

The cytoarchitecture of the human anterior cingulate cortex and its involvement in mood disorder

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“If the human brain were so simple that we could understand it, we would be so simple that we couldn’t.”

Emerson M Pugh

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Abstract

The biological mechanisms proposed to underlie primary mood disorder do not usually include a neuropathological component. Over recent years, a significant imaging literature attests to structural abnormalities in various brain regions in mood disorder, and has encouraged neuropathological investigations. Although the neuropathological understanding of mood disorder is still rudimentary, structural correlates have begun to emerge. The studies described in this thesis investigate the neuropathology of the anterior cingulate cortex in mood disorder.

The anterior cingulate cortex is extremely diverse and complex, particularly in respect to its cytoarchitecture and functional organisation. These details are important when considering the precise localisation and clinical correlates of the neuropathological changes of this region in disease. Accordingly, I performed a detailed analysis of the cytoarchitecture of the human anterior cingulate cortex, as a prelude to investigations of this region in mood disorder. I measured several morphometric parameters within different anatomical levels and both hemispheres of the anterior cingulate cortex. Overall I found a clear distinction in the cellular composition of the supragenual and subgenual regions of the anterior cingulate cortex. The subgenual region demonstrated a lower glial density and smaller neurons in comparison to the supragenual region. A modest difference in neuronal density was also observed, with a higher density in the deep layers of the subgenual cortex compared to the deep layers of the supragenual cortex. Total cortical depth was also thinner in the subgenual region. This work may have important implications for the interpretation of imaging and pathological data in mood disorder.

To assess the cytoarchitecture of this brain region in mood disorder, I examined several morphometric indices in addition to various parameters of gene expression in post mortem brains. I found a range of cytoarchitectural abnormalities in the supragenual anterior cingulate cortex in mood disorder. The most prominent change included a reduction in glial density, which was evident in all layers of the cortex. Glial fibrillary acidic protein was also reduced, providing some evidence for astrocyte involvement. Various neuronal changes were also observed in the mood disorder group. These included layer-specific reductions in pyramidal neuron density and a modest change in the density of calretinin-immunoreactive neurons. I did not find any evidence supporting synaptic pathology in the anterior cingulate cortex in mood disorder.

These findings extend previous evidence of cytoarchitectural alterations in the anterior cingulate cortex in mood disorder and in particular emphasise the prominent involvement of glial cells in the neuropathology of this disease. The origins of the glial (and neuronal) deficits in mood disorder remain to be established, but they are likely to have pathophysiological consequences.

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Abbreviations

ACC	anterior cingulate cortex
ANCOVA	analysis of covariance
ANOVA	analysis of variance
BA	Brodmann area
BDNF	brain derived neurotrophic factor
CaBP	calcium-binding protein
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CB	calbindin
CBF	cerebral blood flow
CE	coefficient of error
CG	cingulate gyrus
CNS	central nervous system
cpm	counts per minute
CR	calretinin
CREB	cAMP regulatory element binding protein
CS	cingulate sulcus
dATP	deoxyadenine triphosphate
DTT	dithiothreitol
DZ	dizygotic
ECS	electroconvulsive shock
ECT	electroconvulsive therapy
fMRI	functional magnetic resonance imaging
GABA	γ -aminobutyric acid
GAD	glutamic acid decarboxylase

GAP-43	growth-associated protein-43
GFAP	glial fibrillary acidic protein
IAR	immunoautoradiography
ISHH	<i>in situ</i> hybridisation histochemistry
MAP	microtubule-associated protein
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MZ	monozygotic
NAA	N-acetyl aspartate
OPC	oligodendrocyte precursor cell
PBS	phosphate-buffered saline
PCC	posterior cingulate cortex
PCG	paracingulate gyrus
PCS	paracingulate sulcus
PET	positron emission tomography
PMI	post mortem interval
PV	parvalbumin
Rnase	ribonuclease
SSC	saline sodium citrate
Tdt	terminal deoxynucleotidyl transferase
TEA	triethanolamine HCL
WMH	white matter hyperintensity

Chapter One: General Introduction

Over recent years, neuropsychological, neuroanatomical and neuroimaging studies have provided evidence supporting the involvement of the anterior cingulate cortex (ACC) in mood disorder. The experiments described in this thesis were designed to investigate the cytoarchitecture of the ACC in mood disorder, by measuring several morphometric indices in addition to various parameters of gene expression in post mortem brains from mood disorder and control subjects. Before these studies were conducted, a thorough assessment of the morphological heterogeneity of the ACC was performed, with particular emphasis given to the division between supragenual and subgenual regions of the ACC. The present chapter provides a background to, and a rationale for, these studies.

1.1 Mood disorders

1.1.1 Symptoms and diagnosis of mood disorder

The mood disorders are so called because their predominant feature is a disturbance of mood. Mood disorders can occur secondary to specific medical or neurological illnesses, other psychiatric disorders, or pharmacological agents; they may also arise in absence of medical or psychiatric antecedents as primary, idiopathic disorders. It is primary, rather than secondary, mood disorders that are the subject of this thesis. Primary mood disorders are classified according to the nature and severity of symptoms during each episode, and by the course of the illness. Standard criterion based systems aim to provide reliable classification: today, the most widely used systems are the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM-IV) and the World Health Organisation's International Classification of Disease (ICD-10).

A basic distinction can be made between the depressive disorders (unipolar depression) and the bipolar disorders. The depressive disorders are distinguished from the bipolar

disorders by the fact that there is no history of ever having had a manic, mixed, or hypomanic episode. There are numerous subtypes within the depressive disorder and bipolar disorder categories - all patients included in this study were diagnosed with either major depressive disorder (termed major depression during this thesis) or bipolar I disorder (termed bipolar disorder) using DSM-IV criteria. The criteria for major depressive episode and manic episode (used in diagnosing major depression and bipolar disorder) are listed in tables 1.1 and 1.2. The DSM-IV classification system also provides 'specifiers' which can be used to describe the most recent mood episode (e.g. with catatonic features, with melancholic features) and the course of recurrent mood episodes (e.g. with rapid cycling). Details regarding specifiers were not available to me and accordingly will not be discussed.

Major depression is characterised by the occurrence of one or more episodes of low mood and/or anhedonia, together with a range of somatic and cognitive symptoms, such as sleep disturbance, loss of appetite, loss of weight, fatigue, psychomotor agitation, impaired concentration and negative thoughts of worthlessness, pessimism and guilt. There may or may not be full recovery between episodes (American Psychiatric Association 1994). In bipolar disorder, depressed and euthymic periods are interspersed with manic episodes, when an abnormally elevated mood is accompanied by associated behaviours and cognitions, such as increased activity, grandiosity, irritability and disinhibition. Delusions and hallucinations may also occur, and they are usually consistent with the mood (American Psychiatric Association 1994).

1.1.2 Epidemiology and aetiology of mood disorder

Recent surveys of mood disorder suggest that the lifetime risk of major depression is around 17% and the lifetime risk of bipolar disorder is approximately 1%. The prevalence in men and women is the same for bipolar disorder, but the rates of major depression are twice as great in women as men; this appears to be consistent across different cultures.

The mean age of onset is around 27 years for major depression and 21 years for bipolar disorder. Both syndromes are highly co-morbid with other disorders, particularly anxiety disorders and substance misuse. Bipolar disorder is a recurrent illness – more than 90% of individuals who have a single manic episode will go on to have future episodes. The course and prognosis of bipolar disorder is variable, the average length of an episode is 6 months and the interval between episodes becomes progressively shorter with age and the number of episodes. Nearly all patients with bipolar disorder recover from acute episodes, but the long-term prognosis is rather poor; for example, less than 20% of bipolar disorder patients achieve a period of 5 years stability with good social and occupational performance. A rapid-cycling pattern (four or more mood episodes in a given year) is associated with a poorer prognosis. The course and prognosis of major depression is also variable. Some people have isolated episodes that are separated by many years without any depressive symptoms, whereas others have clusters of episodes, which may become increasingly frequent with age. Major depressive episodes may end completely, or only partially, or not at all. The average length of a depressive episode is 6 months, though approximately 10-20% of patients may develop a chronic unremitting course. About 5%-10% of individuals with major depression (single episode), subsequently develop a manic episode. The long-term prognosis of major depression may be a little better than that of bipolar disorder, but it is still modest - only about 25% of patients with major depression achieve a period of 5 years of clinical stability. Individuals with mood disorder have a higher than normal mortality rate due, in part, to a higher rate of suicide. The epidemiology of mood disorder is summarised in Gelder *et al.* 2001.

There is much speculation concerning whether major depression and bipolar disorder occupy the same liability continuum, with bipolar disorder being the more severe or deviant form of mood disorder (see Karkowski and Kendler 1997). A recent study comparing the heritability of mood disorder does not support this theory and has proposed that the relationship between major depression and bipolar disorder is better described by

a correlated liability model - whilst significant genetic and nonfamilial environmental correlations were observed between major depression and bipolar disorder, the majority of genetic variance in liability to bipolar disorder was estimated to be specific to the manic syndrome, and did not appear to be shared with major depression (McGuffin *et al.* 2003).

The majority of studies demonstrate aggregation of mood disorder in families (Craddock and Jones 2001; Fava and Kendler 2000). The risk of mood disorder is increased in first-degree relatives of both major depression and bipolar disorder probands, with the risk being approximately twice as great in relatives of bipolar disorder patients (Gelder *et al.* 2001). Relatives of bipolar disorder probands have increased risks of major depression and schizoaffective disorder as well as bipolar disorder; in contrast, relatives of patients with major depression do not have increased rates of bipolar disorder or schizoaffective disorder (Craddock and Jones 2001; Gelder *et al.* 2001; McGuffin and Katz 1989; Sullivan *et al.* 2000).

Familiality does not, of itself, demonstrate that a disease is genetic as family members share environmental as well as genetic factors. Further support comes from twin studies, which compare the concordance rates of mood disorder between monozygotic (MZ) and dizygotic (DZ) twins. The concordance rate for bipolar disorder in MZ twins is around 70%; for DZ twins concordance is around 20%. The concordance rate for major depression is also thought to be greater in MZ twins (~46%) than DZ twins (~20%; see Gelder *et al.* 2001) - though a recent meta-analysis (using rigorous methodological criteria) found that the prevalence of major depression was actually higher in DZ than MZ twins (Sullivan *et al.* 2000). Twin studies can be used to estimate heritability (i.e. the proportion of phenotypic variance in a population attributable to genetic variance). The genetic heritability of major depression is estimated to be in the range of 31%-42% whilst the heritability of bipolar disorder is estimated at 70% (Sullivan *et al.* 2000).

Another strand of evidence for a genetic contribution to bipolar disorder comes from adoption studies, where the biological, but not adoptive, relatives of patients with bipolar disorder have increased rates of the disease (Craddock and Jones 2001). The results of adoption studies for major depression are equivocal, and have only provided qualitative evidence consistent with the existence of genetic factors in the aetiology of major depression (Fava and Kendler 2000; Sullivan *et al.* 2000). Overall, the family, twin and adoption data suggest a genetic component to mood disorder, with a greater genetic influence in bipolar disorder than major depression. Equally, they show that factors other than genetics must also be operating, the twin concordance data being the most important evidence for this.

Identifying environmental correlates for major depression is not straightforward; putative environmental 'risk factors' such as gender, stressful life events, adverse childhood experiences and certain personality traits (i.e. neuroticism) are themselves influenced by genetic factors (Fava and Kendler 2000; Kessler 1997). For example, genetic factors may be involved in the liability of an individual to experience life events since certain individuals seem more prone to select 'risky' environments, and genetic factors may also be involved in how life events are perceived (Kendler *et al.* 1999; Kendler and Karkowski Shuman 1997; Kendler *et al.* 1993). Nevertheless, a wide range of environmental adversities such as job loss, marital difficulties, major health problems and loss of close personal relationships are associated with a substantial risk for the onset of major depression. Difficulties in childhood including physical and sexual abuse, parental discord and divorce are believed to increase the risk of major depression later in life (Fava and Kendler 2000). It is less certain whether bipolar disorder is provoked by life events. It has been suggested that the impact of life events may be more important early in the course of bipolar disorder because once the illness is established environmental precipitants become less important (see Gelder *et al.* 2001). Interestingly, bipolar disorder shares some epidemiological risk factors with schizophrenia, including seasonality of birth,

dermatoglyphic anomalies and perinatal complications. Bipolar disorder may also occur more frequently in higher socio-economic groups (Torrey 1999).

1.1.3 Neuropsychology of mood disorder

Neuropsychological testing of patients with mood disorder during major depressive or manic episodes reveals widespread cognitive impairments, including deficits in attention, learning, memory and executive function. Cognitive deficits tend to be more marked in subjects with bipolar disorder than in subjects with major depression (Murphy and Sahakian 2001). There is disagreement as to whether these cognitive defects are best regarded as global and diffuse, or whether there may be some selectivity to the changes seen; some authors have suggested that deficits in effortful executive tasks may be particularly prominent in depression, which would be consistent with abnormalities seen in medial prefrontal and ACC functional imaging studies (Elliott 1998). It is generally accepted that cognitive deficits are related to the acute state of the illness; however, some deficits (particularly those involving executive and mnemonic function) persist after symptom remission (Austin *et al.* 2001; Elliott 1998; Ferrier *et al.* 1999; Murphy and Sahakian 2001). The presence of persisting neuropsychological deficits is important evidence that enduring brain abnormalities are implicated in the aetiology of mood disorder (see section 7.2.2).

1.2 The anterior cingulate cortex and its involvement in mood disorder

The experiments described in thesis were designed to investigate the cytoarchitecture of the ACC and its involvement in mood disorder. Research in this field was prompted by several lines of evidence indicating a role for the ACC in the expression and modulation of emotion. Classically, the ACC has been related to affect on the basis of electrical stimulation, neuroimaging and lesion studies in animal and humans. In the following

sections I describe the functional organisation of the ACC and its contribution to human behaviour, as a prelude to a detailed discussion of this region in the neuropathology of mood disorder. A comprehensive introduction to the surface features and cytoarchitecture of the ACC is provided in chapter two (section 2.1.1).

1.2.1 The ACC and its contribution to behaviour

The cingulate cortex lies ventral, rostral and dorsal to the corpus callosum. The anatomy of the cingulate cortex is extremely diverse and complex, both in respect to its gross morphology (which can be extremely variable between individuals), and underlying cytoarchitecture (Nimchinsky *et al.* 1997; Vogt *et al.* 1995). In keeping with its anatomical complexity, the cingulate cortex is heterogeneous in terms of its functional organisation and connectivity (Devinsky *et al.* 1995; Paus 2001; Vogt *et al.* 1992). The ACC can be differentiated from the posterior cingulate cortex (PCC) on the basis of cytoarchitecture and patterns of connectivity as well as function. The ACC subserves primarily 'executive' functions related to the emotional control of visceral, skeletal and endocrine outflow, and the PCC subserves 'evaluative' functions such as monitoring sensory events and the organisms own behaviour in the service of spatial orientation and memory (Vogt *et al.* 1992). Functional studies have shown that the ACC does not make a uniform contribution to brain functions and has consequently been further divided into a rostroventral 'affective' division (areas 24a-c, 32 and 25) and a dorsal 'cognitive' division (areas 24b'-c' and 32') (Devinsky *et al.* 1995). The affective division has extensive connections with the amygdala, periaqueductal grey, nucleus accumbens, thalamus, hypothalamus, anterior insula, hippocampus, orbitofrontal cortex and the autonomic brainstem nuclei. In addition to regulating autonomic and endocrine functions, this region is involved in assessing the salience of emotional and motivational information and the regulation of emotional responses. In contrast, the cognitive division maintains strong reciprocal connections with the prefrontal cortex, parietal cortex and premotor and supplementary motor areas. Various functions have been ascribed to this region, including modulation of attention or

executive functions by influencing sensory or response selection; complex motor control, motivation, novelty, response selection and anticipation of cognitively demanding tasks (see Devinsky *et al.* 1995; Vogt *et al.* 1992).

Imaging studies of cognitive and emotional tasks strongly support this functional delineation (Bush *et al.* 2000). Activation in the affective (rostroventral) division is frequently observed during affect-related tasks, including studies of emotional processing in normal healthy volunteers and symptom provocation studies in a number of psychiatric disorders (Dougherty *et al.* 1999; Pardo *et al.* 1993; Rauch *et al.* 1996). It is also activated by induced sadness in normal subjects (George *et al.* 1995; Mayberg *et al.* 1999) and in individuals with major depression (Drevets *et al.* 1997). In further support of the cognitive-versus affective-division distinction, reciprocal suppression of the cognitive (dorsal) component is commonly observed during intense emotional states and has been reported in individuals with major depression and normal subjects experiencing film-induced emotion (Bench *et al.* 1992; Mayberg *et al.* 1999).

Electrical stimulation studies also implicate the ACC in the expression of emotion.

Emotional responses following stimulation include fear, agitation, pleasure, euphoria, and a sense of well-being (Devinsky *et al.* 1995). The functional distinction within the ACC is again revealed by electrical stimulation in conscious patients. For example, Bancaud and Talairach (1992) demonstrated that stimulation of the affective division produced intense fear or pleasure, whereas the cognitive division produced a sense of anticipation of movement.

Structural or functional changes in the ACC can also cause prominent changes in emotion and social behaviour. Indeed, early evidence of behavioural and physiological changes produced by callosal tumours encroaching upon the ACC was used by Papez to argue for the role of the cingulate cortex in emotional experience (Papez 1937). Lesions of the ACC

have produced a host of symptoms, which include apathy, blunted affect, anxiety, impulsivity, aggressive behaviour with minimal or no provocation, psychosis, sexually deviant behaviour, disabling obsessions and compulsions, and impaired social judgement. Many patients can experience difficulty in accurately basing behaviour or affect on experience (see Bancaud and Talairach 1992; Damasio and Van Hoesen 1983; Devinsky *et al.* 1995). By extrapolation, the difficulties that these patients suffer are not entirely dissimilar with the problems of patients with mood disorder. For example, manic patients may engage in expansive behaviours not justified by conditions or experience, and patients with major depression may persist in a negative mood or reaction to events in the face of conditions or experience that would be perceived as positive or neutral by non-depressed individuals. Interestingly, cingulectomy and cingulotomy have been used to treat patients with psychosis and pathological aggression, obsessive-compulsive behaviours and depression (see Ballantine *et al.* 1987; Devinsky *et al.* 1995).

In summary, converging lines of evidence implicate the ACC in the expression and modulation of emotion, information processing and attention. As these functions are seemingly altered in mood disorder, the ACC is a suitable candidate region in which to search for the neuroanatomical substrates of this disorder.

1.2.2 The neuropathology of the ACC in mood disorder

By definition, primary mood disorders do not have any demonstrable, established organic features. From a neuropathological perspective, until recently there has been no reason to question this basic assumption: there were few studies and no convincing data (Jeste *et al.* 1988). Over recent years a significant magnetic resonance imaging (MRI) literature attests to structural abnormalities in various brain regions in mood disorder and has encouraged neuropathological investigations. Although the neuropathological understanding of mood disorder is still rudimentary, and far from contributing in any

diagnostic sense, structural correlates are beginning to emerge. This section describes the neuropathological alterations that have been found in the ACC (see table 1.3).

Neuropathological studies were stimulated by an MRI report that a specific part of the ACC lying ventral to the corpus callosum, the subgenual region (24sg), was 40% smaller in a group of subjects with familial mood disorder. The volume reduction was left-sided and seen in both major depression and bipolar disorder (Drevets *et al.* 1997). Two further MRI studies have extended this finding. Hirayasu *et al.* (1999) reported a volume reduction of 25% in the left subgenual ACC in patients with first-episode psychosis and a family history of mood disorder in first- or second-degree relatives. Lesser and non-significant trends were found in the right hemisphere and in patients without a family history. Botteron *et al.* (2002) also reported left-sided subgenual volume reductions (~20%) in a study of early onset depression.

Drevets and colleagues proceeded to investigate the cellular correlates of the subgenual volume reduction. The resulting paper is noteworthy, being the first large and well-conducted neuropathological study of mood disorder and also because of the striking results. In a preliminary study, Öngür *et al.* (1998) identified a trend towards a decrease in the volume in subgenual area 24, and for a decreased density and number of glia therein. The authors then attempted to verify their preliminary findings using tissue from the Stanley Foundation Neuropathology Consortium. In this series, their original findings were confirmed: a decrease in glial density and glial number was observed in patients with major depression and bipolar disorder. Higher levels of significance were observed in cases with a positive family history. There was no concomitant neuronal loss, and no alterations in glia or neuronal size. Interestingly, the glial deficit was also said to occur in the orbitofrontal cortex, but not in the somatosensory cortex, suggesting that the pattern of pathology is not uniform. This landmark paper was the primary stimulus for the work detailed in this thesis, and largely influenced its design. Although the publication by Öngür

and colleagues (1998) underscored the involvement of the subgenual region of area 24 in mood disorder, it was uncertain whether other regions of the ACC were also implicated. Additional studies (published during the course of my work) have confirmed that cytoarchitectural abnormalities are indeed present in both the subgenual and supragenual regions of the ACC in mood disorder. These studies are detailed below.

Bouras *et al* (2001) conducted a further morphometric study of subgenual area 24 in mood disorder using a large series of brains collected over a 50-year period. Neuronal density was reduced in layers III, V and VI in patients in bipolar disorder compared to controls, but not in patients with major depression. In addition to subgenual area 24, the authors also examined the supragenual region of area 24; however, no morphometric alterations were observed for either diagnostic group. No glial data were presented. Cotter and colleagues (2001a) have also investigated the supragenual region of area 24, and like Öngür *et al* (1998), they used Stanley Foundation tissue. The main positive findings were in major depression, with decreased glial density in layer VI and reduced neuronal size in layers Vb and VI. Neuronal density was unaltered. There were similar but non-significant trends in bipolar disorder. The remaining morphometric studies of the ACC in mood disorder are by Benes' group, who have examined the pregenual region of area 24. In five subjects with bipolar disorder, no differences in neuronal size were seen (Benes *et al.* 2000) but in an expanded group (including 10 bipolar subjects), a decreased density of non-pyramidal neurons in layer II, and a trend towards a lower pyramidal density was found (Benes *et al.* 2001). In contrast, there were no differences in glial density.

Only one experiment to date has attempted to investigate specific neuronal populations within the ACC in mood disorder. Using tissue from the Stanley Foundation, Cotter *et al* (2002a) studied calcium-binding protein expression (permitting the distinction of separate non-pyramidal neuron populations) in the supragenual region of area 24. Reductions in cell density were observed for parvalbumin, calretinin, and calbindin in both major

depression and bipolar disorder, however, only one reduction specific to calbindin-immunoreactive neurons in layer II in bipolar disorder attained statistical significance. This result disappeared after adjusting for multiple comparisons. This study provides partial support for the work by Benes *et al* (2001) (see above).

Given the cytoarchitectural alterations reported in the ACC, alterations in synaptic circuitry might be predicted. In particular, reductions in neuronal size might be suggestive of synaptic pathology, since neuronal size is related to the extent of axonal and dendritic arborisation, and thereby the synaptic organisation which the neuron must support (Esiri and Pearson 2000). There are also close and multifaceted relationships between synapses and glia (Coyle and Schwarcz 2000). Only one study to date has addressed the issue of whether ACC synaptic pathology is a feature of mood disorder. Eastwood and Harrison (2001) studied four synaptic proteins in the supragenual region of area 24 in Stanley Consortium brains. In bipolar disorder, decrements were observed in synaptophysin, complexin II and growth-associated protein-43 (Gap-43) in bipolar disorder, but not complex I. Complexin II was also reduced in major depression. The only data pertaining to dendrites in area 24 are those reported by Bouras *et al* (2001), who in a small set of cases (three in each diagnostic group), found reduced amounts of the microtubule-associated proteins MAP1b and MAP2 in bipolar disorder, but not major depression. In total, these initial findings are consistent with the presence of a synaptic pathology in the ACC to accompany the glial and neuronal alterations in mood disorder.

Overall, the growing body of post mortem data presents a convincing case for cytoarchitectural alterations in the ACC in mood disorder. The discovery of reduced glial density in the ACC in mood disorder is largely unexpected. Glia are usually of interest to neuropathologists in the context of gliosis because it is basic evidence of some form of degenerative or inflammatory process (Berry and Butt 1997; Peters *et al*. 1991). Coinciding with these morphometric observations in mood disorder is a growing

appreciation of the critical roles glia play in manipulating synaptic glutamate levels, central nervous system (CNS) energy homeostasis, liberation of trophic factors and indeed the very existence of synaptic networks of neurons and glia (Coyle and Schwarcz 2000) – all of which suggest that the prominent glial deficit in major depression and bipolar disorder may be integral to the pathophysiology of these disorders, and is worthy of further study. Despite the prevailing evidence indicating glial pathology in mood disorder, it is still uncertain which glial population(s) is involved (see chapter five).

In summary, in all regions of the ACC examined, independent groups have reported decreases in the density of glia, and the density and size of some neurons, accompanied by reductions in indices of synaptic terminal and dendrites, in bipolar disorder and major depression. In this respect there is a consistency and robustness to the observations; nevertheless, notable discrepancies remain. Öngür *et al* (1998) revealed glial pathology without neuronal pathology, Benes *et al* (2001) found the opposite and Cotter *et al* (2001a) found both. Discrepancies also exist between diagnostic boundaries. Öngür *et al* reported cytoarchitectural alterations in major depression and bipolar disorder; Cotter *et al* (2001a), studying the same brains, found them only in the latter, whereas Bouras *et al* (2001) found changes only in bipolar disorder. It is unknown whether this inconsistency is due to anatomical, methodological and/or demographic differences. Clearly, further replication is necessary in order to resolve this variability.

1.3 Other neuropathological findings in mood disorder

Although the focus of this introduction involves the ACC, it is important to consider the other neuropathological findings in mood disorder. The following section will briefly introduce the main results from neuroimaging and neuropathological investigations of the frontal cortex and hippocampus. Like the ACC, these abnormalities involve reductions in regional CNS volume, loss/atrophy of neurons and glia and alterations in molecular

indices of synaptic terminals and dendrites. I will return to these findings, and how they relate to those in the ACC, later on in this thesis. In addition, the white matter lesions which MRI studies indicate are common correlates of mood disorders in the elderly are also considered in the following section. By extrapolation from other diseases, the implied pathology seems likely to be hypoxic/ischaemic in origin, and thus rather different in nature to the cytoarchitectural alterations.

1.3.1 Frontal lobes and hippocampus

The preponderance of the data from recent volumetric neuroimaging studies is suggestive of reduced grey matter volume in the orbital and prefrontal cortex, the ventral striatum, and the mesiotemporal cortex in patients with mood disorder (reviewed in Drevets 2000b; Pearlson 1999; Soares and Mann 1997; Videbech 1997). Several imaging studies have reported hippocampal volume to be reduced in major depression (Bremner *et al.* 2000; Shah *et al.* 1998; Sheline *et al.* 1996), possibly related to duration of illness (Sheline *et al.* 1999). Hippocampal volume may also be reduced, but amygdalar volume increased in bipolar disorder (Altshuler *et al.* 2000; Strakowski *et al.* 1999). Functional brain imaging studies lend support to the structural neuroimaging data; abnormalities in metabolic rate and cerebral blood flow (CBF) have been demonstrated consistently in these same areas in mood disorder (reviewed in Drevets 2000b; Kennedy *et al.* 1997; Videbech 2000).

In addition to the accumulating neuroimaging data, several post mortem studies are now providing direct evidence for reductions in regional CNS volume, cell number and cell size in mood disorder (reviewed in Harrison 2002). Volume reductions have been described in various limbic-system affiliated basal ganglia structures in mood disorder (Baumann *et al.* 1999b). Despite the compelling neuroimaging data concerning the hippocampus, there have been very few neuropathological studies of this region, and no attempt has been made to corroborate the volume reduction reported in the imaging literature.

Glial deficits have been frequently reported in prefrontal regions (Cotter *et al.* 2002b; Rajkowska *et al.* 2001; Rajkowska *et al.* 1999) in major depression and bipolar disorder, and a recent study of the amygdala has also demonstrated glial deficits in major depression (Bowley *et al.* 2002). Neuronal alterations in mood disorder appear to be more subtle than the corresponding glial deficits, and alterations are often only detected when specific morphological size-types of neurons are analysed in individual cortical layers. Nevertheless, several (but not all) studies of the prefrontal cortex have described reductions in neuronal density in mood disorder (Rajkowska *et al.* 2001; Rajkowska *et al.* 1999). Reductions in neuronal size have also been described in the prefrontal cortex (Cotter *et al.* 2002b; Rajkowska *et al.* 1999). In addition to morphometric measurements of cell density and size, synaptic terminals and dendrites have been the focus of several neuropathological investigations of mood disorder. The majority of studies have reported reductions in various synaptic and dendritic indices, particularly in the hippocampus (reviewed in Harrison 2002).

1.3.2 Focal white matter pathology

In addition to the regional brain abnormalities mentioned, many MRI studies have shown an association between mood disorder and focal signal hyperintensities, occurring particularly in the deep white matter and subcortical nuclei. These white matter hyperintensities (WMHs) are seen in excess both in bipolar disorder and major depression, especially in elderly subjects (O'Brien *et al.* 1996; Videbech 1997). In late-life depression, WMHs are linked to risk factors for, and the presence of, vascular disease (O'Brien *et al.* 1996). This finding is consistent with a robust epidemiological association between late-life depression and vascular disease (Alexopoulos *et al.* 1997). WMHs are generally assumed to reflect focal white matter damage, especially ischaemia and infarction, as they do in other diseases (Fazekas *et al.* 1993; O'Brien *et al.* 1996). Consistent with this theory, a recent neuropathological study has reported that WMHs in

elderly subjects with major depression are frequently due to cerebral ischaemia (Thomas *et al.* 2002).

1.4 Aims of the current studies and outline of the remainder of the thesis

There were two main objectives to the studies described in this thesis. The first objective was to quantitatively assess the heterogeneity of the ACC by measuring various morphometric parameters (viz. neuronal density, glial density and neuronal size) in different anatomical regions of Brodmann area 24 using stereological principles. Effects of lateralisation and rostrocaudal differentiation were analysed, and particular emphasis was given to the division between the supragenual and subgenual regions of area 24. A subsidiary aim of this work was to assess the volume of the cingulate gyrus with the Cavalieri estimator. These studies are detailed in **chapter two**. The second objective was to further characterise the extent of cytoarchitectural abnormalities in the ACC in mood disorder. Accordingly, I measured several morphometric indices, in addition to various parameters of gene expression, in ACC brain tissue taken at autopsy from patients with mood disorder (major depression and bipolar disorder) and control subjects.

Chapter three describes a quantitative morphometric investigation of the ACC in mood disorder. A comparison between different methods of neuronal identification (Nissl-staining versus a neuron-specific antibody) is also presented here. **Chapter four** examines the non-pyramidal neuron population by assessing calcium-binding protein expression in the ACC in mood disorder. **Chapter five** pursues the subject of glial pathology in the ACC in mood disorder and considers the specific involvement of each glial cell type. Investigations regarding the synaptic contribution to the cytoarchitectural alterations in the ACC are described in **chapter six**. Finally, a discussion of the main findings obtained and the conceptual issues affecting their interpretation is given in **chapter seven**. Proposals for further research in this field are also provided in the final chapter.

Table 1.1.

DSM-IV criteria for major depressive episode

A. Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest of pleasure.

Note: do not include symptoms that are clearly due to a general medical condition, or mood-incongruent delusions or hallucinations.

- (1) depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful). **Note:** In children and adolescents, can be irritable mood
- (2) markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others)
- (3) significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or a decrease or increase in appetite nearly every day. **Note:** In children, consider failure to make expected weight gains
- (4) insomnia or hypersomnia nearly every day
- (5) psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down)
- (6) fatigue or loss of energy nearly every day
- (7) feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick)
- (8) diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others)
- (9) recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide

B. The symptoms do not meet criteria for a mixed episode.

C. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

D. The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition (e.g., hypothyroidism).

E. The symptoms are not better accounted for by bereavement, i.e., after the loss of a loved one, the symptoms persist for longer than 2 months or are characterised by marked functional impairment, morbid preoccupation with worthlessness, suicidal ideation, psychotic symptoms, or psychomotor retardation.

Table 1.2.

DSM-IV criteria for manic episode

- A. A distinct period of abnormally and persistently elevated, expansive, or irritable mood, lasting at least 1 week (or any duration of hospitalisation is necessary).
- B. During the period of mood disturbance, three (or more) of the following symptoms have persisted (four if the mood is only irritable) and have been present to a significant degree:
 - (1) inflated self-esteem or grandiosity
 - (2) decreased need for sleep (e.g., feels restless after only 3 hours of sleep)
 - (3) more talkative than usual or pressure to keep talking
 - (4) flight of ideas or subjective experience that thoughts are racing
 - (5) distractibility (i.e., attention too easily drawn to unimportant or irrelevant external stimuli)
 - (6) increase in goal-directed activity (either socially, at work or school, or sexually) or psychomotor agitation
 - (7) excessive involvement in pleasurable activities that have a high potential for painful consequences (e.g., engaging in unrestrained buying sprees, sexual indiscretions, or foolish business investments)
- C. The symptoms do not meet criteria for a mixed episode.
- D. The mood disturbance is sufficiently severe to cause marked impairment in occupational functioning or in usual social activities or relationships with others, or to necessitate hospitalisation to prevent harm to self or others, or there are psychotic features.
- E. The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication, or other treatment) or a general medical condition (e.g., hyperthyroidism).

Note: Manic-like episodes that are clearly caused by somatic antidepressant treatment (e.g., medication, electroconvulsive therapy, light therapy) should not count toward a diagnosis of bipolar I disorder.

Table 1.3. Morphometric and synaptic studies of the ACC in mood disorder

Study and Brodmann area	Case/controls	Methods and parameters	Main findings in mood disorder ¹
Öngür <i>et al</i> (1998)	a. 4 BD, 4 MD / 5 CON	a. Nissl stain; volume, neuronal and glia	a. Trend for decreased volume. Decreased glial number and
Area 24 (subgenual), Area 3b	b. 14 BD, 9 MD / 14 CON*	density and number; optical disector	density in MD and familial BD.
		b. Nissl stain; volume, neuronal and glia	b. Trend for decreased glial number and density in MD and familial
		density and number; optical disector	BD. Glial size unchanged. Neuron density and number unchanged
		and rotator	No changes in BA3b.
Benes <i>et al</i> (2000)	5 BD / 12 CON*	Nissl stain; neuronal size	No differences in pyramidal or non-pyramidal neuron size.
Area 24 (pregenual)			
Cotter <i>et al</i> (2001a)	15 BD, 15 MD / 15 CON*	Nissl stain; neuronal and glial density,	In MD: decreased glial density and decreased neuronal size in VI,
Area 24 (supragenual)		neuronal size; optical disector and	with same trend in V. Neuronal density unchanged. No changes in
		nucleator	BD.
Benes <i>et al</i> (2001)	10 BD / 12 CON*	Nissl stain; neuronal density and size,	Reduced density of pyramidal neurons in II. Trend for lower density
Area 24 (pregenual)		glial density	of pyramidal neurons in deeper layers. Pyramidal neurons larger in
			II, non-pyramidal neurons larger in II and III. No change in glia.
Bouras <i>et al</i> (2001)	21 BD, 20 MD / 55 CON	Nissl stain; layer and cortical depth;	In BD: (subgenual) decreased depth of II, V and VI, and reduced
Area 24 (supragenual and subgenual),		neuronal density and size	neuronal density in these layers. No changes in neuronal size. No
Area 18			differences in supragenual 24 or BA18.
			No changes in either area in MD.
Cotter <i>et al</i> (2002a)	15 BD, 15 MD / 15 CON*	PV-, CR-, CB-immunoreactive neuronal	In BD: decreased density of CB neurons in II, and increased
Area 24 (supragenual)		density and size	clustering of PV neurons. No differences in MD.
Eastwood <i>et al</i> (2001)	15 BD, 15 MD / 15 CON*	Immunoblotting; synaptophysin,	Synaptophysin, GAP-43 and complexin II decreased in BD.
Area 24 (supragenual)		Complexin I and II, GAP-43	Complexin II decreased in MD.

BD = bipolar disorder; CB = calbindin; CON = control; CR = calretinin; GAP-43 = growth-associated protein-43; MD = major depression; PV = parvalbumin. *Schizophrenia comparison group

studied as well (data not shown). ¹ Roman numerals refer to cortical layers.

Chapter Two: Quantitative studies of the cytoarchitecture of the human anterior cingulate cortex

2.1 Introduction

The cingulate cortex is located bilaterally on the medial surface of the frontal lobes where it arches around the rostral, dorsal and splenial aspects of the corpus callosum. The cingulate cortex has a particularly rich cytoarchitectonic structure. The surface of the anterior cingulate cortex (ACC) is composed of areas 24 and 25. Area 33 is in the depths of the rostral callosal sulcus. Surrounding the dorsal and rostral borders of areas 24 and 25 is the cingulofrontal transitional area 32, the majority of which is deeply buried in the cingulate sulcus. The surface of the posterior cingulate cortex (PCC) consists predominantly of area 23. Surrounding the dorsal and caudal borders of area 23 is the cinguloparietal transitional area 31 (Garey 1994). The PCC and its underlying cytoarchitecture are extraneous to the remaining content of this chapter and will not be discussed in any further detail.

2.1.1 Surface features of the human anterior cingulate cortex

The extent and pattern of cortical folding within the ACC is highly variable between individuals. The most consistent sulcus on the medial surface of the brain is the cingulate sulcus (CS). The paracingulate sulcus (PCS), when present, runs parallel to the CS in the ACC. The addition of this sulcus creates a 'doubling' of the anterior cingulate region, generating two components. The ventral component is referred to as the cingulate gyrus (CG) and the dorsal component as the paracingulate gyrus (PCG). The PCS can either be continuous along its entire extent, or can show small interruptions along its length and therefore appear segmented (Vogt *et al.* 1995). In addition to sulci on the medial surface, there can be a number of superficial horizontal dimples that create small folds in the cingulate gyrus.

There have been very few detailed studies that have examined the heterogeneity of the ACC in normal subjects, and unfortunately most have been limited in their methodology. Early post mortem studies include those of Eberstaller (1884) and Weinberg (1905), where patterns of ACC morphology were based purely on qualitative judgements of morphological patterns and their frequencies (from Yucel *et al.* 2001). Other post mortem studies have suffered from small sample sizes or have examined only one hemisphere from each subject; with the consequence that cingulate asymmetry has received little attention (Ide *et al.* 1999; Öngür *et al.* 2003; Ono *et al.* 1990; Vogt *et al.* 1995).

More recently, magnetic resonance imaging (MRI) has provided a platform for research into the morphology of the human cingulate in living subjects, where greater sample sizes have permitted statistical investigation into the effects of both hemisphere and gender (Paus *et al.* 1996a; Paus *et al.* 1996b; Yucel *et al.* 2001). The incidence of the paracingulate gyrus (in either its contiguous or segmented form) varies between studies, from 35% (Vogt *et al.* 1995) to nearly 100% (Paus *et al.* 1996b) of cases examined. Most of the aforementioned studies described a significant lateralisation of the PCS, with a greater incidence in the left hemisphere. Correspondingly, the single CS (absent PCS) was more frequently observed in the right hemisphere (Ide *et al.* 1999; Paus *et al.* 1996a; Paus *et al.* 1996b; Yucel *et al.* 2001).

A study into the volume of the intrasulcal grey matter of the cingulate region reported that the PCS was more commonly detected bilaterally, however, when present in only one hemisphere, the left hemisphere exhibited a greater grey matter volume. Interestingly, the same study found females to have a greater CS grey matter volume, but a smaller PCS volume than males (Paus *et al.* 1996a). Previous MRI research from the same group categorised the PCS as either 'prominent' (continuous), 'present' (segmented) or 'absent'. Overall, the right hemisphere demonstrated greater segmentation, with a well-developed PCS being more frequent in the left hemisphere. Considering gender, females more

commonly showed either the prominent or absent form of PCS, with males exhibiting a greater percentage of the segmented type of PCS pattern (Paus *et al.* 1996b). Yucel *et al.* (2001), using the same categorisation, found the PCS to be more commonly 'prominent' in the left hemisphere and more often 'absent' in the right hemisphere. In disagreement with previous work, greater fissurisation was seen in the left hemisphere in males whilst females showed a more symmetric pattern (Yucel *et al.* 2001).

2.1.2 Cytoarchitecture of the anterior cingulate cortex

The ACC is agranular and a distinguishing feature of most areas in this region is a prominent layer V. Area 24 is the major component of the ACC and lies superior (supragenual), anterior (pregenual) and inferior (subgenual) to the corpus callosum. Brodmann's 1909 original account of the cingulate cortex (Garey 1994) does not contain the same levels of detail as more recent assessments; in particular, a recent report presented the cingulate cortex in a flat map format and showed many more cytoarchitectural areas than were originally described by Brodmann (Vogt *et al.* 1995). Area 24 has been divided into three divisions (24a, 24b, 24c) that are progressively more differentiated in the dorsosventral axis (Vogt *et al.* 1995; von Economo 1929). Only areas 24a and 24b are present rostral and ventral to the corpus callosum (Öngür *et al.* 2003; Vogt *et al.* 1995). The cytoarchitecture of area 24 is not uniform along its rostrocaudal extent and accordingly has been divided into rostral (24) and caudal (24') levels (Vogt *et al.* 1995). Studies concerning the chemoarchitecture of the ACC have also reported marked variation along both the rostrocaudal and dorsosventral axes (Nimchinsky *et al.* 1997; Öngür *et al.* 2003). Vogt *et al.* (1995) have comprehensively described the cytoarchitecture and distribution of areas within the ACC and what follows here is a general summary of their work.

Area 24a borders the callosal sulcus and is relatively homogeneous. Layers II and III are definable, but the differentiation between these layers is not as clear as in more dorsal

areas. Layer Va is thin, but prominent and layer Vb is relatively sparse, with clusters of medium to large neurons. More caudal levels of area 24a (i.e., 24a') have a more clearly defined layer II, and a broader layer III. Layer Va is even thinner than in more rostral levels and layer VI is very poorly defined.

Area 24b is generally the most differentiated of all 3 subdivisions. Layer II is well defined as is layer III. Area 24b has the thickest layer Va with large pyramidal neurons. Layer Vb is also at its broadest in this division, although it is still relatively cell sparse with clumps of pyramidal neurons. Caudal levels of area 24b (24b') have thinner layers III and Va compared to more rostral levels.

In area 24c, superficial layers tend to be relatively thick in comparison to the other divisions, although layers II and III are slightly less differentiated than in area 24b. Layer Va is again prominent in 24c, but compared to area 24b, it is thin and has small pyramidal neurons. The aggregations of pyramidal neurons are also more prominent in area 24c as layer Vb is generally more cell sparse. Layer III at more caudal levels of area 24c (24c') is meagre compared to more rostral levels. Layer Vb contains more medium sized neurons and is therefore not as sparse as its more rostral counterpart. Figure 2.4 (following the methods section) shows photomicrographs of the cytoarchitecture of area 24.

It is not clear whether Vogt *et al* (1995) specifically considered tissue from the subgenual region of area 24 (area 24sg), as they appear to focus on the dorsal and rostral aspects of this area. Recent observations suggest that subgenual area 24a (24asg) lies next to the corpus callosum and has a rudimentary, poorly laminated appearance. Subgenual area 24b (24bsg) is much more differentiated and shows a prominent layer V that can be subdivided into a thin dense top layer and a much less densely packed deeper layer. Subgenual area 24a is also much smaller than subgenual area 24b (Öngür *et al.* 2003).

Area 24 is delimited dorsally by the transition to dysgranular area 32. Area 32 has a clear layer Va, which does not reach the thickness and density attained in area 24. Large pyramidal neurons are often present in the deep part of layer III. Area 32 begins in the depths of the CS and continues around onto the surface of the paracingulate gyrus when it is present (Vogt *et al.* 1995); consequently, it is unclear whether the paracingulate gyrus should be included within the strict definition of the cingulate as it is cytoarchitecturally a cingulofrontal transition area (Devinsky *et al.* 1995).

Area 25 lies caudal to subgenual area 24. This area is poorly differentiated, but in similarity with other anterior cingulate areas, layer V is clear and prominent. Layers II and III are difficult to distinguish and layer VI is barely visible.

A special cellular feature of area 24, and more specifically layer Vb, is the presence of an unusual cell type. Termed 'spindle' cells, (due to their spindle like shape), they are thought to represent a subpopulation of pyramidal neurons that are unique to the ACC in humans and great apes (Hof *et al.* 2001; Nimchinsky *et al.* 1999; Nimchinsky *et al.* 1995). The restricted distribution of these cells may indicate that they are associated with functionally distinct areas (Nimchinsky *et al.* 1995). Spindle cells are most prominent in area 24a but are also regularly observed in area 24b, and less frequently in area 24c (Vogt *et al.* 1995).

In summary, post mortem and imaging studies have revealed considerable variability in the location and morphological pattern of the ACC. Investigation into the lateralisation of the PCS revealed a striking asymmetry in the prominence of the PCS in the left hemisphere. Although the work by Vogt *et al.* (1995), Öngür *et al.* (2003) and Nimchinsky *et al.* (1997) has provided invaluable qualitative descriptions regarding the cytoarchitecture and chemoarchitecture of the ACC, no attempt has been made to quantify the observed diversity in cellular composition, either between different layers, levels, hemispheres, or

Brodmann areas. In particular, until recently, little emphasis has been placed on the subgenual region of area 24.

2.1.3 Aims of the current study

The main objective of this study was to characterise several morphometric parameters, viz. neuronal and glial density, neuronal size and lamina depth, in five (bilateral) anatomical levels of area 24b in the ACC in human brain. Two of the five levels were from subgenual areas (area 24sg) and the three remaining levels were from supragenual areas (areas 24 and 24').

This study was designed with three central issues in mind. The first aim was to quantify the cellular composition of area 24 along its rostrocaudal extent. Even though there is considerable heterogeneity within the ACC at both macroscopic and microscopic levels, at present, no study has attempted to quantify the variability in morphometric indices between supragenual areas 24 and 24'. Additionally, research in cognitive neuroscience and neuroimaging has suggested that areas 24 and 24' may be differentially involved in the processes of affect and cognition (see section 1.2.1 and Bush, *et al.* 2000; Devinsky, *et al.* 1995), thus further investigation into the morphometric distinction between these areas is warranted.

The second aim was to explore interhemisphere differences at each level of area 24. As previously discussed, morphological asymmetries have been reported in the ACC, however, asymmetries at the cellular level have not yet been considered in a study of normal human brain tissue. The chief and final aim of this study was to examine whether the morphometry of the supragenual region of area 24 (including areas 24 and 24') differs to that of the subgenual region of area 24. The morphometry of these two related regions has not yet been simultaneously assessed using sections from normal brain tissue.

The study outlined above is particularly pertinent in relation to the remaining content of this thesis, which examines the pathology of the ACC (supragenual area 24) in mood disorder. Both supragenual and subgenual regions have been implicated in imaging and post mortem studies of mood disorder (reviewed in Drevets 2000b; Harrison 2002). The research detailed here will help to indicate how much of the variability between different studies could be introduced at the sampling level, both in terms of anatomical position and hemisphere. This work will equally provide a greater awareness of the morphometric distinction between the supragenual and subgenual levels of area 24. This will be valuable in helping to understand why these two related areas appear to be differentially affected in mood disorder and induced sadness (see section 1.2.1).

In addition to all of the above, a subsidiary aim of this study was to assess the volume of the cingulate gyrus using the post mortem tissue already collected for the aforementioned morphometric analyses.

2.2 Methods

2.2.1 Stereological methods and principles: the Cavalieri estimator, the optical disector and the nucleator

2.2.1.1 *The Cavalieri estimator*

The Cavalieri estimator is the most commonly used stereological method for estimating reference volume (Howard and Reed 1998). The principle of the Cavalieri method is very simple and states that the volume of any object can be estimated from parallel sections separated by a known distance t , by summing up the areas of all cross sections of the object $\sum a$ and multiplying this figure by t (Gundersen *et al.* 1988b):

$$= t * \sum a \quad (1)$$

Equation (1) is an unbiased estimator of volume, provided the position of the first section is random in the object (Gundersen and Jensen 1987). In practice, the cross sectional area of the object being studied is estimated by using a randomly translated point grid that has a known area associated with each point $a(p)$. The area associated with each point on the grid and the number of points lying over all sectional profiles $\sum P_i$ provides an efficient, unbiased estimator of area profile $\sum ai$.

$$\sum ai = \sum Pi * a(p) \quad (2)$$

If the object of interest has been sliced systematically into parallel sections of equal thickness, $\bar{t}$ the volume is then estimated by:

$$V = \bar{t} * \sum^n Pi * a(p) \quad (3)$$

The Cavalieri method is an unbiased estimator of volume and completely independent of the orientation of the set of sections and the shape of the object. It is also a very useful estimator of irregular shaped objects, such as the brain, which would otherwise be difficult and require time-consuming methods (Gundersen *et al.* 1988b). Another noticeable feature of the Cavalieri estimate is its efficiency. Even for an irregular structure such as the brain, a total of around 200 points on 10 to 15 sections will provide an unbiased estimate of volume with an acceptable level of precision. For most practical applications, a coefficient of error (CE) of less than 10% is considered acceptable (Howard and Reed 1998). The Cavalieri method has been employed successfully to estimate the volume of various brain regions using post mortem tissue (Andersen *et al.* 1992; Braendgaard *et al.*

1990; Highley *et al.* 1999; Highley *et al.* 2001; Pakkenberg 1992; West and Gundersen 1990), and scanned images (Howard *et al.* 2003; Pakkenberg *et al.* 1989).

2.2.1.2 *The advantages of stereological investigation for measures of cell density*

The relative merits of 2-D and 3-D cell-counting methods have been the subject of many recent publications (Benes and Lange 2001; Guillery 2002; Hyman *et al.* 1998; Selemon and Rajkowska 2002; Uylings 2002). The main advantage of stereological (3-D) measurement is that particles are counted with a uniform probability, irrespective of their size, shape or orientation (Gundersen *et al.* 1988a). This avoids double counting and the potential problem of undercounting small objects. The use of systematic random sampling, fundamental to all stereological techniques, allows sampling to adequately overcome any heterogeneity within an anatomical region. Another advantage includes the ability to calculate a coefficient of error, which is useful in assessing the adequacy of the sampling scheme (Hyman *et al.* 1998). Although 3-D counting is not always feasible for neuropathological applications (see section 3.2.5.1), in this instance it was decided that as sufficient, suitable tissue was available, the optical disector and nucleator would be employed for the measurement of cell density and size within the ACC.

2.2.1.3 *The physical disector and the optical disector*

In its most basic form, the disector (Sterio 1984) consists of two parallel planes separated by a known distance, which is less than the height of the particles to be counted. Particles transected by one of the planes (the reference plane) and not by the other (the look-up plane) are defined as particles belonging to the reference volume and are counted. Unbiased rules are used for counting the particles (Gundersen 1977). The unbiased counting frame consists of a known area and has an acceptance line and an infinite 'forbidden' line. This ensures that no 2-D object will be counted more than once, providing there is a suitable guard area around the frame (Howard and Reed 1998).

In light microscopy, the optical disector utilises thick transparent sections. By using high numerical aperture lenses, it is possible to make thin optical sections inside the thick section by moving the plane of focus up or down. Consequently, the optical disector operates as a series of disectors in which the distance between the two planes may be arbitrarily great and in which the optical section functions as both reference and lookup sections as it is moved through the section (West 1993). Unbiased rules are again used for counting the particles: any particles touching the left or bottom of the counting frame are not included, whereas any particles touching the top or right sides of the frame are included. As counting is now within a volume and not just an area, the top surface of the counting chamber is excluded, whereas the bottom surface is included. It is also desirable to create guard volumes above and below the disector to avoid edge-of-section artefacts (Hyman *et al.* 1998).

In practice the optical disector is used in conjunction with uniform random, single, thick sections. For each section, the microscope stage is moved stepwise through the region of interest in a meander pattern using systematic sampling. At each sampling position, the numbers of particles coming into focus whilst moving down through the plane of focus are counted. The step distance and frame size need to be optimised for each experiment. In general, for efficient cell counting and an acceptable CE of less than 10%, there needs to be approximately 2-5 particles per disector with no more than 200 particles counted for the entire region (Howard and Reed 1998). Particle density is calculated from the following equation, where $a(fra)$, is the area of the frame, h , is the distance moved through the Z axis, $\sum Q^-$ is the sum of all particles counted and $\sum F$ is the sum of all frames counted (Howard and Reed 1998):

$$\hat{N}_V = \frac{1}{a(fra) * h} * \frac{\sum Q^-}{\sum F}$$

(6)

2.2.1.4 The nucleator

The nucleator is an efficient estimator of number weighted mean particle volume (Gundersen 1988). In practice the nucleator is often used in conjunction with the optical disector method, where particles are selected with uniform random probability (see 2.2.1.3). To successfully use the nucleator method, the particle volume must be estimated from a random point within a uniquely definable subspace of the particle (Howard and Reed 1998). This makes mononucleated cells such as neurons ideal candidates for volume estimation using the nucleator (Gundersen *et al.* 1988a).

For each nucleolus sampled, an isotropic direction is generated from a random point within the nucleolus. The distances in each direction from this point to the cell wall are recorded to estimate cell volume. To increase the efficiency of the nucleator, distance measurements are usually performed in at least two random directions from within the nucleolus (Howard and Reed 1998). From a series of these measurements the mean particle volume is then calculated from equation (7), where l_n refers to the distance between the sampling point within nucleolus to the edge of cell wall (Howard and Reed 1998). Generally, the interactive software manages the task of generating appropriate lines and calculating cell volume.

$$\hat{V}_N = \frac{4\pi}{3 * n} * \sum_{i=1}^n l_{n,i}^3 \quad (7)$$

2.2.2 Calculating coefficients of error

2.2.2.1 Calculation of CE for the Cavalieri method

The CE of the Cavalieri estimate can be calculated by using a predictive formula that takes into account the systematic nature of the sampling (Gundersen and Jensen 1987).

The CE calculation developed by Gundersen and Jensen (1987) will be described below.

Although modified versions of the CE formula can be sought, the predictive formula developed by Gundersen and Jensen has proven useful in many practical applications (Braendgaard *et al.* 1990; Pakkenberg *et al.* 1989; West and Gundersen 1990). In addition to the sum of the point counts on each section (calculated during the Cavalieri estimate), three additional sums are also required for predicting CE:

$$a = \sum_{i=1}^m P_i * P_i$$

$$b = \sum_{i=1}^{m-1} P_i * P_{i+1}$$

$$c = \sum_{i=1}^{m-2} P_i * P_{i+2}$$

(4)

The variable a is the sum of squares of the number of points counted on each section.

The variable b is the sum of the products of the number of points counted on each section and the number of points counted on the next section in the series. The variable c is the sum of the products of the number of points counted on each section and that counted on the second next section in the series. Using these four sums the Gundersen and Jensen (1987) prediction of CE is given by:

$$CE(\sum P) = \frac{\sqrt{(3a + c - 4b)/12}}{\sum P}$$

(5)

2.2.2.2 Calculation of CE for the optical disector method

As for the Cavalieri method, it is possible to calculate a coefficient of error for measures of

cell density, $CE\left(\frac{\sum Q}{\sum F}\right)$, which is again useful in assessing the adequacy of the sampling

scheme. It is, however, a more complicated issue, as both $\sum Q$ (the sum of particles

counted), and $\sum F$ (the sum of frames), have to be considered separately. The

covariance between these two related variables also needs to be determined (Gundersen

and Jensen 1987). Although different versions of the CE formula do exist, I decided to

use the calculation proffered by Gundersen and Jensen (1987), which has been

successfully used in earlier studies of neuronal density (Braendgaard *et al.* 1990;

Pakkenberg and Gundersen 1988; West and Gundersen 1990). The prediction of CE

requires three separate calculations and will be described as clearly as possible below.

The CE of the total number of particles counted, $CE(\sum Q)$, and of the number of frames,

$CE(\sum F)$, are required for the formula and can be calculated in the same manner as

$CE(\sum P)$ (see section 2.2.2.1). In order to determine the covariance between

Q^- and F , three separate variables, D , E and G are calculated:

$$D = \sum Q_i * \sum F_i$$

$$E = (\sum F_i * Q_{i+1} + \sum F_{i+1} * Q_i) / 2$$

$$G = (\sum F_i * Q_{i+2} + \sum F_{i+2} * Q_i) / 2$$

(8)

The variable D is the sum of the products of the number of particles counted on a section and the number of disector samples used to obtain the counts. The variable E is the sum of the means of the product of F from each section and Q^- from the next section in the series, plus the products of Q^- from the section and F from the next section in the series. The variable G is calculated in a similar manner, except that the combinations are made between F and Q^- on section pairs separated by one section (West and Gundersen 1990).

Using these three terms, the covariance between F and Q^- can be calculated:

$$Cov(\sum Q, \sum F) = (3D + G - 4E)/12 \quad (9)$$

Using the equations detailed above, $CE\left(\frac{\sum Q}{\sum F}\right)$ is then predicted by:

$$CE\left(\frac{\sum Q}{\sum F}\right) = \sqrt{CE^2(\sum Q) + CE^2(\sum F) - \frac{2 * Cov(\sum Q, \sum F)}{\sum Q * \sum F}} \quad (10)$$

2.2.3 Tissue collection and preparation

2.2.3.1 Brain selection

After careful consideration, five pathologically normal brains were selected with a fully intact, bilateral cingulate gyrus and were retrieved from the archive. Between autopsy and the present study, the five brains had been stored in formalin for an average of 3.9 years. All brains were characterised macroscopically and histologically by a neuropathologist to confirm the absence of any significant pathology. Medical records were also thoroughly checked to ensure no coincident psychiatric illness. The demographic details of the subjects included in this study are given in table 2.1.

2.2.3.2 Brain collection and photography

Slices from each brain were reassembled into a stack, the length of which was measured and divided by the number of slices to obtain the mean slice thickness. The anterior aspect of each slice was patted dry of excess moisture and photographed using black and white film, ensuring that the planes of the camera lens and the brain slice were parallel. Care was taken to mark the left hemisphere and include a ruler in the field of view.

2.2.3.3 Selection of anatomical levels within the cingulate cortex

As stated in the introduction, one aim of this study was to assess cell density in five anatomically distinct regions of area 24b in the ACC. In order to do this, it was essential to locate five anatomical levels along the rostrocaudal extent of the ACC that were common to all five brains. Careful inspection of photographs led to the identification of three coronal slices in each brain that shared various macroscopic landmarks (see figure 2.1).

Slice 1 - anterior limit of the corpus callosum (**level 2**: rostral subgenual area 24 and **level 3**: rostral supragenual area 24)

The anterior end of the genu of the corpus callosum is evident. The ACC is visible, inferior (subgenual) and superior (supragenual) to the genu of corpus callosum.

Slice 2 - anterior limit of the putamen (**level 1**: caudal subgenual area 24 and **level 4**: rostrocaudal supragenual area 24)

The head of the caudate nucleus is seen in the wall of the lateral ventricle. The rostral end of the putamen is just visible. Again, the cingulate cortex is divided by the corpus callosum, both supragenual and subgenual regions are present.

Slice 3 - anterior limit of the amygdala (**level 5**: caudal supragenual area 24')

The head of the caudate and the putamen are still visible and the internal capsule divides them. The anterior limit of the amygdala and the globus pallidus are observed for the first time. The anterior commissure is also evident. The supragenual ACC is still present.

These descriptions lead to the identification of three supragenual and two subgenual blocks of anterior cingulate tissue that shared common anatomical positions between the brains. Although selected tissue blocks at a given anatomical level were basically equivalent, there were small discrepancies with some blocks presenting slightly more anterior features than others at the same approximate level. The anatomical levels were numbered one to five, from the most caudal level of the subgenual region to the most caudal level of the supragenual region (see figures 2.1, 2.2 and 2.3 and table 2.2).

2.2.3.4 *Tissue sectioning*

In each coronal slice, the cingulate cortex (supragenual and subgenual) was identified and dissected, taking bilateral blocks wherever possible. The left hemisphere was marked using Indian ink. The tissue was then processed on a Leica TP1040 and embedded in paraffin wax. Ten serial 25µm sections were cut from each wax block using a rotary microtome (Leica, Milton Keynes, UK). Sections were floated onto a warm waterbath to remove wrinkles before being mounted onto superfrost slides (VWR International, Poole, UK). The sections were then dried overnight at 37°C and stored at room temperature until use.

Prior to the experiment, sections were stained with 0.75% Cresyl Violet, dehydrated through graded alcohols (70%, 95%, 100% x2), placed in xylene and coverslipped using DPX as a mountant (VWR International). From the ten serial sections, three were systematically, randomly sampled for analysis.

2.2.4 Estimation of grey matter volume in the cingulate gyrus using the Cavalieri method

Photographs of brain slices (see section 2.2.3.2) were used to calculate the volume of the grey matter in the cingulate gyrus. The cingulate gyrus was identified superior, anterior and inferior to the corpus callosum and was measured along its entire length. These coronal slices provided an unbiased estimate of cingulate volume as the very first coronal slice was sectioned randomly; hence, every section of the cingulate had an equal probability of being sampled. Two separate estimates were made; the first estimate included only the cingulate gyrus, and the second estimate also incorporated the paracingulate gyrus (when present). In some cases the PCS was difficult to identify due to problems concerning the differentiation of the double cingulate sulcus from cortical depressions in the fundus of the cingulate gyrus. In these instances the adjacent coronal view was examined in an attempt to follow the course of the CS to see if it was single or

double. Left and right hemispheres were measured separately. A 2mm (4mm²) point counting grid was used to provide the desired 150-200 counts per object. As photographs were used in this study, a correction factor for magnification was applied.

The transparent grid was placed at random on each photograph and the points hitting the grey matter of the cingulate gyrus were counted for each slice and then summated. From these counts, along with the spacing of the grid (corrected for magnification) and the mean slice thickness, the volume of the grey matter was estimated by application of the Cavalieri principle (see equation 3, section 2.2.1.1). The CE for each estimate was calculated using equation 5 given in section 2.2.2.1. All CE values were below 10%, well within the accepted range.

No attempt was made to estimate separate volumes for the anterior and posterior regions of the cingulate gyrus. To make such estimates, it would be of utmost importance to know exactly where the transition between these two regions occurs. After careful inspection of the photographs, it was determined that no single slice gave the ideal transition point between these two areas. Additionally, considering these two regions separately would mean estimating a volume using fewer brain slices. A possible consequence of reducing the number of slices would be to increase the error and produce unreliable results. Hence, volumes are of the entire cingulate gyrus, whereas morphometry measurements were taken from just the ACC (see section 2.2.5 below).

2.2.5 Estimation of neuronal and glial density and neuronal size in the anterior cingulate cortex

2.2.5.1 Identification of area 24b, area 24sg and cortical layers

For each case, Brodmann area 24b was identified in the supragenual and subgenual levels using macroscopic and microscopic criteria (see 2.1.2) (Öngür *et al.* 2003; Vogt *et al.* 1995). As previously stated, the cytoarchitecture of area 24 has been well

documented, which made delineation of the separate lamina relatively straightforward, particularly in the supragenual region (Vogt *et al.* 1995). Unfortunately, layer V in the subgenual region could not be reliably subdivided into layers Va and Vb as it had been in the supragenual levels. Accordingly, to permit comparisons of area 24b between the subgenual and supragenual levels, layers Va and Vb in the supragenual region (levels 3-5) were combined.

In supragenual levels, area 24b was distinguished from adjacent areas 24a and 24c using the following criteria: 1) layers II and III were clearly defined in 24b, unlike 24a where they were quite homogeneous, 2) layers II and III in 24c were well defined, but generally occupied a greater proportion of the cortical depth than in 24b, 3) layer Va was at its thickest and most prominent in 24b, 4) layer Vb contained an intermediate number of spindle cells in 24b; in comparison to 24a which showed the greatest number, and 24c which displayed relatively few of this cell type and 5) area 24c tended to have the most sparse layer Vb. Photomicrographs illustrating the cytoarchitecture of the supragenual region of area 24 are provided in figure 2.4.

Subgenual area 24 was delimited dorsally by the corpus callosum and ventrally by the transition from agranular to granular cortex. This area usually corresponded to the first full subgenual gyrus. Subgenual area 24b displayed a clear and well-laminated arrangement that distinguished it from adjacent subgenual area 24a. Area 24b appeared to occupy most of the subgenual gyrus, whilst subgenual area 24a lied immediately adjacent to the corpus callosum and tended to be very small. As subgenual area 24b corresponded to most of the subgenual gyrus, it will be referred to as subgenual area 24 for the remainder of this chapter. Photomicrographs illustrating the cytoarchitecture of the subgenual region of area 24 are provided in figure 2.4.

2.2.5.2 Counting procedure

All measurements and cell counts were made using an Olympus BX50 microscope (Olympus, UK), connected to a stage motor and a digital vertical depth gauge coupled to a microcator (Heidenhain, Traunreut, Germany). The microscope was mounted with a JVC TK-C1380 colour video camera. This equipment was controlled and viewed on a computer using the Olympus Cast-Grid 2.00.05 stereology sampling software (Olympus DK, Albertslund, Denmark).

For all subsequent cell density measures I was blind to case number. I was also blind to hemisphere apart from when left and right hemispheres had remained intact and were consequently sectioned onto the same slide. As the subgenual and supragenual levels of area 24 required different sampling strategies (see below), it was not possible to blind myself between these two categories.

Sections were viewed with a 100-times oil immersion objective lens to minimise differences in refractive indices (Howard and Reed 1998). Representative high power images of the supragenual and subgenual regions of area 24 are shown in figure 2.5. In order to determine a suitable depth for the optical disector, it was necessary (using a microcator) to calculate the mean thickness of the sections following mounting and staining procedures. Originally sectioned at 25 μ m, the sections had collapsed to a mean depth of approximately 18 μ m. Accordingly, the optical disector depth was set to 12 μ m to allow appropriate guard volumes above and below the disector.

The size of the disector was set at 34% of the total monitor size, which equated to a final disector area of 1896 μ m². This provided a suitable neuron/glia count per disector. There was only one disector per field. Sampling strategies for supragenual and subgenual regions of area 24 were optimised before the definitive experiment. This involved estimating the total number of fields required to provide a count of 150-200 neurons or glia

per layer, per level, hemisphere, and case (neurons and glia falling in the disector volume were recorded separately). Various step sizes were evaluated before this condition was satisfied. Due to the smaller area of the subgenual cortex, a different step size had to be employed to ensure sufficient numbers of cells were counted. The step size for layers within the supragenual levels (levels 3-5) was 330 μ m, whereas the step size for layers in the subgenual levels (1 and 2) was 220 μ m. A meander pattern (of predetermined step distance) was then used to systematically sample the entire region of cortical layers II-VI in each level of area 24.

Before counting commenced, the depth of each cortical layer was determined. Lamina boundaries were identified on each image and the depth of each layer was measured using a 4-times objective lens. These measurements were repeated bilaterally, four times on each of the three sections per level, per case. The percentage of total cortical depth that each layer contributed was then calculated.

At each meander position, the number of neurons or glia that came into focus were counted using the rules of the unbiased counting frame (section 2.2.1.3). Neurons were distinguished by their larger, non-spherical shape, Cresyl Violet stained cytoplasm and a single nucleolus. Glial nuclei were smaller and identified by the absence of stained cytoplasm and the lack of a nucleolus. Other characteristics of glial cells that were considered included the presence of an irregular or ovoid shaped nucleus and a thicker nuclear membrane (see figures 2.5 and 2.6). No attempt was made to distinguish between pyramidal and non-pyramidal neurons, nor to subdivide glial types based on their profiles. A neuron was defined as in focus when the widest and clearest profile of the nucleolus was seen. When the neurons were in focus the nucleator was applied to estimate neuronal cell volume (see 2.2.1.4 and figure 2.6). The stereological software automatically calculated the neuronal volume. Glial cell volume was not assessed as the glial cytoplasm was unstained.

Neuronal and glial densities were calculated using equation 6 (see section 2.2.1.3) and are expressed as cells per $\text{mm}^3/10^3$. Mean neuronal size is expressed in μm^3 . Values of CE for the mean neuronal and glial density estimates in the different layers were calculated using equation 10 (see section 2.2.2.2). All CE values were below 10%; the majority were less than 5% (data not shown). This is well within the accepted range. Density measures were repeated at the end of the experiment for a selection of levels (picked at random) to assess the intra-rater reliability. All values were within 10% of the original measurements.

2.2.6 Statistical Analysis

2.2.6.1 *Analysis of cingulate gyrus volume measures*

The primary aim of this study was to provide a descriptive account into the macroscopic features and to calculate the volume of the grey matter of the cingulate gyrus. Paired t-tests and Pearson's correlations were also used to assess interhemisphere relationships and test for asymmetry.

2.2.6.2 *Analysis of neuronal and glial density and neuronal size measures*

All statistical analyses were carried out using SPSS 10.0 for Microsoft Windows. The data sets to be investigated were: 1) cortical depth, 2) layer depth (as a percentage of total cortical depth), 3) neuronal density, 4) glial density and 5) neuronal size. Although layers Va and Vb had been measured separately in the supragenual levels, this distinction had not been possible in the subgenual levels. Consequently, an average of the two subdivisions was calculated to provide an equivalent figure for layer V in the supragenual levels. Unfortunately, subgenual tissue at anatomical level 1 was missing for one case.

Selecting the appropriate statistical tests for this data set was difficult due to the limited nature of such a small sample ($n=5$). An initial evaluation of the data involved checking

the distributions of points within each data set for normality using the Kolmogorov-Smirnov test. As morphological investigations into the ACC have suggested asymmetries between the hemispheres (see section 2.1.1), an *a priori* decision was made to assess all interhemisphere relationships within each data set using paired-sample t-tests. If no significant patterns emerged, it was decided that the hemisphere data would then be averaged for each data set.

The main purpose of this research was to examine how the cellular composition of the cingulate gyrus varies between different anatomical levels of area 24, and to what extent each cortical layer contributes to this variation. For this reason it was decided that a repeated measures ANOVA, incorporating anatomical level and layer as 'within subject variables', would be employed to analyse each data set. If the repeated measures ANOVA revealed a significant main effect of level ($p \leq 0.01$), paired-sample t-tests were subsequently used to make various level comparisons. If the repeated measures ANOVA also demonstrated a significant interaction between level and layer, paired-sample t-tests were then employed to explore the nature of this relationship, investigating all cortical layers within the levels that reached significance.

Although this manner of multiple testing would usually necessitate a form of correction, such as the Bonferroni method, it was decided that this would be overly rigorous and unsuitable for such a small sample. In respect to this, for all level and layer analyses, a p value of ≤ 0.01 is termed 'significant', and a p value between 0.01-0.05 is discussed as a 'trend' to avoid overstating the significance of these results.

Table 2.1.
Demographic details of each case.

	A	B	C	D	E
Age / years	18	69	34	29	63
Sex	M	M	F	F	F
Cerebrum weight / g*	1480	1350	1465	1245	1155
Duration in fixative / years	6.2	4.8	4.1	4.0	0.4

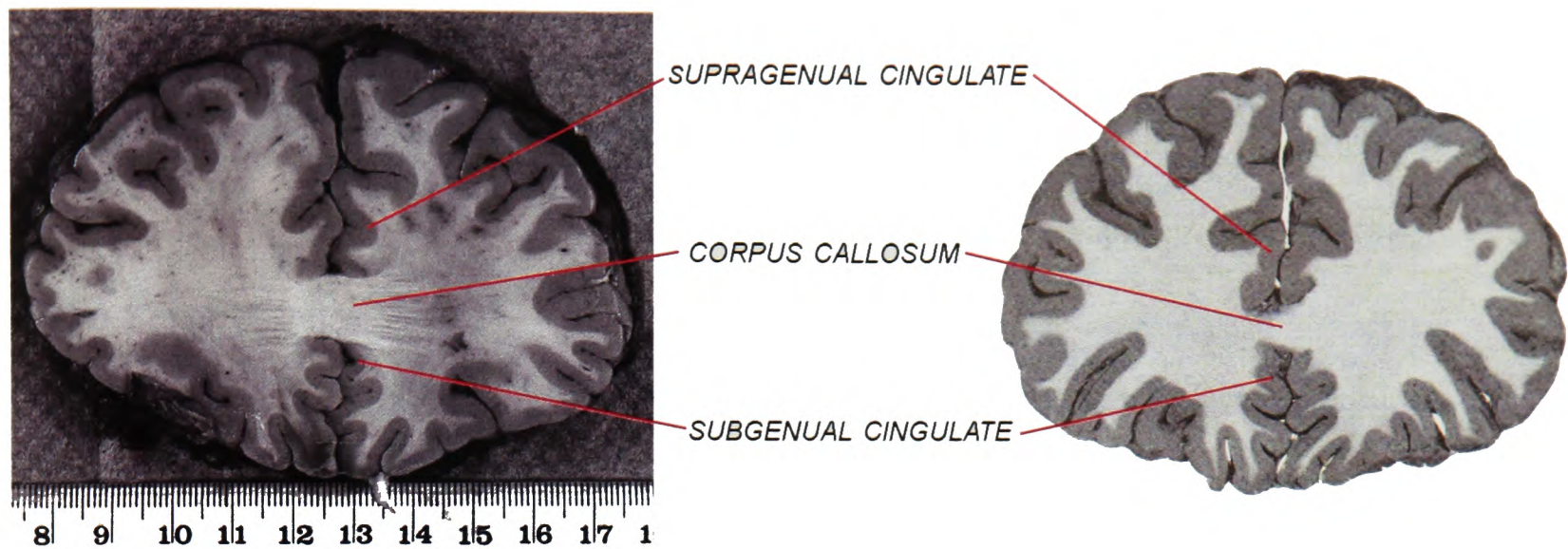
* weight (cerebrum less brain-stem, post-fixation)

Figure 2.1.

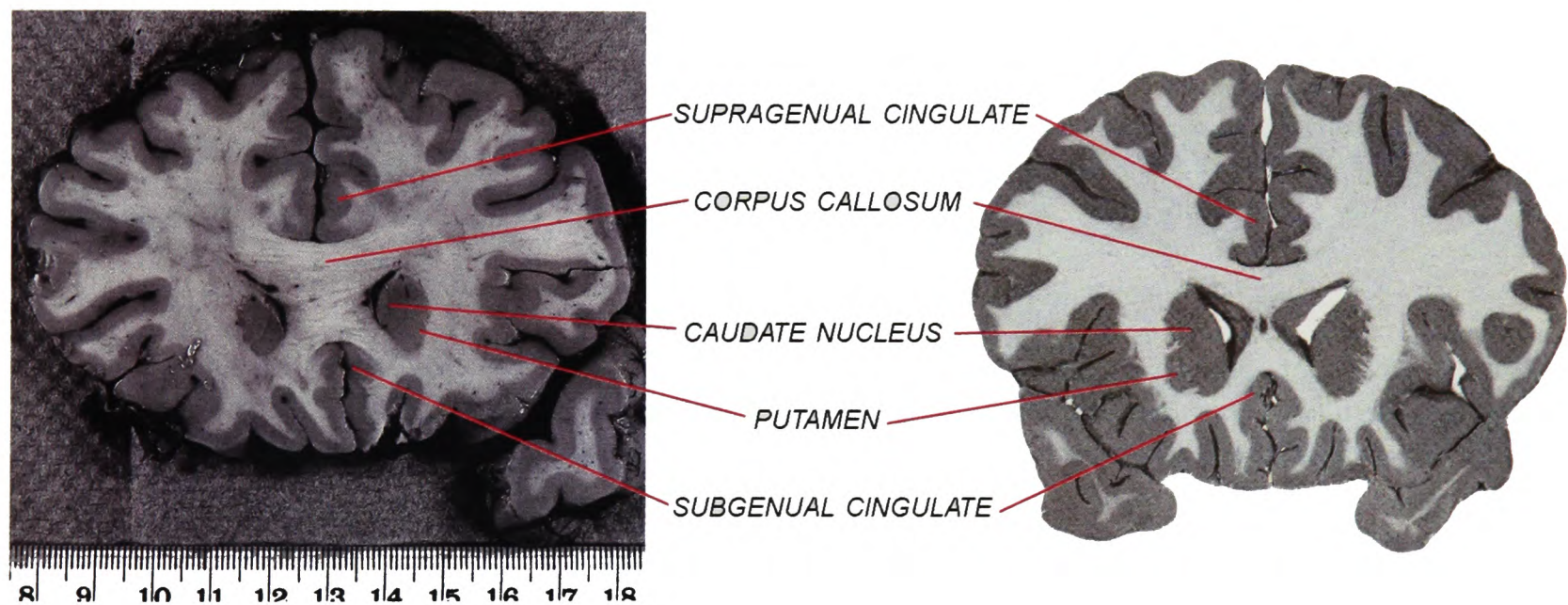
Selection of anatomical levels within the ACC

This figure illustrates the anatomical landmarks that were common to all five brains. These descriptions lead to the identification of three supragenual and two subgenual blocks of ACC tissue that shared common anatomical positions between the five brains. Slice one was identified by the anterior limit of the corpus callosum. The supragenual and subgenual regions of area 24 were both present. Slice two was identified by the anterior limit of the putamen and also contained the supragenual and subgenual regions of area 24. Slice three was identified by the anterior limit of the amygdala – only the supragenual region was present (see section 2.2.3.3). The anatomical levels were numbered one to five, from the most caudal level of the subgenual ACC, to the most caudal level of the supragenual ACC. The coronal slices and corresponding 'anatomical levels' of the ACC are further illustrated in figures 2.2 and 2.3. See also table 2.2.

Slice 1 - supragenual level 3 and subgenual level 2



Slice 2 - supragenual level 4 and subgenual level 1



Slice 3 - supragenual level 5

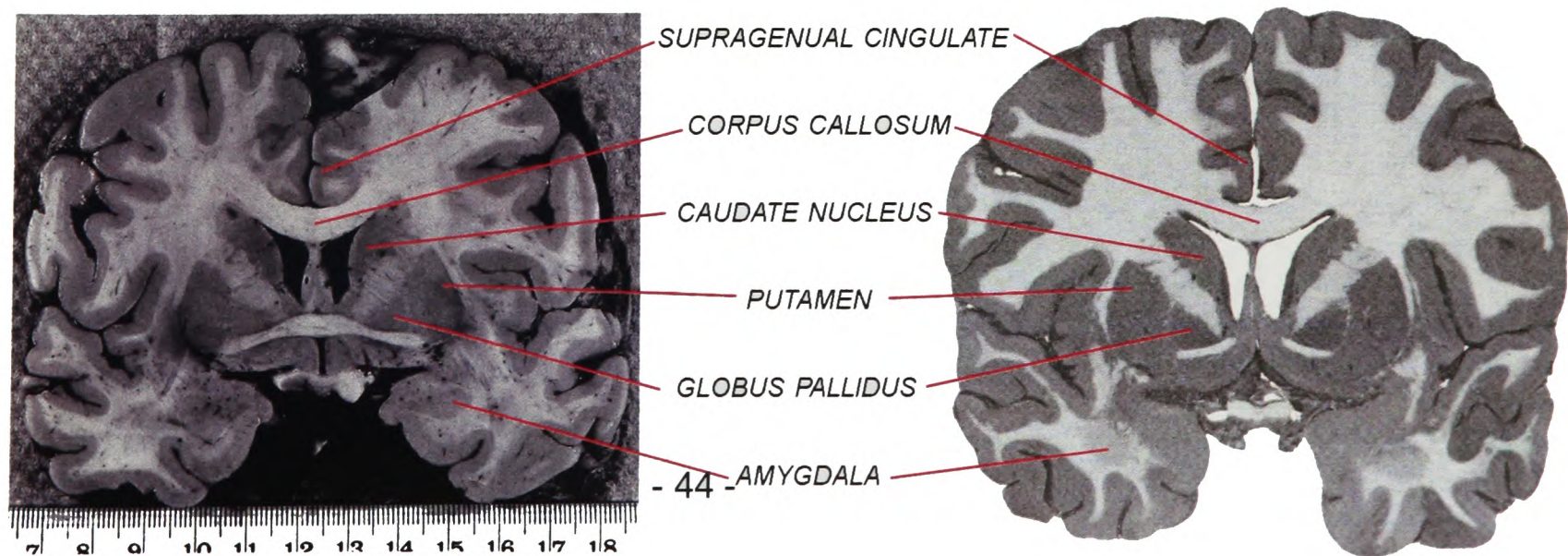


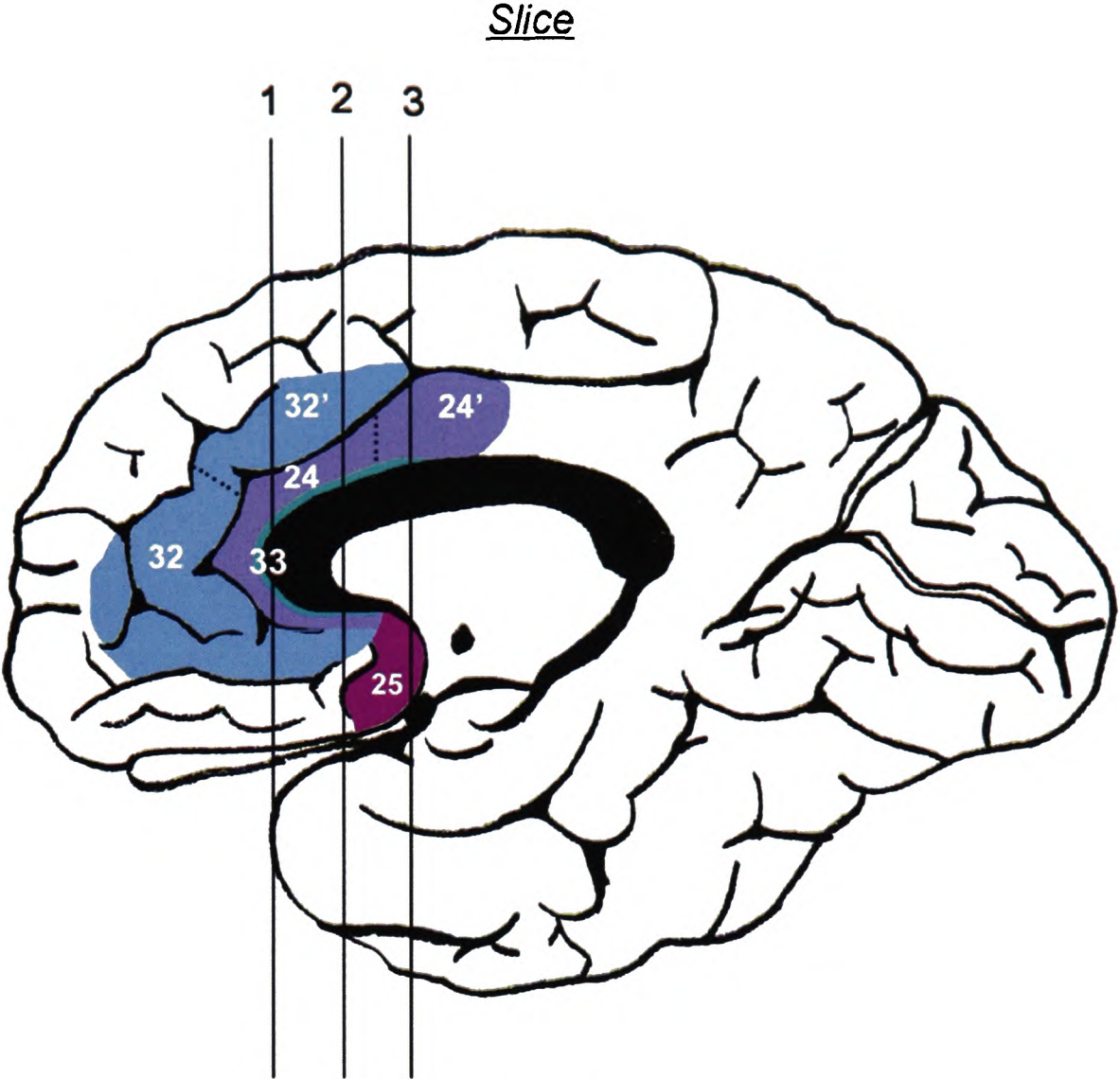
Figure 2.2.

Schematic diagram illustrating the relationship between the three coronal slices and five anatomical levels of the ACC.

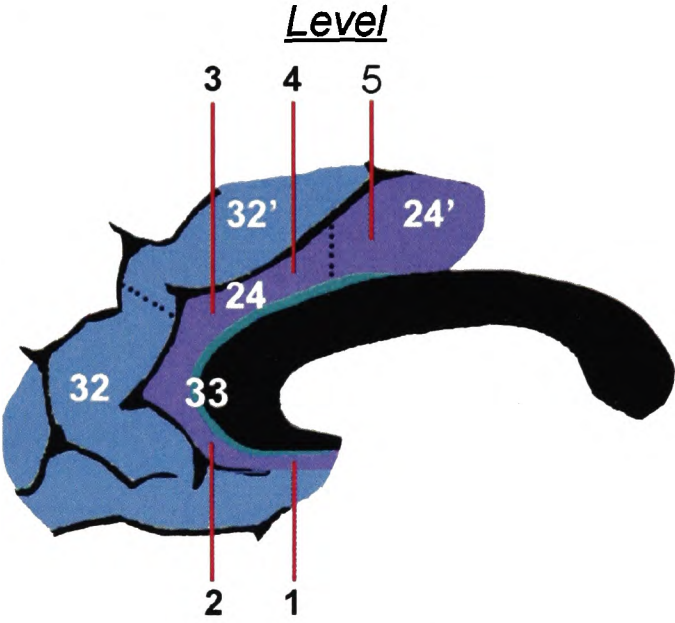
(A, B) Medial and (C) coronal (at the level of each line) views of the cerebral cortex showing Brodmann areas included in the definition of the ACC (see section 2.1). Delineation of Brodmann areas is from Perry (1993). All markings are approximate, because boundaries are defined by cytoarchitecture and not by surface anatomy.

The first coronal slice includes the most rostral levels of the ACC in this study: supragenual level 3 and subgenual level 2. The next coronal slice (2) includes the most caudal level of the subgenual region used in this study (level 1), and intermediate supragenual level 4. The third coronal slice incorporates level 5, the most caudal supragenual region. As illustrated, the five anatomical levels are numbered from the most caudal aspect of the subgenual region (level 1), to the most caudal aspect of the supragenual region (level 5). Further explanation regarding the Brodmann areas included at each level is provided in figure 2.3. See also table 2.2.

A



B



C

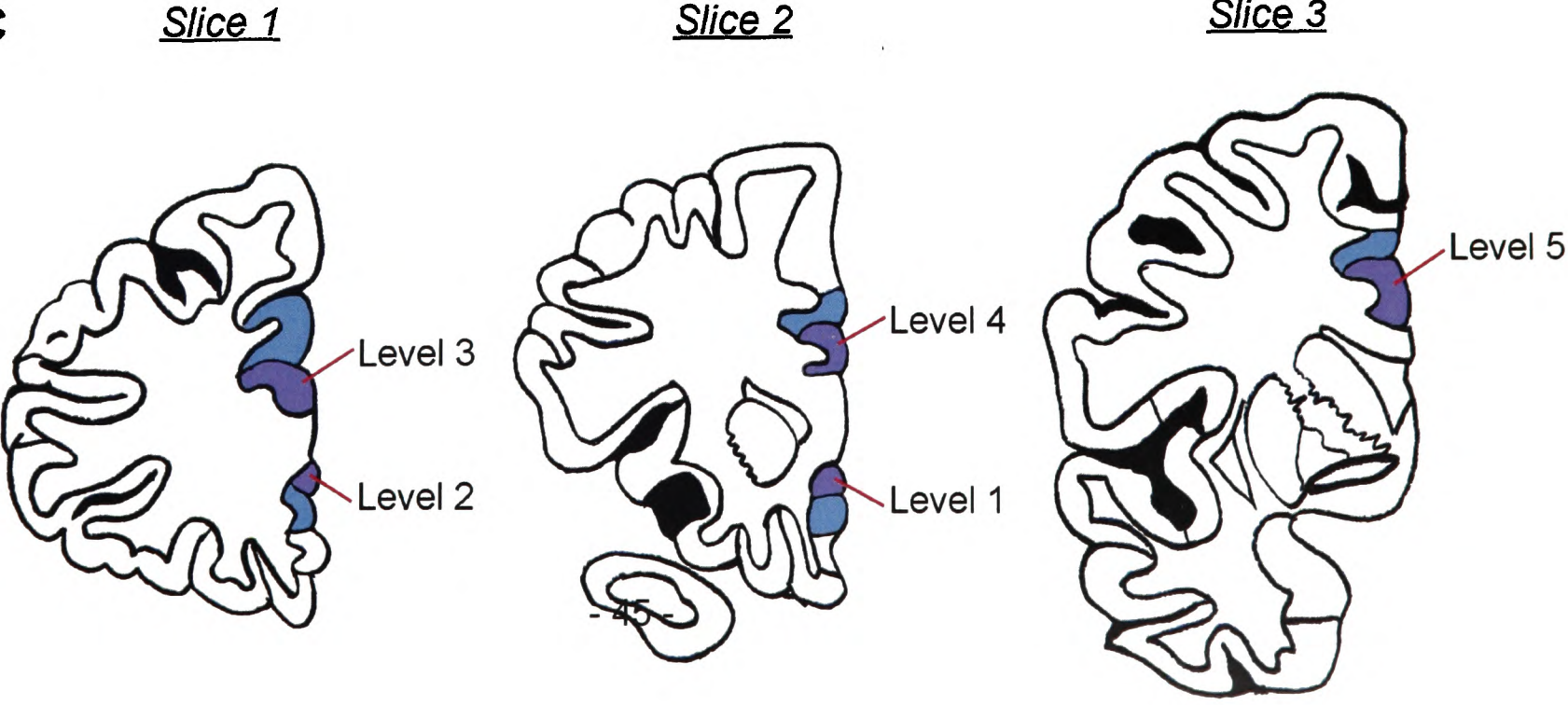


Figure 2.3.

Schematic diagram illustrating the relationship between the five anatomical levels and the Brodmann areas they encompass.

(A) Medial and (B, C) coronal (at the level of each line) views of the cerebral cortex showing all Brodmann areas included in the definition of the ACC (see section 2.1). Delineation of Brodmann areas is from Perry (1993). Brodmann areas 32, 33 and 25 have been included in this diagram as they are all part of the ACC (see section 2.1); however, the descriptions that follow are only concerned with area 24. Rostrocaudal (B) and dorsoventral (C) subdivisions of area 24 (and adjacent area 32) are also shown. All markings are approximate, because boundaries are defined by cytoarchitecture and not by surface anatomy.

In numerical order, **level 1** contains the subgenual region of area 24 (24sg). Dorsoventral subdivisions of the region include 24asg and 24bsg. **Level 2** also contains area 24sg. Again dorsoventral subdivisions include 24asg and 24bsg. Level 2 is more rostral than level 1. **Level 3** contains the supragenual region of area 24. Dorsoventral subdivisions include 24a, 24b and 24c. **Level 4** also contains area 24 (24a, b and c), however, this region is more caudal than level 3. **Level 5** is the most caudal of all supragenual levels and accordingly it is designated as area 24'. Dorsoventral subdivisions of this area include 24a', 24b', and 24c'. Table 2.2 summarises the five levels and the Brodmann areas they contain. The cytoarchitecture of these areas is illustrated in figure 2.4.

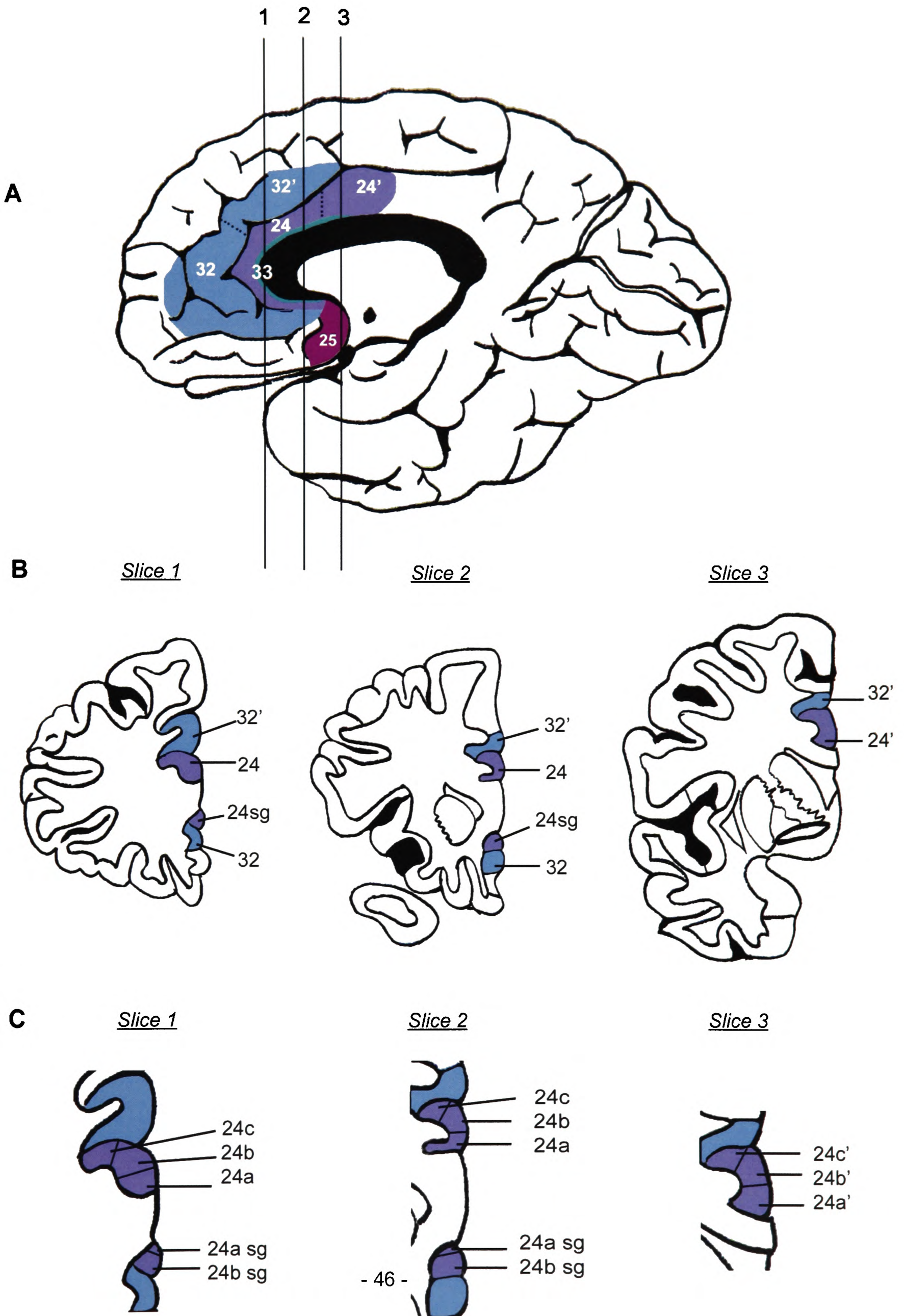


Table 2.2.

This table summarises the terminology used throughout this chapter.

Description	Abbreviation	Comment
Supragenual region of area 24	24 and 24'	Area 24 rostral and superior to the corpus callosum. Includes levels 3, 4 and 5.
Subgenual region of area 24	24sg	Area 24 inferior to the corpus callosum. Includes levels 1 and 2.
Subgenual Level 1	1 24a sg, 24b sg	Caudal level of area 24 inferior to the corpus callosum. Anatomical position: Slice 2, anterior limit of the putamen.
Subgenual Level 2	2 24a sg, 24b sg	Rostral level of area 24 inferior to the corpus callosum. Anatomical position: Slice 1, anterior limit of the corpus callosum.
Supragenual Level 3	3 24a, 24b, 24c	Rostral level of area 24 superior to the corpus callosum. Anatomical position: Slice 1, anterior limit of the corpus callosum.
Supragenual Level 4	4 24a, 24b, 24c	Intermediate level of area 24 superior to the corpus callosum. Anatomical position: Slice2, anterior limit of the putamen
Supragenual Level 5	5 24a', 24b', 24c'	Caudal level of area 24 superior to the corpus callosum. Anatomical position: Slice 3, anterior limit of the amygdala

Figure 2.4.

Photomicrographs of area 24 showing Nissl staining in the rostrocaudal and dorsoventral subdivisions of the supragenual and subgenual regions (scale bar=100µm).

Overall, area 24 is agranular (lacking a definable layer IV); a distinguishing feature of this area is a prominent layer V. A special cellular feature of area 24, and more specifically layer Vb, is the presence of spindle cells (see red arrows). Spindle cells are most prominent in area 24a but are also regularly observed in area 24b, and less frequently in area 24c. See section 2.1.2 for an individual description of each subdivision of area 24.

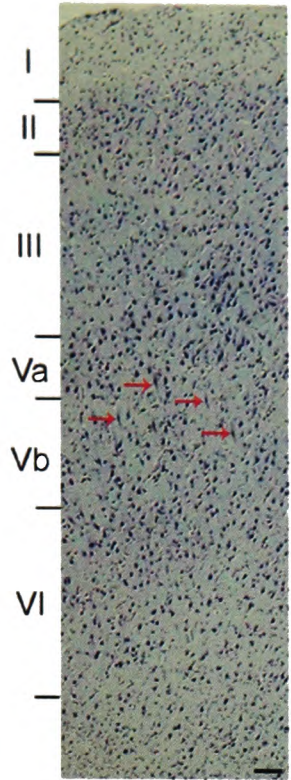
For each case, area 24b was identified in the supragenual and subgenual levels using macroscopic and microscopic criteria from Vogt (1995) (see sections 2.12 and 2.2.5.1). In supragenual levels 3, 4 and 5, area 24b was defined using the following criteria:

1) layers II and III are clearly defined in 24b, unlike 24a where they are quite homogeneous, 2) layers II and III in 24c are well defined, but generally occupy a greater proportion of the cortical depth than in 24b, 3) layer Va is at its thickest and most prominent in 24b, 4) layer Vb contains an intermediate number of spindle cells in 24b; in comparison to 24a which shows the greatest number, and 24c which displays relatively few of this cell type and 5) area 24c tends to have the most sparse layer Vb.

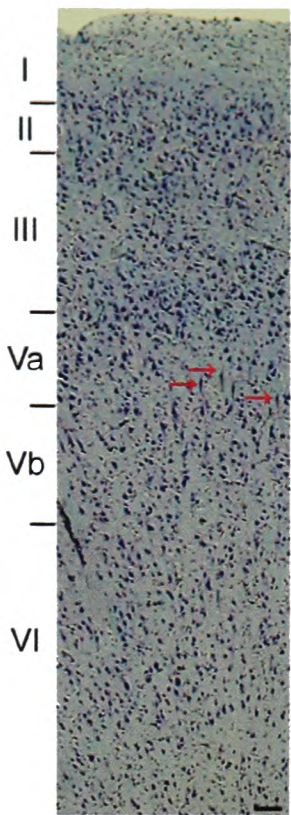
According to macroscopic criteria, supragenual level 5 contains area 24' (see figure 2.3). However, in my material, I found it difficult to distinguish between the more rostral (24) and caudal levels (24') of area 24 using microscopic criteria. Consequently, I can only say that level 5 approximates to area 24'.

Area 24bsg displays a clear and well-laminated arrangement that distinguishes it from adjacent area 24asg, which has a fairly rudimentary lamina appearance. Spindle neurons are present in 24asg and 24bsg.

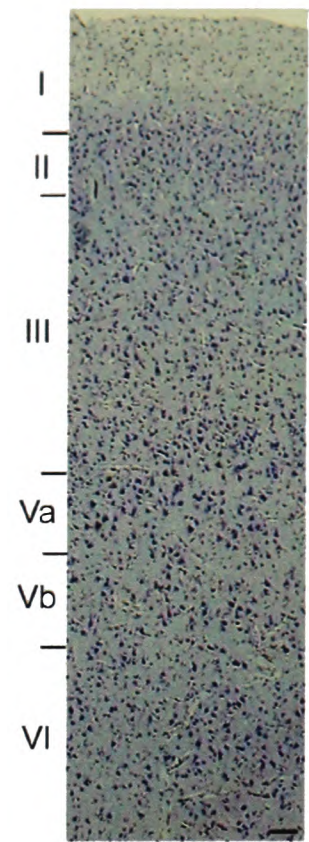
24a



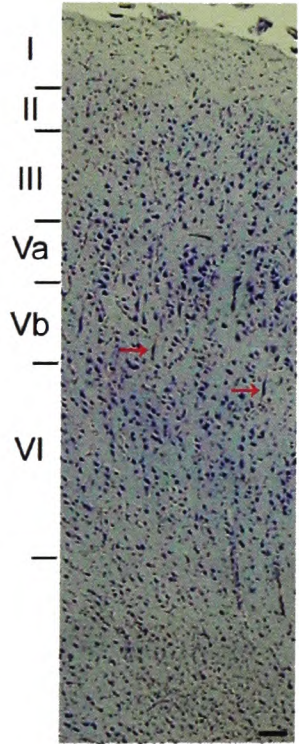
24b



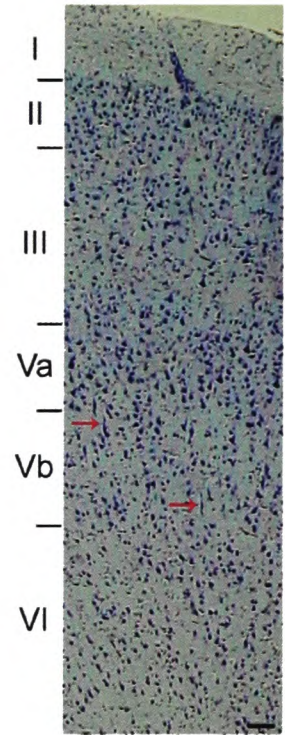
24c



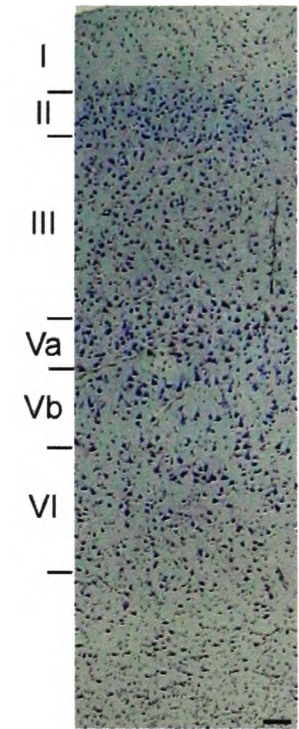
24a'



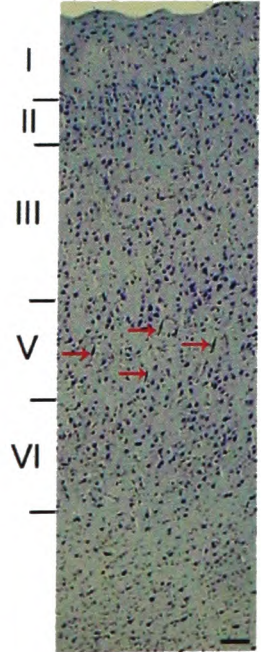
24b'



24c'



24a sg



24b sg

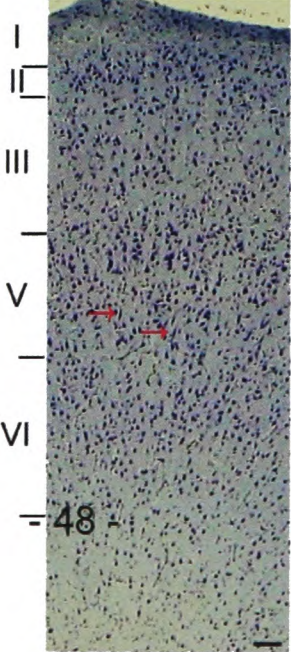


Figure 2.5.

Cell identification, the optical disector and the nucleator (scale bar=10µm).

(A) Neurons were distinguished by their larger, non-spherical shape, cresyl violet stained cytoplasm and a single nucleolus. Glial nuclei were smaller and identified by the absence of stained cytoplasm and the lack of a nucleolus. Other characteristics of glial cells that were considered included the presence of an irregular or ovoid shaped nucleus and a thicker nuclear membrane.

(B) At each meander position, the number of neurons or glia that came into focus were counted. Neurons were defined as 'in focus' when the widest and clearest profile of the nucleolus was seen. Any particles touching the left or bottom of the counting frame were not counted (red 'forbidden' lines), whereas any cells touching the top or right sides of the frame were counted (green 'acceptance' lines).

(C) When neurons were in focus the nucleator was applied to estimate neuronal cell volume. A cursor was used to mark the nucleolus, following which, the software generated lines in an isotropic direction. A cursor was used to mark where each line intersected the neuronal soma wall.

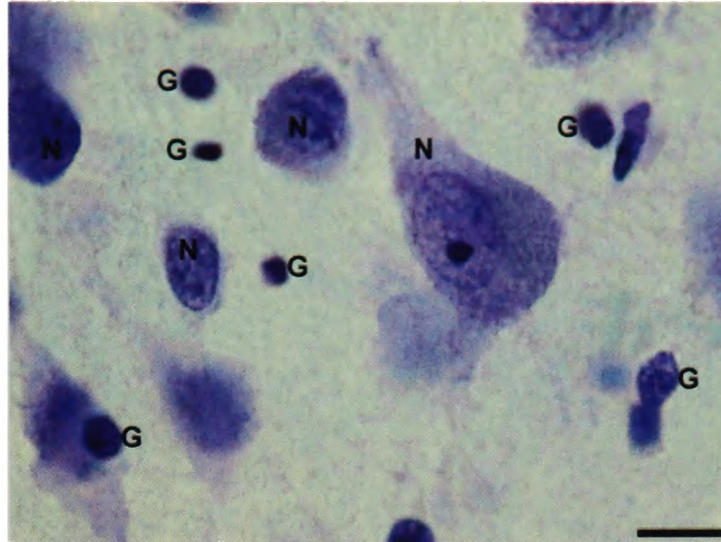
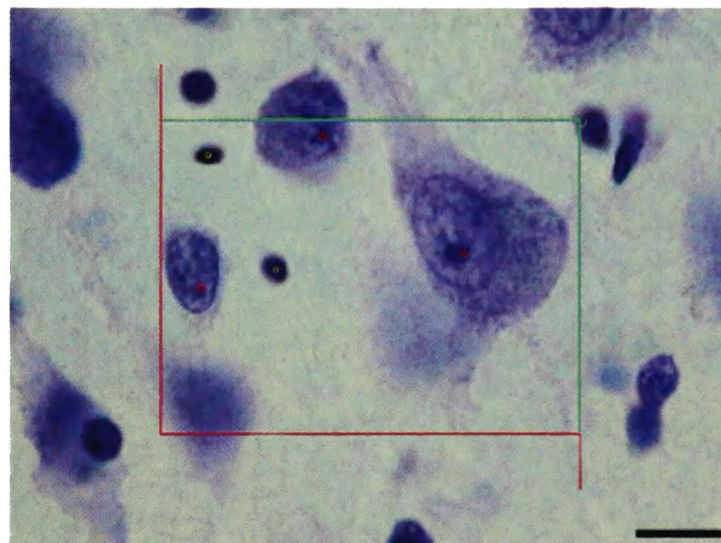
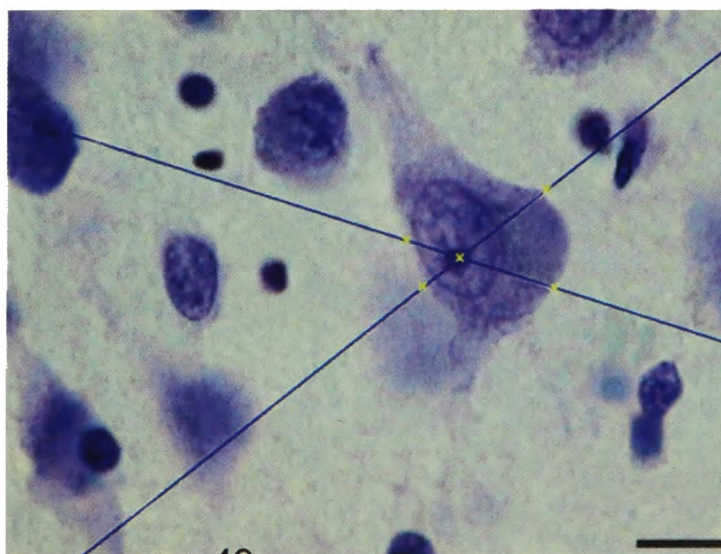
A Glia and neurons in layer V**B Counting with the optical disector****C Estimating neuronal volume with the nucleator**

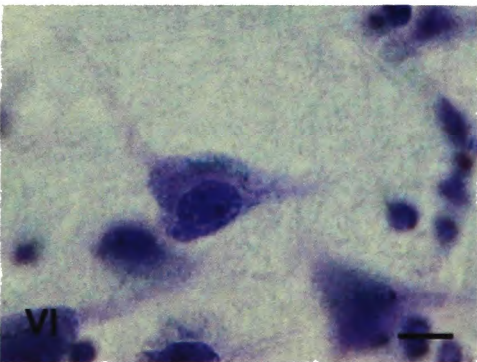
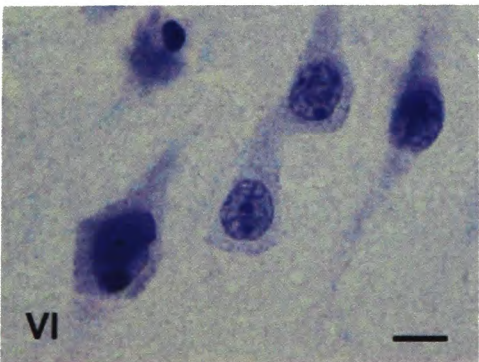
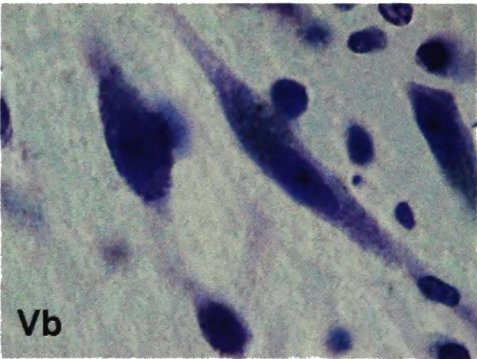
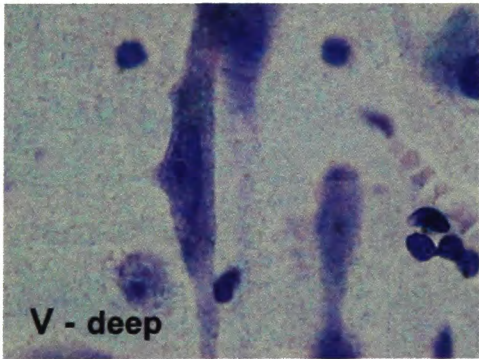
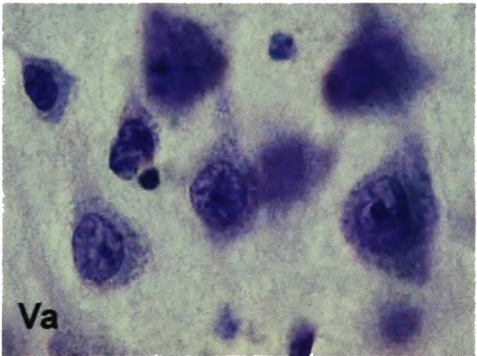
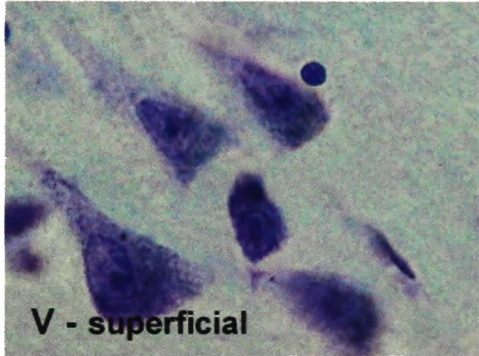
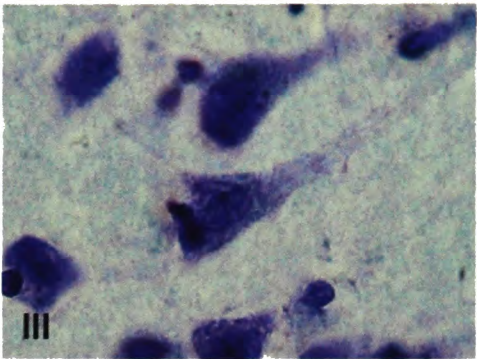
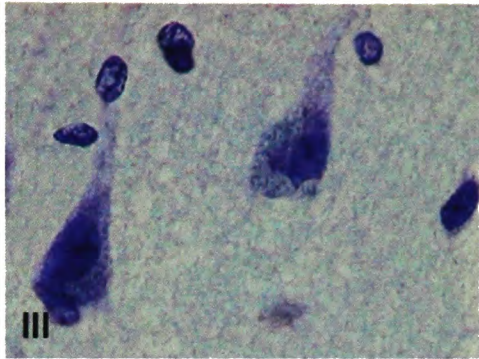
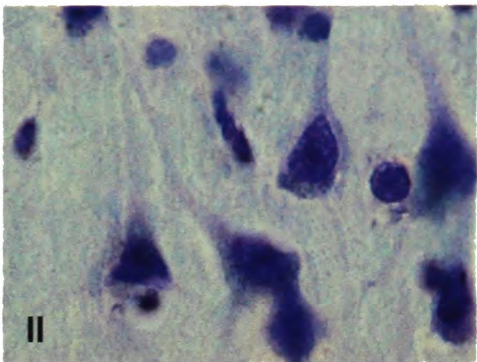
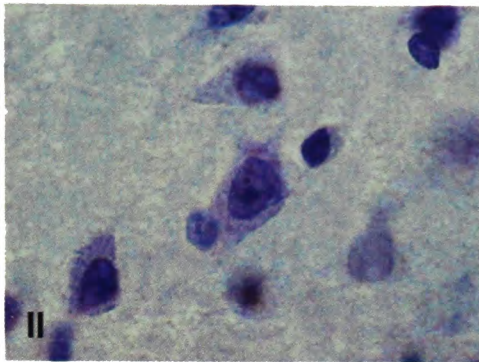
Figure 2.6.

Photomicrographs of neurons and glia in layers II-VI of the subgenual and supragenual regions of area 24 (scale bar=10 μ m).

Glia are small, dark, and generally round with no stained cytoplasm. Neurons are larger, have a nucleolus visible within the nucleus and Nissl-stained cytoplasm. This image also illustrates spindle neurons in deep layer V in the subgenual region, and layer Vb in the supragenual region. As explained in section 2.2.5.1, it was not possible to accurately subdivide layer V in the subgenual region; accordingly, layers Va and Vb were combined in the supragenual region.

Area 24b
Subgenual region

Area 24b
Supragenual region



2.3 Results - Estimation of grey matter volume in the cingulate gyrus

The pattern of cortical folding was highly variable between each subject. The CS and PCS (when present) were relatively easy to distinguish from the other sulci on the coronal photographs. The PCS was present, to varying degrees, in four of the five brains examined. In all four, the PCS was evident in both hemispheres, although it was more prominent on the left side.

The mean (SEM) grey matter volume of the cingulate gyrus was 6.7cm^3 (0.33) and 6.45cm^3 (0.34) for the left and right hemispheres respectively (see table 2.3). Although hemisphere volume varied between cases, no consistent asymmetry was observed ($t(4) = 0.483$; $p = 0.654$). As expected, the inclusion of the paracingulate gyrus increased the overall volume. The mean (SEM) grey matter volume of the cingulate gyrus (including the paracingulate gyrus) was 9.01cm^3 (0.68) and 9.03cm^3 (0.89) for the left and right hemispheres respectively (see table 2.3). Again, no asymmetry in volume was observed between the hemispheres ($t(3) = -0.043$; $p = 0.968$). There were no significant correlations between the left and right hemispheres (CG, $p=0.677$; CG+PCG, $p=0.099$).

2.4 Results - Estimation of neuronal and glial density and neuronal volume in the anterior cingulate cortex

2.4.1 Hemispheric asymmetries

Each of the data sets satisfied the Kolmogorov-Smirnov test for normal distribution. Paired t-tests for comparisons within different levels (collapsing the layer data) revealed no significant asymmetries for any of the variables in levels 1, 2, 3, and 5. At level 4 an asymmetry was observed for neuronal density, but not for any other variable. The left hemisphere demonstrated a greater mean value for neuronal density ($t(4) 4.494$; $p=0.011$). When this finding was explored further by examining the involvement of each

layer, it became apparent that layer V was driving this result ($t(4) = 7.461$; $p = 0.002$), as all other layers failed to reach significance.

Despite this one positive result, the remaining data failed to show any convincing evidence towards left-right asymmetries at any level of area 24. For this reason the decision was taken to average the hemisphere data for all subsequent analyses.

Numerous analyses are reported in the following sections. In an attempt to avoid confusion, whilst the main effects from the repeated measures ANOVA are detailed in full, the individual p values from each paired t -test have been omitted. The exact p values are instead presented in tables 2.6 - 2.11. Similarly, due to the large number of comparisons, significant effects have not been annotated onto the graphs. Again, please refer to each accompanying results table. The mean (SEM) neuronal density, glial density and neuronal size data are summarised in table 2.4.

2.4.2 Analysis of cortical depth and layer depth

The repeated measures ANOVA for cortical depth revealed a significant main effect of level: $F(4,12) = 13.863$; $p < 0.001$ (see table 2.5). Further exploration by way of paired t -tests indicated that the subgenual levels differed significantly from the supragenual levels but not from each other. Comparisons between any of the supragenual levels did not achieve significance. Overall, cortical depth was thinner in the subgenual region than the supragenual region (see table 2.6 and figure 2.7).

The repeated measures ANOVA for layer depth (as a percentage of total cortical depth) failed to reach significance for the main factor of level ($p = 0.253$), although the interaction between level and layer was highly significant: $F(18,48) = 21.891$; $p < 0.001$ (table 2.5). Subsequent paired t -tests, comparing each layer between the different levels, indicated that this effect was due to differences between subgenual (1 and 2) and supragenual (3-5)

levels. No significant layer effects were observed for comparisons within either the subgenual regions, or the supragenual regions. Layer II in subgenual levels differed from layer II in supragenual levels 3 and 5 (but not 4), with a greater percentage layer depth. Layer V percentage depth was also higher in supragenual levels 3-5 compared to subgenual levels 1 and 2. Layer III showed a greater percentage layer depth in subgenual levels, but this failed to reach significance. Layers I and VI were unchanged between the two groups (see table 2.7 and figure 2.7).

2.4.3 Analysis of neuronal density

The repeated measures ANOVA for neuronal density did not find a significant effect of level ($p=0.370$), although again, the interaction between level and layer was significant: $F(12,36) = 5.203$; $p<0.001$ (table 2.5). As there was no overall effect of level, simple t-tests comparing anatomical level were not performed and instead, the individual layers within each level were investigated.

Paired t-tests of individual layers revealed a difference in neuronal density that specifically affected the deep cortical layers in comparisons between subgenual and supragenual regions. Neuronal density was higher in cortical layers V and VI in the subgenual regions in the following level comparisons: levels 1 and 3 (layers V and VI), levels 1 and 4 (layer VI only), and between levels 2 and 3 (layer VI only). Although not reaching any formal measure of significance, all other comparisons between these two regions also pointed to a higher neuronal density in layers V and VI of the subgenual levels (see table 2.8 and figure 2.8).

2.4.4 Analysis of glial density

The repeated measures ANOVA for glial density revealed a significant effect of level ($F(4,12) = 12.547$; $p < 0.001$), but not for the interaction between level and layer ($p = 0.191$) (see table 2.5). Consequently, a simple paired t-test comparing the various anatomical levels was conducted, without any further examination of the separate layers.

Paired analyses revealed that the overall significance was largely due to differences between the subgenual and supragenual levels. Significant results were seen for t-tests between subgenual level 2 and supragenual levels (3-5), where a lower glial density was apparent in the subgenual region. A lower glial density was also observed, to a lesser degree, in comparisons between subgenual level 1 and supragenual levels (3-5).

Analyses within any of the supragenual levels were not significant. A modest difference in glial density was observed between subgenual levels 1 and 2 (see table 2.9 and figure 2.9).

2.4.5 Analysis of neuronal size

The repeated measures ANOVA revealed a significant effect of level ($F(4,12) = 10.016$; $p = 0.001$), and also a significant interaction between level and layer: $F(12,36) = 11.468$; $p < 0.001$ (see table 2.5). Initially, a simple paired t-test was employed to compare mean neuronal size between the different levels of area 24.

Simple paired analyses comparing neuronal size between all anatomical levels again revealed that the subgenual levels were different in respect to the supragenual levels. Smaller neurons were observed in subgenual level 2 compared to supragenual levels 3, 4 and 5. Smaller neuronal sizes also observed, although to a lesser degree, in analyses between subgenual level 1 and all three supragenual levels. No significant results were observed for comparisons within the subgenual regions, or within the supragenual regions (see table 2.10 and figure 2.10)

As a significant interaction between level and layer was also seen in the ANOVA, further paired analyses were used to investigate the individual layers within the levels of interest. Overall, it appeared that layers II, V and VI were altered, albeit to different extents, displaying smaller neuronal sizes in the subgenual levels than in the supragenual levels. Subgenual level 1 showed a fairly modest difference, demonstrating smaller neurons within layers II, V and VI at various level comparisons. Analyses of subgenual level 2 revealed more robust changes, particularly when compared to supragenual level 5. Neuronal size in layer III did not appear to differ between the subgenual and supragenual levels (see table 2.11 and figure 2.10).

2.4.6 Summary of results

Overall, the subgenual region demonstrated less glia and smaller neurons compared to the supragenual region of area 24b. Neuronal density was moderately higher in the deep layers of the subgenual region than in the deep layers of the supragenual region. Cortical depth was thinner in the subgenual region than in the supragenual region. These results are illustrated in figure 2.11.

Table 2.3.

Grey matter volume (cm³) in the cingulate gyrus. CE values are shown in italics.

		A	B	C	D	E	Mean (SEM)
CG	Left	7.02	5.75	7.60	6.19	6.95	6.70 (0.33)
		<i>0.09</i>	<i>0.06</i>	<i>0.10</i>	<i>0.11</i>	<i>0.07</i>	
	Right	7.15	6.11	5.29	6.80	6.89	6.45 (0.34)
		<i>0.08</i>	<i>0.06</i>	<i>0.10</i>	<i>0.09</i>	<i>0.06</i>	
CG + PCG	Left	11.03	-	8.29	8.43	8.28	9.01 (0.68)
		<i>0.07</i>	-	<i>0.08</i>	<i>0.07</i>	<i>0.06</i>	
	Right	11.40	-	7.11	8.74	8.85	9.03 (0.89)
		<i>0.07</i>	-	<i>0.10</i>	<i>0.10</i>	<i>0.05</i>	

CG = cingulate gyrus; PCG = paracingulate gyrus.

Table 2.4.

Mean (SEM) cortical depth, percentage layer depth, neuronal density, neuronal size and glial density in cortical layers II-VI, in the five anatomical levels of area 24 (see table 2.2).

Level	Layer	Cortical depth, μm	Layer depth (%)	Neuronal density, cells / mm ³ , x10 ³	Neuronal size, μm ³	Glial density, cells / mm ³ , x10 ³
Level 1	I		12.4 (1.1)	-	-	-
	II		9.9 (0.1)	105.0 (3.5)	855.7 (91.7)	63.6 (7.9)
	III		30.5 (0.9)	76.4 (1.7)	1417.4 (137.2)	66.1 (5.6)
	V		17.3 (0.1)	99.2 (3.3)	1849.9 (140.2)	70.4 (4.3)
	VI		29.9 (1.0)	81.8 (3.9)	1455.1 (102.6)	87.8 (8.4)
	average	1892.4 (161.8)		90.5 (3.3)	1394.5 (105.9)	72.0 (3.8)
Level 2	I		12.4 (0.6)	-	-	-
	II		9.1 (0.2)	111.0 (1.7)	873.2 (52.5)	60.3 (7.1)
	III		30.3 (1.5)	80.5 (2.3)	1460.5 (68.5)	61.9 (5.5)
	V		18.2 (1.3)	95.7 (5.4)	1957.8 (74.2)	75.6 (5.4)
	VI		30.0 (1.2)	70.6 (2.4)	1574.6 (48.7)	84.3 (7.7)
	average	2170.5 (173.2)		89.4 (3.8)	1466.5 (93.6)	70.5 (3.7)
Level 3	I		11.2 (0.7)	-	-	-
	II		7.9 (0.2)	112.0 (7.5)	1284.3 (72.7)	75.1 (4.3)
	III		27.8 (0.9)	79.7 (1.8)	2049.7 (228.3)	84.1 (3.3)
	V		24.1 (1.1)	79.7 (2.2)	3680.1 (459.6)	94.2 (2.1)
	VI		28.9 (1.3)	57.4 (2.5)	2544.4 (238.6)	101.0 (5.0)
	average	2562.2 (186.6)		81.8 (4.1)	2647.7 (235.7)	89.9 (2.5)
Level 4	I		11.9 (1.0)	-	-	-
	II		9.1 (0.2)	112.0 (5.5)	1207.2 (130.2)	69.9 (2.3)
	III		27.2 (0.9)	77.4 (3.9)	2076.6 (266.7)	83.6 (4.5)
	V		24.5 (0.9)	81.9 (3.8)	3778.5 (374.5)	90.7 (4.2)
	VI		27.4 (1.1)	60.9 (2.5)	2397.3 (246.9)	107.0 (5.7)
	average	2612.1 (130.7)		82.9 (4.03)	2647.6 (237.6)	88.5 (3.1)
Level 5	I		10.3 (0.4)	-	-	-
	II		8.4 (0.2)	111.0 (7.1)	1189.0 (59.1)	73.8 (5.2)
	III		27.8 (0.9)	78.2 (3.2)	2020.8 (146.9)	82.5 (4.3)
	V		25.4 (0.9)	81.6 (4.3)	3682.2 (278.9)	98.1 (5.6)
	VI		28.0 (0.9)	59.3 (3.7)	2355.9 (165.7)	97.8 (2.8)
	average	2522.6 (120.8)		82.3 (4.2)	2586.0 (218.3)	89.9 (2.9)

Table 2.5. Repeated measures ANOVA.

Results of repeated measures ANOVA (cortical layer and anatomical level included as ‘within subject’ factors).

Repeated Measures ANOVA						
	Level		Layer		Level x Layer	
	F	p	F	p	F	p
Cortical Depth	13.86	<.001	-	-	-	-
Layer Depth (%)	1.54	.253	100.32	<.001	9.63	<.001
Neuronal Density	1.176	.370	64.37	<.001	5.20	<.001
Neuronal Size	10.02	.001	48.45	<.001	11.47	<.001
Glial Density	12.55	<.001	18.02	<.001	2.03	.191

See table 2.6 for a level analysis of cortical depth.

See table 2.7 for a level x layer analysis of percentage layer depth

See table 2.8 for a level x layer analysis of neuronal density

See table 2.9 for a levels analysis of glial density

See table 2.10 for a level and table 2.11 for a level x layer analysis of neuronal size.

Table 2.6. Total cortical depth.

Analysis of total cortical depth in different anatomical levels of area 24. Paired t-tests used for comparisons of cortical depth between different anatomical levels. These data are illustrated in figure 2.7.

Total cortical depth			
Level comparisons ¹	Paired t-test		
	t	p	Percentage difference
1 v. 2	-2.73	.072	13%
1 v. 3	-8.69	.003	26%
1 v. 4	-6.05	.009	28%
1 v. 5	-10.04	.002	25%
2 v. 3	-5.65	.005	15%
2 v. 4	-2.86	.046	17%
2 v. 5	-5.57	.005	14%
3 v. 4	-0.36	.738	2%
3 v. 5	0.51	.640	1%
4 v. 5	0.68	.532	4%

¹ subgenual levels (1 and 2), supragenual levels (3, 4 and 5)

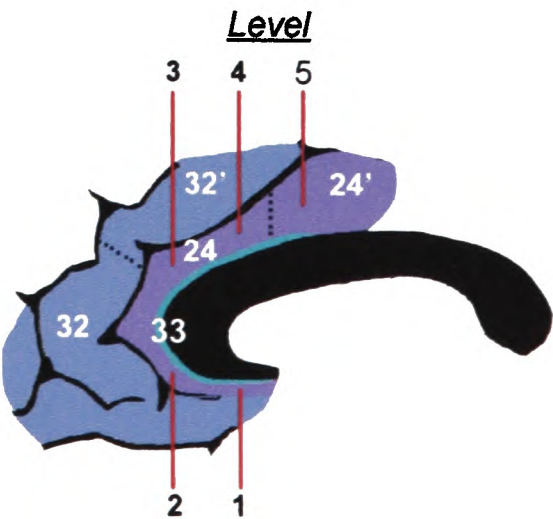


Table 2.7. Cortical layer depth as a percentage of cortical depth.

Analysis of cortical layer depth (as a percentage of total cortical depth) in different anatomical levels of area 24. Paired t-tests used for comparisons of percentage layer depth between different anatomical levels. These data are illustrated in figure 2.7.

Layer depth (expressed as percentage of total cortical depth)										
Level		Paired t-test statistics								
Comparisons ¹	Layer I		Layer II		Layer III		Layer V		Layer VI	
	t	p	t	p	t	p	t	p	t	p
1 v. 2	-0.62	.954	3.08	.055	-0.26	.809	-0.34	.754	0.21	.849
1 v. 3	1.05	.373	37.85	.001	3.91	.030	-4.92	.016	2.44	.092
1 v. 4	0.02	.983	2.19	.006	3.25	.048	-5.74	.011	5.31	.013
1 v. 5	1.43	.248	8.44	.003	3.72	.034	-7.22	.005	1.58	.211
2 v. 3	1.45	.221	5.07	.007	3.06	.038	-4.94	.008	1.16	.311
2 v. 4	0.55	.611	-0.02	.985	3.22	.032	-7.98	.001	2.56	.063
2 v. 5	3.02	.039	3.24	.032	2.70	.054	-6.59	.003	2.83	.047
3 v. 4	-1.54	.199	-3.51	.025	0.98	.383	-0.80	.467	1.99	.116
3 v. 5	1.48	.214	-1.68	.168	-0.03	.980	-2.99	.041	1.09	.338
4 v. 5	1.90	.130	1.79	.147	-0.85	.444	-1.70	.164	-0.58	.594

¹ subgenual levels (1 and 2), supragenual levels (3, 4 and 5)

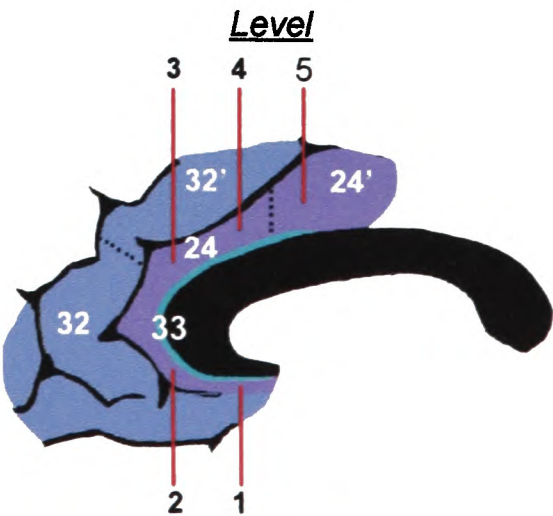


Figure 2.7.

Graphs showing (A) cortical depth and, (B) layer depth (as a percentage of cortical depth) in the five anatomical levels of area 24. See tables 2.5, 2.6 and 2.7 for statistical comparisons between levels.

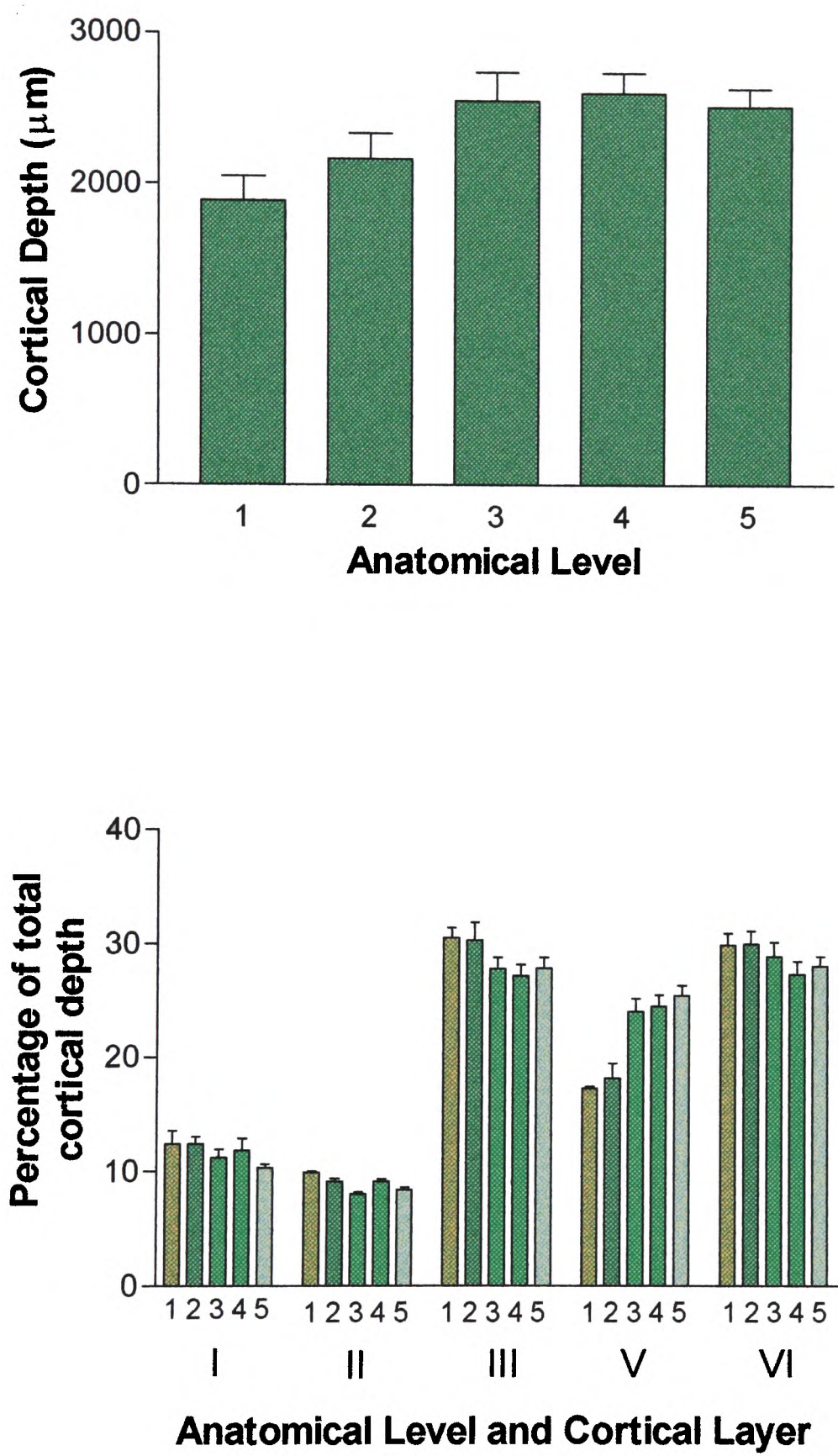


Table 2.8. Neuronal density.

Analysis of neuronal density in layers II-VI in different anatomical levels of area 24. Paired t-tests used for comparisons of neuronal density within individual cortical layers between the different anatomical levels of area 24. These data are illustrated in figure 2.8.

Neuronal Density								
Level	Paired t-test statistics							
Comparisons ¹	Layer II		Layer III		Layer V		Layer VI	
	t	p	t	p	t	p	t	p
1 v. 2	-2.50	.088	-0.67	.550	0.31	.778	5.82	.010
1 v. 3	-0.54	.624	-1.49	.233	3.29	.046	3.67	.035
1 v. 4	-0.83	.470	-0.22	.839	2.43	.093	3.18	.050
1 v. 5	-0.71	.528	-0.84	.462	2.53	.085	2.73	.072
2 v. 3	-0.19	.853	0.26	.808	2.14	.099	3.34	.029
2 v. 4	-0.24	.820	0.55	.609	1.80	.146	2.58	.061
2 v. 5	-0.08	.943	0.47	.665	1.85	.138	2.28	.085
3 v. 4	0.06	.958	0.58	.592	-0.75	.493	-2.57	.072
3 v. 5	0.37	.732	0.61	.574	-0.52	.628	-0.84	.449
4 v. 5	0.30	.778	-0.42	.700	0.13	.905	1.06	.349

¹ subgenual levels (1 and 2), supragenual levels (3, 4 and 5)

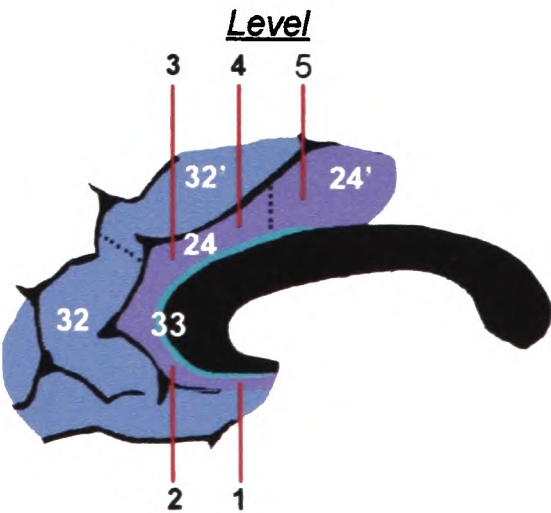


Figure 2.8.

Graphs showing neuronal density in (A) the five anatomical levels of area 24, (B) in cortical layers II-VI in the five anatomical levels of area 24. See tables 2.5 and 2.8 for statistical comparisons between levels.

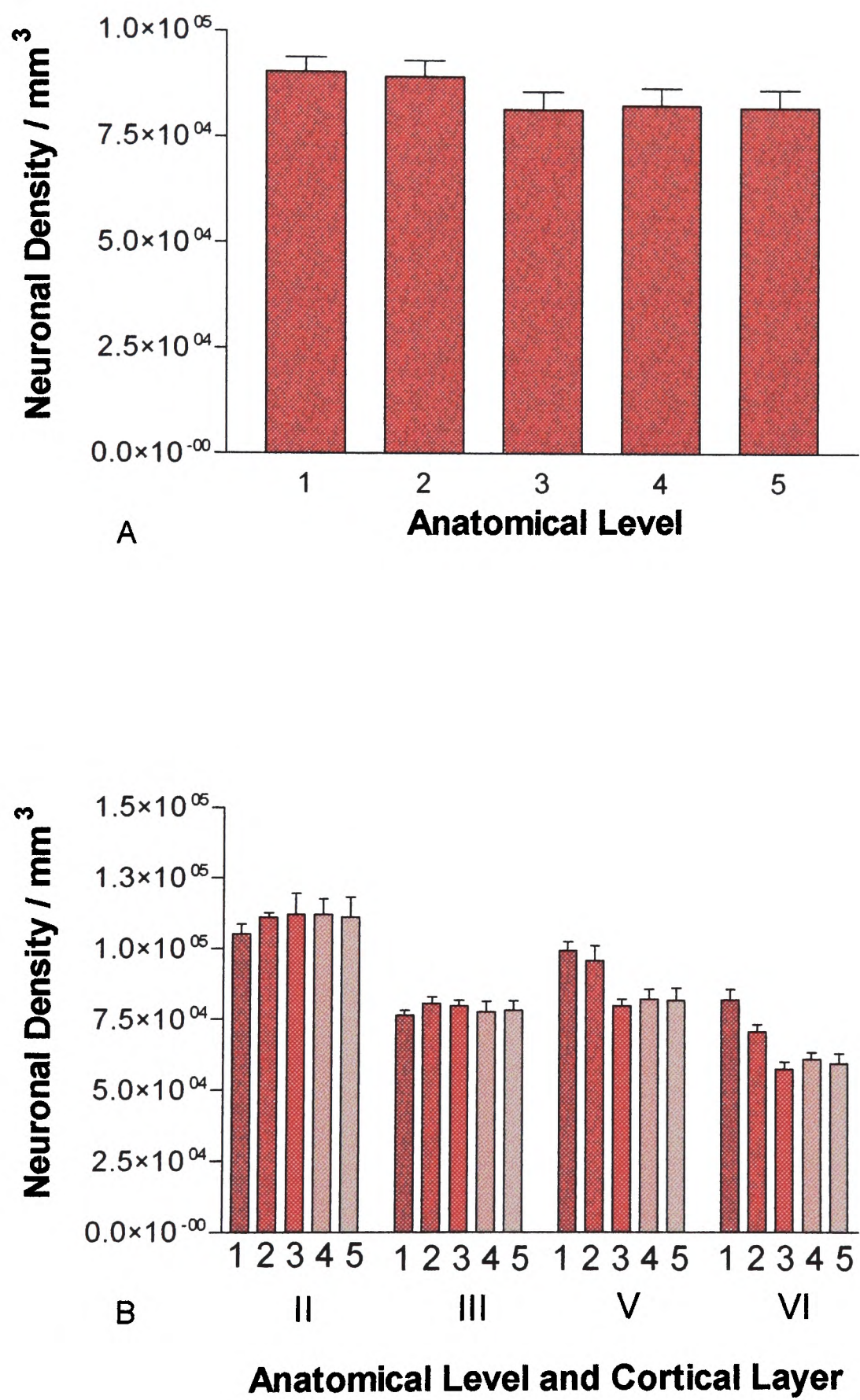


Table 2.9. Glial density.

Analysis of glial density in different anatomical levels of area 24. Paired t-tests used for comparisons of glial density between different anatomical levels. These data are illustrated in figure 2.9.

Glial Density			
Level comparisons ¹	Paired t-test		
	t	p	Percentage difference
1 v. 2	4.02	.028	1%
1 v. 3	-3.25	.048	20%
1 v. 4	-3.64	.036	18%
1 v. 5	-3.25	.048	20%
2 v. 3	-4.49	.011	22%
2 v. 4	-5.49	.005	20%
2 v. 5	-5.39	.006	21%
3 v. 4	0.74	.501	1%
3 v. 5	0.07	.951	0%
4 v. 5	-0.58	.596	1%

¹ subgenual levels (1 and 2), supragenual levels (3, 4 and 5)

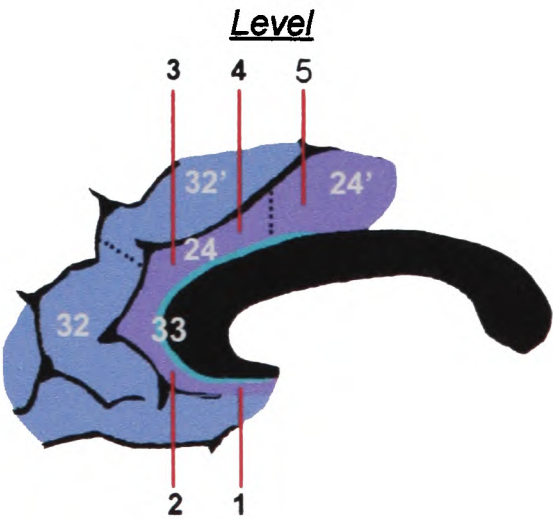


Figure 2.9.

Graph showing glial density in the five anatomical levels of area 24. See tables 2.5 and 2.9 for statistical comparisons between levels.

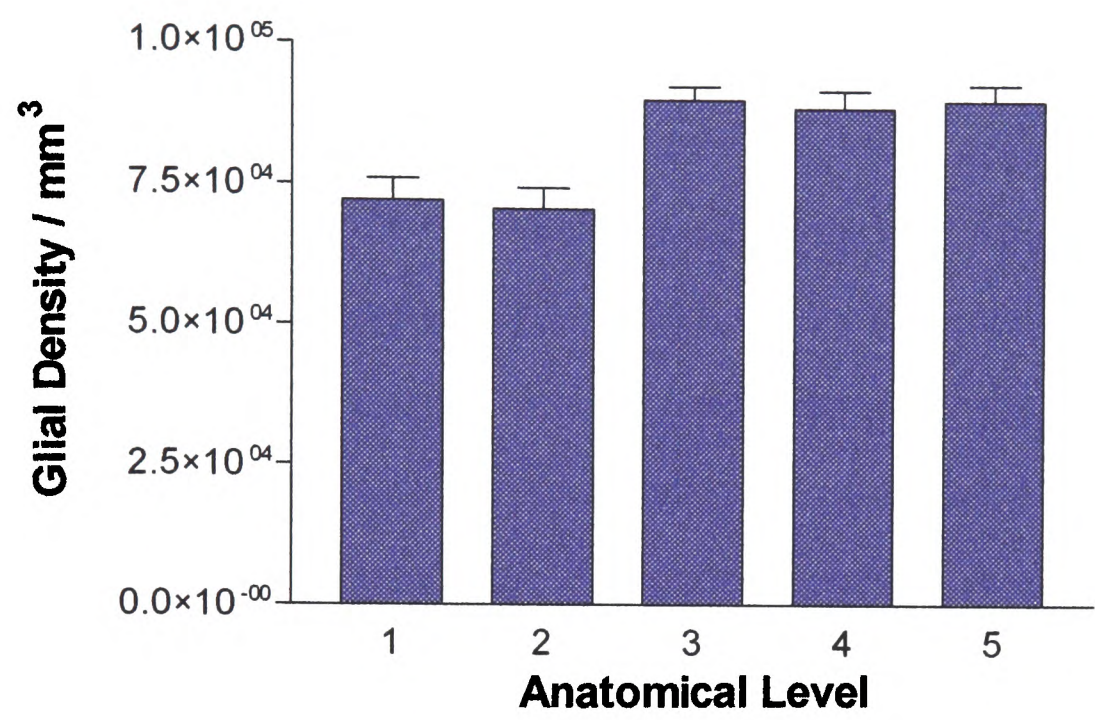


Table 2.10. Neuronal size.

Analysis of neuronal size in different anatomical levels of area 24. Paired t-tests used for comparisons of neuronal size between different anatomical levels. These data are illustrated in figure 2.10.

Neuronal size			
Level comparisons ¹	Paired t-test		
	t	p	Percentage difference
1 v. 2	-0.56	.616	5%
1 v. 3	-3.11	.053	47%
1 v. 4	-3.59	.037	47%
1 v. 5	-4.49	.021	46%
2 v. 3	-4.45	.011	45%
2 v. 4	-4.47	.011	45%
2 v. 5	-6.81	.002	42%
3 v. 4	0.00	.999	0%
3 v. 5	0.53	.625	2%
4 v. 5	0.58	.591	2%

¹ subgenual levels (1 and 2), supragenual levels (3, 4 and 5)

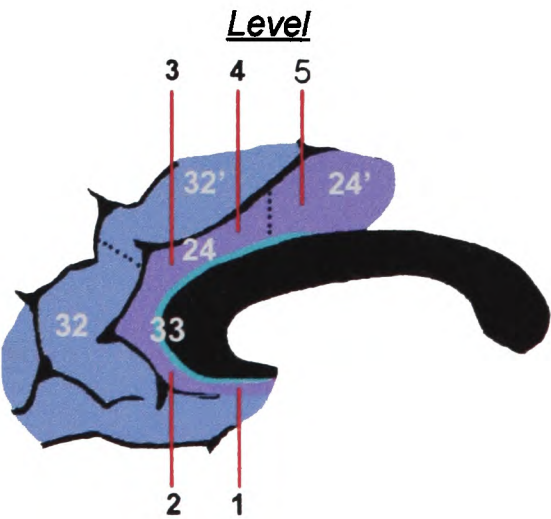


Table 2.11. Neuronal size.

Analysis of neuronal size in layers II-VI in different anatomical levels of area 24. Paired t-tests used for comparisons of neuronal size within individual cortical layers between the different anatomical levels of area 24. These data are illustrated in figure 2.10.

Neuronal Size								
Level	Paired t-test statistics							
Comparisons ¹	Layer II		Layer III		Layer V		Layer VI	
	t	p	t	p	t	p	t	p
1 v. 2	-0.64	.566	-0.41	.710	-0.45	.681	-0.84	.462
1 v. 3	-3.93	.029	-1.76	.177	-3.03	.056	-3.20	.049
1 v. 4	-1.92	.151	-1.89	.156	-4.47	.021	-2.98	.058
1 v. 5	-2.59	.081	-2.23	.103	-5.10	.015	-4.29	.023
2 v. 3	-6.37	.003	-2.28	.085	-4.34	.012	-4.01	.016
2 v. 4	-2.64	.057	-2.13	.101	-5.59	.005	-3.34	.029
2 v. 5	-4.28	.013	-3.39	.027	-7.81	.001	-5.22	.006
3 v. 4	1.05	.352	-0.20	.549	-0.44	.686	1.88	.133
3 v. 5	2.80	.055	0.23	.827	-0.01	.992	1.21	.292
4 v. 5	0.25	.817	0.44	.684	0.74	.502	0.34	.748

¹ subgenual levels (1 and 2), supragenual levels (3, 4 and 5)

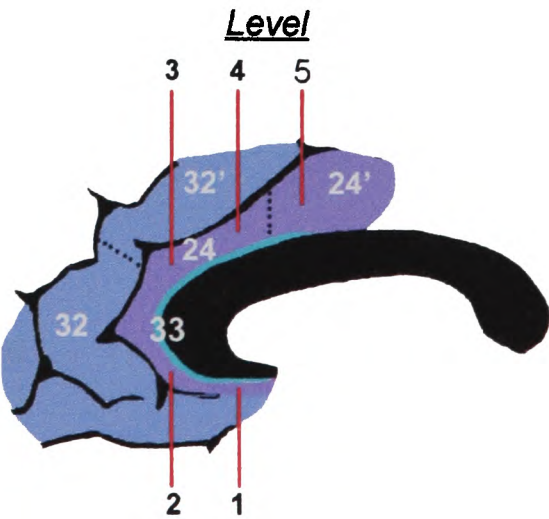


Figure 2.10.

Graphs showing neuronal size in (A) the five anatomical levels of area 24, (B) in cortical layers II-VI in the five anatomical levels of area 24. See tables 2.5, 2.10 and 2.11 for statistical comparisons between levels.

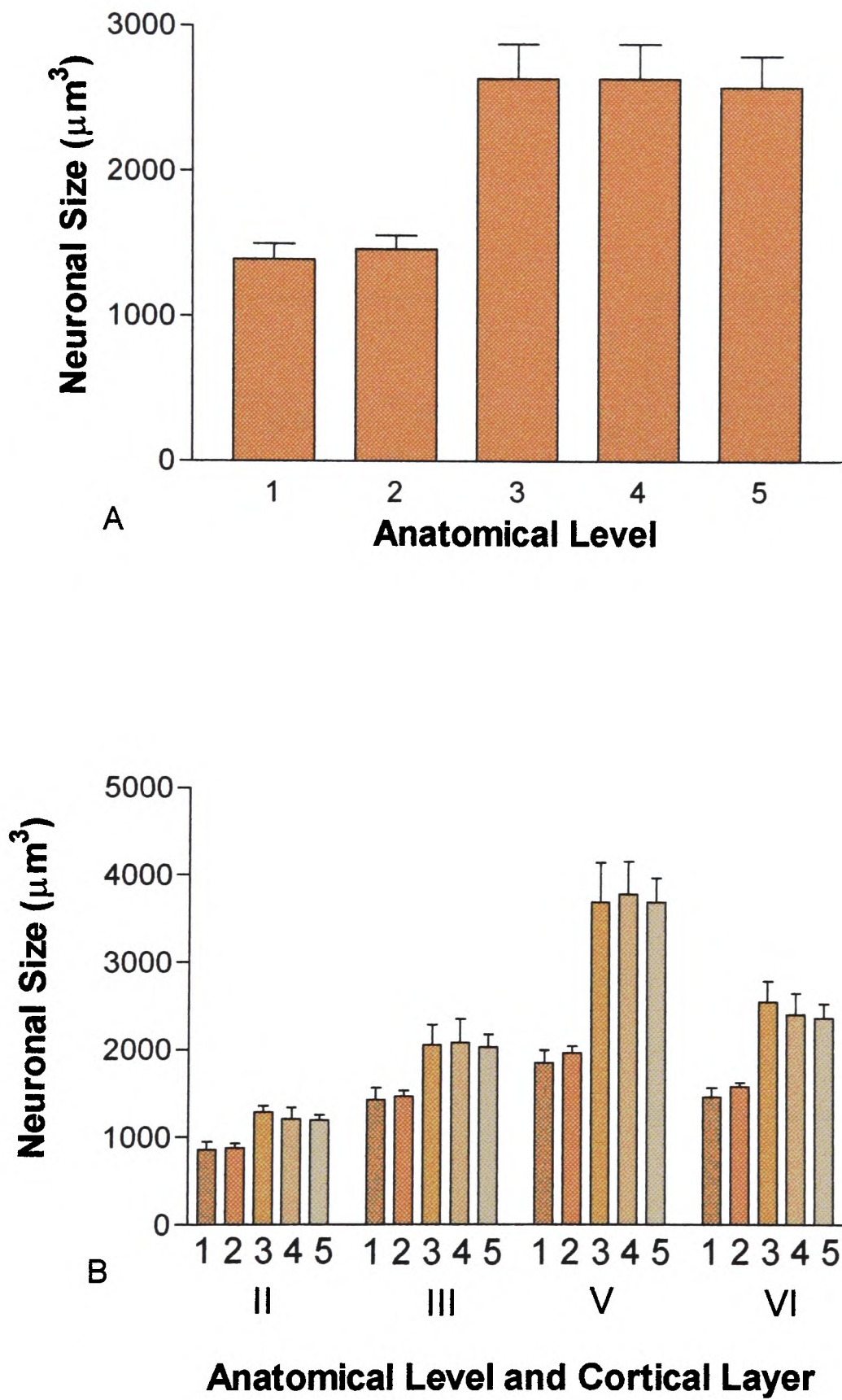
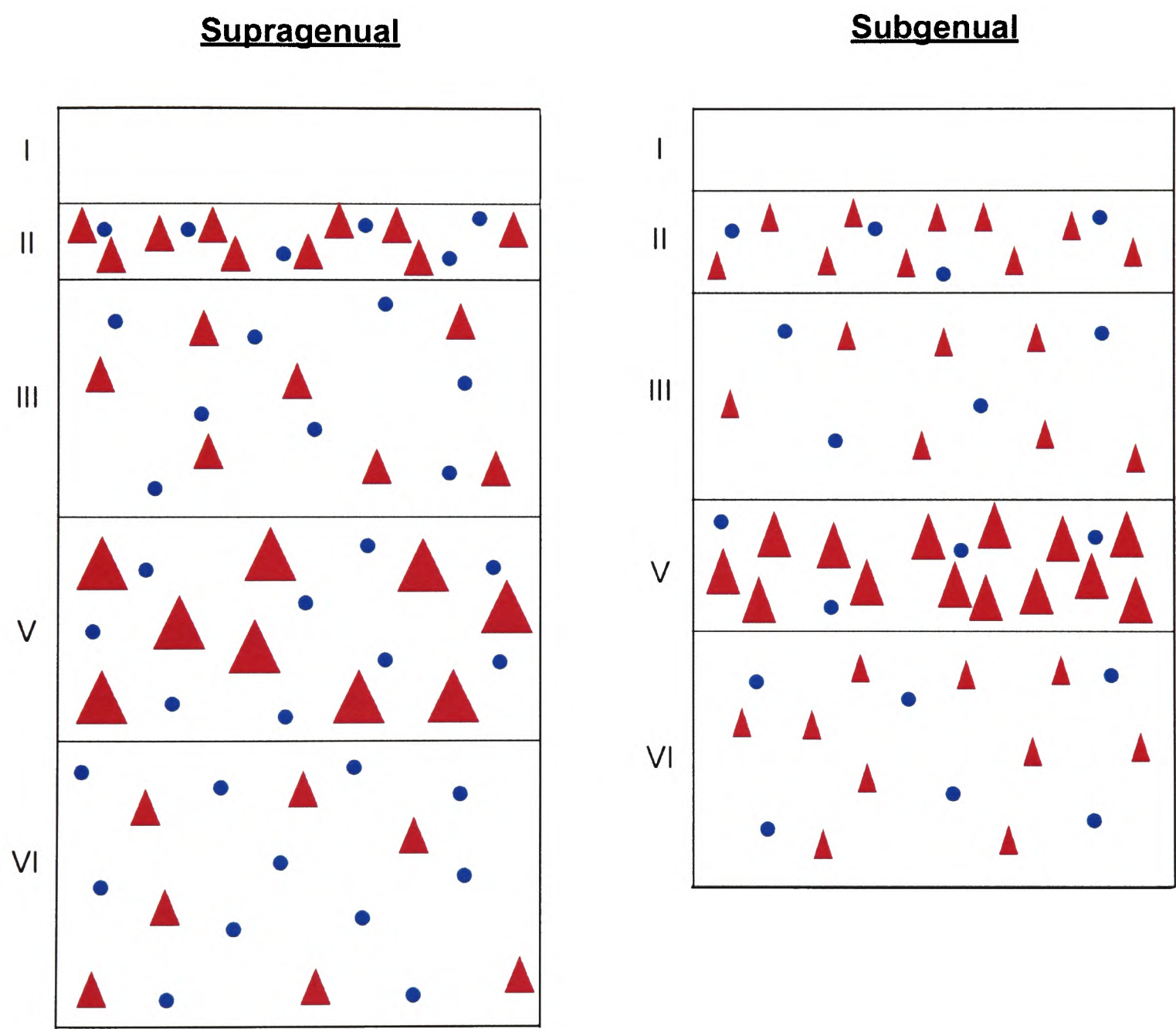


Figure 2.11.

Schematic diagram summarising the main results from the analyses described in this chapter. In comparison to the supragenual region of area 24, the subgenual region demonstrated: 1) thinner cortical depth; 2) higher percentage depth for layer II; 3) lower percentage depth for layer V; 4) greater neuronal density in layers V and VI; 5) lower glial density in all cortical layers; and 6) smaller neurons in all cortical layers.



2.5 Discussion – Grey matter volume in the cingulate gyrus

2.5.1 Reliability of the current findings and comparison with previous studies

The present study employed the Cavalieri principle to obtain volume estimates of the grey matter in the cingulate gyrus. As this study used brain material from which the position of the first cutting plane was uniform random, and the correct mathematical principles were adhered to; the current estimates of volume are free from either systematic or sampling bias. CE values achieved in this study are within the accepted range and therefore indicative of both the adequacy and reliability of the sampling scheme used.

No other post mortem study has performed a normative study investigating the volume of the cingulate gyrus, so comparisons have to be drawn from imaging data, as well as studies investigating cingulate volume within the context of brain disorders. The existing literature shows considerable variation in reported volumes and methodological descriptions are often insufficient; the delineation of anatomical boundaries (particularly, the paracingulate and subgenual cortices and grey/white matter segmentation) frequently remains unclear.

Neuroimaging studies have reported volumes that range from 2-6cm³ for the rostral division and 2-5.5cm³ for the caudal division of the ACC (Crespo Facorro *et al.* 1999; Haznedar *et al.* 2000; Takahashi *et al.* 2002; Takahashi *et al.* 2003). Measured as a single entity, volumes of the ACC range from 5.8–9.5cm³ (Goldstein *et al.* 1999; Highley *et al.* 2001; Szeszko *et al.* 1999). A MRI study assessing the volume of the entire cingulate gyrus (grey and white matter) reported a volume of approximately 15cm³ (Convit *et al.* 2001). In a recent post mortem study, the subgenual cortex was reported to have a mean volume of 0.2cm³ (Öngür *et al.* 1998). The most comparable study to date is that of Highley *et al.* (2001), where the volume of the anterior cingulate gyrus was estimated (using the Cavalieri principle in post mortem tissue) to be approximately 8.6-9.5cm³. This

is nearly equal to the measurements of total volume achieved in the present study; however, it is unclear whether the paracingulate gyrus and/or white matter were included in their measurements.

Although there is considerable diversity between these reports, the volume estimates in the present study are in general agreement with the existing literature. Much of the discrepancy between these studies is probably due to imprecision and differences in approach to anatomical delineation. Volume estimates using planimetric and stereological approaches have been compared (Cotter *et al.* 1999; Howard *et al.* 2003), and both seem to yield very similar results, although the stereological approach is often more efficient and less biased.

Demographic differences between the subjects examined could also contribute to variation between studies. A recent MRI study has reported a global decrease in grey matter volume with declining age, with accelerated loss observed bilaterally in the grey matter of the cingulate gyrus (Good *et al.* 2001). Concerning post mortem tissue, perimortem factors such as death to fixation delay, duration of time in fixative and the mode of embedding can all have effects on tissue volume (Haug 1984; Kretschmann *et al.* 1982; Quester and Schroder 1997; Small and Peterson 1982). Such factors could also contribute to inconsistencies between post mortem and imaging studies.

2.5.2 Limitations of the current study

The most obvious limitation of this work is its small sample size, which would ideally be expanded to allow exploration into the effects of gender and age. As previously discussed, perimortem factors can all potentially influence tissue volume and should ideally be investigated separately and included as covariates where necessary. Due to the small sample size in this study, such analyses were not possible.

As the brains used in this study had been previously sectioned during autopsy, this precluded any decisions regarding slice thickness and also prevented an examination of the medial surface of the brain. Ideally, the mean slice thickness would have been smaller, thus allowing volume be estimated from a greater number of slices and providing greater accuracy. Even though the CE values in this study are acceptable, they could be improved upon. It is also possible that thinner coronal slices would have facilitated the distinction between anterior and posterior cingulate cortices. Unfortunately observations regarding the medial macroscopic features and sulcal patterns, with particular reference to the prominence of the PCS and interhemisphere differences, were not feasible without photographs of the medial surface of the brain.

2.5.3 Future directions – implications for mood disorder research

Recent post mortem studies of mood disorder have revealed altered cell densities in the ACC (Benes *et al.* 2001; Bouras *et al.* 2001; Cotter *et al.* 2002a; Cotter *et al.* 2001a; Öngür *et al.* 1998). Given that differences in morphological features and sulcal patterns are thought to reflect differences in underlying structural organisation and connectivity (Van Essen 1997; Vogt *et al.* 1995); it would be interesting to examine the gross morphology and pattern of sulcal folding in the cingulate gyrus of mood disorder patients. The volume of the cingulate gyrus, and the occurrence/volume of the paracingulate gyrus have already been successfully explored in the brains of schizophrenic patients (Convit *et al.* 2001; Noga *et al.* 1995; Szeszko *et al.* 1999; Takahashi *et al.* 2002; Takahashi *et al.* 2003); morphological and volumetric studies of this nature may be equally applicable to mood disorder research. Recent MRI studies have already revealed significant volume reductions of the subgenual cortex in the brains of mood disorder patients (Botteron *et al.* 2002; Drevets *et al.* 1997; Hirayasu *et al.* 1999). This reduction has been subsequently corroborated by a post mortem study (Öngür *et al.* 1998). Similar comparisons in the supragenual ACC are now warranted.

2.6 Discussion - Neuronal and glial density and neuronal size in the anterior cingulate cortex

2.6.1 Altered cortical depth and cellular composition in different levels of the anterior cingulate cortex

The results of the current study suggest that the subgenual region of area 24b has a subtle, but clearly different, cellular composition to the supragenual region of area 24b. Total cortical depth was thinner in the subgenual cortex, and percentage layer depth was higher for layer II and lower for layer V. A modest difference in neuronal density was also observed, with a higher density in the deep layers of the subgenual cortex compared to the deep layers of the supragenual cortex. The subgenual region also demonstrated less glia and smaller neurons in comparison to the supragenual region of area 24 (see figure 2.11). Concerning variation along the rostrocaudal extent of area 24, no significant differences were observed either between the supragenual levels, or between the subgenual levels. Neuronal density was higher in the left hemisphere at level 4. As this was the only asymmetry observed it is likely to be a chance finding.

These results, both in their specificity and magnitude were unexpected, largely because the supragenual and subgenual regions assessed in this study both belonged to Brodmann area 24. The next two sections consider the reliability of these findings, firstly in comparison to work detailing the cytoarchitecture of the ACC and secondly to stereological studies investigating the cellular composition of the ACC in the context of psychiatric disorders. In the final sections of this discussion I consider the functional implications of this work.

2.6.2 Comparison with previous studies – descriptive studies

In their seminal paper describing the cytoarchitecture of the human cingulate cortex, Vogt *et al* (1995) comprehensively discussed the subtle variations between different Brodmann areas and their subdivisions; however, little emphasis was placed on the differences

between supragenual and subgenual levels of area 24. It is not clear why this is. One explanation could be that the subgenual region of area 24 was not examined separately in this study. An alternative explanation is that no appreciable differences were observed between the subgenual and rostral regions of area 24 and consequently no differences were reported. The supragenual cortex in the present study exhibited a laminated structure and dorsosventral differentiation consistent with the work by Vogt *et al* (1995), and the subdivisions of area 24 and layers therein were relatively straightforward to delineate. The staining characteristics of the subgenual region in this study were largely consistent with a more recent publication (Öngür *et al.* 2003), which reported that subgenual area 24b demonstrated a well-laminated cortex with clear, definable layers. I found the lamina structure of the subgenual cortex to be similar to its supragenual counterpart, although the layers in the subgenual cortex were slightly more difficult to define. The distinction between layers Va and Vb was particularly difficult and consequently I did not attempt to delineate these two divisions of layer V.

Descriptions of the rostral (24) and caudal (24') aspects of the supragenual ACC, and in particular their relative cell densities, are covered in greater detail by Vogt *et al* (1995). Overall, rostral area 24 is described as having a greater neuronal density compared to caudal area 24' and layers III and Va are reportedly thinner in more caudal levels of 24b. In the present study, levels 3 and 4 incorporate rostral area 24b, and level 5 approximates to caudal area 24b' (see figures 2.2 and 2.3). This study did not provide any evidence to support an increased neuronal density in rostral levels of area 24b in the supragenual ACC, nor was there any evidence to support alterations in layer depth between the two areas. Vogt *et al* indicated in their 1995 paper that the greatest differentiation in rostrocaudal levels (particularly in respect to cell density) was observed in areas 24a-24a' and 24c-24c'. Only rostral and caudal levels of area 24b were examined in the present study, and this might go some way in explaining the discrepancy between the two studies. It doesn't, however, explain why I didn't find changes in cortical layer depths between the

rostral and caudal levels of area 24b. A further and perhaps more plausible explanation to the apparent inconsistencies is the small sample size used in the present study. Such small numbers may not have been sufficient to detect subtle progressive changes between the rostral and caudal levels of area 24. Unfortunately, as Vogt *et al* (1995) did not remark on observations concerning glial density or neuronal size, such comparisons cannot be made with my work.

Recent studies have reported the presence of spindle shaped neurons in layer Vb of area 24 (Hof *et al.* 2001; Nimchinsky *et al.* 1995), observed most frequently in 24a, but also consistently seen in area 24b and occasionally in area 24c (Vogt *et al.* 1995). In agreement with these accounts, spindle neurons were readily detected in the present study; they were confined to layer Vb and were seen with increased frequency in the more ventral subdivisions (a>b>c) of area 24. These neurons were most abundant in rostral levels of area 24, although they were intermittently observed at more caudal levels. Interestingly, spindle neurons were not restricted to the supragenual levels of area 24, but were also observed in the subgenual cortex where their distribution was again limited to deep layer V (see figures 2.4 and 2.6). This suggests that spindle neurons are not confined to supragenual levels of area 24, but are in fact characteristic of deep layer V throughout this area, with greatest prevalence in more rostral areas. Unfortunately, these specialised neurons were not assessed separately in the current work (as neurons were counted as a single entity), but this is a logical progression for further research.

2.6.3 Comparison with previous studies – stereological studies

The available quantitative data regarding the cellular composition of the normative ACC are provided by the normal control subjects included in investigations of this region in psychiatric disorders. Although the morphometry of the ACC has been investigated in several studies (Benes 1993; Benes *et al.* 1991; Benes *et al.* 2000; Benes *et al.* 2001; Bouras *et al.* 2001; Cotter *et al.* 2002a; Cotter *et al.* 2001a; Kalus *et al.* 1997; Öngür *et al.*

1998), the discussion here will concentrate on those which used stereological techniques and are therefore comparable to the current study. These data (along with the present study) are summarised in table 2.12.

A recent study of supragenual area 24b (Cotter *et al.* 2001a) reports values in normal subjects for neuronal and glial density that are very similar to the current study. In contrast, neuronal size appears to differ substantially between the two studies, with the current study finding larger neuronal volumes. Individual layer depths (expressed as a percentage of total cortical depth) in the present study are also very comparable to those reported by Cotter and colleagues. Bouras *et al* (2001) also investigated supragenual area 24, but they reported a much lower neuronal density throughout all layers. Additionally, this group assessed neuronal size, but as they measured neuronal diameter it is difficult to make direct comparisons between this and the current study.

Bouras *et al* (2001) also investigated neuronal density in the subgenual cortex. The density values reported for normal subjects are very similar to those achieved in the current study and help to substantiate the values attained here. In contrast, Öngür *et al* (1998) reported much lower values for neuronal density in the subgenual cortex. The same group also reported lower values for glial density than those observed in the present study.

Even though Bouras *et al* (2001) used tissue from both the supragenual and subgenual regions of area 24, no formal comparisons were made between these two regions. Further inspection of their data set reveals that neuronal density is greater in layers III-VI of subgenual area 24 and that the diameter of these neurons is smaller in comparison to supragenual area 24. These findings help to corroborate the results from the current study. Unfortunately, this group did not assess glial density. Another similarity between this and the current study is a thinner layer V in the subgenual region of area 24 compared

to supragenual area 24. Although direct comparisons of supragenual and subgenual areas should not be made between the different studies, it is noteworthy that glial density is much lower in subgenual area 24 (Öngür *et al.* 1998), than in supragenual area 24 (Bouras *et al.* 2001; Cotter *et al.* 2001a) (see table 2.12).

Even though there is considerable variability in the existing literature, the values presented in this study are not dissimilar to previous reports. This study also attained very good CE values for the density measures (data not shown), which helps to validate the adequacy of the sampling scheme. The estimates of cell size are somewhat different between this study and that of Cotter *et al.* (2001a), nevertheless, the range of volumes do overlap. The disparities between different studies are likely to be related to differences inherent to the tissue series, including anatomical, demographic and methodological differences (and in particular, different methods of tissue processing and embedding). Findings from the current study suggest that any inconsistencies within the existing literature are unlikely to have been caused by the varying anatomical positions from which tissue was sampled (within supragenual or subgenual regions), but that the subgenual versus supragenual distinction is critical.

Morphological studies have frequently reported asymmetries in the cingulate cortex, but when interhemisphere differences were investigated at the cellular level in this study, only one significant lateralisation was observed (a greater neuronal density in level 4 of the left hemisphere). Although this result was considered to be a chance finding, it is not unprecedented in the ACC. Cotter *et al.* (2001a) reported an increased neuronal density in the left hemisphere in their study of area 24b – which would be roughly equivalent to level 4 in the present study.

Table 2.12.

Summary of results of this and previous stereological studies measuring neuronal and glial density in the supragenual and/or subgenual regions of area 24.

		Cotter <i>et al</i> (2001a)* ^{1, 2}	Bouras <i>et al</i> (2001)*	Öngür <i>et al</i> (1998)*	Present Study ³
Neuronal Density	Layer				
<i>supragenual cortex</i>	II	192.3	57.2		112.5
	III	96.8	38.6		78.5
	V	102.43	38.7		81.3
	VI	60.6	42.2		59.3
	average	113.0	44.2		82.9
<i>subgenual cortex</i>	II		50.6		85.8
	III		46.5		73.3
	V		48.8		98.2
	VI		49.2		76.3
	average		48.8	25.3	83.4
Glial Density	Layer				
<i>supragenual cortex</i>	II	68.8			73.0
	III	84.4			83.3
	V	97.6			94.3
	VI	148.1			101.9
	average	99.7			88.1
<i>subgenual cortex</i>	II				61.9
	III				63.9
	V				73.1
	VI				85.9
	average			45.7	71.2

* data are from normal comparison subjects; ¹ average of left and right hemisphere data; ² data are median values; ³ supragenual indicates the average of levels 3-5, subgenual indicates the average of levels 1 and 2.

2.6.4 Functional considerations

The cingulate cortex is a highly complicated structure and accordingly, Brodmann's original account of the human cingulate cortex has been modified to accommodate the high level of heterogeneity present within this structure. Vogt *et al* (1995) have reported subdivisions in areas 24 and 23 that are progressively more differentiated in the dorsosventral plane, as well as subtle differences in cytoarchitecture between the rostral and caudal extents of area 24. Furthermore, immunohistochemical analyses have also alluded to areas of transition, with progressive changes along the dorsosventral and rostrocaudal axes of the cingulate cortex (Nimchinsky *et al.* 1997).

The present study further extends this segregation of area 24 and suggests that the subgenual region of area 24 is different to its supragenual counterpart, particularly in regard to neuronal size and glial density, although cortical depth was also different. Given that other researchers have described considerable variation between different aspects of area 24, it is perhaps not surprising that the areas above and below the corpus callosum also appear to have distinctive features of their own. Further credibility to this argument comes from the combination of two separate publications; the first study reported the presence of SMI-32 - immunoreactive pyramidal neurons in layer III of the supragenual cortex (Nimchinsky *et al.* 1997), in comparison, the second study failed to observe SMI-32-immunoreactive neurons in layer III of the subgenual region (Öngür *et al.* 2003). This is particularly interesting given that SMI-32-immunoreactive neurons are thought to represent a subpopulation of ipsilateral corticocortical projection neurons (Campbell *et al.* 1991; Campbell and Morrison 1989). Taken together, these results indicate that the neuronal population in layer III of the supragenual cortex may have different cytoskeletal requirements and/or different connections to the equivalent area of the subgenual cortex. This provides further evidence for differences in the cellular organisation of the supragenual and subgenual regions of area 24. It is quite possible that subsequent

anatomical studies comparing the cellular and chemical features of the supragenual and subgenual regions of area 24 will highlight further distinctions between these two areas.

The main results of the current study suggest that there is a fundamental difference between the cytoarchitecture of area 24 above and below the corpus callosum, which especially involves glial density and neuronal size. Whilst it is difficult to extrapolate these results, some tentative suggestions can be proposed. It has been suggested that the size of a neuron is related to the number, length and diameter of its processes; along with the number of synapses it receives on its surface (Esiri and Pearson 2000). The smaller neuronal size observed in the subgenual cortex in this study could therefore be indicative of each cell supporting fewer processes, reflecting a less extensive synaptic organisation in this region compared to the supragenual region. It could therefore be speculated that the functional networks of connectivity might differ between these two regions.

The finding of reduced glial density in the subgenual cortex is more difficult to interpret, as the glial cells counted in this study are comprised of functionally distinct cells. Overall, glial cells have important roles in synaptic function (Araque *et al.* 1999), synaptic plasticity (Ullian *et al.* 2001), neuronal metabolism (Magistretti 2000; Magistretti *et al.* 1999), and the clearance of extracellular ions and transmitters (Verkhratsky *et al.* 1998). Given their roles in neuronal metabolism and synaptic function, it is at least feasible that the reduced glial density observed in the subgenual region is in some way related to the smaller neuronal size observed in the same area. As the neurons in the subgenual cortex are smaller in size with potentially less extensive dendritic and axonal arbours, they may not require the same degree of glial support and consequently the density of glial cells in this area may be reduced – or vice versa.

2.6.4.1 Implications for neuroimaging research

Sound cytoarchitectural knowledge of the brain is vital for providing a good base for the interpretation of imaging data. As the capability and resolution of neuroimaging becomes ever more precise, it becomes increasingly important that this level of detail can be accurately correlated with the architectonic areas of the human brain. Regarding the areas investigated in the present chapter, there already appears to be some inconsistency concerning the exact location of the subgenual area of the cingulate cortex. This area is associated with selective activity changes during emotional tasks and has been implicated in mood disorders (Botteron *et al.* 2002; Brambilla *et al.* 2002; Drevets *et al.* 1997; Hirayasu *et al.* 1999; Skaf *et al.* 2002) and induced sadness (George *et al.* 1995; Mayberg *et al.* 1999). Each study listed above used comparable anatomical criteria to delineate the subgenual region of the cingulate cortex, anterior and ventral to the corpus callosum; nevertheless, there are discrepancies regarding the designation of cortical areas to this region of interest. Some studies refer to this region as area 25, whilst other authors refer to this region as subgenual area 24 - even though each study seemingly investigates the same region of interest.

Whilst it is conceivable that these juxtaposed ventral areas are both implicated in the aforementioned studies; a recent report from Drevets and colleagues (1999) implied that the different studies had, in fact, all delineated and measured subgenual area 24 – which is the predominant area at this position, and not area 25. A recent study, assessing the cytoarchitecture of the human orbital and medial prefrontal cortices by application of cell body stains and immunohistochemical parcellation, confirms that area 25 is located caudal to subgenual area 24 on the medial wall in humans (Öngür *et al.* 2003). It is plausible that some of this confusion originates from direct comparisons between the cortical maps of monkeys and humans. In the monkey map, area 25 is located more centrally within the medial wall, and area 24b does not extend below the corpus callosum (Carmichael and Price 1994; Öngür *et al.* 2003). Öngür *et al.* (2003) have gone some way in reconciling the

differences between monkey and human orbital and medial prefrontal areas, and although the anatomy of the subgenual area of the human cortex (especially area 24) is substantially different to that of the monkey, the authors do suggest that the subgenual region of area 24 in humans is likely to be equivalent to the anterior parts of 24a and 24b in monkeys. This research will be extremely useful for the interpretation of functional imaging data, as the cortical networks defined within monkeys can now be recognised in the human brain and allocated to discrete cytoarchitectural areas (Carmichael and Price 1994; Öngür *et al.* 2003; Öngür and Price 2000).

2.6.4.2 *Implications for mood disorder research*

The supragenual and subgenual regions of the ACC have been implicated in mood disorder and also in induced sadness (for reviews see Drevets 2000b; Harrison 2002). Interestingly, these two related areas appear to be differentially involved in altered mood states. The subgenual cortex has been especially implicated in neuroimaging studies of depressed mood. Numerous studies have reported a decrease in the volume of the grey matter of the subgenual cortex (Botteron *et al.* 2002; Drevets *et al.* 1997; Hirayasu *et al.* 1999), but not supragenual cortex (Drevets *et al.* 1997) in mood disorder. After correcting for partial volume, most reports indicate that regional cerebral blood flow (rCBF) and metabolism are increased in the subgenual region of depressed patients (Buchsbaum *et al.* 1997; Drevets *et al.* 1997; Skaf *et al.* 2002), and in induced sadness (George *et al.* 1995; Liotti *et al.* 2000; Mayberg *et al.* 1999). Metabolism and rCBF in the subgenual area appear to decrease to normal levels during successful treatment and remission (Drevets 2000a; Mayberg *et al.* 2000; Mayberg *et al.* 1999), although the reduction in volume does not seem to alter during treatment (Drevets *et al.* 1997). Interestingly, the supragenual cortex demonstrates the opposite pattern, with decreases in rCBF observed during induced sadness (Liotti *et al.* 2000; Mayberg *et al.* 1999) and a decrease in blood flow during depression that normalises in remission (Mayberg *et al.* 1999).

In support of the volume reduction described above, a recent neuropathological study has reported a trend towards a decreased subgenual volume in mood disorder patients, along with a reduction in the density and number of glia in this brain region (Öngür *et al.* 1998).

As with the imaging studies, corresponding reports of reductions in the volume of the supragenual cortex have not yet emerged. In the only neuropathological study yet to examine both supragenual and subgenual regions of area 24 simultaneously, reductions in neuronal density and lamina thickness were observed in the subgenual cortex of mood disorder patients – without any accompanying pathology in the supragenual cortex (Bouras *et al.* 2001). A second morphometric study of the supragenual region of area 24 has reported decreases in neuronal size together with decreases in glial density in mood disorder patients (Cotter *et al.* 2001a).

It is clear from these studies that a functional distinction exists between the supra- and subgenual regions of the ACC. In respect to the existing literature, the differential neural responses observed in mood disorder and induced sadness suggests that a reciprocal relationship exists between these two regions of area 24. Equally, the volume reduction of the subgenual cortex appears to be specific, and is yet to be replicated in the supragenual cingulate cortex. The morphometric studies are more ambiguous as both glial and neuronal pathology has been recorded in the supragenual and subgenual regions of area 24, although, when the two regions were investigated concurrently, alterations were only observed in the subgenual region (Bouras *et al.* 2001). It is possible that as further research is undertaken the disparities between these two regions could become even more distinct, or alternatively, disappear altogether.

The results reported in the present chapter provide preliminary evidence for an anatomical distinction between the supragenual and subgenual regions of area 24 in normal subjects – which could conceivably explain why the two regions are differentially affected in mood disorders and induced sadness. It could be speculated that the cytoarchitectural features

reported here; in conjunction with recent evidence suggesting the absence of a specific class of corticocortical projection neurons in layer III of the subgenual area (Öngür *et al.* 2003), may together represent cellular reflections of functional specialisations in distinct domains of area 24. The existence of discrete domains with potentially distinct connections and functions could go some way in explaining the differential neural responses and volumetric alterations observed in the supragenual and subgenual cortices in mood disorder and induced sadness. Further investigation into the cellular composition and functional organisation of the ACC is necessary before this point can be made conclusively.

2.6.5 Limitations of the current study

2.6.5.1 Limitations of the tissue series

The most obvious limitation of this study is its sample size. One problem usually associated with a small series is that of lowered statistical power and the subsequent masking of significant results; nevertheless, marked significant differences between the subgenual and supragenual levels of the cingulate were observed in this study. As previously mentioned, rostrocaudal differentiations within the supragenual region of area 24, as described (albeit qualitatively) by Vogt *et al.* (1995), were not statistically significant in this study. It is feasible that a larger sample size would help to clarify this situation, but as the results were so insignificant it is more probable that area 24b is not particularly variable along its rostrocaudal extent. It is possible that the other dorsoventral subdivisions of area 24 would be more variable (see section 2.6.2).

Perimortem factors such as tissue fixation, processing and embedding can all influence tissue shrinkage and thereby the subsequent measurement of morphometric indices such as cell density and size (Haug 1984; Kretschmann *et al.* 1982; Quester and Schroder 1997; Small and Peterson 1982). Ideally, factors such as brain pH, post mortem interval

(PMI), and fixation time should be incorporated into any analyses. As only limited information was available to me regarding these factors, covariate analyses were not applied in the present study. Repeated measures 'within subject comparisons' were used in this study to compare different cingulate levels; and assuming that all of the levels were equally affected by fixation and embedding, it is unlikely that the addition of covariates would change the overall results. Whilst improbable that the five levels of the anterior cingulate were differentially affected by perimortem factors; research has shown that age at death together with tissue fixation and processing can all have distinctive effects on different brain regions (Haug 1984; Kretschmann *et al.* 1982; Quester and Schroder 1997), accordingly, this caveat should not entirely dismissed.

2.6.5.2 *Methodological considerations – stereological issues*

The stereological approach used in this study also warrants further discussion. The optical disector method was employed as it avoids double counting and makes no assumptions about cell shape or size (Gundersen *et al.* 1988a). Another advantage of this method is that it allowed me to assess the variability introduced by the sampling scheme and to increase the sampling intensity when necessary. Whilst stereological principles for counting and estimating error were adhered to in this study, the sampling and selection of tissue at various levels of the cingulate cortex could be criticised. In its most strict definition, stereology requires that the structure from which measurements are to be made must be clearly definable and sampled in its entirety. The size and complexity of anatomical structures in the human brain can make this difficult and arguably inappropriate (Hyman *et al.* 1998). Moreover, the purpose of this study was to examine morphometric indices at different levels of the same structure. In light of this, a compromise was reached whereby sections were systematically, randomly sampled from defined portions of the anatomical structure of interest.

Two related matters worthy of further discussion include the initial selection of anatomical levels and the delineation of the cortical layers. Although every attempt was made to select tissue blocks that approximated to the same level in each brain, there were slight discrepancies. This may have decreased the probability of detecting any subtle differences between the anatomical levels, although it did not seem to obscure the differences observed between the supragenual and subgenual regions of area 24. The definition of cortical layers was also inevitably subjective and as such must be considered as a potential source of error and bias.

Another relevant issue concerning this study is the measure of cell density, rather than number. In practice, it is necessary to know the volume of an area in order to calculate total cell number. In the current study it was not possible to accurately measure the volume of each cingulate level under examination – and it would have been even more impractical to estimate the volume of each cortical layer. The disadvantage of measuring cell density is well known; it is possible to achieve an increase in cell density that merely reflects the same number of cells in a smaller volume (Harrison and Kleinman 2000). This may be of relevance to the present study as the subgenual cortex does have a much smaller volume than the supragenual regions of the cingulate cortex. However, as a lower glial density was reported in subgenual area 24 (which is of smaller volume), it is improbable that glial number would remain unchanged. Furthermore, measures of cell density provide valuable and important information about the cytoarchitecture of an area, including the status of neuronal processes and synaptic contacts within the neuropil (Harrison and Kleinman 2000).

2.7 Summary

The subgenual region of area 24 demonstrated a lower glial density and smaller neurons in comparison to the supragenual region of area 24. A modest difference in neuronal density was also observed, with a higher density in the deep layers of the subgenual cortex compared to the deep layers of the supragenual cortex. The total cortical depth of this region was thinner relative to its supragenual counterpart. There was no evidence for a rostrocaudal differentiation in the supragenual levels. Overall there was no main effect of lateralisation, although neuronal density was higher in level 4 of the left hemisphere. This work provides preliminary evidence for an anatomical distinction between the supra- and subgenual regions of area 24. Differences in cytoarchitecture could represent cellular reflections of functional specialisations, an interesting supposition in consideration of the apparent differential involvement that exists between these two areas in mood disorders.

Chapter Three: Quantitative studies of the cytoarchitecture of the anterior cingulate cortex in mood disorder

3.1 Introduction

Over recent years, MRI evidence for structural and functional brain abnormalities in mood disorder has steadily accumulated. This evidence has been the primary stimulus behind subsequent investigations into the neuropathological correlates of mood disorder and has also influenced the brain regions selected for such examinations.

The involvement of the ACC in mood disorders is apparent from neuropsychological, neuroanatomical and functional imaging data: (for reviews see: Devinsky *et al.* 1995; Drevets 2000b; Ebert and Ebmeier 1996; Elliott 1998; Kennedy *et al.* 1997; Soares and Mann 1997). The presence of ACC abnormalities in mood disorder is consistent with the known functions of this region in information processing, attention and the expression and modulation of emotion (Arroyo *et al.* 1993; Bush *et al.* 2000; Devinsky *et al.* 1995; Mersulam 1983; Posner and Petersen 1990; Vogt *et al.* 1992). Accordingly, recent reviews have placed the ACC at the interface of motor control, drive, emotion and cognition (Allman *et al.* 2001; Devinsky *et al.* 1995; Paus 2001). As these functions are seemingly altered in mood disorder (Steffens and Krishnan 1998), the ACC is a suitable candidate region in which to search for the presence of histological and cellular correlates of such alterations.

Microscopic investigations of the ACC in mood disorder are in their infancy, nevertheless, the limited number of reports to date all indicate an altered pathology of this region in subjects with mood disorder. In the first neuropathological study of the subgenual region of area 24, Öngür *et al.* (1998), reported a decrease in glial density and glial number throughout the depth of the cortex (without concomitant neuronal loss) in patients with

bipolar disorder and major depression. Higher levels of significance were observed for the subset of cases with a family history of mood disorder. No alterations in glial or neuronal size were reported.

In a second study of subgenual area 24, (Bouras *et al.* 2001), neuronal density was reduced in layers III, V and VI in bipolar disorder patients compared to controls, but not in patients with major depression. Changes in layer depth were also observed in the bipolar group compared to the control group. These authors also examined supragenual area 24; however, no morphometric alterations were observed for either diagnostic group in this region of the ACC. No glial data were presented.

Cotter and colleagues (2001a) also investigated the supragenual region of area 24b. The main positive findings were in major depression, with decreased glial density in layer VI and reduced neuronal size in layers Vb and VI. Neuronal density was unaltered. Benes and colleagues (2001) studied the pregenual region of area 24 in subjects with bipolar disorder. A reduction in the density of non-pyramidal neurons, but not pyramidal neurons, was observed in layer II of subjects with bipolar disorder. Increases in neuronal size were also observed in layers II (pyramidal and non-pyramidal) and III (non-pyramidal). No differences in glial density were reported.

Even though all of these studies have revealed morphometric alterations in the ACC in mood disorder, notable discrepancies remain. Öngür *et al* (1998) revealed glial pathology without concomitant neuronal loss, Benes *et al* (2001) found the opposite and Cotter *et al* (2001a) found both. The layer distribution of significant differences also varies considerably between studies. Discrepancies also exist between diagnostic boundaries. Öngür *et al* (1998) reported differences in both bipolar disorder and major depression; Cotter *et al* (2001a) found them only in the latter group, whereas Bouras *et al* (2001) revealed changes specific to subjects with bipolar disorder. It is likely that these

inconsistencies are related to demographic and/or methodological differences.

Additionally, in consideration of the heterogeneity that exists between different regions of area 24 (see chapter two), it is reasonable that differences in the discrete cytoarchitectonic subdivisions investigated could account for some of the variability. Further replication is necessary in order to resolve the variability between the existing morphometric studies.

3.1.1 Aims of the current study

There were three objectives to the study described in this chapter. My first aim was to measure pyramidal and non-pyramidal neuron density, size and shape together with glial density in a cohort of control and mood disorder subjects. Accordingly, I decided to use Nissl-stained sections from the supragenua region of area 24b and measure cell density, size and shape in layers II-VI. Reductions in neuronal and glial density were predicted on the basis of existing literature. My second aim was to assess pyramidal and non-pyramidal neuronal density, size and shape within the same cohort, by using an antibody that recognises neuron-specific protein, Neu-N (Wolf *et al.* 1996). As glia cells are not marked using this technique, cortical lamination should be enhanced. This technique should also provide a better platform for the correct identification of neuronal cell types and avoid an incorrect distinction between small neurons and glial cells. A third and subsidiary aim of this study was to investigate the relationship between these complementary techniques by comparison of the neuronal counts achieved with both methods.

3.2 Methods

3.2.1 Collection of brains

Brains were collected, with consent, at autopsy from psychiatrically normal control subjects and from individuals meeting criteria for major depression or bipolar disorder as determined by DSM-IV criteria. All brains were characterised macroscopically and

histologically by a neuropathologist to confirm the absence of any significant pathology in excess of normal age related changes. Subjects with a history of alcohol and/or substance abuse were excluded from the study. A corollary of this decision was to reduce the sample size, nevertheless, it was important to try and avoid the potential overlap of neuropathological effects between alcohol abuse and mood disorder (Miguel-Hidalgo *et al.* 2002). In particular, recent studies have reported that alcoholics have fewer glial cells in the hippocampus and prefrontal cortex (Korbo 1999; Miguel-Hidalgo *et al.* 2002), and differences in the morphometry of neurons have also been observed in various brain areas (Harding *et al.* 1997; Harper *et al.* 1987; Kril and Halliday 1999). Although it is not ideal to group subjects with bipolar disorder or major depression together, this was unavoidable due to the strict inclusion criteria and the limited amount of tissue available for this study.

3.2.2 Tissue sectioning and preparation

Blocks of approximately 1cm³ were dissected from the supragenual region of area 24 (roughly 1cm caudal to the tip of the genu of the corpus callosum) and fixed in 10% formalin. Once fixed, the tissue was processed on a Leica TP 1040 and paraffin embedded. 10 µm sections were cut from the wax blocks using a Leica rotary microtome. Sections were floated onto a warm waterbath and collected onto superfrost slides. The sections were then dried overnight at 37°C and stored at room temperature until use. Duplicate sections were used in the Nissl and Neu-N experiments. Prior to the cell-counting requiring Nissl-stained material, two sections from each case were stained with 0.75% Cresyl Violet, dehydrated through graded alcohols (70%, 95%, 100% x2), placed in xylene and coverslipped using DPX as a mountant.

3.2.3 Matching of subject groups

In post mortem research it is important to recognise and account for any potentially confounding factors. Matching the groups in terms of mean and range, and including any significant confounding factors in a covariate analysis, helps to minimise their influence (Hynd *et al.* 2003).

Perimortem factors such as tissue fixation, processing and embedding can all influence tissue shrinkage and thereby the subsequent measurement of morphometric indices such as cell density and size (Haug 1984; Kretschmann *et al.* 1982; Quester and Schroder 1997; Small and Peterson 1982). The shrinkage of brains during embedding is also affected by age at death. Tissue shrinkage in youth tends to be higher than in old age - which could subsequently result in an apparent increase in cell density in younger brains (Haug 1984). It is also possible that age may affect neuronal number and more probably, neuronal size. Glial cells are also reputedly increased in number and size with ageing (Finch and Morgan 1990). Cell size can also be influenced by pH, most likely due to a swelling secondary to acidosis (Harrison and Kleinman 2000).

The integrity and abundance of proteins in post mortem tissue can also be altered by perimortem factors such as PMI and agonal state. Brain pH is a strong correlate of agonal state and has been adopted as a marker for this variable. Although in general, epitopes appear to be more resistant than mRNA to these confounds (Harrison *et al.* 1995), their potential alteration should not be entirely dismissed as different epitopes may be differentially susceptible to the effects of pH and PMI. Neurochemical markers may also decline with age; this may be secondary to neuronal loss, or alternatively, decreases may be due to selective changes in protein synthesis (Finch and Morgan 1990). Agonal state is also thought to influence mRNA abundance (Barton *et al.* 1993), with increasing acidosis frequently associated with reduced mRNA abundance. PMI and tissue freezing may also influence mRNA abundance, to a lesser degree (Harrison *et al.* 1995).

All of the brains used in this study were processed and embedded in an identical manner (see section 3.2.2). Unfortunately, the exact fixation time for each case is not known, though all cases were fixed for a minimum of 6 weeks; consequently, the effect of fixation per se could not be formally assessed in this study. All other aforementioned variables (pH, PMI, age and freezer storage time) were matched as closely as possible between the two subject groups. Regrettably, the hemisphere from which the tissue was dissected was not recorded and therefore interhemisphere effects could not be investigated. The demographic details of the control and mood disorder subjects used in this study are presented in table 3.1.

3.2.4 Immunohistochemistry

3.2.4.1 Principles of immunohistochemistry

Immunohistochemical techniques employ labelled antibodies to visualise antigens in tissue sections. A considerable problem when using tissue that has been fixed in formalin over a prolonged period of time is the masking of antigenic sites, which is presumably related to the formation of methylene bridges between proteins during fixation. Such changes in the secondary and tertiary structure of proteins could result in the inability of the antibody to recognise or even access the antigen. Different techniques such as formic acid pre-treatment, microwave-heating and autoclave-heating have been developed to overcome this problem (Evers and Uylings 1997; Igarashi *et al.* 1994). Although the exact mechanism of antigen retrieval is not fully understood, it is believed that the cross-links between proteins are broken, thus making the antigen accessible to recognisable to the antibody (Evers and Uylings 1997). Microwave-heating was used in the current study to optimise staining; previous research has indicated that it is the heat, and not the microwaves per se, that is necessary to enhance the staining (see Evers and Uylings 1997; Igarashi *et al.* 1994).

For all of the immunohistochemical experiments detailed in this thesis, a commercially available ABC kit (Vectastain Elite ABC, Vector Laboratories, Peterborough, UK) was employed to localise the antigens of interest. After the application of the primary antibody, a biotin-labelled secondary antibody was applied to the section, which binds to the primary antibody. This was then followed by the addition of an avidin-biotin-peroxidase complex (ABC) (Hsu *et al.* 1981). Visualisation of the antigen-antibody complex is achieved by incubating the sections with a suitable substrate for the enzyme. Nickel-enhanced 3'3'-diaminobenzidine (DAB; Perbio, Chester, UK) was used as the colour substrate throughout this thesis.

In general, hydrogen peroxide was used to exhaust any endogenous peroxidase activity that may have been present in the tissue. Normal serum (Sigma, Poole, UK), from the species in which the secondary antibody was raised, was used to block any non-specific sites and reduce the levels of background staining. All diluents contained 0.3% Triton X-100 (Sigma) to aid the penetration of the antibodies into the tissue sections.

3.2.4.2 Neuronal nuclear antigen (Neu-N)

Neuronal nuclear antigen (Neu-N) antibody recognises a neuron-specific nuclear protein in vertebrates (Mullen *et al.* 1992). Its expression has been studied in the developing and adult human brain (Mullen *et al.* 1992; Sarnat *et al.* 1998; Wolf *et al.* 1996). Neu-N immunoreactivity is observed in neuronal nuclei, perikaryon and proximal processes, whilst non-neuronal structures remain unstained. Neu-N is not detected in Purkinje cells, neurons of the internal nuclear layer of the retina, or in the sympathetic chain ganglia (Wolf *et al.* 1996). The function of Neu-N is still unknown.

The use of Neu-N as a sensitive and specific neuronal marker in routinely processed formalin-fixed paraffin embedded human tissue has been previously validated (Sarnat *et*

al. 1998; Wolf *et al.* 1996). In both instances, antigen retrieval was enhanced by microwave heating.

3.2.4.3 *Neu-N immunohistochemistry*

Pilot experiments assessing varying antibody concentrations and incubation times were performed prior to the definitive experiment. Various antigen retrieval buffers were also compared: 0.05M Tris (pH 7.0), Tris-buffered saline (pH 9.0) and 0.01M Citric acid (pH 6.0). Preliminary investigations revealed that microwaving with Citric acid gave optimal results and that microwaving for a longer period of time failed to further enhance the signal (data not shown).

Duplicate sections for each case were de-waxed in three consecutive baths of xylene, followed by rehydration in graded alcohols (4 minutes each in 100% x2, 95%, 70% and distilled water). The sections were then immersed in 3% hydrogen peroxide diluted in phosphate buffered saline (PBS, Sigma, Poole UK) for 30 minutes at room temperature, rehydrated in 95% alcohol and rinsed in PBS. Sections were then heated in 0.01M Citric acid (Sigma) at pH 6.0 in a microwave oven until the solution started to boil. The sections were then removed and allowed to cool for 5 minutes before the process was repeated. Sections were then left to cool to below 60°C before being rinsed in PBS. After microwave treatment, non-specific binding was blocked with 10% normal horse serum (Sigma) in PBS-T for 30 minutes at room temperature. The sections were then briefly rinsed in PBS before application of the primary antibody solution, which contained mouse Neu-N monoclonal antibody (Chemicon International, Hampshire, UK; MAB377) diluted to 1:100 in PBS-T containing 1% normal horse serum. Sections were incubated overnight at 4°C. Sections were then incubated with a biotinylated secondary horse anti-mouse antibody diluted to 1:100 in PBS-T containing 1% normal horse serum at room temperature for one hour. This was followed by incubation with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC, Vector Laboratories) for one hour at room

temperature according to the instructions of the manufacturer. All steps were followed by appropriate washes in PBS. Finally, all sections were developed in a substrate solution of nickel-enhanced DAB, again prepared according to the manufacturers instructions (Perbio). All sections were processed simultaneously and development times for staining were equivalent. Omission of the primary antibody was used as an experimental control.

3.2.5 Morphometric analyses

3.2.5.1 Two-dimensional cell counting

The relative merits of stereological counting were discussed in the previous chapter (see section 2.2.1.2). Although 3-D counting methods have their advantages, they are generally not feasible for studies employing immunohistochemistry. Primary and secondary antibodies are, in most cases, unable to penetrate through thick sections and therefore 2-D counting methodologies are mandatory for immunohistochemical investigations. As it was my intention to compare results from Neu-N and Nissl sections – and to investigate calcium binding protein, GFAP and HLA-DR expression in the same tissue series (see chapters four and five) – it was necessary to employ a 2-D counting approach, thereby enabling me to relate results from immunohistochemical experiments to the basic cell counting data.

A key issue surrounding 2-D counting methodologies is that these methods are not free from assumptions regarding cell shape and size. This can be mathematically controlled for by means of the Abercrombie correction (Abercrombie 1946), however, some investigators believe this correction is inadequate (see Guillery and Herrup 1997; Hedreen 1998). Application of the Abercrombie correction requires the precise measurement of section thickness and cell diameter along the appropriate axis in order to be valuable. Measurement of both parameters in thin tissue sections can be subject to considerable error (Guillery 2002; Selemon and Rajkowska 2002). In essence, the Abercrombie

correction refines the original 'raw' counts and brings them closer to their 'true' value.

Such corrections are of less importance when quantification is aimed at making comparisons between groups (Guillery and Herrup 1997), unless there are concomitant alterations in cell size. The Abercrombie correction can be applied using the following formula:

$$N = n \left(\frac{T}{T + D} \right) \quad (1)$$

where N is the 'true' cell number, n is the estimated cell number, T is the section thickness and D is the mean diameter of the objects (Abercrombie 1946).

3.2.5.2 Identification of area 24b and cortical layers

Using Nissl-stained sections, the supragenua region of area 24b of the ACC was identified and selected for analysis according to macroscopic and microscopic criteria (Vogt *et al.* 1995). This area was delineated using the same criteria as outlined in section 2.2.5.1. This process was then repeated for the Neu-N sections. Nissl and Neu-N sections were then compared to ensure that the delineated areas coincided. Layers II, III, Va, Vb and VI were identified in area 24b using the descriptions provided by Vogt *et al.* (1995), as detailed in section 2.1.2. Layer I was not assessed in this study due to the paucity of neurons in this layer.

As supragenua area 24 is the only Brodmann area examined in this study (and is indeed the only area of the ACC yet to be studied neuropathologically in mood disorder), the terms ACC and area 24 will be used interchangeably throughout the remaining chapters of this thesis, unless differentiation is necessary.

3.2.5.3 *Counting procedure*

The morphometric analyses were undertaken using an image analysis system from Imaging Associates (Bicester, UK) using KS400.3 software, equipped with an Olympus BH40 microscope and colour video camera. Before counting commenced, the depth of each cortical layer in the Nissl-stained sections was determined. Lamina boundaries were identified on each image and the depth of each layer was measured using a 4-times objective lens. These measurements were repeated four times on each of the two sections per case. The percentage of total cortical depth that each layer contributed was then calculated.

Tissue sections were viewed with a 40-times objective lens enabling a reasonable distinction between small neurons and glial cells. Neurons were distinguished from glia using the criteria outlined in section 2.2.5.2, with the one exception that neurons were identified by the presence of a visible nucleus (not nucleolus) in the current study. In addition, pyramidal neurons were distinguished from non-pyramidal neurons using the following criteria 1) pyramidal neurons exhibited a characteristic triangular shape arising from the presence of a single large apical dendrite extending vertically towards the pial surface, 2) non-pyramidal neurons were identified by their bipolar, or multipolar shape and by the presence of two or more small calibre branches. Non-pyramidal neurons also tended to be smaller in size. Methods of cell categorisation were identical between the Nissl and Neu-N experiments.

Sampling strategies for the Nissl-stained and Neu-N sections were optimised before the definitive experiment. Five cases were selected at random and increasing numbers of cells were sampled in each cortical layer until reliable and reproducible density values were obtained. These preliminary tests indicated that sampling approximately 50 cells of each classification, per layer, per section (therefore providing a total of 100 cell counts, per classification, per layer, per case) provided reproducible density estimates. Five cases

(selected at random) were repeated one week later - all values were within 5% of original measurements (data not shown).

An unbiased counting frame with an area of $14,643 \mu\text{m}^2$ was used throughout this study (see section 2.2.1.3). The sampling field was moved systematically through the cortical layer of interest until a sufficient number of each cell type had been counted. Pyramidal neurons, non-pyramidal neurons and glial cells were counted simultaneously in each frame. Pyramidal neuron size, measured as cell body cross-sectional area, was determined by manually tracing around the perimeter of the soma. This measurement also permitted pyramidal neuron shape to be investigated by way of the 'fcircle' function, defined by $4\pi[\text{area}/\text{perimeter}^2]$. Increasing 'fcircle' values indicate objects that are increasingly circular rather than elliptical in shape. Extrapolation of this reasoning to triangular shaped objects (i.e. pyramidal neurons), implies that equilateral, rather than isosceles, triangular shapes have higher 'fcircle' values (Law and Harrison 2003).

The potential influence of cell size on areal density measures can (to a certain degree) be corrected by application of the Abercrombie correction. To enable such a correction in the current study, section thickness was carefully measured using a microcator. The widths of pyramidal neurons and non-pyramidal neurons were measured from 50% of cases in each diagnostic group. Approximately 50 cells were sampled from each cortical layer to provide a reliable estimate of diameter. The index of diameter was used in this study (instead of height) because the majority of neurons lie parallel rather than perpendicular to the plane of coronal sections (Law and Harrison 2003). The Abercrombie correction was then applied to all neuronal measurements using the equation given in section 3.2.5.1. Glial density was not corrected due to the very small size of these cells in relation to section thickness.

Nissl and Neu-N sections were treated identically for all neuronal measurements. All measurements were made blind to diagnosis. Total neuronal density was calculated by summing pyramidal and non-pyramidal neuron counts. All neuronal data is presented in its corrected form. Density values are expressed as cell density/mm². Pyramidal cell size is expressed in μm^2 .

3.2.6 Statistical Analysis

All statistical analyses were carried out using SPSS 10.0 for Microsoft Windows. The data sets to be investigated were: 1) total cortical depth, 2) layer depth (as a percentage of total cortical depth), 3) pyramidal neuron density, 4) non-pyramidal neuron density, 5) neuronal density, 6) glial density, 7) pyramidal neuron size and 8) pyramidal neuron shape for the Nissl-stained sections; in addition to, 1) pyramidal neuron density, 2) non-pyramidal neuron density, 3) neuronal density, 4) pyramidal neuron size and 5) pyramidal neuron shape for the Neu-N sections. An initial evaluation of the data involved checking the distributions of points within each data set for normality using the Kolmogorov-Smirnov test.

The potential confounding effects of age, pH and PMI were evaluated using Pearson's correlations in the control and mood disorder group separately, as well as in both groups combined. The Nissl and Neu-N data sets were considered separately. As it is unlikely that a confounding factor would have differential effects on each cortical layer, any demographic variable found to exert a significant effect was included as a covariate in all layer analyses for a given variable, even if statistical significance was not attained in all layers. Significance was set at $p \leq 0.05$ for all correlation analyses.

There is no single accepted method for analysing data sets of this type – exemplified by the varying statistical methods employed by different research groups (Benes *et al.* 2001; Bouras *et al.* 2001; Cotter *et al.* 2001a; Rajkowska *et al.* 2001); considered in greater

detail in the discussion. Common sense dictates that it is as important to use a rigorous test, as it is to be flexible and adapt statistical methods appropriately in order to accommodate the limitations of the study - such as those imposed by a small sample size. For this reason it was decided that a repeated measures ANOVA, incorporating layer as a 'within subject variable', and diagnosis as a 'between subjects factor' would be employed to analyse each data set, including covariates where necessary. If the repeated measures ANOVA revealed a significant main effect of diagnosis, and/or demonstrated a significant interaction between layer and diagnosis, separate ANOVAs were then employed to explore the nature of this relationship, again including covariates where appropriate. In consideration of the small sample size of this study, a looser interpretation of 'significance' was adopted for the repeated measures ANOVA, and $p \leq 0.1$ was considered as sufficient evidence for further exploration of the data set. For all subsequent ANOVAs, significance was set at $p \leq 0.05$. Again, a Bonferroni correction was not applied in light of the small sample size.

I decided not to carry out separate analyses between bipolar disorder and major depression because of the small subgroups that would result. For the same reason, the effect of suicide within the mood disorder group was not assessed.

Pearson's correlations were used to assess the relationship between the Nissl cell counts and Neu-N cell counts. The relationship between the two techniques was assessed in the control group, the mood disorder group and both groups combined.

Table 3.1.

Demographic details of control and mood disorder subjects included in the morphometric analyses (Nissl and Neu-N). Values are mean $\pm$ S.E.M with range in brackets. There are no significant differences between the two groups ($p>0.2$). ^a major depression (n=5), bipolar disorder (n=2). Diagnoses were made according to DSM-IV criteria. ^b All patients had been treated with antidepressant medication.

Variable	Control (n=9)	Mood disorder (n=7) ^{ab}
Age (years)	58.33 $\pm$ 6.11 (22–79)	64.57 $\pm$ 4.63 (49–75)
Sex (M:F)	5:4	4:3
pH	6.54 $\pm$ 0.06 (6.20–6.72)	6.40 $\pm$ 0.09 (6.06–6.86)
PMI (hours)	32.67 $\pm$ 7.49 (8–72)	34.43 $\pm$ 7.51 (13–75)
No. of suicides	0	4

3.3 Results

3.3.1 Distribution of Neu-N immunoreactivity

Neu-N-immunoreactive neurons were present throughout the cortical layers and in the subadjacent white matter. The staining was present in both nuclei and cytoplasm and extended into the proximal processes. There was no staining of non-neuronal structures such as glia and ependymal cells. The intensity of staining varied both within brains and between brains. Staining with Neu-N greatly enhanced the cortical lamination and therefore made layer definition more straightforward (see figure 3.1). Group means (SEM) for all Nissl and Neu-N morphometric data sets are given in tables 3.2 and 3.3 respectively.

3.3.2 Analysis of cortical depth and lamina depth

Both data sets satisfied the Kolmogorov-Smirnov test for normal distribution. Age, pH and PMI showed no significant correlations with cortical depth or layer depth (expressed as a percentage of total cortical depth), in the mood disorder group, the control group, or both groups combined.

Total cortical depth was unchanged in mood disorder ($p=0.519$). The repeated measures ANOVA for layer depth did not find a significant effect of diagnosis ($p=1.000$); the interaction between diagnosis and layer also failed to reach significance ($p=0.754$). See table 3.4 and figure 3.2. No further analyses were performed.

3.3.3 Analysis of pyramidal neuron density

3.3.3.1 Nissl

Pyramidal neuron density measurements satisfied the Kolmogorov-Smirnov test for normal distribution. Pyramidal neuron density showed no significant correlations with age, pH or PMI in the mood disorder group, the control group, or both groups combined.

The repeated measures ANOVA for pyramidal neuron density failed to show a significant effect of diagnosis ($p=0.980$), but the interaction between diagnosis and layer was significant ($F(4,56)=3.232$; $p=0.019$). See table 3.4. Further analyses indicated a decrease in pyramidal cell density in layer Vb of the mood disorder group ($F(1,14)=11.449$; $p=0.004$). All other layer comparisons were insignificant (layer II, $p=0.239$; Layer III, $p=0.762$; layer Va, $p=0.210$; layer VI, $p=0.479$). These data are presented in table 3.5 and figure 3.3.

3.3.3.2 *Neu-N*

Pyramidal neuron density measurements satisfied the Kolmogorov-Smirnov test for normal distribution. Pyramidal neuron density showed no significant correlations with age, pH or PMI in the mood disorder group, the control group, or both groups combined.

The repeated measures ANOVA for pyramidal neuron density failed to show a significant effect of diagnosis ($p=0.467$), but the interaction between diagnosis and layer was significant ($F(4,56)=2.026$; $p=0.103$). See table 3.4. Subsequent analyses demonstrated that pyramidal neuron density was decreased in layer Va ($F(1,14)=8.886$; $p=0.010$) and layer Vb ($F(1,14)=7.081$; $p=0.019$) in the mood disorder group. All other layer comparisons were insignificant (layer II, $p=0.507$; layer III, $p=0.180$; layer VI, $p=0.808$). These data are presented in table 3.5 and figure 3.3.

3.3.4 Analysis of non-pyramidal neuron density

3.3.4.1 *Nissl*

Non-pyramidal neuron density measurements satisfied the Kolmogorov-Smirnov test for normal distribution. No significant correlations were observed with age, pH or PMI in the mood disorder group, the control group, or both groups combined.

The repeated measures ANOVA did not find a significant effect of diagnosis ($p=0.681$); the interaction between diagnosis and layer also failed to reach significance ($p=0.967$). These data are presented in table 3.4 and figure 3.4. No further analyses were performed.

3.3.4.2 *Neu-N*

Non-pyramidal neuron density measurements satisfied the Kolmogorov-Smirnov test for normal distribution. Non-pyramidal neuron density showed no significant correlations with age, pH or PMI in the mood disorder group, the control group, or both groups combined.

Again, the repeated measures ANOVA did not show a significant effect of diagnosis ($p=0.175$). The interaction between diagnosis and layer also remained insignificant ($p=0.361$). These data are presented in table 3.4 and figure 3.4. No further analyses were performed.

3.3.5 Analysis of neuronal density

3.3.5.1 *Nissl*

Neuronal density satisfied the Kolmogorov-Smirnov test for normal distribution. Age, pH and PMI did not show any significant correlations with neuronal density in the mood disorder group, the control group, or both groups combined.

The repeated measures ANOVA failed to show a significant effect of diagnosis ($p=0.748$), but a significant interaction between diagnosis and layer ($F(4,56)=2.029$; $p=0.103$) was observed. See table 3.4. Subsequent analyses indicated a significant decrease in neuronal density in layer Vb in the mood disorder group ($F(1,14)=9.876$; $p=0.007$). No other layer comparisons were significant (layer II, $p=0.453$; layer III, $p=0.805$; Layer Va, $p=0.212$; layer VI, $p=0.435$). These data are presented in table 3.6 and figure 3.5.

3.3.5.2 *Neu-N*

Neuronal density satisfied the Kolmogorov-Smirnov test for normal distribution. Neuronal density did not show any significant correlations with age, pH or PMI in the mood disorder group, the control group, or both groups combined.

The repeated measures ANOVA showed a significant effect of diagnosis ($F(1,14)=3.531$; $p=0.081$), along with a significant interaction between diagnosis and layer ($F(4,56)=2.441$; $p=0.057$). See table 3.4. Further analyses indicated significant reductions in neuronal density in layers Va ($F(1,14)=19.147$; $p=0.001$) and Vb ($F(1,14)=20.028$; $p=0.001$) in the mood disorder group. No other layer comparisons were significant (layer II, $p=0.774$; layer VI, $p=0.990$), although layer III showed a modest trend towards a reduced neuronal density in the mood disorder group ($p=0.078$). These data are presented in table 3.6 and figure 3.5.

3.3.6 Analysis of pyramidal neuron size

3.3.6.1 *Nissl*

Significant negative correlations were found between pH and pyramidal neuron size in layer III in the control group and the mood disorder group; this correlation remained when the groups were combined. Significant correlations were also observed between pH and pyramidal neuron size in layers Va and Vb in the combined group; this relationship was not significant when the mood disorder group, or the control group were considered individually (see table 3.7). Hence, pH was included as a covariate in the subsequent analyses. No significant correlations were observed for age or PMI.

The repeated measures ANCOVA showed a significant effect of diagnosis ($F(1,13)=4.476$; $p=0.054$), along with a significant interaction between diagnosis and layer ($F(4,52)=6.794$; $p<0.001$). See table 3.4. Subsequent analyses demonstrated that this interaction was due to an increased pyramidal neuron size in the mood disorder group in

the deep layers of the ACC. Pyramidal neuron size was significantly increased in layers Va ($F(1,13)=6.694$; $p=0.023$), Vb ($F(1,13)=6.580$; $p=0.024$), and VI ($F(1,13)=7.678$; $p=0.016$) in the mood disorder group. Pyramidal neuron size was unaltered in layers II ($p=0.890$) and III ($p=0.808$) in this group. These data are presented in table 3.8 and figure 3.6.

Histograms were used to compare pyramidal neuron size distribution in layers Va, Vb and VI between the two subject groups (see figure 3.7). Inspection of these graphs did not suggest that the distribution of pyramidal neuron size was skewed towards larger neurons in the mood disorder group (considering the decreases in pyramidal density observed in mood disorder, this would have provided some evidence for a specific loss of small pyramidal neurons, resulting in an increased group mean in pyramidal size). In fact, both groups demonstrated a relatively 'normal' distribution of pyramidal neuron size in layers Va, Vb and VI, with the distribution curve in the mood disorder group shifted slightly to the right, representing an overall increase in mean pyramidal neuron size in comparison to the control group.

3.3.6.2 *Neu-N*

Significant negative correlations were found between pH and pyramidal neuron size in layer III in the control group, though not in the mood disorder group; this correlation remained significant when the groups were combined. Significant negative correlations were also observed between pH and pyramidal neuron size in layers II and Vb in the control group – but not the mood disorder group or the combined group. Significant positive correlations were found between pH and pyramidal neuron size in layers Va and Vb in the mood disorder group (table 3.7). No other positive correlations were seen between pH and pyramidal neuron size. Consequently, pH was included in the succeeding analyses. No significant correlations were observed for age or PMI.

The repeated measures ANCOVA did not find a significant effect of diagnosis ($p=0.418$); the interaction between diagnosis and layer also failed to reach significance ($p=0.156$). These data are presented in table 3.4 and figure 3.6. No further analyses were performed.

3.3.7 Analysis of pyramidal neuron shape

3.3.7.1 Nissl

Pyramidal neuron shape as defined by the 'fcircle' index satisfied the Kolmogorov-Smirnov test for normal distribution. Significant negative correlations were found between pH and pyramidal neuron shape in layers III and Vb in the control group; the correlation in layer III remained significant when the groups were combined, however, the original effect observed in layer Vb was reduced in magnitude. The combined group also demonstrated a significant negative correlation in layer Va. No significant correlations with pH were observed in the mood disorder group (see table 3.9). No correlations were observed for age or PMI in either the mood disorder group, the control group, or in both groups combined. pH was included as a covariate in the following analyses.

The repeated measures ANCOVA failed to show a significant effect of diagnosis ($p=0.147$), although the interaction between diagnosis and layer was significant ($F(4,52)=2.433$; $p=0.059$). See table 3.4. Subsequent analyses indicated that pyramidal neuron shape was altered in layers Va ($F(1,13)=4.730$; $p=0.049$) and VI ($F(1,13)=7.987$; $p=0.014$) in the mood disorder group. The 'fcircle' index was greater in the mood disorder group, indicating a more equilateral-shaped cell body in comparison to the control group. No other layer comparison reached significance (layer II, $p=0.909$; layer III, $p=0.430$; layer Vb, $p=0.238$). These data are presented in table 3.10 and figure 3.8.

3.3.7.2 *Neu-N*

Pyramidal neuron shape satisfied the Kolmogorov-Smirnov test for normal distribution and did not show any significant correlations with age, pH or PMI in the mood disorder group, the control group, or both groups combined.

The repeated measures ANOVA failed to show a significant effect of diagnosis ($p=0.171$), although the interaction between diagnosis and layer was significant ($F(4,56)=2.993$; $p=0.026$). See table 3.4. Subsequent analyses demonstrated an alteration in pyramidal neuron shape in layer Va in the mood disorder group ($F(1,14)=8.898$; $p=0.010$). Again, the 'fcircle' index was greater in the mood disorder group. All other layer comparisons were insignificant (layer II, $p=0.626$; layer III, $p=0.408$; layer Vb, $p=0.124$), although a modest trend was observed in layer VI ($p=0.086$). These data are presented in table 3.10 and figure 3.8.

3.3.8 Analysis of glial density

Glial density measurements satisfied the Kolmogorov-Smirnov test for normal distribution. Significant negative correlations were found between PMI and glial density in layer II in controls, though not in patients with mood disorder; this correlation remained when the groups were combined. A further negative correlation between PMI and glial density was found in layer III of the mood disorder group, although not in the controls, or the groups combined (see table 3.11). Consequently, PMI was included as a covariate in the analyses. No significant correlations were found for pH or age.

The repeated measures ANCOVA for glial density showed a significant effect of diagnosis ($F(1,13)=6.351$; $p=0.026$), but not for the interaction between diagnosis and layer ($p=0.409$). See table 3.4. Subsequent investigations demonstrated that glial density was significantly reduced in layer II ($F(1,13)=9.877$; $p=0.008$), layer III ($F(1,13)=4.742$; $p=0.048$), layer Vb ($F(1,13)=5.593$; $p=0.034$) and layer VI ($F(1,13)=4.642$; $p=0.050$) in the

mood disorder group. Although not significant ($p=0.164$), layer Va also demonstrated a lower mean value in the mood disorder group compared to the control group. These data are presented in table 3.12 and figure 3.9.

3.3.9 Comparisons and correlations between Nissl and Neu-N techniques

Please refer to table 3.13 and figure 3.10 for results of comparison and correlation analyses.

3.3.9.1 *Pyramidal neuron density*

Positive correlations were found between Nissl and Neu-N in the control group, the mood disorder group and in both groups combined. All correlations were significant with the exception of layer III in the control group and layer Vb in the mood disorder group.

3.3.9.2 *Non-pyramidal neuron density*

Significant positive correlations were found between Nissl and Neu-N in the combined group and the control group. Layer VI showed the only significant correlation in the mood disorder group.

3.3.9.3 *Pyramidal neuron size*

Significant correlations were not consistently observed for pyramidal neuron size. The mood disorder group showed significant positive correlations between Nissl and Neu-N in layers II, III and VI, these correlations remained significant when the groups were combined. In comparison, only layers II, Va and VI showed significant positive correlations in the control group.

3.3.9.4 *Pyramidal neuron shape*

Positive correlations were found between Nissl and Neu-N in the control group, the mood disorder group and in both groups combined. All correlations were significant with the exception of layers Va and Vb in the control group and layer VI in the mood disorder group.

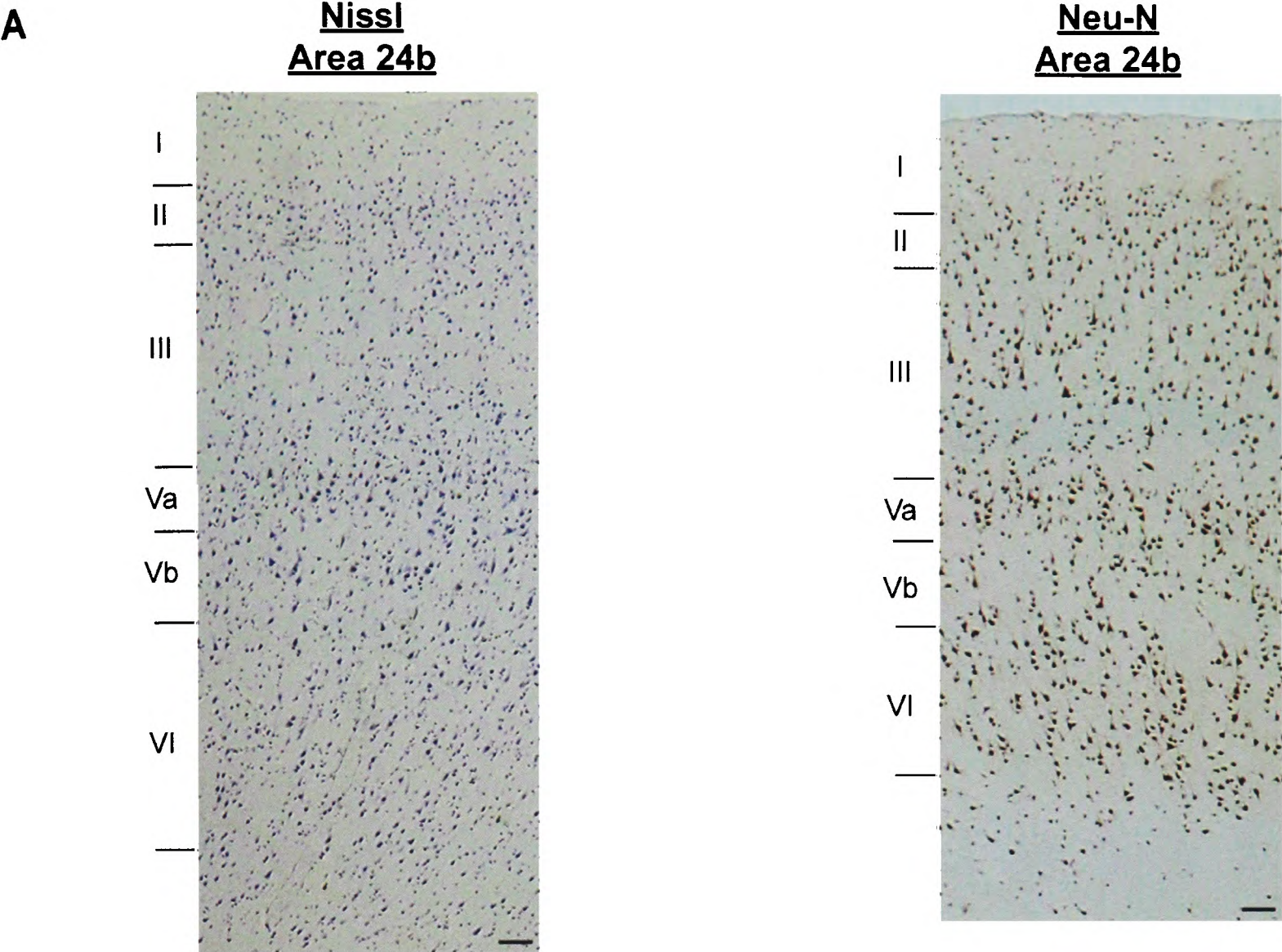
Figure 3.1.

(A) Photomicrographs showing the distribution of Nissl-staining and Neu-N immunoreactivity in area 24b (scale bar = 100µm).

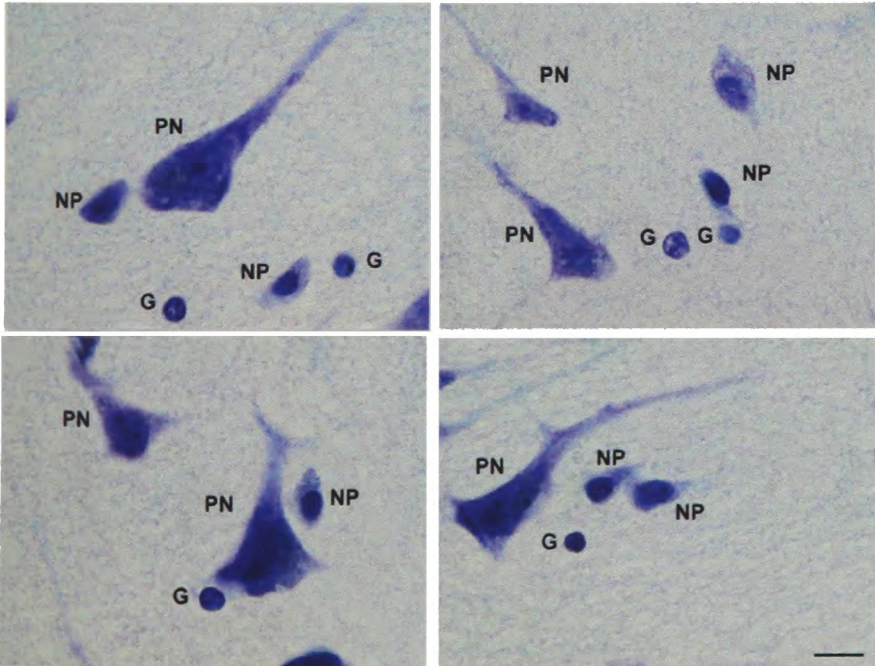
Neu-N-immunoreactive neurons are present throughout the cortical layers and in the subadjacent white matter. Staining with Neu-N greatly enhanced the cortical lamination. The negative control (omission of the primary antibody) did not show any immunoreactivity (data not shown).

(B) High-power photomicrographs showing examples of cells stained with Nissl and Neu-N methods. (scale bar=10µm).

Nissl and Neu-N methods stain pyramidal neurons and non-pyramidal neurons. The Neu-N method does not stain non-neuronal structures such as glia and ependymal cells. This image also shows a spindle neuron immunoreactive for Neu-N. PN = pyramidal neuron; NP = non-pyramidal neuron; G = glia.



B Nissl: Identification of pyramidal and non-pyramidal neurons and glial cells



Neu-N: Identification of pyramidal and non-pyramidal neurons

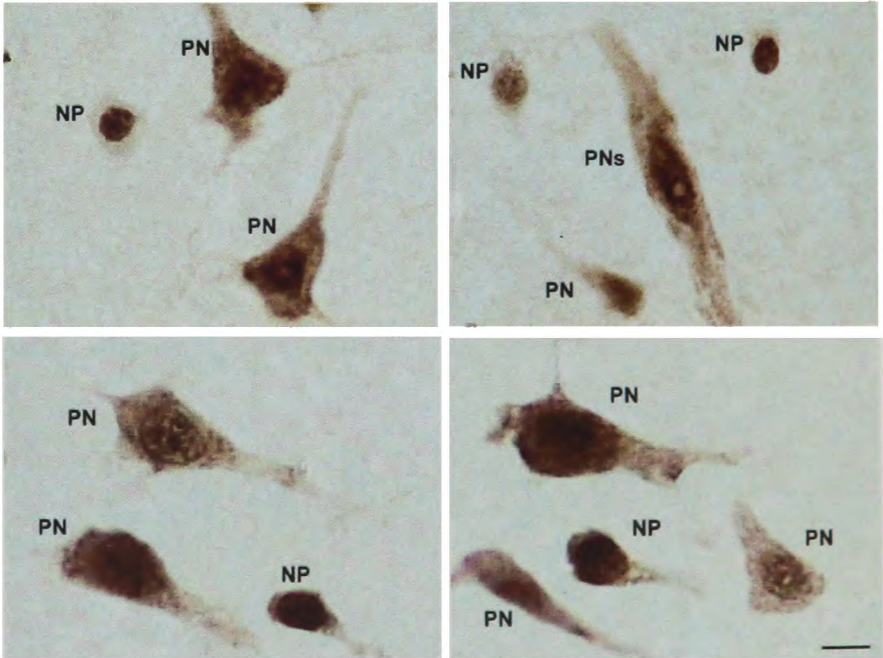


Table 3.2. Group means (SEM) for Nissl morphometric data

Nissl Morphometric Data: Mean (SEM)													
Cortical Layer	Layer depth (%)		Pyramidal neuron density, cells / mm ²		Non-pyramidal neuron density, cells / mm ²		Neuronal density, cells / mm ²		Pyramidal neuron size, μm ²		Pyramidal neuron shape		Glial density, cells / mm ²
	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder	
I	11.3	12.2	-	-	-	-	-	-	-	-	-	-	-
	(0.7)	(1.1)											
II	9.2	9.4	84.9	94.7	68.5	65.8	153.5	160.5	169.2	172.1	.602	.614	243.7
	(0.3)	(0.5)	(5.9)	(4.8)	(5.4)	(1.9)	(6.5)	(5.9)	(10.8)	(6.6)	(0.01)	(0.01)	(16.1)
													(11.4)
III	31.1	32.7	49.6	48.9	24.8	24.4	74.5	73.3	263.6	286.0	.598	.633	267.6
	(0.9)	(1.5)	(1.5)	(1.9)	(2.3)	(2.6)	(2.6)	(3.9)	(22.6)	(13.5)	(0.01)	(0.01)	(10.8)
													(13.3)
Va	9.9	9.3	74.9	69.2	41.1	40.1	115.9	109.3	279.9	357.5	.586	.641	332.2
	(0.2)	(0.4)	(2.4)	(3.9)	(2.5)	(2.4)	(1.7)	(5.5)	(19.7)	(13.8)	(0.01)	(0.01)	(27.1)
													(19.7)
Vb	11.8	10.9	48.1	40.9	22.4	20.7	70.6	61.6	297.3	379.7	.566	.610	352.7
	(0.4)	(0.4)	(1.7)	(1.0)	(1.7)	(2.1)	(1.9)	(2.2)	(19.3)	(17.7)	(0.01)	(0.01)	(19.4)
													(19.5)
VI	26.7	25.5	62.3	65.8	11.2	11.4	73.5	77.2	261.5	321.6	.622	.680	381.5
	(1.5)	(1.9)	(2.3)	(4.5)	(1.2)	(1.5)	(1.9)	(4.6)	(14.4)	(19.1)	(0.01)	(0.01)	(35.5)
													(19.6)

Table 3.3. Group means (SEM) for Neu-N morphometric data

Neu-N Morphometric Data: Mean (SEM)										
Cortical Layer	Pyramidal neuron density, cells / mm ²		Non-pyramidal neuron density, cells / mm ²		Neuronal density, cells / mm ²		Pyramidal neuron size, μm ²		Pyramidal neuron shape ('fcircle' index)	
	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder
II	99.7	106.2	70.5	67.6	170.2	173.8	189.4	181.7	.677	.667
	(7.7)	(4.2)	(6.3)	(2.0)	(10.3)	(4.5)	(8.1)	(7.9)	(0.01)	(0.01)
III	56.4	52.7	30.8	25.7	74.5	73.3	309.4	319.5	.673	.692
	(2.1)	(1.2)	(2.2)	(1.8)	(2.6)	(3.9)	(16.7)	(9.7)	(0.01)	(0.01)
Va	90.8	79.9	49.7	38.9	140.5	118.8	336.4	348.6	.662	.703
	(2.6)	(2.5)	(4.2)	(1.6)	(3.8)	(2.8)	(9.8)	(17.9)	(0.01)	(0.01)
Vb	51.1	45.3	26.6	23.2	77.7	68.6	364.7	383.5	.646	.676
	(1.8)	(0.8)	(1.7)	(0.7)	(1.7)	(0.5)	(11.2)	(19.2)	(0.01)	(0.01)
VI	75.6	79.9	16.7	15.3	92.3	92.2	300.9	324.2	.691	.721
	(3.0)	(4.6)	(1.1)	(2.3)	(3.2)	(4.5)	(13.5)	(19.0)	(0.01)	(0.01)

Table 3.4.

Results of repeated measures ANOVA (layer included as a ‘within subject’ factor).

a) Repeated measures ANOVA: Nissl morphometric data.

Repeated measures ANOVA: Nissl*						
	Diagnosis		Layer		Diagnosis x Layer	
	F	p	F	p	F	p
Layer depth (%)	0.001	1.000	168.104	<.001	0.529	.754
Pyramidal neuron density	0.001	.980	89.303	<.001	3.232	.019
Non-pyramidal neuron density	0.177	.681	192.571	<.001	0.140	.967
Neuronal density	0.107	.748	258.232	<.001	2.029	.103
Pyramidal neuron size ^a	4.476	.054	9.258	<.001	6.794	<.001
Pyramidal neuron shape ^a	2.517	.147	0.551	.699	2.433	.059
Glial density ^b	6.351	.026	10.850	<.001	1.013	.409

* significance set at p≤0.1; ^a repeated ANCOVA with pH; ^b repeated ANCOVA with PMI.

b) Repeated measures ANOVA: Neu-N morphometric data

Repeated measures ANOVA: Neu-N*						
	Diagnosis		Layer		Diagnosis x Layer	
	F	p	F	p	F	p
Pyramidal neuron density	0.559	.467	91.256	<.001	2.026	.103
Non-pyramidal neuron density	2.241	.175	144.527	<.001	1.110	.361
Neuronal density	3.531	.081	168.070	<.001	2.441	.057
Pyramidal neuron size ^a	0.700	.418	4.198	.005	1.738	.156
Pyramidal neuron shape	2.194	.171	8.638	<.001	2.993	.026

* significance set at p≤0.1; ^a repeated ANCOVA with pH

Figure 3.2.

Graphs showing (A) cortical depth and (B) layer depth (as a percentage of cortical depth) in the ACC in controls and mood disorder.

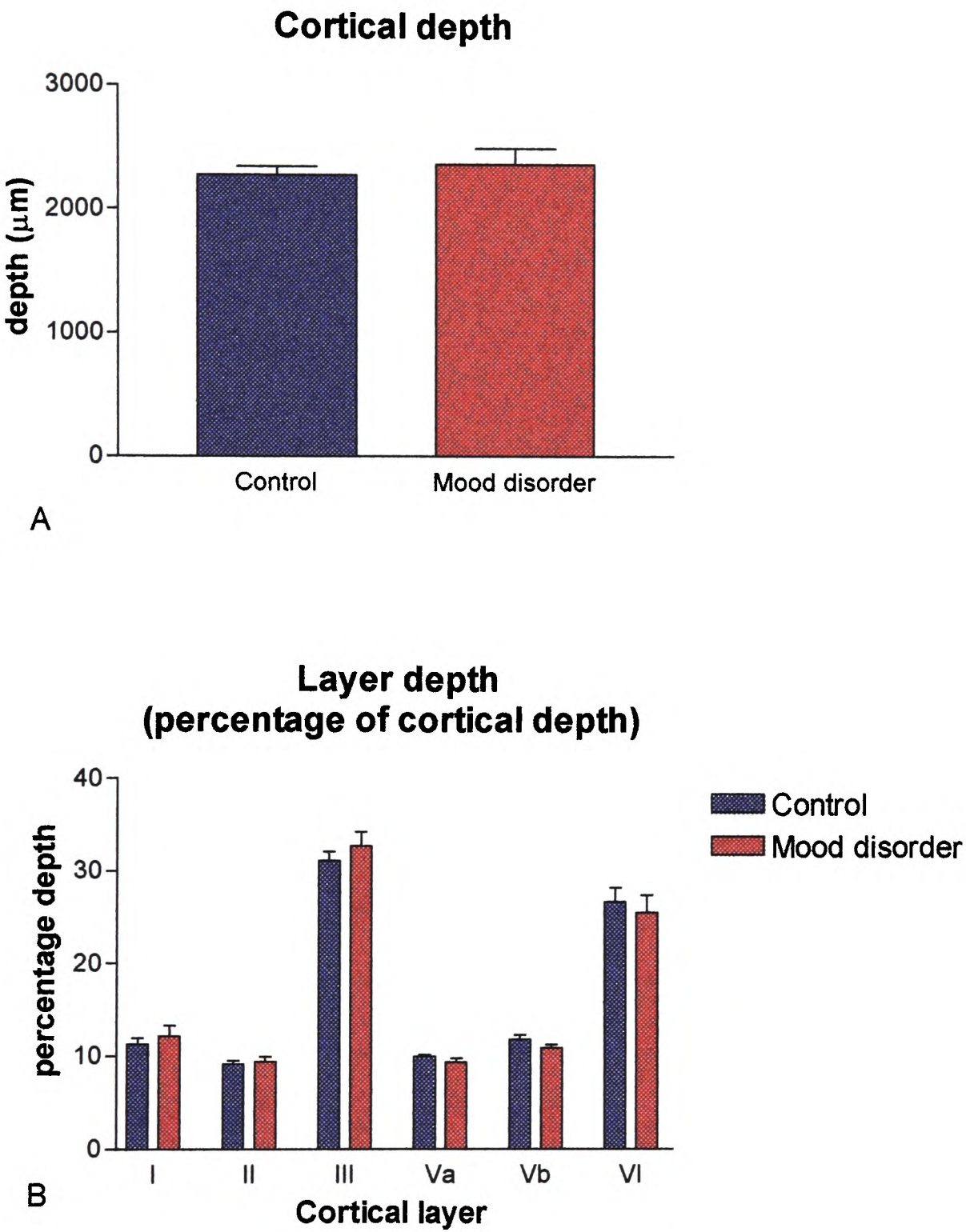


Table 3.5.

Pyramidal neuron density in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method and (B) Neu-N method. ANOVA test used for comparison of pyramidal neuron density between diagnostic groups. These data are illustrated in figure 3.3.

A.

Cortical Layer	Pyramidal neuron density (Nissl)		Test for difference between patient group and control group		
	Mean (S.E.M)		F	p	Percentage difference
	Control	Mood disorder			
II	84.9 (5.9)	94.7 (4.8)	1.51	.239	10.3%
III	49.6 (1.5)	48.9 (1.9)	0.09	.762	1.4%
Va	74.9 (2.4)	69.2 (3.9)	1.73	.210	8.2%
Vb	48.1 (1.7)	40.9 (1.0)	11.45	.004	17.6%
VI	62.3 (2.3)	65.8 (4.6)	0.53	.479	5.3%

B.

Cortical Layer	Pyramidal neuron density (Neu-N)		Test for difference between patient group and control group		
	Mean (S.E.M)		F	p	Percentage difference
	Control	Mood disorder			
II	99.7 (7.7)	106.2 (4.2)	0.46	.507	6.1%
III	56.4 (2.1)	52.7 (1.2)	1.99	.180	7.0%
Va	90.8 (2.6)	79.9 (2.5)	8.89	.010	13.6%
Vb	51.1 (1.8)	45.3 (0.8)	7.08	.019	12.8%
VI	75.6 (3.1)	76.9 (4.6)	0.06	.808	1.7%

Figure 3.3.

Graphs showing pyramidal neuron density in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method and (B) Neu-N method. See tables 3.4 and 3.5 for statistical comparisons between groups. * ≤ 0.05 ; ** ≤ 0.01 .

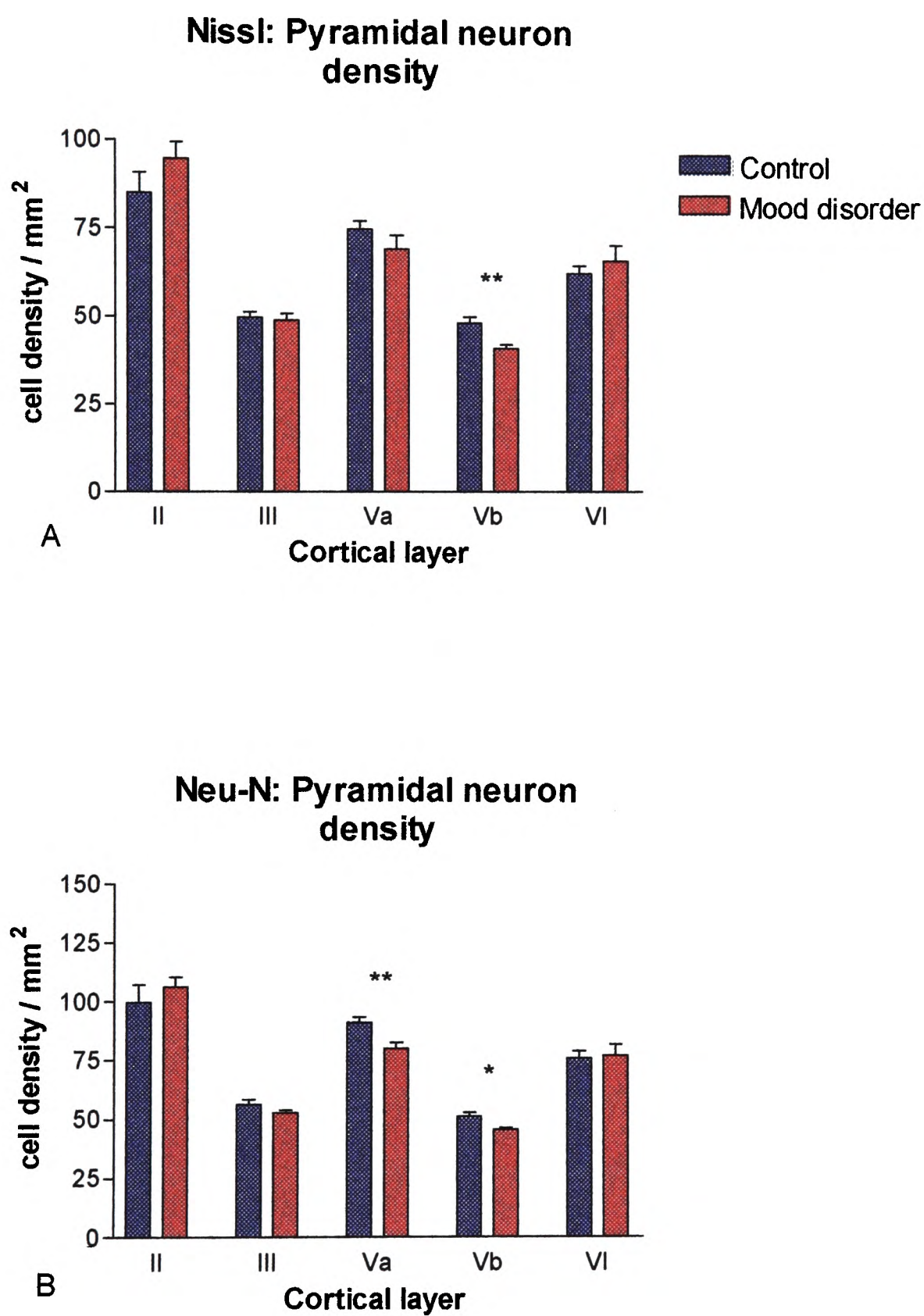


Figure 3.4.

Graphs showing non-pyramidal neuron density in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method and (B) Neu-N method. See table 3.4 for statistical comparisons between groups (separate ANOVAs were not performed, see section 3.3.4)

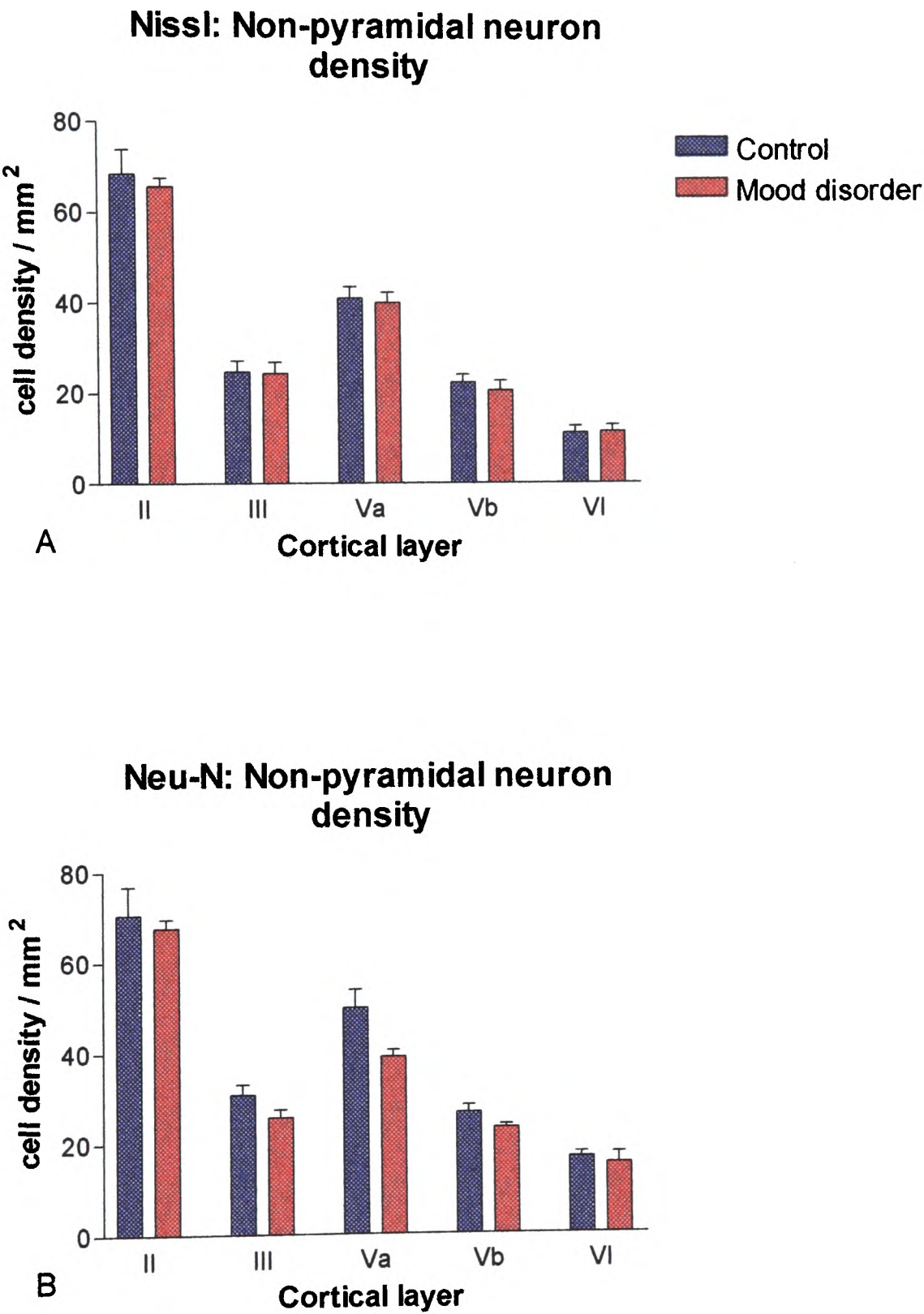


Table 3.6.

Neuronal density in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method and (B) Neu-N method. ANOVA test used for comparison of neuronal density between diagnostic groups. These data are illustrated in figure 3.5.

A.

Cortical Layer	Neuronal density (Nissl)		Test for difference between patient group and control group		
	Mean (S.E.M)		F	p	Percentage difference
	Control	Mood disorder			
II	153.5 (6.5)	160.5 (5.9)	0.59	.453	4.4%
III	74.5 (2.6)	73.3 (3.9)	0.06	.805	1.6%
Va	115.9 (1.7)	109.3 (5.5)	1.71	.212	6.0%
Vb	70.6 (1.9)	61.6 (2.2)	9.88	.007	14.6%
VI	73.5 (5.9)	77.2 (4.6)	0.65	.435	4.8%

B.

Cortical Layer	Neuronal density (Neu-N)		Test for difference between patient group and control group		
	Mean (S.E.M)		F	p	Percentage difference
	Control	Mood disorder			
II	170.2 (10.3)	173.8 (4.5)	0.09	.774	2.1%
III	87.2 (3.7)	78.4 (2.3)	3.61	.078	11.2%
Va	140.54 (3.8)	118.8 (2.8)	19.15	.001	18.3%
Vb	77.7 (1.7)	68.6 (0.5)	20.03	.001	13.3%
VI	92.3 (3.2)	92.2 (4.5)	0.00	.990	0.1%

Figure 3.5.

Graphs showing neuronal density in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method and (B) Neu-N method. See tables 3.4 and 3.6 for statistical comparisons between groups. **** ≤ 0.01 .**

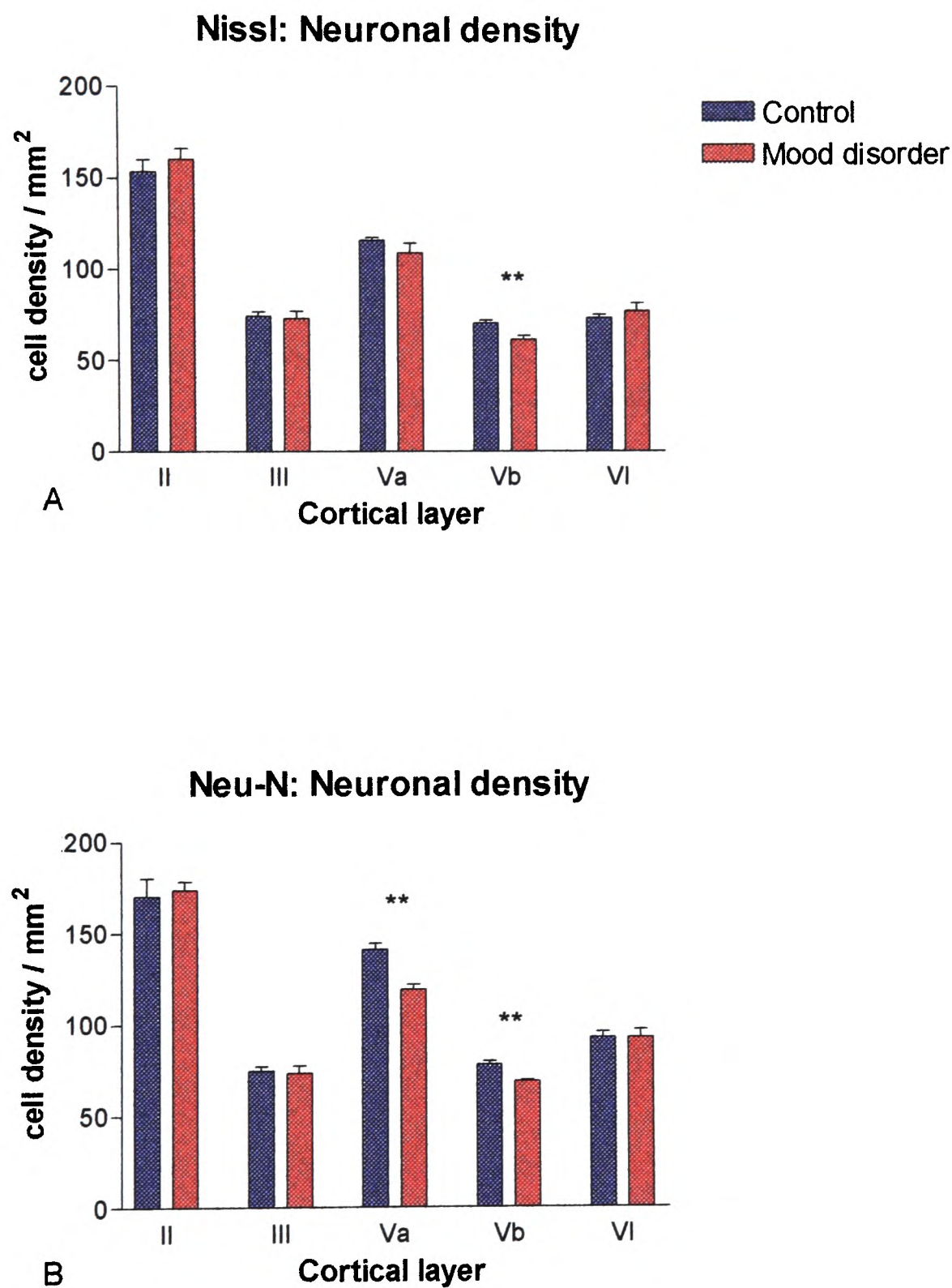


Table 3.7.

The effect of brain pH on pyramidal neuron size in cortical layers II-VI of the ACC.

Numbers are Pearson’s R values. * $p \leq 0.05$; ** $p \leq 0.01$.

Brain pH and pyramidal neuron size						
Cortical layer	Control		Mood disorder		Combined	
	Nissl	Neu-N	Nissl	Neu-N	Nissl	Neu-N
II	-0.51	-0.69*	0.12	0.26	0.10	-0.20
III	-0.86**	-0.66*	-0.76*	-0.29	-0.78**	-0.52*
Va	-0.65	-0.53	-0.49	0.77*	-0.61**	0.17
Vb	-0.53	-0.65*	-0.36	0.75*	-0.53*	0.06
VI	0.04	0.25	0.51	0.64	0.04	0.33

Table 3.8.

Pyramidal neuron size in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method. ANOVA test used for comparison of pyramidal neuron size between diagnostic groups. ANOVAs for Neu-N were not performed, as the repeated measures test was insignificant, (see section 3.3.6). These data are illustrated in figure 3.6.

A.

Cortical Layer	Pyramidal neuron size (Nissl)		Test for difference between patient group and control group ^a		
	Mean (S.E.M)		F	p	Percentage difference
	Control	Mood disorder			
II	169.2 (10.7)	172.1 (6.5)	0.02	.890	1.7%
III	263.6 (22.6)	286.0 (13.5)	0.06	.808	8.5%
Va	279.9 (19.6)	357.5 (13.7)	6.69	.023	27.7%
Vb	297.3 (19.3)	379.7 (17.6)	6.58	.024	27.7%
VI	261.5 (14.3)	321.6 (19.1)	7.68	.016	23.0%

^a ANCOVA with pH

Figure 3.6.

Graphs showing pyramidal neuron size in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method and (B) Neu-N method. See tables 3.4 and 3.8 for statistical comparisons between groups. * ≤ 0.05 .

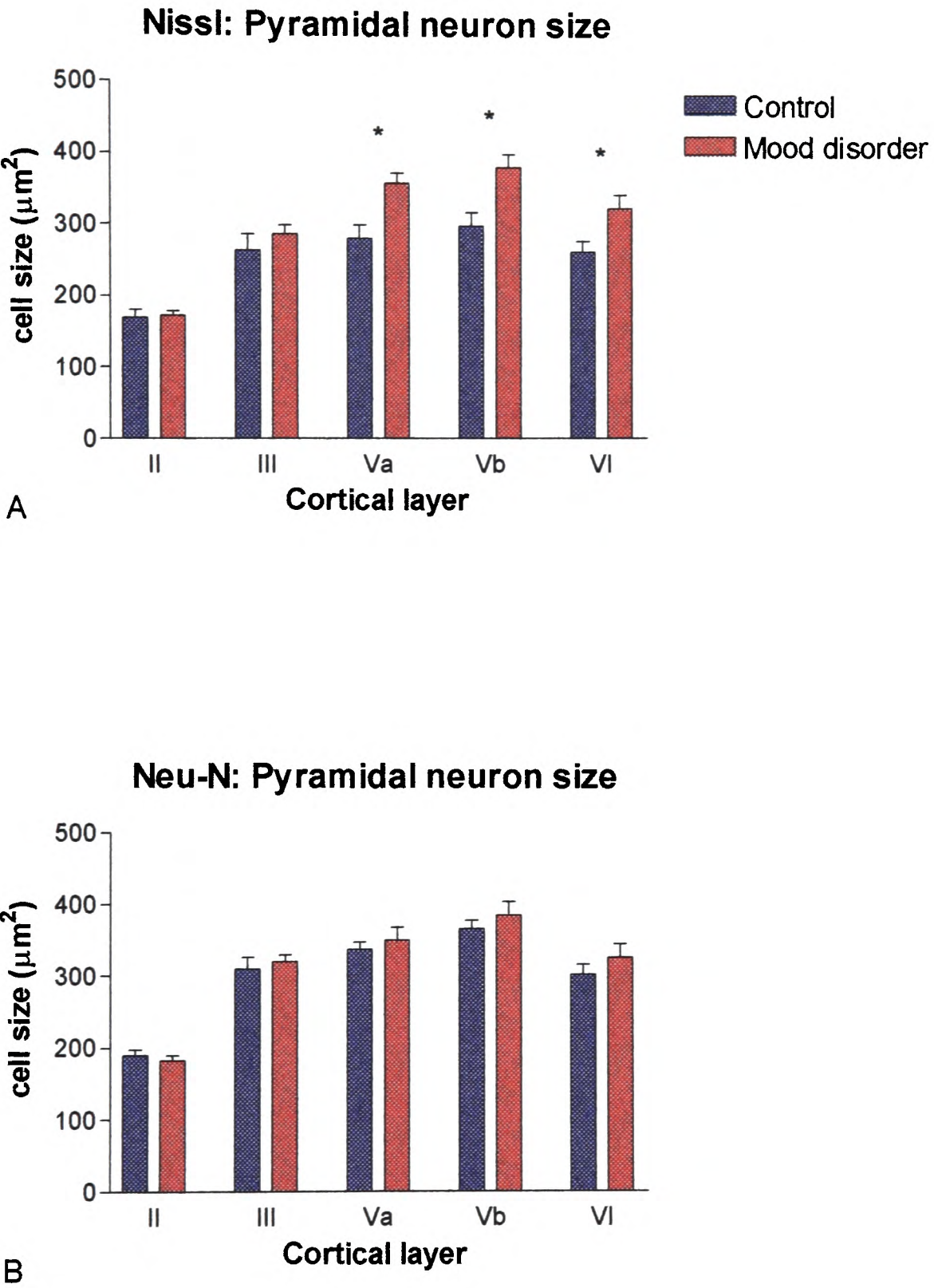


Figure 3.7.

Histograms showing pyramidal neuron size distribution in Nissl-stained sections in (A) layer Va, (B) layer Vb and (C) layer VI of the ACC in controls and mood disorder.

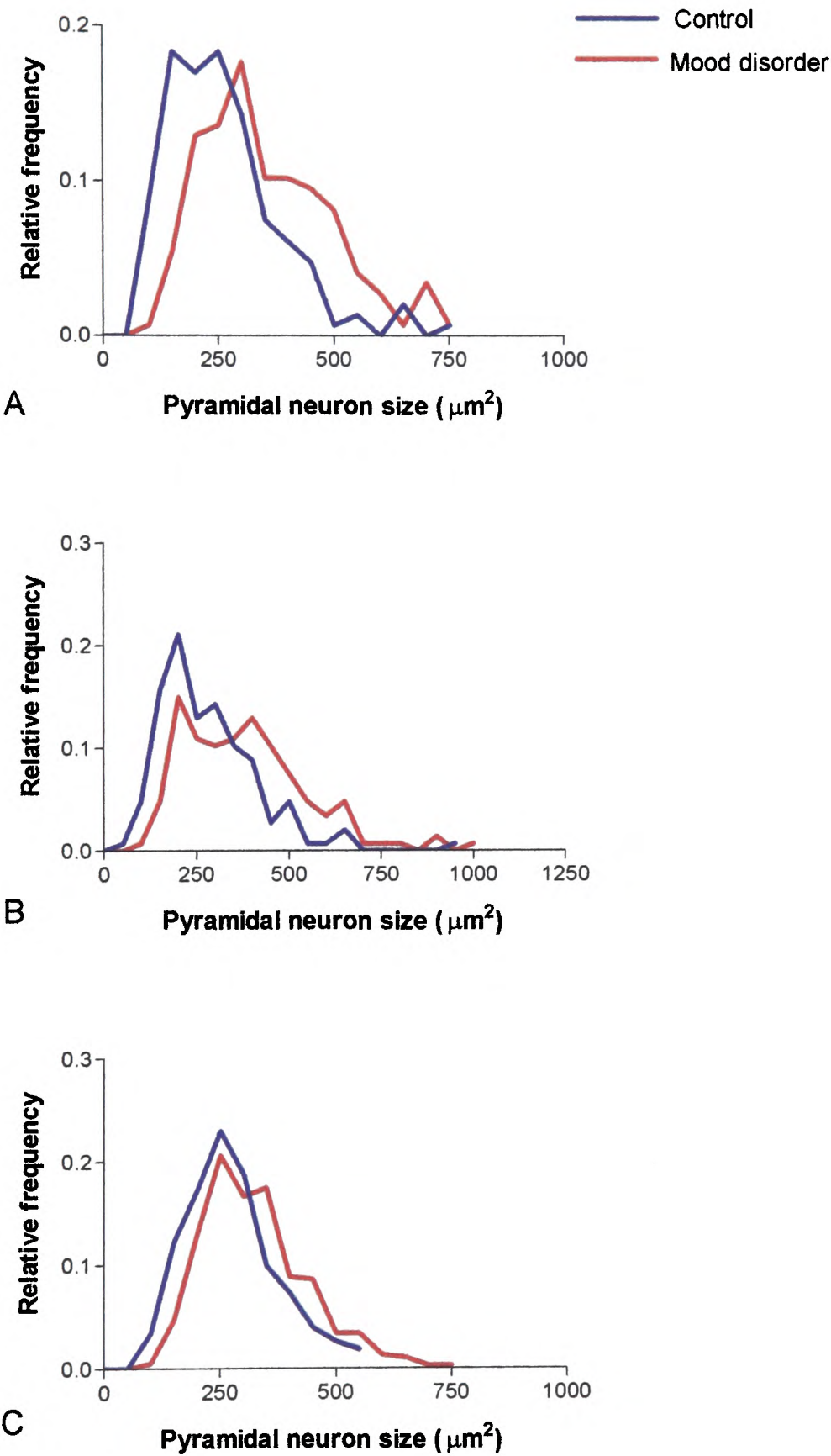


Table 3.9.

The effect of brain pH on pyramidal neuron shape in cortical layers II-VI of the ACC.

Numbers are Pearson's R values. * $p \leq 0.05$; ** $p \leq 0.01$.

Brain pH and pyramidal shape - 'fcircle' (Nissl)			
Cortical layer	Control	Mood disorder	Combined
II	-0.62	-0.06	-0.41
III	-0.75*	-0.31	-0.57*
Va	-0.59	-0.23	-0.51*
Vb	-0.78**	-0.11	-0.48
VI	-0.57	-0.16	-0.47

Table 3.10.

Pyramidal neuron shape in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method and (B) Neu-N method. ANOVA test used for comparison of pyramidal neuron shape between diagnostic groups. These data are illustrated in figure 3.8.

A.

Cortical Layer	Pyramidal neuron shape (Nissl)		Test for difference between patient group and control group ^a		
	Mean (S.E.M)		F	p	Percentage difference
	Control	Mood disorder			
II	.602 (0.01)	.614 (0.01)	0.01	.909	2.0%
III	.598 (0.01)	.633 (0.01)	0.66	.430	5.5%
Va	.586 (0.01)	.641 (0.01)	4.73	.049	8.6%
Vb	.566 (0.01)	.610 (0.01)	1.53	.238	7.2%
VI	.622 (0.01)	.680 (0.01)	7.99	.014	8.5%

^a ANCOVA with pH

B.

Cortical Layer	Pyramidal neuron shape (Neu-N)		Test for difference between patient group and control group		
	Mean (S.E.M)		F	p	Percentage difference
	Control	Mood disorder			
II	.677 (0.01)	.667 (0.01)	0.25	.626	1.5%
III	.673 (0.01)	.692 (0.01)	0.73	.408	2.7%
Va	.662 (0.01)	.703 (0.01)	8.89	.010	5.8%
Vb	.646 (0.01)	.676 (0.01)	2.68	.124	4.4%
VI	.691 (0.01)	.721 (0.01)	3.41	.086	4.2%

Figure 3.8.

Graphs showing pyramidal neuron shape (fcircle) in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method and (B) Neu-N method. See tables 3.4 and 3.10 for statistical comparisons between groups. * ≤ 0.05 ; ** ≤ 0.01 .

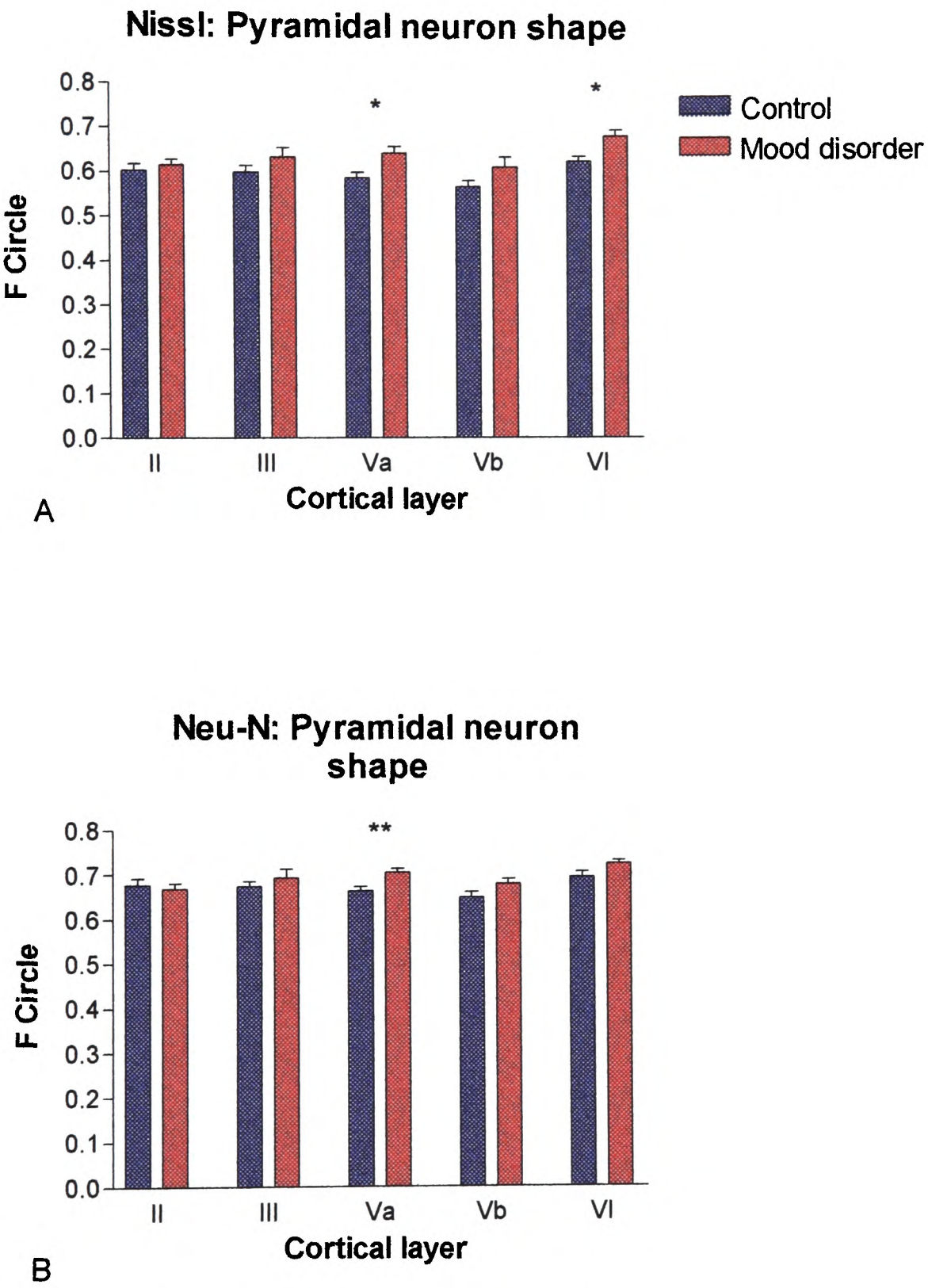


Table 3.11.

The effect of PMI on glial density in cortical layers II-VI of the ACC. Numbers are Pearson's R values. * p≤0.05

PMI and glial density (Nissl)			
Cortical layer	Control	Mood disorder	Combined
II	-0.72*	-0.33	-0.51*
III	-0.72*	-0.07	-0.41
Va	-0.53	0.16	-0.31
Vb	-0.33	-0.48	-0.35
VI	-0.53	-0.38	-0.44

Table 3.12.

Glial density in cortical layers II-VI of the ACC in controls and mood disorder: ANOVA test used for comparison of glial density between diagnostic groups. These data are illustrated in figure 3.9.

Cortical Layer	Glial density / mm ² (Nissl)		Test for difference between patient group and control group ^a			
	Mean (S.E.M)		F	p	Percentage difference	
	Control	Mood disorder				
II	243.7 (16.1)	186.9 (11.4)	9.87	.008	23.3%	
III	267.6 (10.8)	232.2 (13.3)	4.74	.048	13.2%	
Va	332.2 (27.1)	278.8 (19.7)	2.18	.164	16.1%	
Vb	352.7 (19.4)	287.6 (19.5)	5.59	.034	18.4%	
VI	381.5 (35.5)	291.4 (19.6)	4.64	.050	23.6%	

^a ANCOVA with PMI

Figure 3.9.

Graphs showing glial density in cortical layers II-VI of the ACC in controls and mood disorder: (A) Nissl method. See tables 3.4 and 3.12 for statistical comparisons between groups. * ≤ 0.05 ; ** ≤ 0.01 .

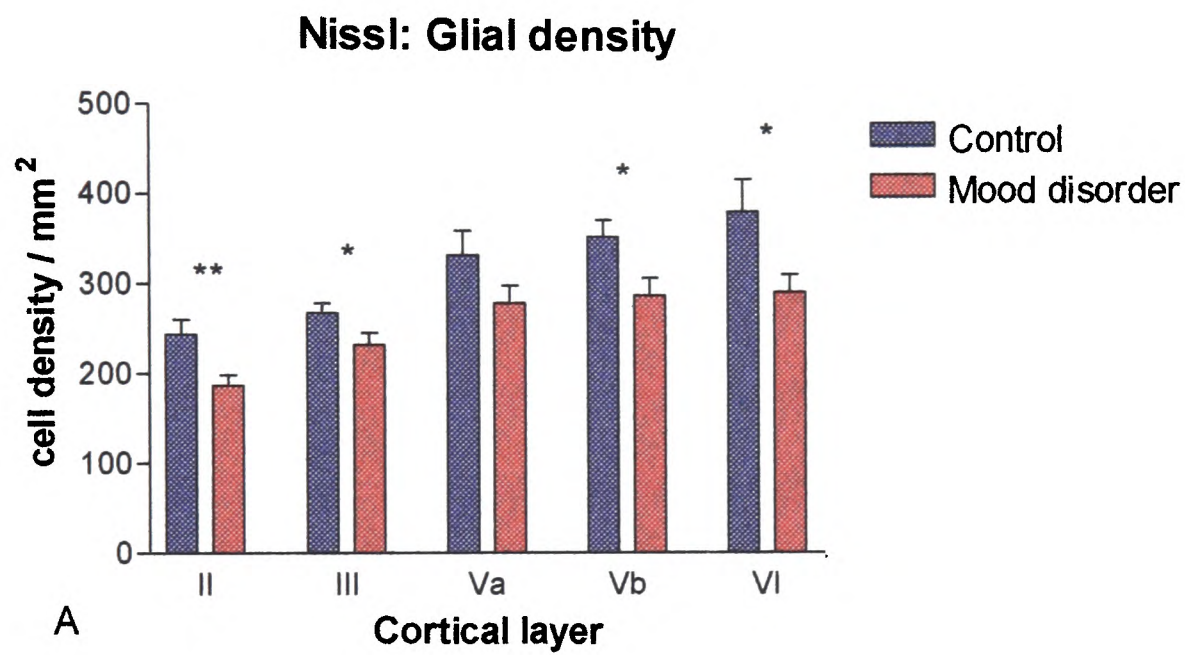


Table 3.13.

Correlations between Nissl and Neu-N methods: (A) pyramidal neuron density, (B) non-pyramidal neuron density, (C) pyramidal neuron size and (D) pyramidal neuron shape. Numbers are Pearson's R values. * $p \leq 0.05$; ** $p \leq 0.01$.

A.

Nissl and Neu-N correlations: Pyramidal neuron density			
Cortical Layer	Control	Mood disorder	Combined
II	0.94**	0.85*	0.89**
III	0.39	0.92**	0.51*
Va	0.87**	0.93**	0.85**
Vb	0.71**	-0.36	0.72**
VI	0.67*	0.82*	0.75**

B.

Nissl and Neu-N correlations: Non-pyramidal neuron density			
Cortical Layer	Control	Mood disorder	Combined
II	0.86**	0.48	0.84**
III	0.65*	0.59	0.58**
Va	0.87**	0.53	0.70**
Vb	0.54*	0.42	0.48*
VI	0.52*	0.81*	0.66**

Table 3.13. (continued).

C.

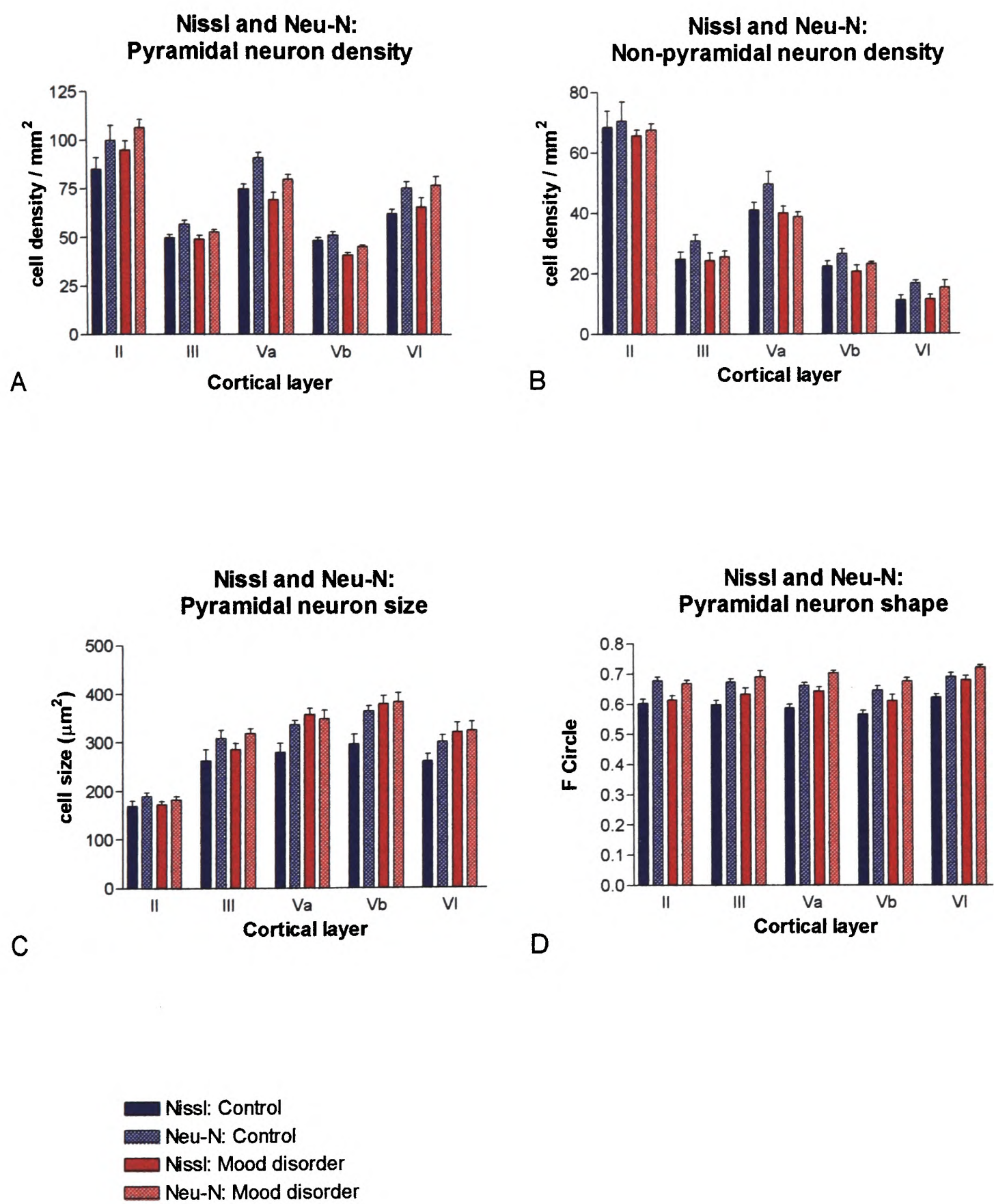
Nissl and Neu-N correlations: Pyramidal neuron size			
Cortical Layer	Control	Mood disorder	Combined
II	0.38	0.96**	0.50*
III	0.84**	0.76*	0.83**
Va	0.78*	-0.26	0.34
Vb	0.46	-0.48	0.15
VI	0.78*	0.85*	0.80**

D.

Nissl and Neu-N correlations: Pyramidal neuron shape (fcircle)			
Cortical Layer	Control	Mood disorder	Combined
II	0.68*	0.71*	0.65**
III	0.73*	0.96**	0.86**
Va	0.43	0.64*	0.68**
Vb	0.27	0.74*	0.54*
VI	0.55*	0.34	0.61*

Figure 3.10.

Graphs showing comparisons between Nissl and Neu-N methods: (A) pyramidal neuron density, (B) non-pyramidal neuron density, (C) pyramidal neuron size and (D) pyramidal neuron shape.



3.4 Discussion

3.4.1 Reduced neuronal and pyramidal neuron density in the ACC in mood disorder

Pyramidal neuron density was reduced in layer Va (Neu-N: 13%) and layer Vb (Nissl: 17%; Neu-N: 12%) in mood disorder. Total neuronal density was also reduced in layers Va (Neu-N: 18%) and Vb (Nissl: 14%; Neu-N: 13%) in mood disorder.

Existing studies of neuronal density in the ACC in mood disorder are equivocal. The first morphometric study of the subgenual region of the ACC failed to observe any alterations in neuronal density (Öngür *et al.* 1998), however, this study is limited as the authors did not investigate each cortical layer individually and results from the current study are certainly suggestive of layer-specific changes. Bouras *et al.* (2001) found reduced neuronal density (of a similar magnitude to those in the current study, 15-20%) in layers II-VI in the subgenual region of the ACC in bipolar subjects; however, unlike the results presented here, no differences were reported for the supragenual region of the ACC, or in major depression. A study of the pregenual ACC reported a significant reduction in non-pyramidal neurons in bipolar disorder (Benes *et al.* 2001). Another study of the supragenual ACC failed to observe any deficits in neuronal density in either major depression, or bipolar disorder (Cotter *et al.* 2001a).

Prefrontal and limbic regions have also been investigated in mood disorder. Reductions in neuronal density have been reported for large neurons in area 9 (layers II, III and IV), with concomitant increases in the density of small neurons in major depression. A decreased density of large neurons was also observed in area 10/47 (layers IIIa and Va) (Rajkowska *et al.* 1999). In a study of area 9 in bipolar subjects, the same authors report a reduction in total neuronal density and pyramidal neuron density in layers II, III and V (9-30%). Non-pyramidal neuron density was unchanged (Rajkowska *et al.* 2001). A second study of area 9 failed to report any differences in neuronal density in subjects with major

depression, or bipolar disorder (Cotter *et al.* 2002b). A recent study of the amygdala in mood disorder also failed to find any significant alterations in neuronal density (Bowley *et al.* 2002). The layer-specific reduction in pyramidal neuron density observed in the present study provides some support for these existing morphometric investigations and substantiates not only the direction of alteration, but also the magnitude of change.

3.4.2 Reduced glial density in the ACC in mood disorder

Glial density was significantly reduced in layers II (23%), III (13%), Vb (18%), and VI (23%) in the mood disorder group. A reduction of similar magnitude was also observed in layer Va (16%), but this failed to reach significance.

Reductions in glial density have been somewhat consistently reported in mood disorder research. In the one study of glial density in the supragenual ACC to date, Cotter *et al.* (2001a) reported a significant decrease in glial density in layer VI (22%) in major depression, but not bipolar disorder. Although not significant, layers III, Va and Vb also showed a similar trend in major depression. In a study of the subgenual region of the ACC, glial density and number were reduced in major depression (20%) and bipolar disorder (41 %, familial cases only) (Öngür *et al.* 1998). Glial size remained unchanged. In comparison, a study of the pregenual ACC failed to observe any significant alterations in glial density (Benes *et al.* 2001).

Investigations into the prefrontal and limbic regions of the brain have also pointed to deficits in glial density. In a study of major depression, reduced glial density was reported in area 9 (layer III and IV, 20-30%), and area 47 (15%). Area 10/47 also showed a trend for an overall decrease in glial density (Rajkowska *et al.* 1999). Differential changes in glial size were also observed; an increased density of glia with large nuclei was observed in layer III in area 9, whereas areas 47 and 10/47 showed a decreased density of medium and large sized glia (Rajkowska *et al.* 1999). Reductions in glial density have also been

observed in area 9 in bipolar disorder, with significant changes confined to layers IIIc and Vb. Glial cell enlargement and alterations in glial cell shape were also observed (Rajkowska *et al.* 2001). Other analyses of area 9 have indicated a reduced glial density in major depression, but not bipolar disorder. Changes were specific to layer V (30%), and did not involve any alterations in glial cell size (Cotter *et al.* 2002b). A recent study of the amygdala has also demonstrated decreases in glial density in major depression (29%), but not bipolar disorder (Bowley *et al.* 2002). The glial deficits observed in the present study provide support for the existing morphometric literature; confirming both the direction and magnitude of change. The implications of reduced glial density will be considered in greater detail later in the discussion (section. 3.4.4).

3.4.2.1 *Factors affecting glial density*

Significant negative correlations between glial density and PMI were observed. As staining with the Nissl method tends to be fairly robust and not severely affected by a moderate delay in fixation, it is unlikely that differences in PMI accounted for the morphometric differences between the diagnostic groups. Nevertheless, PMI was included as a covariate in the statistical analyses. Although significant correlations were not observed in the mood disorder group, it is unlikely that PMI would differentially affect the two groups; unless the disease (or factors relating to the disease) counteracts normal PMI associated changes in the ability to detect glial cells. It is more likely that high variability and increased noise within the mood disorder group concealed any significant correlations. It is possible that the significant correlations observed here occurred by chance, however, negative correlations between glial density and PMI have been previously reported (Benes *et al.* 2001). Interestingly, when PMI was included as a covariate in a study of the amygdala in mood disorder, the significant effect of decreased glial density disappeared (Bowley *et al.* 2002).

3.4.3 Alterations in pyramidal neuron size and shape in mood disorder

In the Nissl material, pyramidal neuron size was increased in layers Va (27.7%), Vb (27.7%) and VI (23.0%). This was not replicated in the Neu-N material, although inspection of the data suggests a similar pattern of alteration (see figure 3.6). Pyramidal neuron shape was also altered, with significant differences in layers Va (Nissl and Neu-N) and VI (Nissl). Overall, the *fcircle* values indicate that the mood disorder subjects have a more 'equilateral-shaped' triangular cell body in comparison to the control subjects.

The findings of increased pyramidal neuron size in conjunction with altered shape in the current study were largely unexpected, mainly because the majority of studies have reported smaller neurons in mood disorder. Smaller neurons have been observed in layer VI (22%) in a study of the supragenual region of the ACC, with a similar trend in layer V (18%) (Cotter *et al.* 2001a). In contrast, a study of the subgenual and supragenual regions of the ACC did not report any alterations in neuronal size in major depression, or bipolar disorder (Bouras *et al.* 2001).

Decreases in neuronal size have also been reported in prefrontal regions: reductions have been observed in layers III and IV of area 9, layer II of area 47, and layers II and III in area 10/47 in a study of major depression (Rajkowska *et al.* 1999); no alterations in neuronal size were observed in bipolar disorder (Rajkowska *et al.* 2001). Cotter *et al.* (2002b) have also reported decreases in neuronal size in both major depression (6%, layer VI) and bipolar disorder (layers V and VI; 14% and 18% respectively) in area 9.

Although not predicted prior to commencing this study, increases in pyramidal neuron size are not unprecedented in the ACC. In a study of the pregenual region of the ACC, Benes *et al.* (2001) reported larger pyramidal neurons in layer II. Although not significant, similar increases were observed in other cortical layers. Non-pyramidal neurons also showed an

increased cell size in layers II and III. All increases were in the region of 20% - comparable to those reported in the present study.

As increases in pyramidal cell size were not predicted, and were significant only in the Nissl material, the results of the current study should be interpreted cautiously until confirmed in a larger series. Overall, the size data are inconclusive.

3.4.3.1 *Factors affecting pyramidal neuron size and shape*

Significant negative correlations were found with pH and pyramidal neuron size in the Nissl series, with more acidic brains having larger cells. This effect of pH is presumably due to osmotic swelling, secondary to acidosis. Interestingly, although the majority of correlations were negative in the Neu-N series, all of the significant correlations in the mood disorder group showed a positive relationship between the two variables. Again, it is unlikely that pH would differentially affect the two groups and therefore this finding is more likely to reflect increased variability in the mood disorder group, resulting in a chance occurrence - especially in consideration of the Nissl series, which did not show a similar pattern. Correlations between pH and cell size have been previously reported (see Harrison 2000), including specific correlations between glial cell size and pH in the ACC (Cotter *et al.* 2001a).

Cell shape also showed a significant negative correlation with pH in the Nissl series, but not the Neu-N series, with more acidic brains having higher 'fcircle' values. The mechanism behind such a relationship is unclear but may again be related to osmotic pressure – with subsequent swelling creating a fatter and shorter cell body. The reason for the discrepancy between the Nissl and Neu-N data is unclear, although it is conceivable that the immunohistochemical procedure, per se (including microwaving), may have affected the relationship between cell shape and pH. Negative correlations between cell shape and pH have been previously reported (Law and Harrison 2003).

3.4.4 Functional implications

The cellular changes reported here indicate that glial density and neuronal density are abnormal in mood disorder. In respect to layer specificity, the glial cell deficit was observed across all cortical layers in the ACC (layer Va showed the same percentage decrease, even though it was not statistically significant). In comparison, changes in neuronal density were more subtle, with alterations in total neuronal and pyramidal neuron density restricted to layers Va and Vb (considering the Nissl and Neu-N results in unison). Interestingly, no alterations in non-pyramidal neuron density were observed. The changes observed in both pyramidal neuron size and shape were also confined to the deeper cortical layers, although changes in neuron size should be interpreted cautiously as they were not replicated in the Neu-N series. Whether the reductions in neuronal and glial density represent cell loss is not clear, as neither the volume of the ACC nor the volume of each cortical layer was (or could have been) assessed.

The findings of this study help to corroborate the existing morphometric studies of mood disorder. As discussed in sections 3.4.1 and 3.4.2, although glial and/or neuronal deficits have been consistently reported in the ACC (Benes *et al.* 2001; Bouras *et al.* 2001; Cotter *et al.* 2001a; Öngür *et al.* 1998), other prefrontal and limbic brain regions also show specific alterations in mood disorder, particularly in respect to glial deficits (Bowley *et al.* 2002; Cotter *et al.* 2002b; Rajkowska *et al.* 2001; Rajkowska *et al.* 1999); nevertheless, a uniform pathology of mood disorder seems unlikely given the negative results in the somatosensory (Öngür *et al.* 1998) and visual cortices (Bouras *et al.* 2001). In this respect, the neuropathology of mood disorder is coherent with anatomical and functional imaging data that seem to suggest a preferential involvement of the frontolimbic areas in mood disorder (see Drevets 2000a; b).

3.4.4.1 Interpretation of glial and neuronal deficits

The finding of decreased glial density, along with layer-specific reductions in pyramidal neuron density in this study supports the view that neurodegeneration with accompanying gliosis is not a feature of mood disorder (Rajkowska *et al.* 1999). Whilst decreases in glial density appear to be a prominent feature of mood disorder (see Cotter *et al.* 2001b), which specific type of glial cell (i.e., astrocytes, oligodendrocytes, or microglia) is involved in this pathology remains to be determined by immunohistochemical methods. To date, immunohistochemical studies of glial fibrillary acidic protein (GFAP – used as a marker of astrocytes) remain inconclusive (Miguel Hidalgo *et al.* 2000). A recent proteomic study has reported decreases in various GFAP isoforms in major depression and bipolar disorder (Johnston-Wilson *et al.* 2000). Oligodendrocytes have also been the focus of recent attention and in a recent ultrastructural study were reported to show signs of apoptosis and necrosis in area 10 in bipolar disorder (Uranova *et al.* 2001). A reduction in the density of oligodendrocytes has also been reported (in preliminary form) in layers III and VI in the dorsolateral prefrontal cortex in major depression and bipolar disorder (Orlovskaya *et al.* 2000; Uranova *et al.* 2002). An investigation and discussion in to which of these glial populations are particularly affected in mood disorder is the subject of chapter five.

For many years, the role of glial cells in cortical and neuronal function has been underestimated (Coyle and Schwarcz 2000); and it is only recently that their vital role in normal cortical functioning has been realised and their potential involvement in the neurobiology of psychiatric disorders fully appreciated (Cotter *et al.* 2001b). Glial cells have important roles in synaptic function, synaptic plasticity, neurotransmission, neuronal metabolism, and the clearance of extracellular ions and transmitters (Araque *et al.* 1999; Bezzi and Volterra 2001; Chvatal and Sykova 2000; Magistretti 2000; Magistretti *et al.* 1999; Olie *et al.* 2001; Pfrieger and Barres 1996; Ullian *et al.* 2001; Verkhratsky *et al.* 1998). Given their close association with neurons and their involvement in the

maintenance of neuronal structure and synapse number (Ullian *et al.* 2001), a popular model has evolved where deficits in a glial population beget a 'neuronal pathology', with downstream effects on neuronal, synaptic and dendritic morphology (Rajkowska 2000).

The general decreases in glial density, accompanied by subtle layer-specific decreases in neuronal density observed in the present study certainly seem to be coherent with this theory. Although speculative, it is reasonable that glial deficits are the central pathological event, and as this pathology worsens (perhaps with increasing duration of illness), specific neuronal deficits arise. Conversely, it is equally feasible that disease-related reductions in the activity of neurons with altered morphology would require less glial support, which may be reflected in a reduced number or density of glial cells. Such a theory is less consistent with the present findings, as one would expect to observe glial deficits only in those cortical layers with specific neuronal deficits – and not the generalised deficit in glial cells found in the present study. Although practically impossible considering the restraints of post mortem research, it would be very interesting to conduct a temporal investigation into the sequence of neuropathological events in mood disorder.

The finding of increased pyramidal neuron size in mood disorder in this study is not entirely compatible with the proposed theories. Given that glial cells are vital for the metabolic and structural support of neurons (Magistretti *et al.* 1999; Pfrieger and Barres 1996; Ullian *et al.* 2001); glial cell loss could conceivably result in neurons being more vulnerable to excitotoxic damage, and less capable to supporting the metabolic demands of an extensive dendritic arborisation and a normal cell size (Cotter *et al.* 2001b).

Therefore, if neuronal pathology were secondary to glial pathology, one would expect to observe neuronal atrophy – and not increases in neuronal size. Likewise, other non-glial, disease-related effects on neuronal function would probably be expected to reduce neuronal size. Indeed, the majority of the existing literature seems to be in support of smaller neurons in mood disorder (Cotter *et al.* 2002b; Cotter *et al.* 2001a; Rajkowska *et*

al. 2001; Rajkowska *et al.* 1999), rather than an increase in neuronal size (Benes *et al.* 2001).

Given that decreases in pyramidal neuron density were observed in layers Va and Vb in the mood disorder group, it could be hypothesised that an overall increase in pyramidal neuron size may simply reflect a specific loss of the smaller pyramidal neurons in this group. However, histograms illustrating the distribution of pyramidal neuron size did not provide any support for this theory, as the distribution curves were not skewed towards larger cells in the mood disorder group (see figure 3.7). Nevertheless, the occurrence of decreased cell density together with increased cell size does not have to be mutually exclusive. It is at least possible that increases in pyramidal neuron size occur in response to, and compensate for, the overall deficits in pyramidal neuron density. In this respect, it is interesting that the decreases in pyramidal neuron density observed in this study were confined to layers Va and Vb - which are the same layers that demonstrated significant changes in cell size and shape. However, this is entirely speculative, and my findings of increased pyramidal neuron size are not substantiated by the existing literature. Furthermore, as significant increases in cell size were not replicated in the Neu-N series (although similar patterns were observed), this finding may in fact be a chance result and therefore any interpretation should be made with caution until such findings have been replicated in a large and well-characterised brain series.

It is also interesting to speculate that glial pathology might be related to the dysfunction of various neurotransmitter systems implicated in mood disorder (see Charney 1998). Glial cells may play a role in neurotransmission via serotonergic, adrenergic and glutamatergic receptors distributed on their cell bodies and processes (Azmitia *et al.* 1996; Gallo and Ghiani 2000; Porter and McCarthy 1997) and therefore any pathological involvement of these cells might contribute to deficits in these neurotransmitter systems in mood disorder (see Drevets 2000b; Drevets *et al.* 2000).

Additionally, it is noteworthy that since glial cells provide the energy requirements of neurons (Magistretti *et al.* 1999), and are the major contributors to PET and fMRI signals (Magistretti 2000; Magistretti and Pellerin 1996); any differences in the various functional imaging parameters observed in mood disorder (Drevets 2000b; Videbech 2000) may be (at least in part) attributable to the glial pathology consistently observed in this illness.

3.4.4.2 *What causes the glial and neuronal deficits in mood disorder?*

Questions regarding the origins of the glial and neuronal deficits in mood disorder still remain to be answered. It is feasible that patients with mood disorder are genetically predisposed for the cellular changes detected post mortem, and have fewer glia cells (and possibly fewer neurons) at birth; although it is also possible that the cellular changes are a consequence of other mechanisms underlying mood disorder. A recent review has suggested that glucocorticoids might play a pivotal role in the pathology of mood disorder (Cotter *et al.* 2001b). The authors suggested that elevated levels of glucocorticoids, due to continuous hypersecretion of cortisol as part of the pathogenesis of the illness— or to illness related stress; may act to reduce glial cell activity and possibly instigate glial cell death, in addition to causing excitotoxic damage to neurons, thereby predisposing, or exacerbating psychiatric illness (Cotter *et al.* 2001b). However, as a comprehensive study of cellular stress and apoptosis has not been performed in the prefrontal and cingulate regions of the brain (and recent studies in the hippocampus did not find any evidence of cellular stress and only minor evidence of apoptosis; Lucassen *et al.* 2001); such hypotheses, whilst motivating, remain to be confirmed by post mortem research.

Despite all this speculation, there is not yet sufficient, consistent data available to substantiate any of these theories and therefore the pathophysiological significance of decreased glial density, or the link between neuronal and glial deficits, remains elusive. It is still not clear whether glial cell reduction is a reaction to other mechanisms underlying mood disorders, or whether glial cells play a causative role in the manifestation of

depressive symptoms. Further investigations are required to determine whether a specific glial population is involved, whether reductions in glial density are the primary pathological event – and whether neuronal pathology is a even a consequence of glial pathology.

Although the neuropathology of mood disorder appears to have a primarily frontolimbic focus, questions regarding the regional (and diagnostic) specificity of the neuropathological alterations also remain to be addressed (see section 7.2).

3.4.5 Correlations between Nissl and Neu-N

Overall, robust positive correlations were consistently observed between the Nissl and Neu-N techniques (see table 3.13), indicating that Neu-N is a valuable and reliable neuronal marker in human post-mortem tissue. In general, the control, mood disorder and combined groups all demonstrated similar relationships between Nissl and Neu-N methods; with the single exception of non-pyramidal neuron density in the mood disorder group, where layer VI showed the only significant correlation (though other layers did approach statistical significance).

Even though significant correlations were observed between the two techniques, the Neu-N method repeatedly produced higher density counts (see figure 3.10). Tissue shrinkage (due to immunohistochemical processing) is unlikely to explain this finding, as final section thickness did not differ between the two techniques, for either subject group (see section 3.4.6). On the contrary, it is quite plausible that the unavoidable, subjective nature of this study did contribute to this discrepancy. Neuronal staining with the Neu-N method was noticeably superior to that produced by the Nissl method; cells were stained with a greater intensity, the contour of each neuron was more defined and the overall clarity of staining was greatly improved. This enhanced staining may have resulted in a sampling bias, with a greater number of neurons being ‘unconsciously’ included in each count with the Neu-N material. In addition, as the Neu-N technique afforded greater confidence regarding cell categorisation – it is possible that the lower density counts obtained with the Nissl series

represent a more cautious density estimate (due to difficulties in categorising with certainty small cells that may be neurons or glia). Consequently, absolute values for pyramidal neuron and non-pyramidal neuron density are not comparable between the two methods, though reassuringly, robust correlations are observed between the two techniques.

The Neu-N method frequently demonstrated greater size and 'fcircle' values when compared to the Nissl method. It could be speculated that the immunohistochemical procedure itself, which included microwaving, may have affected the size and shape of these cells, resulting in augmented values in the Neu-N study. Interestingly, the mood disorder group does not show the same pattern as the control group with regard to pyramidal neuron size. Although cells are slightly larger in layers II and III in the Neu-N study, the figures for layers Va, Vb and VI are somewhat equivalent between the two methods. It is difficult to explain why cell size is differentially affected in the two subject groups; although, if differences in cell size between the two methods are related to the immunohistochemical protocol, it is possible that the disease itself, or related factors such as medication, may have a confounding effect on the 'normal' immunohistochemical process.

Overall, the Nissl and Neu-N methods showed robust correlations, suggesting that the two techniques are reasonably comparable. Whilst density counts tended to be higher in the Neu-N material; both diagnostic groups were similarly affected, and consequently this should not pose a problem when quantification is aimed at making comparisons between groups. In summary, the two techniques complement one another (and can help to substantiate results), however, neither technique was clearly superior. The Neu-N method greatly improved cortical layer definition, and also removed any ambiguity potentially caused by the misclassification of small neurons as glia (and vice versa). Conversely, the quality of Neu-N staining varied considerably between brains, which suggests that this technique may not be appropriate for all post mortem studies.

3.4.6 Methodological considerations

The findings of decreased pyramidal neuron density and glial density in mood disorder in the present study are in accordance with the existing literature. The discovery of larger neurons in mood disorder is less compatible, though not unprecedented in the ACC.

Although there are many similarities between the existing morphometric studies of the ACC in mood disorder, significant discrepancies remain. These discrepancies could be related to the heterogeneity of this region, and in particular, the anatomical differences between the subgenual and supragenual regions of the ACC, such as those described in chapter two. Differences in the nature of the patient sample, including variables such as age and sex together with more complicated issues concerning patient diagnosis, duration of illness and medication status, may also contribute. The manner in which the tissue has been collected, including tissue processing and embedding is also likely to effect any subsequent morphometric measurements.

Additionally, the use of fundamentally different cell-counting approaches (with their own inherent strengths and weaknesses) could contribute to the differences between studies. From this point of view it is reassuring that both 2-D and 3-D counting approaches have reported reductions in glial density (3D: Öngür *et al.* 1998; Rajkowska *et al.* 2001; Rajkowska *et al.* 1999, and 2D: Cotter *et al.* 2002b and the present study) and neuronal density (3D: Bouras *et al.* 2001; Rajkowska *et al.* 2001; Rajkowska *et al.* 1999, and 2D: Benes *et al.* 2001 and the present study) in mood disorder.

The findings of this study are subject to the usual caveats associated with two-dimensional morphometric analyses. The first issue pertains the identification of cell types and layers. As distinguishing small neurons from glia can be particularly difficult; a 40-times objective lens was employed, (rather than a 25-times objective as used by other researchers; see Benes *et al.* 2001; Uylings 2002), providing an adequate spatial resolution for the correct distinction of these cell types. The positive correlations observed between Nissl and Neu-

N techniques also afford some confidence regarding the classification of small neurons and glia in the Nissl material. The definition of cortical layers was inevitably subjective and as such should be considered as another potential source of error and bias.

I employed the Abercrombie equation to correct for the potential bias of neuronal density estimates. As neurons vary in size between different layers, a separate correction was applied to each layer; even so, this may not have been sufficient since neurons also vary in size within cortical layers (see Uylings 2002). The Abercrombie correction was not applied to glial measurements (due to the very small size of these cells in relation to section thickness). Whilst it is feasible that glial cell size could differ between the two subject groups, given that glial cell size was unchanged in a study of the subgenual ACC in mood disorder (Öngür *et al.* 1998), in concert with other data from prefrontal regions reporting increases in the size of glial cells in mood disorder (Cotter *et al.* 2002b; Rajkowska *et al.* 1999); I believe that the reductions in glial density observed in this study are not due to biased cell counting.

The possibility that reductions in neuronal and glial density in mood disorder are due to differential shrinkage of brain tissue between the two groups also warrants consideration. Generally speaking, this possibility seems remote as in most cases the blocks of tissue from the mood disorder and control cases were processed simultaneously; accordingly, the same shrinkage conditions should apply to both groups. Another indication that shrinkage does not account for the differences between groups is that cortical thickness and percentage layer depths were not reduced in the mood disorder group. Moreover, further shrinkage produced by staining and immunohistochemical procedures does not appear to have caused differential shrinkage, as final section thickness did not differ between the mood disorder or control group, for either technique (Nissl: control, $5.75 \pm 0.14\mu\text{m}$; mood disorder, $5.63 \pm 0.13\mu\text{m}$; ANOVA, $p=0.537$ and Neu-N: control, $5.88 \pm 0.13\mu\text{m}$; mood disorder, $6.00 \pm 0.20\mu\text{m}$; ANOVA, $p=0.620$).

3.4.6.1 *Limitations of this study*

The most obvious limitation of this study is its sample size. As previously mentioned, one problem usually associated with a small series is that of lowered statistical power and the subsequent masking of significant results. However, significant (and predicted) reductions in neuronal and glial density were observed in the mood disorder group. Although other researchers have employed more complex and sophisticated statistical analyses (Cotter *et al.* 2001a), it was decided that such an approach was not valid for the small sample size of the present study. Other groups, with considerably larger data sets, have used Bonferroni corrections in their morphometric analyses (Bouras *et al.* 2001). In contrast, other researchers have taken a slighter more relaxed approach to their statistical analyses. For example, Benes *et al.* (2001) used t-tests in their analysis of morphometric changes in the ACC in three diagnostic groups. Öngür *et al.* (1998) also employed t-tests (without any covariate analyses) – and used 1-tailed t-tests in their second, hypothesis driven study. The statistical approach adopted in this study aimed to be vigilant, but at the same time took into consideration the limited nature of the sample.

A second issue pertaining to the sample itself is the grouping of subjects with bipolar disorder and major depression. This is not ideal, but could not be avoided due to the limited supply of tissue. Inspection of each data set suggests that both of the subjects with bipolar disorder displayed values very close to the mean of the group, and it therefore seems unlikely that including these subjects altered the results. Questions regarding whether these two diagnostic groups are distinct at the neuropathological level still remain to be addressed. This topic is considered in greater detail in chapter seven (section 7.2.4).

The relationship between mood disorder and suicide is also problematic and needs to be considered in the context of this study as four of the seven mood disorder subjects committed suicide. Suicide could complicate matters for three main reasons. Firstly,

suicide may have its own neuropathological associations (Bachus *et al.* 1997; Baumann *et al.* 1999a; Salib and Tadros 2000); secondly, it may indicate a more severe form of mood disorder (Ahearn *et al.* 2001); and thirdly, suicide may produce artefacts secondary to the mode of death. For example, brain pH is higher on average after suicide, than after deaths from natural causes, presumably relating to the shorter agonal states associated with most methods of suicide (Harrison 2002; Harrison *et al.* 1995). Brain pH is known to effect morphometric parameters (see section 3.2.3). In particular, recent experimental research has indicated that hypoxia and acidosis may influence glial counts (Bondarenko and Chesler 2001). Whilst it seems unlikely that the significant findings in this study are entirely attributable to suicide, the close association between mood disorder and suicide should not be forgotten.

3.4.6.2 *Confounding factors*

Any demographic variables found to exert significant effects were included in the analyses and are therefore unlikely to contribute to, or account for, the morphometric alterations observed in this study. Unfortunately, information was not available regarding formalin fixation time, and therefore this must be considered as a potential source of error.

Reassurance can be sought from inspection of the existing morphometric literature, which generally suggests that fixation time does not have any significant bearing upon neuronal or glial density, or the size of these cells in both the ACC and prefrontal areas (Bouras *et al.* 2001; Cotter *et al.* 2001a; Rajkowska *et al.* 2001; Rajkowska *et al.* 1999). In comparison, a recent study has shown that formalin fixation time did affect the shape of pyramidal cells in the prefrontal cortex, such that increasing fixation was associated with higher 'fcircle' values (Law and Harrison 2003), and therefore must be considered a potential confounding effect for measurements of cell shape. Importantly, alcoholism is unlikely to be a confounding factor in this study.

3.4.6.3 Neuropathological effects of mood disorder treatments

It is possible that the results of this study are associated with drug treatment, as all of the mood disorder subjects used in this study had received either antidepressant and/or mood stabilising medication. In addition, two subjects had received electroconvulsive therapy (ECT) in the weeks prior to death (see appendix one). Unfortunately, substantive clinical information regarding medication status was not available and therefore clinico-pathological correlations could not be performed. Cotter *et al* (2001a) did not find any evidence that pharmacological treatments had predictive effects on cell density or neuronal size. Öngür and colleagues (1998) also argued that the observed reduction in glial number and density was not attributable to medication effects. In contrast – two recent studies have provided weak correlational evidence for effects of lithium treatment on cell populations (Bowley *et al.* 2002; Rajkowska *et al.* 2001). The potential neuropathological effects of ECT, lithium and antidepressant treatment will each be considered in the following paragraphs.

The majority of the literature seems to support the view that ECT does not have any demonstrable neuropathological effects (Zachrisson *et al.* 2000; Devanand *et al* 1994). Notwithstanding, there have been reports of cytoarchitectural alterations after electroconvulsive shock (ECS) in rats, including increased expression of GFAP (Steward 1994), and synaptic (Jorgensen and Bolwig 1979) and dendritic markers (Pei *et al.* 1998), as well as enhanced hippocampal neurogenesis (Madsen *et al.* 2000). Further consideration to the effects of ECS on synaptic organisation is given in chapter 6 (see section 6.4.2).

Lithium appears to have more pronounced effects in the brain. Recent studies have demonstrated increases in cortical grey matter volume (Moore *et al.* 2000b) and N-acetyl aspartate signal (NAA, a marker of neuronal viability and function - Moore *et al.* 2000a) after four weeks of lithium treatment. The authors suggested that the increases probably

occurred secondary to neurotrophic effects (i.e. expansion of neuropil content) rather than cell swelling (Manji *et al.* 2000a). There has only been one morphometric study to date investigating lithium treatment in rats; no alterations in cortical neuronal number, density or size were reported (Licht *et al.* 1994), and so the morphometric correlates of the *in vivo* findings remain unclear. Conversely, increases in GFAP (Rocha and Rodnight 1994) and astrogliosis (Rocha *et al.* 1998) have been reported in the rat hippocampus after lithium treatment.

There are no neuropathological studies of the effects of other mood stabilisers, antidepressants, or minor tranquillisers. Experimental studies in the rat have indicated that antidepressants may affect neuronal morphology (Smialowska *et al.* 1988), promote neurogenesis (Malberg *et al.* 2000) and regenerate monoaminergic neurons (Kitayama *et al.* 1997). Furthermore, Norrholm and colleagues have reported that antidepressants alter dendritic spine density in the hippocampus (Norrholm and Ouimet 2000) and that antidepressant treatment prevents the loss of dendritic spines associated with some animal models of depression (Norrholm and Ouimet 2001).

Although the extrapolation of results from experiments in animals to the human situation is questionable, and may not be of clinical relevance, the present data do seem to suggest that the findings of reduced neuronal and glial density in mood disorder is unlikely to be a consequence of pharmacological treatments. If anything, glial density is potentially increased after ECT and lithium treatment, which suggests that such treatments have the potential to mask the neuropathological signal being sought. In addition, a recent investigation has reported that chronic neuroleptic treatment increases glial density in the prefrontal cortex (Selemon *et al.* 1999). It is interesting to speculate that the actions of pharmacological agents on glial cells may, in part, be responsible for their therapeutic effect in mood disorder.

3.5 Summary

The main finding of this study was a reduction in glial density in layers II, III, Vb and VI in the ACC in mood disorder. A similar trend was observed in layer Va. More subtle and specific reductions were observed in pyramidal neuron density in layers Va and Vb in mood disorder. Differences in non-pyramidal neuron density remained insignificant. Surprisingly, increases in pyramidal neuron size were also observed in the deep layers of the ACC in mood disorder. Differences between total cortical depth and individual layer depths were insignificant between the two subject groups. This study provides further evidence supporting a glial deficit in mood disorder, and it is postulated that this deficit may result in a 'downstream' neuronal pathology. Significant correlations were found between Nissl and Neu-N preparations, suggesting that Neu-N is a suitable and reliable neuronal marker for neuropathological studies of mood disorder.

Chapter Four: Calcium-binding protein expression in the anterior cingulate cortex in mood disorder

4.1 Introduction

Although Nissl-stained preparations continue to be useful and are a necessary starting point for many morphometric investigations, such work can be complemented and expanded upon by employing immunohistochemical techniques. These techniques utilise antibodies that specifically label discrete neuronal populations, thereby enabling distinctions that would not have been possible with a Nissl stain alone. A useful set of neuronal markers are the calcium-binding proteins (CaBPs) parvalbumin, (PV), calretinin (CR) and calbindin-D28K (CB). The majority of neurons that contain these proteins are non-pyramidal γ aminobutyric acid (GABA)ergic neurons. Negative findings from colocalisation studies with PV, CR and CB have indicated that they are contained in non-overlapping subpopulations of neurons (DeFelipe 1997). Studies have revealed that the most characteristic morphological types of neurons immunoreactive for PV are chandelier cells and large basket cells. Calretinin is expressed predominantly in bipolar cells and bitufted cells and CB is expressed primarily in bitufted cells (see DeFelipe 1997). Pyramidal cells stained for PV, CR and CB have also been described, but usually they are few in number, weakly stained and not present in all areas and species (Andressen *et al.* 1993; DeFelipe 1997; Nimchinsky *et al.* 1997).

The function of CaBPs in neurons is largely unknown, however, PV, CR and CB are considered to play an important role in calcium homeostasis, and are generally thought of as calcium buffering proteins (Andressen *et al.* 1993). It has also been suggested that CaBPs may play a role in the protection of neurons. Experimental evidence has shown that neurons expressing CaBPs are selectively resistant to excitotoxins, show attenuated levels of apoptosis and are protected from oxidative stress (see Eyles *et al.* 2002).

Although the presence of CaBPs may indeed confer a survival advantage to the neuron, this is by no means proven (Andressen *et al.* 1993).

Recent neuropathological studies of psychiatric disorders have utilised PV, CR and CB as markers of discrete GABAergic subpopulations (Beasley *et al.* 2002b; Cotter *et al.* 2002a; Daviss and Lewis 1995; Kalus *et al.* 1997; Reynolds *et al.* 2001; Zhang and Reynolds 2002). Their dispersal throughout the cytoplasm and fine neuronal processes, (Baimbridge *et al.* 1992), coupled with a highly distinctive distribution in a subpopulation of neurons within the same cortical region make them extremely suitable for detailed morphometric analyses. In addition, such biochemical approaches to cytoarchitecture allow a chemical subclassification of neurons that transcends cell shape. It is possible that these chemical singularities are related to differences in the connectivity of neurons bearing different CaBPs and therefore any examination of these proteins in psychiatric illness is potentially interesting as variations in expression may be indicative of subtle alterations in the intrinsic organisation of a cortical area.

Studies of CaBP expression in mood disorder are limited and only one study to date has focused on the supragenual cingulate cortex. Reductions in cell density were observed for PV, CR and CB in both major depression and bipolar disorder, however, only one reduction specific to CB-immunoreactive neurons in layer II in bipolar disorder attained statistical significance (Cotter *et al.* 2002a). This result disappeared after adjusting for multiple comparisons. Morphometric studies have also reported reduced numbers of small, presumed inhibitory, non-pyramidal neurons in layer II in the ACC in bipolar disorder (Benes *et al.* 2001).

Reductions in CaBP neurons have also been reported in prefrontal regions in mood disorder. A study of prefrontal area 9 indicated that the density of CB-immunoreactive neurons was reduced in layers II and III in bipolar disorder; however, this significant effect

did not withstand Bonferroni correction. No other significant changes were reported (Beasley *et al.* 2002b). A second study by the same group reported a decrease in CR-immunoreactive neurons in layer I in area 46 in major depression, again this result did not remain significant after correction. This paper also reported alterations in the entorhinal cortex. CR density in layers V and VI was reduced in bipolar disorder and the density of PV-immunoreactive neurons was reduced in layer II in major depression (Reynolds *et al.* 2002). Decreases in PV-immunoreactive neurons have also been reported in the CA1 subfield of the hippocampus in bipolar disorder. Again, all significant comparisons were lost after Bonferroni correction (Zhang and Reynolds 2002). Although not specific to CaBPs, other deficits in the GABAergic system have been reported in mood disorder. There are indications that expression of glutamic acid decarboxylase mRNA (GAD – the synthesising enzyme for GABA) and the density of GAD-immunoreactive terminals are reduced in the prefrontal cortex and ACC in bipolar disorder (Benes *et al.* 2000; Guidotti *et al.* 2002).

Even though all of the studies investigating CaBPs in mood disorder have revealed alterations in the density of PV-, CR- and CB-immunoreactive neurons, notable discrepancies exist between these studies. These preliminary data seem to suggest that bipolar disorder and major depression may be differentially affected. Decreases in all three markers have been observed in bipolar disorder (Beasley *et al.* 2002b; Cotter *et al.* 2002a; Reynolds *et al.* 2002; Zhang and Reynolds 2002), whereas reductions in major depression are less frequently reported and involve reductions in PV- and CR-, but not CB-immunoreactive neurons (Reynolds *et al.* 2002). The layer distribution of significant differences also varies considerably between studies. Differences between the brain regions examined rather than demographic differences are most likely to explain these disparities since all of the aforementioned studies have used tissue from the Stanley Foundation Neuropathology Consortium brain collection (see Torrey *et al.* 2000). Further replication is required to authenticate these preliminary results.

4.1.1 Aims of the current study

The primary objective of this study was to investigate the density of non-pyramidal neurons labelled by PV, CR and CB in the ACC in mood disorder. Although significant alterations were not observed in non-pyramidal neuron density in the previous chapter, it is feasible that subtle changes in a specific subpopulation of non-pyramidal neurons may have been masked by a 'total non-pyramidal neuron' count that does not permit discrimination between different neuronal populations. Given that qualitative studies in the normative ACC have reported marked variation in the distribution of CaBP-immunoreactive neurons and neuropil labelling patterns along the rostrocaudal and dorsoventral axes of the supragenual ACC (Hof *et al.* 1993; Nimchinsky *et al.* 1997; Öngür *et al.* 2003), I decided to investigate the density of PV-, CR- and CB-immunoreactive neurons in all subdivisions of area 24 (24a, 24b and 24c). The intention of this work was to replicate and expand upon the study by Cotter and colleagues (2002a), which focused exclusively on area 24c in the ACC. In the current study, density measurements were made in those cortical layers where the immunoreactive neurons were principally observed, namely: layer III for PV, layer II for CR and layer II for CB. A subsidiary aim of this study was to qualitatively examine the distribution of CaBP expression along the dorsoventral axis of the ACC.

4.2 Methods

4.2.1 Tissue collection and preparation

The collection and preparation of tissue used in this study has been previously described in sections 3.2.1, 3.2.2 and 3.2.3. The demographic details of the individuals included in the experiments are shown in table 4.1.

4.2.2 Immunohistochemistry for PV, CR and CB

The principles of immunohistochemistry were outlined in section 3.2.4.1. Pilot experiments assessing varying antibody concentrations, incubation times, and antigen retrieval techniques were performed prior to the definitive experiment (data not shown). These preliminary results revealed that microwaving on high power for 15 minutes in 0.01M Citric acid (pH 6.0) greatly improved staining for each antibody. Microwaving for longer periods of time failed to further enhance the results.

Duplicate sections for each case were de-waxed in three consecutive baths of xylene, followed by rehydration in graded alcohols (4 minutes each in 100% x2, 95%, 70% and distilled water). Sections were then heated in a microwave oven in 0.01M Citric acid (Sigma, Poole, UK) at pH 6.0 for 15 minutes. After microwave treatment, non-specific binding was blocked with 10% normal serum (Sigma) in PBS-T for 30 minutes at room temperature. The sections were then briefly rinsed in PBS before application of the primary antibody solution (PV, 1:5000; CR, 1:5000; CB, 1:15000 – all Swant, Bellinzona, Switzerland), diluted in PBS-T containing 10% normal serum. Sections were incubated for 3 nights at 4°C. Sections were then incubated with the appropriate biotinylated secondary antibody diluted to 1:100 in PBS-T containing 10% normal serum at room temperature for 4 hours. This was followed by incubation with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC, Vector Laboratories) for 2 hours at room temperature. All steps were followed by appropriate washes in PBS. Finally, all sections were developed in a substrate solution of nickel-enhanced DAB, prepared according to the manufacturers instructions (Perbio, Chester, UK). Omission of the primary antibody was used as an experimental control.

4.2.3 Morphometric analysis

4.2.3.1 Identification of Brodmann areas 24a, 24b and 24c

Areas 24a, 24b and 24c were identified and defined in adjacent Nissl-stained sections according to macroscopic and microscopic criteria (Vogt *et al.* 1995). These criteria are outlined in section 2.2.5.1. These boundaries were then extrapolated onto the CaBP slides. The distributions of cell types and neuropil staining patterns in the different subdivisions and cortical layers of the ACC were confirmed using descriptions provided by Nimchinsky *et al.* (1997). Although the sections were not counterstained, the cortical layers of interest were easily delineated. CR and CB showed a high density of neurons in layer II, which diminished in layer III. PV-immunoreactive neurons were readily identified in layer III, which was defined by a dense band of immunoreactive neuropil.

4.2.3.2 Counting procedure

All morphometric analyses were undertaken using an image analysis system from Imaging Associates (Bicester, UK) using KS400.3 software, equipped with an Olympus BH40 microscope and colour video camera. Tissue sections were viewed with a 20-times objective lens for all counting procedures.

Parvalbumin-, CR- and CB-immunoreactive non-pyramidal neurons were identified as labelled if clear immunopositivity was apparent in the cytoplasmic profile of a cell. Some neurons showed a very light stain that barely exceeded background levels and were therefore not assessed. Any lightly stained pyramidal neurons were also excluded.

Sampling strategies were optimised before the definitive analyses. PV, CR and CB sections were optimised separately. For each antibody, five cases were selected at random and increasing numbers of frames were sampled in each cortical layer of interest until reliable and reproducible density values were attained. These preliminary tests indicated that sampling 30 frames per layer, per subdivision, per section (therefore

providing a total of 60 frames per subdivision) provided reproducible density estimates for PV, CR and CB. Five cases (selected at random) were repeated at the end of the analysis for each antibody - all values were within 5% of original measurements (data not shown). An unbiased counting frame with an area of $57,600 \mu\text{m}^2$ was used throughout this study (see section 2.2.1.3). The sampling field was moved systematically through the cortical layer of interest until a sufficient number of frames had been sampled.

The potential influence of cell size on areal density measures (see section 3.2.5.1) was assessed and corrected by means of the Abercrombie formula. To enable such a correction in the current study, the thickness of each section was carefully measured using a microcator. The widths of PV-, CR- and CB-immunoreactive neurons were measured from 50% of cases in each diagnostic group. Approximately 50 cells were sampled per case to provide a reliable estimate of diameter (see section 3.2.5.3). The Abercrombie correction was then applied to all neuronal measurements using the equation given in section 3.2.5.1.

All measurements were made blind to diagnosis. All data is presented in its corrected form. Density values are expressed as cell density/ mm^2 .

4.2.3.3 *Statistical analysis*

All statistical analyses were carried out using SPSS 10.0 for Microsoft Windows. The data sets to be investigated were: 1) PV-immunoreactive neuronal density, 2) CR-immunoreactive neuronal density and 3) CB-immunoreactive neuronal density. An initial evaluation of the data involved checking the distributions of points within each data set for normality using the Kolmogorov-Smirnov test.

The potential confounding effects of age, pH and PMI were evaluated using Pearson's correlations in the control and mood disorder group separately, as well as in both groups

combined. Each of the CaBP-immunoreactive subpopulations was considered separately. As it is unlikely that a confounding factor would have differential effects on each subdivision of area 24, any demographic variable found to exert a significant effect was included as a covariate in all subdivision analyses, even if statistical significance was not attained for all subdivisions. Significance was set at $p \leq 0.05$ for all correlation analyses.

Statistical analyses were performed in an identical manner to those outlined in section 3.2.6, substituting subdivision for layer as the 'within subject variable'.

Table 4.1

Demographic details of control and mood disorder subjects included in the experiments.

Values are mean $\pm$ S.E.M with range in brackets. There are no significant differences between the two groups ($p>0.2$). ^a major depression (n=5), bipolar disorder (n=2).

Diagnoses were made according to DSM-IV criteria. ^b All patients had been treated with antidepressant medication.

Variable	Control (n=9)	Mood disorder (n=7) ^{ab}
Age (years)	58.33 $\pm$ 6.11 (22–79)	64.57 $\pm$ 4.63 (49-75)
Sex (M:F)	5:4	4:3
pH	6.54 $\pm$ 0.06 (6.20–6.72)	6.40 $\pm$ 0.09 (6.06–6.86)
PMI (hours)	32.67 $\pm$ 7.49 (8–72)	34.43 $\pm$ 7.51 (13–75)
No. of suicides	0	4

4.3 Results

4.3.1 Distribution of calcium-binding protein immunoreactivity: qualitative observations

Three of the brains used in this study, (two control subjects and one mood disorder subject) were devoid of any CaBP immunoreactivity in area 24a. Reasons for this are unclear as their demographic details were unremarkable by comparison with the remainder of the sample, as was the histological appearance of adjacent Nissl-stained sections. In comparison, areas 24b and 24c displayed neuronal and neuropil labelling consistent with the expected distribution and morphology. Consequently, these cases were only excluded from analyses of area 24a. Additionally, numbers for the analyses of CB and CR in area 24c are reduced as a result of damage to the tissue sections in the region of layer II. Table 4.2 shows the final group demographics for each experiment.

All qualitative observations and discussions pertaining to descriptions of the layer and subdivisional distributions of CaBP immunoreactivity are specific to the control cases only.

4.3.1.1 *Parvalbumin*

Parvalbumin-immunoreactive neurons in the ACC were typically intensely stained.

Immunoreactive neurons were present predominantly in layer III and were also observed in layers II, V and VI, but not in layer I or the white matter. All neurons immunoreactive for PV appeared to be non-pyramidal and consisted of a variety of morphologies, including bitufted cells, small and large multipolar cells and small bipolar cells. In the majority of cases area 24a exhibited an equivalent staining pattern to area 24c, with sparser staining observed in area 24b (see figure 4.1).

A dense plexus of immunoreactive neuropil, consisting of stained processes and puncta, was observed in layer III and extended through to layer V. In the majority of cases

neuropil staining appeared to mirror the cell body staining, with areas 24a and 24c exhibiting a greater intensity of staining than area 24b. However, in a minority of cases, the neuropil staining increased gradually, from ventral to more dorsal areas, such that in area 24c it was very intense (see figure 4.2).

4.3.1.2 *Calretinin*

Calretinin-immunoreactive neurons were present throughout the cortical layers and in the subadjacent white matter, and were predominantly observed in layers II and superficial III. Neurons positive for CR were darkly stained and appeared to be non-pyramidal neurons exhibiting a variety of morphologies, including bitufted and bipolar cells. Cell body staining was again roughly equivalent between the three subdivisions, although area 24b frequently displayed a sparser, less intense staining pattern (see figure 4.3).

Neuropil was stained throughout the cortical width, although layer I regularly displayed intense neuropil labelling. Neuropil labelling was consistent across all three subdivisions of area 24 (see figure 4.3).

4.3.1.3 *Calbindin*

Calbindin-immunoreactive neurons were present throughout the cortical width. These neurons were found principally in layers II and superficial III, whereas a lower density was observed in layers V and VI. The majority of CB-immunoreactive neurons appeared to be densely stained non-pyramidal neurons with round to ovoid cell bodies comprised predominantly of bitufted neurons. Occasionally, lightly stained pyramidal neurons were observed in layers II and III. The cellular patterns were comparable between areas 24a, 24b and 24c, although staining increased gradually from ventral to more dorsal areas (see figure 4.4).

The neuropil demonstrated an incremental staining pattern, from layer II through the deep portion of layer III. Layer I, V and VI were largely unstained by comparison. Again, the neuropil labelling pattern mirrored the cell body staining, such that area 24c showed a greater intensity than area 24a (see figure 4.4).

4.3.2 Analysis of PV-immunoreactive neuronal density

PV-immunoreactive density measurements satisfied the Kolmogorov-Smirnov test for normal distribution. Significant positive correlations were observed between the density of PV-immunoreactive neurons and pH in area 24a of the control group and the mood disorder group, but not the groups combined. A significant positive correlation with pH was also observed for area 24b in the control group, though not in the mood disorder group or in the groups combined (see table 4.3). No other significant correlations were observed. pH was included as a covariate for all subsequent analyses.

Although reductions in PV-immunoreactive neuron density were observed for the mood disorder group relative to the control group (see table 4.5 and figure 4.5), these comparisons did not achieve significance. The repeated measures ANCOVA did not find a significant effect of diagnosis ($p=0.120$) or area ($p=0.611$); the interaction between diagnosis and area also failed to reach significance ($p=0.776$). No further analyses were performed (see table 4.6).

4.3.2.1 Abercrombie measurements - section thickness and cell size

Final section thickness did not differ between the control or mood disorder group ($p=1.000$). There were no significant differences in cell size (measured as cell diameter) between the two groups ($p=0.658$) (data not shown).

4.3.3 Analysis of CR-immunoreactive neuronal density

CR-immunoreactive density measurements satisfied the Kolmogorov-Smirnov test for normal distribution. The density of CR-immunoreactive neurons did not show any significant correlations with age, pH or PMI in the mood disorder group, the control group, or both groups combined.

The repeated measures ANOVA showed significant effects of area ($F(2,14)=7.309$; $p=0.007$) and diagnosis ($F(1,7)=18.332$; $p=0.004$), but the interaction between area and diagnosis remained insignificant ($p=0.670$) (see tables 4.5 and 4.6, and figure 4.5).

Further analyses indicated significant reductions in CR-density in areas 24a ($F(1,11)=5.303$; $p=0.042$) and 24b ($F(1,13)=28.907$; $p<0.001$) in the mood disorder group.

Although the density of CR-immunoreactive neurons in area 24c also appeared to be decreased in the mood disorder group, the ANOVA did not attain statistical significance ($p=0.194$) (see table 4.7).

4.3.3.1 Abercrombie measurements - section thickness and cell size

Final section thickness did not differ between the control or mood disorder group ($p=0.620$). There were no significant differences in cell size (measured as cell diameter) between the two groups ($p=0.124$) (data not shown).

4.3.4 Analysis of CB-immunoreactive neuronal density

CB-immunoreactive density measurements satisfied the Kolmogorov-Smirnov test for normal distribution. Significant positive correlations were observed between the density of CB-immunoreactive neurons and pH in area 24a of the control group, but not the mood disorder group; this correlation remained significant when both groups were combined. A significant positive correlation with pH was also observed in area 24b in the combined group, but not when the control or mood disorder groups were considered separately (see

table 4.4). No significant correlations were observed for age or PMI. Consequently, pH was included as a covariate in the succeeding analyses.

Although reductions in CB-immunoreactive density were observed for the mood disorder group relative to the control group (see table 4.5 and figure 4.5), these comparisons did not attain significance. The repeated measures ANCOVA did not find a significant effect of diagnosis ($p=0.544$) or area ($p=0.568$); the interaction between diagnosis and area also failed to reach significance ($p=0.499$) (see table 4.6). No further analyses were performed.

4.3.4.1 Abercrombie measurements - sections thickness and cell size

Final section thickness did not differ between the control or mood disorder group ($p=0.620$). There were no significant differences in cell size (measured as cell diameter) between the two groups ($p=0.286$) (data not shown).

Table 4.2.
Demographic details of control and mood disorder subjects included in the final CaBP analyses.

Variable	PV: area 24a		PV: area 24b		PV: area 24c	
	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder
	(n=6)	(n=6) ^a	(n=9)	(n=7) ^a	(n=7)	(n=6) ^a
Age (years)	58.67 ± 8.62	63.33 ± 5.28	58.33 ± 6.11	64.57 ± 4.63	58.33 ± 6.11	62.83 ± 5.08
Sex (M:F)	4:2	3:3	6:3	4:3	6:3	4:2
pH	6.51 ± 0.08	6.39 ± 0.10	6.54 ± 0.06	6.40 ± 0.09	6.54 ± 0.06	6.42 ± 0.10
PMI (hours)	20.33 ± 4.18	34.17 ± 8.88	32.67 ± 7.49	34.43 ± 7.51	32.67 ± 7.49	33.50 ± 8.82
No. of suicides	0	3	0	4	0	4

Variable	CR: area 24a		CR: area 24b		CR: area 24c	
	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder
	(n=7)	(n=6) ^a	(n=8)	(n=7) ^a	(n=7)	(n=5) ^a
Age (years)	60.57 ± 7.53	63.33 ± 5.28	60.38 ± 6.52	64.57 ± 4.63	65.14 ± 4.66	64.40 ± 5.91
Sex (M:F)	5:2	3:3	6:2	4:3	5:2	3:2
pH	6.54 ± 0.08	6.39 ± 0.10	6.56 ± 0.07	6.40 ± 0.09	6.56 ± 0.06	6.43 ± 0.10
PMI (hours)	22.43 ± 4.10	34.17 ± 8.88	28.63 ± 7.14	34.43 ± 7.51	33.71 ± 9.60	37.60 ± 9.56
No. of suicides	0	3	0	4	0	4

Variable	CB: area 24a		CB: area 24b		CB: area 24c	
	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder
	(n=7)	(n=6) ^a	(n=6)	(n=7) ^a	(n=6)	(n=5) ^a
Age (years)	60.57 ± 7.53	62.83 ± 5.08	67.00 ± 4.64	64.57 ± 4.63	66.17 ± 5.38	64.40 ± 5.91
Sex (M:F)	5:2	4:2	5:1	4:3	4:2	3:2
pH	6.54 ± 0.08	6.42 ± 0.10	6.60 ± 0.06	6.40 ± 0.09	6.54 ± 0.07	6.43 ± 0.10
PMI (hours)	22.43 ± 4.10	33.50 ± 8.82	20.00 ± 3.92	34.43 ± 7.51	27.33 ± 8.49	37.60 ± 9.56
No. of suicides	0	4	0	4	0	4

Values are mean ± S.E.M. There are no significant differences between the two groups in any of the CaBP's in any subdivisions of area 24 (p>0.2). ^a All patients had been treated with antidepressant medication.

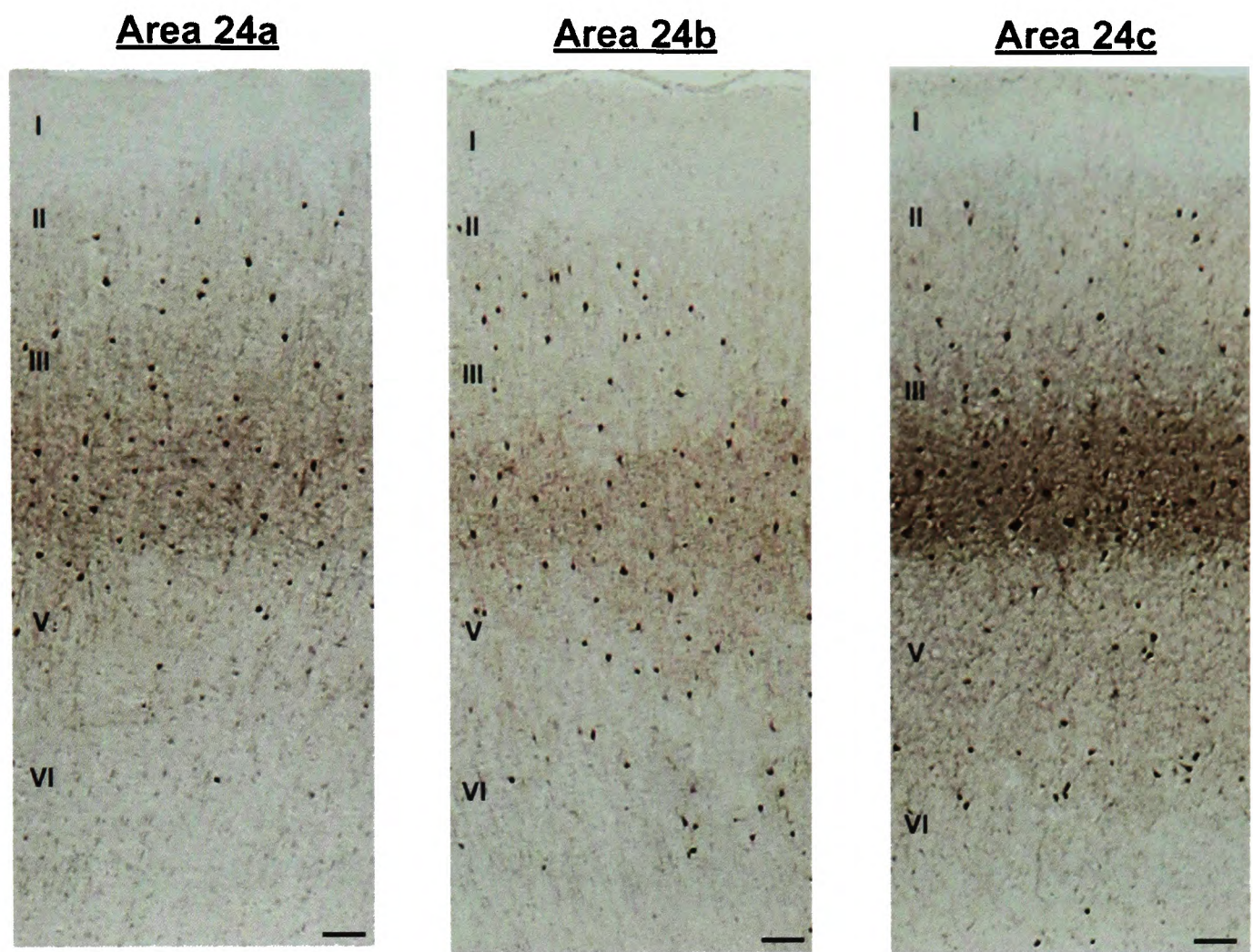
Figure 4.1.

(A). Photomicrographs showing the distribution of parvalbumin immunoreactivity in area 24 (scale bar=100µm).

Staining is present throughout the cortical width, though immunoreactive neurons are present predominantly in layer III. A dense plexus of immunoreactive neuropil is also present in deep layer III. Staining is most intense in area 24c. The negative control (omission of the primary antibody) did not show any immunoreactivity (data not shown). See figure 4.2 for further photomicrographs demonstrating the distribution of parvalbumin immunoreactivity.

(B). Photomicrographs showing bipolar, bitufted and multipolar non-pyramidal neurons and processes immunoreactive for parvalbumin in cortical layer III (scale bar=30µm).

A Distribution of parvalbumin immunoreactivity



B Bipolar, bitufted and multipolar neurons immunoreactive for parvalbumin in cortical layer III

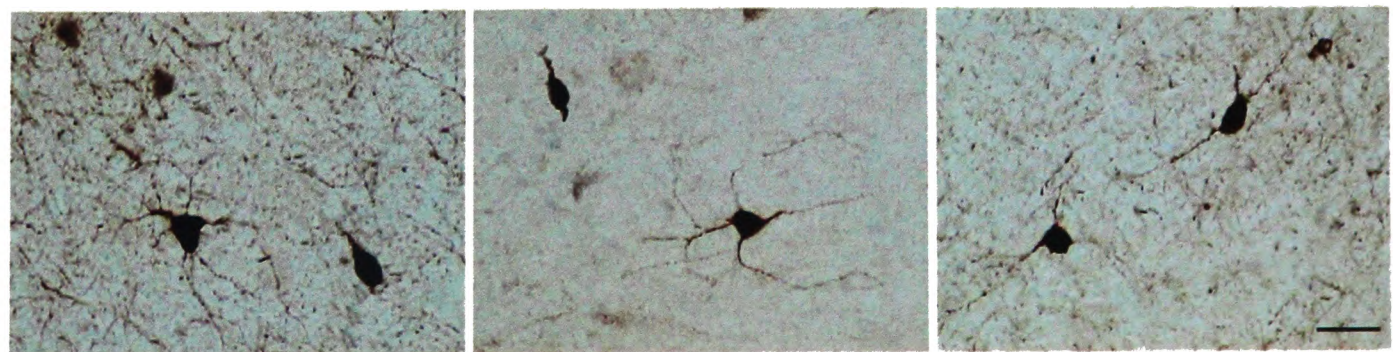


Figure 4.2.

Photomicrographs showing intersubject variation in the distribution of parvalbumin immunoreactivity in area 24 (scale bar=100µm).

In a minority of cases, the neuron and neuropil labelling increased gradually, from ventral to more dorsal areas, such that in area 24c it was very intense (A). Based on the existing literature, this would be the expected pattern of distribution; however, in the majority of my cases, area 24a exhibited an equivalent staining pattern to area 24c, with sparser staining observed in area 24b (B).

Distribution of parvalbumin immunoreactivity

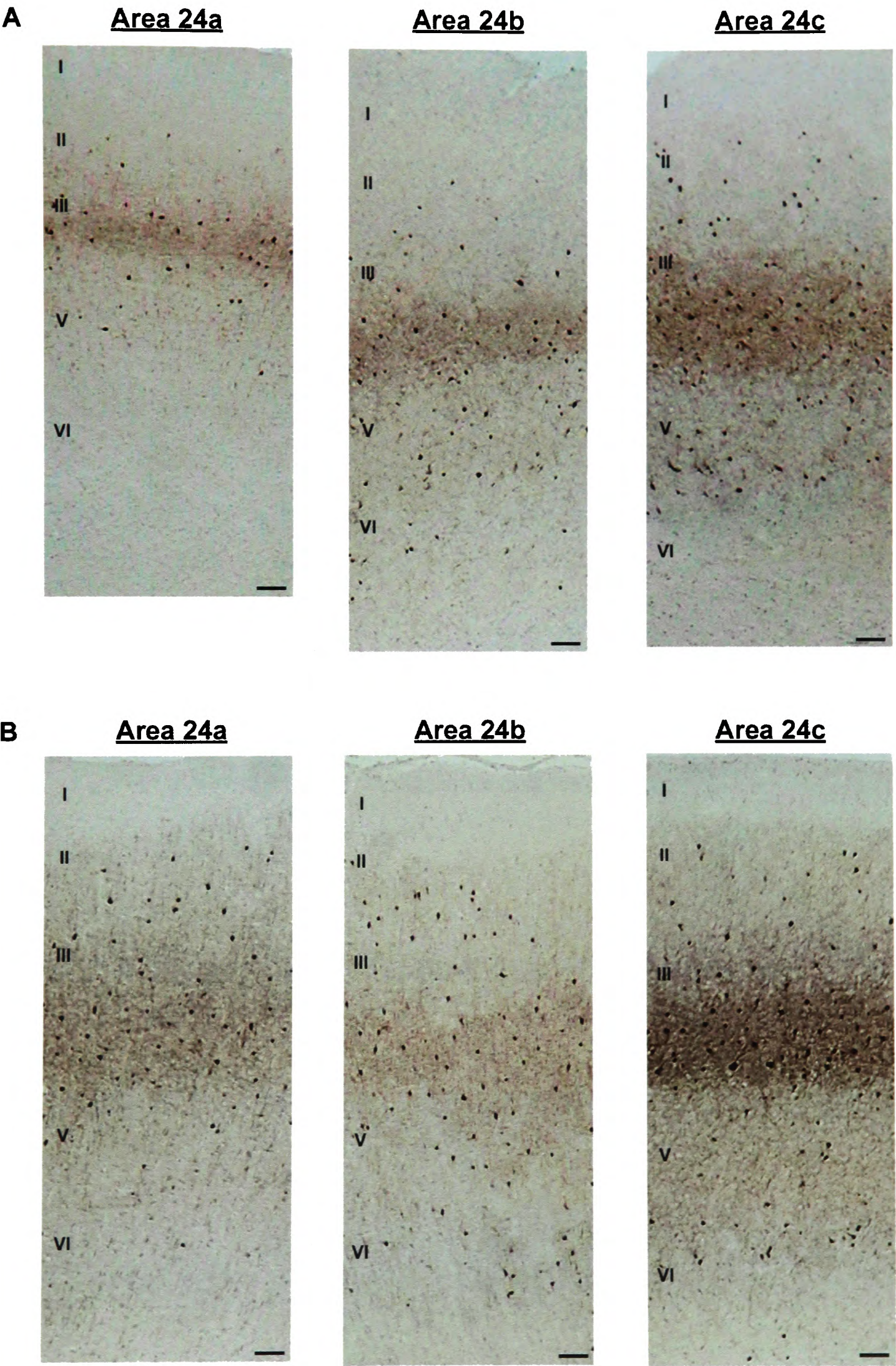


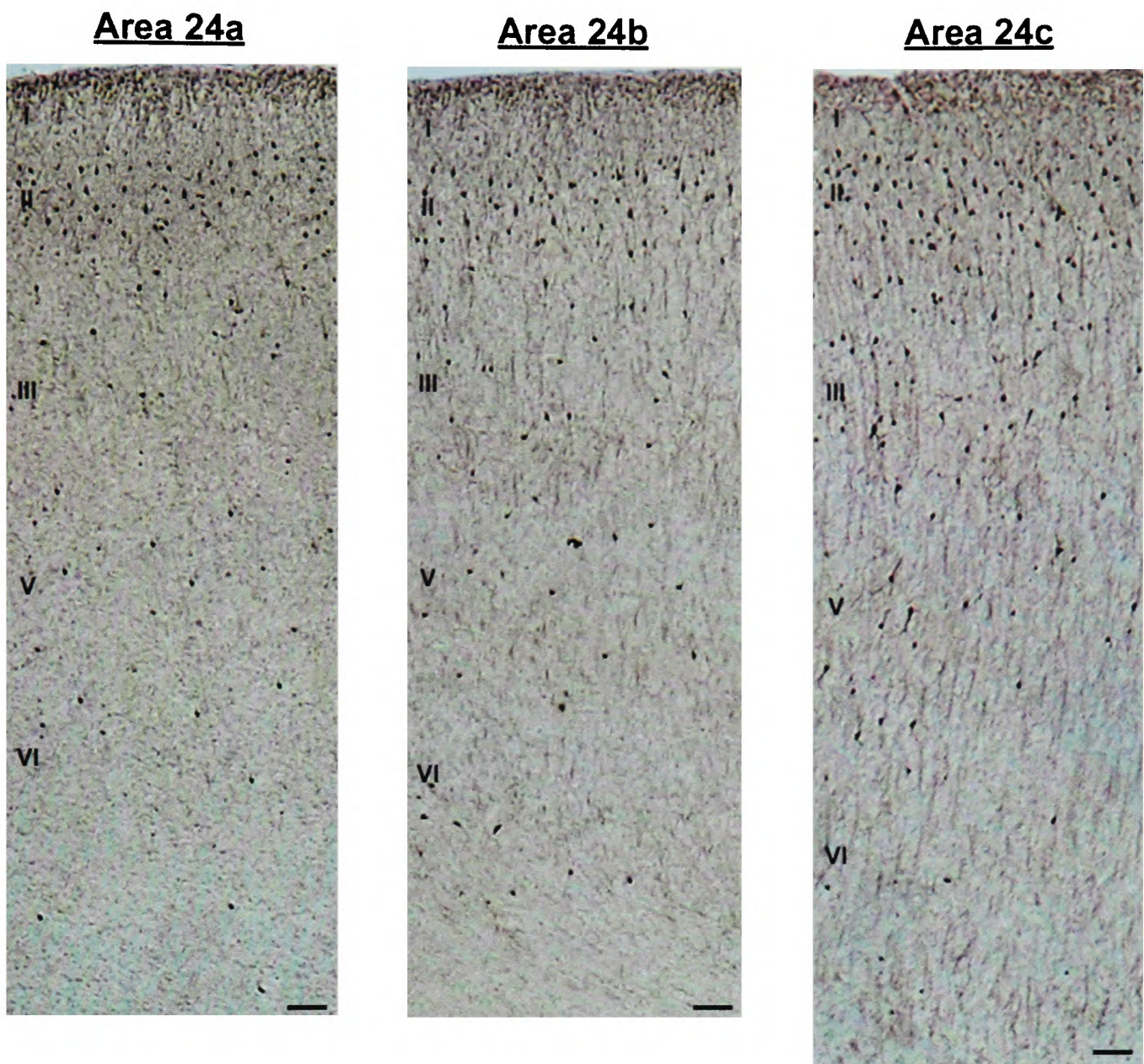
Figure 4.3.

(A). Photomicrographs showing the distribution of calretinin immunoreactivity in area 24 (scale bar=100µm).

Staining is present throughout the cortical width, but the majority of immunoreactive neurons are present in layer II. Calretinin neuronal and neuropil staining is roughly equivalent in all subdivisions of area 24, however, neuronal staining is often sparser in area 24b. The negative control (omission of the primary antibody) did not show any immunoreactivity (data not shown).

(B). Photomicrographs showing bipolar and bitufted non-pyramidal neurons and processes immunoreactive for calretinin in cortical layer II (scale bar=30µm).

A **Distribution of calretinin immunoreactivity**



B **Bipolar and bitufted neurons immunoreactive for calretinin in cortical layer II**

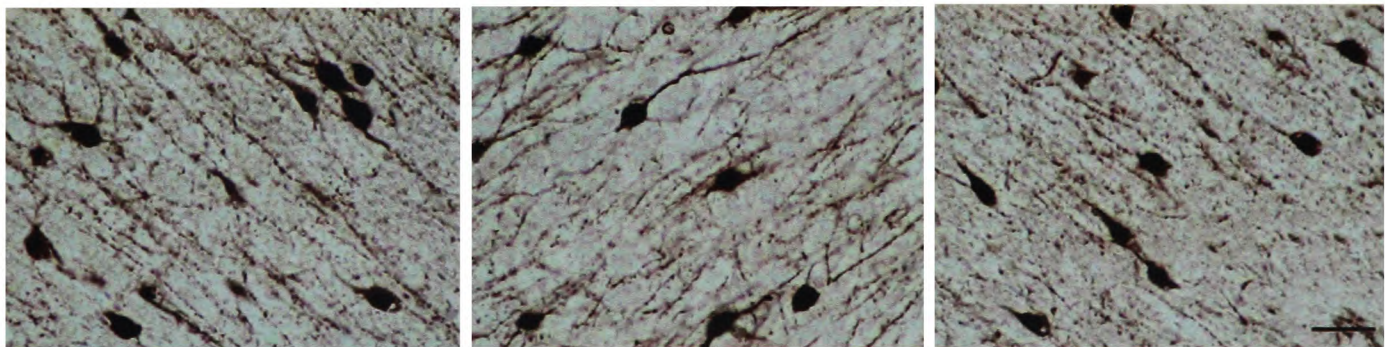


Figure 4.4.

(A). Photomicrographs showing the distribution of calbindin immunoreactivity in area 24 (scale bar=100µm).

Staining is present throughout the cortical width, though immunoreactive neurons are present predominantly in layer II. Calbindin neuronal and neuropil staining patterns are comparable between areas 24a, 24b and 24c, although staining increases gradually from ventral to more dorsal areas. The negative control (omission of the primary antibody) did not show any immunoreactivity (data not shown).

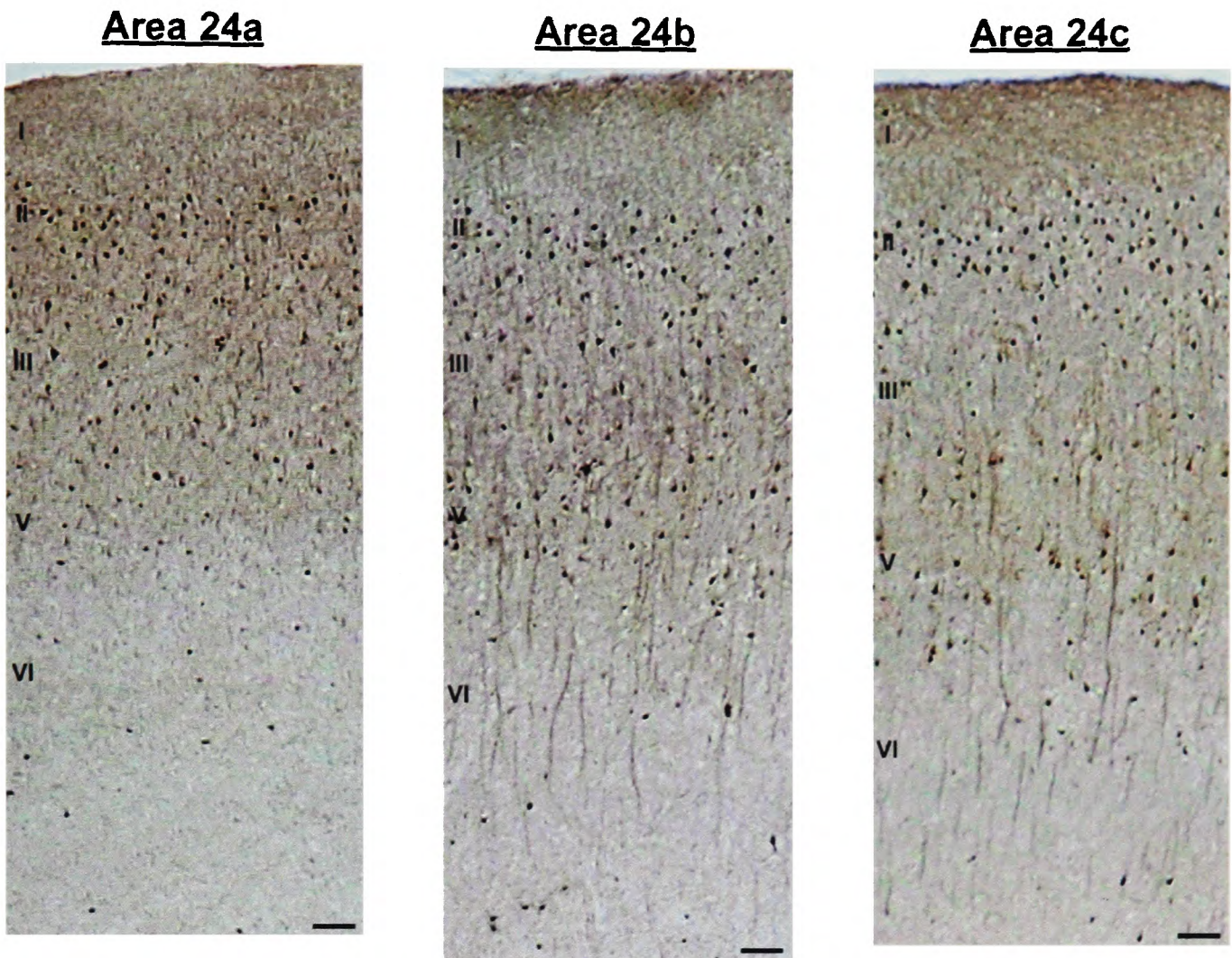
(B). Photomicrographs showing bipolar and bitufted and pyramidal neurons and processes immunoreactive for calretinin in cortical layer II (scale bar=30µm).

The majority of calbindin-immunoreactive neurons are densely stained non-pyramidal cells.

Occasionally, lightly stained pyramidal cells are observed in layer III (indicated with red asterisk).

Pyramidal neurons were not assessed in the morphometric analyses.

A Distribution of calbindin immunoreactivity



B Bitufted, bipolar and pyramidal neurons immunoreactive for calbindin in cortical layer II

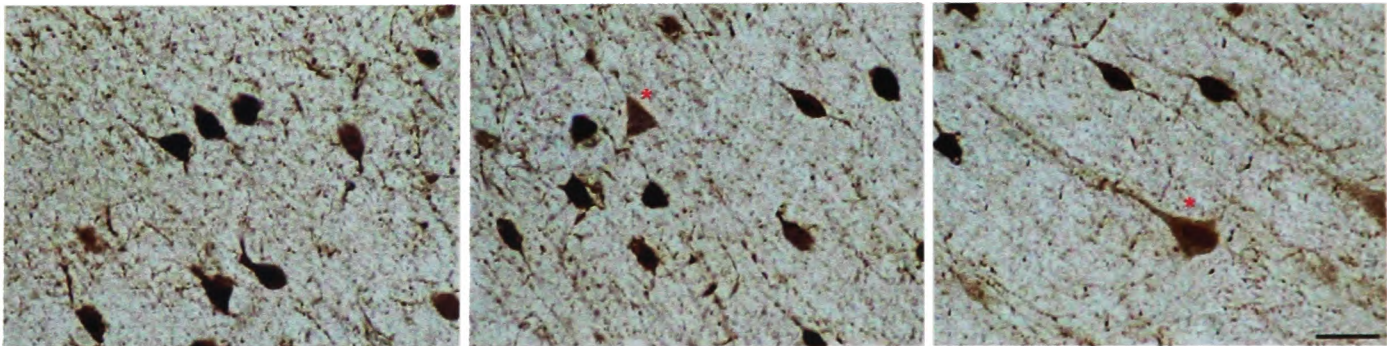


Table 4.3.

The effect of brain pH on parvalbumin immunoreactivity in the ACC. Numbers are Pearson's R values. * $p \leq 0.05$

Density of PV-immunoreactive neurons and pH			
Subdivision of area 24	Control	Mood disorder	Combined
24a	0.88*	0.83*	0.30
24b	0.72*	0.34	0.36
24c	0.55	0.16	0.45

Table 4.4.

The effect of brain pH on calbindin immunoreactivity in the ACC. Numbers are Pearson's R values. * $p \leq 0.05$

Density of CB-immunoreactive neurons and pH			
Subdivision of area 24	Control	Mood disorder	Combined
24a	0.79*	0.72	0.76*
24b	0.43	0.37	0.50*
24c	0.49	0.64	0.52

Table 4.5.

Density of CaBP-immunoreactive neurons in each subdivision of the ACC in controls and mood disorder. These data are illustrated in figure 4.5.

Calcium-binding protein data: Mean (S.E.M)						
Subdivision of area 24	Density of PV-positive neurons, cells / mm ²		Density of CR-positive neurons, cells / mm ²		Density of CB-positive neurons, cells / mm ²	
	Control	Mood disorder	Control	Mood disorder	Control	Mood disorder
24a	17.06 (2.3)	12.64 (1.2)	47.23 (1.4)	39.08 (3.5)	32.72 (1.4)	28.88 (2.9)
24b	10.62 (1.5)	9.52 (0.7)	43.67 (1.4)	34.35 (0.9)	41.53 (4.2)	33.74 (2.7)
24c	15.26 (0.7)	13.03 (0.8)	48.91 (2.3)	43.14 (3.8)	43.77 (3.6)	38.56 (1.9)

Table 4.6.

Results of repeated measures ANOVA (subdivision of area 24 included as a ‘within subject’ factor).

Repeated measures ANOVA*						
Calcium-binding protein	Diagnosis		Area		Diagnosis x Area	
	F	p	F	p	F	p
Parvalbumin ¹	3.022	.120	0.508	.611	0.259	.776
Calretinin	18.332	.004	7.309	.007	0.413	.670
Calbindin ¹	0.407	.544	0.588	.568	0.730	.499

* significance set at p≤0.1; ¹ repeated measures ANCOVA with pH

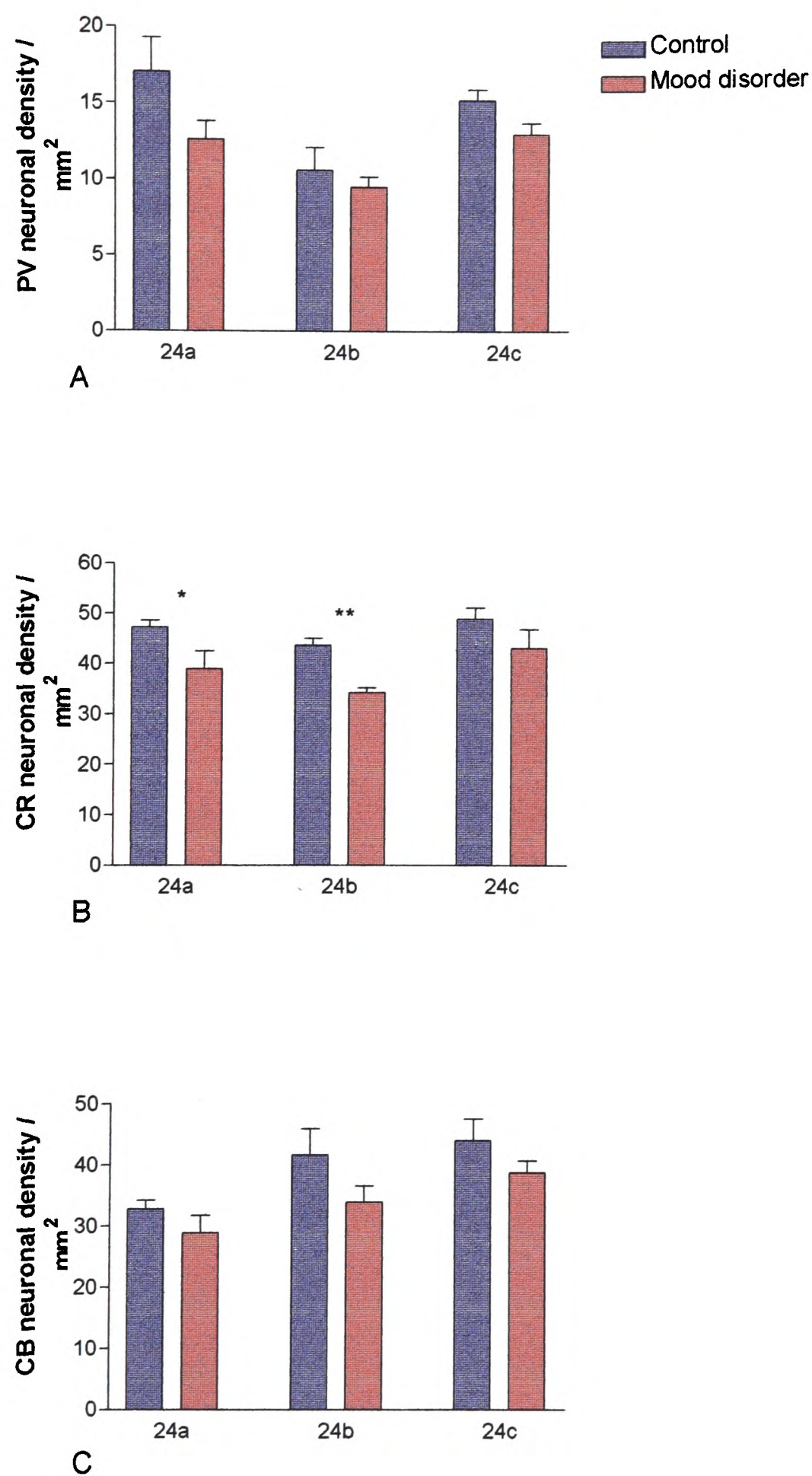
Table 4.7.

Density of calretinin-immunoreactive neurons in each subdivision of the ACC in controls and mood disorder. ANOVA test used for comparison of calretinin immunoreactivity between diagnostic groups. These data are illustrated in figure 4.5 (B).

Density of calretinin-positive cells			Test for difference between		
Mean (S.E.M)			patient group and control group		
	Control	Mood disorder	F	p	Percentage difference
Area 24a	47.2 (4.4)	39.1 (3.5)	5.30	.042	20.7%
Area 24b	43.7 (1.4)	34.3 (0.8)	28.91	<.001	27.4%
Area 24c	48.9 (2.3)	43.1 (3.8)	19.4	.194	13.4%

Figure 4.5.

Graphs showing density of (A) parvalbumin-, (B) calretinin-, and (C) calbindin-immunoreactive neurons in areas 24a, 24b, and 24c of the ACC in controls and mood disorder. See tables 4.6 and 4.7 for statistical comparisons between groups. * ≤ 0.05 ; ** ≤ 0.01 .



4.4 Discussion

4.4.1 Distribution of calcium-binding protein immunoreactivity: qualitative observations

4.4.1.1 Parvalbumin

The layer distribution of neuronal immunoreactivity and neuropil labelling patterns and the various neuronal morphologies (bipolar, multipolar and bitufted) reported in the current chapter are in accordance with previous studies of PV immunoreactivity in the normative ACC (Hof *et al.* 1993; Kalus and Senitz 1996; Nimchinsky *et al.* 1997). The observed variation in PV immunoreactivity along the dorsoventral axis of the ACC in the current study is not entirely consistent with previous work, since Nimchinsky *et al.* (1997) reported an incremental staining pattern along the dorsoventral axis of area 24, with PV immunoreactivity being most intense in area 24c. In the present study, a third of the cases did demonstrate a gradual increase in the intensity of neuronal and neuropil immunoreactivity from ventral to dorsal areas of the ACC, however, the remaining cases did not show this pattern and instead exhibited equivalent labelling patterns in areas 24a and 24c, with slightly less immunoreactivity in area 24b (see figure 4.2).

This could reflect a genuine variation in the distribution of PV-immunoreactive neurons and neuropil in the ACC - although Nimchinsky *et al.* (1997) did not find high levels of immunoreactivity within area 24a, intense staining of this area has been reported elsewhere (Kalus and Senitz 1996; Kalus *et al.* 1997). Furthermore, the subjects used by Nimchinsky and colleagues were much older (82.3 ± 9.5 years) than those in the present investigation and therefore demographic differences between the two studies may go some way in explaining the discrepancies. Alternatively, the qualitative work by Nimchinsky and colleagues (1997) could provide an alternative explanation for such differences. They observed that during the transition between areas 24 and 24' a progressive change in the emphasis of the neuronal and neuropil labelling occurred, with

the intensity of labelling shifting gradually from dorsal to more ventral regions. In relation to the present study, it is feasible that any intersample variation in the distribution and intensity of PV immunoreactivity may be related to the varying anatomical positions (along the rostrocaudal axis) from which tissue was originally sampled. Interestingly, since rostral regions of area 24 reportedly possess low levels of CaBP immunoreactivity in area 24a (Nimchinsky *et al.* 1997), it is possible that all three brains devoid of immunoreactive signal in area 24a in this study (see 4.3.1) were originally sampled from a more rostral region of area 24.

4.4.1.2 *Calretinin*

The layer distribution of CR-immunoreactive neurons and neuropil labelling patterns reported in the current chapter are in general agreement with existing studies of CR in the normative ACC (Gabbott *et al.* 1997; Hof *et al.* 1993; Nimchinsky *et al.* 1997). The various morphologies observed for CR-immunoreactive neurons are also consistent with these studies. In the present study, although neuronal staining was approximately equivalent across all three subdivisions of area 24, the majority of cases appeared to have fewer CR-immunoreactive neurons in area 24b in comparison to areas 24a and 24c. This pattern was not as pronounced as those differences previously described for PV-immunoreactive neurons. In contrast, Gabbott *et al.* (1997) reported very equivalent density values for CR-immunoreactive neurons in the three subdivisions of area 24 – however, this observation was based on just three cases. Nimchinsky *et al.* (1997) did not describe any differences (or similarities) in CR distribution in the subdivisions of area 24. It is unclear why this is – one explanation could be that no appreciable changes were observed between the varying subdivisions and consequently no differences were documented. The differences between these investigations and the current study are small, and are likely to relate to differences in demographic details between the studies and/or differences in the region of the ACC from which tissue was sampled (as previously suggested for PV).

4.4.1.3 Calbindin

The layer distribution of CB immunoreactivity and neuropil labelling patterns reported in the present study are substantiated by the previous studies of CB immunoreactivity in the normative ACC (Hof *et al.* 1993; Nimchinsky *et al.* 1997). The primarily bitufted morphology of CB-immunoreactive neurons observed in the present study is also corroborated by existing literature. Cell density in areas 24a, 24b and 24c increased gradually from ventral to more dorsal areas, though staining in areas 24b and 24c was quite comparable. This is again consistent with the existing literature, which suggests that cell density in areas 24b and 24c is fairly constant, but greater than in area 24a (Hof *et al.* 1993; Nimchinsky *et al.* 1997).

4.4.2 Reductions in CR, but not PV or CB immunoreactivity in the ACC in mood disorder

Significant reductions in CR immunoreactivity were observed in areas 24a (17%) and 24b (21%). Similar, but non-significant reductions were observed for PV and CB. In contrast, Cotter *et al.* (2002a) did not report any significant changes in CR density in mood disorder, however, their study focused on area 24c, which was also insignificant in the current study. Although significant reductions in CR density have not been previously reported in the ACC, investigations of prefrontal regions have also indicated that CR density is reduced in layer I of area 46 in major depression and in layers V and VI in the entorhinal cortex in bipolar disorder (Reynolds *et al.* 2002).

Previous studies of mood disorder have reported significant reductions in CB density in both the ACC (layer II) (Cotter *et al.* 2002a) and area 46 (layers II and III) (Beasley *et al.* 2002b) in bipolar disorder, but not major depression. Deficits in PV density have also been observed in CA1 subfield of the hippocampus in bipolar disorder (Zhang and Reynolds 2002). The current study did not find any statistical evidence to support these findings, however, it is noteworthy that the non-significant reductions in the present study

ranged between 12%-25%, which is similar in magnitude to the significant and non-significant reductions in PV and CB reported previously in the ACC in mood disorder (Cotter *et al.* 2002a). The lack of significance in this study may possibly reflect a type II error (false-negative) given the small sample size and the spread of data.

Further evidence supporting a GABAergic deficit in the ACC in mood disorder is provided by a recent morphometric study that reported reduced numbers of small, presumed inhibitory, non-pyramidal neurons in layer II in the ACC in bipolar disorder (Benes *et al.* 2001). Furthermore, an immunohistochemical study has reported a reduced density of GAD-immunoreactive terminals in layers II-VI in the ACC in bipolar disorder; these differences were most significant in layers II and III (Benes *et al.* 2000).

Due to the incomplete staining and tissue damage resulting in a small 'n' for each subject group in this study, this study is likely to be underpowered and as such should be interpreted cautiously. This investigation needs to be replicated in a larger and better characterised brain series to address whether the reductions in CR density really are specific to subdivisions 24a and 24b, or whether high variability in the current study (due to its small size and/or the heterogeneity of staining in the ACC) obscured any 'significant' reductions in area 24c. Likewise, high variability may have masked statistically significant alterations in PV and CB. It is unlikely that such variability would have resulted in spurious significant findings, although this possibility should not be entirely dismissed. In consideration to the heterogeneity of CaBP immunoreactivity in the ACC, it is noteworthy that the qualitative account by Nimchinsky and colleagues (1997) suggests that CR immunoreactivity is not as variable along the rostrocaudal extent of the ACC as PV and CB immunoreactivity. Consequently, it is feasible that any potential intersample variability introduced at the sampling level in the current study did not greatly influence CR labelling by comparison to PV and CB labelling; possibly explaining why only CR showed statistically significant results.

4.4.2.1 Factors affecting CaBP immunoreactivity

In accordance with previous studies of CaBP immunoreactivity in mood disorder, pH showed significant positive correlations with PV and CB immunoreactivity, but not CR immunoreactivity (Beasley *et al.* 2002b; Cotter *et al.* 2002a; Reynolds *et al.* 2002). The mechanism for this is unclear, although it could be speculated that progressively more acidic conditions result in a conformational change in the structure of the protein that conceals the epitope, thereby decreasing immunoreactivity. CR appears to be selectively resistant to the deleterious effects of decreasing pH.

Age and PMI did not show significant correlations with any of the CaBPs. The potential confounding effect of fixation time could not be investigated in the present study, however, reassurance can be sought from inspection of the existing literature, which suggests that fixation time does not have any significant bearing upon CaBP immunoreactivity (Beasley *et al.* 2002b; Cotter *et al.* 2002a; Reynolds *et al.* 2002; Zhang and Reynolds 2002).

4.4.3 Interpretation of CR deficits in mood disorder

Decreases in CR can be interpreted in various ways; deficits may be secondary to a general or selective loss of non-pyramidal neurons, or may even reflect a decrease in CR protein expression. The present study has the distinct advantage that 'total' non-pyramidal neuron counts had been previously performed on adjacent tissue sections, consequently permitting a more comprehensive interpretation of the results. If reductions in CR were due to non-pyramidal neuron loss, then one would expect to see concomitant evidence for this in the Nissl-stained sections. Interestingly, the study reported in the previous chapter failed to find any deficits in non-pyramidal neuron density in mood disorder (see section 3.3.4) – however, it is conceivable that this 'total' non-pyramidal neuron count may have obscured any significant changes in a discrete subpopulation of non-pyramidal neurons, which is an appealing theory considering that only reductions in CR were significant in the present study. Whilst it is possible that specific alterations may

have been concealed by a total count, this is perhaps unlikely given that reductions (albeit insignificant for PV and CB) were observed in all three CaBPs and therefore any theories proposing that deficits in CaBPs reflect cell loss are not entirely supported or confirmed by the morphometric evidence from the previous chapter.

As aforementioned, cell loss is only one explanation, and an alternative interpretation is that the observed deficits in CR are, potentially reversible, changes in protein expression reflecting the functional activity of the cell, which itself is likely to be dependant on other factors, such as the activity of other neurons with which it forms synapses. This theory is certainly more compliant with the present findings. A caveat of this conclusion is that 'total' non-pyramidal neuron deficits have been previously reported in the ACC in mood disorder. In a study of bipolar patients, significant reductions in density were found in layer II of the ACC (Benes *et al.* 2001). However, this study cannot be directly compared to the present work because it exclusively concerned bipolar patients and utilised tissue from the pregenual region of area 24. Reductions in the expression of GABAergic markers, in the absence of cell loss are not without precedent. Akabarian *et al.* (1995) and Volk *et al.* (2000) have both reported a reduction in the density of GAD-labelled cells – without an accompanying reduction in non-pyramidal neurons in the frontal cortex of schizophrenic patients. Equivalent studies have not yet been performed in mood disorder, though my data are certainly suggestive of reduced CR protein expression in the ACC in mood disorder. Quantitative protein analysis techniques using unfixed tissue would help to clarify the extent to which various CaBP content is altered.

Given the discrepant evidence for and against non-pyramidal neuron deficits in the ACC in mood disorder, it is quite feasible that interpretations of neuron loss and/or reductions in protein expression are true to differing extents in patient groups that differ between post-mortem studies in terms of a wide variety of parameters including the brain region under investigation, demographic details, symptom profile and aetiological factors.

4.4.3.1 *Implications of CR deficits in mood disorder*

Although the findings of the present study can only be taken as preliminary evidence for CR deficits in the ACC in mood disorder, a brief discussion will be made as to the consequences of such a deficit. Experimental evidence has indicated that diminishing intraneuronal CaBP content renders the neuron in a less excitable state (presumably due to its reduced ability to buffer intracellular calcium transients); thereby affecting the events of depolarisation and repolarisation. Prolonged firing in such a circumstance could lead to dormancy in these neurons, potentially disinhibiting control over pyramidal output (see Eyles *et al.* 2002). Reductions in CR-containing neurons, or deficits in CR expression could presumably have the same disinhibitory effect over pyramidal function.

CaBP-containing neurons are also speculated to have a protective role; experimental evidence has shown that neurons expressing CaBPs are selectively resistant to excitotoxins, show attenuated levels of apoptosis and are protected from oxidative stress (see Eyles *et al.* 2002). It is therefore feasible that reductions in CR density and/or expression may render neurons unable to cope with cellular stress. Nevertheless, the neuroprotective properties of CaBPs have not been consistently demonstrated and their protective role in the diseased human brain remains a moot point (Andressen *et al.* 1993).

4.4.3.2 *Selectivity in CaBP changes*

This study reported significant reductions in CR density in areas 24a and 24b, but not 24c in mood disorder. Reductions in PV and CB remained insignificant. These preliminary data suggest that GABAergic deficits may be selective, both in relation to the CaBP, and the subdivision of area 24, affected in mood disorder. In considering why selective GABAergic neurons are affected, it might be important to consider the individual properties of each CaBP, such as their kinetics of calcium chelation. For example, Eyles *et al.* (2002) have suggested that differences in CaBP kinetics may render neurons differentially susceptible to calcium-mediated toxicity in schizophrenia.

The chemical subclassification of non-pyramidal neurons revealed by CaBPs might permit an alternate explanation. It is possible that selective changes in these proteins might reflect vulnerable connections that are specifically affected in mood disorder. Similarly, as variations in the distribution of CaBPs along the dorsoventral axis of the ACC might represent cellular reflections of differences in the intrinsic organisation and connectivity of these areas (Nimchinsky *et al.* 1997); selective changes in the subdivisions of area 24 may be indicative of specific connections that are vulnerable in mood disorder.

Importantly, it should be restated that this discussion is largely speculative. This study is likely to be underpowered and therefore significant changes in other CaBPs and subdivisions of area 24 cannot be ruled out. Consequently, this study provides only rudimentary evidence for selective changes in CR immunoreactivity in the ACC in mood disorder and the findings of the present study must be considered preliminary pending replication.

4.4.4 Methodological considerations and limitations of the current study

Many of the limitations and considerations relevant to the current study, including discussions pertaining to the sample itself (i.e. demographics, diagnostic heterogeneity, suicide), together with issues surrounding two-dimensional counting methodology have been considered in chapter three (section 3.4.6) and will therefore not be reiterated here.

A considerable limitation of this study concerns the small sample size, as a result of incomplete staining across all subdivisions of area 24, coupled with damaged tissue in the region of area 24c in various sections. This resulted in the diminution of an already small brain series. Nevertheless, this study was unprecedented in its assessment of CaBP immunoreactivity in areas 24a, 24b and 24c in the ACC in mood disorder and therefore provided valuable preliminary data. As the subdivisions of area 24 are related, I decided to use a repeated measures ANOVA with subdivision as a 'within subject variable'

(mirroring the cortical layer analyses of chapter three). A corollary of this decision was to further reduce the 'n' of each subject group, as only those subjects with data values for all three subdivisions of area 24 were included in each test. In consideration to this, a looser interpretation of 'significance' was adopted for the repeated measures ANOVA, and $p \leq 0.1$ was considered as sufficient evidence for further exploration of the data set. For all subsequent ANOVAs (which included all data points), significance was set at $p \leq 0.05$. The statistical approach adopted in this study could be criticised, however, even with small numbers, the repeated measures ANOVA was considered to be an appropriate test due to its more conservative nature and suitability regarding related variables.

Another limitation of this study relates to the variation in dorsoventral staining patterns achieved with each CaBP. Whilst this variation may reflect 'true' intersample variation, it is possible that slight inconsistencies in tissue sampling may also be a factor since tissue was (unavoidably) collected over a protracted period of time and different pathologists were responsible for the dissection of different brains. As previously discussed (section 4.4.1.1), any intersample variation concerning the distribution and intensity of CaBP immunoreactivity may be related to the varying anatomical positions from which tissue was originally sampled. Although one would hope that such added 'noise' is likely to be minimal and affect each diagnostic group equally, it remains possible that any variation introduced at the sampling level may have obscured (or produced) significant findings. Either way, this study has further exemplified the complexity of the ACC and has also provided preliminary evidence suggesting that all three subdivisions of area 24 should be investigated in any future studies of CaBP expression in mood disorder. Moreover, this study focused on those cortical layers that are principally labelled by PV, CR and CB, partly due to time constraints, but also because these layers appeared to be primarily affected in studies of the CaBPs in mood disorder (Beasley *et al.* 2002b; Cotter *et al.* 2002a; Reynolds *et al.* 2002); however, this by no means excludes the involvement of

other cortical layers and therefore any replication of this work should ideally assess all cortical layers in each subdivision of area 24.

Significant demographic variables were included in the covariate analyses it is therefore unlikely that these potential confounds contribute to, or account for, the reduction in CR density observed in this study. Unfortunately, it is possible that the results of this study are associated with drug treatment, as all of the mood disorder subjects had received medication. There is little evidence with regard to the potential effects of antidepressant medication on the expression of PV, CR or CB. Previous investigations of CaBP expression in mood disorder have not reported any significant correlations with antidepressant dose, or lifetime medication load (Beasley *et al.* 2002b; Cotter *et al.* 2002a; Zhang and Reynolds 2002). In addition, correlations have not been observed between CB cell density and lithium treatment (Cotter *et al.* 2002a). Sufficient clinical information regarding medication status was not available in the present study and therefore equivalent tests could not be performed; nevertheless, the existing literature does suggest that medication is unlikely to have influenced cell density, although this point cannot be made categorically.

4.5 Summary

The distribution of PV, CR and CB-immunoreactive neurons and neuropil labelling patterns was highly variable in the dorsoventral axis of area 24. This study provided preliminary evidence for significant reductions in CR immunoreactivity in areas 24a and 24b, but not 24c in mood disorder. Similar (but insignificant) reductions were observed for PV and CB. Since deficits in non-pyramidal neuron density were not observed in chapter three, it is suggested that these reductions reflect changes in CaBP content, rather than neuron density.

Chapter Five: GFAP and HLA-DR expression in the anterior cingulate cortex in mood disorder

5.1 Introduction

5.1.1 Glia

Glia represent the most numerous group of brain cells, nevertheless, their role in cortical functioning has been grossly neglected in comparison to the neuronal population (Coyle and Schwarcz 2000). Over the past decade an increasing number of observations have progressively challenged the classical view that places glial cells in a subservient position to neurons; recent research has demonstrated that glial cells are far from passive but are intimately involved in the active control of neuronal activity, synaptic formation and function and neurotransmission. Consequently, it is only recently that the potential involvement of these cells in the neurobiology of psychiatric disorders has been fully appreciated (reviewed in Cotter *et al.* 2001b).

The glial populations of the CNS comprise three functionally distinct cell types: oligodendrocytes, microglia and astrocytes. In the white matter, oligodendrocytes are aligned in rows as interfascicular cells between nerve fibres. They are also associated with myelinated fibres in the grey matter. Many grey matter oligodendrocytes are also closely associated with neuronal cell bodies (so called 'satellite' oligodendrocytes). The main function of oligodendrocytes concerns the myelination of axons in the CNS, and as such they are crucial for the conduction of action potentials along axons (Peters *et al.* 1991). They are also implicated in the guidance of developing neurites through release of growth inhibitors. Stabilising functions of these inhibitors (i.e. restriction of sprouting) have also been proposed (Schwab 1990). Additionally, oligodendrocytes, in conjunction with astrocytes, are known to influence the formation and efficacy of synapses (Pfrieger and Barres 1997). Interestingly, it has been suggested some of the satellite oligodendrocytes

may in fact be undifferentiated oligodendrocyte precursor cells (OPCs) (Berry and Butt 1997; Levine *et al.* 2001). The function of OPCs in the CNS is largely unknown, but their processes are in contact with nodes of Ranvier and synapses, suggesting a regulatory role at these structures. It has been proposed that these cells may function as stem- or stem-like cells, providing oligodendrocytes for remyelination by migration to interfascicular sites (Levine *et al.* 2001).

Microglia are the resident macrophages of the CNS with a capacity for immune surveillance and control (Kreutzberg 1996; Thomas 1992). The transformation of microglia from a ramified (resting) to a phagocytic state occurs as a graded response with proliferation, migration, morphological, immunophenotypical and functional changes (Kreutzberg 1996). It is still debated whether microglia can revert from an activated form to a resting form (Nelson *et al.* 2002). Activated microglia can destroy invading micro-organisms, remove potentially deleterious debris and through the secretion of various neurotrophins (Elkabes *et al.* 1996; Nakajima *et al.* 1998) they promote tissue repair and neuronal regeneration and thus facilitate the return to tissue homeostasis (Kreutzberg 1996). In addition, neurotrophins and cytokines released by ramified microglia may support and regulate normal glial and neuronal function (Elkabes *et al.* 1996; Kreutzberg 1996; Nelson *et al.* 2002). In normal adult tissue ramified microglial cells also serve a constitutive role in cleansing the extracellular fluid (Thomas 1992). These roles underscore the fact that microglia play a dynamic functional role in the working CNS and hence should not be primarily seen as a nidus of disease (Nelson *et al.* 2002).

Astrocytes are the predominant glial cell type and due to their involvement in numerous and complex functions they deserve special consideration. Astrocytes found in the grey and white matter are morphologically distinct. White matter astrocytes have numerous fibrils and are therefore referred to as fibrous astrocytes, whereas grey matter astrocytes generally contain fewer fibrils and consequently have been called protoplasmic astrocytes

(Peters *et al.* 1991). Their classical roles in neurodegenerative diseases and inflammatory processes are well recognised (Berry and Butt 1997). In response to injury, astrocytes increase their number and size and change their pattern of gene expression, such that GFAP is up-regulated (Berry and Butt 1997). Astrocytes are also important for the ionic and metabolic homeostasis of the neuropil (Peters *et al.* 1991). Astrocytes, together with microglia, synthesise and release various neurotrophins thought to be important for the support and regulation of neuronal and glial function (Connor and Dragunow 1998).

Recent research has suggested an important role for astrocytes in the formation, efficacy and plasticity of synapses (Pfrieger and Barres 1996; 1997; Ullian *et al.* 2001). They are also important for other aspects of synaptic function including the clearance of glutamate from the synaptic cleft, and thus modulate the duration of synaptic current and prevent excitotoxic damage (Oliet *et al.* 2001; Pfrieger and Barres 1997). Moreover, a growing body of research indicates that neuron-to-glia signalling and glia-to-neuron signalling occurs in the brain (Bezzi and Volterra 2001; Gallo and Ghiani 2000). Glutamate release from neurons can stimulate metabotropic glutamate (mGlu) receptors on astrocytes and induce Ca^{2+} -regulated neurotransmitter release. Glutamate released from astrocytes can then access either neuronal NMDA or mGlu receptors in order to facilitate or depress the synapse. These experiments indicate that astrocytes can potentially modulate synaptic neurotransmission and plasticity. (Araque *et al.* 1999; Gallo and Ghiani 2000). Furthermore, Ca^{2+} waves can propagate through neighbouring astrocytes, thereby permitting astrocytes to modulate synapses distant from the synapse that was originally active (Araque *et al.* 1999).

Finally, astrocytes are crucial for the energy requirements of neurons (Magistretti and Pellerin 1996; Tsacopoulos and Magistretti 1996). As previously discussed, astrocytes are responsible for removing glutamate from the synapse. Glutamate is transported into the cell along with extracellular sodium where it is converted to glutamine and

subsequently released and taken up by neurons. An increase of intracellular sodium in glial cells activates the sodium-potassium pump, which promotes glucose intake and glycolysis. This results in the release of lactate as an energy substrate for neurons.

Astrocytes therefore couple the activity of glutamatergic synapses with glucose utilisation via the sodium-potassium pump. Moreover, the aggregate interaction of glucose uptake, glycolysis and intraneuronal oxidative metabolism provides the signal in functional brain imaging studies (Magistretti 2000; Magistretti *et al.* 1999).

5.1.2 Glial subtypes in mood disorder

Reductions in glial density have been fairly consistently reported in mood disorder research (see section 3.4.2); the glial deficit reported in chapter three supports and extends this literature. As the Nissl method does not allow ready differentiation between the different glial subpopulations, it is not yet known whether one type of glia is accounting for the deficit observed in mood disorder or whether there is a more generalised reduction across all glia subpopulations. Recent research has employed proteomic, immunohistochemical and ultrastructural techniques in an attempt to clarify this issue.

To date, only one study has tackled this issue in the ACC. In an immunohistochemical study of GFAP in late-life major depression, Davis *et al* (2002) did not find any significant alterations in the supragenual region of the ACC, although a significant increase in GFAP staining was observed in layer I in the prefrontal cortex in late-life depression. The relevance of this study to other research focusing on younger patients is unclear given that late-life depression is associated with vascular disease (see section 1.3), which may independently trigger astrogliosis (Alexopoulos *et al.* 1997; Davis *et al.* 2002; Thomas *et al.* 2002).

All remaining investigations of the glial subpopulations in mood disorder pertain to the prefrontal cortex. In a follow-on study to their research in area 9 in mood disorder,

Rajkowska and colleagues failed to find any evidence for reduced GFAP staining or GFAP-immunoreactive astrocyte density in the sample considered as a whole; however, a specific decrease was observed in the younger (30-45) major depression cases compared with a subgroup of age-matched controls (Miguel Hidalgo *et al.* 2000). Alterations in GFAP in mood disorder are also suggested by three further publications. Johnston-Wilson *et al.* (2000) recently demonstrated reduced levels of various GFAP isoforms in major depression and bipolar disorder. The same group then went on to perform an immunohistochemical investigation into the localisation of phosphorylated GFAP in the same brain collection. Major depression, but not bipolar disorder, was characterised by decreased labelling of astrocytes adjacent to blood vessels in the white matter of the gyrus (Webster *et al.* 2001a). An immunoblotting technique for total GFAP in this same brain collection also found significant reductions in the prefrontal cortex in major depression and bipolar disorder (Knable *et al.* 2001).

Oligodendrocytes have also been the focus of recent attention, and in an ultrastructural study were reported to show reactive, progressive and regressive changes in area 10 in bipolar disorder (Uranova *et al.* 2001). A reduction in the density of oligodendrocytes in area 9 has also been reported (in preliminary form) in layers III (Uranova *et al.* 2002) and VI (Orlovskaya *et al.* 2000) in major depression and bipolar disorder.

Microglia have not been systematically investigated in mood disorder. The only existing publication is a qualitative study of microglia which demonstrated their activation in mood disorder (Bayer *et al.* 1999); however, the antibody used by the authors to detect 'activated' microglia (HLA-DR) has subsequently been employed to label ramified and activated microglia (Radewicz *et al.* 2000; Thomas *et al.* 2002) and therefore the authors' interpretation of neuropathological activation may be inappropriate (see discussion).

5.1.3 Aims of the current study

The primary objective of this study was to systematically assess the individual contribution of astrocytes, oligodendrocytes and microglia to the overall glial deficit observed in mood disorder in chapter three. Accordingly, immunohistochemical and/or *in situ* hybridisation techniques were employed which permitted the detection of each glial cell type. Again, this study focused on area 24 of the ACC.

5.2 Methods

5.2.1 Tissue collection and preparation

5.2.1.1 Paraffin embedded tissue

Paraffin-embedded tissue sections were used for the immunohistochemistry and immunoautoradiography protocols. The collection and preparation of paraffin-embedded tissue has been previously described in sections 3.2.1, 3.2.2 and 3.2.3. The demographic details of the individuals included in the experiments are shown in table 5.1.

5.2.1.2 Frozen tissue

Frozen tissue sections were used in the *in situ* hybridisation histochemistry (ISHH) protocol. Blocks of approximately 1cm³ were dissected from the supragenual region of area 24, roughly 1cm caudal to the tip of the genu of the corpus callosum. The blocks were placed in plastic moulds and surrounded by OCT compound (Agar Scientific, Stanstead, UK). The moulds were placed in a mixture of dry ice and alcohol to freeze (this method of freezing helps to avoid ice crystals) and subsequently stored at -70°C. Prior to sectioning, frozen tissue was allowed to warm to -20°C. 18µm sections were cut from the frozen blocks using a Shandon cryostat (Cheshire, UK), thaw mounted onto superfrost microscope slides and immediately stored at -20°C. The demographic details of the individuals included in the ISHH experiment are shown in table 5.2.

5.2.2 Immunohistochemistry

5.2.2.1 Immunohistochemistry: microglia

There are many different antibodies commercially available for the detection of microglia, many of which can differentiate between ramified and activated microglia. For the purposes of this study I wanted to detect all microglial populations and therefore I decided to use an antibody against HLA-DR, which is a major histocompatibility complex class II marker of microglia that is constitutively expressed, but markedly increased when microglia are activated (Gehrmann *et al.* 1993). Again, the effectiveness of this antibody in formalin fixed human post mortem tissue has been recently validated (Radewicz *et al.* 2000; Thomas *et al.* 2002).

Pilot experiments assessing various antigen methods, antibody concentrations and incubation times were performed prior to the definitive experiment (data not shown). The final immunohistochemical and antigen retrieval protocol adopted for HLA-DR was identical to that outlined in section 3.2.4.3. The primary antibody solution contained mouse HLA-DR monoclonal antibody (Dako, Cambridge, UK; M0775) diluted to 1:30 in PBS-T containing 1% normal serum. The experiment was performed on duplicate sections; omission of the primary antibody was used as an experimental control.

5.2.2.2 Immunohistochemistry: astrocytes

The principal method for identifying astrocytes is immunohistochemistry with antisera raised against GFAP, an intermediate filament present in the cell body and processes of all astrocytes (Eng *et al.* 2000). The use of GFAP as a marker of astrocytes in routinely processed formalin-fixed paraffin embedded human tissue has been previously validated (Halliday *et al.* 1996). A polyclonal rabbit antibody against GFAP (Dako, Cambridge, UK; Z0334) was selected to visualise astrocytes.

Pilot experiments indicated that GFAP staining was, as expected (see Halliday *et al.* 1996), highly variable between brains, with some cases presenting minimal immunoreactivity in the grey matter. Consequently, it was decided that cell counting for GFAP would not be performed, and instead GFAP immunoreactivity would be measured across the entire width of the cortex. In consideration to this, immunautoradiography was employed to provide a more reliable and quantitative method for assessment of GFAP immunoreactivity (see section 5.2.3).

5.2.2.3 Immunohistochemistry: oligodendrocytes

Two antibodies against oligodendrocytes (Chemicon International, Hampshire, UK; MAB328 and MAB1580) were tested under various conditions in paraffin-embedded and frozen tissue sections. Unfortunately, all attempts at specific staining were unsuccessful. An account of all experimental conditions applied during the pilot investigation is beyond the scope of this thesis.

5.2.3 Immunoautoradiography

5.2.3.1 Principles of immunoautoradiography

Immunoautoradiography (IAR) is conceptually similar to immunohistochemistry, except that the secondary antibody is radioactively labelled (^{35}S in this case), allowing the sites of antigen-antibody binding to be assessed autoradiographically. This overcomes a major concern with the use of traditional immunohistochemical methods in a quantitative mode, namely the variability in the intensity of the staining reaction produced with DAB or other chromogen. The use of immunoautoradiography as a quantitative tool was developed and validated in rat tissue (Blanchard *et al.* 1993; Raisman Vozari *et al.* 1991), and has been used successfully in human post mortem tissue (for examples see Eastwood *et al.* 1995; Eastwood and Harrison 1995).

5.2.3.2 *Immunautoradiography for GFAP*

Triplicate sections were dewaxed and rehydrated in graded alcohols. Non-specific binding was blocked with 10% normal serum in PBS-T for 30 minutes at room temperature. The sections were briefly rinsed in PBS before application of the primary antibody solution, which contained rabbit polyclonal GFAP antibody (Dako; Z0334) diluted to 1:1000 in PBS-T containing 1% normal serum. Sections were incubated overnight at 4°C. Sections were then incubated with ^{35}S labelled secondary donkey anti-rabbit antibody diluted to 0.5 $\mu\text{Ci/ml}$ (Amersham Biosciences, Little Chalfont, UK) in PBS-T with 1% normal serum at room temperature for one hour. The sections were then washed in PBS (3x 10 minutes), rinsed in distilled water and allowed to air dry. Sections were placed against Kodak BioMax MR film, coexposed with ^{14}C microscales (Amersham Biosciences). Omission of the primary antibody was used as an experimental control. Appendix four provides details regarding the development of autoradiograms.

5.2.4 *In situ hybridisation histochemistry*

5.2.4.1 *Principles of in situ hybridisation histochemistry*

In *in situ* hybridisation histochemistry (ISHH), a labelled nucleic acid probe complementary to the target RNA is allowed to hybridise to its target directly within a tissue section. Oligonucleotide probes were used throughout this thesis. The stability of the hybrids formed is governed by a number of factors, including the length of the probe, the percentage of GC pairings, the absolute degree of homology between the probe and the target mRNA, along with the temperature and composition of the hybridisation buffer (Farrell 1998). Oligonucleotides used herein were between 30-40 bases in length and had a GC/AT ratio between 50 and 65. The GC content is an important consideration, since too high a percentage of GC can lead to non-specific binding, while a low GC percentage may cause inefficient binding (due to the relative stability of GC and AT hydrogen bonds, see Farrell 1998).

The melt temperature (T_m) of the probe, at which 50% of the hybrids will spontaneously dissociate, is given by the following formula (Farrell 1998):

$$T_m(^{\circ}C) = 16.6 \log[M] + 0.41[P_{gc}] + 81.5 - P_m - B/L - 0.65[P_f] \quad (1)$$

where M is the molar concentration of Na^+ , to a maximum of 0.5, P_{gc} is the GC percentage, P_m is the percentage mismatch between the probe and the target mRNA (this was 0 in all cases), P_f is the percentage of formamide in the buffer, B is 675 for synthetic oligonucleotide probes and L is the length of the probe. Hybridisation is generally carried out $15^{\circ}C$ below the melt temperature. After hybridisation, slides are washed to remove unbound probe (thereby decreasing non-specific signal). The wash temperature (T_w) is calculated according to the following formula (Farrell 1998):

$$T_w(^{\circ}C) = 16.6 \log[M] + 0.41[P_{gc}] + 81.5 - P_m - B/L \quad (2)$$

5.2.4.2 Pre-treatment

All sections were pre-treated within two weeks of sectioning. The pre-treatment protocol (see appendix three) was designed to preserve the mRNA, ensure an adequate preservation of tissue morphology and to minimise non-specific binding.

5.2.4.3 Selection of an oligonucleotide probe for GFAP

The probe was chosen from within the coding region of the published cDNA sequence (Kumanishi *et al.* 1992) and was 36 bases in length with a GC content of 53%. The antisense (5'-TCT CCA CGG TCT TCA CCA CGA TGT TCC TCT TGA GGT-3', bases 1222-1257; (Kumanishi *et al.* 1992) and sense probe (5'-ACC TCA AGA GGA ACA TCG TGG TGA AGA CCG TGG AGA-3') sequences were checked for homology with known genes using the EMBL databank before being purchased from Oswel DNA Services (Southampton).

5.2.4.4 Probe labelling

Oligonucleotides were labelled by incubation with [α ^{35} S]dATP (~1200 Ci/mmol, NEN Dupont, Hounslow, UK) in a 1:10 molar ratio, 2 units terminal deoxynucleotidyl transferase and buffer (500mM cacodylate buffer pH 6.8, 5mM CoCl₂, 0.5mM dithiothreitol; Promega, Southampton, UK) at 37°C for 30 minutes, and purified using NICK columns according to the manufacturers instructions (Amersham Biosciences).

5.2.4.5 Hybridisation and washing

Tissue sections (in triplicate) were allowed to warm to room temperature before the probe was applied. Purified probe was diluted in hybridisation buffer (see appendix two) to give a final concentration of 1×10^6 counts per minute per section hybridised (based on 150 μ l per section). Dithiothreitol, which prevents the formation of disulphide bonds, was added at a final dilution of 2% to increase the signal to noise ratio (Dagerlind *et al.* 1992). Slides were covered with coverslips and incubated overnight in trays humidified with 50% formamide and 4x saline sodium citrate (SSC) at 33°C.

After incubation the coverslips were removed and post hybridisation washes were carried out in 1x SSC, at 56°C for 3x 20 minutes, followed by 2x 60 minute washes at room

temperature. Slides were then rinsed in water to remove excess salts and air dried. Additional controls to determine non-specific binding were obtained by adding excess unlabelled probe to the slide prior to the addition of the hybridisation mix. When dry, sections were placed against Kodak BioMax MR film, coexposed with ^{14}C microscales. A Northern analysis of human brain RNA probed with the oligonucleotide was also performed to ensure that the oligonucleotide was targeting mRNA of the predicted size.

5.2.5 Morphometric analysis – HLA-DR immunoreactivity

5.2.5.1 *Identification of Brodmann area 24b*

Area 24b was identified and defined in adjacent Nissl-stained sections according to macroscopic and microscopic criteria (Vogt *et al.* 1995). These criteria are outlined in section 2.2.5.1. Boundaries were then extrapolated onto the HLA-DR slides.

5.2.5.2 *Counting procedure*

The counting procedure used in this study was analogous to that outlined in section 4.2.3.2, with the one exception being that cell diameter and section thickness were not measured (microglial density was not corrected due to the very small size of these cells in relation to section thickness). Tissue sections were viewed with a 20-times objective lens. Microglial cells were counted manually using a cursor to mark cells, selected only where the cell body was clearly evident. Microglial cells were counted in area 24b and in the adjacent white matter.

Sampling strategies were optimised before the definitive analysis, as outlined in section 4.2.3.2. These preliminary tests indicated that sampling 20 frames per section, per region (area 24b/white matter), provided reproducible density estimates for HLA-DR-immunoreactive microglia. Five cases (selected at random) were repeated one week later - all values were within 5% of original measurements (data not shown). An unbiased

counting frame with an area of $57,600 \mu\text{m}^2$ was used throughout this study (see section 2.2.1.3). The sampling field was moved systematically throughout the entire cortical width of area 24b and the gyral white matter until a sufficient number of frames had been sampled in each region, taking care to avoid the subpial regions as well as the border zone between white and grey matter.

All measurements were made blind to diagnosis. Density values are expressed as cell density/ mm^2 .

5.2.6 Image analysis

Autoradiograms were illuminated upon a light box and the images were visualised using a high-resolution monochrome video camera. Signal intensity on the autoradiograms was measured by comparison with a calibration curve using the ^{14}C microscale images (a conversion factor of three was used to translate these values into ^{35}S equivalents).

The areas to be measured were delineated by hand. Measurements were taken through the depth of the grey matter in area 24 as well as in the gyral white matter. Multiple readings were taken to ensure that a representative value was obtained for each section. All measurements were corrected for non-specific signal (arising from sense strand or cold displacement hybridisation for ISHH and omission of the primary antibody for IAR). Again, all measurements were made blind to diagnosis. The relative abundance of GFAP protein and mRNA is expressed in terms of ^{35}S nCi/g tissue equivalents.

5.2.7 Statistical analysis

All statistical analyses were carried out using SPSS 10.0 for Microsoft Windows. An initial evaluation of the data involved checking the distributions of points within each data set for normality using the Kolmogorov-Smirnov test. Each data set was analysed separately.

The potential confounding effects of age, pH, PMI and freezer storage time (for frozen tissue) were evaluated using Pearson's correlations in the control and mood disorder group separately, as well as in both groups combined. Any demographic variable found to exert a significant effect was included as a covariate in any subsequent analysis.

Significance was set at $p \leq 0.05$ for all correlation analyses. Differences between control and mood disorder subjects were investigated using one-way ANOVA, including covariates where appropriate. Again, significance was set at $p \leq 0.05$.

Table 5.1.

Demographic details of control and mood disorder subjects included in the HLA-DR immunohistochemical and GFAP IAR experiments. Values are mean $\pm$ S.E.M with range in brackets. There are no significant differences between the two groups ($p>0.2$). ^a major depression (n=5), bipolar disorder (n=2). Diagnoses were made according to DSM-IV criteria. ^b All patients had been treated with antidepressant medication.

Variable	Control (n=10)	Mood disorder (n=7) ^{ab}
Age (years)	59.70 $\pm$ 5.63 (22–79)	64.57 $\pm$ 4.63 (49–75)
Sex (M:F)	6:4	4:3
pH	6.50 $\pm$ 0.07 (6.14–6.72)	6.40 $\pm$ 0.09 (6.06–6.86)
PMI (hours)	37.20 $\pm$ 8.09 (8–78)	34.43 $\pm$ 7.51 (13–75)
No. of suicides	0	4

Table 5.2.

Demographic details of control and mood disorder subjects included in the GFAP ISHH experiment. Values are mean $\pm$ S.E.M with range in brackets. There are no significant differences between the two groups ($p>0.1$). ^a major depression (n=7), bipolar disorder (n=2). Diagnoses were made according to DSM-IV criteria. ^b All patients had been treated with antidepressant medication.

Variable	Control (n=8)	Mood disorder (n=9) ^{ab}
Age (years)	63.00 $\pm$ 4.66 (42–79)	60.44 $\pm$ 4.90 (43–86)
Sex (M:F)	5:3	5:4
pH	6.52 $\pm$ 0.07 (6.14–6.72)	6.33 $\pm$ 0.05 (6.06–6.50)
PMI (hours)	38.75 $\pm$ 10.10 (8–78)	36.67 $\pm$ 6.38 (13–75)
No. of suicides	0	5

5.3 Results

5.3.1 Analysis of HLA-DR-immunoreactive microglial density

5.3.1.1 *HLA-DR immunoreactivity: qualitative observations*

Three of the brains used in this study were devoid of any HLA-DR immunoreactivity. Reasons for this are unclear, since their demographic details were unremarkable by comparison with the remainder of the sampled, as was the histological appearance of adjacent Nissl-stained sections. All three brains were found to be controls after de-coding.

HLA-DR-immunoreactive glial cells exhibited morphologies consistent with activated and ramified microglia (Gehrmann *et al.* 1993). 'Ramified' microglia had small oval cell bodies and multiple long thin processes and were evident throughout the cortex and white matter. Microglia with a ramified morphology were predominant. 'Activated' microglia appeared to be more darkly stained and possessed an enlarged cell body with thickened or protracted processes. Again, cells with an activated morphology were observed in the cortex and white matter. Examples of presumed ramified and activated microglia are illustrated in figure 5.1. It is important to point out that these pictures represent two opposite ends of the spectrum, and that many microglial cells displayed an intermediate morphology. HLA-DR staining was fairly uniform across the width of the cortex and was greater in white matter than in grey matter.

5.3.1.2 *Group comparisons*

HLA-DR-immunoreactive density measurements in area 24b and the white matter satisfied the Kolmogorov-Smirnov test for normal distribution. Significant positive correlations were observed between the density of grey matter HLA-DR-immunoreactive microglia and age in the control group, but not the mood disorder group, this correlation remained significant when both groups were combined. A significant positive correlation was also observed in the white matter between HLA-DR-immunoreactive density and age in the combined

group, though not when either the control or mood disorder group were considered separately (see table 5.3). No other significant correlations were observed. The ANCOVA, with age included as a covariate, failed to find a significant effect of diagnosis (grey matter, $p=0.527$; white matter, $p=0.171$) (see table 5.4 and figure 5.2).

5.3.2 Analysis of GFAP protein expression

5.3.2.1 *GFAP protein expression: qualitative observations*

GFAP IAR signal was diffusely distributed throughout the grey matter, but frequently demonstrated a greater intensity in layer I. The extent and intensity of GFAP signal in grey matter was highly variable between brains. Some cases showed minimal GFAP signal, whilst others demonstrated a much stronger signal. GFAP signal was consistently greater in white matter than in grey matter. Negligible signal was seen after omission of the primary antibody. All observations were comparable with the pattern of GFAP immunoreactivity observed in the pilot immunohistochemical experiments (see figure 5.3).

5.3.2.2 *Group comparisons*

Measurements of GFAP expression in the grey and white matter satisfied the Kolmogorov-Smirnov test for normal distribution. Significant positive correlations were observed in the grey and white matter between GFAP expression and pH in the control group, but not the mood disorder group, this correlation remained significant when both groups were combined (see table 5.5). No other significant correlations were observed. The ANCOVA (including pH as a covariate) revealed that GFAP was significantly reduced in the mood disorder group in the white matter ($F(1,14)=5.313$; $p=0.037$); but failed to show a significant effect of diagnosis in the grey matter ($p=0.291$) (see table 5.6 and figure 5.4).

5.3.3 Analysis of GFAP mRNA expression

5.3.3.1 *GFAP mRNA expression: qualitative observations*

GFAP mRNA expression was comparable to the pattern of protein expression described in section 5.3.2.1. Northern blots demonstrated that the oligonucleotide for GFAP detected a single band of the predicted molecular weight (see figure 5.5 and Galea *et al.* 1995; Le Prince *et al.* 1993). Negligible signal was seen with the sense strand probe or with excess cold probe (see figure 5.5).

5.3.3.2 *Group comparisons*

Measurements of GFAP mRNA expression in the grey and white matter satisfied the Kolmogorov-Smirnov test for normal distribution. There were no significant correlations with age, pH, pmi or freezer storage time in the control group, the mood disorder group or both groups combined. The ANOVA did not show a significant effect of diagnosis (grey matter, $p=0.913$; white matter, $p=0.981$), (see table 5.7 and figure 5.6).

Figure 5.1.

Photomicrographs showing HLA-DR immunoreactivity in the grey matter and white matter of area 24 (scale bar=100µm).

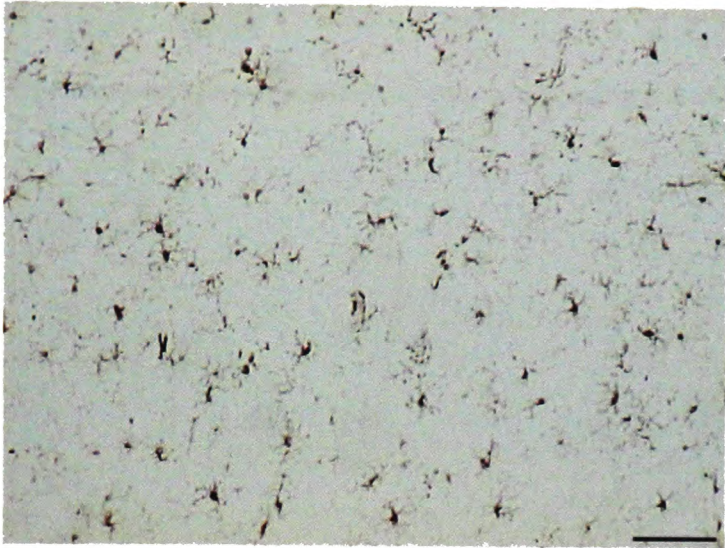
HLA-DR immunoreactivity is evident in the grey and white matter; considerably more staining is seen in the white matter. The negative control (omission of the primary antibody) did not show any immunoreactivity (data not shown).

Photomicrographs showing the morphology of HLA-DR microglia (scale bar=30µm).

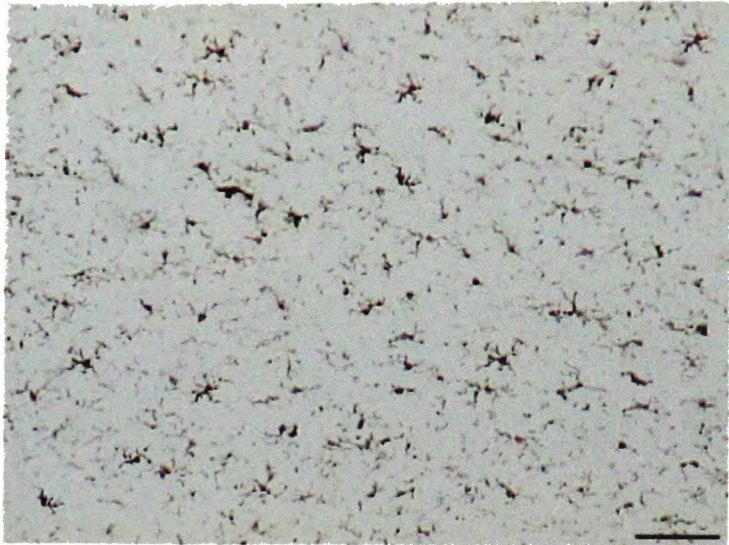
HLA-DR-immunoreactive glial cells exhibited morphologies consistent with activated and ramified microglia. 'Ramified' microglia have small oval cell bodies and multiple long thin processes (A-C). 'Activated' microglia are more darkly stained and possess an enlarged cell body with thickened or protracted processes (D-F). Images were taken from the grey matter of area 24, however, microglial cells consistent with 'ramified' and 'activated' cells were also present in the white matter. Microglia with a 'ramified' morphology are predominant in both regions.

HLA-DR immunoreactivity in anterior cingulate cortex

Grey matter



White matter



Ramified and activated microglia immunoreactive for HLA-DR

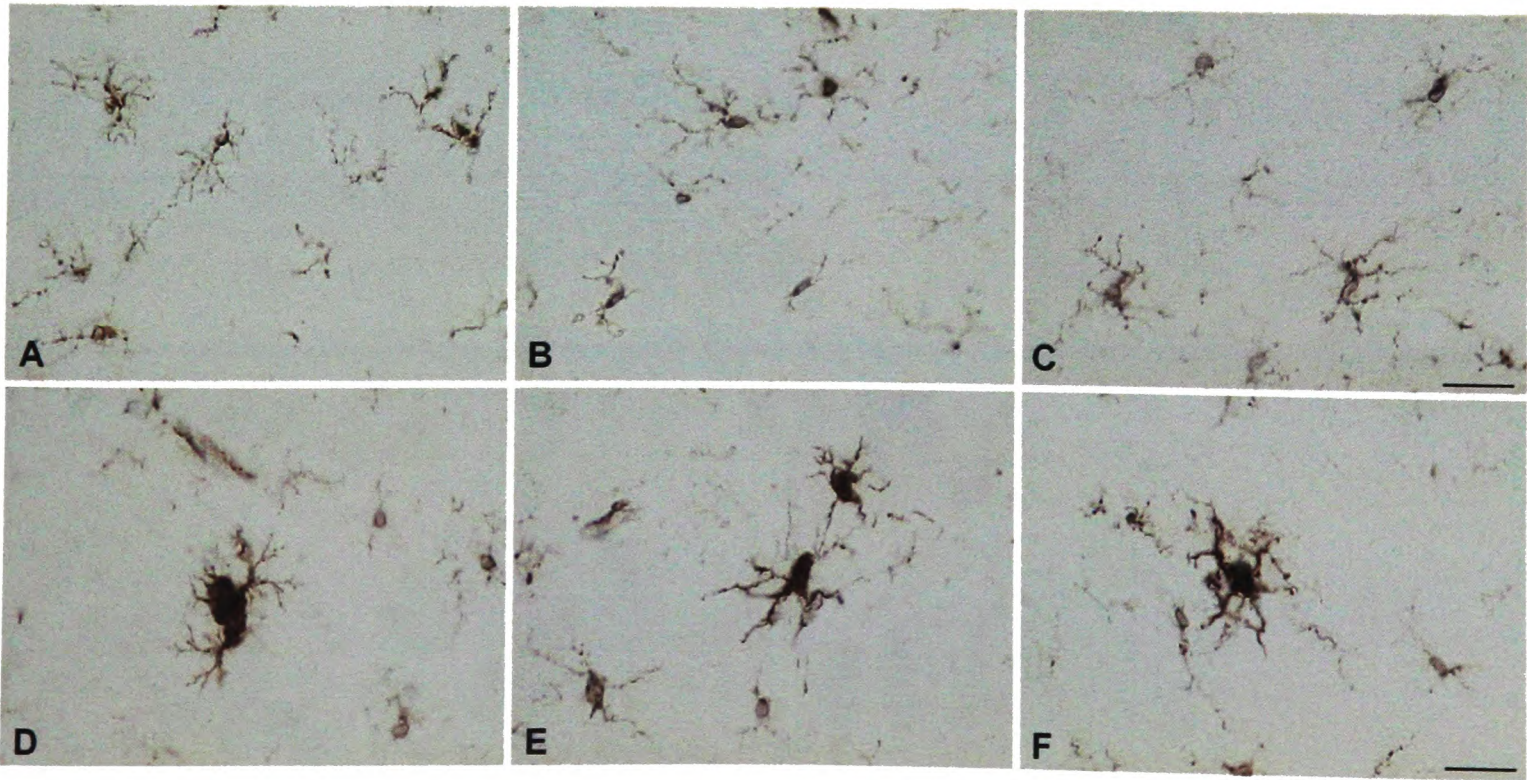


Table 5.3.

The effect of age on HLA-DR immunoreactivity in the ACC. Numbers are Pearson's R values. * $p \leq 0.05$

Density of HLA-DR-immunoreactive microglia and age			
	Control	Mood disorder	Combined
Grey matter	0.83*	0.46	0.58*
White matter	0.46	0.51	0.50*

Table 5.4.

Density of HLA-DR-immunoreactive microglia in the ACC in controls and mood disorder. ANOVA test used for comparison of HLA-DR immunoreactivity between diagnostic groups. These data are illustrated in figure 5.2.

Region	Density of HLA-DR-immunoreactive microglia		Test for difference between		
	Mean (S.E.M)		patient group and control group ^a		
	Control	Mood disorder	F	p	Percentage difference
Grey matter	105.3 (6.9)	105.1 (9.9)	0.43	.527	0.1%
White matter	193.7 (7.8)	223.5 (14.4)	2.15	.171	13.3%

^a ANCOVA with age

Figure 5.2.

Scatterplots showing grey matter (GM) and white matter (WM) density of HLA-DR immunoreactive microglia in the ACC in controls and mood disorder. Bars represent the mean for each group. See table 5.4 for statistical comparisons between groups.

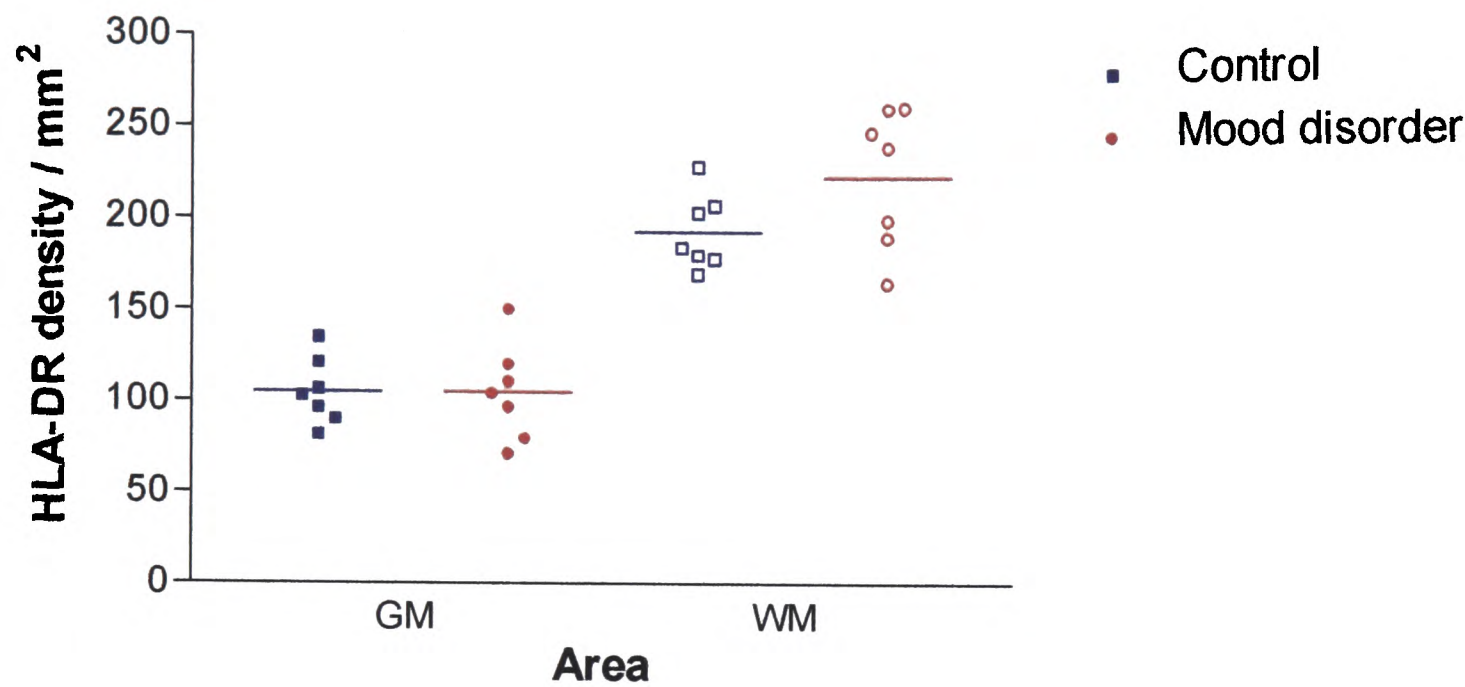


Figure 5.3.

Photomicrographs showing GFAP immunoreactivity in the grey matter and white matter of area 24 (scale bars: low power=100µm; high power=30µm).

GFAP immunoreactivity prominently reveals astrocyte cell bodies and thread-like processes.

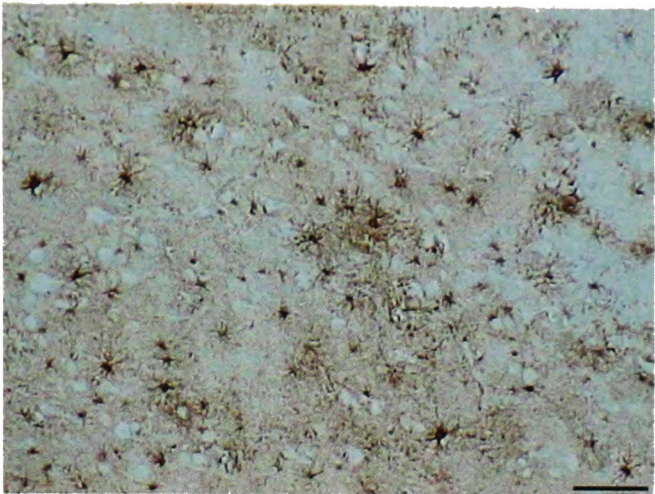
Staining is evident in the grey and white matter, although more immunoreactivity is seen in the white matter.

Autoradiograms showing GFAP expression in area 24.

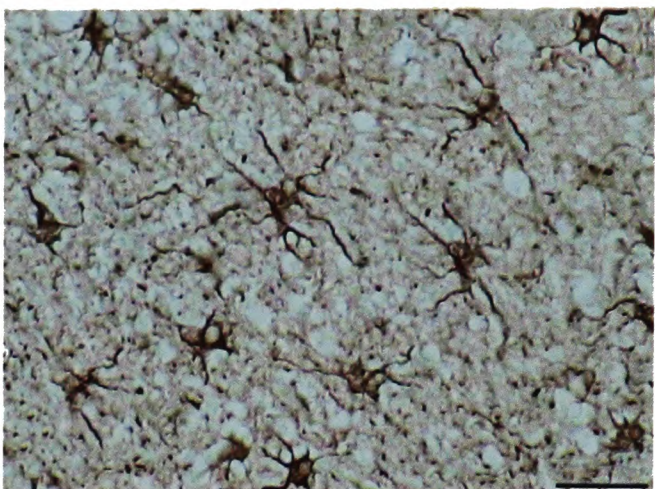
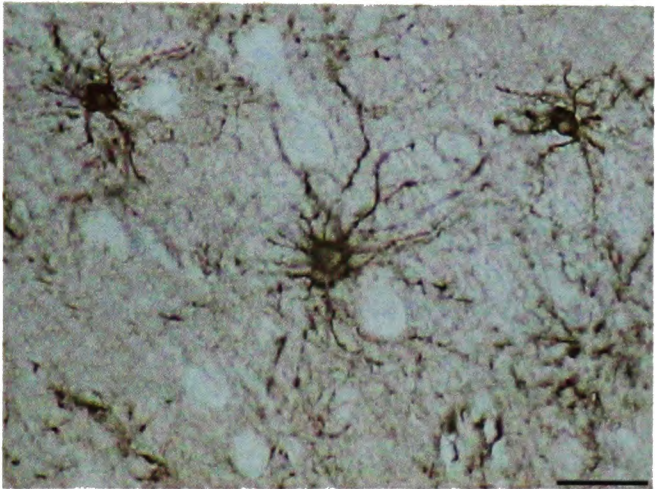
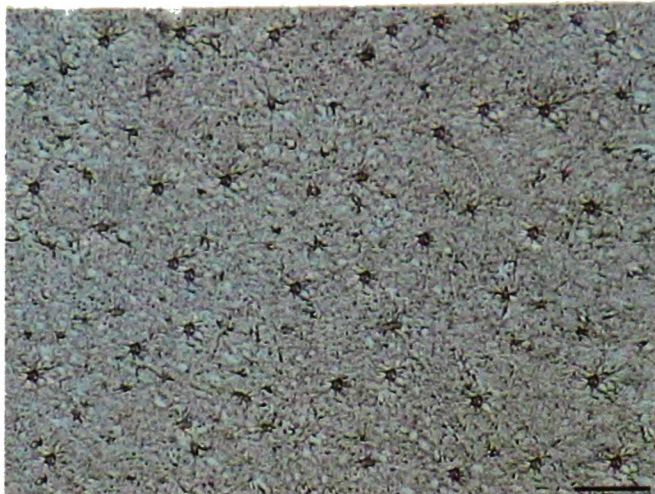
GFAP IAR signal is diffusely distributed throughout the grey matter. GFAP signal is consistently greater in white matter than in grey matter. The extent and intensity of GFAP signal in grey matter is highly variable between brains (as shown in panels A and B). A negative control omitting the primary antibody is also included (C).

GFAP immunoreactivity in anterior cingulate cortex

Grey matter



White matter



Autoradiograms of GFAP

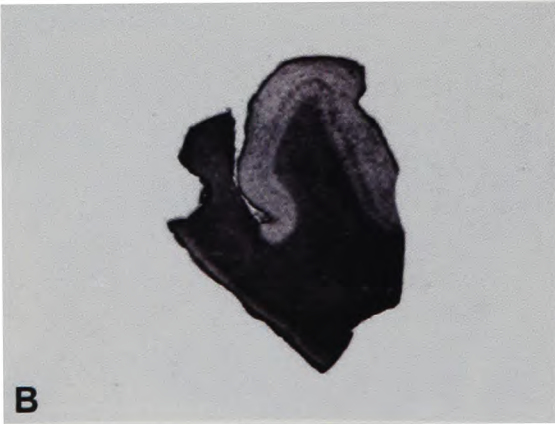
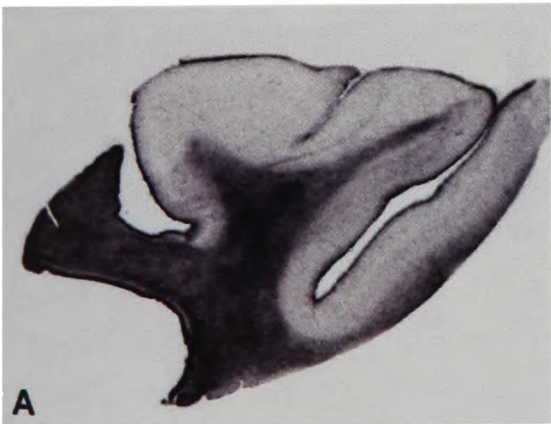


Table 5.5.

The effect of brain pH on GFAP protein expression in the ACC. Numbers are Pearson’s R values. ** p≤0.01

GFAP protein expression and pH			
	Control	Mood disorder	Combined
Grey matter	0.83**	0.57	0.72**
White matter	0.87**	0.64	0.78**

Table 5.6.

GFAP protein expression in the ACC in controls and mood disorder. ANOVA test used for comparison of GFAP between diagnostic groups. These data are illustrated in figure 5.4.

Region	GFAP protein expression, ³⁵ S nCi/g		Test for difference between			
	Mean (S.E.M)		patient group and control group ^a			
	Control	Mood disorder	F	p	Percentage difference	
Grey matter	1324.9 (123.0)	1021.2 (184.9)	1.21	.291	29.7%	
White matter	2445.8 (172.2)	1887.1 (177.4)	5.31	.037	29.6%	

^a ANCOVA with pH

Figure 5.4.

Scatterplots showing the relative abundance of GFAP in grey matter (GM) and white matter (WM) in the ACC, in controls and mood disorder. Bars represent the mean for each group. See table 5.6 for statistical comparisons between groups. * ≤ 0.05

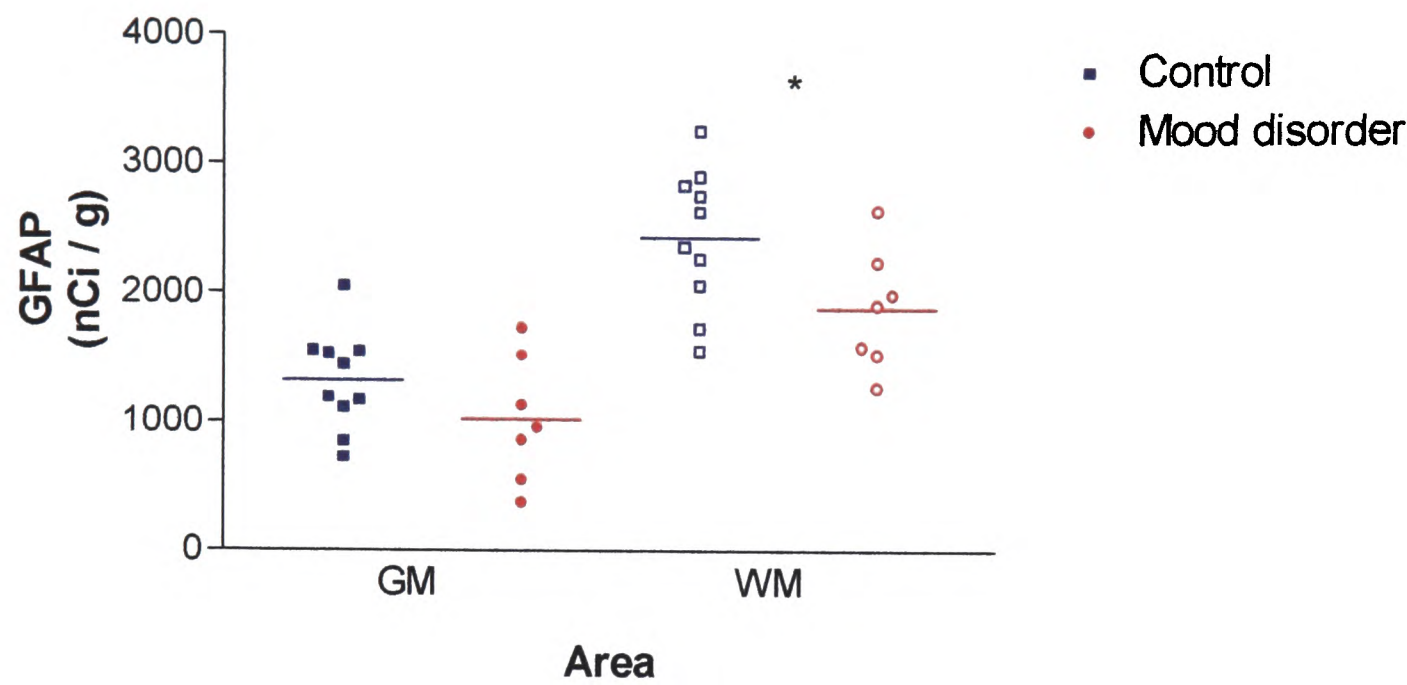


Figure 5.5.

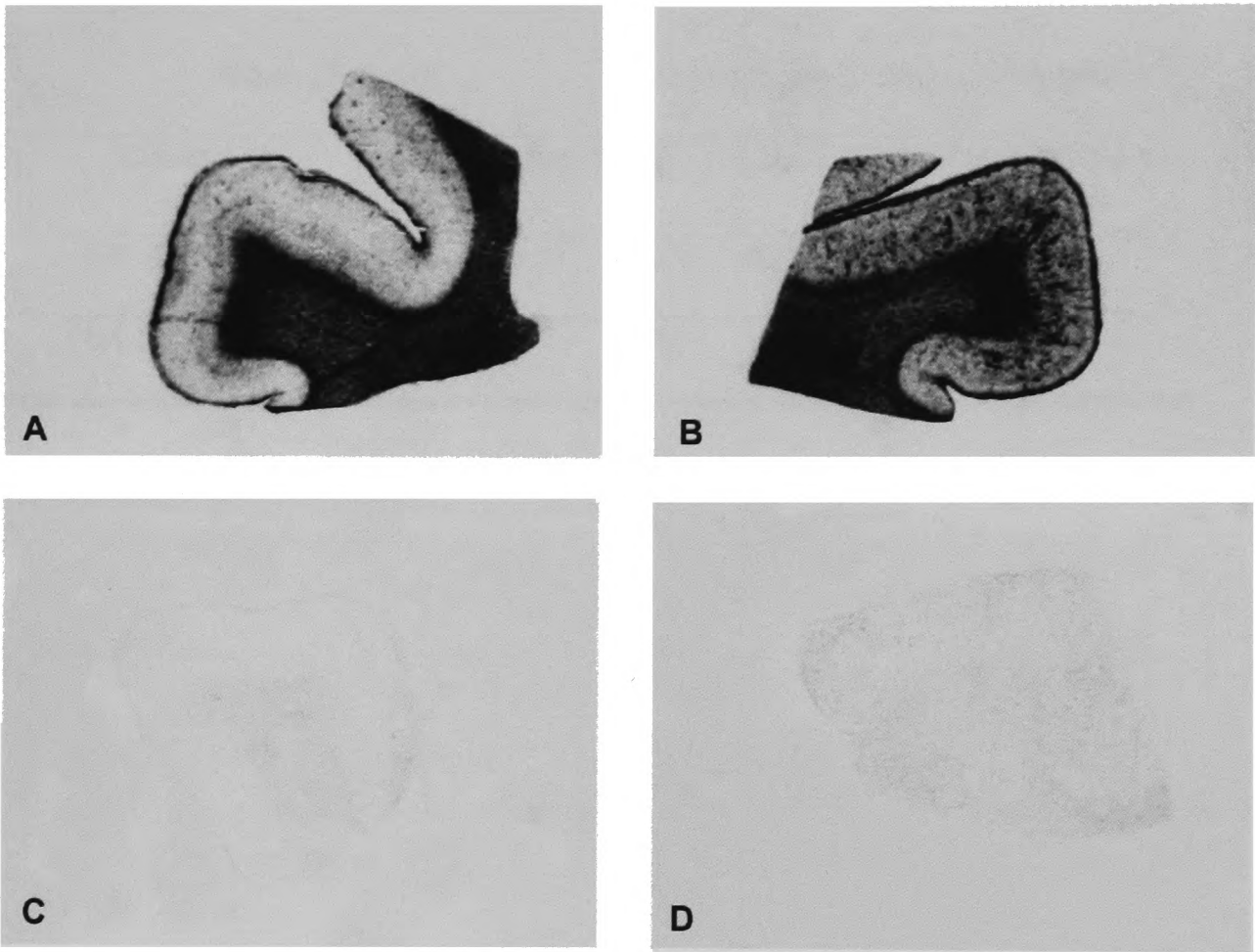
Autoradiograms showing GFAP mRNA expression in area 24.

GFAP mRNA signal is diffusely distributed throughout the grey matter. GFAP signal is consistently greater in white matter than in grey matter. The extent and intensity of GFAP mRNA signal in grey matter is highly variable between brains (as shown in panels A and B). Non-specific hybridisation signals are shown in C (excess cold probe) and D (sense strand hybridisation).

Northern blot of GFAP mRNA

Northern blots demonstrated that the oligonucleotide for GFAP detected a single band of the predicted molecular weight (2.7kb) in the hippocampus (HC) and ACC.

Autoradiograms of GFAP mRNA



Northern blot of GFAP mRNA

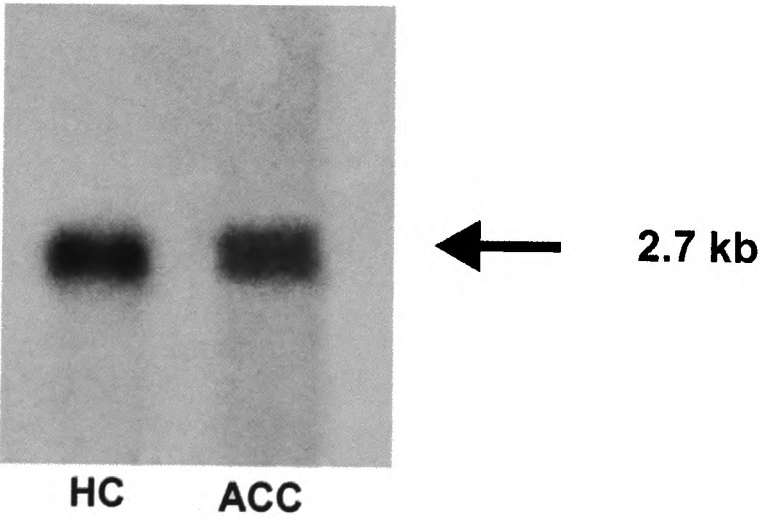


Table 5.7.

GFAP mRNA expression in the ACC in controls and mood disorder. ANOVA test used for comparison of GFAP mRNA between diagnostic groups. These data are illustrated in figure 5.6.

Region	GFAP mRNA expression, ³⁵ S nCi/g		Test for difference between			
	Mean (S.E.M)		patient group and control group			
	Control	Mood disorder	F	p	Percentage difference	
Grey matter	351.4 (51.9)	359.1 (46.1)	0.02	.913	2.1%	
White matter	964.3 (126.4)	958.9 (174.4)	0.01	.981	0.5%	

Figure 5.6.
Scatterplots showing the relative abundance of GFAP mRNA in grey matter (GM) and white matter (WM) in the ACC in controls and mood disorder. Bars represent the mean for each group. See table 5.7 for statistical comparisons between groups.

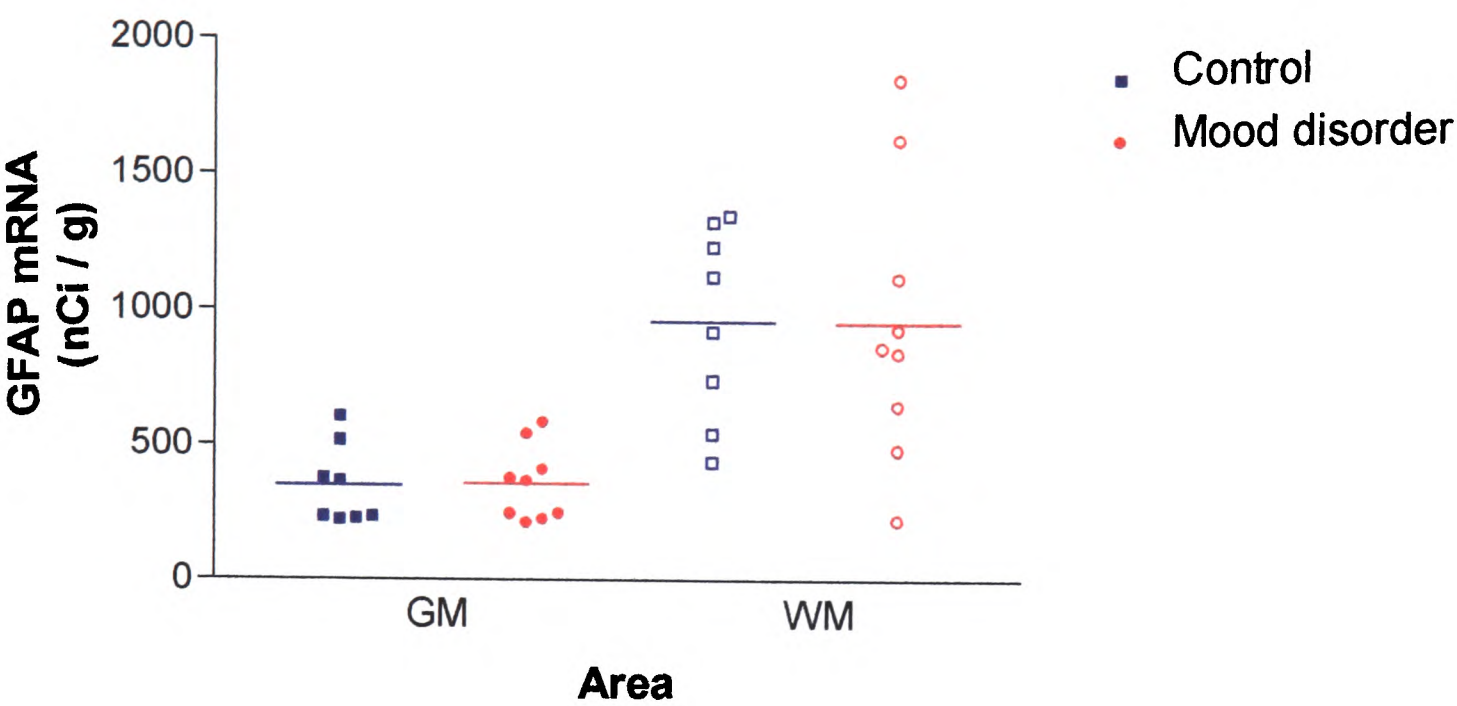
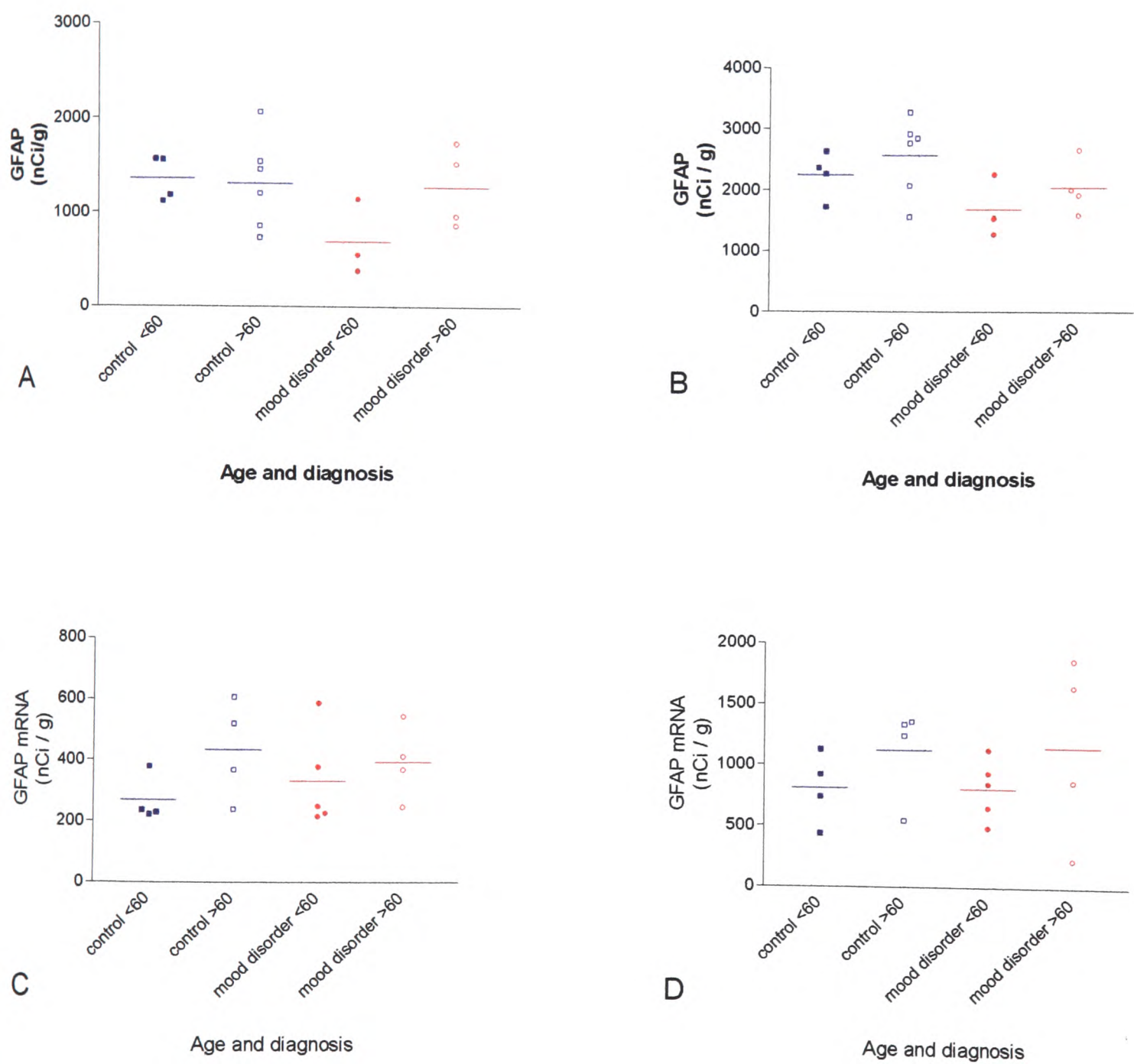


Figure 5.7.

Scatterplots illustrating the relationship between age and the relative abundance of (A) GFAP in grey matter, (B) GFAP in white matter, (C) GFAP mRNA in grey matter, and (D) GFAP mRNA in white matter in the ACC in controls and mood disorder. Bars represent the mean for each group.



5.4 Discussion

5.4.1 No alterations in HLA-DR-immunoreactive microglia in mood disorder

The distribution of HLA-DR immunoreactivity and the various cell morphologies (ramified and activated) observed in the grey and white matter are in accordance with a previous study of HLA-DR immunoreactivity in the human ACC (Radewicz *et al.* 2000). The association between microglial density and age has also been previously reported in the ACC (Radewicz *et al.* 2000).

No significant alterations were observed in the density of HLA-DR-immunoreactive microglia in grey or white matter in mood disorder. This result rather suggests that microglia are not contributing to the glial deficit observed in this brain series in mood disorder (see section 3.4.2). Moreover, as HLA-DR was employed as a marker of all microglial populations (ramified and activated), the data implies that microglia are not significantly up-regulated in mood disorder, which is consistent with the supposition that neurodegeneration (with accompanying microglial proliferation) is not a feature of mood disorder (Rajkowska *et al.* 1999). Additionally, the majority of microglial cells detected in this study exhibited a resting ramified morphology rather than an activated one, again indicating a lack neurodegenerative change.

This contrasts somewhat with the findings of Bayer *et al.* (1999), who reported microglial activation in mood disorder; however, their findings were purely qualitative and microgliosis was only reported in one of six subjects with mood disorder. Furthermore, they employed HLA-DR as a marker of activated microglia (hence concluding that specific staining was a sign of microgliosis). There is now a general consensus that microglia in the human brain constitutively express HLA-DR molecules (Gehrmann *et al.* 1993; Perlmutter *et al.* 1992; Radewicz *et al.* 2000), and recent publications have used HLA-DR as a marker of ramified and activated microglia (Radewicz *et al.* 2000; Thomas *et al.*

2002). The qualitative observations of the present study are certainly in agreement with this view. Cell morphologies consistent with both ramified and activated microglia were detected in control and mood disorder cases (see figure 5.1), though the ramified population of microglial cells was predominant. No attempt was made to assess each subpopulation separately since the objective of the current study was to obtain a 'total' microglial count. This would be an interesting focus for future work.

5.4.2 Reductions in GFAP protein in mood disorder

The distribution and variability of GFAP IAR signal observed in the current study is consistent with previous immunohistochemical reports of the ACC (Davis *et al.* 2002) and various prefrontal regions (Halliday *et al.* 1996; Miguel Hidalgo *et al.* 2000). The distribution of GFAP mRNA expression was, as expected, similar to the distribution of protein expression. GFAP expression was significantly reduced in the white matter of area 24 in mood disorder. An equivalent (but non-significant) reduction was also observed in the grey matter of area 24.

The findings of the current investigation provide partial support for the existing, albeit equivocal, studies of GFAP expression in mood disorder. In the one study of GFAP immunoreactivity in the ACC to date, Davis *et al.* (2002) failed to observe any significant alterations in GFAP protein expression in late-life depression, although as previously mentioned (section 5.1), it is unclear whether studies of late onset depression are relevant to those of early onset depression. The remaining literature pertains to the prefrontal cortex. Miguel-Hidalgo *et al.* (2000) conducted the first immunohistochemical study of GFAP based on their findings of decreased glial density in area 9 in mood disorder. Their results were largely inconclusive, and although a specific decrease was observed in the younger (30-45) major depression cases compared with a subgroup of age-matched controls, there was no evidence for reduced GFAP staining or GFAP-immunoreactive astrocyte density in the sample considered as a whole. In a further immunohistochemical

investigation, Webster *et al* (2001a) revealed a reduction in the labelling of astrocytes adjacent to blood vessels in the white matter of the gyrus in major depression, but not bipolar disorder. A non-specific immunoblotting technique for total GFAP in this same brain collection found significant reductions in the prefrontal cortex in major depression and bipolar disorder (Knable *et al*. 2001). A reduction in various GFAP isoforms in major depression and bipolar disorder has also been revealed using proteomic techniques (Johnston-Wilson *et al*. 2000).

The significant reduction of GFAP expression in the white matter found in the present study supports the findings of Webster *et al* (2001a), who reported a reduction only in those astrocytes adjacent to blood vessels in the white matter in major depression. Although the immunoblotting and proteomic studies were conducted on homogenates of prefrontal cortical samples, it is not possible to determine if the significant reductions in GFAP were produced solely by the inclusion of white matter material.

Questions regarding whether astrocytes in the grey and white matter are differentially and specifically altered in mood disorder cannot be answered without further experimental replication. The high variability of GFAP staining observed within cortical regions (see figure 5.3) may, to a certain extent, account for the lack of significant findings in the grey matter in mood disorder. With particular reference to the current study, it is possible that the heterogeneous staining observed within the grey matter introduced substantial noise, which may have obscured any 'statistically significant' alterations, even though a comparable reduction in GFAP protein was observed. Repeating this experiment in a larger brain series would help to clarify this issue.

5.4.3 No alteration in GFAP mRNA in mood disorder

The results of the present study, in concert with those detailed above, certainly suggest that the glial deficit in mood disorder is, at least in part, attributable to a decrease in the population of astrocytes. Unfortunately, the mRNA data of the current study is resoundingly insignificant and therefore does not support this hypothesis. If reductions in GFAP expression were secondary to a loss of astrocytes, one would anticipate a similar reduction in GFAP mRNA. The occurrence of decreased GFAP together with unchanged GFAP mRNA somewhat implies that GFAP content (and cell activation), rather than astrocyte number, is altered in mood disorder. Further experiments are required before this point can be made conclusively. Alternative reasons explaining the divergent protein and mRNA data are explained below.

5.4.3.1 *Discrepancy between GFAP mRNA and protein data*

The observed divergence between GFAP protein and mRNA data may be a methodological artefact, or a real feature of the disease. For example, ISHH and IAR methods may differ in their sensitivity to detect changes, particularly when multiple isoforms of GFAP mRNA (Galea *et al.* 1995) and GFAP protein (Inagaki *et al.* 1990) exist. With particular regard to the two isoforms of GFAP mRNA, the GFAP oligonucleotide used in the present study was purposefully designed within the coding region of human GFAP cDNA and should therefore detect both the alpha and beta isoforms of GFAP mRNA (Galea *et al.* 1995). Another important methodological consideration relates to the tissue used in both experiments. GFAP IAR was performed on fixed paraffin tissue sections whereas GFAP ISHH was performed on fixed frozen sections. Moreover, the cases used in each experiment were not identical since paraffin and frozen tissue was not available for each case (four mood disorder and six control cases were shared between the two experiments - see appendix one). This may limit the extent to which comparisons can be drawn between the two experiments.

Alternatively, there are many points along the pathway from mRNA to protein at which gene expression can be regulated, including translational control, post-translational modifications (phosphorylation, acetylation, glycosylation) and protein degradation (Inagaki *et al.* 1990). It is therefore conceivable that levels of GFAP protein will not necessarily mirror those of mRNA expression.

Although only conjecture, the data may also be indicative of a disease-related uncoupling of the relationship between levels of GFAP mRNA and that of the protein. It is feasible that disease-specific alterations in pathways controlling post-translational modification may lead to a reduction in GFAP protein, (or impair the recognition of the GFAP epitope(s) by immunohistochemical techniques), without affecting mRNA expression. This theory is partially supported by a recent proteomic investigation, which reported reductions in specific GFAP isoforms (most likely relating to various post-translational modifications) in major depression and bipolar disorder (Johnston-Wilson *et al.* 2000). It was not possible for me to directly assess the relationship between GFAP protein and mRNA expression in each diagnostic group due to the small number of control and mood disorder subjects shared between the two experiments and accordingly this theory remains entirely speculative.

5.4.4 Factors affecting GFAP expression

Significant positive correlations were observed between GFAP expression and pH. It is likely that this relationship reflects the relative ability to detect the GFAP epitope(s), rather than a change in astrocyte density per se, given that glial density (section 3.3.8) and GFAP mRNA expression were not affected by pH. Although difficult to explain, it is possible that progressively more acidic conditions result in a conformational change in the structure of the protein that conceals the epitope, thereby decreasing GFAP immunoreactivity.

GFAP did not show any significant correlations with age. This is surprising given that both GFAP protein and mRNA have been reported to increase with age in brain tissue from normal human subjects (David *et al.* 1997; Nichols *et al.* 1993), while GFAP immunoreactivity increases with age in subjects with major depression (Miguel Hidalgo *et al.* 2000). The reasons for such a discrepancy are unclear, but are likely to relate to technical differences, such as the specific antibody used in each study (see Halliday *et al.* 1996). In support of this theory, another group using the Z0334 GFAP antibody (see 5.2.3.2) also failed to observe any significant relationship between GFAP and age in the ACC (Davis *et al.* 2002). Alternatively, these differences may reflect the variability that has been observed in the GFAP content of human cerebral cortex between distinct brain regions and different people (David *et al.* 1997). Interestingly, inspection of figure 5.7, clearly illustrates that although the association between GFAP and age was not significant, GFAP protein and mRNA levels were increased in the older subgroup (>60) for each diagnostic group. Separate analyses were not performed between the younger and older subgroups due to the small number of cases involved.

5.4.5 Implications of reduced GFAP in mood disorder

As previously mentioned, a significant reduction in GFAP IAR signal in the white matter, along with a non-significant reduction in the grey matter in mood disorder may be either indicative of a reduction in astrocyte density, a reduction in astrocyte activity, or a reduction in GFAP content. If the former explanation were true (which is perhaps unlikely given the insignificant mRNA data), then astrocytes may partially account for the glial deficit observed in chapter three, though the potential involvement of oligodendrocytes (which could not be investigated in this study) should not be overlooked (Orlovskaya *et al.* 2000; Uranova *et al.* 2001; Uranova *et al.* 2002). The prospective implications and functional consequences of a glial deficit in mood disorder have been extensively discussed in section 3.4.4.

A reduction in GFAP content, whilst not explaining the glial deficit reported in chapter three, may nonetheless have considerable impact over normal cortical functioning. GFAP determines the complex astrocyte morphology that includes multiple processes that contact blood vessel walls, ensheath neuronal synapses and abut nodes of Ranvier. Attempts at pinpointing specific functions of GFAP have largely relied on inferences from changes in astrocyte properties or secondary effects on other cell types in GFAP-null mice (Messing and Brenner 2003). Such experiments have suggested that GFAP is necessary for the integrity of CNS white matter architecture and that its absence may lead to dysmyelination (Liedtke *et al.* 1996). Other researchers have indicated that the absence of GFAP may impair the ability of the astrocyte to induce or maintain the blood-brain barrier in endothelium - possibly explained by the alleged importance of GFAP in determining the capacity of astrocytes to form end-feet structures (Pekny *et al.* 1998). Importantly, changes in synaptic transmission have also been documented, including impairment of LTD in the cerebellum and enhanced LTP in the hippocampus (see Messing and Brenner 2003). Takemura *et al.* (2002) have demonstrated that filament formation and stability were altered in GFAP phosphorylation-deficient mice, and have projected that the phosphorylation of GFAP regulates the structural plasticity of glial filaments and ultimately astrocyte function. Whilst such experiments have helped to underscore the importance of GFAP in astrocyte function (and therefore cortical function), it is not yet clear whether these genetic manipulations in mice have any bearing upon the human situation, and therefore any inferences should be made with caution. Notwithstanding, it is quite apparent that GFAP plays a significant role in normal astrocyte function and therefore reductions in this protein could conceivably be involved in the neurobiology of mood disorder.

5.4.6 Interpretational considerations

Many of the limitations and considerations relevant to the current study (sample size, 2-D methodology, medication effects) are similar to the methodological discussions in chapter three and shall not be reiterated here.

The use of GFAP as a ubiquitous marker of astrocytes is a substantial issue requiring further discussion. Although the presence of GFAP has long been seen as the gold standard for defining astrocytes, some reservations have recently appeared with reports of GFAP negative astrocytes in the grey matter (Jabs *et al.* 1997; Walz and Lang 1998). Accordingly, it must be questioned whether the experiments in the present study were capable of detecting all astrocyte populations. Furthermore, there is increasing experimental evidence that suggests astrocytes are heterogeneous with respect to their antigen profile, receptor expression and electrophysiological properties (D'Ambrosio *et al.* 1998; Jabs *et al.* 1997; Matthias *et al.* 2003; Walz and Lang 1998). With particular regard to GFAP expression, questions remain as to whether this heterogeneity is a reflection of distinct astrocyte subtypes or whether the differences represent a spectrum of many possible levels of GFAP in a single cell type. The latter case would suggest a dynamic cell population that could alter its GFAP content in response to the surrounding environment (Walz 2000). Both explanations would have considerable impact upon neuropathological investigations, both in terms of immunohistochemical detection and succeeding interpretations. That distinct astrocyte subtypes exist and could be differentially involved in normal cortical functioning, and therefore its dysfunction, has to be considered a serious hypothesis.

Similar, but less complex issues surround the use of HLA-DR as a ubiquitous marker of microglia. Whilst I am confident that HLA-DR immunohistochemistry detected both ramified and activated microglia in the present study, I cannot be certain that this antibody detected both populations in their entirety. Indeed, despite the premise that microglia

constitutively express HLA-DR molecules (Gehrmann *et al.* 1993; Perlmutter *et al.* 1992), many researchers use this antibody as a marker of activated microglia only (Bayer *et al.* 1999; Overmyer *et al.* 1997), and a further study revealed that HLA-DR immunohistochemistry detected only a small fraction of the total microglial population (when compared to leukocyte common antigen, Tooyama *et al.* 1990). It is possible that studies reporting microglia as HLA-DR-negative may have used less sensitive immunostaining techniques; alternatively, the variability in HLA-DR immunostaining may be attributed to differences in tissue processing, (see Mattiace *et al.* 1990; Perlmutter *et al.* 1992; Radewicz *et al.* 2000). Conversely, it must be considered that some microglia cells are indeed HLA-DR negative. As mentioned in section 5.3.1, three control cases in this study were devoid of any HLA-DR immunoreactivity. This is likely to be explained by the difficulties associated with antigen detection in paraffin fixed post mortem tissue. Although I tried to optimise the antigen retrieval procedure, it may not have been sufficient for all cases. Problems similar to this are likely to add to the general confusion surrounding immunohistochemical detection of microglia. Indeed, if I had included the HLA-DR negative cases in my analyses, I would have reached the same conclusion as Bayer *et al.* (1999), namely, an increase in HLA-DR immunoreactivity in mood disorder.

Greater confidence in the detection of various glial populations will require a larger panel of well-characterised molecular probes, and the use of complementary methods such as double-labelling techniques. During the course of my work, immunostaining of a member of the human glucose transporter family (hGLUT5) has been validated as a useful method for the evaluation of resting and activated microglia in paraffin sections (Horikoshi *et al.* 2003). Given the contradicting reports regarding the specificity of HLA-DR; immunohistochemical techniques employing hGLUT5 may benefit future studies investigating the neuropathology of mood disorder.

Examination of the oligodendrocyte population in mood disorder should also be a priority area for future research. The present study found no evidence of microglia involvement in mood disorder, and only limited evidence of astrocyte involvement. It is therefore reasonable to suggest that oligodendrocytes may be contributing to the glial deficit reported in chapter three. Certainly, recent evidence from post mortem research demonstrating reductions in oligodendrocyte density in schizophrenia and bipolar disorder supports this premise (Hof *et al.* 2003; Orlovskaya *et al.* 2000; Uranova *et al.* 2001; Uranova *et al.* 2002). Concerning the issue of oligodendrocyte detection – novel work by Hof and colleagues (2003) suggests that a myelin associated enzyme, CNPase (2', 3'-cyclic nucleotide 3'-phosphodiesterase), may be a suitable marker of near-mature and mature oligodendrocytes in human post mortem tissue. This may provide a useful method for future studies investigating the oligodendrocyte population in mood disorder.

5.5 Summary

The density of HLA-DR-immunoreactive microglia was unaltered in mood disorder, indicating that microglia are not accounting for the glia deficit reported in chapter three. GFAP expression was significantly reduced in the white matter of area 24 in mood disorder. An equivalent (but non-significant) reduction was also observed in the grey matter of area 24. This suggests that the glial deficit may be partly attributable to the astrocyte population. In contrast, GFAP mRNA expression was unchanged in the mood disorder group; accordingly the potential implications of reduced GFAP content, rather than astrocyte number, are discussed in relation to the neuropathology of mood disorder.

Chapter Six: Synaptic protein expression in the anterior cingulate cortex in mood disorder

6.1 Introduction

Studies of synapses and dendrites complement investigations of the neuronal cell body. Direct visualisation of synapses is problematic in post mortem tissue and so most studies measure gene products localised to these neuronal compartments. This approach has become widely used in other disorders, such as Alzheimer's disease and schizophrenia (Eastwood *et al.* 1995; Eastwood *et al.* 2001; Masliah and Terry 1993). It is now being applied to mood disorders, in part to inform about possible alterations in synaptic connectivity, which are plausible given the nature of the morphometric data (Harrison 2002). The purpose of this study was to assess synaptic pathology in the ACC in mood disorder. Alterations in ACC synaptic circuitry might be predicted in consideration of the various cytoarchitectural alterations reported in this region in major depression and bipolar disorder. In particular, reductions in neuronal size might be suggestive of synaptic pathology, since neuronal size is a correlate of axonal arborisation (Esiri and Pearson 2000). Likewise, reductions in ACC metabolism (see Drevets 2000b) might also be indicative of synaptic perturbation as much of cerebral metabolism is devoted to synaptic activity (Raichle 1998).

Three synaptic proteins and their encoding mRNAs were measured: synaptophysin, complexin I and complexin II. These proteins are particularly useful as they provide information regarding somewhat different aspects of synaptic organisation.

Synaptophysin is a synaptic vesicle protein (Wiedenmann and Franke 1985) and is the most established and widely used synaptic protein for molecular neuropathological studies. Synaptophysin is present in virtually all synapses and its measurement has been shown to provide a reliable estimate of synaptic density in Alzheimer's disease and ageing

(Masliah and Terry 1993; Masliah *et al.* 1990; Masliah *et al.* 1989). Although the exact functions of synaptophysin remain elusive, it is thought to be involved in calcium-dependant exocytosis, synaptic plasticity and clathrin-independent endocytosis (Daly *et al.* 2000; Damer and Creutz 1994; Jahn and Sudhof 1994; Janz *et al.* 1999; Sudhof 1995).

Complexin (cx) I and II are small cytosolic proteins and are considered to be important constituents of the docking/fusion complex in pre-synaptic terminals (Ishizuka *et al.* 1995; Takahashi *et al.* 1995). Again, the molecular role of the complexins remains to be fully elucidated, however, regulatory roles in neurotransmitter release and SNAP receptor function have been suggested (McMahon *et al.* 1995; Ono *et al.* 1998). Their utility arises by virtue of the fact that cx I is expressed predominantly in axosomatic synapses, whereas cx II is accentuated in axospinal and axodendritic synapses (Takahashi *et al.* 1995; Yamada *et al.* 1999); which suggests that cx I and II might be involved in phenotypically different synapses. Supporting this theory, Eastwood and colleagues have shown that cx I is expressed by inhibitory more than excitatory neurons, and conversely, cx II is expressed by excitatory more than inhibitory neurons in various regions of the brain (Eastwood *et al.* 2000; Eastwood *et al.* 2001; Eastwood and Harrison 2000; Harrison and Eastwood 1998), including the ACC (Eastwood and Harrison 2001).

In view of the accumulating evidence supporting morphometric abnormalities in the ACC in mood disorder, it is perhaps surprising that only one study thus far has investigated synaptic pathology in area 24. Decrements in synaptophysin, GAP-43 and cx II in bipolar disorder, and cx II in major depression, have been reported in a Western blot study of the supragenual ACC (Eastwood and Harrison 2001). In the one synaptic study of the prefrontal cortex to date, Honer *et al.* (1999) reported that synaptophysin was not significantly altered in subjects with major depression.

In contrast, the hippocampus has been the focus of several synaptic investigations.

Overall, these studies are suggestive of alterations in the synaptic circuitry, especially in bipolar disorder. Measurements of synaptic density in the hippocampus are equivocal; SNAP-25 was increased in major depression, but decreased in bipolar disorder (Fatemi *et al.* 2001). Another study showed that synaptic density was unchanged in a cohort that included subjects with bipolar disorder and major depression (Muller *et al.* 2001). In a study of complexin mRNA, transcripts for cx I and II were reduced in various hippocampal subfields in bipolar disorder, but not major depression (Eastwood and Harrison 2000). Synaptophysin mRNA was also decreased in the hippocampus in mood disorder (mixed BPD and MDD subjects; Webster *et al.* 2001b).

6.1.1 Aims of the current study

The primary objective of this study was to assess the synaptic contribution to the cytoarchitectural alterations of the ACC in mood disorder. I decided to evaluate the expression of synaptophysin and cx I and II in mood disorder using quantitative immunohistochemistry and *in situ* hybridisation histochemistry. This approach permitted an evaluation of the 'total' synaptic contribution, as well as a differential assessment of excitatory and inhibitory pathways.

6.2 Methods

6.2.1 Tissue collection and preparation

The collection and preparation of frozen tissue used in this study has been previously described in section 5.2.1.2. The demographic details of the individuals included in the experiments are shown in table 6.1.

6.2.2 Immunoautoradiography

Pilot immunohistochemical experiments were performed to determine the optimal primary antibody concentration (data not shown).

Quantitative immunoautoradiography was carried out essentially as described in chapter five (see section 5.2.3.2). However, as frozen (rather than paraffin) sections were used in the present study, a slight modification of this protocol was necessary. The frozen sections were postfixed in a freshly prepared solution of 4% (w/v) paraformaldehyde (Sigma, Poole, UK) in PBS for 5 minutes. After fixation the sections were rinsed in PBS for 3x 5 minutes. The immunoautoradiography protocol outlined in chapter five was then followed. All antibody (primary and secondary) concentrations are provided in table 6.2. Omission of the primary antibody was used as an experimental control.

Synaptophysin IAR was carried out using a mouse monoclonal synaptophysin antibody (Dako, Cambridge, UK; SY38). This antibody has been extensively characterised (Eastwood and Harrison 2001; Masliah and Terry 1993; Masliah *et al.* 1990; Wiedenmann and Franke 1985). Complexin IAR was carried out using mouse monoclonal antibodies (cx I: SP33; cx II: LP27; gifts from S. Takahashi, Kochi Medical School, Japan). Both antibodies have been extensively characterised in mouse and rat brain tissue (Ono *et al.* 1998; Takahashi *et al.* 1995). The use of cx I and II as markers of inhibitory and excitatory circuits in human ACC tissue has been previously validated (Eastwood and Harrison 2001); however, it should be stated that this distinction is far from absolute, thus constraining data interpretation.

6.2.3 *In situ* hybridisation histochemistry

In situ hybridisation histochemistry was carried out essentially as described in chapter five (see section 5.2.4). Additional sections were incubated with sense-orientation probe to

determine (and correct for) non-specific signal in each experiment. Antisense probe sequences together with incubation and wash temperatures are given in table 6.3. The specificity of the synaptophysin (Eastwood *et al.* 1994) and complexin (Eastwood *et al.* 2001; Harrison and Eastwood 1998) probes has been previously demonstrated in human post mortem tissue by Northern blot analysis and a series of ISHH controls (sense-orientation probe, ribonuclease treatment and hybridisation in excess unlabelled probe).

6.2.3.1 *Emulsion dipping*

Following film autoradiography, the location of the target mRNA was determined by coating the slides with photographic emulsion (see appendix five). These sections were intended primarily for descriptive and illustrative purposes; accordingly, the relative abundance of the target mRNA within individual cell populations was not determined.

6.2.4 **Image analysis**

The film autoradiograms were analysed blind to diagnosis as outlined in section 5.2.6. Measurements were taken throughout the depth of the grey matter of area 24 and corrected for non-specific signal.

6.2.5 **Statistical analysis**

Statistical analyses were performed in an identical manner to those outlined in section 5.2.7.

Table 6.1.

Demographic details of control and mood disorder subjects included in the synaptic protein experiments. Values are mean $\pm$ S.E.M with maximum and minimum values in brackets. There are no significant differences between the two groups ($p>0.1$). ^a major depression (n=8), bipolar disorder (n=2). Diagnoses were made according to DSM-IV criteria. ^b All patients had been treated with antidepressant medication.

Variable	Control (n=9)	Mood disorder (n=10) ^{ab}
Age (years)	61.56 $\pm$ 6.20 (22–79)	62.20 $\pm$ 4.72 (43–86)
Sex (M:F)	4:5	6:4
pH	6.48 $\pm$ 0.07 (6.14–6.75)	6.35 $\pm$ 0.05 (6.06–6.53)
PMI (hours)	39.22 $\pm$ 8.77 (8–78)	35.30 $\pm$ 5.87 (13–75)
No. of suicides	0	6

Table 6.2.

Primary and secondary antibodies employed in IAR experiments.

Target protein	1° antibody and concentration	2° antibody and concentration
Synaptophysin	Mouse monoclonal, SY 38; 1:100	³⁵ S sheep anti-mouse; 0.2 µCi/ml
Complexin I	Mouse monoclonal, SP33; 1: 5000	³⁵ S sheep anti-mouse; 0.5 µCi/ml
Complexin II	Mouse monoclonal, LP27; 1:1000	³⁵ S sheep anti-mouse; 0.5 µCi/ml

Table 6.3

Oligonucleotide probes employed in ISHH experiments.

Target mRNA	Probe sequence (5'-3')	Incubation temperature	Wash temperature
Synaptophysin	TAG CCT TGC TGC CCA TAG TCG CCC TGA GGC CCG TAG CCA ^a	34°C	58°C
Complex I	TTC TTG ATG CCG TAC TTG TCT CGG ATG CCC TGG CGC ^b	35°C	60°C
Complexin II	GGC TGC TCC AGG GCT GCT TTC TCC TCT GCT TCC TTC ^b	35°C	60°C

^a from Eastwood *et al.*, 1984; ^b from Harrison and Eastwood 1998

6.3 Results

Negligible signal was seen with the sense strand probes (ISHH), or after omission of the primary antibody (IAR), demonstrating the specificity of detection of each gene product. The final 'n' of each group was slightly reduced (by one or two cases) for some of the analyses as a result of tissue damage during the ISHH and/or IAR protocol. Please refer to the individual graphs to ascertain the final number of cases included in each analysis.

6.3.1 Analysis of synaptophysin expression

6.3.1.1 *Synaptophysin protein expression*

Synaptophysin IAR signal was prominent over the grey matter, with minimal signal in the white matter (see figure 6.1).

Measurements of synaptophysin satisfied the Kolmogorov-Smirnov test for normal distribution. There were no significant correlations with age, pH, pmi or freezer storage time in the control group, the mood disorder group or both groups combined. The ANOVA did not reveal a significant effect of diagnosis ($p=0.185$) (see table 6.5 and figure 6.4).

6.3.1.2 *Synaptophysin mRNA expression*

Synaptophysin mRNA was abundant in the grey matter, with minimal signal in the white matter. Dipped sections confirmed that synaptophysin mRNA was expressed by all neuronal populations (see figure 6.1).

Measurements of synaptophysin mRNA satisfied the Kolmogorov-Smirnov test for normal distribution. Significant negative correlations were observed between synaptophysin mRNA and PMI in the mood disorder group, but not the control group, this correlation remained significant when both groups were combined (see table 6.4a). ANCOVA with PMI did not show a significant effect of diagnosis ($p=0.131$) (see table 6.6 and figure 6.4).

6.3.2 Analysis of complexin I expression

6.3.2.1 *Complexin I protein expression*

Complexin I IAR signal was prominent over the grey matter. Some signal was detected in the white matter (see figure 6.2).

Measurements of cx I satisfied the Kolmogorov-Smirnov test for normal distribution. There were no significant correlations with age, pH, pmi or freezer storage time in the control group, the mood disorder group or both groups combined. The ANOVA did not reveal a significant effect of diagnosis ($p=0.535$) (see table 6.5 and figure 6.4).

6.3.2.2 *Complexin I mRNA expression*

Complexin I mRNA was abundant in the grey matter, with minimal signal in the white matter. The dipped sections confirmed that cx I mRNA was expressed primarily by inhibitory neurons (non-pyramidal neurons). Occasional pyramidal neurons were weakly labelled for cx I mRNA (see figure 6.2).

Measurements of cx I mRNA satisfied the Kolmogorov-Smirnov test for normal distribution. Significant positive correlations were observed between cx I mRNA and PMI in the mood disorder group, but not the control group, this correlation remained significant when both groups were combined. Further significant positive correlations were observed between cx I mRNA and pH in the control group, but not the mood disorder group; this correlation also remained when both groups were combined (see table 6.4b). PMI and pH were included as covariates in the succeeding analysis. The ANCOVA failed to show a significant effect of diagnosis ($p=0.118$) (see table 6.6 and figure 6.4).

6.3.3 Analysis of complexin II expression

6.3.3.1 *Complexin II protein expression*

Complexin II IAR signal was prominent over the grey matter. Some signal was detected in the white matter (see figure 6.3).

Measurements of cx II satisfied the Kolmogorov-Smirnov test for normal distribution.

Significant positive correlations were observed between cx II and PMI in the mood disorder group, but not the control group; the correlation remained significant when both groups were combined (see table 6.4c). ANCOVA with PMI did not reveal a significant effect of diagnosis ($p=0.252$) (see table 6.5 and figure 6.4).

6.3.3.2 *Complexin II mRNA expression*

Complexin II mRNA was abundant in the grey matter, with minimal signal in the white matter. The dipped sections confirmed that cx II mRNA was expressed abundantly by excitatory neurons (pyramidal neurons). Occasional non-pyramidal neurons were weakly labelled for cx II mRNA (see figure 6.3).

Measurements of cx II mRNA satisfied the Kolmogorov-Smirnov test for normal distribution. Significant positive correlations were observed between cx II mRNA and age in the control group, the mood disorder group and in both groups combined (see table 6.4d). Age was included as a covariate in the following ANCOVA, which failed to show a significant effect of diagnosis ($p=0.219$) (see table 6.6 and figure 6.4).

Figure 6.1.

Synaptophysin protein expression in area 24.

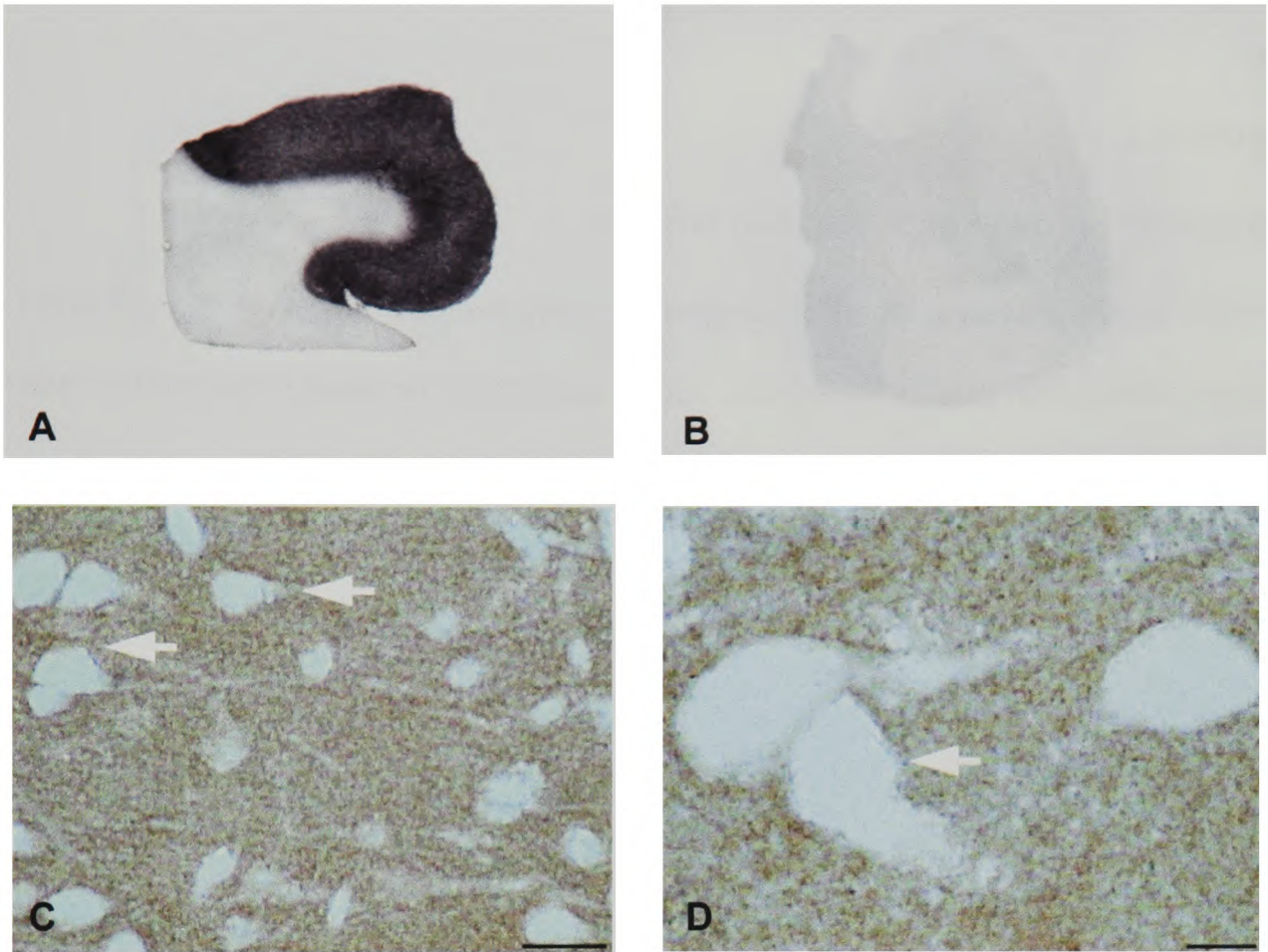
Panel A shows an example autoradiogram of synaptophysin expression. Synaptophysin shows a fairly uniform distribution across the cortex, with minimal reactivity in the white matter. A negative control omitting the primary antibody is also included (B). Photomicrographs illustrating synaptophysin immunoreactivity are shown in panels C and D. It can be seen that neurons are free of labelling (white arrows) in contrast to the punctate staining in the neuropil. Scale bar for photomicrograph in panel C=30µm, and panel D=10µm.

Synaptophysin mRNA expression in area 24.

Panel E shows an example autoradiogram of synaptophysin mRNA expression. Again, signal is abundant in the grey matter, with minimal signal over the white matter. Although synaptophysin mRNA distribution is reasonably uniform – a dense band of signal is often detected over layer V. Non-specific signal from sense strand hybridisation is shown in F.

Emulsion dipped ISHH sections showing synaptophysin mRNA in layers III and V of area 24 are shown in panels G and H, respectively. Labelling is observed over pyramidal and non-pyramidal neurons (black arrows). Glial cells are not labelled. Non-specific hybridisation in layer V of area 24 is shown in I. Scale bar for photomicrographs in panels G, H and I=10µm.

Synaptophysin protein expression



Synaptophysin mRNA expression

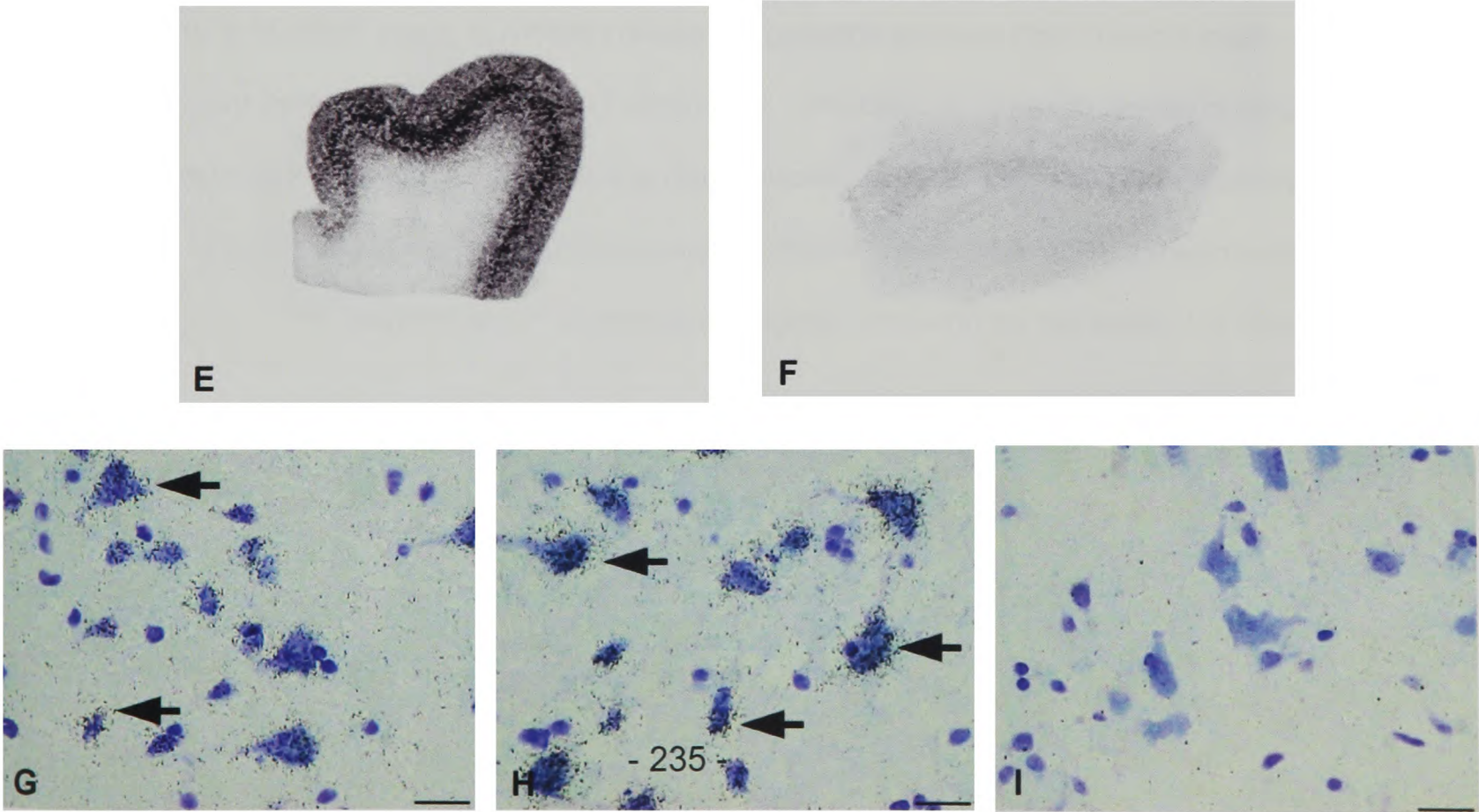


Figure 6.2.

Complexin I protein expression in area 24.

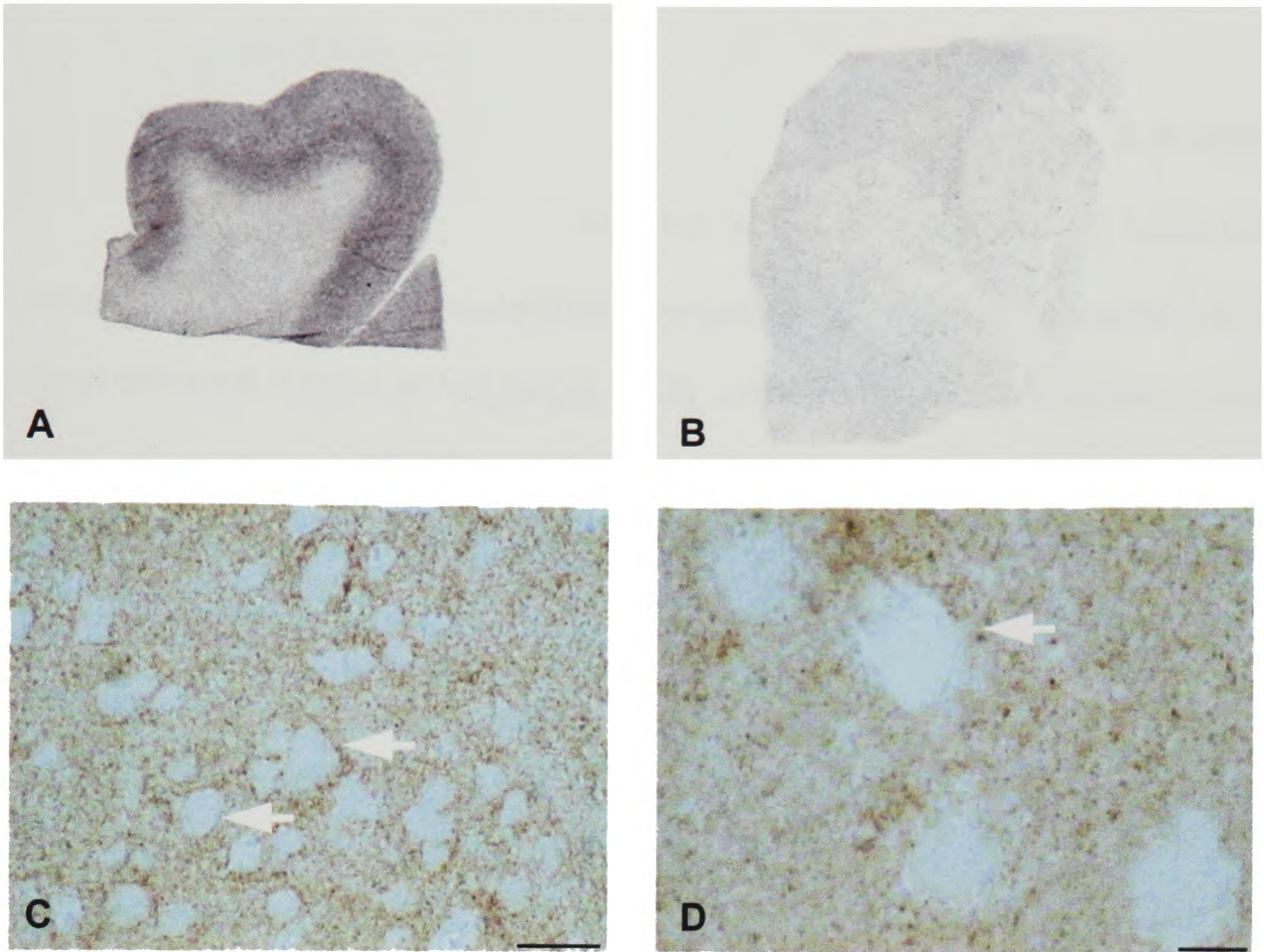
Panel A shows an example autoradiogram of complexin I expression. Complexin I shows a fairly uniform distribution across the cortex. Some signal can be seen in the white matter. A negative control omitting the primary antibody is also included (B). Photomicrographs illustrating complexin I immunoreactivity are shown in panels C and D. It can be seen that neurons are free of labelling (white arrows) in contrast to the punctate staining in the neuropil. Scale bar for photomicrograph in panel C=30µm, and panel D=10µm.

Complexin I mRNA expression in area 24.

Panel E shows an example autoradiogram of complexin I mRNA expression. Signal is abundant in the grey matter, with minimal signal over the white matter. Non-specific signal from sense strand hybridisation is shown in F.

Emulsion dipped ISHH sections showing complexin I mRNA in layers II and III of area 24 are shown in panels G and H, respectively. Complexin I labelling is concentrated over inhibitory non-pyramidal neurons (black arrows). Weak labelling is sometimes observed over pyramidal neurons (arrow heads). Glial cells are not labelled. Non-specific hybridisation in layer III of area 24 is shown in I. Scale bar for photomicrographs in panels G, H and I=10µm.

Complexin I protein expression



Complexin I mRNA expression

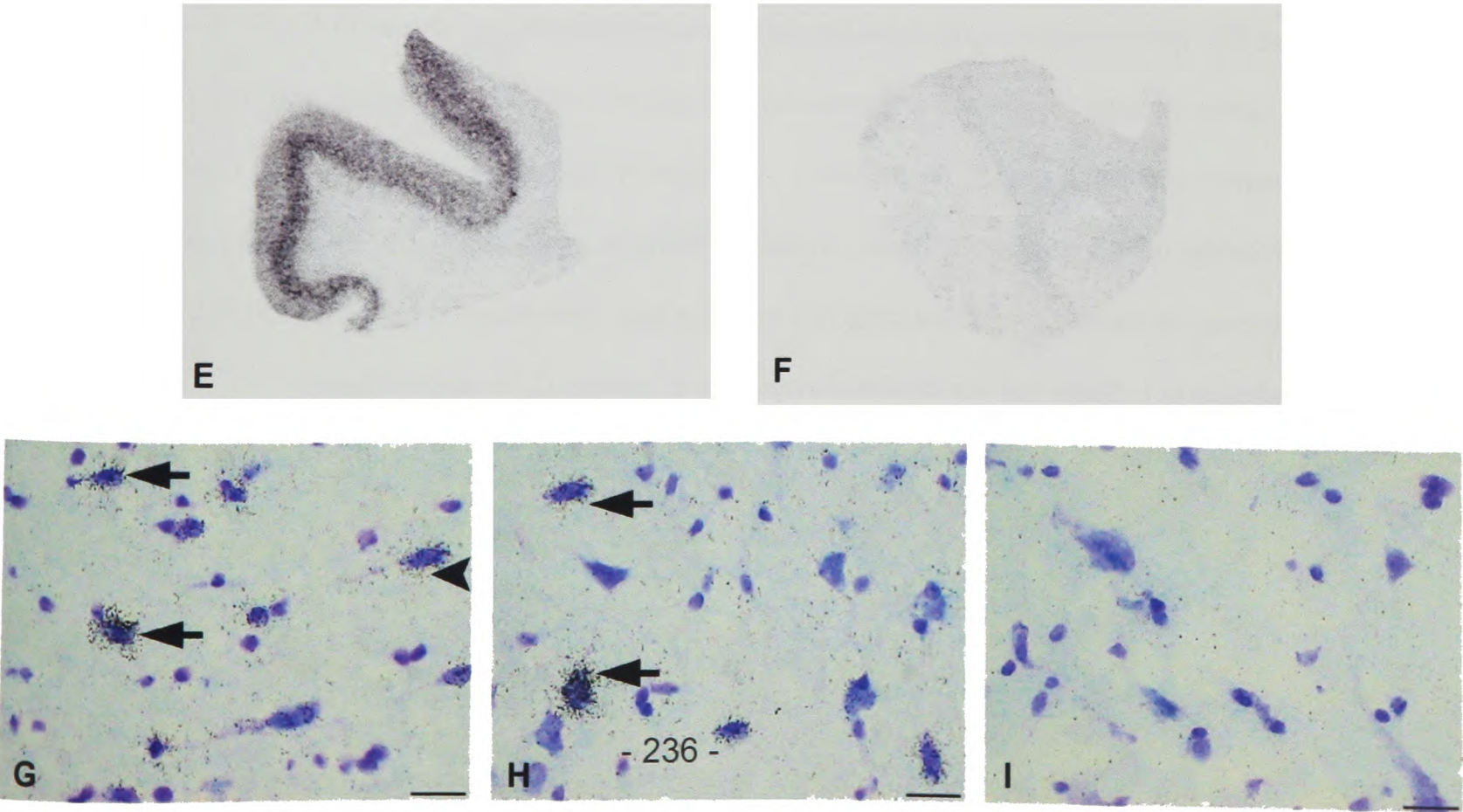


Figure 6.3.

Complexin II protein expression in area 24.

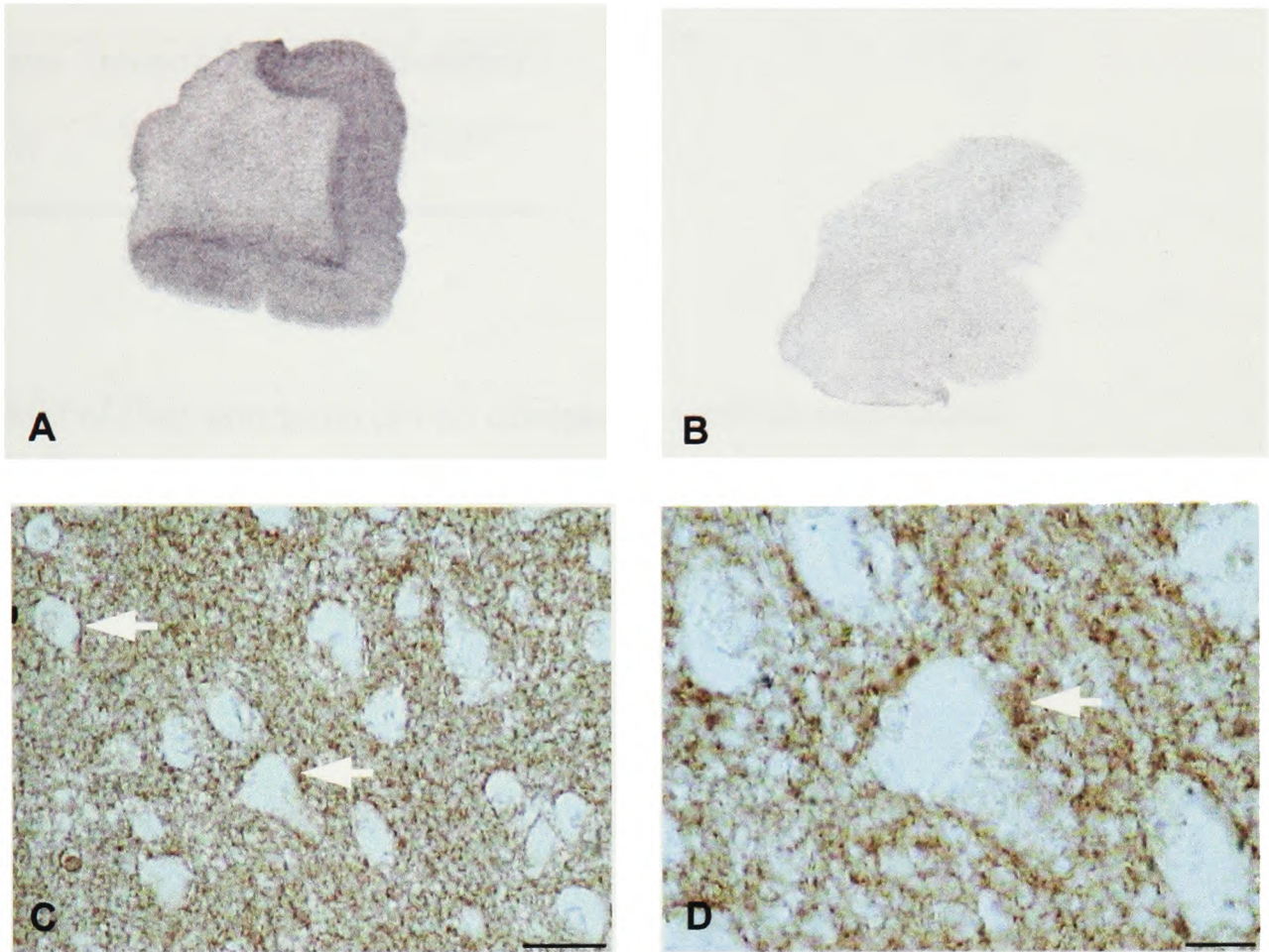
Panel A shows an example autoradiogram of complexin II expression. Complexin I shows a fairly uniform distribution across the cortex. Some signal can be seen in the white matter. A negative control omitting the primary antibody is also included (B). Photomicrographs illustrating complexin II immunoreactivity are shown in panels C and D. It can be seen that neurons are free of labelling (white arrows) in contrast to the punctate staining in the neuropil. Scale bar for photomicrograph in panel C=30µm, and panel D=10µm.

Complexin II mRNA expression in area 24.

Panel E shows an example autoradiogram of complexin II mRNA expression. Signal is abundant in the grey matter, with minimal signal over the white matter. Complexin II mRNA distribution is fairly uniform, although a dense band of signal can sometimes be observed in the deeper cortical layers (see E). Non-specific signal from sense strand hybridisation is shown in F.

Emulsion dipped ISHH sections showing complexin II mRNA in layers III and V of area 24 are shown in panels G and H, respectively. Complexin II labelling is concentrated over excitatory pyramidal neurons (black arrows). Weak labelling is sometimes observed over non-pyramidal neurons (arrow heads). Glial cells are not labelled. Non-specific hybridisation in layer V of area 24 is shown in I. Scale bar for photomicrographs in panels G, H and I=10µm.

Complexin II protein expression



Complexin II mRNA expression

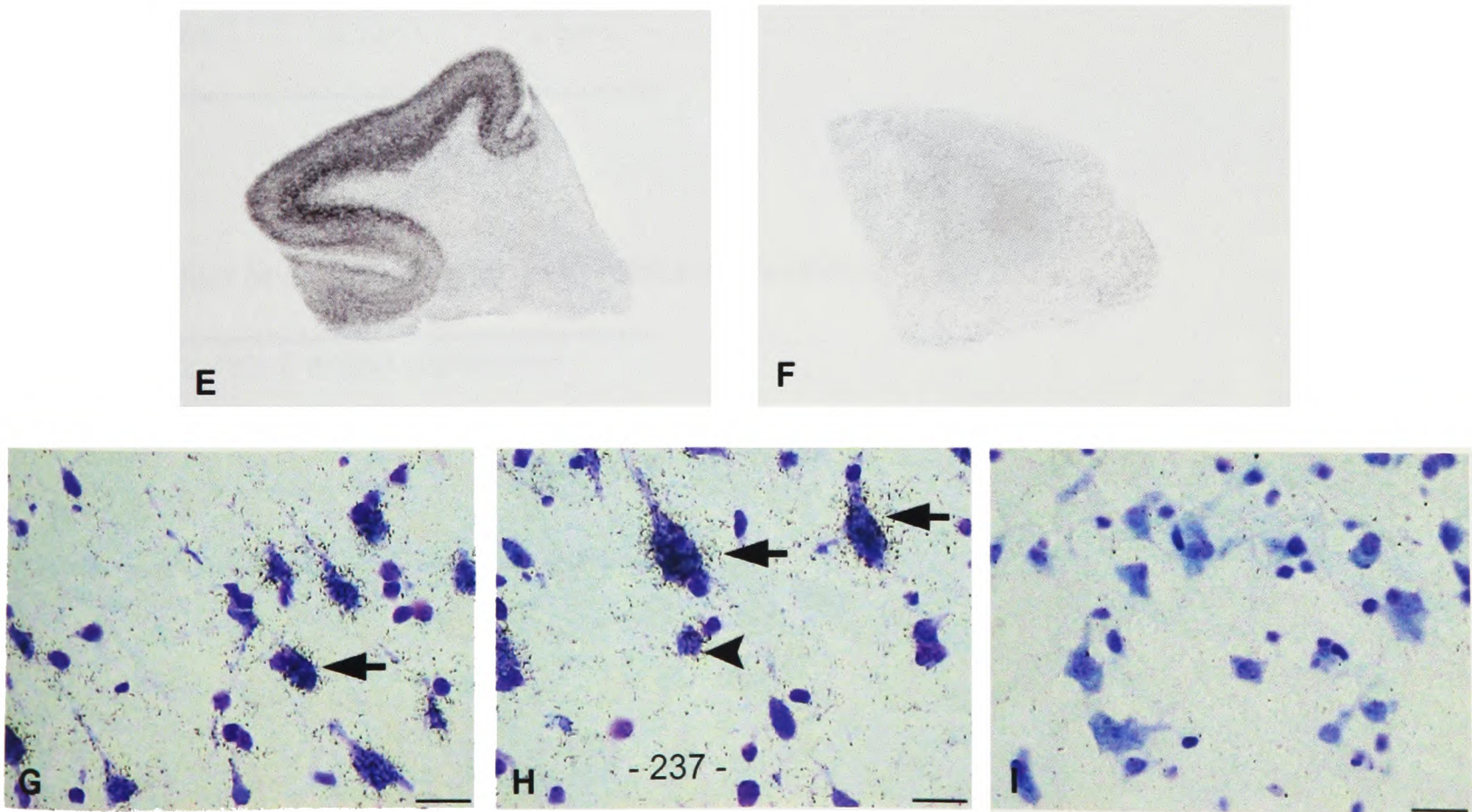


Table 6.4.

Pearson’s correlations. All numbers are Pearson’s R values. * $p \leq 0.05$; ** $p \leq 0.01$

a) The effect of PMI on synaptophysin mRNA expression.

Synaptophysin mRNA expression			
	Control	Mood disorder	Combined
PMI	-0.19	-0.80**	-0.57*

b) The effect of PMI and brain pH on complexin I mRNA expression.

Complexin I mRNA expression			
	Control	Mood disorder	Combined
PMI	-0.50	-0.75*	-0.62**
pH	0.85**	0.61	0.65**

c) The effect of PMI on complexin II expression.

Complexin II protein expression			
	Control	Mood disorder	Combined
PMI	0.63	0.74*	0.66**

d) The effect of age on complexin II mRNA expression.

Complexin II mRNA expression			
	Control	Mood disorder	Combined
age	0.87**	0.71*	0.76**

Table 6.5.

Synaptic protein expression in the ACC in controls and mood disorder. ANOVA test used for comparison of synaptic protein expression between diagnostic groups. These data are illustrated in figure 6.4 (A), (C), (E).

	Synaptic protein expression, ³⁵ S nCi/g		Test for difference between		
	Mean (S.E.M)		patient group and control group		
	Control	Mood disorder	F	p	Percentage difference
Synaptophysin	708.8 (18.3)	669.43 (21.4)	1.91	.185	5.8%
Complexin I	749.9 (11.8)	725.9 (30.1)	0.40	.535	3.3%
Complexin II ^a	218.6 (16.9)	186.6 (17.8)	1.44	.252	17.2%

^a ANCOVA with PMI

Table 6.6.

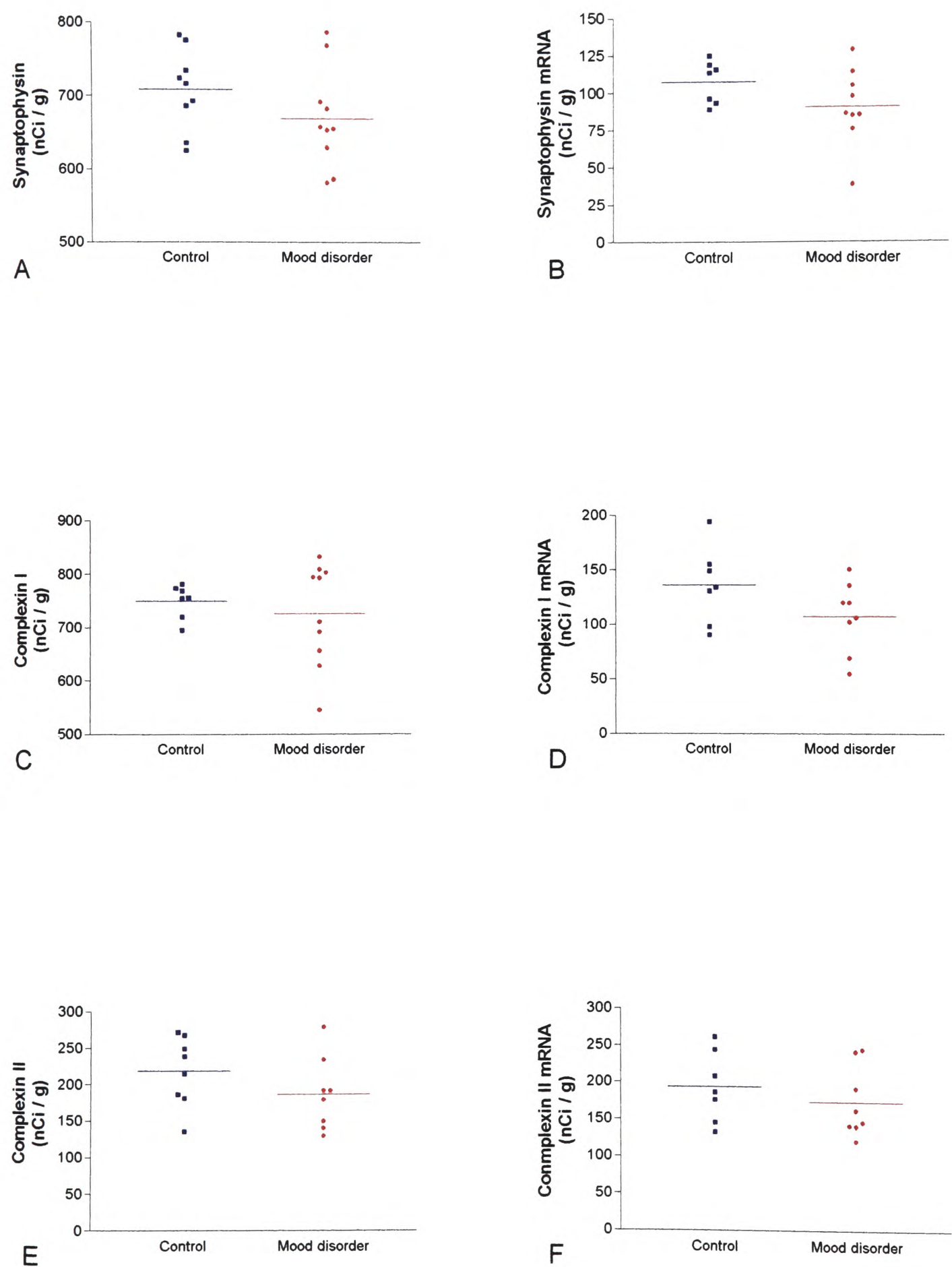
Synaptic protein mRNA expression in the ACC in controls and mood disorder. ANOVA test used for comparison of synaptic protein mRNA expression between diagnostic groups. These data are illustrated in figure 6.4 (B), (D), (F).

	Synaptic protein mRNA expression, ³⁵ S nCi/g		Test for difference between		
	Mean (S.E.M)		patient group and control group		
	Control	Mood disorder	F	p	Percentage difference
Synaptophysin ^a	107.4 (5.4)	90.2 (8.5)	2.59	.131	19.1%
Complexin I ^b	136.1 (13.3)	106.9 (11.4)	1.83	.204	27.3%
Complexin II ^c	193.8 (18.1)	173.4 (16.8)	1.68	.219	11.8%

^a ANCOVA with PMI; ^b ANCOVA with PMI and pH; ^c ANCOVA with age

Figure 6.4.

Scatterplots showing the relative abundance of (A) synaptophysin, (B) synaptophysin mRNA, (C) complexin I, (D) complexin I mRNA, (E) complexin II, and (F) complexin II mRNA in the ACC in controls and mood disorder. Bars represent the mean for each group. See tables 6.5 and 6.6 for statistical comparisons between groups.



6.4 Discussion

6.4.1 No evidence for synaptic alterations in the ACC in mood disorder

Synaptophysin, cx I and cx II and their encoding mRNAs were not significantly changed in the ACC in mood disorder. These data suggest that ACC synaptic circuitry is not altered in mood disorder. However, it is noteworthy that modest (albeit non-significant) reductions in synaptophysin mRNA, cx I mRNA and cx II were observed in the mood disorder group (see tables 6.5 and 6.6). The lack of significance may possibly reflect a type II (false-negative) error given the small sample size and the spread of data. Replication of these experiments in a larger and well characterised brain series would help to clarify this issue.

The findings of the present study are at variance with a recent investigation of synaptic protein expression in the ACC. Eastwood and Harrison (2001) reported reductions in synaptophysin, GAP-43 and cx II expression in bipolar disorder, and a significant reduction in cx II expression in major depression. However, important disparities exist between these investigations; firstly, different techniques were employed (Western blotting versus IAR and ISHH), secondly, the sample size was considerably smaller in the current study, and thirdly, the mood disorder cases used in the present study comprised a mixed group of major depression and bipolar disorder subjects. The issue of diagnosis is particularly relevant given that the majority of subjects in the current study were diagnosed with major depression (8 out of 10). In this respect, the only real discrepancy between the two studies involved cx II expression in major depression. Although I was unable to replicate the findings of Eastwood and Harrison (2001), it is noteworthy that a 17% reduction in cx II was observed in mood disorder in the present study. Further evidence for synaptic perturbations in the ACC in mood disorder comes from a recent study investigating the expression of dendritic microtubule-associated proteins (MAP). Bouras *et al* (2001) reported reductions in MAP1b and MAP2 in bipolar disorder, but not major

depression. Changes in the expression of MAP proteins may be suggestive of abnormal afferent synaptic innervation or activity.

As mentioned in the introduction, the majority of the synaptic protein literature to date has focused on the hippocampus. Rosoklija *et al* (2001) found decreased arborisation of apical dendrites, and a reduced density of dendritic spines on subicular pyramidal neurons in six mood disorder subjects (mainly bipolar disorder). These findings are consistent with the reduced expression of synaptic protein genes in the hippocampus, especially in the subiculum, in bipolar disorder (Eastwood and Harrison 2000). The studies of hippocampal synaptic pathology in major depression (or mixed 'mood disorder' groups) are more ambiguous, with reports of increased (Fatemi *et al.* 2001), decreased (Fatemi *et al.* 2001; Webster *et al.* 2001b) and unaltered (Eastwood and Harrison 2000; Muller *et al.* 2001) synaptic protein expression.

Overall, the various synaptic studies to date suggest that synaptic perturbations may be more prominent in bipolar disorder than major depression. Whether such differences are merely a matter of degree, or are the hint of a diagnostically specific pattern of alterations in bipolar disorder versus major depression remains to be determined. Furthermore, the majority of the existing synaptic protein literature relates to the hippocampus – where very few morphometric changes have been reported in mood disorder (see Harrison 2002). In contrast, the regions most consistently demonstrating significant morphometric alterations in mood disorder (prefrontal and limbic areas) have been relatively neglected. This apparent contradiction emphasises the need to expand this area of research and in particular to concentrate on the synaptic contribution to the cytoarchitectural alterations observed in the ACC, amygdala and prefrontal brain regions.

6.4.1.1 *Factors affecting synaptic protein expression*

In agreement with Harrison *et al* (1995), the various synaptic epitopes investigated in this study proved to be more resistant than their encoding mRNA transcripts to the deleterious effects of pH and PMI. Corroborating previous work, I found significant negative correlations between synaptophysin mRNA and PMI (Eastwood *et al.* 1995; Eastwood *et al.* 1994; Webster *et al.* 2001b). The negative correlation observed between PMI and cx I mRNA has also been previously recorded (Harrison and Eastwood 1998). Significant correlations were also observed between cx I mRNA and pH, again supporting previous studies which have indicated that a lower brain pH can lead to a decrease in the quality and quantity of cx mRNA in post mortem brain tissue (Eastwood and Harrison 2000; Harrison and Eastwood 1998). The protein expression of cx II was also affected by PMI, and is consistent with an earlier report of cx II protein in the ACC (Eastwood and Harrison 2001). Complexin II mRNA was affected by age, with inspection of the data showing that this reflected a tendency for the mRNA to increase with age in the ACC. The relationship between cx mRNA and age has also been documented formerly (Eastwood and Harrison 2000; Harrison and Eastwood 1998). Considered together, these results indicate yet again the importance of performing demographic correlations and demonstrate that both related and unrelated mRNAs can be differentially sensitive to the effects of PMI and pH (Hynd *et al.* 2003). Importantly, Eastwood and Harrison (2001) did not perform covariate analyses (as non-parametric statistical analyses were used), highlighting another difference between this study and the present work.

6.4.2 **Methodological considerations and limitations of the current study**

Many of the methodological considerations pertinent to this chapter have been addressed in earlier chapters and will not be restated here.

The techniques employed in this study (ISHH and IAR) were specifically selected to provide a quantitative measure of synaptic protein expression. While these methods

afford greater anatomical resolution than homogenate based methods they are still not ideal. In particular, given the layer-specific patterns of glial and neuronal alterations reported in the ACC in mood disorder (see chapter three), it is quite feasible that a 'total' grey matter measurement was neither adequate nor suitable to detect subtle differences in synaptic protein expression. Although not used for quantitative purposes in the present study, the emulsion dipped ISHH sections would provide a suitable medium for more detailed analyses. Furthermore, dipped sections would allow the relative abundance of mRNA within individual cells to be measured. As reductions in neuronal density and neuronal size have been reported in the mood disorder it will be important to ascertain whether changes in mRNA expression are merely reflecting alterations in neuronal density rather than alterations in synaptic circuitry. Any replication of this work should ideally assess grain density over individual neuronal populations.

Once again the potential effect of medication needs to be considered. Although I did not have sufficient clinical information to explore clinico-pathological correlations, previous studies (with adequate clinical data) have indicated that antidepressants, antipsychotics and mood stabilisers did not affect synaptophysin or complexin expression in the ACC (Eastwood and Harrison 2001) or the hippocampus (Eastwood and Harrison 2000).

Experimental animal studies have revealed that the effects of antipsychotic medication on synaptophysin and complexin expression are primarily restricted to the basal ganglia (Eastwood *et al.* 2000; Eastwood *et al.* 1994); equivalent studies assessing the neuropathological effects of antidepressant and mood stabilising medication have not yet been performed.

Taken together, these data would suggest that the results of the present study are unlikely to be confounded by medication; notwithstanding, considerable caveats remain to be addressed. Chronic ECS alters synaptic efficacy as evidenced by the development of LTP in the dentate gyrus (Stewart *et al.* 1994). Increases in synaptic markers have also been

recorded in rat forebrain and occipital cortex after ECS treatment (Jorgensen and Bolwig 1979).

Recent studies have demonstrated that chronic ECS induces sprouting and synaptic reorganisation of the mossy fibre pathway (Vaidya *et al.* 1999) and promotes the sprouting of serotonergic axons (Madhav *et al.* 2000) in the rat hippocampus. Changes in the expression of hippocampal MAP2 have also been observed after ECS treatment in rats (Pei *et al.* 1998). Furthermore, Norrholm and colleagues reported that antidepressants alter dendritic spine density in the hippocampus (Norrholm and Ouimet 2000) and have subsequently demonstrated that antidepressant treatment prevents the loss of dendritic spines associated with some animal models of depression (Norrholm and Ouimet 2001).

Interestingly, brain derived neurotrophic factor (BDNF) has been proposed to play a role in the antidepressant and ECS-induced sprouting and remodelling (Duman 1998; Vaidya *et al.* 1999). This theory stems from recent studies, which have demonstrated that both ECS and the chronic administration of a variety of antidepressants increase the expression of cAMP regulatory element binding protein (CREB, which regulates BDNF gene expression), BDNF and its receptor, trkB, in certain populations of neurons in the hippocampus (Nibuya *et al.* 1995; Nibuya *et al.* 1996; Thome *et al.* 2000). Similar effects of antidepressant treatment (increases in CREB and BDNF) have been recently described in post mortem samples from subjects with mood disorder (Chen *et al.* 2001; Dowlatsahi *et al.* 1998).

Changes in neuropil structure are not limited to ECT or antidepressant treatment. Recent studies have reported increases NAA signal (Moore *et al.* 2000a) and grey matter volume (Moore *et al.* 2000b) after lithium treatment, suggesting that lithium has neurotrophic properties (Manji *et al.* 2000a). Since NAA is believed to be located primarily in dendrites, it has been predicted that the increase in signal is likely to reflect an expansion of the

neuropil (Manji *et al.* 2000a) – thus implicating mood stabilising medication in synaptic reorganisation.

These studies clearly demonstrate the potential capacity of antidepressants, lithium and ECT to produce changes in synaptic organisation, though considerable caution is needed in the extrapolation of these results to the treatment of humans. Although these studies provide experimental evidence of synaptic remodelling following ECS and antidepressant treatment, the impact of remodelling on presynaptic protein expression is still unclear since only one study has actually assessed the abundance of synaptic proteins after ECS (Jorgensen and Bolwig 1979). Nevertheless, it is prudent to bear these studies in mind as all of the subjects included in this study were prescribed antidepressant and/or mood stabilising medication, and two subjects also received ECT in the weeks prior to death (see appendix one).

6.5 Summary

No significant changes were found in synaptophysin, complexin I, or complexin II expression in the ACC in mood disorder. This suggests that marked alterations in synaptic circuitry do not accompany, or contribute to, the ACC cytoarchitectural abnormalities reported in chapter three. The experiments described in this chapter need to be replicated and confirmed in a larger series before this point can be made conclusively.

Chapter Seven: General discussion

The ACC has long been implicated in mood, emotion and their disorders because of neuropsychological, anatomical and functional imaging data; consequently, the involvement of the ACC in mood disorder has been the focus of many recent neuropathological investigations. The aim of this work was to provide a detailed analysis of the cytoarchitecture of area 24 in the human ACC, followed by an investigation of this brain region in subjects with mood disorder. To fulfil this aim I measured several morphometric indices, in addition to various parameters of gene expression in post mortem brains from mood disorder and control subjects. In this chapter I summarise and discuss the main findings obtained and consider some of the conceptual issues affecting their interpretation. Some suggestions for further studies in this field are also presented. The limitations of this work have been considered extensively throughout this thesis and will not be reiterated here.

7.1 Summary and discussion of results

7.1.1 The cytoarchitecture of the ACC

In this cytoarchitectural investigation, several morphometric parameters (viz. neuronal density, glial density and neuronal size) were measured in five anatomical levels of area 24b in the ACC. These data were analysed for significant effects of lateralisation, rostrocaudal differentiation and supragenual - subgenual differentiation. Overall, there was no effect of lateralisation, and no evidence for rostrocaudal differentiation in the supragenual levels; in contrast, I found the subgenual - supragenual distinction to be critical. Total cortical depth was lower in the subgenual region, with a higher percentage depth for layer II, and a lower percentage depth for layer V. Neurons were considerably smaller in most layers of the subgenual cortex and glial density was substantially lower throughout the subgenual region.

This study provides quantitative evidence of an anatomical distinction between the supragenual and subgenual regions of area 24. Previous studies investigating the cytoarchitecture of the ACC have not made quantitative comparisons between these two regions (see section 2.6.2); nevertheless, it is noteworthy that a specific class of corticocortical projection neurons are present in layer III of the supragenual cortex (Nimchinsky *et al.* 1997), but absent in the subgenual cortex (Öngür *et al.* 2003); thus providing further evidence of an anatomical distinction within area 24. All told, these results may represent cellular reflections of functional specialisations in distinct domains of the ACC. The potential existence of discrete functional domains may go some way in explaining the apparent differential involvement of the subgenual and supragenual cortices in mood disorder (Drevets 2000b; Harrison 2002).

7.1.2 Glial pathology in the ACC in mood disorder

Glial density was reduced in all cortical layers of the ACC in mood disorder. This finding adds further credence to the recent morphometric investigations that have convincingly demonstrated cortical glial pathology in subjects with major depression (Bowley *et al.* 2002; Cotter *et al.* 2002b; Cotter *et al.* 2001a; Öngür *et al.* 1998; Rajkowska *et al.* 1999) and bipolar disorder (Öngür *et al.* 1998; Rajkowska *et al.* 2001). Interestingly, in a recent investigation (published after the completion of chapter three), Cotter and colleagues failed to replicate their earlier work in the ACC (Cotter *et al.* 2001a) and did not find a significant reduction in glial density in either major depression or bipolar disorder (Chana *et al.* 2003). This is surprising as the study focused on an adjacent area (area 24c) in the same brain collection as their earlier study. The authors have suggested that the basis of this apparent contradiction may be methodological in nature; their most recent study, unlike their initial stereological investigation, was a 2D assessment and therefore may have been biased regarding cell size. In their latest assessment, the authors reported an increase in glial cell size, which may in turn have overestimated and normalised the glial cell density values (Chana *et al.* 2003).

It cannot be concluded from my results whether the glial deficit in mood disorder is a cause or consequence of the disease process, and indeed, it was not an aim of the study to reach such a conclusion. Similarly, the consequences of altered glial density remain to be established; nevertheless, given the fundamental roles that glial cells play in neuronal structure, synaptic function and plasticity, and neurotransmission, it is reasonable to predict that shortfalls within the glial population will have a marked effect on normal cortical function and are likely to contribute to the pathophysiology of mood disorder. Figure 7.1 depicts a scheme of hypothetical sequences of cellular changes in mood disorder.

Glial cells represent a heterogeneous population comprising oligodendrocytes, microglia and astrocytes, and this underlies a considerable problem concerning the interpretation of results. I endeavoured to clarify this issue by assessing the individual contribution of astrocytes, oligodendrocytes and microglia to the reductions in glial density.

Unfortunately, I was not able to assess oligodendrocyte density, and the specific involvement of this glial type needs to be examined in any future work.

Microglia density was not altered in the mood disorder group, which suggests that these glial cells are not contributing to the overall deficit. In contrast, GFAP expression was significantly reduced in the white matter and a similar (though non-significant) reduction was observed in the grey matter. This work is in agreement with previous studies that have implicated GFAP in the pathophysiology of mood disorder (Johnston-Wilson *et al.* 2000; Knable *et al.* 2001; Miguel Hidalgo *et al.* 2000; Webster *et al.* 2001a); however, it is still not clear whether the alterations in GFAP are indicative of a reduction in the astrocyte population, or conversely, a reduction in GFAP content.

7.1.3 Neuronal pathology in the ACC in mood disorder

Pyramidal neuron density was significantly reduced in layers Va and Vb in mood disorder; total neuronal density was also reduced in these cortical layers. I did not find any significant changes in non-pyramidal neuron density. The existing studies of neuronal density in mood disorder are equivocal. The majority of studies assessing ACC architecture have not found any evidence for alterations in neuronal density in either major depression or bipolar disorder, although decreases in neuronal density have been observed in the subgenual, but not the supragenual region of area 24, in subjects with bipolar disorder (Bouras *et al.* 2001). Benes and colleagues (2001) have also reported reductions in non-pyramidal, but not pyramidal, neuron density in a pregenual region of the ACC. A reduction in the density of large neurons has also been described in the prefrontal cortex (Rajkowska *et al.* 2001; Rajkowska *et al.* 1999).

As discussed in the previous section, it is not possible to determine whether the alterations in neuronal density play a causative role in the manifestation of depressive symptoms, or are merely a consequence of the disease process. The pattern of neuronal pathology certainly appears to be more subtle than the corresponding glial alterations, and it is appealing to speculate that deficits in the glial population may be the central pathological event, with specific neuronal deficits arising as the glial pathology worsens (see figure 7.1). The majority of neuropathological studies to date have reported smaller neurons in mood disorder - in comparison, I found increases in pyramidal cell size together with alterations in pyramidal cell shape. As larger neurons were not predicted, and changes were significant only in the Nissl material, these data are inconclusive.

Since the completion of chapter three, an additional study examining the cytoarchitecture of the ACC in mood disorder has emerged. The authors report decreases in neuronal size and increases in neuronal density in area 24c major depression and bipolar disorder (Chana *et al.* 2003). This pattern of pathology is interesting as the complementary

changes of increased neuronal density and reduced neuronal size might imply that the interneuronal neuropil is reduced in mood disorder, as has been suggested for the prefrontal cortex in schizophrenia (Selemon and Goldman-Rakic 1999). Notwithstanding, the majority of studies have reported decreases in cell density in mood disorder and accordingly do not support this view. Clearly, this area of research requires further replication before the pattern of neuronal pathology in mood disorder becomes fully resolved.

To investigate the non-pyramidal neuron population in mood disorder I also studied CaBP expression. Although PV-, CR- and CB-immunoreactive neurons were reduced in the ACC, only reductions in CR immunoreactivity were significant. This work provides further support for the recent investigations that have reported various GABAergic deficits in mood disorder (Beasley *et al.* 2002b; Benes *et al.* 2000; Benes *et al.* 2001; Cotter *et al.* 2002a; Reynolds *et al.* 2002; Zhang and Reynolds 2002). As 'total' non-pyramidal neuron density was unchanged in mood disorder, it is unclear whether the reductions in CR expression reflect cell loss, or diminished CR content.

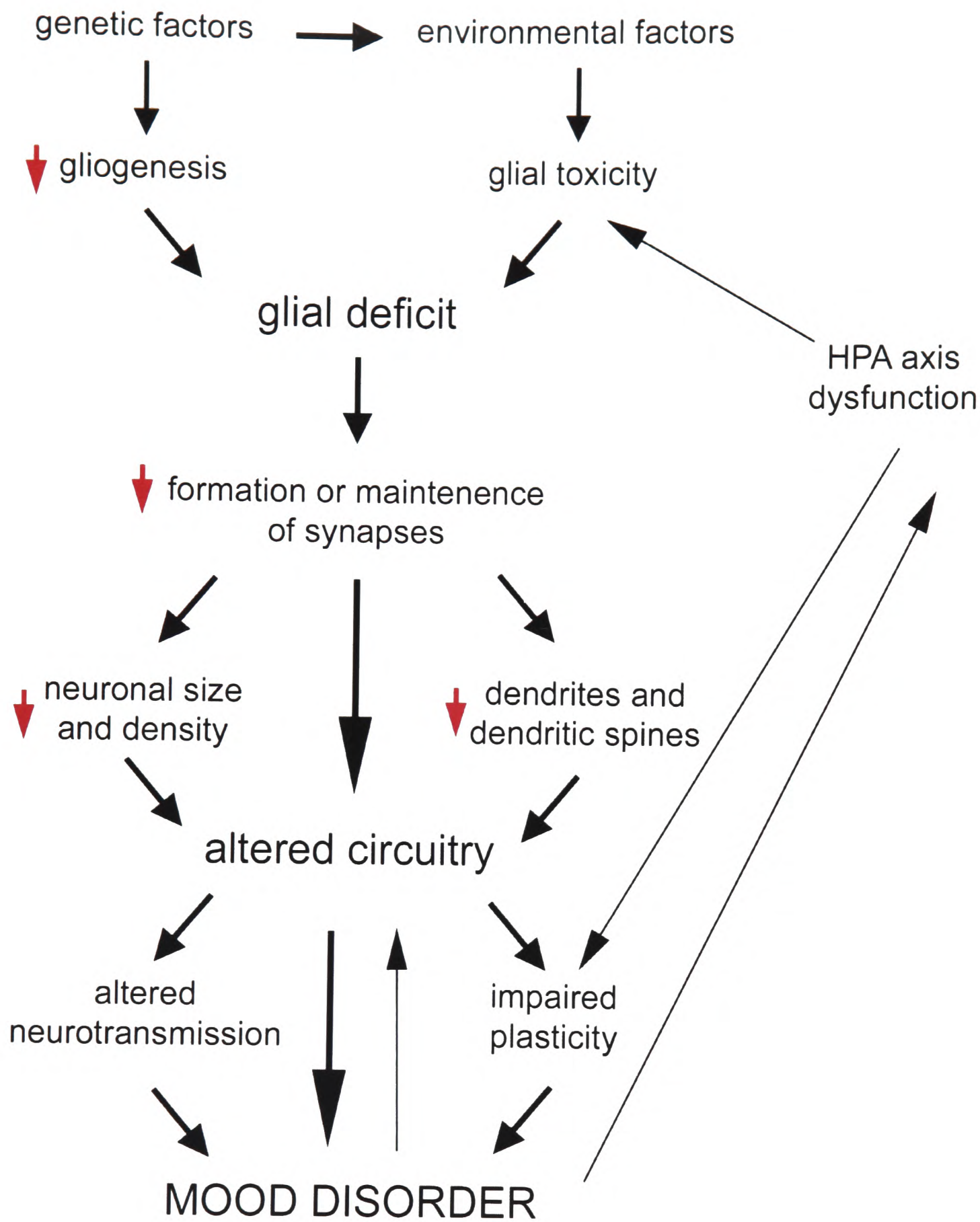
7.1.4 Synaptic pathology in the ACC in mood disorder

No significant alterations in synaptic protein expression were observed in the mood disorder group; nevertheless, notable (albeit non-significant) reductions were observed in cx II protein, synaptophysin mRNA and cx I mRNA expression. In view of the accumulating evidence proposing ACC cytoarchitectural abnormalities in mood disorder (including the present work), it is perhaps surprising that measures of synaptic protein expression were not significantly changed. Clearly, more studies are required to determine whether synaptic pathology is a feature of the ACC in mood disorder.

Figure 7.1

Scheme of hypothetical sequence of cellular changes in mood disorder.

This scheme proposes that glial changes precede neuronal changes. Mood disorder patients may have either less glia at birth, or may exhibit a greater vulnerability to environmental factors that detrimentally effect glial cells. The interaction between genetic factors and environmental factors is also indicated. Lack of sufficient glial support (e.g., less structural support of synapses, shortage of glucose at synapses) may lead to neuronal pathology. Neurons would become smaller in size, reflecting the reduced extent of axonal and dendritic arborisation. Pathology of both neurons and glia may worsen with increasing duration of illness and recurrent episodes, and this pathology may eventually lead to cell loss. The association between elevated levels of glucocorticoids, glial toxicity and neural plasticity is also illustrated. I have suggested that the alterations in glial and/or neuronal density will ultimately lead to altered circuitry. Although there was no convincing evidence of ACC synaptic pathology in this study, other reports have demonstrated alterations in synaptic circuitry in the ACC (Eastwood and Harrison 2001), and it is difficult to envisage in what way the reported neuropathological alterations could be significant other than in terms of aberrant connectivity of some kind.



7.2 Conceptual issues

7.2.1 Is the neuropathology anatomically specific?

Within the cerebral cortex, a uniform pathology of mood disorder seems unlikely given the negative findings in the somatosensory (Öngür *et al.* 1998) and visual cortices (Bouras *et al.* 2001). The majority of the neuropathological findings to date imply a preferential involvement of the frontolimbic areas in mood disorder – a view shared by anatomical and functional imaging data. Nevertheless, morphometric analyses could be extended to other brain regions implicated in the imaging literature, such as the striatum and hippocampus (see Drevets 2000b; 2001; Soares and Mann 1997). Curiously, the hippocampus has been the focus of relatively few systematic morphometric investigations; specifically, glial density has not been quantitatively examined in this brain region. This is particularly surprising given the well-studied model linking depression, via growth factors and second messengers, to the deleterious effects of stress on cell morphology and neurogenesis in the hippocampus (Brown *et al.* 1999; Duman *et al.* 1997; Jacobs *et al.* 2000; Sapolsky 2000). Recently, Lucassen *et al.* (2001) reported the first neuropathological examination of the hippocampal stress-atrophy hypothesis. In the mood disorder group, only minor increases in apoptotic cells were seen, and cell stress markers were negative. Moreover, the positive findings were not in the hippocampal subfields at risk for glucocorticoids damage. These results cast some doubt over the validity of the stress/atrophy model of major depression. In a further qualitative study of the same brains, they did not find any evidence of differences in neuronal density, orientation or clustering, and only minor localised alterations in GFAP immunoreactivity (Muller *et al.* 2001). Given the recent publication from Bowley *et al.* (2000), reporting a decrease in glial density, the amygdala is also a priority area for future study.

It will also be pertinent to consider the uniformity of pathology within a given brain region, especially in complex anatomical regions such as the ACC. The heterogeneity of ACC

cytoarchitecture and connectivity is well documented (Carmichael and Price 1994; 1995; Freedman *et al.* 2000; Nimchinsky *et al.* 1997; Vogt *et al.* 1995); and it is feasible that individual areas (and/or connections) within the ACC could be specifically and selectively affected in mood disorder. The differential involvement of dorsal and rostroventral regions of the ACC in cognition and emotion (see Bush *et al.* 2000; Devinsky *et al.* 1995 and section 1.2.1), and the selective volume decrements in the subgenual cortex in mood disorder (Drevets *et al.* 1997; Öngür *et al.* 1998), provide some support for this premise. In addition, the cytoarchitectural investigation in chapter two revealed a morphometric distinction between the supragenual and subgenual regions of area 24, further illustrating the anatomical heterogeneity within the ACC. Future neuropathological studies of the ACC in mood disorder should ideally assess both the supragenual and subgenual regions of area 24 – and perhaps even the subdivisions within these regions.

A further aspect of regional localisation is hemispheric asymmetry. Although I was not able to investigate laterality effects, the possibility that changes in the ACC are lateralised has been raised by some of the findings in mood disorder (Botteron *et al.* 2002; Drevets *et al.* 1997; Hirayasu *et al.* 1999), along with the demonstration that the ACC is structurally asymmetrical (see section 2.1.1 and Ide *et al.* 1999; Paus *et al.* 1996a; Paus *et al.* 1996b; Vogt *et al.* 1995; Yucel *et al.* 2001) - although importantly, I did not find any convincing evidence of lateralisation at the cellular level (see section 2.4.1). Post mortem and neuroimaging reports of lateralisation in mood disorder have not been entirely consistent; decreases in grey matter volume, glucose metabolism, and blood flow have been reported in the left subgenual cortex in major depression and bipolar disorder (Drevets *et al.* 1997); whereas a stereological investigation of this brain region showed a trend towards a volume reduction only in the group with bipolar disorder, and in addition reported glial deficits in both the left and right cortices in major depression and bipolar disorder (Öngür *et al.* 1998). Interestingly, Cotter *et al.* (2001a) found a hemispheric difference, and a hemisphere by diagnosis interaction, for some of their glial density data. Ideally, future

studies should aim to use tissue from both cortical hemispheres enabling the direct assessment laterality effects within subjects.

7.2.2 What are the clinical correlates of the neuropathology?

The aetiology and pathophysiology of primary mood disorder remain poorly understood. Neuropathology has the potential to enhance our understanding of the illness; nonetheless, despite recent advances in elucidating the pathological changes in mood disorder, it is still not known whether these abnormalities cause a vulnerability to mood disorder, or whether they are compensatory changes to other pathogenic processes or sequelae of recurrent illness.

It is possible that the clinical correlates of the neuropathology are the emotional features of mood disorder. Attempts at coupling particular elements of the neuropathology with specific symptoms in mood disorder have proved interesting. For example, the amygdala is thought to be involved in the recall of emotional or arousing memories, and accordingly it has been suggested that abnormalities in amygdala metabolism observed in mood disorder may be related to anxiety symptoms and depressive ruminations. Similarly, the dopaminergic projection to the ventral striatum, ventral tegmentum and medial prefrontal cortex (including the ACC) may be especially relevant to anhedonia and amotivation in depression and euphoria and hypermotivation in mania (Drevets 2001).

Whilst this is an interesting proposition, it is perhaps more probable that the clinical correlates of the neuropathology are the neuropsychological characteristics of mood disorders, not their symptoms. Since post mortem studies include patients dying at all phases of illness, neuropathology is likely to detect enduring brain changes, i.e. those alterations that are primarily trait, rather than state related. It is therefore pertinent that numerous neuropsychological abnormalities have been reported in mood disorder (including impairments in executive functions, memory, attention and concentration), and

while there is substantial improvement in cognitive performance on symptomatic recovery, many studies report the presence of residual impairments relative to controls (Austin *et al.* 2001; Ferrier *et al.* 1999; Murphy and Sahakian 2001; van Gorp *et al.* 1998). Interestingly, many of the putative anatomical substrates of cognitive function (including the ACC and frontal brain regions) have consistently shown abnormal function in mood disorder (Drevets 2000b), and in analogy with the neuropsychological data, there are persistent abnormalities after treatment and recovery (Drevets *et al.* 1992; Ebert and Ebmeier 1996; Mayberg *et al.* 2000). Again, individual facets of the neuropathology might connect to specific neuropsychological deficits (Bench *et al.* 1993; Dolan *et al.* 1994; Elliott 1998).

Conversely, the neuropathology may not be directly related to the behavioural or neuropsychological characteristics of mood disorder, but may simply reflect a vulnerability to mood disorder. Prospective, longitudinal imaging studies including the relatives of subjects with mood disorder, in conjunction with neuropsychological studies, might be informative in this respect. Unfortunately, equivalent post mortem studies are not a realistic possibility.

Finally, it is feasible that the neuropathology may be secondary to other disease-related processes. For example, some aspect of medication or institutional care (including a deprived environment) could potentially lead to these changes. It has been suggested that glial cell loss is not diagnostically specific (see section 7.2.4) and may therefore be a general feature of psychiatric illness, possibly related to the elevated glucocorticoids levels observed in major depression, bipolar disorder and schizophrenia (Cotter and Pariante 2002; Cotter *et al.* 2001b). Whilst this is an intriguing argument, it is equally plausible that glial cell loss may represent a primary vulnerability factor, which predates or predisposes to psychiatric illness generally.

7.2.3 Is the neuropathology static, progressive, or reversible?

Questions regarding the timing of the neuropathological alterations and whether these alterations are static, progressive, or reversible remain to be addressed. Various imaging studies of the ACC have shown the volume decrements are present at first episode (Hirayasu *et al.* 1999), and in adolescence (Botteron *et al.* 2002) and also suggest that cingulate neurochemical alterations are present relatively early in the disease process (Auer *et al.* 2000; Winsberg *et al.* 2000). These studies are important as they indicate that decrements in cortical activity and volume, and therefore the morphometric changes that putatively underlie them, are present early in the illness and are not merely the consequence of chronic drug treatment.

In addition, these studies, along with other evidence suggesting delayed developmental milestones in pre- mood disorder cases (see Jones and Tarrant 1999) raise the possibility that the neuropathology of mood disorder may reflect a neurodevelopmental process.

Some support for this premise comes from two cytoarchitectural investigations, where dysplasia and heterotopias (indicative of prenatal migration disturbances) were described in the entorhinal cortex in bipolar disorder (Beckmann and Jacob 1991), and in a mixed group of mood disorder subjects (Bernstein *et al.* 1998). Changes in developmental gene expression in mood disorder have also been used to infer a developmental process. In particular, expression of reelin (which is involved in neuronal migration, cortical lamination and synaptogenesis) is reportedly decreased in mood disorder (Fatemi *et al.* 2000; Guidotti *et al.* 2002) and changes in developmentally regulated isoforms of nerve cell adhesion molecule have also been described in bipolar disorder (Vawter *et al.* 2000).

Nevertheless, such interpretations should be made with caution since many 'developmental' genes are now believed to have persisting functions in the adult brain.

Alterations in the density and distribution of interstitial white matter neurons are also presumed to be a sign of aberrant neuronal migration or programmed cell death. In the one study of white matter neurons to date, the authors did not find any evidence for

abnormalities in major depression or bipolar disorder in the frontal cortex (Beasley *et al.* 2002a).

Importantly, a (theoretical) developmental aetiology may not preclude a progressive element to mood disorder. It is entirely feasible that ongoing stress-induced neurotoxicity, in conjunction with other environmental and behavioural events (see Fuchs and Gould 2000; Kolb and Whishaw 1998; Lee *et al.* 2000), may play a role in emergent neuropathological changes and therefore the progressive pathophysiology of mood disorder. Whether the neuropathological alterations are progressive has not yet been investigated, though a recent study examining synaptic protein expression in the ACC in mood disorder found a correlation between the extent of synaptic protein reduction and the duration of illness (Eastwood and Harrison 2001). Establishing the casual sequence and progression of morphological changes in mood disorder will be particularly challenging area for future research.

7.2.4 Is the neuropathology diagnostically specific?

Investigations into the neurobiology of mood disorder struggle with the limitation that psychiatric nosology remains at a syndromatic level, in which non-specific behavioural signs and symptoms (e.g. insomnia, fatigue, low mood, impaired concentration, overactivity) rather than pathophysiology, are used to define major psychiatric disorders. Consequently, specific links between syndrome and pathophysiology may not exist; and it is therefore unsurprising that a degree of neuropathological continuity is observed across the major psychiatric phenotypes.

The question of whether major depression and bipolar disorder are distinct at a pathological level is troublesome, and is particularly pertinent to the present study since the mood disorder cohort comprised subjects with major depression or bipolar disorder. To date, the similarities appear greater than the differences; reductions in neuronal

density, neuronal size and glial density have been reported in both diagnostic groups (see Harrison 2002). Additionally, by virtue of the fact that several of the key mood disorder studies have used tissue from the Stanley Foundation Consortium, comparisons can be made between schizophrenia and mood disorder, adding a further level of complexity to data interpretation. Features reported in schizophrenia as well as in mood disorder include decreased neuronal density in the ACC, reduced synaptic and dendritic markers in the prefrontal cortex and hippocampus and glial deficits (see Cotter *et al.* 2001b; Harrison 1999). Additionally, increased neuronal density has been reported in the prefrontal cortex in schizophrenia (Selemon and Goldman-Rakic 1999), and a recent publication has suggested that increases in neuronal density may also be a feature of mood disorder (Chana *et al.* 2003). Whilst there are similarities between schizophrenia and mood disorder, the changes are by no means identical, leading some authors to emphasise the differences, rather than the similarities (see Baumann and Bogerts 1999; Benes *et al.* 2001; Rajkowska *et al.* 2001). Overall, it seems reasonable that schizophrenia and mood disorder (and subcategories therein) will have both shared, and unique neuropathological signatures.

Although the alterations in bipolar disorder and major depression are essentially comparable, there are subtle differences; for example, in the prefrontal cortex the densities of both large and small neurons are reputedly decreased in bipolar disorder, whereas in major depression the reduction in large neurons is accompanied by increases in the population of small neurons – leading some authors to suggest that major depression is characterised by neuronal atrophy, while the pattern of change in bipolar disorder is indicative of neuronal loss (Manji *et al.* 2000b; Rajkowska 2000). However, this theory is far from conclusive, and such interpretations are likely to be premature considering the inadequacies of the available data and the fact that few studies have been suitably powered to address this issue. Moreover, even if this proposition is applicable to the prefrontal cortex, the situation in the ACC appears to be rather different; firstly,

neuronal alterations have not been consistently observed in this brain region, and secondly, these alterations encompass both increases and decreases in neuronal density in both diagnostic groups (see section 7.1.3).

The reductions in glial density and number are far more convincing and appear to be consistent across diagnostic groups and brains regions. Interestingly, whilst glial deficits have been reported (to differing extents) in all morphometric studies examining the ACC and prefrontal regions in major depression, only two studies have reported changes in bipolar disorder (see table 1.3) – though again, this may simply reflect inadequacies of the existing data. Initially, the glial deficits appeared to be more prominent and even a defining feature of mood disorder; however, this distinction appears to be waning as research in this field expands. There are now numerous reports that indicate glial deficits are also a feature of schizophrenia (see Cotter *et al.* 2001b), particularly in the oligodendrocyte population (see section 5.4.6). Indeed, it has been proposed that the lack of diagnostic specificity may indicate that the glial deficits are a feature of psychiatric illness generally.

Considering synaptic and dendritic markers, the various studies of mood disorder to date intimate that synaptic perturbations may be more prominent in bipolar disorder compared with major depression (Bouras *et al.* 2001; Eastwood and Harrison 2000; 2001; Fatemi *et al.* 2001; Vawter *et al.* 1999); however, the majority of these studies have focused on the hippocampus and in view of the paucity of data regarding the ACC and prefrontal regions, it would be premature to argue (yet) that synaptic pathology distinguishes major depression from bipolar disorder.

In conclusion, there is not yet sufficient evidence to support a neuropathological distinction between major depression and bipolar disorder (or arguably even schizophrenia). Even though there are similarities between the different diagnostic categories, the origins,

complementarity, and clinical correlates of the neuropathology remain unknown. Moreover, even if the putative neuropathology does span diagnostic categories, the underlying biological mechanisms may be quite different for each illness.

7.3 Future work

7.3.1 The cytoarchitecture of the ACC

This work will need to be extended in a larger series in order to authenticate the findings. As this preliminary study does not find any evidence for rostrocaudal differentiation in area 24b, it will be possible to extend the main findings of this work by using just one supragenual level and one subgenual level from each brain.

Although significant rostrocaudal differentiation was not observed in area 24b in respect to cell density and size, it will be pertinent to extend this investigation to areas 24a and 24c, especially in consideration of the work by Vogt *et al* (1995), which describes a more discernible variation between the rostral and caudal levels of these subdivisions compared to area 24b. In addition, this would permit a subsequent analysis of cell density and size in the dorsosventral plane of the ACC.

As the glial cells investigated in the current study represent a heterogeneous population of oligodendrocytes, microglia and astrocytes it is not possible to assess whether the reduction observed in subgenual area 24 is common across all glial types, or whether a specific subpopulation has been affected. This will need to be addressed in future work. Likewise, it will be valuable to distinguish pyramidal neurons from non-pyramidal neurons in any subsequent work. As they were not assessed separately in the current study it is possible that alterations in a specific neuronal population were masked and therefore affected the probability of detecting any differences in neuronal density between the two

regions. Similarly, it is possible that the reduction in neuronal size observed in subgenual area 24 reflects a change within a specific population of neurons.

The spindle cells of layer Vb should also receive specific attention in any future work. It has recently been suggested that these cells may participate in the neuronal circuitry responsible for focused attention and emotional expression (Allman *et al.* 2001). Strong evidence suggests that there is a functional distinction between the dorsal (area 24') and rostroventral (area 24, including area 24sg) regions of the ACC, the dorsal division appears to be associated with cognition whereas the rostroventral division is more associated with emotion (Bush *et al.* 2000; Devinsky *et al.* 1995; Dougherty *et al.* 1999; Pardo *et al.* 1993; Rauch *et al.* 1994; Rauch *et al.* 1995; Rauch *et al.* 1996). With this in mind, it is possible that the distribution, abundance and size of spindle cells may differ between these functionally distinct divisions.

Further evaluation of the cytoarchitecture of the supragenual and subgenual regions of area 24 could also be achieved by employing molecular markers of synapses and dendrites together with immunocytochemical measurements of glia and neurons. To this end, I endeavoured to examine four synaptic proteins (synaptophysin, GAP-43, complexin I and complexin II) together with GFAP, in the five levels of area 24 using immunautoradiography. Unfortunately, these experiments were not informative as the quality of staining was very variable and in some cases specific signal was not detected above background. It is likely that the low levels of immunoreactivity were related to the extended fixation period of the tissue used in this study. Accordingly, conclusions regarding effects of level or hemisphere could not be inferred from this data. Questions concerning whether molecular markers of synaptic and glial density reflect the observed morphometric variation between the supra- and subgenual regions of area 24 remain to be addressed.

Although Nimchinsky and colleagues (1997) have gone some way in detailing the chemoarchitecture of the ACC, further knowledge could be gained from quantifying the subsets of neurons marked by PV, CR, CB and neurofilament proteins. In light of the findings presented here, it would be particularly pertinent to address the potential variation between the subgenual and supragenual cortices. As these neuronal markers have the capacity to reflect differences in the intrinsic organisation and connectivity of cortical areas (Nimchinsky *et al.* 1997), it is feasible that quantification of these specific neuronal populations would be informative regarding the functional specialisations in distinct regions of the ACC, and in particular the functional distinction between the rostroventral and dorsal divisions of the ACC (Bush *et al.* 2000; Devinsky *et al.* 1995).

7.3.2 The neuropathology of the ACC in mood disorder

The work outlined in this thesis has demonstrated a range of cytoarchitectural abnormalities in mood disorder, but has left many more questions than answers. The most obvious limitation of this work concerns the small sample size, and consequently replication is necessary in a larger and better-characterised series to determine which findings are robust, and which are not. Ideally, any replication should allow variables implicated by recent work (family history, age at onset and clinicopathological correlations) to be investigated more critically. Data interpretation will also be aided by parallel animal studies investigating the neuropathological effects of antidepressant and mood stabilising medication.

The most fruitful avenues for pursuing the subject of glial pathology in mood disorder will involve the elucidation of the glial subtype, or subtypes, that are abnormal in number using stains that are selective for astrocytes, oligodendrocytes and microglia. Although I attempted to address this issue in chapter five, the study was unavoidably hampered by the insufficiency of suitable markers for each glial subpopulation, thereby limiting data interpretation. Greater confidence in this respect will require a larger panel of well-

characterised molecular probes in conjunction with the measurement of multiple parameters of gene expression. A search for apoptotic glial cells and studies of relevant cytokines, chemokines and growth factors (which all influence glial proliferation and migration) may also be helpful. As mentioned earlier, oligodendrocytes should be a priority area for future work, especially given their apparent involvement in the neuropathology of bipolar disorder and schizophrenia. When individual glial types can be consistently and reliably distinguished from one another (using immunohistochemical techniques), powerful methodologies such as laser capture microdissection are available which will facilitate investigations into the molecular characteristics of single antigenic positive cells in mood disorder. Until additional studies of specific glial populations in mood disorder are performed, further interpretation of glial reductions will be difficult.

Similarly, the extent and nature of neuronal pathology in mood disorder needs to be established. It will be important to ascertain whether neuronal perturbations in the cortex represent neuronal loss or atrophy. The increase in neuronal density in the ACC demonstrated by Chana *et al* (2003) will need to be confirmed; although increased neuronal density in mood disorder has been encountered previously, it is generally thought to reflect neuronal atrophy, resulting in an apparent increase in the density of smaller neurons (see Rajkowska 2000). Immunohistochemical markers of cellular stress and apoptosis have been studied in the hippocampus in mood disorder (Lucassen *et al.* 2001), and similar investigations are warranted in the frontal and limbic brain regions.

Analogous to discussion above concerning glia, it will be equally important to examine individual neuronal populations. A Nissl stain, used in most studies, does not permit unequivocal distinction of pyramidal from non-pyramidal neurons, let alone one neuronal subpopulation from another. The calcium-binding proteins, PV, CR and CB are frequently employed to study individual cell types in the non-pyramidal cell population, but equivalent markers are not readily available to study the pyramidal neuron population. A recent study

has utilised various neurofilament antibodies to define three, largely separate, pyramidal neuron populations in area 9 in mood disorder and schizophrenia (Law and Harrison 2003). Although the comparisons between diagnostic groups were largely inconclusive, this methodological approach may be of value for investigating the differential neuropathological involvement of pyramidal neuron subpopulations in the ACC.

Ultimately, it will be of utmost importance to elucidate the biological mechanisms that underlie the neuropathology of mood disorder. There has been much speculation that alterations in cell number or density revealed in post mortem studies of mood disorder may be relevant to stress-induced changes in gene expression related to signal transduction pathways and cell survival (neurotrophic/neuroprotective) factors (Duman *et al.* 1997; Duman *et al.* 2000; Manji *et al.* 2000b; Rajkowska 2000). Such models posit that the disruption of intracellular mechanisms, crucial for the normal function and survival of neurons, may produce impairments of neuroplasticity and cellular resistance, thereby resulting in cell death and atrophy.

Support for this hypothesis is provided by studies indicating that factors involved in neuronal atrophy and survival are a target of antidepressant and mood stabilising treatments. Antidepressant treatment upregulates the cAMP-CREB cascade and expression of BDNF (thereby increasing cell survival), and has been shown to increase hippocampal neurogenesis (Duman 1998). Lithium has been reported to increase the expression of Bcl-2 (an anti-apoptotic factor) in brain and cultured cells and inhibits apoptosis of cerebellar cultured granule cells (Manji *et al.* 2000a).

It is conceivable that the reductions in cell density observed in mood disorder could result from alterations in the factors that control programmed cell death (e.g. increased Bad or decreased Bcl-2 proteins), and the pathways that control the function and expression of these proteins (e.g. decreased CREB). The relative expression and abundance of these

proteins and transcription factors in mood disorder need to be investigated in post mortem tissue – though the marked effect of antidepressant medication on the abundance of these factors might limit data interpretation. A recent post mortem study of the temporal cortex has reported higher CREB concentrations in patients treated with antidepressants than in untreated patients with major depression. Furthermore, the untreated patients had lower CREB concentrations than controls (Dowlathahi *et al.* 1998). Additional studies are necessary to confirm and extend this finding to other brain regions, and to examine other proteins involved in cell survival and plasticity.

The preceding discussion focused on the possibility that regional reductions in cell number in mood disorder are primarily due to cell death, however, ongoing impairment of neurogenesis may also play a role. Hypotheses and experimental investigations regarding the deleterious effects of stress on cell morphology and neurogenesis have centred largely on the hippocampus (Brown *et al.* 1999; Duman *et al.* 1997; Jacobs *et al.* 2000; Sapolsky 2000); though, there are no reports that directly demonstrate a reduction in the number of neurons in the hippocampus in mood disorder. This point needs to be addressed in future post mortem investigations of cell number, preferably using unbiased stereological procedures. In contrast, numerous cytoarchitectural alterations have been observed in the cerebral cortex regions, especially the ACC and prefrontal cortex. A recent study demonstrating that neurogenesis also occurs in the cerebral cortex, (Gould *et al.* 1999), raises the possibility that the cell loss in the cerebral cortex of patients with mood disorder could result from decreased neurogenesis. However, the finding of cortical neurogenesis has not been fully substantiated, leading some authors to suggest that the newly generated cells in the neocortex are in fact oligodendrocytes (Kornack and Rakic 2001; Rakic 2002). Future studies need to determine the influence of stress and antidepressants on cortical neurogenesis, gliogenesis and cell morphology.

Finally, the pattern of neuropathological alterations in mood disorder may provide innovative strategies and targets for the treatment of this illness. It will be important to determine whether manipulation and up-regulation of different glial populations by pharmacological treatments can normalise cortical function. Additionally, factors implicated in the CNS cell survival and cell death pathways (i.e. CREB, BDNF, MAP kinase, Bcl-2) may provide important novel therapeutic targets. Such treatment modalities have the potential to enhance neuroplasticity and cellular resistance, and thereby modulate the long-term course and trajectory of mood disorder (Manji *et al.* 2000b).

7.4 Final Summary

The opening result chapter of this thesis reported a clear distinction in the cellular composition of the subgenual and supragenual regions of area 24; this may have important implications for the interpretation of imaging and pathological data in mood disorder and further studies are necessary to fully characterise the cytoarchitectural heterogeneity of this brain region. The remaining result chapters were devoted to examining the involvement of the ACC in mood disorder. I demonstrated a range of cytoarchitectural abnormalities; the most prominent of which included reductions in glial density and GFAP, along with a reduction in pyramidal neuron density. These cytoarchitectural changes are unlikely to be a consequence of confounding demographic variables or alcohol abuse, although medication effects cannot be entirely excluded. Together, these changes are suggestive of an alteration in the cellular composition and neural circuitry in the ACC in mood disorder, which are likely to be developmental or plasticity-related in origin rather than neurodegenerative. Besides confirming and extending the morphometric alterations in mood disorder, future neuropathological studies should examine elements of the signal transduction pathways and cell survival factors that influence cell density and plasticity. In addition, imaging studies could begin to explore the possibility of using probes that identify intracellular targets and regulatory factors. These approaches will ultimately lead to a more complete characterisation of the molecular and cellular determinants of mood disorder.

Appendix One: Demographic details of all subjects

Subject	Age/sex	Brain pH	PMI	*FST	Paraffin	Frozen	Clinical details
Mood disorder							
P947	55M	6.36	13	105	•	•	BPD (AD, Li, BZ)
P950	75M	6.06	25	105	•	•	MDD, S (AD)
P956	51F	6.42	26	103	•	•	MDD, S (AD, BZ)
P957	75F	6.28	40	-	•		MDD (AD, BZ)
P962	75M	6.86	26	-	•		MDD, S (AD)
P963	49F	6.36	75	97	•	•	BPD (Li)
P984	48M	6.29	30	84		•	MDD, S (AD, Li)
P992	86F	6.10	60	80		•	MDD (AD)
P995	72M	6.47	36	76	•	•	MDD, S (AD, BZ, ECT)
P101	65F	6.42	29	60		•	MDD (AD)
P103	43M	6.50	36	53		•	MDD, S (AD, Li, ECT)
P104	78M	6.53	23	52		•	MDD, S (AD)
Control							
P938	47M	6.72	21	110	•	•	-
P942	22F	6.20	37	-	•		-
P948	71M	6.68	25	-	•		-
P949	61M	6.67	19	105	•	•	-
P952	75F	6.75	27	104		•	-
P953	72F	6.54	12	104	•	•	-
P964	72M	6.71	35	96	•	•	-
P972	79M	6.30	8	91	•	•	-
P987	59M	6.69	72	83	•	•	-
P988	72F	6.14	78	83	•	•	-
P997	42F	6.35	65	72	•	•	-

PMI, post mortem interval; FST, freezer storage time; *at time of the first experiment; MDD, major depressive disorder; BPD, bipolar disorder; S, suicide; AD, antidepressant; Li, lithium; BZ, benzodiazepine; ECT, electroconvulsive therapy. Filled circles indicate tissue availability

Appendix Two: Preparation of buffers and solutions

All chemicals were purchased from VWR International, (Poole UK) unless otherwise stated. Solutions were made using double-distilled water.

Hybridisation buffer (for ISHH)

5ml 100% deionised formamide ^a

2ml 20X SSC ^{b #}

0.5ml 0.5M sodium phosphate buffer*

0.1ml 0.1M sodium pyrophosphate ^c

1ml 50X Denharts solution ^d (Sigma, Poole, UK)

0.2ml salmon sperm DNA ^e (Sigma)

0.2ml polyadenylic acid ^f (Sigma)

10µl heparin

1g dextran sulphate ^g

make up to 10mls with DEPC H₂O

^a *reduces energy required for hybridisation*

^b *high salt concentration promote hybridisation rates*

^c *competes with any remaining free nucleotides*

^d *blocks non-specific binding to repetitive sequences*

^e *blocks non-specific binding to repetitive sequences*

^f *competes with the polyA tail of the labelled probe*

^g *forms matrix which reduces effective volume, creating favourable reaction kinetics*

#20X saline sodium citrate (SSC)

175g NaCl

88g tri-sodium citrate

in 1 litre dH₂O

pH 7.4

***Sodium phosphate buffer**

50% 0.5M Na₂HPO₄

50% 0.5M NaH₂PO₄

pH 7.0

Phosphate buffered saline (PBS)

80g NaCl

2g KCl

11.5g Na₂HPO₄

2g KH₂PO₄

in 1 litre dH₂O

pH 7.5

NB for *in situ* hybridisation experiments, PBS was made up from tablets (Sigma) in order to minimise contamination with RNases or fragmented DNA.

4% paraformaldehyde (PFA)

40g paraformaldehyde (Sigma)

make up to 1 litre using PBS

Heat whilst stirring to <60°C, add a few drops of conc. NaOH solution to aid dissolving, correct pH to 7.5

TE buffer (for ISHH probe purification with NICK columns)

10mM Tris-HCl

1mM EDTA (Sigma)

pH 7.5

TEA/AA (pre-treatment of slides for ISHH)

19.5g triethanolamine

9g sodium chloride

in 1 litre ddH₂O

pH 8.0

2.5ml acetic anhydride to be added immediately prior to use. The solution is useable for 2 hours following addition of acetic anhydride.

Appendix Three: Pre-treatment protocol for ISHH

Sections were brought up to room temperature and then passed through a series of solutions as follows:

- i. freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS) for 5 minutes *(to lightly fix the tissue, thus preserving cellular morphology and RNA)*,
- ii. two rinses in PBS,
- iii. 100mM triethanolamine HCL / 164mM sodium chloride solution containing 0.25% acetic anhydride, for 10 minutes *(which coats the slide with negative charge, thereby preventing non-specific binding of negatively charged probes)*,
- iv. graded alcohols (70%, 1 minute; 80%, 1 minute; 95%, 2 minutes; 100%, 1 minute) to dehydrate the tissue sections,
- v. chloroform for 10 minutes *(to remove lipids, which might otherwise contribute to non-specific binding)*,
- vi. 100% ethanol for 1 minute, followed by 95% ethanol for 1 minute, to partially rehydrate the tissue,

All sections were air dried and stored at -20°C.

Appendix Four: Development of autoradiograms

Sections were apposed to Kodak BioMax MR film for a time period determined in each pilot study. The film was then processed by hand under safelight conditions as described below:

- i. 5 minutes in a 20% solution of PQ universal developer (Ilford)
- ii. 30 seconds in a 2% solution of glacial acetic acid
- iii. 5 minutes in a 20% solution of Hypam fixer (Ilford)
- iv. 10 minute wash in tap water

All solutions were made using double-distilled water. Each film was hung to air dry.

Appendix Five: Emulsion dipping protocol and development of sections

The dipping procedure was carried out under safelight conditions. Each slide was dipped into liquefied photographic emulsion (EM-1 Hypercoat emulsion, Amersham Biosciences, Little Chalfont, UK), which had been diluted 2:1 with 2% glycerol in double-distilled water. The back of each slide was then wiped dry and carefully placed into a bioassay tray. Each tray was wrapped in several layers of aluminium foil to protect the sections from light exposure. The next day the slides were racked up and placed into light proof boxes, which were again covered with aluminium foil. The slides were kept at 4°C until developed.

Sections were developed after an exposure time 2-4 times as long as was necessary for autoradiography. Slides were allowed to warm up to room temperature and then developed (again under safelight conditions) by passing the slides through the following solutions:

- i. 2 minutes in a 20% solution of Phenisol developer (Ilford)
- ii. 30 seconds in 2% acetic acid
- iii. 5 minutes in a 30% w/v solution of sodium thiosulphate
- iv. 30 minutes in cold running tap water
- v. brief rinse in double-distilled water

Sections were then lightly counterstained with 0.25% cresyl violet.

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