Examining Potential Cellular Alterations within the Anterior Cingulate Cortex in Major Depression and Suicide

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“Nothing can bring you peace but yourself”
- Ralph Waldo Emerson
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Abstract

Representing a major public health concern, suicide is a leading cause of death worldwide. Generally regarded as a behavior with a multitude of state and trait dependent risk factors (e.g. psychiatric disorders, substance abuse, genetics), explanations as to why certain individuals commit suicide while others do not are complex. Of interest is in studying potential trait dependent variables involved in the neurobiology of suicide, particularly at the cellular level. Knowledge of the cellular integrity may aid in explaining the observed macroscopic alterations and ultimately the behavioral correlates associated with suicidality. Therefore we set out to summarize extant knowledge of the cellular alterations occurring in the brains of major depressive and suicide individuals. Following this, we conducted our own cellular investigation in a region known to be altered in major depression and suicide, a supracallosal area of BA24a. Neuronal and glial cell densities as well as neuronal cell sizes were assessed in upper and lower cortical layers between sudden-death controls and MDD suicide subjects. Secondary analyses were also conducted to examine the effect of alcohol on depressed suicides. Analyses of cell densities and neuronal soma sizes between controls and MDD suicide subjects did not reveal any significant differences. Further analyses showed increased glial cell densities in alcoholic depressed suicides. Future studies are necessary to examine explicit changes in the cellular compositions occurring in alcoholic dependent individuals. Staining techniques aimed at targeting specific subtypes of neurons and glial cells will help determine if these cell populations do in fact have an influential role in suicide and MDD.
Résumé

Chapter 1: Introduction

1.1 DEFINING SUICIDAL BEHAVIOR

Being a multifaceted behavior, no simple explanations exist regarding the “state of mind” of suicidal individuals. While suicidal completion refers to the most definitive and unequivocal act of completed suicide, heterogeneity exists amongst suicide attempters. Among the latter, the spectrum ranges from highly lethal and failed suicide attempts, in which high intention and planning are evident, and survival is the result of good fortune, to low-lethality attempts whereby attempts are often impulsive and triggered commonly by an interpersonal conflict and often contain a strong element of an appeal for help (Beek et al., 1976). Thus, Beck and colleagues (1976), suggested that the act of suicide encompasses two dimensions, intent and lethality, which are positively correlated with each other. Intent refers to the degree of an individual’s wish to die which is assessed by the extent of the desire to die and the likelihood of discovery. Lethality constitutes the degree of medical damage an individual suffered as a result of their suicide attempt. While an individual may have high intent to die, he/she may underestimate the lethality of the chosen method, resulting in survival. Because of this, suicide attempters represent a heterogeneous group of individuals with a diverse range of behaviors. Similarly, suicide and attempted suicide rarely occur outside a context of a psychiatric disorder. It has been estimated that over 90% of suicide cases have had a previous diagnosable psychiatric illness, with mood disorders being among the most commonly associated to suicide or serious suicide attempt
(Rich et al., 1988; Beautrais et al., 1996). Among the latter, major depressive disorder (MDD) is a significant precursor where it has been inferred to underlie most suicides (Black et al., 1985). Specifically, MDD is observed to be diagnosed in more than half of completed suicides (Murphy, 1986). In addition, it is believed that close to 10% of patients with a depressive disorder will commit suicide, and a reported 16% of depressives will have attempted suicide at some point in their lives (Chen & Dilsaver, 1996; Kessler et al., 1999). Consequently, it remains a challenge to fully define and classify individuals whose attempt will result in a fatal act.

1.2 POSSIBLE MODELS FOR SUICIDALITY

Like most psychiatric disorders there are no simple explanations as to why certain individuals choose to end their own lives while others do not. In trying to understand this devastating act, putative models for suicide have been proposed focusing on identifying and classifying potential risk factors. One model referred to as the stress-diathesis model, previously put forth by John Mann (2002), has classified risk factors for suicide in terms of belonging to one of two domains: the trigger domain and the threshold domain. The trigger or stress aspect in this model is state-dependent and encompasses most of the features assessed clinically such as acute drugs/alcohol, medical illness and family/social stress. On the other hand, the threshold domain or diathesis include factors such as genetic makeup, biological variability, chronic illnesses such as chronic substance or alcohol, and dietary factors leading to low cholesterol levels. According to John Mann, the overlap between an increased predisposition to suicide (from threshold domain factors) in addition to stressors
from the trigger domain may make a person more susceptible to committing suicide. Therefore, a propensity for more severe suicidal ideation together with a greater likelihood of acting on powerful feelings place some individuals at a greater risk for suicide.

A second model has been referred to as the process model of suicidal behavior. Again this model is based on a state-trait interaction approach but additionally describes the development and progression of suicidality as a process occurring within individuals and in interaction with their surroundings (Van Heeringen, 2001). According to this model, suicidality evolves from the initial thoughts of focusing on taking one’s life to recurrent suicide attempts, with increasing lethality and suicide intent, and finally ends with the completed act of suicide. The process model describes 3 levels of risk factors: trait-dependent and state-dependent factors, described in psychological and biological terms, and threshold dependent factors. Studies conducted in the cognitive psychological domain have singled out 3 main characteristics constituting the trait-dependent predisposition to suicidal behavior (Williams & Pollock, 2001). First, when faced with psychosocial stressors there is a tendency to see oneself as a loser and have perceptions of “defeat”. Second, perceptions of “no escape.” Such perceptions are often associated with 1) problem-solving deficits instigating a perception of entrapment and 2) impairments in memory, particularly, autobiographical. Lastly, perceptions of “no rescue” or feelings of hopelessness.

Taken together, these models stress the importance of identifying potential risk factors which would make certain individuals more susceptible to
suicide. Over the past 30 years, prospective studies have identified an array of predictive characteristics that place individuals at an increased risk for suicide.

1.3 RISK FACTORS FOR SUICIDAL BEHAVIOUR

One of the best predictors of suicidal behavior is a history of suicide attempts and current suicidal ideation (Leon et al., 1990; Brent et al., 1994). However, studies have shown that the overall rate of suicide at first attempt can be as high as 75%, often employing more fatal methods (Maris, 1981; Isometsa & Lonnqvist, 1998). Therefore a history of suicide attempts is not sufficient to predict most suicides; additional risk factors need to be considered.

1.3.1 Personality Correlates

Representing emotional, behavioral, motivational, interpersonal, experiential and cognitive styles, personality traits help us relate to and cope with the world (McCrae & Costa, 1997; Krueger et al., 2000). Personality disorders are diagnosed in approximately 9% to 28% of completed suicides, with the significance of a personality disorder as a risk factor for attempted suicide being even greater, with rates as high as 55% among attempters (Soloff et al., 1994). Among the personality traits often associated with those who attempt suicide are impulsivity and aggressiveness whereby suicide attempters are not only more aggressive toward others and their environment but also display more impulsive behaviors in, for example, relationships or personal decisions about a job or purchases (Mann et al., 1999; Conner et al., 2003). Arguments have been made suggesting that it is the association between impulsivity with aggressive behaviors that puts an individual at an increased
risk for suicide (Apter et al., 1993; Turecki, 2005). Additional personality features shown to cluster in suicidal individuals include those persons who are introverted, negativistic, avoidant, dependent, neurotic, hostile, and antisocial (Engstrom et al., 1997; Rudd et al., 2000).

1.3.2 Behavioral Correlates

Behaviorally, suicide attempters, in comparison to non-attempters, experience more subjective depression and greater measures of hopelessness, in particular, having more severe suicidal ideation (Beck et al., 1974; Kim et al., 2003). The development of hopelessness is often related to interpersonal factors, such as burdensomeness and failed belongingness (Perez-Smith et al., 2002). An Interpersonal-Psychological Theory of Attempted and Completed Suicide has recently been put forth by Joiner (2002). Based on this theory, suicidal individuals feel hopeless specifically about feelings of being a burden on others and of failed belongingness. Suicidal persons also show fewer reasons for living, lower self-esteem (Daskalopoulou et al., 2002; Osvath et al., 2004) and social isolation (Trout, 1980; Joiner et al., 2005). Defined as “a state in which interpersonal contacts and relationships are disrupted or nonexistent” (Trout 1980), social isolation has been consistently related to suicidal behavior. Joiner (2002) suggests that this need to be social is so powerful that, when satisfied, it can prevent suicide; however, when the need for social connection is extinguished, the risk for suicide greatly increases. Lastly, though findings tend to be mixed, suicide attempters and victims often show more anxious behaviors
whereby suicide rates for those with an anxiety disorder range from 6% to 60% (Noyes, 1991).

1.3.3 Clinical Correlates

Clinical features shown to increase the risk for suicidal behavior consist largely of comorbid substance abuse and alcoholism (Murphy, 1988; Roy et al., 1990; Murphy et al., 1992) whereby approximately 18% of alcoholics will eventually die by suicide (Roy & Linnoila, 1986). However, reviewing epidemiological literature, Murphy and Wetzel (1990) found that the lifetime risk of suicide among individuals with alcohol dependence treated in out-patient and in-patient settings was 2.2% and 3.4%, respectively. Nonetheless, alcoholics are seven times more likely, compared to the general population, to be at a lifetime risk for suicide (Gorwood, 2001). Individuals at risk for suicide also tend to suffer more stressful life events including interpersonal conflict, interpersonal loss, legal and/or disciplinary problems and family discord (Orbach, 1989; Brent et al., 1993). A history of childhood abuse and neglect are also among stress-related factors contributing to an increased vulnerability to suicide where it has been suggested that childhood trauma may play a role in the age of onset along with the frequency of suicide attempts (Brodsky et al., 2001; Roy, 2004).

1.3.4 Familial and Genetic Correlates

There is mounting evidence suggesting a familiar and genetic link to suicidal behavior. Suicide attempters and completers are reported to have an
increased rate of suicidal acts within their families (Roy, 1983; Turecki, 2001; Mann et al., 2005). Specifically, first- and second-degree relatives of adolescent and adult suicide attempters (Murphy & Wetzel, 1982; Pfeffer et al., 1994) and completers (Tsuang, 1983; Brent et al., 1996; Kim et al., 2005) are at an increased risk for suicidal behaviors compared to relatives of non-suicidal individuals. Striking evidence suggests that relatives of suicide completers are over 10 times more likely than relatives of comparison subjects to attempt or complete suicide, even after controlling for psychopathology (Kim et al., 2005). From family studies we are unable to differentiate between genetic and environmental causes (e.g. separation or loss, imitation of a family member's suicide and the presence of violence or hostility within the family) for transmission of suicide. However, findings from twin and adoption studies grant us the ability to better understand the genetic contribution to suicidal acts.

1.3.4a Twin and Adoption Studies

There have been consistent reports indicating that monozygotic twins, in comparison to dizygotic twins, show higher concordance rates for suicides (Roy et al., 1991; Statham et al., 1998) and for suicide attempts (Roy et al., 1995). While both monozygotic and dizygotic twins are thought to be exposed to similar environmental settings they differ genetically. Monozygotic twins are genetically identical while dizygotic twins share about fifty percent of their genome, therefore higher concordance rates for suicides in monozygotic twins (13.2%), in comparison to dizygotic twins (0.7%), would indicate a greater genetic influence on suicidal behavior (Roy et al., 1995). Similarly, adoption studies have also been
applied in order to distinguish genetic from environmental influences with respect to suicidal behavior. While adopted individuals and their adoptive parents share common environmental experiences, they do not share genetic material. Opposed to this, individuals who were adopted early in life share genetic material with their biological parents, however they lack common environmental experiences. Therefore, an adoptee’s suicidal behavior that is linked to a familial history of suicide in their biological family would lead to inference that the suicidal behavior must be at least partly due to genetics factors. Original adoption studies come from a Danish population whereby a six-fold increase in suicide rates was reported among the biological relatives of suicide adoptees in comparison to control adoptees (Schulsinger, 1979). Intriguingly, in both control and suicide adoptees, there were no reported cases of suicide among adoptive relatives. Such findings provide further support for a genetic influence (possibly suggesting a predisposition to the act of suicide) rather than an environmental cause on suicidal behavior.

As previously mentioned, a strong association exists between suicide and other psychiatric disorders. Therefore such observations from familial, twin and adoption studies could be the result of mental illness and not completely due to an independent genetic influence on suicide. Several studies have addressed this issue and in large part, findings hold true even after accounting for rates of psychoses and mood disorders. For example, studies of familial transmission have shown that parents of youth suicide victims have an increased rate of suicidal behavior which was independent of the presence of psychopathology (Brent et al., 1996). Similarly, the suggestion that suicide may be independent of
psychiatric diagnosis comes from an Amish study by Egeland & Sussex, (1985). They looked at 26 confirmed suicides in an Amish community and found that 92% of the suicides were diagnosed with a major affective disorder, and 73% of the suicides were from four multigenerational families, who also had a very high genetic loading for bipolar, unipolar, and other affective disorders. However the reverse was not true. Some families had strong loading for major affective disorder but not higher suicide rates thus suggesting that the high rate of suicide may be independent of psychiatric diagnosis. Support for such suggestions can also be found within twin studies, whereby a history of serious suicide attempts in one twin was found to be a powerful predictor in monozygotic pairs, increasing the risk for suicide attempts by ten-fold (Statham et al., 1998). This prediction existed even after considering and controlling for psychosocial and psychiatric variables. In summary, familial, twin, and adoption studies seem to suggest the notion that suicidal behavior is heritable and independent from psychiatric diagnoses. Unfortunately, the specific genetic factors that could be contributing to the suicide risk remain largely unknown.

1.3.4b Candidate Genes Investigated in Suicide

While the exact genetic factors implicated in suicide still need to be defined, the serotonergic system (see section 1.4.1) has been the major focus of attention in candidate gene studies of suicidal behavior. The most commonly investigated serotonin (5-HT) genes include the tryptophan hydroxylase gene (TPH), serotonin 1B receptor gene (5-HT1B), serotonin receptor 2A (5-HT2A) and 1A (5-HT1A) genes, and the serotonin transporter (5-HTT) gene. Due to the
large amount, and often discrepant findings, such candidate gene studies will only be briefly discussed.

TPH is the rate-limiting biosynthetic enzyme for 5-HT synthesis. There are two TPH isoforms; TPH1, expressed in peripheral tissues; and the more recently discovered TPH2, expressed predominantly in the brain and in serotonergic neurons (Walther et al., 2003). Researchers have linked TPH to suicidal behavior suggesting a relationship between an intronic polymorphism in the TPH1 gene and suicide attempt behavior and lower serotonergic function (Nielson et al., 1994; Bellivier et al., 2004; but see Mann et al., 1997). The focus of much current research is now towards understanding the possible associations of TPH2 to suicide and other psychopathologies. Haplotype studies suggest association of the TPH2 gene to completed suicide (Zill et al., 2004) and suicide attempt (de Lara et al., 2007) however others disagree finding no associations (De Luca et al., 2006; Mann et al., 2008).

The 5-HT1A receptor is both a somatodendritic autoreceptor and postsynaptic heteroreceptor involved in controlling the firing rate of serotonergic neurons, thus determining the availability of serotonin in the synaptic cleft (Kamali et al., 2001). Unfortunately, there is a rather vague understanding with respect to associations between the 5-HT1A receptor gene and suicide reports (Nishiguchi et al., 2002; Lemonde et al., 2003; Wasserman et al., 2006).

Promising associations have been made between the 5-HT2A receptor gene allele 102C, and major depression, specifically in patients with suicidal ideation and behavior (Du et al., 2000; Arias et al., 2001; Vaquero-Lorenzo et al.,
2007). However independent research groups have been unsuccessful in reporting such associations (Turecki et al., 1999; Bondy et al., 2000b; Ertugrul et al., 2004).

The 5-HT$_{1B}$ receptor controls the release of serotonin and non-serotonin neurotransmitters and acts as both a presynaptic autoreceptor and a postsynaptic heteroreceptor, respectively, (Barnes & Sharp, 1999; Zifa & Filllon, 1992). In large part, many groups have failed to find associations between this receptor gene and suicide (Nishiguchi et al., 2001; Stefulj et al., 2004; Tsai et al., 2004) however there have been several findings associating the 5-HT$_{1B}$ receptor gene and a family history of suicide attempts in patients with personality disorders (New et al., 2001) and other psychiatric disorders (Moret & Briley, 2000; Mundo et al., 2001). Interestingly, this receptor gene has also been related to impulsive aggressive behavior and suicide (Zouk et al., 2007).

The gene for 5-HTT is considered to be of importance as the serotonin transporter (SERT) plays a critical role in the process of 5-HT reuptake. While some studies have shown an association between the short allele and suicide (Bondy et al., 2000a; Wasserman et al., 2007), others have reported an association between the long allele and suicide (Du et al., 1999), or a lack thereof between this polymorphism and suicide (Fitch et al., 2001; Courtet et al., 2003; de Lara et al., 2006). Such discrepancies may be partially explained by evidence suggesting that the 5-HTT gene may have a greater role in violent traits and thus show more of an association in violent suicides as opposed to non-violent suicides (Courtet et al., 2001; 2003; Zalsman et al., 2001).

Unfortunately results from candidate gene studies are somewhat inconclusive owing in part to differences in the phenotypic characterization of
subjects. Most suicide victims have an accompanying psychiatric disorder therefore it remains a challenge to dissociate such disorders from suicidal behavior. Nonetheless, studying candidate genes remains an interesting avenue to gain insight into possible mechanisms involved in predisposition to suicidality.

In summary, each of the aforementioned categories of risk is underlain at least to some degree by specific genetic and neurobiological factors. While evidence for the serotonergic system in suicide has been known for a long time, there is emerging consensus that several other neurobiological systems are implicated in this tragic behavior.

1.4 NEUROBIOLOGICAL SYSTEMS INVOLVED IN SUICIDE

Three neurobiological systems have been evidenced to play prominent roles in the pathophysiology of suicidal behavior. These include dysfunctions of the serotonergic and noradrenergic systems, and hyperactivity of the hypothalamo-pituitary-adrenal axis. The majority of the evidence implicating these three systems comes from postmortem studies honing in on neurochemical alterations, as well as cellular changes. While the former will be briefly discussed in this section, the latter will be more extensively reviewed in Chapter 2. It can be hypothesized that such neurobiological dysfunctions mediate the incidence of suicidal behavior via the altered modulation of basic neuropsychological functions, such as feeling, thinking and behaving.

A monoamine-deficiency hypothesis of depression has been put forth which postulates a deficiency in serotonin (5-HT) or norepinephrine (NE) neurotransmission in the brain (Schildkraut, 1965). Evidence for this hypothesis comes from the use of early antidepressants which blocked the reuptake of NE
and 5-HT by the presynaptic neuron (Hirschfeld, 2000). The effects of this pharmacologic action are to increase the availability of NE and 5-HT in the synapse and to increase stimulation of the postsynaptic neuron. Additionally, inhibitors of the enzyme monoamine oxidase, an enzyme responsible for catabolizing NE and 5-HT in their respective presynaptic neurons, were also discovered to have antidepressant effects (Crane, 1956; Iversen, 1965). Inhibitors of this enzyme could be expected to increase the availability of neurotransmitters. Taken together, such discoveries led to a major theory of depression known as the monoamine-deficiency hypothesis.

1.4.1 Serotonergic System

Perhaps one of the most heavily studied neurobiological systems involved in suicide has been the serotonergic system. 5-HT can be found in neurons, platelets, mast cells, and the enterochromaffin cells. Only a small amount of 5-HT is actually found in the brain (approximately 1–2%) and because it cannot cross the blood-brain barrier, it has to be synthesized in the central nervous system (Kamali et al., 2001). The primary step in its synthesis is the uptake of TPH from the plasma, arising for the most part from the diet. A constant supply of TPH is needed to the brain otherwise within a few short hours; brain serotonergic levels will be significantly diminished (Delgado et al., 1990). General evidence suggests there to be a serotonergic hypofunction occurring in the brains of suicide individuals, particularly in more lethal suicidal acts (Mann et al., 1992; Malone et al., 1996). It has been proposed that such a low serotonergic function may be considered a trait marker for suicide. This observation was supported by several
research groups whom documented that low cerebrospinal fluid (CSF) levels of 5-HT's major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), predicted future suicide and suicide attempts in psychiatric patients with mood disorders (Roy et al., 1989; Nordstrom et al., 1994). Additionally, reduced serotonergic levels have been reported in suicide victims (Cheetham et al., 1989) with studies showing alterations specific to the orbital frontal cortex of suicide victims (Mann et al., 2000; Arango et al., 1995). 5-HT receptor populations have also been evidenced to be altered in the brains of suicide victims showing increases in 5-HT$_{1A}$ postsynaptic receptors in the ventral prefrontal cortex (PFC), in the temporal and entorhinal cortex in schizophrenic suicides (Joyce et al., 1993; Arango et al., 1995) and recently in the dorsal raphe nuclei (Boldrini et al., 2007), although the evidence has been inconsistent (Mann & Arango, 1999; Cheetham et al., 1989). Of interest, Joyce and colleagues (1993) reported, in more than 90% of the areas under investigation, a negative correlation between 5-HT$_{1A}$ and serotonin transporter binding. This suggests that a common influence on the 5-HT$_{1A}$ and serotonin transporter binding results in upregulation of the post-synaptic 5-HT$_{1A}$ and reduction of the nerve terminal SERT in the suicide group.

As a number of antidepressants are potent antagonists of the 5-HT$_{2}$ receptor it has become the focus of much attention in postmortem studies of suicide along with the more recent 5-HT$_{2A}$ subtype. While again, findings are not uniform (Lowther et al., 1994; Stockmeier et al., 1997) overall there appears to be an increase in 5-HT$_{2A}$ receptor density in certain areas of the PFC (Stanley & Mann, 1983; Hrdina et al., 1993; Turecki et al., 1999). It has been hypothesized that the increase in 5-HT$_{2A}$ receptor density within the PFC might be in part, a
consequence of decreased 5-HT release, suggesting the presence of a possible compensatory mechanism for this reduced serotonergic activity (Arango et al., 1997). In a more recent study, significantly lower binding potential of frontal 5-HT\textsubscript{2A} receptors were shown in attempted suicide patients when compared with control subjects (Van Heeringen et al., 2003). Interestingly, the patients who had attempted suicide also had higher levels of hopelessness, higher scores on the temperament dimension harm avoidance, and lower scores on the character dimensions self-directedness and cooperativeness. The authors also found significant correlations between harm avoidance, hopelessness and binding index in this same group.

One index of 5-HT nerve terminal innervation of cortical regions is through assessing SERT binding (Zhou et al., 1995). Therefore studying SERT in brains of suicide victims has been of interest. Findings seem to be discrepant which may partially be the consequence of different ligands used and more importantly findings tend to differ based on the brain region under investigation. There is evidence to suggest reductions in SERT binding in suicide completers which may be specific to the PFC regions (Mann et al., 2000) while increases in SERT have recently been shown in bipolar patients in more limbic areas such as the thalamus, the dorsal cingulate cortex, medial prefrontal cortex and insula (Cannon et al., 2006). Specifically, it was found that subjects with a history of suicide attempts showed an increase in binding within the anterior cingulate cortex (ACC) while reductions in SERT binding were shown in the forebrain.

From these postmortem studies findings seem to be region specific implicating largely the PFC, the medial prefrontal cortical (mPFC) areas and the
brainstem. The majority, but not all of studies, indicates that 5-HT receptor populations are altered in suicide victims. Decreases in presynaptic binding sites (e.g. reduced SERT) within the PFC and increases in such binding sites in the mPFC (e.g. ACC) and the brainstem (e.g. raphe nuclei) have been largely observed as well as an upregulation of the postsynaptic receptors, 5-HT$_{2A}$ and 5-HT$_{1A}$. Although abnormalities in serotonergic function have been the primary focus for studies on suicidal behavior, there is building evidence indicating that abnormalities of noradrenergic function may also be involved in the pathophysiology of suicide.

1.4.2 Noradrenergic System

Since the 1960s, noradrenaline (NE) has been recognized to play an integral role in MDD and thus suicidal behaviors. The noradrenergic hypothesis was based largely upon observations of the effects of drugs on mood in humans. Studies revealed that when drugs were given, known to deplete NE, depressive symptoms were observed in the patients and likewise, drugs that elevated the synaptic availability of NE alleviated the depressive state (Prange, 1964). Within the brain, the principle site for synthesis of NE comes from the locus coeruleus (LC), a structure located in the floor of the fourth ventricle. In humans, the LC consists of approximately 45,000 cells whereby these noradrenergic cells project to nearly all regions of the brain, including limbic structures such as the amygdala, hippocampus, and thalamus (Moore & Bloom, 1979). The activity of LC neurons is modulated by various sensory inputs and environmental factors such as stress, a known causal agent to suicidal behavior (Valentino et al., 1983;
Weiss et al., 1994). Thus it is not surprising that research has begun to focus on this brain region in suicide individuals. Although not as extensive as 5-HT, NE neurotransmission dysregulations have also been identified in suicide victims.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of NE. The expression of TH in LC neurons can be altered by environmental and pharmacological stimuli that affect the activity of LC neurons (Melia & Duman, 1991). Therefore TH is an ideal candidate protein to detect changes in LC neurochemistry in suicide. The majority of evidence suggests that TH content in the brains of suicide victims is increased compared to control individuals (Ordway et al., 1994a; Zhu et al., 1999 but see Biegon & Fieldust, 1992; Baumann et al., 1999). The observed elevated expression of TH in the LC in suicide individuals may imply a pre-mortem over-activity of these neurons; similarly, such increases could be the consequence of a deficiency of the cognate transmitter, NE.

There are three major classes of adrenergic receptors: $\alpha_1$, $\alpha_2$ and $\beta$-adrenoceptors. $\alpha_2$-adrenoreceptors are of particular interest because a small but relevant proportion (20-40%) in the brain appear to be presynaptic inhibitory autoreceptors (U'Prichard, 1984; Simson & Weiss, 1989). Therefore, these receptors play a critical role in regulating the release and synthesis of NE. Initial findings suggest significant increases in $\alpha_2$-adrenergic receptors within the LC of suicide victims relative to controls (Ordway et al., 1994b). Elevated binding to $\alpha_2$-adrenergic receptors has also been documented in other brain regions, notably the PFC (Meana et al., 1992; González et al., 1994), temporal lobe (De Paermentier et al., 1991a), and hippocampus (González et al., 1994). However, similar to 5-HT, inconsistencies exist within the literature (Arango et al., 1993;
Gross-Isseroff et al., 2000). Taken together such observations indicate that the high-affinity state of the $\alpha_2$-adrenergic receptor is upregulated within the LC and in other areas of the brain among suicide victims.

There have been a limited number of studies attempting to quantify $\beta$-adrenoreceptor binding in the brains of suicide victims. These receptors are also of importance as it has been shown, in animals, that chronic administration of antidepressant drugs results in adaptive changes in central noradrenergic receptors. Specifically, when antidepressants were given, a down-regulation of $\beta$-adrenoreceptor binding sites was observed in the rat frontal cortex (Banerjee et al., 1977). This reduction of central $\beta$-adrenoreceptors has a similar time course to that of the clinical improvement in depressed patients receiving antidepressant therapy. Such observations have initiated speculation that the former effect could be involved in the mechanism of action of antidepressants and those abnormalities in $\beta$-adrenoceptor function might be associated with depressive illness and thus suicide. Again, reports are conflicting and at present there seems yet to be a determined consensus as to whether these receptors are increased (Mann et al., 1986; Biegon & Israeli, 1988; Arango et al., 1990), decreased (De Paermentier et al., 1991b) or remain unaffected (Ferrier et al., 1986; Stockmeier & Meltzer, 1991) within suicide individuals.

Explaining such neurochemical postmortem evidence, has led some to hypothesize that suicide victims experience chronic activation of the LC (Ordway, 1997). The mechanism behind this activation could be through exposure to chronic stress or through inefficiency of the LC to over-respond to normal daily stressors. Consequently, this long-term activation could lead to a depletion of
synaptic NE and a resulting increase in the expression of TH and an upregulation of α and β adrenergic receptors within the LC.

1.4.3 Hypothalamo-Pituitary-Adrenal-Axis

Chronic stress and its biological consequences are considered to be trait-dependent risk factors in major depression and in an individual’s likelihood to commit suicide. Clinical research has consistently demonstrated that stressful life events precede the onset, maintenance, and relapse of depressive symptomology (Billings & Moos, 1982; Swindle et al., 1989). Physiological responses to chronic stress are mediated by the hypothalamo-pituitary-adrenal (HPA) axis. When a person undergoes a stressful situation a number of hormonal changes occur in the body altering the HPA axis. Briefly, in response to stress the hypothalamus, first, releases corticotrophin-releasing hormone /factor (CRH/CRF) and arginine vasopressin (AVP) which acts upon the pituitary gland. The pituitary gland then secretes adrenocorticotropic hormone (ACTH) which signals to the adrenal glands to release cortisol. When concentrations of cortisol have reached an appropriate level, negative feedback mechanisms signal the pituitary gland to stop releasing ACTH. A second fast feedback process is also used and is dependent on the rate of change in concentration of cortisol. This feedback system involves interactions with both mineralcorticoid and glucocorticoid receptors in the hypothalamus and hippocampus (Varghese & Brown, 2001). Functioning as the “executive” within the HPA axis, the hippocampus participates in suppressing a stress response through glucocorticoid (GC)-mediated negative feedback which in turn inhibits the HPA axis (Sapolsky et al., 1986). Consequently, in a chronic state of stress,
the hippocampus becomes overexposed to GCs. Such saturation is damaging to
the hippocampus due to the fact that cortisol is neurotoxic. Therefore, damage to
the hippocampus causes a disinhibition of GC feedback further leading to
activation of the HPA axis. Subsequently, GC levels rise, further damaging the
hippocampus. This vicious cycle is known as the glucocorticoid cascade
(Sapolsky et al., 1986).

A common test used to examine HPA axis functioning is the
dexamethasone suppression test (DST). In this test a dose of synthetic
corticosteroid dexamethasone is given in the evening. Cortisol samples are then
taken the following day. A normal response is inhibition of cortisol release. If
cortisol is not decreased below a certain level, the patient is said to have DST
nonsuppression. This failure to suppress cortisol is considered evidence for HPA-
axis hyperactivity (Coryell & Schlesser, 2001). Often paired with this test is a
CRH challenge test where subjects are immersed in a stressful situation and levels
of cortisol are measured through saliva samples. When individuals undergo a
stress task increased cortisol secretion, as well as an increased corticotropin
release are seen in the combined dexamethasone suppression/CRH stimulation
test (Nemeroff, 2002). As stress is a factor involved with depression and suicidal
behavior, this combined test has been useful for these individuals. Much
research has indicated that depression is accompanied by deregulations of the
HPA axis resulting in elevated cortisol levels. Gibbons & McHugh (1962)
provided the first report in humans, with depression, showing that these
individuals secrete excessive quantities of cortisol and exhibit insensitivity to GC
feedback inhibition. Building from this, current work has shown that both an
excess of cortisol and DST nonsuppression are observed in patients with mood disorders where dexamethasone nonsuppression occurs in approximately half of individuals with major depression (Arana & Mossman, 1988). Further, depressed subjects show a strong increase in the activity of the CRH neurons (Raadsheer et al., 1994) along with increased CRH CSF levels (Nemeroff, 1984; Banki et al., 1987). These observations have lead some to the “cortico-releasing factor hypothesis of depression” suggesting that increased CRH levels or that impairment in the negative feedback of cortisol could account for HPA overactivity, leading to depression and in some cases suicide (Nemeroff, 1996; Young et al., 1991). A number of studies have also found evidence for the involvement of the HPA axis in suicide victims. It has been predicted that dexamethasone nonsuppression confers a four-fold increase in risk for eventual suicide (Coryell et al., 2006). Additionally, the adrenal glands of suicide victims were shown to be larger than those of matched violent controls (Dorovini-Zis & Zis 1987; Szigethy et al., 1994), fewer binding sites of CRH receptors were found in the frontal cortex (Nemeroff et al., 1988), and an elevated level of CRH was found in the CSF (Arato et al., 1989) as well as in the frontopolar cortex (Merali et al., 2004) of suicide individuals. Recently, increased levels of CRH were observed in the LC and raphe nuclei in depressed suicide subjects (Austin et al., 2003). Merali and colleagues (2006) also found increased CRH levels along with other stress-related neuropeptides in the LC, the paraventricular hypothalamic nucleus, and several frontal cortical regions in suicide victims. Contrarily, other studies have failed to document such changes in CRH levels in CSF both in major depressive subjects (Roy et al., 1987; Pitts et al., 1995) and suicide individuals (Charlton et
al., 1988). Unchanged CRH receptor binding sites have also been reported in the cortex of depressed suicides (Hucks et al., 1997). Thus such observations would go against the CRH hyperactivity hypothesis of depression and consequently suicide. While it is important to recognize discrepancies, to date, the majority of studies provide evidence for the occurrence of stress related changes in major depressives and suicide victims. Interestingly, animal studies have eloquently illustrated that chronic levels of stress can alter 5-HT release (Keeney et al., 2006; Leonard, 2005). Specifically, decreased 5-HT$_{1A}$ (Crayton et al., 1996), and 5HT$_{2A}$ receptor binding (Takao et al., 1997) as well as decreased levels of 5-HT (Luine et al., 1993) have been observed when chronic corticosterone is administered. Additionally, CRH has been implicated in mediating the effects of stress on NE neurotransmission, specifically in the LC at the level of the noradrenergic neurons (Valentino et al., 1991; Melia & Duman, 1991). Such findings add further support to both, the serotonergic hypothesis for suicidal behavior suggesting that 5-HT receptors may play a partial role in controlling affective states (Chaouloff et al., 1999) as well as the noradrenergic hypothesis. Their modulation by corticosteroids provides a potential mechanism by which these hormones could regulate mood. These data may also provide a biological understanding of how stressful events may increase the risk for suicide in vulnerable individuals.

The interplay between these three systems further supports the suggestion that the etiology of suicide is unlikely due to one dysfunctional neurobiological system, but rather is the consequence of abnormalities occurring within numerous biological networks. Therefore it is not surprising that several brain regions have been targeted in studies investigating suicide. With the advent of novel imaging
techniques it has been possible to view key structures involved in major depression whereby inferences from these imaging studies can be made towards suicide cases.

1.5 BRAIN REGIONS IMPLICATED IN SUICIDE: FOCUS ON THE ANTERIOR CINGULATE CORTEX

Macroscopic evidence firmly implicates six major areas as sites of functional and structural abnormalities in major depression and therefore suicide; prefrontal cortex, ACC, amygdala, hippocampus, raphe nucleus and the LC. While these brain regions will be discussed in chapter 2, at this time, attention will be paid to the ACC.

1.5.1 Anterior Cingulate Cortex (BA24)

The ACC is part of the brain’s limbic system and is located in the medial prefrontal cortex. It lies on the medial wall of the cerebral hemisphere and wraps around the corpus callosum. The ACC is a heterogeneous region in terms of its cytoarchitecture, connectivity and function. Classically, the ACC has been related to affect, on the basis of lesion studies in both humans and animals (Corkin et al., 1979; Kennard, 1954). However, in the late 1980’s, came the introduction of more precise methods for studying anatomical connectivity, cytoarchitecture and function, making clear that the ACC encompasses numerous specialized subdivisions sub-serving a vast array of cognitive, emotional, motor, nocioceptive and visuospatial functions (Devinsky et al, 1995; Bush et al, 2000). Several cytoarchitecturally distinct regions have been identified within the ACC. These include a dorsal cognitive division (Brodmann areas (BA) 24a, 23b, 24c and 32).
and a rostral–ventral affective division (rostral areas 24a–c and 32, and ventral areas 25 and 33) (Vogt et al., 1995). The dorsal cognitive subdivision has reciprocal connections with the dorsolateral prefrontal cortex (BA46/9), parietal cortex (BA7), premotor and supplementary motor areas and is part of a distributed attentional network (Devinsky et al., 1995). Specifically, this subdivision is involved in a number of different functions, including detection of response conflict and processing of cognitively demanding information, monitoring competition, complex motor control, motivation, novelty, error detection and working memory (Vogt et al., 1992; Devinsky et al., 1995; Drevets & Raichle, 1998). In contrast, the affective subdivision has extensive connections to the amygdala, periaqueductal gray, nucleus accumbens, hypothalamus, anterior insula, hippocampus, and orbitofrontal cortex. These connections allow the ACC to act as a bridge between the limbic structures and the frontal lobe. Therefore, the affective subdivision functions to integrate cognitive activity with affective experience being primarily involved in assessing the salience of emotional and motivational information and in the regulation of emotional responses (Vogt et al., 1992; Devinsky et al., 1995; Drevets & Raichle, 1998). There is mounting evidence displaying abnormalities within the affective subdivision of major depressive individuals and suicide completers.

1.5.2 Role for the ACC in Major Depression and Suicide

The rostral ACC has been implicated as a neural correlate of MDD. Macroscopically, abnormal function and structure have been observed in neuroimaging studies whereby depressed patients display decreases in regional
cerebral blood flow, (Kennedy et al., 2001; Gonul et al., 2004), volume reductions (39-48%) in the subgenual (Drevets et al., 1997) and supracallosal areas of BA24 (Caetano et al., 2006), significant bilateral gray and white matter volume reductions (approximately 20%) and sulci widening (Ballmaier et al., 2004). The ACC also acts as a therapeutic target for deep brain stimulation (DBS) where chronic stimulation of white matter sustained remission of depression in treatment resistant depressive patients (Mayberg et al. 2005). Anterior cingulotomies are also gaining in popularity for treating extreme cases of MDD (Steele et al., 2007). Dysfunctions within the ACC and its circuitry have also been described behaviorally in depressed individuals, showing impaired problem solving, maladaptive behavioral regulation, abnormal error monitoring, and increased sensitivity to sad events (Davidson et al., 2002). Additionally, the ACC is thought to be involved in mediating many behaviors influenced by stress considering it acts as a major target for GC’s which are involved in the stress response (Diorio et al., 1993; Devinsky et al., 1995). As previously mentioned (see section 1.4.3), it has been well documented that the hippocampus plays a major role in stress responses via its involvement with the HPA axis (McEwan et al., 1979; Sapolsky, 2000; Gusnard et al., 2001). Considering stressors are postulated to act as trait-dependent risk factors in MDD and suicide, one could reason that other structures connected to the hippocampus, i.e.: the ACC, also show abnormalities particularly at the cellular level. Indeed, MDD patients are reported to show reductions in glutamatergic concentrations, specifically glutamine and glutamate, suggesting alterations within the glutamatergic system (Auer et al., 2000; Mirza et al., 2004). Following from such studies, recent microscopic investigations have begun to
look at potential cellular alterations within MDD and suicide which may offer fine-detailed explanations for the observed macroscopic changes.
OBJECTIVES

The current thesis aims to investigate possible cellular alterations occurring in the brains of suicide individuals, focusing on those who met criteria for MDD. Of particular interest was a region located within the affective subdivision of the ACC - BA24a - as subdivisions within this region have shown evidence for cellular changes occurring in MDD subjects (see Chapter 2). However, in most studies, only a portion of the psychiatric groups died by suicide so it remains unclear as to the histopathology occurring within these individuals. Therefore, there are essentially two driving questions guiding the thesis, first, to date, what does the literature suggest regarding cellular alterations, not only within the ACC but in the other suicide relevant brain regions? Second, are there density and soma size differences, with respect to neurons and glial cells, occurring in BA24 between a complete depressed suicide cohort and control individuals?

HYPOTHESIS

We hypothesized that cellular compositions, specifically, glial density and neuronal soma size and density would be altered in BA24a, between suicide completers diagnosed with MDD and control subjects.
References


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Chapter 2:  
Through the Looking Glass: Examining Neuroanatomical Evidence for Cellular Alterations in Major Depression and Suicide.


2.1 PREFACE TO MANUSCRIPT IN PREPARATION

Recently there has been a surge of investigations focusing on possible microscopic alterations occurring in the brains of suicide and major depressed individuals. While such studies include neurochemical and biochemical changes, the role for cellular alterations has been of particular interest as they offer potential anatomical explanations for the observed abnormalities within neuroimaging studies. In this chapter we examine the commonalities and differences amongst various literatures in human subjects, and show the involvement for morphometric parameters in neurons and glial cells in the key structures associated with major depression and suicide.

2.2 ABSTRACT

A number of neuromodulatory pathways, largely targeting frontal-limbic brain structures, have been implicated in major depressive disorder (MDD) and thus in the often unfortunate consequence of suicide. Understanding possible neuroanatomical alterations, particularly at the cellular level, in MDD and suicide subjects have been fairly novel and exciting areas of research, aiding to explain gross structural changes often observed in these individuals. Our purpose in this review is to summarize extant knowledge on the microscopic alterations occurring in the main brain regions involved in MDD and suicide. The evidence suggests
that changes in the various morphometric parameters often assessed (cell densities and somal sizes), are generally region specific, and involve certain subtypes of neurons (e.g. serotonergic, GABAergic) and glial cells (e.g. astrocytes). There is a definite need to expand upon these original insights to fully understand the cellular and molecular underpinnings of MDD and suicide.

2.3 INTRODUCTION

Suicide represents a major public health concern, as it is one of the leading causes of death in Western societies. In this perspective, a major challenge in the field of psychiatry is to understand the neurobiological factors in the etiology of major depressive disorder (MDD), a condition strongly predisposing to suicidal behavior (Cavanagh et al., 2003). According to the World Health Organization, MDD affects approximately 121 million people worldwide and is ranked as the main cause of disability. Epidemiological studies indicate that at least 50% of all adult suicides have had a previous diagnosis of depression (Henriksson et al., 1993; Balazs et al., 2003), whereas in adolescents (11-19 years of age), the rate increases to 76% (Shafii et al., 1988). Furthermore, 15% of individuals with a lifetime diagnosis of MDD admit to have attempted suicide at some point in their lives (Chen & Dilsaver, 1996). In addition, the majority (60–70%) of acutely depressed patients experience suicidal ideation, and 10–15% of those with a major depressive episode will end their lives by committing suicide (Moller, 2003).

While advancements have been made towards delineating MDD, a clear understanding as to why certain individuals with MDD commit suicide and others do not remains elusive. A stress-diathesis model which classifies risk
factors for suicidal behavior into those that are trait-dependent and those that are state-dependent has been proposed by Mann (1998). Briefly, the stress aspect in this model is state-dependent and encompasses factors such as acute drugs/alcohol, medical illness and family/social stress. Possible factors influencing a predisposition include: genetics, biological variability, chronic illnesses such as alcohol and/or substance abuse, early life experiences and diet. According to this model, the overlap between increased predisposition to suicide in addition to stressors from the stress domain may increase an individual’s susceptibility to commit suicide (Mann, 1998).

There is epidemiological evidence for a genetic component to suicidal behavior, and progress has recently been made in identifying gene variants which can predispose to MDD and suicide. In this context, the most extensively studied genes are those related to the serotonin (5-HT) system, with genetic variants for various proteins involved in 5-HT transmission having been implicated in MDD (e.g. Caspi et al., 2003; Lemonde et al., 2003; Zill et al., 2004; Zhang et al., 2005). Other neuromodulatory pathways, such as the polyamine (Sequeira et al., 2006) and catecholamine systems (Malafosse et al., 1997; Persson et al., 1997; Hattori et al., 2006) have also been associated to MDD and suicide on the basis of genetic studies. Furthermore, there is evidence that an alteration in the expression of at least one of the genes coding for proteins mediating the stress response is linked to suicidal behavior. Indeed, Wasserman and colleagues (2008) recently associated a single nucleotide polymorphism in the main corticotrophin-releasing hormone receptor (CRHR1)
gene to suicide attempt. Taken together, these promising studies strongly argue for a genetic predisposition to both MDD and suicidal behavior.

What these genetic findings also highlight is that neuromodulatory systems, well-known to facilitate cellular plasticity, are functionally altered in MDD. This is consistent with the now widespread view that plasticity is reduced in key areas of the depressed brain (e.g. Nestler et al., 2002). This view was further supported by recent reports showing dysregulation of the cortical fibroblast growth factor (FGF) system in MDD (Evans et al., 2004), and decreased brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) concentrations in the brains of suicide victims (Dwivedi et al., 2003, 2005). These neurotrophins are involved in key aspects of neuronal and glial development and function, such as cellular proliferation, migration and synaptic transmission (Lewin & Barde, 1996; McAllister, 2001). To gather further evidence that the plasticity of brain cells is challenged in MDD and suicide, fine neuroanatomical studies have been conducted in several brain regions known from neuroimaging analyses to display functional or gross structural alterations in MDD. In addition to offering a much higher spatial resolution than other approaches, studying the microscopic features of postmortem human brain tissues allows to scrutinize subcellular compartments (dendrites, axons, synapses, etc...) and differentiate cell types based on phenotype (neurons versus glial cells) and neurochemical features.

To this day, studies investigating the fine neuroanatomical characteristics of depressed brains have mainly examined neuronal and glial cell densities, and only a few of these descriptions have focused on suicide. Here, we
systematically review this literature for each of the main regions associated with MDD: the prefrontal cortex (PFC) (Baxter et al., 1989; Coffey et al., 1993; Sheline et al., 1998), medial prefrontal cortex (mPFC) (Drevets et al., 1997; Mayberg et al., 2005), amygdala (Drevets & Raichle, 1992), hippocampus (Bremner et al., 2000), raphe nucleus (Boldrini et al., 2005; see review by Stockmeier, 1997), and locus coeruleus (LC) (Klimek et al., 1997; Ordway et al., 1994, 2003). These structures are illustrated in figures 1 and 2. For each of these brain regions, evidence for an involvement in MDD will be briefly presented, followed by a review of the available cell-specific densitometric and morphometric data.

2.4 PREFRONTAL CORTEX

Several lines of evidence support a dysfunctional involvement of the PFC in major depression and suicide. Neuroimaging studies of MDD subjects have shown volume reductions in both gray and white matter, sulcal widening, decrease in glucose metabolism, and alterations in blood flow in the PFC (Cohen et al., 1989; Drevets et al., 1997; Elkis et al., 1996). Recent studies have revealed that subjects with a history of suicide attempts present deficits in PFC-related executive functions, such as impairments in visuospatial conceptualization (planning), visual attention (or reading fluency) and cognitive inhibition and impulsivity (Keilp et al., 2001; Swann et al., 2005; Raust et al., 2007). When reviewing the fine neuroanatomical studies aimed at understanding the cellular substrates possibly underlying these gross morphological changes and functional deficits, it becomes apparent that the different PFC subregions can display important differences, and may thus
contribute differentially to MDD and suicide. Perhaps more intriguingly, alterations in PFC glial cell numbers and densities have been reported by different groups, suggesting a prominent role for these cells. As detailed below, two main PFC subregions have been scrutinized: the dorsolateral PFC (DLPFC) (mainly Brodmann area 9, or BA9) and the orbitofrontal cortex (mainly BAs 10 and 47), including both rostral (rORB) and caudal (cORB) portions.

2.4.1 Dorsolateral Prefrontal Cortex (BA9)

The first study to present evidence of changes in numbers of neuronal and glial cells in the brain of subjects with MDD (the majority of which had died by suicide) was led by Rajkowska and collaborators (1999). Focusing on BA9, these authors reported variations in neuronal densities in layers II, III and VI of MDD subjects compared to healthy controls; the former presenting significant reductions and increases in the densities of the largest and smallest neurons, respectively. These results raise the possibility that pyramidal layers, at least in BA9, are more critically involved in MDD and suicide. Layer V pyramidal cells are the principal source of efferents to subcortical structures, such as the basal ganglia and thalamus. In this context, dysfunctions in these brain regions contribute as well - at least indirectly - to severe mood disorders on the basis of reduced cortical input. Neuroanatomical data showing that there are significantly more neurons (26-37%) in the postmortem limbic thalamus of MDD subjects compared to controls (Young et al., 2004) support this hypothesis, at least histopathologically. Although some investigators have not observed any significant difference in BA9 neuronal densities between MDD
and control subjects (Cotter et al., 2002a), others have provided further evidence for changes in pyramidal neuron densities in this cortical area (Law and Harrison, 2003). These authors reported that MDD subjects displayed the lowest density of pyramidal neurons, especially in BA9 layer III, when compared to other psychiatric disorders, thus suggesting a particular deficiency in cortico-cortical glutamatergic synapses. However, when using the neurofilament protein NF200 as a marker of pyramidal neurons, Miguel-Hidalgo and colleagues failed to replicate this finding (Miguel-Hidalgo et al., 2005). From these results, these authors reasoned that their previously reported large neuron density reductions in MDD BA9 (Rajkowska et al., 1999) should be accounted for by other populations of pyramidal (i.e. non-NF200-IR) or even by nonpyramidal cells. In addition, such observations indicate that neurofilament-related cytoskeleton deficiencies of pyramidal neurons do not occur in prefrontal cortical circuits in MDD, consistent with the idea that the prefrontal cognitive impairments observed in this mood disorder are not a result of neurodegenerative mechanisms such as those characterizing Alzheimer’s disease.

Emerging clinical evidence suggests that MDD may be associated with reduced levels of γ-aminobutyric acid (GABA) within the brain (Sanacora et al., 2003, 2004). Few post-mortem morphometric studies have been conducted on GABA neurons. Recently, Bielau and colleagues (2007) have reported a highly significant increase in density of GABA neurons (GAD 65/67-immunoreactive (IR)) in BA9 of MDD subjects versus controls. This increase was also observed in other regions, namely the orbitofrontal cortex, superior temporal cortex, and
hippocampus (Bielau et al., 2007), suggesting that increased GABA transmission is a widespread phenomenon in MDD. However, these results need to be confirmed independently, as other studies have indicated a somewhat opposite trend. Thus, by focusing on subpopulations of cortical GABA interneurons based on their co-expression of neuropeptides, significant reductions in both neuron somal size and density were reported by Rajkowska and colleagues for BA9 calbindin (CB)-IR neurons in MDD versus control subjects (Rajkowska et al., 2007). In addition, a similar trend was observed in another prefrontal cortical area, the orbitofrontal cortex (BA10,47; see below). In contrast, these authors found no differences in the density and somal size of parvalbumin-immunoreactive (PV-IR) interneurons between the same cohorts (Rajkowska et al., 2007). These findings have interesting functional implications, as they suggest that different PFC interneuronal circuits are selectively affected in MDD. Morphologically, PV-IR interneurons correspond mainly to basket or chandelier cells. By making contact on the soma and axonal initial segment of pyramidal neurons, these cells can strongly regulate cortical output activity (Lund & Lewis, 1993; Conde et al., 1994; DeFelipe, 1997). On the other hand, CB-IR interneurons present a double bouquet morphology, extending dense axon collaterals which synapse on pyramidal neuron apical dendrites in layer I (Lund & Lewis, 1993; Cauil et al., 1997; DeFelipe, 1997; Zaitsev et al., 2005). These cells are thus well positioned to modulate cortical input, and their reduced density and somal size in PFC could thus contribute to the functional deficits previously associated with this region in MDD subjects. It remains to be determined whether the large varicose 5-HT axons which
terminate preferentially on cortical CB-IR interneurons (Smiley & Goldman-Rakic, 1996; Hornung, 2003) are involved in these morphological changes.

Data regarding glial cells in BA9 are somewhat more consistent than those for neurons, with reductions in glial densities reported to be mostly targeted to layers III and V in MDD subjects (Rajkowska et al., 1999; Cotter et al., 2002a). The detailed analysis of Rajkowska and colleagues (1999) further revealed that layer III glial cells with the largest nuclei were significantly increased in MDD patients; exactly the opposite of what these authors observed for large neurons in the same layer. Based solely on nuclear size, this population of glial cells is likely to be constituted mainly of astrocytes. Historically, these cells have been viewed as support elements in the CNS, but in recent years, tremendous progress has allowed to understand that they are much more versatile, being central to many structural and functional aspects of cerebral activity. Thus, astrocytes have been described to play roles in injury response, immune defense, ionic homeostasis of the neuropil, cerebral glucose metabolism and maintenance of synaptic transmission (Magistretti et al., 1999; Coyle & Schwarcz, 2000). Astrocytes are also known to express transporters and receptors for the neurotransmitters glutamate, GABA, 5-HT, and norepinephrine (NE) (Kimelberg & Katz, 1985; Blankenfeld & Kettenmann, 1992; Sutin & Shao, 1992; Russ et al., 1996). The recent finding that in MDD astrocytic glutamate transporters and GABAA receptor subunits are respectively down- and up-regulated in prefrontal and anterior cingulate cortical areas suggests a key role for astrocytes in the pathophysiology of depression (Choudary et al., 2005).
Expanding from their initial morphological study showing that glial cell densities and glial nuclear size are altered in BA9 of MDD subjects (Rajkowska et al., 1999), Miguel-Hidalgo and collaborators (2000) refined their approach by examining, in the same region, the packing densities of cells immunoreactive for glial fibrillary acidic protein (GFAP), a specific marker of astrocytic phenotype in the adult cerebral cortex. These authors found that, compared to control subjects, only the younger (≤ 45 years old) MDD subjects significantly differed with regards to the areal fraction occupied by GFAP-immunoreactivity. In fact, areal fraction values in the youngest MDD subjects were lower than the lowest values measured in the control group. This led to the hypothesis that a yet to be identified age-related alteration in cortical astrocytic plasticity occurs in MDD (Miguel-Hidalgo et al., 2000). In support of this hypothesis, there is growing evidence suggesting that elderly MDD subjects differ from younger individuals in terms of etiology, phenomenology, and cerebrovascular pathology (Kumar et al., 1998; Lee et al., 2003; Taylor et al., 2003). Furthermore, one report has highlighted significant reductions in the overall (layers I–VI) packing density of pyramidal neurons, as well as layer-specific alterations in layers IIIc and V in the PFC of elderly MDD patients (Rajkowska et al., 2005). It was further observed that the subjects with the highest neuronal densities were the youngest (< 50 years old) whereas those with the lowest were the oldest (> 60 years old). Overall, these few studies suggest that astrocytes undergo age-related morpho-functional changes in the PFC of MDD subjects. However, some inconsistencies between the layer-specific data on GFAP-IR astrocytes (Miguel-Hidalgo et al., 2000) and those previously obtained for glial cells identified on the basis of
nuclear morphology (Rajkowska et al., 1999) suggest that other types of glial cells, namely oligodendrocytes and microglial cells, are also affected in MDD.

Oligodendrocytes provide support to neurons as neuronal satellites in grey matter, and by producing the myelin sheath which isolates axons in white matter. One study has shown prominent reductions in the numerical density of oligodendrocytes in BA9 layer VI - but not in adjacent white matter - of psychiatric subjects (Schizophrenia, Bipolar disorder, MDD) compared to controls (Uranova et al., 2004), suggesting that oligodendroglial subpopulations are differentially affected. The authors of this study interpreted their results as a disturbance in glia-neuron interactions that may account for the atrophy of pyramidal neurons previously documented for PFC layer VI in these disorders. Thus, morphological changes observed in PFC glial cells are likely to be accounted for by changes in astrocytes as well as oligodendrocytes. However, the respective implication of these cells in MDD (and other psychiatric conditions) remains to be further explored. To our knowledge, a single study has examined microglial cells in relation to mood disorders (Steiner et al., 2008). This recent report showed a robust increase of immunolabeled microglia in all brain regions examined (DLPFC, ACC, hippocampus, medial thalamus) of subjects suffering from MDD (or schizophrenia), and which had committed suicide. The immunomarker used in this investigation (HLA-DR) is a well-established marker of neuroinflammation and neurodegeneration, raising the interesting possibility that such processes might be specifically linked with suicidal behavior.
2.4.2 Orbitofrontal Cortex (BAs10,47)

Similar to the observations in BA9, layer-specific alterations have also been documented in MDD for both rostral and caudal portions of BAs10,47 (Rajkowska et al., 1999). In the rORB region of MDD subjects, this study found reductions in the neuronal size and densities of the largest neurons in layers II-IV, whereas increases in the density of small neurons were observed in layer III. When focusing on glial cell densities, decreases were also observed in MDD individuals, particularly in the case of medium- and large-sized glial cells in layers IIIa and IV, respectively. Caudally, the cORB cortex in MDD displayed significant reductions in mean neuronal soma size in layer II, and the density of large-sized neurons were decreased in layers IIIa and Va (Rajkowska et al., 1999). The examination of cORB glial cells in the same subjects led these authors to report important reductions (greater than in rORB) of cell sizes and densities in layers III, V and VI in MDD (Rajkowska et al., 1999). Taken together, these findings in BAs10,47 suggest that there exists a rostro-caudal rather than dorso-ventral pattern of morphometric changes in the PFC of MDD subjects. In contrast, Cotter and colleagues (2005) did not observe any significant difference in ORB cortex cell densities between psychiatric and controls subjects. Only a decrease in layer III neuronal soma size in MDD was reported. In addition to possible methodological differences (see section 2.10), one possible explanation for this discrepancy could reside in the fact that Cotter and colleagues investigated a more rostral portion of ORB cortex than those studied by Rajkowska and colleagues (1999).
2.5 MEDIAL PREFRONTAL CORTEX

Considered part of the limbic system, the mPFC mediates many behaviors influenced by stress due to it being a major target for glucocorticoids, hormones mediating the stress response (Diorio et al., 1993; Devinsky et al., 1995). The mPFC displays reciprocal connections with the parahippocampal gyrus, and thus is in close relation to the hippocampus *(discussed in section 2.6)*, which plays a major role in the response to stress (McEwan et al., 1979; Sapolsky, 2000; Gusnard et al., 2001). Considering that chronic stress could act as a trait-dependent risk factor in MDD and suicide (Mann, 1998), one might predict that structures strongly connected to the hippocampus, such as the mPFC, are affected at the cellular level.

Most cellular studies on the mPFC have focused on the affective subdivision of this region, which includes BAs 25, 32, 33, and rostral area 24 (anterior cingulate cortex; ACC). An executive control system centered on the ACC and the PFC is implicated in governing information processing and response selection in situations involving planning, error monitoring and correction, and response inhibition (Posner & Dehaene, 1994; Miller & Cohen, 2001). Dysfunctions within this network have been described in depressed individuals (Davidson et al., 2002) which could lead to impaired problem solving, maladaptive behavioral regulation and abnormal error monitoring. In this context, it is interesting to note that the subgenual ACC has been a therapeutic target for deep brain stimulation (Mayberg et al., 2005).
2.5.1 Anterior Cingulate Cortex (BA24)

MDD subjects show gross structural (Ballmaier et al., 2004), neurochemical (Auer et al., 2000) and functional (Bremner et al., 2004; George et al., 1997; Kumari et al., 2003) abnormalities within BA24. However, few studies have been conducted to explore possible cellular alterations in this region in depressed subjects and suicide completers. Similar to studies conducted in the PFC, observations within the mPFC are difficult to interpret and slightly inconsistent. Initial reports found no evidence for morphological alterations in the mPFC subgenual region, however, by further examining the clinical history of subjects, those with familial mood disorders were shown to have significant reductions in glial cell number and density (Ongur et al., 1998). More recent evidence has also generally indicated reductions in glial cell density and neuronal soma size in BA24 (Cotter et al., 2001; Chana et al., 2003). However, results differ depending on the particular anatomical subdivision of BA24 being analyzed. It is known that the ACC is cytoarchitecturally (Vogt et al., 1995; Ongur & Price, 2003; Gittens & Robbinson, 2004) and functionally (Devinsky et al., 1995; Vogt et al., 1995; Bush et al., 2000) heterogeneous, and this has led to three regional subdivisions: BA24a, b, and c.

Within BA24b, layer VI was most affected in MDD subjects and showed diminished numbers of glial cells as well as reduced neuron somal size (Cotter et al., 2001), whereas in the more dorsal BA24c, significant reductions in neuronal soma size were observed in layer V, together with an increase in neuronal density. Glial nuclear sizes were also found to be increased in layers I and II between MDD and control groups (Chana et al., 2003). However, these results
were not reproduced by a stereological analyses of BA24a, an area ventral to BA24b, which showed no significant difference in neuronal density or somal size in both upper and lower BA24a cortical layers between MDD and sudden-death (control) subject groups (Bouras et al., 2001). Current results from our group have also failed to observe any significant difference between depressed suicide and control subjects in terms of glial and neuronal densities as well as neuron somal size within BA24a (Hercher et al., 2008).

Two independent groups have attempted to examine specific mPFC neuron types in MDD (Cotter et al., 2001b; Bielau et al., 2007). These authors found no significant differences in the densities of GABAergic CB-IR or PV-IR interneurons between MDD and control groups. As discussed in section 2.4.1, a significant increase in the number of activated microglia was reported in the ACC of MDD subjects having committed suicide (Steiner et al., 2008).

2.6 HIPPOCAMPUS

MDD has not generally been regarded as a hippocampal disorder. There is however growing evidence suggesting that this region is affected in MDD/suicide individuals (Sheline, 2000; Vakili et al., 2000; Neumeister et al., 2005). Interest in the hippocampus with regards to mood disorders has recently been fuelled by the hypothesis that the biological and cellular basis of MDD is intimately related to dysfunctional adult hippocampal neurogenesis (AHN; see reviews of Kempermann & Kronenberg, 2003; Dranovsky & Hen, 2006). Briefly, this argument is based on two lines of reasoning. The first comes from recent morphological and morphometric analyses of the hippocampus in depressed patients showing structural changes in terms of volume loss, gray
matter alterations, and neuropil reductions (Sheline, 2000; Stockmeier et al., 2004). According to Jacobs (2002), changes in AHN could be responsible for such structural changes. The second line comes from accumulating evidence that various antidepressant treatments stimulate the proliferation of hippocampal progenitor cells. Interestingly, the latency of antidepressive effects corresponds well to the maturation period of newborn neurons in the adult hippocampus (Dranovsky & Hen, 2006). Moreover, AHN in animals is robustly inhibited by stress, a significant causal agent in the etiology of MDD (Gould et al., 1997, 1998). Unfortunately, all of these studies have been conducted in animal models, and it remains to be determined if AHN is relevant to MDD and suicide.

A single, recent study in humans, has examined proliferation markers within the hippocampus of depressed versus control cases, reporting no differences between groups (Reif et al., 2006). However, this study presents important limitations in that only a few sections (of unknown periodicity) per hippocampus could be analyzed.

In terms of morphological studies, the human hippocampus is only beginning to be explored in the context of MDD. The limited number of publications on the topic reveals contrasting results in terms of neuronal and glial cell size and density. Surprisingly, one group has shown increases in the mean densities of pyramidal neurons and glial cells in CA regions and in the granule cell layer of the dentate gyrus, with accompanying reductions in the mean somal size of these cells in MDD subjects versus controls (Stockmeier et al., 2004). In a previous histological study, Muller and colleagues (2001) had reported significant reductions in the density of GFAP-IR astrocytes in
hippocampal CA1 and CA2 regions of MDD subjects. In addition, the expression of GAP-43, a neuron-specific phosphoprotein essential for neurite growth and plasticity (Benowitz & Routenberg, 1997) was measured to be lowest in the same areas (Muller et al., 2001). Whether these observations are related to the increased microgliosis reported for MDD suicide subjects (Steiner et al., 2008) remains to be explored.

2.7 AMYGDALA

The amygdala plays a key role in the formation and storage of emotional events. Being prominently implicated in the regulation of emotion, particularly impulsivity and aggression, and heavily connected with the orbital and mPFC, the amygdala should be a targeted area for cellular studies in MDD and suicide.

Neuroimaging approaches have provided evidence that the amygdala is dysfunctional in MDD. In general, increases in cerebral blood flow and glucose metabolism have been observed in the amygdala of depressed patients (Drevets et al., 2000; Ketter et al., 2001). In addition, changes in amygdalar volume in MDD subjects seem to be a consistent feature reported by MRI investigations (Sheline et al., 1998; Monkul et al., 2007).

Possibly due to the difficulties in dissecting the human amygdala, few morphological studies have been conducted on this limbic region. Bowley and colleagues (2002) reported reductions in amygdalar glial cell density, particularly in the left hemisphere of MDD subjects. The hypothesis that cellular changes may not be generalized but rather specific to certain vulnerable brain regions in MDD were supported by their comparative analysis of the entorhinal cortex, which displayed no significant differences in glial densities
between MDD and control subject groups. However, a significant increase in neuronal density was observed in this cortical area in MDD subjects, a finding which was proposed to arise from the slightly smaller tissular volume measured in MDD subjects, although this difference was not significant (Bowley et al., 2002).

In a follow-up study, Price and colleagues then scrutinized the different glial cell subpopulations in the amygdala, and found that the density of oligodendrocytes was significantly lower in MDD than in control subjects (Hamidi et al., 2004). Interestingly, no changes were observed in the case of astrocytes or microglia. These findings point to a possible deficiency in the myelination process in this region, but also in neuronal extracellular homeostasy, a function of satellite oligodendrocytes.

### 2.8 RAPHE NUCLEUS

The raphe contains the 5-HT neurons that innervate the entire central nervous system. Although this remains to be established, two rapheo-cortical projection types have been suggested on the basis of morphological features and 5-HT nucleus of origin. Thus, in the cortex of primates, Kosofsky and Molliver (1987) reported that the dorsal raphe nucleus (DRN) gives rise to fine, non-varicose 5-HT axons, while the medial raphe nucleus (MRN) projects highly varicose cortical 5-HT axons. Additionally, these authors reported that each nucleus projected to different PFC layers. Fine non varicose axons are present in all layers but more abundantly in layers III-VI, while highly varicose axons are mainly observed in layers I-II. Interestingly, and as described above, PFC cellular alterations in humans have been mainly observed in layers III-VI, thus
suggesting that DRN dysfunction might account for the wide-ranging alterations in 5-HT transmission well-documented in MDD and suicide. Direct evidence has recently come from the study of Bach-Mizrachi and colleagues, showing that there is a 33% increase in expression (mRNA) of tryptophan hydroxylase-2 (TPH2), the neuronal isoform of the rate-limiting 5-HT biosynthesis enzyme, in the raphe of suicide victims compared to controls (Bach-Mizrachi et al., 2006).

Although the overall neuronal density in the DRN was reported unchanged between depressed suicides and control subjects (Baumann et al., 2002), investigations focusing on TPH-IR cells in this nucleus have generally shown significant differences in neuron numbers between groups. The first of these studies found, on average, a significantly higher number and density of DRN TPH-IR neurons in MDD subjects compared to controls (Underwood et al., 1999). More recently, TPH-IR neurons in the DRN were found to be more numerous in depressed suicide subjects compared to controls (Boldrini et al., 2005). Using immunoautoradiography, Bonkale and colleagues initially reported that the number of TPH-IR neurons in the DRN was comparable between depressed suicides and controls (Bonkale et al., 2004). However, using the same approach, these authors recently showed that the number of TPH-IR neurons in this nucleus is increased in alcohol-dependent depressed suicides, but that this phenomenon is restricted to the dorsal subnucleus (Bonkale et al., 2006).

Although they must be considered with caution because TPH expression might be confounded by state dependent factors such as drugs, psychopathology and suicidal behaviour (Baumann et al., 2002), most studies in the literature suggest an increase in TPH expression in the DRN. This might seem
counterintuitive; decreases in cell number and downregulated TPH expression could have been expected based on the multiple lines of evidence indicating an overall reduction in 5-HT transmission in both cortical and subcortical regions of MDD and suicide subjects. However, an increase in number of TPH-IR neurons suggests a compensatory mechanism to alleviate cortical 5-HT deficits, whereby more 5-HT neurons would be synthesizing increased levels of TPH in an attempt to restore the reductions in the synthesis and release of 5-HT in the innervated territories (Underwood et al., 2004). Such results may also be explained as a response to stress, given that MDD suicide subjects are usually under considerable amounts of stress and often show alterations within the stress response system (Pfennig et al., 2005; Bao et al., 2007). In support of this hypothesis, TPH protein and mRNA levels are both increased in response to stress (Chamas et al., 2004) and following glucocorticoid replacement in adrenalectomized animals (Azmitia et al., 1993).

Alternatively, a predisposing neurodevelopmental phenomenon may exist which increases the number of 5-HT neurons in the DRN, as suggested by the observation of significantly higher numbers of TPH-IR neurons documented across the lifespan, even at a young age, in suicide subjects (Underwood et al., 1999). A possible mechanisms accounting for such an increase would be an alteration in the programmed cell death normally occurring during brainstem development.

2.9 LOCUS COERULEUS

It has been postulated that the pathophysiology of major depression involves, at least in part, a disruption of the central noradrenergic system. The

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LC contains a large group of noradrenaline (NA) neurons innervating almost all brain regions, including limbic structures such as the amygdala, hippocampus, and thalamus (Foote et al., 1983; Moore & Bloom, 1979). Early evidence showed that drugs which deplete NA could induce depressive symptoms, whereas drugs that enhanced NA transmission alleviated depression (Prange, 1964). This led to the catecholamine theory of depression, which proposes that an absolute or relative deficiency in catecholamines, particularly NA, occurs in the brains of depressive individuals (Schildkraut, 1965). More recent evidence has shown that the LC displays some of the highest antidepressant binding site densities in the brain (Biegon & Rainbow, 1983; Crespi et al., 1980; Richards et al., 1992) and that this nucleus presents neurochemical alterations in MDD and suicide subjects (Klimek et al., 1997; Merali et al., 2006).

The central NA system is known to interact with the stress response through a feed-forward mechanism. Briefly, a stressor activates the release of corticotropin-releasing factor (CRF) within the LC, thereby stimulating this nucleus. This activation in turn brings about the release of NA in forebrain terminal regions, further stimulating the release of CRF. This feed-forward mechanism within such a fundamental brain-activating system is thought to be centrally involved in a variety of psychiatric disorders, including MDD and suicide, which are accompanied by abnormal responses to stressors. In MDD, elevated LC concentrations of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of adrenaline, NA and dopamine, may also represent an adaptive response to chronic stress associated with depression. This is supported by animal studies showing that stress activates the LC, resulting in elevated NA.
release (Pavcovich et al., 1990) accompanied by increases in TH mRNA protein
in the LC and other noradrenergic nuclei (Nankova et al., 1996; Wang et al.,
1998). Finally, a further line of evidence linking a stress-induced dysregulation
of this system comes from the genetic study of Bissette and colleagues (2003)
showing an increase in CRF mRNA in depressed suicides.

In general, and compared to the other brain regions discussed in this
review, there is less compelling morphological evidence suggesting that the LC
is altered in MDD/suicide subjects. Furthermore, studies conducted on the LC
often had a subject group consisting entirely of suicide completers.
Unfortunately, this group is generally composed of individuals with various
mood disorders, such as schizophrenia, bipolar disorder and MDD. Therefore,
the absence of more homogeneous subject groups in most of these studies
should be taken into consideration when interpreting the findings.

In an original study examining the effect of age on LC neurons, Chan­
Palay and Asan (1989) reported on an isolated case where a depressive elderly
subject showed a 55% reduction in LC cell numbers compared to age-matched
controls. This study sparked a series of morphological reports which focused on
the LC in MDD and suicide. While most studies failed to find differences
between MDD and control cases in the number of neuromelanin containing cells
(Baumann et al., 1999a, 1999b; Syed et al., 2005; Zhu et al., 1999; Ordway,
1999), one report indicated significant reductions in the total number and
average density of pigmented LC neurons in suicide completers (Arango et al.,
1996), and another the inverse trend (Ordway et al., 1994). Interestingly, the
reduced number of LC neurons reported in the former study concerned only the
left brain hemisphere in suicide victims (Arango et al., 1996). Recent studies have indicated similar hemispheric differences for other brain regions (Bowley et al., 2002; Hamidi et al., 2004). Thus, in MDD/suicide victims, the left frontal lobe may display greater alterations due to a disproportionate reduction in NA innervation. Unfortunately, there are currently no comparative inter-hemispheric data on most brain regions involved in MDD/suicide which could help verify this hypothesis.

Most morphological studies on the LC have focused on the number of TH-IR neurons, and in this respect, no differences have been reported between MDD/suicide and control subjects (Biegon & Fieldust, 1992; Baumann et al., 1999a; Syed et al., 2005). Interestingly, while Baumann and colleagues (1999a) did not find differences between bipolar, unipolar and control groups, further examination revealed that the patients with mood disorders not committing suicide had significantly fewer TH-IR neurons. Arguably, this observation suggests that the LC undergoes similar increases in local neurotransmitter synthesis than those in the DRN (see above section) in subjects with mood disorders which are prone to suicide. In line with this argument, increased levels of TH protein have been observed in the LC of suicide subjects (Ordway et al., 1994; Zhu et al., 1999; but see Biegon & Fieldust, 1992).

2.10 DISCUSSION

By revealing that the cellular composition is often altered in brain regions implicated in MDD and suicide, the studies reviewed above add an important dimension to our comprehension of these psychiatric conditions. However, they also raise a number of important methodological issues, all of which need to be
carefully considered when interpreting quantitative neuroanatomical data. These issues mainly revolve around the quantitative approach used to compare a given cell population between subject groups. Briefly, the most commonly used approach in earlier studies was 2D counting. This method assesses the numbers of cell profiles in a tissue section across the x-y plane, and presents the advantage of employing very large microscopic fields allowing each and every cell of interest observed in large areas to be counted. The alternative 3D approach, known as stereology, is nowadays more widely accepted. Stereology exploits an optical probe across the depth of sections (z axis) which provide cell counts that are unbiased, as they are not influenced by differences in cell size, shape, or orientation (Williams and Rakic, 1988). Importantly, the data provided by stereology also take into account changes in tissue volume. This is clearly a major parameter to consider, knowing that routine tissue processing for histology involves several steps resulting in highly significant changes in tissue volume (Dorph-Petersen et al., 2001). Obviously, this methodological artefact can strongly distort values such as cell densities. Stereology can thus add a higher degree of precision (but see Benes & Lange, 2001); it also entails a more careful and thorough planning of all experimental steps, from tissue preservation and sectioning to establishing the parameters for a truly random sampling. For a comprehensive review of the different stereological concepts, the reader is referred to Mouton (2002).

Some of the studies presented in this review have used 2D and others 3D cell counting approaches. This might account for some of the discrepant data reported by different groups. Another important factor to consider which may
also generate variability is that brain regions are rarely homogeneous structures, and thus need to be sampled throughout, whichever counting approach is chosen. For instance, within the LC, cell density increases and cell size decreases along the rostrocaudal axis (Chan-Palay & Asan, 1989). Similarly, within the neocortex, there are characteristic layer-specific differences in cell densities (Vogt et al., 1995). This emphasizes the need to systematically sample throughout an entire region of interest or, if the region is only partially available as is often the case when studying human brain tissues, an awareness of such heterogeneity should be acknowledged when interpreting results. Finally, the importance of internal controls should also be stressed here, particularly since morphological studies very rarely examine in parallel, from the same subjects, brain regions for which there is no evidence of an implication in mood disorders.

Finally, another important methodological consideration is that care should be taken to gather for all subjects under study the most complete clinical, psychological and demographical information possible, because factors such as alcohol consumption or number, length and severity of depressive episodes could significantly alter neuronal or glial morphology. Ideally, neuroanatomical studies focused on MDD and suicide should be conducted on individuals who were free of medications, drugs and even alcohol. However, controlling for such factors usually leads to very small sample sizes (thus jeopardizing statistical power) since there is high co-morbidity between drug abuse and psychiatric conditions. In consequence, it is probably more informative (and realistic) to obtain data from large subject groups as long as all possible confounding factors have been taken into consideration.
It remains elusive as to whether the cellular alterations displayed in MDD brains are causes or consequences of the disorder, and if suicide is a contributing factor. Some of the changes observed may suggest a developmental deficiency. For instance, although the smaller than average neuronal sizes described in the PFC of MDD subjects could be due to cell shrinkage, they may also be the result of diminished growth during ontogeny. However, this hypothesis remains to be supported by experimental evidence. A single study examining interstitial neurons, early-born neurons derived from the embryonic subplate, did not provide any evidence showing a difference in distribution nor density between MDD and control subjects (Beasley et al., 2002). This does not exclude that the differentiation of later-generated neurons may be affected by yet to be discovered obstacles to developmental programs. In this perspective, future investigations of MDD would gain in exploring the expression, distributional features and genetic variants of proteins involved in developmental plasticity.

Interestingly, some of the cell morphological alterations reviewed here can also be related to findings on the distribution of both afferent and efferent systems implicated in MDD, particularly in laminated regions such as the neocortex. Thus, in the rORB, neuron somal size alterations were mostly reported in layers II-IV, in which 5-HT axons terminate predominantly on non-pyramidal neurons (Lewis, 1992). Furthermore, layer II in humans presents a high density of 5-HT receptors (Pazos et al., 1987). In BA9 and in the cORB, the above-mentioned findings of cell alterations in the lower cortical lamina could implicate the major cortical descending glutamatergic pathways (Selemon & Goldman-Rakic, 1985).
The cell density changes reported for the hippocampus and other brain areas in MDD have been proposed to arise from neuropil reductions (Stockmeier et al., 2004). Neuropil consists of dendrites, the lattice of glial cells and their processes, and proximal axons surrounding neuronal cell bodies. Thus a possible explanation for the tissular changes observed in depression may reside in important losses of dendritic branches and spines, bringing about increased cell densities because of accompanying volume reductions such as those documented in imaging studies (Stockmeier et al., 2004). Dendrites are the main input sites from other cells, and the geometry of the dendritic arbor has been proposed to determine many functional properties of neurons (Koch & Segev, 2000). It is thus likely that dendritic alterations could result in important functional changes by playing a role in mediating behaviors.

While the great majority of anatomical studies in MDD and suicide have focused on the size and density of cell bodies, the reduced neuropil hypothesis clearly calls for future studies to scrutinize the morphology of dendritic arborizations in the same brain regions. There exists however a limitation in that the methods and analyses involved are generally labour intensive, particularly when applied to human brain tissues. To our knowledge, a single such study has been conducted in the context of psychiatric disorders, which examined dendritic structures in the subiculum (Rosoklija et al., 2000). By using Sholl analysis, which reveals the branching pattern of dendrites, these authors showed significant decreases in apical dendritic arborizations. In addition, decreases in the number of spines borne by dendrites of internal subicular pyramidal cells were found in psychiatric subjects compared to controls (Rosoklija et al., 2000).
Adding support to the idea that individuals may be predisposed to MDD was the finding that individuals with the lowest spine counts had at least two first-degree relatives with definite or probable mood disorders. However, these results will need to be replicated with a larger sample size.

### 2.11 CONCLUSION

The neuroanatomy of mood disorders is a relatively young field of study and, clearly, more research is needed to establish the nature and localize precisely the different manifestations of cellular alterations occurring in the brains of MDD and suicide subjects. This will be accomplished all the more rapidly as morphometric studies are being increasingly conducted with unbiased sampling approaches. Since MDD is considered by many to be a disorder of reduced brain plasticity, it would also be beneficial if the focus of these studies shifted somewhat towards other neuronal compartments than the soma, namely dendritic trees, the major site of intercellular communication in the brain.

Similarly, the glial contribution to brain cellular alterations in mood disorders will need to be defined by studying specific features of the different CNS glial subpopulations. As illustrated in this review, recent morphological analyses of post-mortem brain tissues have led to novel insights as to the cellular and molecular underpinnings of MDD and suicide, and laid ground for future investigations in this – and other - fields. In the future, two main research avenues will likely need to be explored in tandem. The first will be to scrutinize the respective regional contributions of different cell compartments and populations to the morphological alterations observed in the brains of MDD.
subjects. The second will be to consider suicide as a contributing factor to such alterations.
Figure 1: Saggital section through the midline showing prefrontal and medial prefrontal areas in the right hemisphere. Modified with permission from, Digital Anatomist Project at the University of Washington. http://da.biostr.washington.edu
Figure 2: Horizontal section illustrating posterior brain areas involved in MDD and suicide. Modified with permission from, Michigan State University: Brain Biodiversity Bank. http://www.brains.rad.msu.edu Supported by the National Science Foundation.
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Chapter 3:
Stereological Investigation of Anterior Cingulate Cortical Cells in Depression and Suicide


3.1 PREFACE TO MANUSCRIPT IN PREPARATION

Upon reviewing the cellular literature, it becomes evident that post-mortem tissue sampling studies have laid the foundations for insights into possible morphological alterations in suicide and MDD. Thus studying tissue from these individuals presents a potential informative approach for revealing the neurobiology of this psychiatric disorder. In this chapter we build upon the existing evidence and report a cellular study investigating potential glial cell density and neuronal soma size and density alterations in depressed suicide subjects.

3.2 ABSTRACT

Changes in glial and neuronal soma sizes and densities have been reported in the postmortem anterior cingulate cortex (ACC; Brodmann area 24, BA24) of subjects with major depressive disorder (MDD). A study assessing such possible cellular alterations in BA24 has yet to be conducted with a cohort composed entirely of subjects with MDD having committed suicide. Therefore, the purpose of this study was to compare BA24 cell densities and sizes between sudden-death controls and MDD suicide subjects. We examined a 1cm³ tissue block from BA24a of the supracallosal ACC in 26 postmortem brain specimens (13 controls and 13 suicides). Neuronal and glial cell densities as well as neuronal cell sizes
were assessed in the upper and lower cortical layers using the optical fractionator and nucleator three-dimensional stereological probes. Morphometric parameters (glia density, neuronal soma size and density) did not significantly differ between control and MDD/suicide subjects in BA24a. Secondary analyses showed a significant increase in glial cell densities in both upper and lower cortical layers of alcoholic-dependent depressed suicides compared to non-alcoholic depressed suicides (38%) and to a lesser extent in control subjects (30%). The present investigation indicates that the soma sizes of neurons as well as neuronal and glial cell densities in BA24a are unaffected in depressed suicide subjects. The significant increases in glial cell densities in alcohol-dependent depressed suicide subjects suggest that alcohol dependence brings about important changes in the cellular composition of this cortical area, and warrant further studies to identify the glial cell population(s) involved in this phenomenon.

3.3 INTRODUCTION

A major challenge in the field of psychiatry is to understand the etiology of major depressive disorder (MDD) and suicide, the latter being a frequent consequence of the former (Cavanagh et al., 2003). Accounting for 10-12% of annual deaths in North America (WHO), suicide represents a major public health concern. However, a clear understanding as to why certain depressive individuals commit suicide and others do not remains elusive. In order to understand the neurobiological underpinnings of MDD and suicide, recent studies have begun to look at possible cellular alterations in certain brain regions known to be involved in these disorders. One such region is the anterior cingulate cortex (ACC) (Brodmann area 24, BA24). Being “the seat of dynamic vigilance by which
environmental experiences are endowed with an emotional consciousness" (Papez, 1937), the ACC acts as a bridge between limbic structures and the frontal lobe, integrating cognitive activity with affective experience. Dysfunctions within this network have been described in depressed individuals showing impaired problem solving, maladaptive behavioral regulation and abnormal error monitoring (Davidson et al., 2002). Macroscopic alterations within BA24 in MDD and suicide individuals have also been reported in neurochemical (Auer et al., 2000), structural (Ballmaier et al., 2004), and functional studies (Bremner et al., 2004; George et al., 1997; Kumari et al., 2003). Microscopically, there have been few morphological studies on BA24, and a clear picture regarding possible cellular alterations within this brain region in MDD and suicide has yet to emerge. While initial reports have indicated reductions in BA24 glial densities and mean neuronal soma size (Ongur et al., 1998; Cotter et al., 2001), subsequent studies showed increased neuron densities (Chana et al., 2003), or no differences in neuronal densities and soma sizes (Bouras et al., 2001). Further comprehensive knowledge of BA24 cell populations altered in MDD and suicide may aid in explaining morphometric alterations occurring in other brain regions connected to the ACC. Indeed, there is evidence for cellular changes occurring in structures having direct connections with the ACC, critically the hippocampus (Rosoklija et al., 2000; Stockmeier et al., 2004), amygdala (Bowley et al., 2002; Hamidi et al., 2004) and prefrontal cortical regions (Rajkowska et al., 1999, 2007; Miguel-Hidalgo et al., 2000, 2005; Cotter et al., 2002a, 2005; Law & Harrison, 2003; Uranova et al., 2004).
While the majority of studies include suicide victims as only a minor subgroup within psychiatric groups, it is of interest to study these individuals as an independent cohort, as there is building evidence suggesting a predisposition to suicide (Roy et al. 1997; Mann, 1998; Turecki, 2001; Kim et al., 2005). Therefore, this investigation was undertaken to explore potential cellular alterations within the ACC in MDD subjects having committed suicide compared to sudden-death controls. Due to the sizeable number of suicides meeting criteria for alcohol dependence, we also sought to ascertain whether differential cellular alterations occur in suicide subjects with alcohol dependence on the basis that chronic alcohol consumption results in a plethora of neurochemical (Adinoff et al., 2003; Underwood et al., 2004), cellular (Kril & Harper, 1989; Arango et al., 1994; Miguel-Hidalgo et al., 2006) and behavioral (Sullivan et al., 2000) adaptations associated with various brain regions. For this purpose, neurons and glial cells were assessed in BA24a using an unbiased 3D (stereological) method.

3.4 METHODS AND MATERIALS

3.4.1 Human Subjects

All procedures were approved by the Douglas Institute’s ethical review board, and written informed consent from next-of-kin was obtained for each subject. Postmortem brain tissues were obtained from the Quebec Brain Bank (QBB; Douglas Institute), which benefits from a special collaboration with the Quebec Coroner’s Office. The sample was composed exclusively of male subjects, ranging in age between 18-58 years. In each case, the cause of death was ascertained by the Coroner’s office, and a toxicological screening was performed with body fluid or tissue samples to obtain information on drug and
alcohol use at the time of death. For all subjects, psychological autopsies were performed as described previously (Dumais et al., 2005). This allowed to generate detailed case information on psychiatric history, medical history and other relevant clinical and sociodemographic data. In brief, a trained interviewer administered the *Structured Clinical Interview for DSM-IV Psychiatric Disorders* (SCID-I) to one or more informants of the deceased. A panel of clinicians reviewed SCID-I assessments, case reports, coroner’s notes and medical records to obtain consensus psychiatric diagnoses.

The depressed suicide group consisted of 14 subjects having died by suicide and who had previously been diagnosed with a major depressive episode at some point in their lives. One subject in this group was diagnosed with a minor depressive episode. Additionally, these subjects were considered on the basis that they had not been diagnosed with any other psychiatric disorder such as bipolar disorder or schizophrenia, and were reported to be medication-free at least three months prior to death. The control group was comprised of 14 subjects with no previous psychiatric diagnosis and who died suddenly from natural causes that had no direct influence on brain tissue. Finally, groups were matched, as best as possible, for mean subject age, post-mortem interval (PMI), tissue pH, and storage time. Two subjects (one per group) yielded extreme values that were more than two standard deviations above the group mean. These subjects were removed from the analyses, thus bringing the number of subjects per group to 13. Demographics, as well as histological and clinical data are summarized in Table 1.
3.4.2 Tissue Preparation

Tissue samples were removed from the left hemisphere, which had been flash-frozen and kept at -80° C upon arrival at the QBB. An anterior region within the ACC immediately dorsal to the genu of the corpus callosum (Figure 1) was identified based on previously defined macroscopic criteria (Vogt et al., 1995; Gittins & Harrison, 2004). One cm³ tissue blocks of BA24 and the genu of the corpus callosum were removed and immersed in formalin overnight, embedded in paraffin and cut on a microtome into serial 25 μm-thick sections. Sections were systematically and randomly sampled, and between 3-7 sections per subject were stained with cresyl violet according to standard methods. Slides were coded in order for the investigator to remain blind to subjects and groups during the analysis.

3.4.3 Identification of Cortical Laminae

In each section, BA24a of the ACC was identified and selected for analysis according to established microscopic criteria (Gittens & Harrison, 2004). In addition, the genu of the corpus callosum served to orient and specifically identify this region, which is located immediately dorsal to this white matter landmark. Under a 5X magnification, two areas within BA24a were delimited for quantification purposes: an upper cortical contour consisting of layers I - III, and a lower cortical contour containing layers V (Va, Vb) and VI. The division between these upper and lower cortical contours was based on the upper limit of a prominent layer V, which displays a high density of medium to large neurons, and characteristic spindle pyramidal neurons located in layer Vb of BA24a (Figure 2).
3.4.4 Morphometric Analyses

Cells were analyzed at a final magnification of 40X (NA 0.75) using a Leica microscope (DM4000B) connected to a stereology workstation (StereoInvestigator; MBF Bioscience, Williston, VT, USA). Neurons were identified by the presence of a cresyl violet-stained cytoplasm, a single nucleolus, and a large nonspherical outline. Glial cells were identified by the absence of stained cytoplasm, the presence of a thicker nuclear membrane, and more heterogeneous chromatin within the nucleus (Figure 3). Cell densities were estimated using the “Optical Fractionator” probe allowing for three-dimensional (3D) quantification of both neuron and glial cells. This unbiased 3D counting method estimates the total number of objects in a unit of tissue volume with an optical probe providing counts through the z axis that is not distorted by differences in cell size, shape, or orientation (Williams & Rakic, 1988). Among the primary advantages of using the optical fractionator method is that cell counts are independent of section thickness, as they are generated in a defined volume contained entirely within this thickness. In the present study, the distance between section surfaces (top and bottom) and the limits of the unbiased virtual counting spaces (dissector) was set at 10% of the section thickness (guard zone) and the height of the dissector was fixed at 9 μm. In each section, ten to fifteen 3D counting boxes (225 μm x 150 μm) were randomly distributed within each of the upper and lower cortical contours, thus yielding a total of approximately 120-180 counting boxes per subject. Grid size dimensions were generally defined as 400 μm x 350 μm, but in some subjects for which fewer sections of quality could be obtained, a correction was introduced by altering grid size dimensions. This
allowed to ensure that the targeted cell count was met, and a reliable coefficient of error (CE) achieved. Using the average of Gunderson \((m=1)\) and the 2nd estimated CE (Schmitz-Hof) yielded CEs ranging between 0.02 and 0.05 for neurons and glia within each of the defined contours. Counting of neurons and glial cells within the boxes was performed according to stereological rules of the dissector probe (Gundersen et al., 1988). Section thickness was measured at each sampling site, yielding small, non-significant differences in the average mounted thickness between the control and suicide groups \((16.74 \mu m \pm 3.60 SD \text{ vs } 14.85 \mu m \pm 5.09 SD \text{ respectively})\). On average, 1500 glia and 800 neurons were counted per subject. The Cavalieri method was used to obtain an unbiased estimate of the volume of sections analyzed (Mouton, 2002). Cell density values were obtained by dividing total cell number counts by the corresponding tissue volume estimates.

To assess neuronal soma size, the stereological isotropic nucleator probe was applied. This probe allows for the mean neuronal area and volume of sampled objects to be estimated from the intersection with the cell boundaries of a set of rays \((4 \text{ per cell in this study})\) which emerge from a consistent point of origin within the cell (usually the nucleolus) (Gundersen et al., 1988). The nucleator was applied only to neurons that displayed an identifiable nucleolus as well as a complete and intact cell membrane. Glial cells were not assessed, as their cytoplasm is unstained by cresyl violet. Ideally, the nucleator probe should be used to estimate neuronal morphometrics by sampling in tissues cut at random orientations. Due to technical limitations in the present study, only sections cut in a coronal plane were used, possibly introducing a sampling bias in neuronal soma.
size estimates. However, given that both plane of cutting and sampling methods were identical for all subjects throughout the study, any introduced bias would likely affect group values in the same fashion.

3.4.5 Statistical Analyses

All data are expressed as mean ± SEM. The objective of the statistical analysis was to compare, within upper and lower cortical areas, the three morphometric parameters (neuronal density, glial density, neuronal soma size) between the depressed suicide and control groups. Pairwise comparisons were performed with the Independent samples Student’s t-test between groups for neuronal density, glial cell density and neuronal soma size in both upper and lower cortical layers. The significance of group differences was set at $p < 0.05$.

A secondary analysis, using univariate analysis of variance (ANOVA) was carried out to examine the effect of alcohol dependence on all three parameters in depression and suicide. Multiple correlation analyses were used to examine the potential influence of age, PMI, tissue pH, and storage time on the dependent variables studied. These variables were considered to be potential confounders of group differences if they differed between the psychiatric group and the control group according to $t$-tests, or were shown to empirically predict cell densities and soma size at the 5% significance level according to Pearson’s correlation. Additional group comparisons were then conducted using analysis of covariance (ANCOVA), and confounders identified in this way with significance set at $p < 0.05$. 
3.5 RESULTS

As mentioned above, care was taken to match groups for most parameters. Thus, on average, the depressed suicide and control groups did not differ in age \( t_{(24)} = -0.140, p > 0.05 \), PMI \( t_{(24)} = -0.064, p > 0.05 \), nor storage time \( t_{(24)} = -1.389, p > 0.05 \). However, there was a significant difference in pH between groups \( t_{(24)} = -3.245, p = 0.003 \), with the depressed suicide group samples presenting, on average, a higher pH compared to the control group (6.65 vs. 6.32). When all subjects were grouped together, only one slightly significant correlation could be found, whereby age was positively correlated with glial cell densities in lower cortical layers only \( r = 0.393, p = 0.047 \). Storage time, tissue pH and PMI did not have any influence on the remaining morphometric parameters.

3.5.1 Glial Densities

No significant differences were found between depressed suicide and control groups with respect to BA24a glial cell densities, both in upper \( t_{(24)} = -1.950, p = 0.063 \) and lower cortical layers \( t_{(24)} = -1.754, p > 0.092 \) (Figure 4a). Analyzing the influence of alcohol dependence on glial cell densities revealed significant differences between alcoholic depressed suicides and non-alcoholic depressed suicides as well as control subjects in both upper \( F_{(2,21)} = 5.605, p = 0.011 \) and lower cortical layers \( F_{(2,21)} = 8.867, p = 0.002 \). Pairwise comparison tests showed alcoholic depressed suicide subjects to have higher BA24a glial cell densities than control subjects [upper, \( p = 0.012 \); lower, \( p = 0.002 \)] and non-alcoholic depressed suicides [upper, \( p = 0.052 \); lower, \( p = 0.007 \)]. There was no significant difference between control and non-alcoholic depressed
suicide subjects (Figure 5a). ANCOVAs were then conducted for glial density values taking into account tissue pH and age, and no significant differences were found between groups. However, comparing the effect of alcohol significance persisted between groups in both upper \[ F (2,20) = 4.802, p = 0.020 \] and lower cortical layers \[ F (2,19) = 5.244, p = 0.015 \]. These differences were attributed to the alcoholic suicides having significantly higher glial cell densities than non-alcoholic suicides in both upper (37%, \( p = 0.031 \)) and lower cortical layers (38%, \( p = 0.017 \)).

3.5.2 Neuronal Densities

No significant difference between control and depressed subjects was observed with regards to neuronal densities in both upper \[ t (24) = -0.372, p = 0.713 \] and lower BA24a layers \[ t (24) = -0.766, p = 0.451 \] (Figure 4b). No significant group differences in neuronal densities were found when comparing control subjects to depressed suicide subjects with and without alcohol dependence, in both upper \[ F (2,21) = 0.117, p = 0.891 \] and lower cortical layers \[ F (2,21) = 1.341, p = 0.283 \] (Figure 5b). Using tissue pH as a covariate, no significant differences in neuronal densities were found between control and depressed suicide subjects in upper \[ F (1,23) = 0.550, p = 0.466 \] and lower cortical layers \[ F (1,23) = 0.008, p = 0.930 \]. Likewise, ANCOVAs comparing controls and suicide subjects with and without alcohol dependence, led to no significant differences in neuronal densities in upper \[ F (2,20) = 0.417, p = 0.665 \] and lower cortical layers, \[ F (2,20) = 1.459, p = 0.256 \].
3.5.3 Nucleator Estimates

As illustrated in Figure 4c, no significant differences emerged from nucleator measurements between control and suicide subjects for upper \( t(24) = 1.024, p = 0.316 \) and lower BA24a layers \( t(24) = 1.166, p = 0.255 \). Neuron somal dimensions between control subjects and depressed suicide groups with and without alcohol dependence remained similar in lower cortical layers \( F(2,21) = 2.645, p = 0.094 \), but significantly differed in upper layers \( F(2,21) = 3.993, p = 0.034 \) (Figure 5c). Following pairwise comparisons, this effect was only marginal when comparing alcoholic suicides to non-alcoholic suicides \( p = 0.062 \) and with controls \( p = 0.057 \). After taking pH into account however, there remained no significant difference between groups with regards to neuronal size in both upper and lower BA24a layers \( \text{upper, } F(1,23) = 1.937, p = 0.177; \text{lower, } F(1,23) = 1.302, p = 0.266 \). Finally, when analyzing alcohol dependence as an additional factor, ANCOVAs showed significant group differences only in upper layers \( \text{upper, } F(2,20) = 4.316, p = 0.028; \text{lower, } F(2,20) = 2.772, p = 0.087 \). This difference was due to alcoholic suicides having significantly smaller neuron somal sizes than control individuals \( 21\%, p = 0.043 \).

3.6 DISCUSSION

In this investigation of BA24a, we found no significant evidence for differences in glial and neuronal densities nor in neuronal soma sizes between depressed suicide subjects and sudden-death controls. Seeing that, on average, groups differed significantly with regards to tissue pH, and that age was found to be variably correlated with glial cell densities in lower cortical layers, it remains possible that these confounding variables may be masking group differences in
cell morphometrics and densities. Nonetheless, even after correcting for such covariates values remained similar, as no significant differences emerged between control and depressed suicide groups.

The few morphometric studies that have been conducted on BA24 in the context of mood disorders have focused on slightly different subdivisions of this region. In particular, an earlier study found no evidence for morphological alterations in a subgenual region of BA24, but further examination showed that subjects with familial mood disorders had significant reductions (20%) in glial cell numbers (Ongur et al., 1998). In our sample, a similar analysis could not be performed as we lacked complete information of a familial history of depression for a number of subjects and because the seven depressed suicide subjects with history of familial depression were also those that did not meet criteria for alcohol dependence. The other studies conducted on BA24 have mainly examined supracallosal regions. Employing a more detailed method than initial studies, Bouras and colleagues (2001) used a 3D technique to investigate BA24a. Similar to our findings, these authors reported no significant changes in neuronal densities or soma sizes when comparing large samples of major depressed and controls subjects. These authors, however, did not analyze glial densities nor are we aware of how many MDD subjects died by suicide. Analyzing BA24b, a region dorsal to BA24a, Cotter and colleagues (2001a) also used a 3D method and examined individual cortical layers. These authors reported that, in comparison to controls, major depressed subjects displayed significant decreases in glial cell density (22%) and reductions in neuronal soma size which were limited to layer VI. More recently, using a two-dimensional (2D) approach in their study of BA24c, an area
directly dorsal to BA24b, Chana and colleagues (2003) found in layer V decreases (9%) in neuronal soma sizes which were accompanied by increases (24%) in neuronal density. In addition, these authors also reported increases in neuronal soma size (30%) and glial nuclear size (10-13%) in supragranular layers I-II.

In contrast to these studies, our findings and those of Bouras and collaborators (2001) suggest that BA24 glial and neuronal cell populations remain largely unaltered in depression and suicide. Discrepancies between reports could be due to the heterogeneity of the ACC, which displays important cytoarchitectural differences throughout its rostral-caudal and dorsal-ventral extents (Vogt et al., 1995; Gittens & Harrison, 2004). Specifically, one study showed important cellular differences between subgenual and supracallosal BA24 regions, with glial densities being about 25% lower in the former and neuronal densities higher in the latter (Gittens & Harrison, 2004). Thus, differences between quantitative morphological studies within supracallosal regions of BA24 may be due, at least in part, to differential sampling of a heterogeneous structure.

It is important to underline here that cresyl violet staining does not allow discriminating between specific subtypes of neurons (e.g. classes of interneurons) or glial cells (oligodendrocytes, astrocytes, microglia). Consequently, alterations of specific glial or neuronal subtypes in depressed suicide subjects may have gone unnoticed in the present experimental conditions. In this perspective, two independent groups have specifically examined γ-aminobutyric acid (GABA) neurons within BA24 (Cotter et al., 2001b; Bielau et al., 2007). These groups did not report any significant difference in BA24 GABA neuron densities between controls and major depressed subjects, suggesting that in this cortical region, the
morphology of interneurons is unaffected by mood disorders. More recently, increased microgliosis was reported in multiple brain regions (including ACC) of suicide victims, irrespective of psychiatric diagnosis (Steiner et al., 2008). These authors interpreted such observations as an involvement of immunological factors in the etiology of suicide. Although these results will need to be reproduced independently, they support the trend set by other studies which have illustrated that in depression, glial cell populations display the most robust morphological and densitometric changes (Rajkowska et al., 1999, Cotter et al., 2002; Hamidi et al., 2004).

Due to the relatively large number of alcohol-dependent depressed suicides in the present study, secondary analyses were conducted investigating the potential influence of alcohol on cell densities and neuron somal size. In the current literature, there is little information regarding cellular pathological alterations in alcoholic subjects, particularly those who died by suicide. Furthermore, the majority of cellular investigations in alcoholic subjects have been conducted within the PFC or the hippocampus, and to our knowledge, BA24 has yet to be investigated with this respect. There is evidence suggesting that specific subcortical neuronal subtypes are altered in alcoholic suicides. Notably, lower numbers of noradrenergic and serotonergic neurons have been reported in such subjects (Arango et al., 1994; Underwood et al., 2007). In our current investigation, BA24a neuron cell densities were not significantly altered between groups. These findings are in agreement with previous literature suggesting that neuronal loss is not a generalized phenomenon in alcoholics (Harper, 1998; Jensen & Pakkenberg, 1993).
In the present study, glial cell densities were increased (38%) in alcoholic
depressed suicides compared to non-alcoholic suicides and, to a lesser extent, with
controls (30%) in both upper and lower cortical layers. It is important to note that
the lack of information concerning the age of onset and duration of alcohol
dependence represents a limitation to the analysis, as the influence of these
parameters could not be assessed. While reductions have previously been
observed in alcoholic subjects (Korbo, 1999; Miguel-Hidalgo et al., 2006) and in
depressed alcoholics subjects (Miguel-Hidalgo et al., 2002), earlier studies in
alcoholic subjects also described increases (Harper et al., 1987; Kril & Harper,
1989). The authors of these studies proposed that such increases could reflect
glial cell proliferation in response to progressive degeneration and death of
cortical neurons. Such a mechanism, may explain the cerebral shrinkage observed
in these patients (Kril & Halliday, 1999; Pfeffererbaum et al., 1997).

Although the present experimental conditions prevented a precise
identification of the glial cell type(s) responsible for the observed density
increases in alcohol-dependent depressed suicides, it is tempting to speculate that
this phenomenon results from an immune response leading to microgliosis, as
previously reported in suicide brains (Steiner et al., 2008). However, as these
authors point out, caution should be exerted in interpreting such findings, as
increases in microglia could be the result of stress-related changes independent of
suicidal behavior. Thus, a similar mechanism could be occurring in alcohol-
dependent suicides whereby a combination of immunomodulatory effects and
stress-related factors would contribute to the observed increases in glial cell
densities. There is evidence suggesting that chronic alcohol-dependent subjects
experience continuously increased concentrations of cortisol, the main hormone mediating the stress response (Adinoff et al., 2003). Furthermore, current studies have shown that low concentrations of ethanol promote inflammatory processes in brain and in glial cells by up-regulating certain responses to inflammatory injury, such as release of cytokines and inflammatory mediators, activation of associated signaling pathways and transcriptional factors (Blanco & Guerri, 2007; Crews et al., 2006).

3.7 CONCLUSION

In conclusion, we have found no evidence for cellular alterations present in BA24a between depressed suicides and controls possibly suggesting other neurobiological factors are having more of an influential role in suicide. The increases in glial cell densities observed in alcohol-dependent depressed suicides warrants further studies to scrutinize changes in the cellular compositions occurring in alcoholic dependent individuals. Additional studies are needed to determine the specific glial cell subtypes responsible for these alterations occurring in BA24.

Acknowledgements

The authors gratefully thank Dr. Cecilia Flores for granting the use of her microscope in order to carry out the analysis. We would also like to acknowledge the technical aid of Simon Spanswick. The excellent assistance of the Quebec Coroner’s Office, Montreal, Quebec is also appreciated, as is the cooperation and support of the next of kin of the deceased. We are also indebted to the Quebec Brain Bank in providing tissue samples, supported in part by Fonds de la Recherche en Sante du Quebec (N. M.). This research was supported by the Canadian Institute for Health Research (G. T.).
Figure 1: Saggital view of the dissection of interest. A 1cm³ tissue block of BA24a was taken above the genu of the corpus callosum. Modified from, Digital Anatomist Project at the University of Washington. http://da.biostar.washington.edu
Figure 2: Observed cortical layers within BA24a. Upper layers encompassed layers I, II and III, while lower layers included Va, Vb, and VI. Paraffin, 25μm-thick section. 5x. Scale bar = 250 μm.
Figure 3: Differentiation between neurons (black arrow) and glial cells (white arrow) within BA24a. Paraffin, 25 μm section. 40x. scale bar = 25 μm
Figure 4: Morphometric parameters observed in BA24a between control and suicide subjects for a) glia cell density, b) neuronal cell density and c) neuronal somal size. Mean bars are shown.
Figure 5: Comparisons between control subjects and suicides with and without alcohol dependence in BA24a among a) glial cell densities b) neuronal densities and c) neuronal soma size. Mean bars are shown. Note: significant increases in glial cell densities in alcohol-dependent depressed suicides and reduced neuronal somal sizes in upper cortical layers were observed, $p < 0.05$. 
Table 1. Group Summaries of Demographic, Histological, and Clinical Information

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n=13)</th>
<th>Suicide (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
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<td></td>
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<tr>
<td>Age (years)</td>
<td>37.85 ± 10.48</td>
<td>38.46 ± 11.93</td>
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<tr>
<td>Postmortem interval (PMI) (hours)</td>
<td>28.88 ± 13.28</td>
<td>29.19 ± 11.26</td>
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<tr>
<td>Brain tissue pH</td>
<td>6.32 ± 0.30</td>
<td>6.65 ± 0.21</td>
</tr>
<tr>
<td>Storage Time (months)</td>
<td>101.54 ± 50.82</td>
<td>107.08 ± 20.47</td>
</tr>
<tr>
<td>Cause of death</td>
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<tr>
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<td>8</td>
<td>10 hanging</td>
</tr>
<tr>
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<td>1 CO Poisoning</td>
</tr>
<tr>
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<td>Lorazepam</td>
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<td>Minor Depression</td>
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<tr>
<td>Alcohol (abuse: dependence)</td>
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<tr>
<td>Drug Use (abuse: dependence)</td>
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<tr>
<td>Lifetime axis I diagnosis **</td>
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<td>Major Depression</td>
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<td>Alcohol (abuse: dependence)</td>
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<tr>
<td>Drug Use (abuse: dependence)</td>
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<tr>
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<td>1</td>
</tr>
</tbody>
</table>

*Mean ± SD are shown.
*Past = medication prescribed in the past, excluding the last 3 months before death.
*Present = medication prescribed within the last 3 months before death.
*Family history was defined as the number of subjects who had a relative with a confirmed diagnosis of MDD or suicide, negative if there was no history, and unknown if insufficient information was available.
*Diagnosis of at least 1 axis I disorder within the last 6 months before death.
**Diagnosis of at least 1 axis I disorder in the past, excluding those with a present diagnosis.
References


Chapter 4: Discussion

4.1 Summary

The broad aim of this thesis was to address whether there is indeed evidence for cellular alterations occurring within the brains of suicide individuals with a previous MDD diagnosis. Extensive review of the literature indicated that frontal, limbic and brainstem regions experience morphological changes in major depression and suicide, largely implicating the: PFC, ACC, hippocampus, amygdala, raphe nucleus, and LC. As detailed in chapter 2, post-mortem tissue studies involving MDD and suicide individuals provided fine neuroanatomical evidence for cellular compositions (glial and neuronal densities and somal sizes, dendrites and spines) to be altered in these individuals. The extent of the changes in the morphometric parameters was often region specific and involved several neuromodulatory systems, notably, GABAergic, serotonergic, noradrenergic and glutamatergic pathways. Across all brain areas, a reduction in glial cell densities appeared to be a consistent trend, particularly concerning astrocytes (Miguel-Hidalgo et al., 2000; Choudary et al., 2005) and oligodendrocytes (Uranova et al., 2004) suggesting these specific cell types to be playing a key role in the pathophysiology of depression and suicide.

Interestingly, cell parameters were observed to be increased in MDD and suicide individuals in both the hippocampus (Stockmeier et al., 2004) and to an even greater extent within the raphe nucleus, principally serotonergic neurons in the latter (Underwood et al., 1999). It remains to be determined if the observed increases in densities are the result of reduced neuropil, alterations in
programmed cell death that normally occur during brain development, or the consequence of a compensatory mechanism attempting to alleviate cortical deficits. Upon reviewing the literatures, it becomes evident that recent morphological studies have laid foundations as to the cellular underpinnings of MDD and suicide. Nevertheless, there is a need for further research, conducted in an unbiased manner, to understand the nature and pinpoint precisely the different manifestations of cellular changes occurring in these individuals. Recognizing this necessity and the fact that inconsistencies exist between reports, prompted us to conduct an independent cellular investigation.

We choose an area located within the limbic system, the ACC (BA24a), which has been previously shown, by both macroscopic (Ballmaier et al., 2004; Bremner et al., 2004) and microscopic (Auer et al., 2000; Cotter et al., 2001) evidence, to be involved in suicide and major depression. The main conclusion from our study was that we failed to find differences for glial cell densities or neuronal morphometrics and densities between a complete depressed suicide cohort and control subjects. While this result opposes our initial hypothesis, the results are not entirely surprising as they are in agreement with previous literature (Bouras et al., 2001; but see Cotter et al., 2001; Chana et al., 2003). These findings suggest that neurobiological factors, other than alterations in cell populations might have more of an influential role in MDD and suicide. In this regard, studying potential differences at a molecule level such as targeting candidate genes or assessing protein levels of specific cell subtypes may provide fruitful evidence for alterations occurring in the brains of suicide victims in comparison to controls.
Of surprise were the findings from our secondary analysis which showed an increase in glial cell densities of alcohol-dependent depressed suicides compared to non-alcoholic depressed suicides (38%) and to a lesser extent in controls (30%). While it remains unclear by which mechanism(s) glial cell densities would be increased, the possibility of an immune response causing microgliosis is enticing. As presented in chapter 3 (section 3.6), a recent study revealed exciting observations suggesting that microgliosis is perhaps responsible for increases in glial cell densities in suicide victims (Steiner et al., 2008). These findings may implicate immunological factors, which to date, have played an underestimated role in suicide. Indeed, microglia are known to release neuroendocrine factors, cytokines, and nitric oxide (Frank et al., 2006); all of which modulate noradrenergic (Labuzek et al., 2005) or serotonergic (Linden et al., 2002) neurotransmission and thus may trigger suicidality. Seeing that alcohol-dependent individuals also show abnormalities in regulating stress responses (Adinoff et al., 2003; Dai et al., 2007), it is tempting speculate that a combination of both immunomodulatory effects and stress-related factors are occurring in alcohol-dependent suicides and thus be partially accountable for the observed increases. Additionally, it has become increasingly known that major disorders of the central nervous system, such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease, are not solely characterized by the emergence of dysfunctional neurons, but are in large part orchestrated by glial-cell-controlled inflammatory processes. Seeing that increases were observed between alcoholic-dependent depressed suicides and with both, non-alcoholic depressed suicides and controls suggest that these increases are related to changes in the cellular
compositions of alcoholic dependent individuals. As there was a slightly larger effect occurring within the suicide group, it would be intriguing for future studies to determine whether differential glial pathologies are occurring between alcoholic depressed suicides and non-alcoholic depressed suicides within BA24.

4.2 Limitations and Future Directions

Constraints of the present experiment together with the challenging task of working with human tissue, may explain why our primary finding was not in agreement with several past studies. One possibility for the discrepancies, as discussed in chapters 2 (section 2.10), are variances in methodological techniques. With respect to the ACC, both 2D and 3D techniques have been utilized in addition to differential sampling of distinctive, heterogeneous subdivisions within BA24. While each counting method has its advantages and disadvantages (Benes & Lange, 2001), it is important to interpret results accordingly while also having an awareness of the heterogeneity of the region of interest. In our current investigation we employed a 3D technique consistent with that of Bouras and colleagues, (2001). Care was taken to sample BA24a tissue as systematically and randomly as possible, furthermore morphometric parameters were thoroughly examined. Therefore we were confident in the cell density values and nucleator measurements obtained. Additionally, density measurements for our control subjects are comparable with previous cellular reports (Rajkowska et al., 1999; Bouras et al., 2001; Miguel-Hidalgo et al., 2006).

A limitation to our study, presented in chapter 3 (section 3.6), was that we did not analyze individual cortical layers, alternatively we divided our region into upper and lower cortical contours consisting of layers I, II, III and Va, Vb, VI.
respectively due to technical difficulties. In large part, within the ACC, significant laminar differences have been observed (Cotter et al., 2001; Chana et al., 2003). Therefore, it appears crucial for future studies to delineate and subsequently explore possible layer specific alterations. Studies conducted in monkeys have shown that neurons located in lower cortical layers, within BA24, send output projections to deeper brain regions and also express high levels of dopamine and glucocorticoid receptors (Vogt & Pandya, 1987). Recognizing that different layers within the ACC are associated with specific neurobiological pathways it would be intriguing to determine which system (e.g. serotonergic, noradrenergic, dopaminergic, HPA) has a greater influence in suicide and major depression. However, it may be more reasonable and likely to assume that these disorders yield irregularities in multiple pathways. Thus the challenge would be to gain an understanding on exactly how these systems are working together in suicide and major depression. To address this issue, morphological studies should exploit specific stains targeting certain subtypes of neurons and glial cells. In contrast, the present study employed a non-specific stain, labelling all classes of cells, possibly contributing to the inability to detect potential alterations. A final limitation worth considering for any cellular study is that it remains unclear whether the cellular alterations reported are a cause or a consequence of suicide and MDD. The morphological changes may represent an early neurobiological marker of suicide and MDD. Alternatively, such alterations might be an epiphenomenon of the underlying psychopathology of illness such as being long term consequences of stress and depression. Unfortunately, this dissociation is incredibly difficult to tease apart, as most suicides have had a previous psychiatric
diagnosis, as discussed in chapter 1. Acquiring tissue across the lifespan of individuals (from early adolescence into elderly) may aid in this distinction.

4.3 Concluding Remarks

Taken together, post-mortem studies can provide the fine detail needed for exploring possible alterations in specific populations of cells (neurons and glial cells) and their components (dendritic trees, axons, synapses) occurring in MDD and suicide. Therefore studying tissue from depressed suicide victims presents a potential informative approach for revealing the neurobiology of this psychiatric disorder. This thesis presented an extensive review displaying evidence, and a lack thereof, for cellular changes occurring within fronto-limbic structures and brainstem regions previously shown to be associated with suicide and MDD. Conclusions from our own investigation demonstrated that within a specific sub-region of the ACC - BA24a - cellular alterations were not evident when comparing a depressed suicide cohort to control individuals. Conceivably these findings suggest that the behavioral correlates, in addition to macroscopic alterations associated with BA24 in suicide and MDD, may not be greatly affecting the cellular anatomy of this region. However, before such conclusions can be firmly drawn, detailed staining, targeting specific subtypes of neurons and glial cells should be conducted. Additional studies are also necessary to examine explicit changes in the cellular compositions occurring in alcoholic dependent individuals. These would aid in providing knowledge for the morphological sensitivity occurring within the ACC and thus contribute to understanding the etiology of this devastating act of suicide.
References


