producing phosphorylated CREB (pCREB), is required for the transcriptional activation of CREB (Lee et al., 1990). Genes whose expression is dependent on cAMP contain a cAMP-response element (CRE), which interacts with CREB (Meinkoth et al., 1991). Although, CREB can bind to the CRE in its unphosphorylated state, only pCREB can initiate transcription. It has been hypothesized that since the binding of CREB alone cannot initiate transcription, its phosphorylation may result in structural changes resulting in the association with transcriptional machinery. Evidence indicates that the CREB binding protein (CBP) interacts with Ser133 phosphorylated-CREB (Flint & Jones, 1991). CBP then recruits the transcriptional machinery required for CREB-regulated gene expression. Therefore, events linking cAMP signalling with downstream gene expression include the phosphorylation of CREB, its subsequent binding to CRE, and its interaction with CBP.

Temporal cortex CRE-binding was measured using the electophoretic mobility shift assay (detailed methodology in *Appendix A*). Immunoblotting of temporal cortex pCREB levels was not possible due to low signal. Prefrontal cortex slices were also available from the Stanley Foundation Neuropathology Consortium and it was possible to consistently measure pCREB levels in this tissue using the more sensitive immunohistochemistry assay. Unfortunately, temporal cortex and hippocampal slices were not available at the time for this assay.

Temporal cortex CRE-binding results

There were no statistical differences in age, sex or postmortem delay between the groups. Regression analysis revealed a significant increase in CRE-binding with increasing age ($P = 0.01$, $r^2 = 0.1$). There was no effect of postmortem delay or sex on CRE-binding. All subsequent analyses were covaried for age.

There were no overall effects of diagnosis, treatment or suicide on temporal cortex CRE-binding. However, subgroup analysis revealed a significant decrease in CRE-binding in MDD subjects that died from suicide, relative to MDD subjects that died of other causes ($p = 0.038$).

Prefrontal cortex phosphorylated CREB results

There was no statistical difference in age, sex or postmortem delay between the groups. Regression analysis revealed a significant decrease in pCREB levels with increasing postmortem delay ($p=0.007$, $r^2=0.12$). There was no effect of age and sex on pCREB levels. All subsequent analyses were covaried for postmortem delay.

Mean pCREB levels (±SEM) were: control 7.8±0.4, BD 8.7±0.5, MDD 8.5±0.4, and SCZ 10.1±0.6. ANOVA revealed a significant effect of diagnosis on prefrontal cortex pCREB [$F = 2.87$, $df(3,59)$, $p=0.04$]. One way Dunnett's ANOVA revealed a significant increase in prefrontal cortex pCREB levels in subjects with SCZ as compared to controls ($p=0.0015$).

There were no overall effects of suicide or drug treatments. Subgroup analysis revealed a significant decrease in pCREB levels in SCZ subjects treated with neuroleptics, as compared to SCZ subjects not treated with neuroleptics at the time of death [$T = -5.48$, $p = 0.0054$).

2. TUNEL staining

In the human hippocampus, a proliferative region exists in the dentate gyrus supragranular zone where granular cells are born throughout life in a process known as neurogenesis (McEwen, 1999). These cells migrate to the granular layer of the dentate gyrus and ultimately undergo apoptosis, resulting in a constant turnover of dentate gyrus neurons. Recent evidence suggests that BDNF and other neurotrophic factors are important in regulating the equilibrium between hippocampal neurogenesis and apoptosis (Lee et al., 2000; Mattson, 2000; Ward & Hagg, 2000; Linnarsson et al., 2000; Young et al., 1999).

Factors that stimulate hippocampal neurogenesis, such as diet and environmental enrichment, are associated with increased dentate gyrus CREB phosphorylation (Young et al., 1999) and increased BDNF and GDNF levels (Lee et al., 2000; Young et al., 1999). Enrichment-induced increases in BDNF levels were also associated with a decrease in

apoptosis in the dentate gyrus(Young et al., 1999), possibly because BDNF has been shown to protect neurons against apoptosis induced by oxidative, metabolic and excitotoxic stresses (Mattson, 2000).

Conversely a decrease in BDNF activity is associated with increases in apoptosis. One study found BDNF knockout mice have increased apoptosis of cholinergic neurons innervating the hippocampus (Ward & Hagg, 2000). Another study reported BDNF knockout mice had an increase in apoptosis in subgranular and granular layers of the dentate gyrus (Linnarsson et al., 2000).

These studies suggest that altered BDNF activity can shift the equilibrium between neurogenesis and apoptosis in the granular cells of the dentate gyrus. In MDD, the blunted cAMP signalling may lead to decreased BDNF activity, ultimately resulting in a shift towards apoptosis in the dentate gyrus. Interestingly, recent MRI studies of subjects with MDD revealed significant atrophy of hippocampal volume relative to controls (Bremner et al., 2000; Sheline et al., 1999; Sheline et al., 1996). These findings would be concordant with an underlying increase in apoptosis of dentate gyrus cells in MDD. Furthermore, it would follow that AD treatment, which has been shown to increase cAMP signalling and BDNF levels, may promote the survival of the dentate gyrus neurons.

Apoptosis is associated with a variety of morphological and biochemical characteristics which distinguish it from the passive process of necrosis. An end stage marker of apoptosis is the fragmentation of DNA into multimers of approximately 180bp nucleosomal units. The terminal dUTP nick-end labelling (TUNEL) technique has been developed to stain for DNA-fragmentation, and has been used to detect apoptosis in human

postmortem brain (Ferrer, 1999; Zhu et al., 1999). Detailed methodology is provided in Appendix A.

Results

Preliminary covariate analysis showed no significant differences in age, sex and post-mortem delay between the groups. DNA-fragmentation was expressed as apoptotic index (AI), which was calculated by dividing the number of TUNEL positive cells by the total cell number in the dentate gyrus. There was no effect of age or sex on AI. Regression analysis showed a significant effect of postmortem delay interval on AI ($p = 0.015$, $r^2 = 20.7$). All subsequent analyses were covaried for postmortem delay.

AI for the groups (±SEM) were: control 0.2% (±0.2), bipolar disorder 0.6% (±0.1), major depressive disorder 0.8% (±0.45), and schizophrenia 0.8% (±0.3). The AI was not statistically different in these groups [$F = 1.22$, $df(3, 27)$, $p = 0.32$]. Similarly, there was no effect of suicide or drug treatments on these subjects.

In addition to the subjects used for the above analyses, there were several single hippocampal slices available from other subjects. When these were added to the experiment, the final subject numbers were 10 controls, 12 BD, 9 MDD and 7 SCZ. Analysis of these subjects changed the AI values to: control 0.17%, bipolar disorder 0.55%, major depressive disorder 0.99%, and schizophrenia 0.71%. The larger sample size revealed a greater A.I. difference between controls and depressed subjects. However, we chose not to perform statistics on this data, as our initial experimental design required a minimum of two slices per subject in order to obtain a conservative estimate of DNA-fragmentation.

In summary, we did not find any significant change in DNA-fragmentation in hippocampal slices obtained from patients with mood disorders, as compared to controls. The study was limited by small sample sizes, which resulted in low statistical power to detect differences in A.I. However, the experiment demonstrated the feasibility of TUNEL staining in postmortem hippocampus to quantify DNA-fragmentation. Data obtained from this experiment will be stored until more hippocampal brain tissue is available to increase sample size and statistical power.

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