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Table of Contents

ABSTRACT

Background: Despite increasing evidence supporting the neuroinflammatory theory of depression, little is known about the neuroinflammatory environment in the brain of individuals suffering from depression. The major aim of this thesis was to compare the fine morphometric properties of astrocytes and microglia, two glial cell types involved in neuroinflammation, in the dorsal anterior cingulate cortex (dACC) of depressed suicides versus matched nonpsychiatric controls. This region was chosen on the basis that it has been repeatedly implicated in mood disorders, but also because it modulates the behavioural responses to inflammation.

Methods: Morphometric analyses of 3D-reconstructed astrocytes were carried out from Golgi-impregnated dACC samples with the aid of the Neurolucida software. Similar analyses were performed for microglia in sections immunostained for the macrophage marker Ionized calcium binding adaptor molecule 1 (IBA1) after an initial characterization of microglial phenotypes in the dACC of nonpsychiatric controls. The distribution of IBA1-immunoreactive (IBA1-IR) cells was also assessed using stereology, and blood vessels were characterized as being intimately associated with either a high or a low density of IBA1-IR amoeboid-like cells. Furthermore, the expression of cytokines and of a subset of macrophage-specific genes were measured by quantitative PCR.

Results: Significant cellular and molecular changes between groups were observed in the dACC white matter. First, fibrous astrocytes displayed a significantly larger cell body, as well as longer, more ramified processes in depressed suicides compared to controls. Total densities of IBA1-IR microglia did not differ between depressed suicides and controls. However, a finer analysis examining relative proportions of microglial phenotypes revealed that the ratio of primed over ramified microglia was significantly increased in depressed suicides. Strikingly, the proportion of blood vessels surrounded by a high density of macrophages was more than twice higher in depressed suicides than in controls, and this difference was strongly significant. Consistent with these

observations, expression of IBA1 and MCP-1, a chemokine involved in the recruitment of circulating monocytes, was significantly upregulated in the dACC white matter of depressed suicides. Furthermore, mRNA for CD45, a marker enriched in perivascular macrophages, was also significantly increased in samples from depressed suicides. **Conclusions**: Altogether, these findings suggest the presence of low-grade neuroinflammation in the dACC white matter of depressed suicides, and provide further support to the neuroinflammatory theory of depression.

RÉSUMÉ

Contexte: Malgré les données appuyant la théorie neuroinflammatoire de la dépression, on en sait peu sur l'environnement neuroinflammatoire au sein du cerveau de personnes souffrant de dépression. L'objectif principal de cette thèse était de comparer les propriétés morphométriques fines d'astrocytes et de microglies, deux types de cellules gliales impliquées dans la neuroinflammation, dans le cortex cingulaire antérieur doral (dCCA) de dépressifs suicidés par rapport à des témoins appariés. Cette région a été choisie parce qu'elle a déjà été impliquée dans les troubles de l'humeur, mais aussi parce qu'elle module les réponses comportementales à l'inflammation.

Méthodes: Des analyses morphométriques d'astrocytes reconstruits en trois dimensions à l'aide du logiciel *Neurolucida* ont été réalisées à partir d'échantillons marqués par la méthode de Golgi dCCA. Des analyses similaires ont été effectuées pour la microglie à partir de coupes immunomarquées pour la protéine spécifique aux macrophages *Ionized calcium binding adaptor molecule 1* (IBA1) suite à une première caractérisation des phénotypes microgliaux observables dans des échantillons dCCA témoins. La distribution des cellules IBA-immunoréactives IBA1 (IBA1-IR) a également été étudiée par stéréologie, et les vaisseaux sanguins ont été caractérisés comme étant intimement associés soit à une forte ou à une faible densité de cellules IBA1-IR amiboïdes. De plus, l'expression de cytokines et d'un certain nombre de gènes spécifiques aux macrophages a été mesurée par PCR quantitative.

Résultats: Des changements cellulaires et moléculaires significatifs ont été mesurés entre les groupes de sujets au sein de la substance blanche du dCCA. Premièrement, chez les dépressifs suicidés, les astrocytes fibreux affichaient un plus gros corps cellulaire ainsi que des processus plus ramifiés que chez les témoins. Bien que les densités totales de microglies ne différaient pas entre les groupes de sujets, une analyse plus fine examinant les proportions relatives des phénotypes microgliaux a révélé que la proportion de microglies présentant un phénotype « stimulé » (*primed*) était plus important chez les dépressifs suicidés que chez les témoins. La proportion des vaisseaux

sanguins entourés d'une forte densité de macrophages au sein de la matière blanche du CCA était plus de deux fois plus élevée chez les dépressifs suicidés que chez les témoins, et cette différence était fortement significative. En accord avec ces observations, l'expression de IBA1 et de MCP-1, une chimiokine impliquée dans le recrutement des monocytes circulants, était significativement régulée à la hausse dans la substance blanche CCA des suicides déprimés. L'ARNm codant pour la protéine CD45, un marqueur enrichi au sein des macrophages périvasculaires, était également augmenté de manière significative dans le même région chez les dépressifs suicidés.

Conclusions: Prises dans leur enseble, ces données originales suggèrent la présence d'une neuroinflammation de faible intensité au sein de la substance blanche du dCCA chez les dépressifs suicidés et, plus généralement, soutiennentla théorie neuroinflammatoire de la dépression.

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LIST OF PUBLICATIONS

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3. **Torres-Platas SG**, Cruceanu C, Chen GG, Turecki G, Mechawar N (2014) Evidence for increased microglial priming and macrophage recruitment in the dorsal anterior cingulate white matter of depressed suicides. *Brain, behavior, and immunity* 42:50-59.

4. Cruceanu C, Cheng Tang PP, Rogic S, Lopez JP, **Torres-Platas SG**, Gigek CO, Alda M, Rouleau GA, Pavlidis P, Turecki G (2015) Transcriptome sequencing of the anterior cingulate in bipolar disorder: dysregulation of G proteincoupled receptors. *American Journal of Psychiatry*: in press.

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6. Huh C, Amilhon B, Ferguson K, **Torres-Platas SG**, Manseau F, Peach J, Scodras S, Mechawar N, Skinner F, Williams S (2015) Excitatory inputs determine phase-locking strength and spike-timing of CA1 stratum oriens/alveus parvalbumin and

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ABBREVIATIONS

- 5-HIAA - 5-hydroxy-indoleacetic acid
- ACC - Anterior Cingulate Cortex
- ATP - Adenosine triphosphate ACC - Anterior cingulate cortex
- ACTH - adrenocorticotrophic hormone-releasing factor
- BA - Brodmann area
- BBB - Blood Brain Barrier
- CCR2- chemokine (C-C motif) receptor 2
- CD - Cluster Differentiation
- CMS - chronic mild stress
- CMS - central nervous system
- CRP - c-reactive protein
- CSF - Cerebro-spinal fluid
- COX - cyclooxygenase
- DAMPs - Damage danger-associated molecular patterns
- DLPFC - Dorsolateral Prefrontal Cortex
- EAAT - Excitatory amino acid transporter
- GFAP - Glial fibrillary acidic protein
- HLA-DR- human leukocyte antigen
- HPA- Hypothalamic-Pituitary-Adrenal
- ICAM-1 - Cellular Adhesion Molecule
- IDO - Indoleamine-2,3-dioxygenase
- IL - Interleukin
- IL-1RA - Interleukin-1 receptor antagonist
- K^+ - Potassium
- LPS - Lipopolysaccharide
- MAO - monoamine oxidase
- MCP-1/CCL2 - Monocyte chemoattractant protein
- MDD - Major depressive disorder
- MHC - Major histocompatibility complex
- NFκB - Nuclear Factor Kappa-light-chain-enhancer of activated B cells
- NMDA - N-methyl-D-aspartate
- PAMPs - Pathogen danger-associated molecular patterns
- PFC - Prefrontal Cortex
- STAT3 - Signal Transducer and Activator of transcription 3
- Th - T helper cell
- TLR - Toll-like receptor
- TGF- β - Transforming growth factor beta
- GABA - γ-aminobutyric acid
- TNF - Tumor Necrosis Factor
- US - United States

- VCAM-1 - Vascular Cellular Adhesion Molecule

CHAPTER 1.0: INTRODUCTION

1.1 NEUROBIOLOGY OF DEPRESSION AND SUICIDE

1.1.1 Introduction

Major Depressive Disorder (MDD) is a psychiatric condition characterized by the persistent lowering of a person's mood that is often accompanied by anxiety. MDD is clinically diagnosed if at least five of the following symptoms are present every day for at least two weeks: significant weight loss or weight gain, disturbed sleep, psychomotor agitation or retardation, fatigue or decreased energy, feelings of worthlessness or guilt, diminished ability to think or concentrate or suicidal ideation (Black et al., 2014). In the United States (US) alone, MDD affects approximately 15 million adults per year (Kessler et al., 2005) and, worldwide, it currently affects 350 million people and is the leading cause of disability according to the World Health Organization (WHO, 2012).

MDD results too frequently in suicide completion, which ranks among the top ten causes of death for individuals of all ages. Worldwide, almost 1 million people die yearly by suicide, which means that on average, one person commits suicide every 40 seconds. Additionally, for every person who completes a suicide, 20 or more may attempt to commit suicide (WHO, 2014). Psychological autopsy studies indicate that at least 50% of all adult suicides have had a previous diagnosis of depression (Kim et al., 2003) or bipolar disorder (Arsenault-Lapierre et al., 2004). Furthermore, up to 15%-19% of individuals with a lifetime diagnosis of major depressive disorder (MDD) (Chen and Dilsaver, 1996) and 50% of individuals with bipolar disorder have a history of attempted suicide (Jamison, 2000).

As detailed below, this global burden has given rise to an increasingly multidisciplinary research field aimed at understanding the biological causes underlying depression and suicide. Based on different lines of evidence, several hypotheses have

been posited to explain the pathophysiology of depression. Among the more prominent, which will be discussed further below, the monoamine theory of depression was one of the first to be proposed and was intimately linked to the discovery of the first antidepressant drugs. Another major hypothesis is the corticotropin hypothesis of depression, which is based on evidence of abnormal hormonal levels in depressed patients. Lastly, the neuroinflammatory theory of depression, supported by several converging lines of evidence, has been gaining significant momentum in the past twenty years.

1.1.2 Monoamine Hypothesis of Depression

In the 1950´s, the monoamine hypothesis of depression was first proposed and rapidly became the leading theory regarding the underlying biological causes of depression for numerous decades. This hypothesis, which is intimately linked to the serendipitous finding of the first antidepressant drugs, states that depression is caused by an hypoactivity of monoamine systems in the brain (Baumeister et al., 2003). The first depression-inducing molecule, reserpine, was isolated from the dried root of the plant *Rauwolfia Serpentina*, which had traditionally been used in India as a soothing agent and to treat snakebites (Glynn, 1955). In the 1950's, reserpine was found by Indian scientists to be very effective to lower blood pressure (reviewed in Baumeister et al., 2003). After its extensive use and increasing popularity, the active component of the root was marketed in tablet form as a sedative antihypertensive drug under the name "Serpasil" (Glynn, 1955). However, increasing concerns about patients developing depression due to reserpine, rapidly decreased its popularity (Cahn, 1955). This psychotropic property, however, led to many research efforts directed at understanding reserpine´s mode of action. After a few years, it was found that, pharmacologically and chemically, reserpine was very similar to serotonin and that reserpine causes prolonged depletion of cerebral serotonin (Shore et al., 1955, Brodie et al., 1956) and catecholamines, resulting in hypokinesia (Carlsson et al., 1957). It was then discovered

that reserpine causes a blockade of the vesicular monoamine transporter, leading to increased catabolism of monoamines by monoamine oxidase (Henry and Scherman, 1989, Baumeister et al., 2003).

A second major serendipitous event contributing to the monoamine hypothesis of depression was the discovery of the positive side effects of isoniazid and ipronazid, which were both designed to treat tuberculosis (Fox, 1952). These drugs caused euphoria, pain relief, increased appetite, weight gain, reversed apathy, and increased the patients' sense of well-being (Robitzek et al., 1952). All these effects were initially thought to be the result of the improvement of the tuberculosis symptoms (e.g. pulmonary symptoms), however, other side effects indicating central and autonomic nervous system stimulation (e.g. drowsiness and dry mouth) were also noticed (Selifoff et al., 1952), which lead to the speculation that this drug may be acting directly on the nervous system (Maxwell and Eckhardt, 1990).

In 1953, 70% of the patients with depressive disorders who were treated with isoniazid for one to six months, showed mood improvement (Baumeister et al., 2003) and five years later, around 380,000 patients in the US were treated with iproniazid (Bosworth et al., 1955, Floody et al., 1958). In the early 1960s, research efforts to understand iproniazid´s mode of action resulted in the discovery that iproniazid is a potent inhibitor of the enzyme monoamine oxidase (MAO) (Lecocq and Linz, 1952). However, in 1961 the popularity of iproniazid decayed rapidly after it was found that hepatitis was a common secondary effect of the drug and, therefore, it was withdrawn from the US Market (Baumeister et al., 2003).

Based on the observations that (1) reserpine or drugs that decrease monoamines concentrations have depressogenic effects and that (2) iproniazid or drugs that increase monoamines have antidepressant effects, Everett and Toman postulated the first version of the monoamine hypothesis of depression. They stated that: "An excess of

central amines might result in irritability, restlessness and aggressiveness. In the opposite direction, a deficiency of these substances would result in depression." (Society of Biological Psychiatry. and Masserman, 1959 reviewed in Baumeister et al., 2003). However, the credit of the first postulation of the monoamine hypothesis of depression was mainly given to Joseph Schildkraut, who wrote a compelling and comprehensive review of all the lines of evidence supporting this theory. This paper, published in the American Journal of Psychiatry in 1965 became the most frequently cited paper ever published in this journal (Schildkraut, 1965).

Following this period, pharmaceutical companies began competing to develop new compounds to treat mental illnesses. The discovery of the first tricyclic antidepressant, imipramine, was made by Roland Kuhn, a Swiss psychiatrist working at the Cantonal Mental Hospital of Münsterlingen in Switzerland, while searching for a chlorpromazine-like substance for the treatment of schizophrenia. When testing the compound "G 22,355" which has a very close structural resemblance to chlorpromazine, Kuhn found it ineffective for the treatment of schizophrenia (Ban, 2006). However, he decided to test this compound on one of his female patients who suffered from severe depression, and the treatment improved her symptoms. Thereafter, Kuhn administered G 22,355 to a total of 43 depressed patients and he concluded that the drug is effective to treat depression. The drug was given a generic name of imipramine and Kuhn's first paper on the treatment of depressive states was published in the *Swiss Medical Journal* in 1957 (Ban, 2006).

In 1965, the mechanism of action of imipramine was becoming clear and it was demonstrated by Dr. Lapin and his co-workers that it enhanced the effect of serotonin in the central nervous system (CNS), suggesting that increased central levels of serotonin could account for mood elevation and that the noradrenergic processes were responsible for the hyperkinetic component of the clinical antidepressant effect (Oxenkrug, 2013). Imipramine was shown to block serotonin reuptake into platelets

(Marshall et al., 1960) and shortly thereafter, Axelrod and colleagues showed that imipramine blocks the reuptake of norepinephrine into presynaptic terminals (Axelrod et al., 1961). Therefore, it was shown that imipramine effectiveness was due to the inhibition of the uptake of monoamines and the enhancement of noradrenergic transmission (Wong et al., 2005). Since it was found that imipramine was more effective to inhibit the reuptake of serotonin versus the reuptake of noradrenaline in the CNS, it was proposed that serotonin reuptake inhibition specifically contributed to the mood elevation produced by tricyclic antidepressants (Wong et al., 2005). In addition to these pharmacological studies, increasing evidence suggested that serotonin and its metabolite 5-hydroxy-indoleacetic acid (5-HIAA) were decreased in postmortem samples of suicide completers (Shaw et al., 1967, Bourne et al., 1968). These and other studies suggested that indoleamines and, more specifically, serotonin, were involved in depression and represented a good therapeutic target.

The biology of serotonergic synthesis, degradation, storage, uptake and release was characterized in the 1960s (Wong et al., 2005). At the time, the antidepressant drugs available were inhibitors of the enzyme MAO or inhibitors of the uptake of monoamines. Research efforts were focused on the development of the first selective serotonin reuptake inhibitor (SSRI), and in 1982, the drug zimelidine was developed by the Swedish pharmaceutical company Astra. The clinical use of this drug was short lived, however, because several patients developed Guillain–Barré syndrome. Fluoxetine, marketed with the name of Prozac in 1987 by Eli Lilly (Perez-Caballero et al., 2014), was one of the first members of the SSRI class of antidepressants and became the most prescribed antidepressants (Blier and El Mansari, 2013). After fluoxetine, several other antidepressants targeting monoamine reuptake were developed, such as paroxetine, sertraline, citalopram and escitalopram (the S-enantiomer of citalopram) (Blier and El Mansari,2013).

To this day, many laboratories worldwide have characterized the biological and chemical phenotypes associated with monoaminergic system that contribute to depression and suicide. There are numerous factors that alter the synaptic concentration of serotonin such as genetic variation in tryptophan hydroxylase 2, 5-HT_{1A} receptors (Albert et al., 2014), 5-HT_{1B} autoreceptors, serotonin transporters (Caspi et al., 2003), and the polymorphism of a variety of postsynaptic 5-HT receptors. However it has been suggested that, since all these associations are weak, these anomalies in the serotonergic system confer a predisposition to depression rather than being the cause (Prins et al., 2011). Additionally, the fact that antidepressants targeting the serotonergic system require several weeks to relieve depressive symptoms when the monoaminergic neurotransmission is enhanced immediately after administration is not consistent with this hypothesis (Prins et al., 2011) and suggests the involvement of other systems in the pathophysiology of depression.

1.1.3 Glucocorticoid Hypothesis of Depression

Before the appearance of antidepressants, there were substantial reports of the alterations of stress system hormones in depressed patients (Holsboer, 2000). However, for many years, these changes were seen as a result of the stressful experience of suffering from depression. In 1919, the hormonal therapy was first proposed as potential antidepressants treatment (reviewed in Holsboer, 2000). Given that the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis not only regulates body peripheral functions such metabolism and immunity, but also has profound effects on the brain, the glucocorticoid hypothesis of depression was proposed. This hypothesis states that dysregulation stress hormones causes depression and antidepressants may act through normalizing the HPA axis (Pariante and Lightman, 2008).

The Stress system or the HPA axis activity is orchestrated by the secretion of corticotropin releasing factor (CRF) and vasopressin from the parvocellular neurons of

the hypothalamus, which in turn activate the secretion of adrenocorticotrophic hormone (ACTH) and vasopressin from the pituitary, which finally stimulates the secretion of the glucocorticoids (cortisol in humans and other primates, and corticosterone in rodents) from the adrenal cortex. Then, glucocorticoids bind to the glucocorticoid receptor (GR) outside the brain (multiple target tissues) or inside the brain (hippocampus and hypothalamus) to produce a feedback inhibition signal both on the hypothalamus and directly on secretion of ACTH from pituitary, causing a reduction of HPA axis activity (Pariante and Lightman, 2008).

Postmortem studies of suicide completers have showed increased activity of CRF in the paraventricular nucleus (Raadsheer et al., 1994, Raadsheer et al., 1995) (Austin et al., 2003), reduced number of CRF binding sites in the frontal cortex (Nemeroff et al., 1988), and reduced GR in the hippocampus (McGowan et al., 2009), which suggest an alteration on the HPA axis of suicide completers. These alterations in the HPA axis, often lead to a chain of events such as enlargement of the pituitary gland, enlargement of the adrenal cortex (Nemeroff et al., 1992) and increased baseline cortisol levels (Gold et al., 1986) and vasopressin (von Bardeleben et al., 1989). Although hypercortisolemia resulting from in impairment of GR mediated negative feedback is also known to be a feature of a subset of depressed individuals (Stetler and Miller, 2011), it is likely to be influenced by many factors such as the history of early life adversity, or the specific type of depression (melancholic, psychotic or atypical depression) (Stetler and Miller, 2011). In fact, this association is suggested to be more strongly linked to suicide independently of the psychiatric diagnosis since it is enhanced in violent suicide attempts (Roy, 1992), and may be used to predict suicide completion (Coryell and Schlesser, 2001 reviewed in Turecki, 2014).

Over the last few years, one of the most interesting findings is that early life events can directly influence the predisposition of increased activity of the HPA axis. Several lines of research have demonstrated in rodents (Meaney, 2001), non-human

primates (Higley et al., 1991), and humans (Heim et al., 2000, Heim et al., 2008) that neonatal maternal separation from their mothers for long periods or childhood adversity, abuse or maltreatment, cause HPA axis changes that persist into adulthood. These changes resemble those present in depressed individuals (Anacker et al., 2014) and are characterized by permanent changes in gene expression that are important for the stress response. Suicide victims with a history of childhood abuse/trauma, presented decreased levels in the GR expression in the hippocampus and increased levels of methylation in the nuclear receptor subfamily 3 group C member (NR3C1) promoter, which codes for the receptor for glucocorticoids (McGowan et al., 2009). These findings suggest that childhood maltreatment produces changes in the expression of GR causing reactivity in the HPA axis in humans that have been exposed to childhood maltreatment. These long lasting changes have been propose to confer a predisposition to depression and suicide.

1.1.4 Inflammatory Hypothesis of Depression

Cytokines are small soluble proteins (≅15-30 kDa) released by host cells to regulate immune system responses and the normal function of many cell types (Opal and DePalo, 2000). A broad range of cells in the body produces cytokines. However, these are secreted mainly and most commonly by immune cells such as T-cells in the periphery and astrocytes and microglia in the brain. A substantial amount of evidence suggests that depression can be caused by external or internal stressors, which through activation of multiple signaling pathways, result in increased expression of inflammatory markers that contribute to depressive symptoms (Maes, 2008). It has been shown that excessive and/or prolonged pro- inflammatory cytokine activity (which also regulates peripheral inflammatory responses) results in depressive symptoms. Therefore, it has been suggested that activity of the immune system may itself contribute to depression (Miller et al., 2009a). Patients diagnosed with MDD display elevated plasma levels of cytokines including interleukin-1 (IL-1), IL-2, IL-6, IL-8, IL-12, interferon-α (IFN- α) and

tumor necrosis factor-α (TNFα) (McNally et al., 2008, Maes et al., 2009), as well as acute phase proteins, chemokines and adhesion molecules (Miller et al., 2009). Data regarding IL-1, TNF-α, IL-6 c-reactive protein (CRP) appear to be some of the most reproducible findings in this regard and are sufficiently robust that these cytokines have been proposed as peripheral biomarkers for major depression (Maes, 1995, Miller et al., 2009a). In spite of some inconsistencies in the literature, which may be due to sample heterogeneity, several meta-analyses argue strongly for the association of depression and increased peripheral levels of pro-inflammatory cytokines IL-1, IL-2, IL-6, IL-8, IL-12, INF-α and TNFα (Dowlati et al., 2010, Liu et al., 2012, Black and Miller, 2014).

Evidence derived from immunotherapies with pro-inflammatory cytokines further implicate cytokines in depression. In patients with hepatitis C, administration of exogenous IFN-α as immunotherapy results in a 45-70% incidence of clinical depression (Capuron et al., 2005, McNally et al., 2008, Miller et al., 2009a) and altered activation of the dorsal ACC (Capuron et al., 2005). Additionally, depression is comorbid with many diseases or conditions with an inflammatory component, such as rheumatoid arthritis, fibromyalgia, inflammatory bowel disease and coronary artery disease. These findings are consistent with the notion that depression and inflammation may share common mechanisms (Aggarwal et al., 2006; Bisoendial et al., 2007; Bouzakri and Zierath, 2007).

Additional support for this hypothesis has come from animal models showing that peripheral administration of pro-inflammatory cytokines (or inducers such as lipopolysaccharide [LPS]) produces depressive-like behaviors in rodents (Merali et al., 2003). Different behavioral tests/models of depression have been associated with alterations in plasma and cerebral IL-1β, IL-2, TNF-α (Kubera et al., Kubera et al., 1996, Grippo et al., 2005) and IL-6 expression (Zhou et al., 1993, Takaki et al., 1994, Shizuya et al., 1997). This depressive-like behavior can also be observed after acute or chronic treatment with inflammatory challenges or 24 hours after a systemic injection of LPS (Frenois et al., 2007, Dantzer et al., 2008). Furthermore, increased cerebral IL-1 after

chronic mild stress (CMS; a well-accepted model of depression) is not only necessary but also sufficient to activate the downstream signalling pathways that cause the symptoms typically seen in depression (Goshen et al., 2008). Transgenic mice over-expressing the IL-1 receptor antagonist (IL1-Ra) and a knock out (KO) mice of the IL-1 family receptor type 1 (IL-1r) show no depressive-like behaviour following CMS (Goshen et al., 2008). The same study showed that depressive-like behaviour induced by CMS in IL-1Ra overexpressing transgenic mice is mediated preferentially by the central nervous system. Interestingly, chronic intraventricular IL-1β administration is sufficient to produce depressive-like behaviors, and administration of anti-inflammatory cytokines such as IL-1ra or IL-10 can block the behavioral effects of treatment with IL-1 and/or LPS in laboratory rodents. Furthermore, chronic treatment with antidepressants can reduce or even abolish the depressive-like effects induced by peripheral, but not central, LPS or IL-1β administration in rats (Castanon et al., 2001).

In humans, SSRIs were found to reduce depressive symptoms as well as peripheral levels of IL-1β and IL-6 (but not TNFα), thus suggesting that the treatment with antidepressant drugs may exert selective effects on the expression of cytokines (Hannestad et al., 2011). Furthermore, administration of drugs that inhibit the action of cytokines and their signalling pathways (e.g. cyclooxygenase inhibitors) have been shown to improve mood in patients with inflammatory disorders, and increase the responsiveness to antidepressant treatment in patients with depression (Mendlewicz et al., 2006; Muller et al., 2006; Tyring et al., 2006). These results complement the notion that cytokines contribute to the regulation of mood and that successful antidepressant treatment is associated with the normalization of IL-6 and IL-1β levels, and that this may be mediated by the recovery of serotoninergic homeostasis in the brain.

Taken together, these lines of evidence suggest that elevated peripheral levels of pro-inflammatory cytokines are a characteristic of depression. But the question of the precise effect these cytokines have on the brain remains elusive. Cytokines from the

periphery cannot freely cross the blood-brain barrier (BBB), but they can nevertheless influence brain physiology and function through at least five mechanisms:

(1) Cytokines can access the brain through leaky regions of the BBB, including the choroid plexus and circumventricular organs such as the organum vasculosum laminae terminalis and the area postrema (Ericsson et al., 1994, Banks et al., 1995, Turnbull and Rivier, 1999), preoptic area and the median eminence (Turnbull and Rivier, 1999). Several of these circumventricular organs are located in the hypothalamus (the median eminence, organum vasculosum laminae terminalis and preoptic area), while others have direct connections to the hypothalamus (Pace et al., 2007).

(2) A small percentage of circulating cytokines can be actively transported to the brain through saturable cytokine-specific transporters expressed by endothelial cells (Pace, Miller, 2012).

(3) Cytokines can indirectly affect the brain by stimulating the production of second-messenger signals from endothelial cells in the BBB, which in turn leads to an excess production of cytokines and other inflammatory mediators. Activation of IL-1 receptors on endothelial cells induce cyclooxygenase, which in turn results in the production of prostaglandin E2 which can freely cross the BBB. Furthermore, prostaglandin E2 can activate microglial cells, and can activate PVN-CRF cells in the hypothalamus, and thus the HPA axis (Rivest, 2001, Pace and Miller, 2009, Bellavance and Rivest, 2014).

(4) Cytokines can act directly or indirectly on peripheral neurons that project afferent nerve fibers in the brain, such as the vagus and the trigeminal nerves. The vagus contains neurons that project to the brain stem that, in turn, project to the hypothalamus. IL-1 can influence the brain by activating such afferents (Goehler et al., 2000, Pace and Miller, 2009).

(5) Cytokines can be synthesized by infiltrating activated monocytes that are recruited from the periphery into the brain (Pace and Miller, 2009). It has been recently demonstrated that stress and other factors can cause the recruitment of monocytes to the brain parenchyma (Wohleb et al., 2014).

Evidence of increased production of local pro-inflammatory cytokines in the CNS of depressed patients is scarce. Just a handful of studies have successfully reported increased levels of pro-inflammatory cytokines in the brain or cerebro-spinal fluid (CSF). In a study of suicide teen-agers, Dwivedi and collegues reported a significant increase in IL-6, Il-1β and TNF-α mRNA and protein expression in Brodmann area 10 (BA10) prefrontal cortical gray matter (Pandey et al., 2012). Other investigations performed in the orbitofrontal cortex of suicide completers, where gender was taken into account, found increased expression of IL-4 in samples from women and of IL-13 in samples from men (Tonelli et al., 2008). No significant differences were found in expression levels of IL-6, Il-1β and TNF-α (Tonelli et al., 2008). In a study where CSF of suicide attempters compared with healthy controls was analyzed, levels of vascular endothelial growth factor (VEGF) and of IL-8 were measured to be significantly lower in suicide attempters, with no significant difference in IL-6 being detected between groups (Isung et al., 2012). Interestingly, plasma levels of IL-6 have been associated with impulsivity and the choice of a violent suicide attempt, and it has been suggested that IL-1β and IL-6 are most robustly associated with suicidality (Black and Miller, 2014). These results suggest that different types of cytokines may influence methods of suicide, although the underlying mechanisms remain to be described.

1.1.5 Reconciling the main hypotheses

Hyperactivity of the HPA axis, as evidenced by elevated circulating concentrations of cortisol, is a highly consistent observation in patients suffering from MDD (Pariante, 2009). When an organism is facing a stressful event, the duration of the HPA response depends on the degree and type of the stressor (physical, emotional, immunological, etc.) and on the precise circadian phase at which stress occurs (Bellavance and Rivest, 2014). This response is controlled by regulatory feedback loops involving the adrenal glands, pituitary, PVN, and upstream cortico-limbic structures such

as the hippocampus, amygdala, and medial prefrontal cortex (Bellavance and Rivest, 2014). The HPA axis requires a very precise and subtle balance regulation for an optimal and healthy performance, and factors such as chronic stress, which is a precipitating factor in depression, is thought to alter this balance.

Clinical and experimental evidence has shown that antidepressant treatment is associated with the resolution of the impairment in the negative feedback in the HPA axis (Anacker et al., 2014). This antidepressant normalization of the HPA axis is achieved by an increase of GR expression and function, and therefore, GR-mediated HPA axis feedback inhibition resulting in a reduction of HPA-axis activity (Pariante and Lightman, 2008). This normalization of GR function by antidepressant treatment is a significant predictor of long-term clinical outcome. Additionally, successful treatment with imipramine can significantly inhibit the production of some inflammatory mediators such as nitric oxide and TNF-α in vitro and normalize C-reactive protein levels (Miller et al., 2009j, Maes et al., 2012). This suggests that antidepressants, which can modulate the serotoninergic levels in the brain, successfully improve symptoms of depression by restoring the HPA axis and decreasing the levels of inflammation.

1.1.5.1 The relationship between Inflammation and HPA axis activation

The relationship between the immune system and the HPA axis is not bidirectional, since pro-inflammatory cytokines can activate the HPA axis, but cortisol is largely known for being one of the most potent anti-inflammatory and immunosuppressive hormones in the body (Rhen and Cidlowski, 2005). Therefore, the fact that increased levels of pro-inflammatory cytokines and a parallel excess of glucocorticoid is characteristic of depression suggests a more complex regulatory mechanism of these two systems (see below).

Evidence has shown that IL-6, IL-1α, IL-1β, and TNF-α, can acutely activate the HPA axis (Pace et al., 2007), through four different mechanisms: (1) by inducing several inflammatory signaling molecules including NF-kB, p38 MAPK and STAT5, that disrupt GR translocation from the cytoplasm to nucleus through GR phosphorylation, (2) through nuclear protein-protein interactions which inhibit GR-DNA binding, (3) GR binding to its DNA response element and, (4) by causing a decrease in the GR alpha, the active form of the receptor, and increased GR beta, a relatively inactive GR isoform (Pace et al., 2007).

In animal models, IL-1 when administered peripherally in low doses, produces elevated plasma concentrations of ACTH and corticosterone (Besedovsky et al., 1986), and increases the cerebral levels of IL-1β (Goshen et al., 2008), which in turn may perturb the peripheral immune system and increase the cytokine plasma concentrations (Maes, 1995, Miller et al., 2009a). Not only can an increase in cerebral IL-1β can be a causal candidate of depression symptomatology, but also the effects of IL-1β, which can be centrally originated by astrocytes and microglia, have also been shown to be essential and sufficient to trigger the mentioned pathway in animal models of depression.

Glucocorticoids dampen the inflammatory and neuroendocrine responses to a variety of challenges including pathogen invasion and stress (Pace et al., 2007) and directly modulate the phenotypes, survival and function of immune cells given that the GR is ubiquitously expressed in nearly all cells of the immune system. They promote the migratory activity back to the bone-marrow, secondary lymphoid organs (Besedovsky et al., 2014) and survival of myeloid cells. Glucocorticoids also efficiently suppresses the production of pro-inflammatory cytokines, chemokines, and reactive oxygen species (Ehrchen et al., 2007, Besedovsky et al., 2014 reviewed in Bellavance and Rivest, 2014), and trigger T cell apoptosis (Purton et al., 2004). Despite this strong anti-inflammatory effect that glucocorticoid has over immune cells, under certain circumstances, namely

stress, they can have the opposite effect and activate the immune system. Monocytes subjected to a chronic stress develop a “transcriptional fingerprint” characterized by a decreased expression of glucocorticoids response elements (transcriptional activity mediated by GR), and increased activity of pro-inflammatory transcription factor NF-κB. These changes occur despite the similar circulating levels of cortisol than controls, suggesting that stress causes a resistance to glucocorticoids in monocytes, which allows activation of pro-inflammatory phenotype (Miller et al., 2008, O'Donovan et al., 2011). These findings have been validated in *ex vivo* studies, where monocytes from chronically stressed individuals are stimulated with LPS, in the presence of cortisol, and produce more pro-inflammatory cytokines. Further to this, and their immune cells are less inhibited by cortisol when compared to control subjects (Miller et al., 2002, Rohleder, 2012).

Exposure of peripheral blood immune cells to inflammatory cytokines modifies the number of GR and their function (Pariante and Miller, 2001). IL-1 and IFN-α reduce GR function through downstream signalling molecules such as the p38 mitogen activated protein kinase, which interacts on GR translocation, and signal transducer and activator of transcription (STAT) 5. STATS5 interacts with the GR DNA binding (Wang et al., 2004, Pace et al., 2007, Hu et al., 2009). Additionally, it has been shown that acute exposure of TNF-α on T cells causes a decreases in T cell proliferation (Miller, 2010). Further to this, chronic exposure modulates cell cycle, proliferation, ubiquitination, cytokine synthesis, calcium signaling, and apoptosis genes as well as impairs NF-κB and adaptor protein 1 transactivation (Lee et al., 2008).

It has been suggested that the proliferation and activation of peripheral blood mononuclear cells (lymphocytes, monocytes or macrophages) is blunted in patients suffering from depression (Hickie et al., 1993, Zorrilla et al., 2001, Irwin and Miller, 2007, Miller, 2010). Interestingly, studies have revealed that T-helper cells (CD4+) from depressed patients present a significantly increased expression of Fas (CD95), and thus

undergo spontaneous apoptosis (Ayala et al., 1999, Szuster-Ciesielska et al., 2008), which has been proposed to be a function of chronic stress (Miller, 2010). GCs promote the differentiation of regulatory T cells (Treg), which are key suppressors of immune functions (Tischner and Reichardt, 2007, Baschant and Tuckermann, 2010).

Thus, chronic stress and exposure to pro-inflammatory cytokines renders T cells unresponsive to the anti-inflammatory action of glucocorticoids, and triggers the production of more pro-inflammatory cytokines. Increased pro-inflammatory cytokines, which are tightly regulated with the stress hormones, are associated to the modulation of mood and act centrally in different parts of the brain.

1.1.5.2 Kynurenine Pathway

A number of animal studies indicate that acute exposure to pro-inflammatory cytokines and cytokine-inducers such as LPS, can alter the turnover of serotonin in multiple brain regions (Dunn and Wang, 1995, Dunn et al., 1999, Anisman et al., 2005). Activation of the enzyme indoleamine 2,3-dioxygenase (IDO), depletes the amino acid tryptophan available, which is precursor of serotonin, causing a down regulation of serotonin levels (Schwarcz and Pellicciari, 2002). A number of pro-inflammatory cytokines, alone or in combination can activate the IDO pathway. Conversely, IDO expression is inhibited by anti-inflammatory cytokines, such as IL-4 and IL-10. IDO is the rate-limiting enzyme that produces numerous neuroactive molecules, including quinolinic and kynurenic acid, both of which are an NMDA receptor agonist and antagonist, respectively. Therefore, an increase of pro-inflammatory cytokines would activate IDO, which in turn, would increase the quinolinic to kynurenic acid ratio causing a depletion of tryptophan available to produce serotonin. Production of quinolinic acid would increase the NMDA receptor agonism and glutamate release (McNally et al., 2008). Of note, tryptophan stimulates the proliferation of effector T cells, therefore,

depletion of tryptophan would cause T cells to undergo apoptosis (Mellor et al., 2003, Beissert et al., 2006, Miller, 2010).

Suicide attempters showed increased levels of quinolinic acid in the CSF that was significantly correlated with higher levels of IL-6. Interestingly, the levels of quinolinic acid correlated positively with the total scores of the Montgomery-Asberg Depression Rating Scale (MADRS), suggesting that higher symptoms of depression and suicidal ideation are accompanied by increased levels of IL-6, quinolinic acid, and therefore, glutamatergic agonism (Erhardt et al., 2013, Bay-Richter et al., 2015).

Indeed, aberrant levels of glutamate have also been linked to depression and suicide. Several studies have reported increased concentrations of glutamate in plasma (Altamura et al., 1993, Mauri et al., 1998, Kucukibrahimoglu et al., 2009) and increased concentration of glutamine in cerebrospinal fluid (Levine et al., 2000). Studies using proton magnetic resonance spectroscopy have found reduced metabolic glutamate/glutamine exchange in the hippocampus and anterior cingulate cortex of patients suffering from MDD (Auer et al., 2000, Pfleiderer et al., 2003, Mirza et al., 2004, Block et al., 2009). Interestingly, antidepressant drugs have been found to reverse symptoms of depression by reducing the serum and plasma glutamate concentrations (Maes et al., 1998, Kucukibrahimoglu et al., 2009) as well as cerebrospinal fluid glutamine concentrations (Garakani et al., 2013). These findings suggest that these monoamine-based antidepressant drugs are modulating the glutamatergic system (Hillhouse and Porter, 2015) that may be affected by pro-inflammatory cytokines. Moreover, ketamine is a novel glutamatergic-based antidepressant drug that has produced rapid and sustained antidepressant effects in patients with depression, and is well known for having a very rapid anti-suicidal effect. Ketamine itself is a NMDA receptor antagonist that binds to the phencyclidine site inside the ion channel of the NMDA receptor blocking it (Hillhouse and Porter, 2015).

The implication of the inflammatory and neuroendocrine system in depression and suicide have been mainly been reported in the periphery. Therefore, it is important to understand how these irregularities may alter the brain function. The inflammatory system in the brain has been known to be orchestrated by glial cells such as astrocytes and microglia (see chapter 1.2).

Glial cells are important for the general brain function, as they play an important role in immune response. Abnormalities in glial cell function could have deleterious effects on neuronal circuitry and be the result or the cause of dysregulation in the immune system contributing to the etiology of major depression.

1.2 GLIAL CELLS, MOOD DISORDERS & INFLAMMATION

Glial cells were first described in 1856 by Rudolf Virchow as passive elements that fill the space not occupied by neurons, and providing shape to the nervous system (Kettenmann and Verkhratsky, 2008). A subset of these glial cells were later recognized in 1893 by Michael von Lenhossek as astroglia, and later in 1919 by Pio del Rio-Hortega as oligodendrocytes and microglia. It was not until 1921 that glial cells were officially subdivided into oligodendrocytes, microglia, as well as fibrous and protoplasmic astrocytes (Garcia-Marin et al., 2007). Thereafter, Santiago Ramon y Cajal anticipated that the function of these cells would remain unknown until the proper methods to study them were developed (Kettenmann and Verkhratsky, 2008), and it was not until the last couple of decades that the diversity of functional properties displayed by glial cells began to be recognized and elucidated. Nowadays, glial cells are recognized as fundamental and necessary elements for normal brain function since they are involved in vital processes such as brain development and homeostasis, immune functions, neurotransmission, neuroplasticity, etc. (Bernardinelli et al., 2011, Jones et al., 2011, Lee et al., 2015, MacVicar and Newman, 2015, Rose and Chatton, 2015, Villacampa et al., 2015) Astrocytes and microglia are of particular interest in the context of the neuroinflammatory hypothesis of depression, since these cells are central mediators of

the brain´s immune system. They will therefore be the main focus of this chapter. For a complete review on the evidence supporting an implication of oligodendrocytes in mood disorders, see review written by Edgar and Sibille in 2012 (Edgar and Sibille, 2012).

Glial cells have been repeatedly implicated in the pathophysiology of MDD. Studies have reported decreases in glial cell densities in the prefrontal cortex, accompanied by a decrease in cortical thickness in patients with mood disorders (Ongur et al., 1998, Rajkowska et al., 1999, Cotter et al., 2001a, Cotter et al., 2002, Webster et al., 2005, Drevets et al., 2008b). Increases in the size and density of glial cell nuclei in MDD have also been reported in layer III of dorsolateral prefrontal cortex (Rajkowska et al., 1999). In the anterior cingulate cortex (ACC; limbic cortex), however, results have varied, with some groups reporting decreases in glial cell densities (Ongur et al., 1998, Cotter et al., 2001a, Cotter et al., 2002, Drevets et al., 2008b) between MDD and nonpsychiatric controls, and others found no differences (Cotter et al., 2001a, Hercher et al., 2009b). Unfortunately, all of these studies have been conducted with Nissl-stained tissues, thus making it impossible to identify possible changes specific to glial subtypes (astrocytes, oligodendrocytes or microglial cells).

Among the many functions ascribed to glial cells, the specific roles played by microglia and astrocytes are crucial for brain viability as these cells are key players in the inflammatory responses occurring in the CNS. Microglia, which respond rapidly to fine changes in the structural integrity of the brain, such as subtle alterations in the microenvironment (Kreutzberg, 1996), mediate the brain's immune responses, including the release of pro-inflammatory cytokines. This is known as the T helper cell type 1 (Th1) response or a polarized M1 activation. Classically, these cytokines activate astrocytes which, in many cases and with longer latency, trigger the T helper cell type 2 (Th2) immune response or, alternatively, macrophages trigger the macrophage M2 activation.

This Th2 or M2 activation results in antibody and anti-inflammatory factor production which, through various feedback mechanisms, then inhibits the Th1 response (McNally et al., 2008). However, it is worth noting that activated astrocytes can exert both pro- and anti-inflammatory effects on microglial cells (Koppenol, 1991, Eddleston and Mucke, 1993), and both microglia and astrocytes can have both M1 and M2 activation. A precise balance between these alternative forms of activation and a subtle modulation of these responses between astrocytes and microglial cells is indispensable for a healthy inflammatory response.

Pro-inflammatory cytokines such as IL-1β, IL-2, IFN-α and TNF-α, which are preferentially released by microglia, induce the activity of indoleamine-2,3-dioxygenase (IDO), the first enzyme in the kynurenine pathway that converts tryptophan into quinolinic and kynurenic acid, which are N-methyl-D-aspartate (NMDA) receptor agonist and antagonist, respectively (McNally et al., 2008). Kyneurin is preferentially converted to kynurenic acid in astrocytes and quinolinic acid in microglia (Schwarcz and Pellicciari, 2002). Inflammatory cytokine activity results in an increased quinolinic acid to kynurenic acid ratio, leading to excessive NMDA activity and to excitotoxicity (McNally et al., 2008, Maes et al., 2009). A precise balance between Th1 and Th2 activity is critical to maintain the accurate pro-inflammatory cytokine levels and brain activity.

1.2.1 Astrocytes

Astrocytes represent a heterogeneous population of cells that vary morphologically and, presumably, functionally across the brain regions and among different species (Emsley and Macklis, 2006, Oberheim et al., 2009). Mainly from animal studies, it is known that astrocytes play a key role in maintaining extracellular homeostasis by regulating extracellular K^+ concentrations. Astrocytes express K^+ channels that allow them to have very low resting potentials. They are extensively and tightly coupled by connexin 43 forming a syncytium of coupled cells (Lin and Bergles,

2004). This K^+ reuptake mechanism plays a fundamental role in determining astrocyte contribution to buffering of extracellular K^+ and uptake of potentially toxic neurotransmitters (McKhann et al., 1997). Indeed, one of the major functions of astrocytes in the CNS is glutamate uptake through transporter proteins such as the EAAT2 that supports excitatory neurotransmission, prevents exitotoxicity (Nedergaard et al., 2002) and mediate plasticity of dendritic spines (Verbich et al., 2012).

Unlike neurons, astrocytes are not electrically excitable, but they can nonetheless respond to a plethora of chemical signals such as glutamate (Bernardinelli et al., 2011) norepinephrine (Bekar et al., 2008, Schummers et al., 2008), adenosine triphosphate (ATP) (Bernardinelli et al., 2011), γ-aminobutyric acid (GABA), acetylcholine, prostaglandins and endocannabinoids (Porter and McCarthy, 1996, Kang et al., 1998, Araque et al., 2002). Astrocytes respond to the synaptic release of neurotransmitters by changing their membrane potential through the increase of intracellular calcium concentration. This allows, on a milliseconds scale, the propagation of signals through gap junctions to non-stimulated astrocytes across relatively long distances (Porter and McCarthy, 1996, Bernardinelli et al., 2011). The mechanisms of propagation of such calcium waves differ between the gray and white matter, being transmitted through gap junctions in the former and by ATP in the latter, in which the astrocytic networks are not organized into syncytia. (Schipke et al., 2002, Haas et al., 2006 reviewed in Oberheim et al., 2012). It has been proposed that astrocytes form a network that constitutes an extra-neuronal pathway for rapid long-distance signal transmission within the CNS (Scemes and Giaume, 2006). Additionally, astrocytes directly interact with the synapses influencing their consolidation or elimination (Verbich et al., 2012). Astrocytes actively modulate neuronal signalling via calcium-mediated non-linear integration of input signals and gliotransmitter release (Araque, 2008). These molecules released by glial cells are released via exocytosis such as D-Serine (Henneberger et al., 2010, Zhuang et al., 2010), ATP (Zhang et al., 2003) or glutamate (Araque et al., 2014). This concept of astrocytes being the protagonists on the

regulation of the signals between neurons is well known as the “tripartite synapse” (Zhuang et al., 2010).

Astrocytes interact directly with the circulatory system, fulfilling nutritive and protective functions through contacts with blood vessels, and acting as a fundamental constituent of the blood brain barrier (BBB). The BBB is made up of blood vessel endothelial cells, pericytes, astrocytic end feet and axon terminals (Tajes et al., 2014). Astrocytes express water channel aquaporin 4 and the inwardly rectifying K^+ channel Kir4.1K+, which mediates K^+ buffering, and therefore regulates ion concentration (Tajes et al., 2014). These cells exert effects over endothelial cells (and vice-versa) by releasing a wide range of molecules that influence vascular blood flow, glycogen and oxygen demands, as well as the permeability of the tight junctions (Gordon et al., 2011). They can also mediate paracellular and transcellular transport, and receptor-mediated transcytosis (Tajes et al., 2014). Up to 10% of blood glucose is taken from the blood through the BBB and stored exclusively in astrocytes in form of glycogen (Brown and Ransom, 2007) that serves as an endogenous source of energy for the brain (Benarroch, 2010).

During neuronal development, astrocytes supply trophic support, and contribute to neuronal growth, guidance, differentiation, survival, and synaptogenesis (Araque, 2008). Furthermore, following injury, they contribute to limiting tissue damage by forming glial scars to isolate pathogens (Sofroniew, 2009), releasing pro-inflammatory and anti-inflammatory cytokines (McNally et al., 2008) and communicating with the peripheral immune system across the BBB (Volterra and Meldolesi, 2005) (for more information on the topic, see *Astrocytes and Inflammation* (section 1.2.3).

In terms of morphological features, specialized astrocytes have been recognized in specific compartments in the nervous system such as Müller cells that reside in the retina or Bergmann glia and velate astrocytes in the cerebellum (Matyash and

Kettenmann, 2010). Additionally, seven different morphologies of specialized astrocytes have been recognized in the mammalian brain such as tanycytes in the ventricles extending their projections to the hypothalamus, marginal glia and perivascular glia that form the glia limitans in the pia surface, ependymal glia that line the ventricles and fibrous and protoplasmic astrocytes in the white and gray matter, respectively (Emsley and Macklis, 2006). Besides these phenotypes, in recent years, the morphological and functional diversity of human astrocytes have been shown to be several fold more complex and more diverse than their rodent counterparts. Four distinct astrocytic populations have been described in human neocortex: three in the gray matter (interlaminar, protoplasmic and varicose projection or polarized astrocyte) and one in the white matter (fibrous astrocyte) (Oberheim et al., 2006, Oberheim et al., 2009). Interlaminar astrocytes reside in cortical layer I and extend one or two processes to deeper layers (I/II). Varicose projection astrocytes are localized in cortical layers V/VI and extend 1 to 5 long varicose processes with up to 1 mm in length. Both of these cell types are unique to humans and higher order primates. Compared to rodents, protoplasmic astrocytes in humans are 2.6-fold larger in diameter and extend 10-fold more primary processes contacting an estimated of 270,000–2 million synapses (compared to 20,000–120,000 in rodents) (Oberheim et al., 2009). Additionally, protoplasmic astrocytes propagate calcium waves 4 times more rapidly. These differences are hypothesized to be the result of an evolution of human astrocytes that confers higher brain processing (Han et al., 2013, Zhang and Barres, 2013). Supporting this hypothesis, Han and colleagues recently conducted a study where they engrafted human glial progenitor cells into neonatal immune-deficient mice. Upon maturation, these mice displayed glial progenitor cells as well as mature and functional astrocytes coupled to the resident astrocytes by gap junctions. These cells preserved all their hominid features as well as the 4-fold faster conduction of calcium waves. Moreover, in these engrafted mice, learning and memory were enhanced, suggesting that human astrocytes can enhance cognition in mice (Han et al., 2013).

1.2.2 Astrocytes in Depression

Multiple lines of evidence suggest that astrocytes are implicated in mood disorders. One of the most used astrocytic-specific markers is the glial fibrillary acidic protein (GFAP), an intermediate filament and a fundamental part of the astrocytic cytoskeleton that has been widely used to label and assess astrocytic integrity in the CNS. Several histological studies conducted in postmortem brain samples of subjects having suffered from MDD have suggested that the density of astrocytes is altered in depression and suicide. Thus, the areal fraction occupied by astrocytes in the gray matter was reported to be decreased in the dorsolateral prefrontal cortex (DLPFC) of middle-aged subjects (Miguel-Hidalgo et al., 2000) and in the orbitofrontal cortex in a mixed group of middle-aged and elderly subjects compared to controls (Miguel-Hidalgo et al., 2010), while being increased in elderly subjects with late-onset depression compared to controls (Miguel-Hidalgo et al., 2000, Davis et al., 2002). Immunohistochemical studies have been conducted in the anterior cingulate white matter, and subjects with affective disorders were found to display significant reductions in GFAP-immunoreactivity compared to controls (Gittins and Harrison, 2011). In terms of GFAP gene and protein expression, decreases have been reported in PFC by independent groups (Miguel-Hidalgo et al., 2000, Webster et al., 2001, Si et al., 2004, Nagy et al., 2014). Interestingly, decreased GFAP gene and protein expression in subjects suffering from depression have also been measured in the locus coeruleus (Chandley et al., 2013) and amygdala (Altshuler et al., 2010, Kekesi et al., 2012). These findings raise the possibility that depression-associated astrocytic dysfunctions occur non-specifically throughout the brain.

Other astrocyte-specific markers have also been implicated in depression and suicide. The T1 isoform of the BDNF receptor tropomyosin-related kinase B (TrkB.1), a truncated isoform expressed exclusively by astrocytes that mediates BDNF-induced calcium signalling, was found to be significantly down-regulated in the frontal cortex of suicide completers (Ernst et al., 2009). Similarly, genes coding for the astrocyte-specific

gap junction proteins connexin 30 and connexin 43 were recently reported to be significantly downregulated in the DLPFC (Ernst et al., 2011) and locus coeruleus (Bernard et al., 2011) of suicide completers (reviewed in Rajkowska and Stockmeier, 2013).

Glutamatergic transmission also seems to be affected in depression and suicide, and this phenomenon is likely influenced by astrocytes. Indeed, reduced levels of the excitatory amino acid transporter-1 and -2 (EAAT1 and EAAT2) and glutamine synthetase mRNA in the dACC and DLPFC (Bernard et al., 2011) and protein in the orbitofrontal cortex (Miguel-Hidalgo et al., 2010) were also reported in samples from MDD patients. Additionally, glutamate signalling and astrocyte-associated genes were found to be downregulated in the locus coeruleus in MDD (Bernard et al., 2011, Ordway et al., 2012, Chandley et al., 2013). Other studies have complemented these findings with neuroimaging studies performed in patients with MDD, showing a decrease in glutamate in plasma (Altamura et al., 1995), the ACC (Auer et al., 2000, Rosenberg et al., 2005), PFC (Hasler et al., 2007), amygdala (Michael et al., 2003) and hippocampus (Milne et al., 2009 reviewed in Rajkowska and Stockmeier, 2013). Despite accumulating evidence indicating that astrocyte-specific markers are dysregulated in depression and suicide, it remains unclear if this phenomenon reflects overall changes in astrocytic numbers.

Supporting evidence of an implication of astrocytes in the etiology of mood disorders has also come from animal model studies. For example, Wistar Kyoto rats, which display natural depressive-like behavior, display significantly lower numbers of GFAP-immunoreactive cells in the PFC compared to Sprague-Dawley rats (Gosselin et al., 2009), and selective pharmacological ablation of prefrontal cortical glia in rats produces depressive-like behavior (Banasr and Duman, 2008). Similarly, animal models of stress have shown that these paradigms reduce GFAP-immunoreactivity in rodent PFC (Braun et al., 2009), hippocampus (Czeh et al., 2006) and basolateral amygdala (Leventopoulos

et al., 2007). Antidepressant treatments in animal models have also proven to be effective in preventing GFAP downregulation. Thus, treatment with the SSRI fluoxetine prevents the decrease in astrocytic numbers caused by psychosocial stress (Czeh et al., 2006), and riluzole, a glutamatergic presynaptic terminal release inhibitor, also prevents the downregulation of GFAP in rat PFC following exposure to CMS (Banasr et al., 2010).

1.2.3 Astrocytes & Inflammation

Astrocytes display a graded response to immune challenges that includes changes in gene expression (such as upregulation of GFAP), cellular structure (progressive cellular hypertrophy) and, in more severe reactions, cell proliferation and scar formation (John et al., 2003, Sofroniew, 2005, 2009, Sofroniew and Vinters, 2010). GFAP is not essential for the normal appearance and function of most astrocytes. However, it is essential for reactive astrogliosis and scar formation (Sofroniew and Vinters, 2010). Astrogliosis, a term generally used to designate an abnormal increase in astrocytic size and proliferation, is also accompanied by hypertrophy as well as increased GFAP expression by these cells.

Astrogliosis is elicited in response to a wide variety of molecules involved in the immune response, including growth factors, lipopolysaccharides and other Toll-like receptor ligands (mediators of innate immunity), cytokines, neurotransmitters such as glutamate and noradrenaline, purines such as ATP, reactive oxygen species, oxygen or glucose deprivation, molecules associated with metabolic toxicity and some regulators of cell proliferation (Sofroniew and Vinters, 2010). The morphological, molecular and functional changes of reactive astrocytes depend on the context of the stimuli and reflect different responses to the graded spectrum of activation. Different signalling pathways such as NFκB, SOCS3, Nrf2, cAMP that ultimately lead to the activation of the STAT3 pathways, produce a different response in the astrogliosis response. Under strong inflammatory conditions, activated astrocytes grow scars to delimitate the area

of extravasation and leukocyte infiltration, promoting BBB repair and sustaining neuronal survival after an inflammatory insult (Farina et al., 2007). Additionally, astrocytes have a strong influence on endothelial cells, and thus have a strong impact on the integrity and transport control across the BBB in normal and inflammatory conditions (Abbott et al., 2006).

Thus, astrocytes play an important role in the regulation of the BBB and are fundamental elements for a controlled inflammatory response. They can also delimitate the site of an injury and prevent infiltration of immune cells in healthy tissue.

1.2.4 Microglial Cells

Microglial cells are probably the most versatile cells in the CNS. They are formed from migrating myeloid cells from the yolk sac to the nervous system early in development and thus have a mesodermal origin. During cerebral development, these cells migrate using the white matter tracts with an ameboid shape that changes into the ramified morphology in a more mature brain (Kettenmann et al., 2011).

As the main resident immune cells in the brain, microglial cells are constantly and dynamically scanning their environment in a resting/surveillance state (Nimmerjahn, 2012). When a stimulus triggers activation, these cells undergo a graded morphologically and functional change ranging from a very ramified phenotype to an ameboid morphology with phagocytic/inflammatory properties (Kettenmann et al., 2013). This amoeboid morphology allows the rapid locomotion that allows migration to the site of lesion or infection through chemotactic signals. At the site of injury or infection, microglial cells can also display phagocytic activity and/or proliferate to provide increased defense against pathogens. After the injury has been healed or the pathogens removed, microglial cells turn off the inflammatory signals, restore the tissue to recover homeostasis, and clear debris and damaged cells (Kettenmann et al., 2013).

As part of their "activated repertoire", microglia release and synthesize a variety of cytokines, reactive oxygen species, growth factors, cell adhesion molecules, extracellular matrix proteins, transcription factors, proteins of the major histocompatibility complex (MHC) and other molecules that modify the healing or the course of pathology of the CNS (Kettenmann et al., 2013). The cues that can trigger microglial activation are molecules associated to bacterial walls such as pathogen- and damage/danger-associated molecular patterns (PAMPs/DAMPs), viral capsules or the DNA or RNA of pathogens that can bind to the Toll-like receptor (TLR) family (Kettenmann et al., 2011). Additionally, microglial cells sense impaired or excessive neuronal activity that can be perceived as signs of lesion and therefore, trigger microglial activation. Similarly, microglial activation can be triggered by the release of molecules from damaged tissue and intracellular proteins that have normal physiological functions, but leave their respective physiological compartments upon stress, or after having been biochemically altered (Lotze et al., 2007, Rubartelli and Lotze, 2007, Lehnardt et al., 2008).

In order to be able to mount an inflammatory response, then recover tissue homeostasis and clear the cellular debris and damaged cells, microglial change from an pro-inflammatory to an anti-inflammatory phenotype (Gordon, 2003). These phenotypes, also called M1 and M2, represent the two extremes of the whole spectrum of functional profiles that can be displayed by microglia (Cherry et al., 2014). It has been proposed that microglial resting state is closer to the M2 phenotype. In a normal brain, "resting microglia" limit the expression of some surface receptors that stimulate an inflammatory response (such and MHCII, CD45 and FC receptors) and it is biased towards an M2 anti-inflammatroy phenotype promoting immune homeostasis and maintaining the immunosuppressive environment in the healthy brain (Olah et al., 2011). M2 is characterized by a more rapid and acidic phagosome for a more efficient removal of debris (Cherry et al., 2014). This phagosome can be seen in the normal

synaptic pruning performed by microglial cells, which is characterized by the rapid elimination of developing synapses (Garcia-Vallejo et al., 2014). Depending on the milieu in which microglial gets activated, they can display three different profiles: “classical activation,” “alternative activation,” and “acquired deactivation”. Classical activation is represented by an M1 phenotype and is characterized by the production and secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, superoxide, nitric oxide, reactive oxygen species, and certain proteases (Colton and Wilcock, 2010). The remaining two classifications lean more towards the M2 side of the spectrum. Alternative activation state is limited by the production of the anti-inflammatory cytokines IL-4 or IL-13 and the transcription of genes that promote, tissue repair, and extracellular matrix reconstruction (Colton, 2009, Colton and Wilcock, 2010). Acquired deactivation lessens acute inflammation and is characterized by the uptake of apoptotic cells or exposure to the anti-inflammatory cytokines IL-10 and transforming growth factor (TGF)-β; a pleiotropic cytokine that promotes angiogenesis (Tang and Le, 2015).

Activated microglia interact with neurons influencing either positively or negatively their survival and synaptic architecture. They can sense neuronal activity since they virtually express all the receptors for neurotransmitters, neuropeptides and neuromodulators (reviewed in Pocock and Kettenmann, 2007, Kettenmann et al., 2013). In normal conditions, microglial cells make repetitive contacts with synapses at a frequency of once per hour. These contacts last 5 minutes and seem to be activity-dependent since they are decreased with drops in neuronal activity (Wake et al., 2009). When a neuron is injured, microglial cells can remove the synaptic input of these cells in a process called “synaptic stripping” (Blinzinger and Kreutzberg, 1968, Moran and Graeber, 2004, Yamada et al., 2008). Furthermore, microglial cells have been shown to transform their morphology and activity as a result of a sensory experience. Microglial in a normal mouse visual cortex display direct apposition with synaptic clefts and are surrounded by extracellular space. When mice are deprived from light, microglial cells change their morphology, show altered distributions, display phagocytic structures and

appose synaptic clefts more frequently. Re-exposure to light reverses all these effects (Tremblay et al., 2010). Microglia can also influence synaptic pruning, adult neurogenesis and the development of the nervous system.

1.2.5 Microglia & Inflammation

The physiological response accompanying morphological changes in activated microglia is highly complex, as it can be associated with a range of functions, such as housekeeping or phagocytosis. These graded responses depend on the type of stimuli as well as interactions with other cells (i.e. astrocytes, T cells). Many factors can change microglial physiology such as necrotic or apoptotic debris from other cells (i.e. DNA, RNA), neurotransmitters or neuromodulators (i.e. ATP, glutamate), abnormal folded proteins and other intracellular material (Kettenmann et al., 2011).

Activation of microglial Toll-like and Nod-like receptors triggers a classical M1 activation that is characterized by the activation of mitogen-activated protein kinase (MAPK) (ERK1/2 and p38), secretion of pro-inflammatory cytokines (IL-1β, IL-12, IFN-□ and TNF-α) increase of CC-chemokine ligand 2, and production of reactive oxygen species (ROS) (Fernandes et al., 2014). In addition, upregulation of inducible nitric oxide synthase (iNOS or NOS2), glutaminase and inducible cyclooxygenase (COX-2) leads to an increase of nitric oxide (NO), glutamate and prostaglandins, respectively (Fernandes et al., 2014). ROS, and NO, and play a crucial role in bacterial and viral infection (Nakagawa and Chiba, 2014). This can lead to the adaptive immune response such as the up-regulation of the MHC class II and interaction with T- cells (Shastri et al., 2013). Microglial activation triggers the upregulation of certain receptors involved in phagocytosis (such as MHC class I, MHC class II and CD68), complement receptors (CR1, 2, and 4), class A and B type scavenger receptors (including CD36), co-stimulatory molecules (CD80, CD86) and intercellular adhesion molecule-1 (ICAM-1). Additionally, they also upregulate opsonic receptors such as immunoglobulin Fcγ receptors (FcγRIII)

as well as receptors involved in the clearance of apoptotic products such as the low density lipoprotein receptor (LRP, CD91). CD45, CD40, CD11b. These receptors are constitutively expressed on the cell surface of microglial cells an then get upregulated in face of a inflammatory insult (Lynch, 2009).

Th2 activation has been paired with the M2 macrophage polarization that parallels an alternative activation (Gordon and Martinez, 2010). The signature cytokines of the alternative activation are IL-4 and IL-13 that trigger the expression of cell surface receptors such as arginase-1, heparin-binding lectin (Ym1), cysteine-rich protein FIZZ-1, CD36, CD163, and CD206, and produce anti-inflammatory cytokine such as IL-10, which can suppress M1 inflammation (Mosser and Edwards, 2008). *In vitro* studies have shown that IL-4 decreases iNOS activity, superoxide and TNF-α production (Zhao et al., 2006). Additionally, IL-4 also increases the phagocytic activity of microglial cells through the scavenger receptor CD36 (Shimizu et al., 2008). IL-13 and IL-10 increased microglial secretion of the TGF-β superfamily member that are neuroprotective and also have been shown to promote oligodendrocyte differentiation (Shimizu et al., 2008). It is suggested microglial–neuronal cross-talk in a healthy brain microenvironment skews microglial phenotype toward M2 (Ponomarev et al., 2007 reviewed in Fernandes et al., 2014).

These alternative forms of activation represent the extreme end of a spectrum rather than discrete states and represent the initiation, development and cessation of the inflammation. The cells in the M1 phenotype can change into the M2 phenotype, and vice versa, depending on their environmental cues and the ability of these cells to shift between these phenotypes may be fundamentally important for the function of a healthy CNS. Alterations on these balances are thought to be a key player in many neurodegenerative diseases.

1.2.6 Microglial Cells in Depression

Depression has been regarded for at least a few decades as a condition with an inflammatory component (Maes, 1995). As principal mediators of the brain immune system, microglial cells are the target for abnormal brain-immune communication in depression and suicide. However, clinical evidence of microglial activation in depression and suicide has only recently begun to be reported. In 1999, the first report of microglial activation was described in one of six patients with affective disorder that presented evidence of activated microglia in the hippocampus, as imaged with the MHC class II (HLA-DR) antigen (Bayer et al., 1999). However, this study was limited by the fact that the diagnosis was not clear, that its onset occurred at 75 years old, and that the study lacked statistical power. Nearly a decade later, Steiner and colleagues conducted an immunohistochemical study using anti-HLA-DR antibody on postmortem samples of psychiatric patients and matched controls, and found no significant difference in microglial densities in any of the brain areas investigated. However, these authors reported a significant increase in microglial densities and cluster organization in the DLPFC, ACC and mediodorsal thalamus of suicide completers, regardless of diagnosis, suggesting that suicide is associated with microglial activation (Steiner et al., 2008). A subsequent postmortem study conducted by the same group showed that depressed suicides present a significant increase in number of cells positive to quinolinic acid in the subgenual and anterior mid-cingulate cortex (Steiner et al., 2011). Since microglial cells are the only cells in the CNS to express the entire enzymatic pathway to produce quinolinic acid (Saito et al., 1993), these results suggest a local increase in inflammation mediated by microglial cells (Steiner et al., 2011).

In animal models, depressive-like behaviors induced by chronic stress have been associated with microglial activation. Following three days of chronic unpredictable stress, microglia undergo stress-induced activation and proliferation and subsequent apoptosis (Maes, 1995). Blockage of this stress-induced microglial activation by

minocycline (a potent anti-inflammatory agent), imipramine or IL-1 receptor antagonist (IL-1RA) rescues the microglial activation, apoptosis and depressive-like behaviors (Kreisel et al., 2014). Indeed, prolonged stress has been associated with changes in the density and morphology of microglial cells in different brain regions such PFC, amygdala and hippocampus (Tynan et al., 2010, Wohleb et al., 2011, Hinwood et al., 2013). Very interestingly, a recent study showed that repeated social defeat caused the priming of circulating monocytes and the recruitment of these monocytes in the perivascular space, PFC, amygdala and hippocampus. This recruitment is mediated chemokine receptor-2 and also coincides with the appearance of anxiety-like behaviors (Wohleb et al., 2013 reviewed in Wohleb et al., 2014). Altogether, these studies suggest that microglia and, more generally, macrophages, may be implicated in depression, and further support the inflammatory hypothesis of depression.

1.2.7 Peripheral Infiltration to the CNS in Neuroinflammation

More than a decade ago, the CNS was believed to be entirely immune-privileged, since it contains everything for immune defense and it is protected by barriers that prevent free passage of peripheral immune cells. However, it is now clear that this view is no longer accurate (Carson et al., 2006). Neuroinflammatory processes are highly and carefully modulated, given that the typical symptoms of inflammation can be deleterious to the brain and may compromise the viability of the organism when exacerbated (Rezai-Zadeh et al., 2009). Peripheral immune cells derived from bone marrow precursors can infiltrate the CNS in their surveillance activities at very low and controlled rates. Upon disease or extreme inflammatory conditions, this rate of peripheral infiltration can increase significantly. This infiltration to the brain is tightly regulated by the BBB (Rezai-Zadeh et al., 2009) and is very similar to a typical infiltration of immune cells to peripheral organs. Peripheral cells are recruited and infiltrate the brain through chemo-attraction, cellular rolling, adhesion, and diapedesis across the vascular wall. Additionally, infiltration to the CNS requires crossing the BBB, which

involves transmigration of endothelial cells into the perivascular space and progression through astrocytic feet processes into the brain parenchyma (Owens et al., 2008). Although the passage of immune cells to the brain parenchyma is tightly controlled, peripherally borne cells constantly cross the intact BBB in healthy individuals, to replace the population of perivascular macrophages and pericytes (Matsumoto and Fujiwara, 1987, Hickey and Kimura, 1988, Bechmann et al., 2001).

Endothelial cells are bound to each other and form a physical barrier of elaborate network of tight junctions. These tight junctions are transmembrane proteins that form homophilic interactions in a pericellular ziplock-like adhesive structure sealing the intercellular cleft. BBB tight junctions belong to a different family of proteins such as the occludins, claudins, cadherin, an endothelium-specific member of the cadherin family, Jams and actin binding accessory proteins such as zona occludens-1 (Coisne and Engelhardt, 2011). These junctions are presumed to prevent the indiscriminate entry of blood cells into the perivascular space and disruption of these tight junction networks can contribute to a breakdown of the BBB (Carson et al., 2006). Disruption of the BBB by abnormal function of the tight junctions causes infiltration of toxins and other cellular discharges and debris that can cause a strong neuroinflammatory response and deleterious effects in neurons. In normal conditions, patrolling T cells are able to cross successfully across the layer of the BBB without any disruption of the tight junctions (Wolburg et al., 2005). Transmigration of leukocytes across the endothelial cells is tightly regulated by the expression of endothelial adhesion molecules such as E-selectin, platelet-endothelial cell adhesion molecule, vascular cellular adhesion molecule-1 (VCAM-1), and ICAM-1 (Carman and Springer, 2004, Millan et al., 2006 reviewed in Carson et al., 2006).

The recruitment of immune cells to any peripheral tissue organ or the brain takes place through chemoattraction. Following microglial and astrocytic activation, chemokines released by these cells diffuse into the bloodstream, thereby attracting

leukocytes to the site of inflammation. These cells upregulate different molecules and receptors that allow them to adhere to the wall of the capillaries and transmigrate to the perivascular space. In this space, known as the Virchow-Robin space, cells are retained unless they are also recruited by elements of the CNS (Rezai-Zadeh et al., 2009). Astrocytes are an important source of chemokines that regulate the entry of molecules and immune cells to the CNS and, therefore, are key elements that control the integrity of the BBB (Voskuhl et al., 2009, Toft-Hansen et al., 2011 reviewed in Finsen and Owens, 2011). During neurodegenerative diseases, monocytes and microglia are the main recruited cells at the site of lesion in the CNS, and these cells are usually recruited by the monocyte chemoattractant proteins (MCPs), which belong to the beta chemokine family, and especially CCL2 (MCP1).

Astrocytes express MCP-1, which is a CC-chemokine that controls the inflammatory response and plays a crucial role in the recruitment of T cells and monocytes (Traynor et al., 2002). This chemokine is capable of altering both the Th1 and Th2 response since MCP-1 may affect innate and adaptive immunity through monocyte regulation and the TH systems (Banisadr et al., 2005). MCP-1 is also regulated by the HPA axis. It has been shown that stress causes increases in the levels of MCP-1 (Madrigal et al., 2010). However, acute treatment with corticosterone reduces significantly the production of MCP-1 in astrocytes (Madrigal et al., 2010). In rats, when the production of glucocorticoids is blocked after stress paradigms, the concentration of MCP-1 was largely increased in the cortex. Similarly, the treatment with the antidepressant desipramine resulted in an increase in cortical MCP-1 expression (Madrigal et al., 2010).

The immune system of the brain is composed of many dynamic elements that interact with each other. Not only are astrocytes and microglial cells are in charge of the brain immune system but they also have a dynamic interaction with the peripheral immune system through the BBB and the circumventricular organs. Disruption of the

balance between Th1 and Th2 is thought to be involved in the course of many neurodegenerative and psychiatric disorders such as MDD, in which a persistent activation of the Th1 response may lead to elevated expression of inflammatory cytokines (McNally et al., 2008).

1.3 THE ANTERIOR CINGULATE CORTEX

1.3.1 Anterior Cingulate Cortex: Anatomy and Location

The anterior cingulate cortex (ACC), which lies ventral, rostral and dorsal to the corpus callosum forming a "cingulum" or collar, is a key component of the limbic system (Allman et al., 2001). One of the characteristics of the ACC and what makes it different from other cortical areas is the lack of layer IV and a very developed layer V, which mainly contains outputs to subcortical regions. Additionally, layer V of the ACC is characterized by the presence of spindle cells, which were thought to be exclusively found in humans and great apes (Allman et al., 2001). However recent research has suggested that spindle cells may be found in other regions (Allman et al., 2011).

The ACC can be subdivided into subgenual (below the genu of the corpus callosum), pregenual or rostral (anterior and dorsal to the genu of the corpus callosum) (Palomero-Gallagher et al., 2009) and the dorsal part of the ACC. The subgenual ACC includes BA25, and most ventral portions of BA24 and BA32 (Pizzagalli, 2011). The pregunal ACC is composed of BA24 and BA32 and the most posterior part of the ACC that includes caudal area 24' and 32', and cingulate motor area (Pizzagalli, 2011). BA24 in the subgenual ACC has a thinner layer III (Palomero-Gallagher et al., 2009), and BA24 and BA32 display a thinner gray matter and a lower glia-to-neuron ratio than the pregenual ACC (Palomero-Gallagher et al., 2009). In this thesis, we selected the pregenual ACC, more specifically BA24, which is referred as the dorsal ACC (dACC).

Functionally, the ACC is involved in the modulation of many functions such as emotion, attention, cognition, arousal, motivation, problem-solving, error detection, task anticipation, recognition, and adaptive response (Devinsky et al., 1995), reading, word generation, episodic recall and, working memory, among others (Wang et al., 2005). However, the anatomical and architectural heterogeneity of the ACC suggest that each of the above-mentioned functions reside in different parts of the ACC (Gittins and Harrison, 2004, Palomero-Gallagher et al., 2009). For example, it is known that the dACC has a role in reward-based decision-making (Sheth et al., 2012), and support cognition and motor control (Bush et al., 2002). Additionally, the dACC provides the prediction of expected cognitive demand to optimize future behavioral responses. In situations of changing demands, it produces accuracy by delaying responses, whereas it promotes signal efficiency by hastening responses in situations with stable cognitive demands (Sheth et al., 2012). The rostral ACC has also been shown to be involved in the perception of emotions and the storing of emotional memories. Another distinction between pregenual and subgenual ACC is that the former is associated with positive emotions whereas the former is associated with negative emotions (Vogt, 2005).

1.3.2 Anterior Cingulate Cortex: circuits and connections

The ACC receives afferent projections from the hypothalamus, the prefrontal cortex (PFC) parietal cortex and the premotor regions and has strong bidirectional connections with the dorsolateral prefrontal and temporal cortices (Pandya et al., 1981). Additionally, the ACC is directly related to the subicular complex (and thus hippocampus), posterior orbital cortex, the anterior thalamic and the brainstem nuclei (Wang et al., 2005). One of the key features of the ACC is that it samples more thalamic nuclei than any other brain region, including the paraventricular nuleus (Devinsky et al., 1995). The ACC also has a unique specialized contribution to motor function, as documented by the substantial projections from the ACC to different parts of the

striatum: caudate nucleus, ventral striatum, nucleus accumbens and putamen (Devinsky et al., 1995). BA 24, 25 and 32 are specially implicated in affect and cognition and these areas project to the amygdala (Vogt, 2009).

Based on positron-emission-tomography studies, a meta-analysis identified several regions within the frontal cortex that were co-activated with distinct subdivisions of the ACC across a range of tasks (Koski and Paus, 2000). The subgenual ACC was more frequently co-activated with the medial orbitofrontal gyrus. The dorsal portions of the ACC were consistently co-activated with dorsolateral prefrontal regions, and middle frontal gyrus was more frequently co-activated with the supracallosal ACC (Koski and Paus, 2000). In a recent connectome study, it was shown that the insula connects directly to the ACC and that the activity of the caudal part of the ACC was correlated with sensorimotor circuits, while the activity of the rostral portions was correlated with prefrontal regions (Margulies et al., 2007). Other studies have suggested that the ACC takes into account "task complexity", and thus, sensorimotor circuits are represented in the posterior part of the caudal ACC and higher order executive function circuits are located in more anterior parts. Between these two rostral and caudal parts of the ACC there were transition regions, which are associated with a combination of ventral and dorsal brain systems (Botvinick et al., 2004).

1.3.3 Anterior Cingulate Cortex and Depression

In addition its intrinsic importance in providing new information about normal cognition and motor control, the ACC is connected to brain structures that regulate mood and it is responsible for emotional evaluation of error, conflict detection, autonomic and visceral responses and is able to discern between emotional valence of thought. All of the above functions are disturbed in many neuropsychiatric disorders, and the ACC has been repeatedly implicated in mood disorders and suicide (Bush et al., 2002).

Evidence from neuroimaging studies has consistently indicated reductions in ACC activation in patients suffering from MDD (Ebert and Ebmeier, 1996, Chana et al., 2003, Sacher et al., 2012). Additionally, it is a well-documented finding that subjects suffering from depression show reductions in ACC gray matter (Botteron et al., 2002, Sharma et al., 2003, Coryell et al., 2005, Du et al., 2012, Depping et al., 2015). In recent meta-analyses of studies employing voxel-based morphometry, it was suggested that the most robust structural abnormality in MDD patients compared to healthy controls was a reduction in ACC gray matter (Bora et al., 2012, Du et al., 2012 reviewed in Price and Drevets, 2010).

The degree of improvement in depressive symptoms following cognitive behavioral therapy, which is widely used to treat MDD, is correlated with the recovery of gray matter volume loss in the caudal portion of the anterior cingulate cortex (Fujino et al., 2015). Similarly, deep brain stimulation, which is an invasive but effective treatment for depression, is applied to the subgenual ACC and is increasingly used in cases of treatment-resistance depression (Lozano et al., 2008, Ramasubbu et al., 2013, Berlim et al., 2014 reviewed in Hamani et al., 2011). In terms of the effects of antidepressant drugs, in a study using 4-T 1H proton magnetic resonance spectroscopy, it was suggested that ketamine, an NMDA receptor antagonist fast-acting antidepressant drug (Zarate et al., 2006) may act by increasing the glutamine levels in the ACC (Rowland et al., 2005). Thus, it is proposed that MDD is characterized by impaired modulation of the activity of cortico-limbic circuitries along with the modulation of other brain regions associated with emotional processing (Diener et al., 2012, Sacher et al., 2012). Cortico-limbic areas process emotional states, stimuli and supports the adaptation of the human to the environment (Comte et al., 2014). Several brain structures such as amygdala, ACC and different prefrontal areas compose this system, and the ACC has a strategic position be connected to both prefrontal cortex and amygdala (Comte et al., 2014).

1.3.4 Anterior Cingulate Cortex and Neuroinflammation

Systemic inflammation triggers an adaptive behavioral response called the sickness behavior that is characterized by lethargy, depression, anorexia, and reduction in grooming. This adaptive behavior is believed to have evolved to allocate the organism's resources to elicit an effective immune response and better cope with the infection (Hart, 1988). Sickness behaviors are triggered by inflammatory mediators produced at the site of infection by activated immune cells. These mediators are pro-inflammatory cytokines that include interleukin IL-1α and IL-1β, TNF-α and IL-6. These cytokines not only mediate the systemic immune system response against and infection, but they also act on the brain to produce sickness behaviors. In a double-blind randomized crossover study, it was shown that subjects that received a typhoid vaccination presented an inflammatory response characterized by increased levels of circulating IL-6 and significant mood reduction (Harrison et al., 2009a). These molecular and behavioural changes correlated with enhanced activity within subgenual ACC. Indeed, inflammation causes mood changes through the alteration of subgenual ACC activity and mesolimbic connectivity. Furthermore, this inflammation-associated mood change reduced connectivity of subgenual ACC to amygdala, medial prefrontal cortex, nucleus accumbens, and superior temporal sulcus; a phenomenon that was modulated by peripheral IL-6 (Harrison et al., 2009a). Similarly, functional magnetic resonance imaging studies have focused on patients undergoing treatment with IFN-α, which can cause mood or cognitive symptoms, including disturbances in attention and memory. This treatment is also associated with a significant activation of the dorsal ACC (Capuron and Miller, 2004 reviewed in Capuron and Miller, 2004). These and other studies support the fact that cytokines can influence the activity of different brain regions, including the basal ganglia and frontal cortex, with increased activity in the dorsal ACC being more consistently observed (Capuron and Miller, 2004). Thus, increased activity in

the ACC during innate immune system activation may underlie behavioural mechanisms that allow a wounded or sick animal to cope with energetic demands, and maintain vigilance in order to survive. ACC abnormalities have also been associated with psychiatric disorders, and are presumed to account for some of the behavioral changes seen in depression and suicide. This evidence strongly suggests that depression and inflammation elicit behavioral changes through circuits involving the ACC.

1.5 RATIONALE AND OBJECTIVES

The purpose and goal of my doctoral research was to explore the inflammatory profile of glial cells and their possible association to depression and suicide. More specifically, the objectives of my research were:

§ To conduct comparative morphometric analyses of protoplasmic and fibrous astrocytes in postmortem d ACC samples from depressed suicides and compare them to matched nonpsychiatric controls.

§ To perform a morphometric analysis of the different microglial phenotypes found in postmortem samples of dACC from adult individuals having died with no history of inflammatory, neurological nor psychiatric illness.

§ To examine the distribution and morphology of macrophages in well-characterized dACC white matter samples from depressed suicides and matched psychiatrically healthy controls.

CHAPTER 2.0: ASTROCYTIC HYPERTROPHY IN ANTERIOR CINGULATE WHITE MATTER OF DEPRESSED SUICIDES

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2.1. ABSTRACT

Background: Increasing evidence suggests that cortical astrocytic function is disrupted in mood disorders and suicide. The fine neuroanatomy of astrocytes, however, remains to be investigated in these psychiatric conditions. In this study, we performed a detailed morphometric analysis of 3D-reconstructed gray and white matter astrocytes in Golgi-impregnated dorsal anterior cingulate cortex (dACC) samples from depressed suicides and matched controls. **Methods:** Postmortem dACC samples (BA24) from 10 well-characterized depressed suicides and 10 matched sudden-death controls were obtained from the Quebec Suicide Brain Bank. Golgi-impregnated protoplasmic astrocytes (gray matter, layer VI) and fibrous astrocytes (adjacent white matter) were reconstructed, and their morphometric features analyzed with the *Neurolucida* software. For each cell, the soma size as well as number, length and branching of processes were determined. Densities of thorny protrusions found along the processes of both astrocytic subtypes were also determined. **Results:** Protoplasmic astrocytes displayed no significant difference between groups for any of the quantified parameters. However, fibrous astrocytes had significantly larger cell bodies, as well as longer, more ramified processes in depressed suicides, with values for these parameters being about twice as high as

those measured in controls. **Conclusions:** These results provide the first evidence of altered cortical astrocytic morphology in mood disorders. The presence of hypertrophic astrocytes in BA24 white matter is consistent with reports suggesting white matter alterations in depression, and provides further support to the neuroinflammatory theory of depression.

Key words: Depression, suicide, limbic, cerebral cortex, glia, inflammation

2.2 INTRODUCTION

Mood disorders affect more than 20 million adults per year in the United States alone (Kessler et al., 2005). These often severe conditions result too frequently in suicide completion, with psychological autopsy studies indicating that at least 40% of all adult suicides have had a previous diagnosis of depression or bipolar disorder (BPD) (Arsenault-Lapierre et al., 2004). Furthermore, up to 15% of individuals with a lifetime diagnosis of major depressive disorder (MDD) (Chen and Dilsaver, 1996) and 50% of individuals with BPD have a history of attempted suicide (Jamison, 2000).

In the search for biological factors underlying depression, neuroanatomical investigations of fronto-limbic cortical circuitries have suggested altered densities and distribution patterns of glial cells (Ongur et al., 1998, Rajkowska et al., 1999 reviewed in Cotter et al., 2001e, Hercher et al., 2009g), although other studies have not reached the same conclusions (Bouras et al., 2001, Hercher et al., 2009b). Unfortunately, all of these studies are limited by the fact that they are based on a simple discrimination of neurons and glia according to differences in cresyl violet staining of the cytoplasm and nucleus, and thus cannot account for changes in specific glial cell subpopulations. Work focused on the specific astrocytic marker glial fibrillary acidic protein (GFAP) (Miguel-Hidalgo et al., 2000, Si et al., 2004) and on the excitatory amino acid transporters (EAAT1-2) (Miguel-Hidalgo *et al*, 2010) has suggested that cortical astrocytic function may be perturbed in depression. Moreover, expression of the astrocyte-specific tropomyosin-

related kinase B receptor (TrkB.1) isoform and of astrocyte connexins 30 and 43 were recently found to be downregulated, respectively, in orbitofrontal cortex and dorsolateral prefrontal cortex of suicide completers (Ernst et al., 2009). Taken together, these studies lend support to the hypothesis that communication within cortical astrocytic networks is affected in mood disorders, and are consistent with neuroimaging evidence indicating that both structure and function of fronto-limbic cortical areas are altered in mood disorders (Drevets et al., 2008a). Indeed, astrocytes provide crucial metabolic support to neuronal networks in the CNS (Allaman et al., 2010), and their activity is a major determinant of fMRI signals (Prior et al., 2004, Schummers et al., 2008, Sibson et al., 2008). Furthermore, it is now well established that astrocytes are directly involved in many facets of neuronal function, such as neurotransmission and neuroplasticity (Auld and Robitaille, 2003, Araque, 2008, Fellin, 2009, Allaman et al., 2010). By assembling in syncytial networks through gap junctions, astrocytes are also well positioned to rapidly convey information within and between brain regions (Cornell-Bell and Finkbeiner, 1991, Goldberg et al., 2010).

Largely on the basis of fine anatomical criteria, two main subtypes of cortical astrocytes have been traditionally recognized in mammals: protoplasmic and fibrous astrocytes, residing in the gray and white matter respectively. However, recent work conducted by Oberheim and colleagues has revealed a greater diversity and complexity of cortical astrocytes in humans (Oberheim et al., 2009). Human astrocytes were found to be proportionally larger and their processes more elaborate than in rodents. Furthermore, these investigators described a cortical astrocytic subtype unique to man, the “varicose” astrocyte, which can project a single varicose process across several cortical columns (Oberheim et al., 2009). The physiology of cortical astrocytes in humans also displays distinctive features, namely the propagation of fast intracellular calcium waves (Oberheim et al., 2009). Despite these recent advances, little is known about the fine morphological features of cortical astrocytes in humans, and nothing in the context of psychiatric disorders. The major aim of this study was to conduct

comparative morphometric analyses of protoplasmic and fibrous astrocytes in Golgi-stained postmortem dorsal anterior cingulate cortex (dACC) samples from depressed suicides and matched nonpsychiatric controls. Our working hypothesis was that astrocytes would display altered features in depressed suicides, in line with the putative disorganization of cortical astrocytic networks in mood disorders.

2.3 MATERIALS AND METHODS

Subjects

This study was approved by the Douglas Hospital Research Ethics Board, and written informed consent from next-of-kin was obtained for each subject. Postmortem brain samples from depressed suicides (n = 10) and matched sudden-death controls (n = 10) were provided by the Quebec Suicide Brain Bank (www.douglasrecherche.qc.ca/suicide). All psychiatric subjects committed suicide in the context of a major depressive episode (see Table 1 for diagnosis distribution), and controls died suddenly without any psychiatric nor neurological illness. For each individual, the cause of death was ascertained by the Quebec Coroner's office, and psychological autopsies were performed by proxy-based interviews, as described previously (Dumais et al., 2005). In brief, a trained interviewer conducted the *Structured Clinical Interview for DSM-IV* Psychiatric Disorders (SCID-I) with one or more informants of the deceased, after which a panel of clinicians reviewed SCID-I assessments, case reports, coroner's notes and medical records to obtain consensus psychiatric diagnosis. Subject groups were matched for age ($p = 0.97$), tissue pH ($p = 0.26$), tissue storage time ($p = 0.71$) and postmortem interval (PMI; $p = 0.13$). Furthermore, the delay between death and refrigeration of the bodies at the morgue was also matched between groups (averages of 8.82 and 10.97 hours for depressed suicides and controls, respectively; $p = 0.481$).

Golgi Impregnations

Formalin-fixed dACC samples (1 cm^3) adjacent to the dorsal part of the genu of the corpus callosum (BA24) (Vogt et al., 1995, Gittins and Harrison, 2004) were dissected from the right hemisphere and silver-impregnated according to a modified Golgi protocol described previously (Hercher et al., 2009a). Briefly, tissue blocks were processed separately, in the dark and in constant agitation at room temperature. Samples were first immersed in a solution of 3% $K_2Cr_2O_7$ and 10% formalin for 24 h, followed by 24 h in a fresh solution of 3% $K_2Cr_2O_7$, then washed in distilled water and in 2% $AgNO_3$ until the solution ran transparent. Samples were next placed in 2% $AgNO_3$ for 48 h before being dehydrated through a graded series of ethanol solutions, cleared in xylene, embedded in paraffin, and cut on a microtome in serial 50 µm-thick sections.

Morphometric Analyses

All samples were coded and analyzed randomly by a researcher blinded to subject number and diagnosis. A total of 200 astrocytes were reconstructed and analyzed in this study. For each subject, five protoplasmic astrocytes were analyzed in the gray matter, and five fibrous astrocytes in the white matter. Data acquired for each cell population were averaged per subject. Due to the greater diversity of astrocytic subtypes in upper cortical layers and the higher density of astrocytes in lower cortical layers (Miguel-Hidalgo et al., 2000, Oberheim et al., 2006, Oberheim et al., 2009), protoplasmic astrocytes were selected exclusively from layer VI. When scanning through randomly selected subsets of serial sections, using random observational patterns, the first five astrocytes encountered per layer (i.e. layer VI or white matter) that fulfilled the following criteria were selected for analysis: (1) location of the cell body in lower layer VI or in adjacent white matter, for protoplasmic and fibrous astrocytes, respectively; (2) full impregnation of the cell body and its processes; (3) astrocytic processes unobscured by background staining or by other cells; (4) characteristic bushy

morphology for protoplasmic astrocytes (layer VI), and presence of relatively unbranched processes for fibrous astrocytes (white matter) (**Fig. 1**). In addition, the great majority of selected cells were readily observed to contact blood vessels, a canonical attribute of astrocytes (**Fig 1**).

Astrocytes were traced under a 100X (NA 1.40) oil immersion objective (Olympus BX51 light microscope) and their processes analyzed in three dimensions within single sections using a computer-based tracing system (*Neurolucida* v. 8.10.2, MBF Bioscience, Williston, VT) (**Fig. 2**). Only cell bodies were analyzed in two dimensions (area at its largest cross-sectional diameter) because of limitations associated with tracing and measuring Golgi-stained somas in 3D with this software. Thus, for each astrocyte, the cell body area as well as the number, length, diameter and branching points (nodes) of its processes were measured. We further observed thorny protrusions along the length of processes extended by all protoplasmic and fibrous astrocyte **(Fig. 3)**. These structures were similar in appearance to dendritic spines, with an approximate length of 0.5-1.5 µm. The density of these protrusions was also determined for each astrocytic process, and expressed as numbers of astrocytic spines per µm. In order to further assess the spatial distribution of astrocytic processes, a three dimensional version of the Sholl analysis (Sholl, 1956) was used. In this analysis, a series of increasing 10 µm concentric circles around the soma allowed for quantification of the distribution of each parameter within a given radius, and thus in relation to the cell body.

Statistical Analyses

Statistical analyses were performed using PASW Statistics 18 (Statistical Product and Service Solutions, Chicago, IL, USA). All measurements were expressed as mean ± standard error of the mean (SEM), and $p \leq 0.05$ was considered significant in all statistical tests. Normality was assessed using Shapiro-Wilk tests, and parametric between subjects two-tailed t-tests were applied for normally distributed data sets.

Two-tailed U-tests were used for non-normally distributed data. For Sholl analyses, two-way mixed design between subject (controls/depressed suicides) and within subject (distance from cell body) ANOVAs were performed. This was followed by simple effects test to determine specific points of statistical significance. Multiple correlation analyses followed by ANCOVAs were used to examine the influence on measured variables of potential confounders. The latter comprised age, PMI, brain pH, tissue storage time, medication use, alcohol dependence, and smoking.

2.4 RESULTS

Cell body size No significant difference was found between layer VI protoplasmic astrocytes in controls (110.3 ± 11.5 μm^2) and depressed suicides (134.6 ± 21.1 μm^2) (**Fig. 4a**). However, the cell body size of white matter fibrous astrocytes was significantly larger in depressed suicides (150.7 ± 11.6 μm^2) than in controls (114.5 ±10 μm^2) (*t(18)= -2.319 , p = 0.032*) (**Fig. 4b**).

Astrocytic processes The number of primary processes radiating from the cell body did not differ between depressed suicides and controls for either protoplasmic astrocytes (13.6 ± 0.7 vs 14.7 ± 0.5 processes, respectively) or fibrous astrocytes (19.8 ± 1.0 vs 19.1 ± 1.1 processes, respectively). Similarly, branching of these primary processes was similar between depressed suicides and controls in the case of gray matter protoplasmic astrocytes, with very similar numbers of branch ends (33.2 ± 2.2 vs 34.4 ± 2.7 ends, respectively) and nodes (18.6 ± 1.6 vs 18.6 ± 2.3 nodes, respectively) (**Fig. 5a**). In contrast, white matter fibrous astrocytes displayed a highly significant, more than two-fold, increase in average number of nodes in depressed suicides compared to controls (39.8 ± 4.0 vs 18.3 ± 1.9 nodes, respectively**)** (*t(18)= -4.644, p= 0.0002*) (**Fig. 5b**). As expected, the average number of branch ends was also significantly increased in fibrous astrocytes (63.3 ± 4.9 vs 39.0 ± 2.8 ends, respectively) (*t (18) = -4.268, p= 0.0004*). To evaluate the effect of this increased branching on the length of processes in

depressed suicides, total branch length was then measured and found to be similar between protoplasmic astrocytes in depressed suicides and controls (697.3 ± 50.0 vs 717.1 ± 59.3 µm, respectively) (**Fig. 6a**), but significantly increased in fibrous astrocytes of depressed suicides compared to controls (1557.0 ± 174.0 vs 797.1 ±129.0 µm, respectively) (*t(18)= -3.493, p= 0.002*) (**Fig. 6b**). The average length of processes (total length divided by number of primary processes) projected by protoplasmic astrocytes was consequently similar between depressed suicides and controls (52.3 ± 2.4 vs 49.6 ± 3.8 µm, respectively) but significantly increased for fibrous astrocytes in the former group (80.2 ± 9.3 vs 41.2 ± 5.3 µm, respectively) (*t (18)= -3.625, p= 0.001*). Similarly, the total volume of processes per astrocyte was on average significantly higher in depressed suicides compared to controls for fibrous astrocytes (1111.8 ± 99.7 vs 576.3 ± 41.8 μm^3, respectively**)** (*t (18)= - 4.953, p = 0.0001)* but not for protoplasmic astrocytes (1159.1 ± 235.8 vs 1121.9 ± 400.8 μm^3, respectively).

Astrocytic spines On average, the total number of spines per protoplasmic astrocyte was similar between depressed suicides and controls (21.8 ± 3.9 vs 22.1 ± 2.7 spines, respectively) (**Fig.7a**), but significantly higher for fibrous astrocytes in depressed suicides (57.3 ± 16.0 vs 20.5 ± 3.8 spines, respectively; p = 0.003) (*U (18) = 80 p= 0.023*) (**Fig. 7b**). This likely resulted from the increased process length per cell in the latter group, as supported by the fact that the density of spines borne by astrocytic processes did not differ between groups for protoplasmic astrocytes (0.035 ± 0.006 vs 0.034 ± 0.007 spines/µm) nor fibrous astrocytes (0.034 ± 0.008 vs 0.027 ± 0.004 spines/µm) in depressed suicides compared to controls, respectively.

Sholl Analyses The following parameters were quantified by Sholl analysis: length and volume of processes, intersections, nodes, and astrocytic spines. As expected from the data presented above, Sholl analyses did not reveal any significant difference in any of these parameters when comparing layer VI protoplasmic astrocytes in depressed suicides and controls (not shown). The measured increase in process length per white

matter fibrous astrocyte in depressed suicides was also evidenced by Sholl analysis, in terms of number of intersections, number of nodes, as well as process length. Thus, compared to fibrous astrocytes in controls, increases in process volume and process length (**Fig. 8**) were highly significant in all radii between 20 and 50 μm ($p < 0.005$). Furthermore, intersections and nodes formed by fibrous astrocytes in depressed suicides were significantly more numerous than in controls between 30 and 50 μm away from the soma ($p < 0.022$; **Fig. 9**). Finally, more astrocytic spines were generally found between 30 μm and 50 μm from the fibrous astrocyte soma in depressed suicides compared to controls ($p < 0.012$; **Fig. 10**).

Potential confounders None of the potential confounders was found to significantly influence any of the measured variables.

Taken together, these data reveal that fibrous astrocytes in BA24 white matter present hypertrophic features in depressed suicides compared to controls (**Fig. 11**).

2.4 DISCUSSION

This is the first postmortem study to provide a detailed fine neuroanatomical assessment of cortical astrocytes in both healthy and psychiatric subjects. Morphometric analyses confirmed the complex arborization patterns displayed by human protoplasmic and fibrous astrocytes (Oberheim et al., 2009). Despite clear differences (e.g. branching patterns), many similarities were found between these two astrocytic subtypes. One of the common features was the presence, along processes, of protrusions reminiscent of dendritic spines. Thorny processes were recently mentioned as a feature of protoplasmic astrocytes in human temporal cortex (Oberheim et al., 2009). Here, we show that thorny processes are also characteristic of fibrous astrocytes, and that their density along processes is similar in both astrocytic subtypes. We currently ignore the functional role of these spines which, to our knowledge, have never been described in other species, but it is tempting to speculate that they represent

specialized postsynaptic structures analogous to dendritic spines. Future work using electron microscopy will be required to gain a better understanding of their ultrastructural features and microenvironment.

A major finding of this study arose from the comparison of BA24 astrocytes in depressed suicides and matched controls. Although morphometric features of protoplasmic astrocytes were in all points comparable between groups, fibrous astrocytes were found to be larger and to extend longer and more ramified processes in depressed suicides (**Fig. 11**). These results further highlight the distinctiveness of astrocytic subtypes within the same cortical region, and suggest that a morphological remodeling may occur in fibrous astrocytes independently of adjacent gray matter protoplasmic astrocytes. Importantly, this is the first report of selective cellular changes occurring in the white matter in depression. Alterations in dACC white matter circuitry are likely to impact on communication between this cortical area and other intimately associated brain regions implicated in depression, such as the amygdala and prefrontal cortex (Vogt et al., 1995, Allman et al., 2001).

The cortical area investigated here was previously described by our group to display similar Nissl-stained (upper and lower cortical) glial densities in young adult depressed suicides compared to controls (Hercher et al., 2009b). Interestingly, in an elegant quantitative analysis published by Miguel-Hidalgo and colleagues, the packing density and areal fraction occupied by GFAP-immunoreactive astrocytes in dorsolateral prefrontal cortex were found to differ between MDD subjects and matched controls, but only when younger (30-45 years old) and older (46-86 years old) subjects were considered independently (Miguel-Hidalgo et al., 2000). A subsequent study by the same group showed a strongly significant positive correlation between GFAP protein levels and age at time of death in depressed subjects (Si et al., 2004). Furthermore, GFAP protein levels were also found to be positively correlated with the areal fraction occupied by GFAP-immunoreactivity in BA9 (Si et al., 2004). The results of the present

study, showing similar anatomical features between BA24 protoplasmic astrocytes in middle-aged depressed suicides and matched controls, suggest that if alterations in GFAP expression also occur in this region in depressed suicides, they are not accompanied by morphological changes. However, this does not preclude the possibility that astrocytic function and signalling are affected in depression and suicide, as suggested previously (Miguel-Hidalgo et al., 2000, Si et al., 2004, Ernst et al., 2009).

It can be hypothesized that the hypertrophic fibrous astrocytes described here in depressed suicides reflect local inflammation in the white matter. Strong lines of evidence support the neuroinflammatory theory of depression (Maes et al., 2009). In particular, it has been well documented that patients suffering from depression have significantly higher levels of circulating pro-inflammatory cytokines (Miller et al., 2009a), and that administration of interferon-? leads to depressive-like symptoms (Malaguarnera et al., 1998 reviewed in Horsmans, 2006) that are accompanied by increased dACC activation (Capuron et al., 2005). Expression of brain cytokines seems altered in suicides (Tonelli et al., 2008), and pro-inflammatory cytokines have been implicated in the development of stress-induced depressive symptoms (Goshen et al., 2008, Audet et al., 2010). The differences between cortical gray and white matter astrocytes highlighted in the present study need to be explored further, but may have to do with an increased facility of cytokines to diffuse within the brain along white matter tracts (Konsman et al., 2000). Interestingly, white matter hyperintensities (WMHs) (Debette and Markus, 2010), which are thought to represent regions of acute astrocyte activation (Simpson et al., 2007) or astrogliosis (Fazekas et al., 1993), increase the risk of developing MDD (de Groot et al., 2000, Bae et al., 2006, Iosifescu et al., 2007, Li et al., 2007 reviewed in Tham et al., 2010) and are strongly associated with suicide (Grangeon et al., 2010). WMHs, have been proposed to arise from inflammation and oxidative stress (Xu et al., Wright et al., 2009 reviewed in Rosenberg, 2009) both of which are well documented to be increased in depression (Maes, 2008, Miller et al., 2009a).

When interpreting results of this study, two particular limitations need to be kept in mind. First, given that the reconstruction and morphometric analysis of each astrocyte constituted a lenghty and labour-intensive process, the sample size was relatively modest (20 subjects, 200 cells). Multiple correlation analyses followed by ANCOVAs were performed to evaluate the influence on measured variables of potential confounders such as alcohol dependence, smoking and medication. Although none of these factors were found to significantly influence any of the variables, it is acknowledged that the statistical power to detect such confounders was somewhat limited due to sample size. Nevertheless, the specificity and statistical significance of the findings are reassuring. Second, we cannot establish whether our observations are due to depression, suicide, or to a combination both. Future work could clarify this issue by including samples from non-depressed suicides and depressed non-suicides.

In summary, this first morphometric characterization of human cortical gray and white matter astrocytes shows unexpected features such as spines, found along processes extended by both protoplasmic and fibrous astrocytes. It also reveals highly significant morphological changes displayed by BA24 white matter fibrous astrocytes in depressed suicides compared to controls. These changes, which were not found in adjacent layer VI protoplasmic astrocytes, consisted of larger cell bodies as well as longer and more ramified processes. Taken together, these results suggest significant differences between dACC cortical gray and white matter astrocytic activation that may reflect a state of chronic inflammation affecting the white matter compartment of this limbic region in depression and suicide (Torres-Platas et al., 2011).

2.6 AKNOWLEDGEMENTS

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2.7 TITLES AND LEGENDS TO FIGURES

Table I Subject information

	Controls ($n = 10$) Mean ± SEM	Depressed suicides ($n = 10$) Mean ± SEM
Age (years)	48.3 ± 5.9	48.6 ± 5.3
Gender	8M:2F	7M:3F
Tissue pH	6.5 ± 0.13	6.6 ± 0.5
PMI (h)	57.0 ± 5.7	41.0 ± 6.4
Cause of death	5 cardiovascular	7 hanging
	3 road accident	2 intoxication
	2 falling	1 jumping
Clinical information		
Unipolar depression	—	7
Bipolar depression	—	3
Smoking	3	4
Alcohol dependence	—	4
Antidepressants	—	7 (1)*

*Presence of antidepressants was only detected by toxicological analysis in 1/7 subjects.

Figure 1

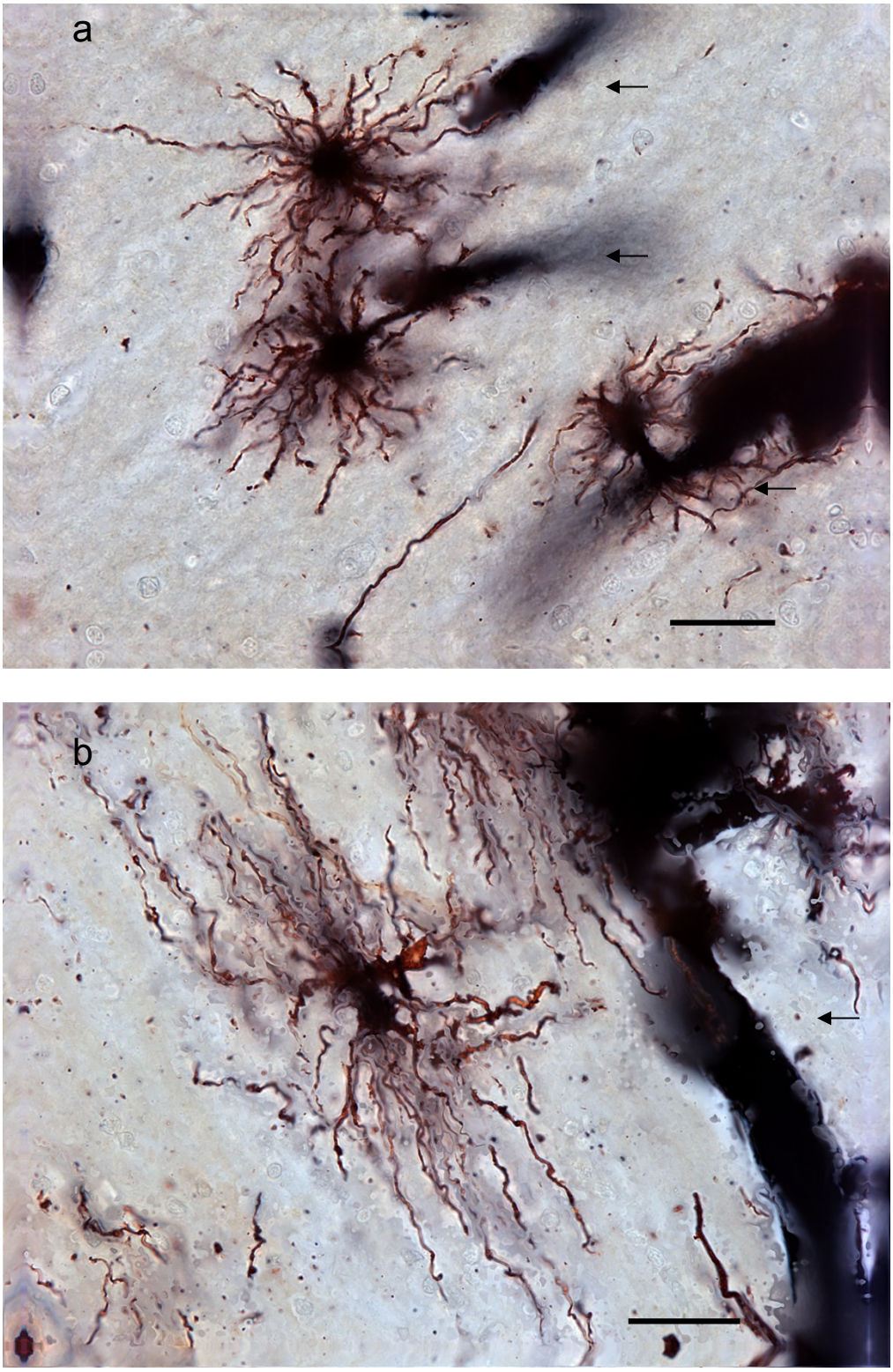

Fig. 1. *Representative examples of Golgi-stained protoplasmic and fibrous astrocytes in human dACC.* In the gray matter layer VI, protoplasmic astrocytes from a control subject (a) displayed a characteristic spherical cell body with tortuous varicose and thorny processes radiating in all directions. Fibrous astrocyte in the white matter (b) of the same subject presented a more oblong cell body, with relatively long and unramified varicose and thorny processes extended mainly in two opposite directions. Micrographs taken at three different focal points were merged to produce the image in (b). Processes extended by both astrocytic subtypes were often seen contacting blood vessels (arrows). Scale bar in (a): 10 µm, and in (b): 25 µm.

Figure 2

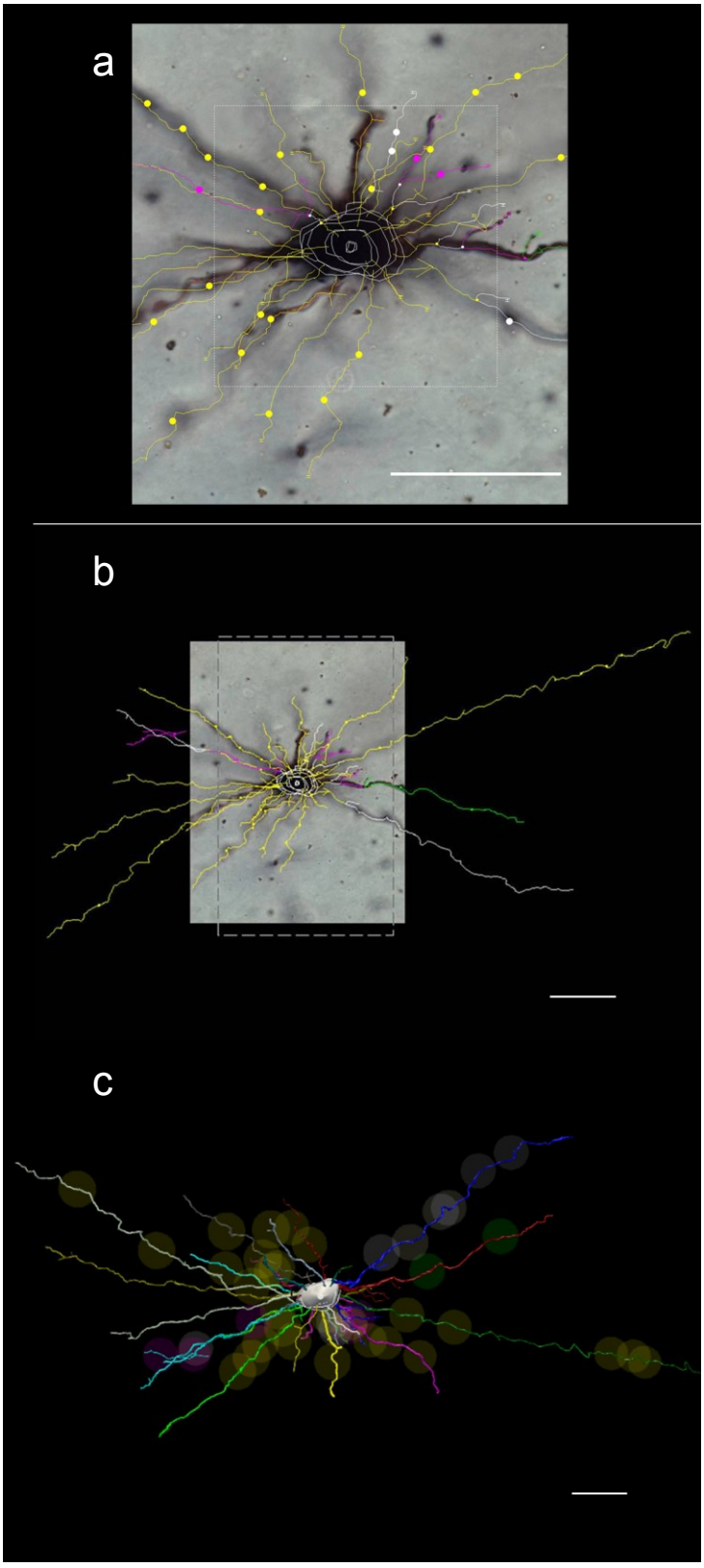
a
b
c

Fig. 2. *Three-dimensional reconstruction of processes extended by astrocytes.* Reconstructing processes extended by astrocytes and visualized across the depth of histological sections with the *Neurolucida* software involved several steps that are summarized here in the case of a BA24 white matter fibrous astrocyte. The image in (b) is a lower magnification of the one shown in (a). First, the outline of the cell body is traced at its largest cross-sectional diameter to measure its area. For the purpose of this illustration, the soma outline was drawn at different focal points to generate a three dimensional image. Second, the course of each process was tracked and traced along its full length within the tissue section, as shown by coloured segments extended by the cell body in (a) and (b). Colour codes were used to identify primary and higher-order branches, and markers (e.g. small circles) to label particular features such as astrocytic spines or nodes. The reconstructed cell is shown in (c), with each primary process and its branches coded by a different colour. Scale bars in (a), (b) and (c): 25 µm.

Figure 3

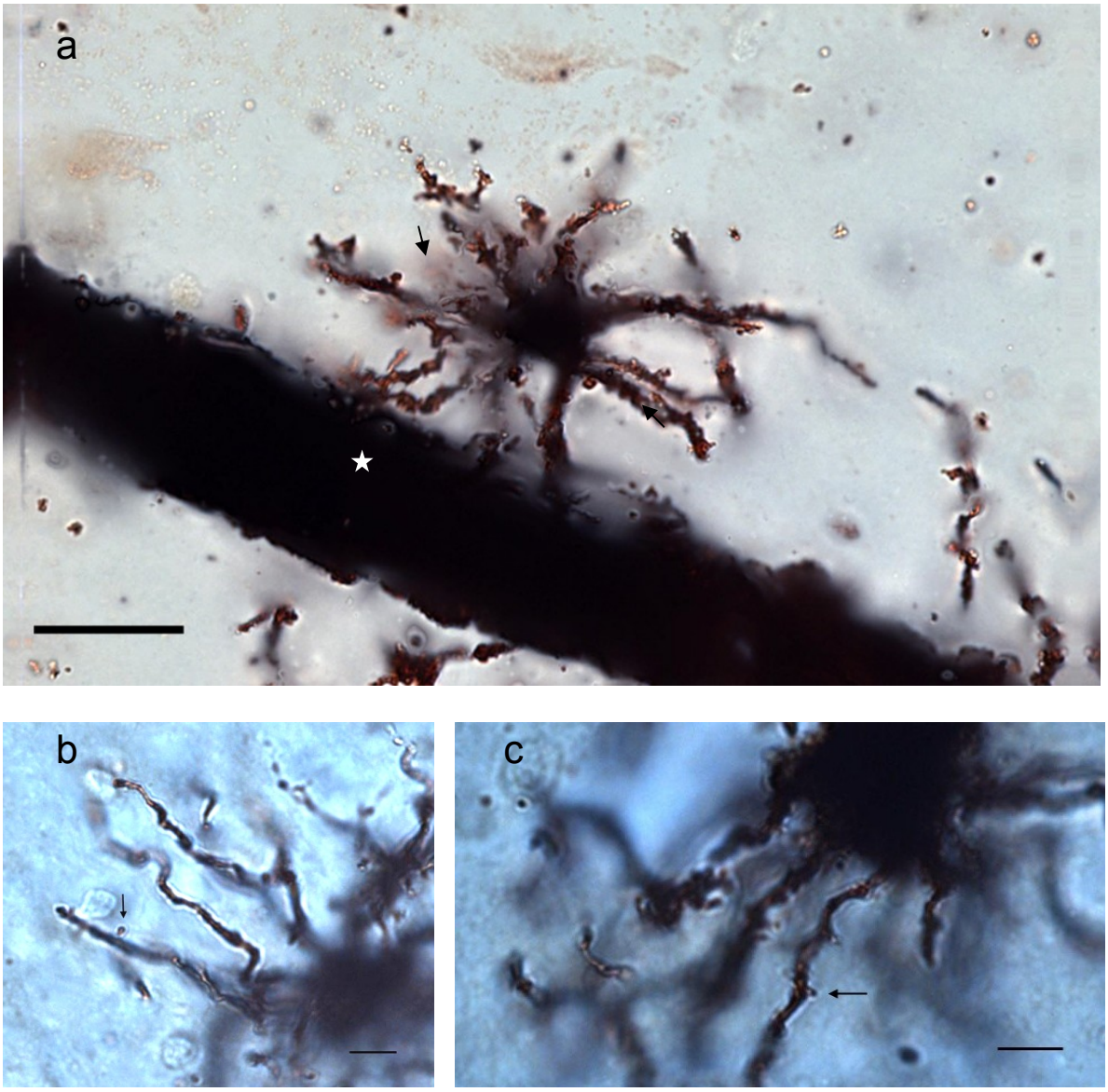

Fig. 3. *Spines are a feature of protoplasmic and fibrous astrocytic processes in human dACC.* Golgi-stained astrocytic processes were always observed to be thorny. This was due to the presence of spine-like structures (black arrows) found along processes extended by protoplasmic (a) and fibrous astrocytes (b, c) alike. Micrographs taken at three different focal points were merged to produce the image in (a). Several processes of the protoplasmic astrocytes illustrated in (a) contact a large blood vessel (white star). Scale bars in (a): 10 µm, and in (b) and (c): 25 µm.

Figure 4

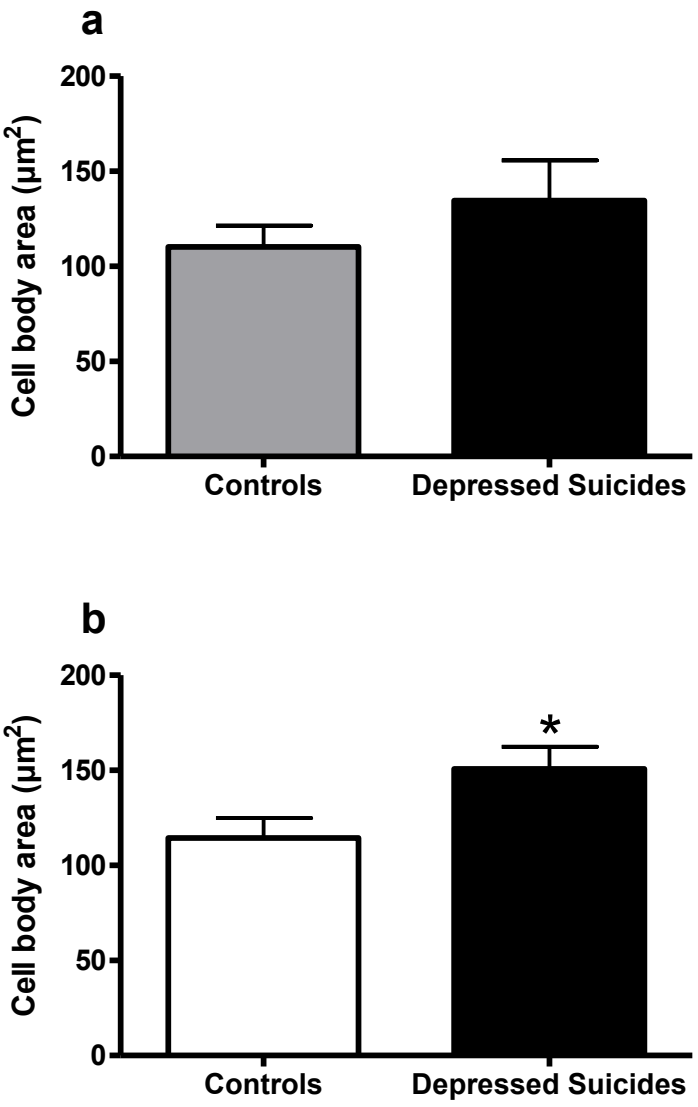

Fig. 4. *Fibrous astrocytes in BA24 of depressed suicides present a significantly larger cell body area than in controls.* Both layer VI protoplasmic (a) and white matter fibrous (b) astrocytes showed a greater cell body area in depressed suicides than in matched controls. However, this increased soma size was significant only in the case of fibrous astrocytes. * $p < 0.05$

Figure 5

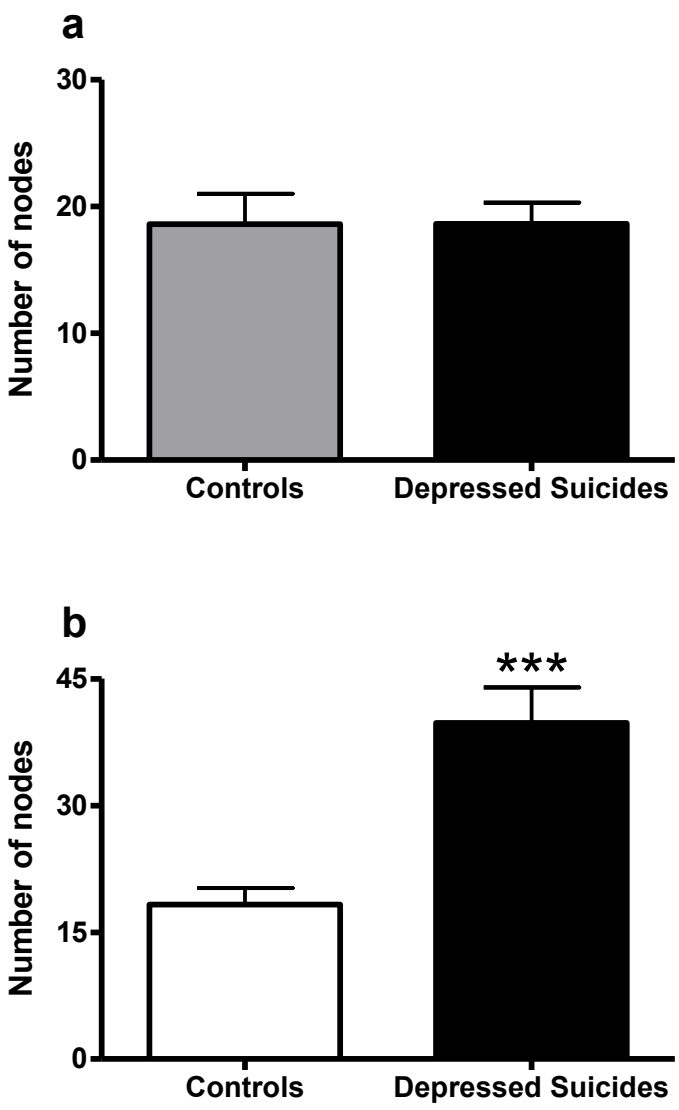

Fig. 5. *Fibrous astrocytes in BA24 of depressed suicides present significantly more branching points than in controls.* The number of nodes made by processes was similar between groups for layer VI protoplasmic astrocytes (a) but more than twice higher in depressed suicides in the case of fibrous astrocytes (b). *** $p < 0.0005$

Figure 6

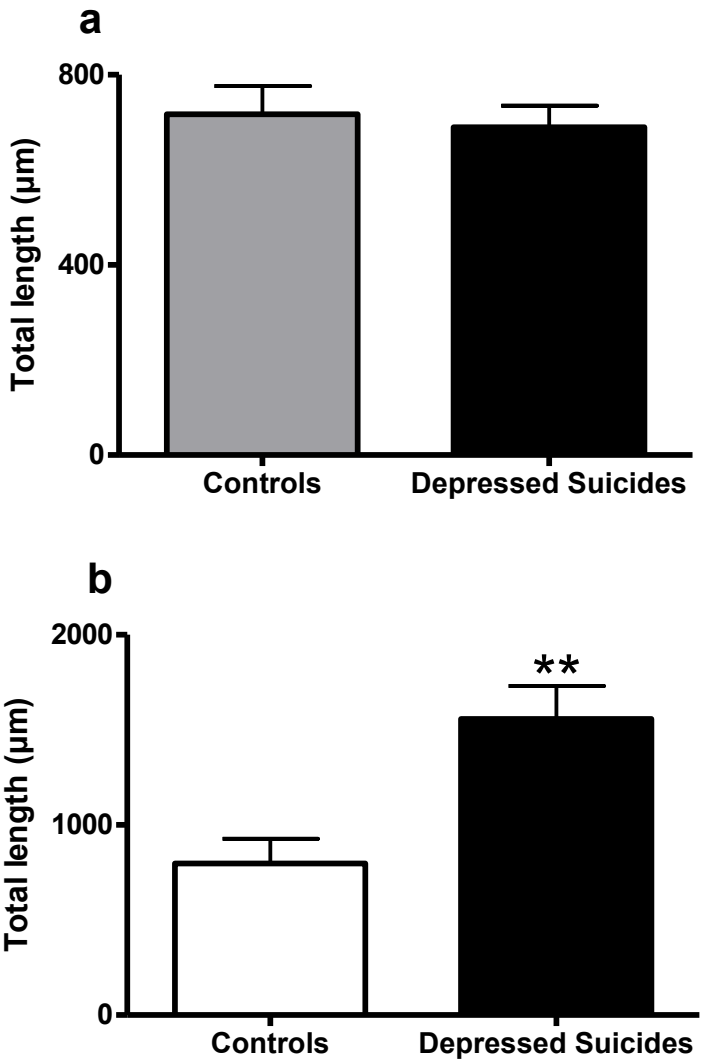

Fig. 6. *Fibrous astrocyte projections in BA24 are significantly longer in depressed suicides than in matched controls.* Numbers of processes emerging from the cell body were found to be similar between groups for both protoplasmic and fibrous astrocytes (see *Results* section). However, the total length of processes per cell was significantly different between groups in the case of fibrous astrocytes, with a value almost twice as high in depressed suicides than in controls (b). As expected, with an absence of group difference in number of nodes per cell, total process length was very similar between groups for protoplasmic astrocytes (a). ** $p < 0.005$

Figure 7

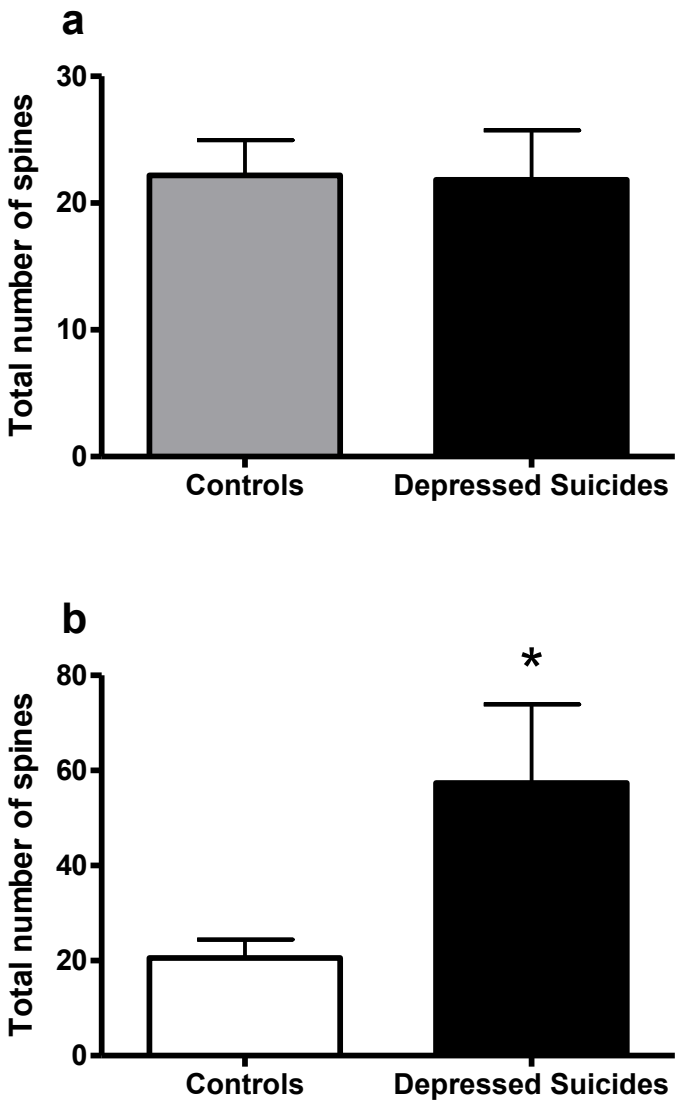

Fig. 7. *Astrocytic spines in fibrous astrocytes are significantly increased in depressed suicides due to the increase in process length per cell.* The density of spines per process length was similar for both astrocytic subtypes and did not present any group differences (see *Results* section). In relation to total process length per cell, however, the total number of spines per cell was significantly higher in depressed suicides compared to controls in the case of fibrous (b) but not of protoplasmic astrocytes (a). * $p < 0.05$

Figure 8

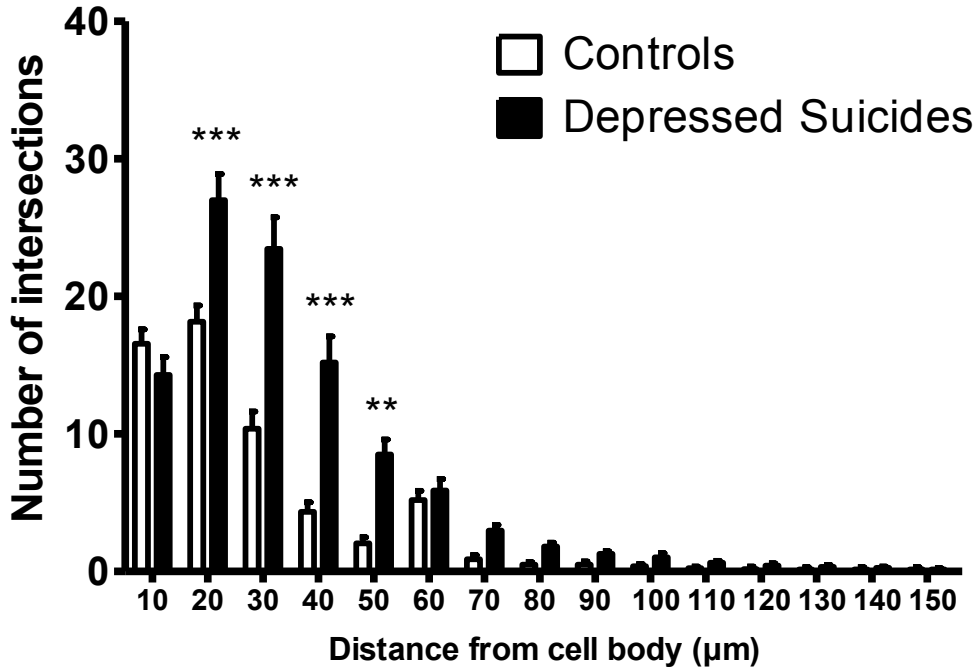

Fig. 8. *Sholl analysis of intersections made by processes of fibrous astrocytes.* Sholl analysis confirmed that process length and branching pattern of fibrous astrocytes is more elaborate in depressed suicides than in controls, with increased intersections being found within almost all concentric radii between 20 and 50 µm around the cell body. ** $p < 0.005$, *** $p < 0.0005$.

Figure 9

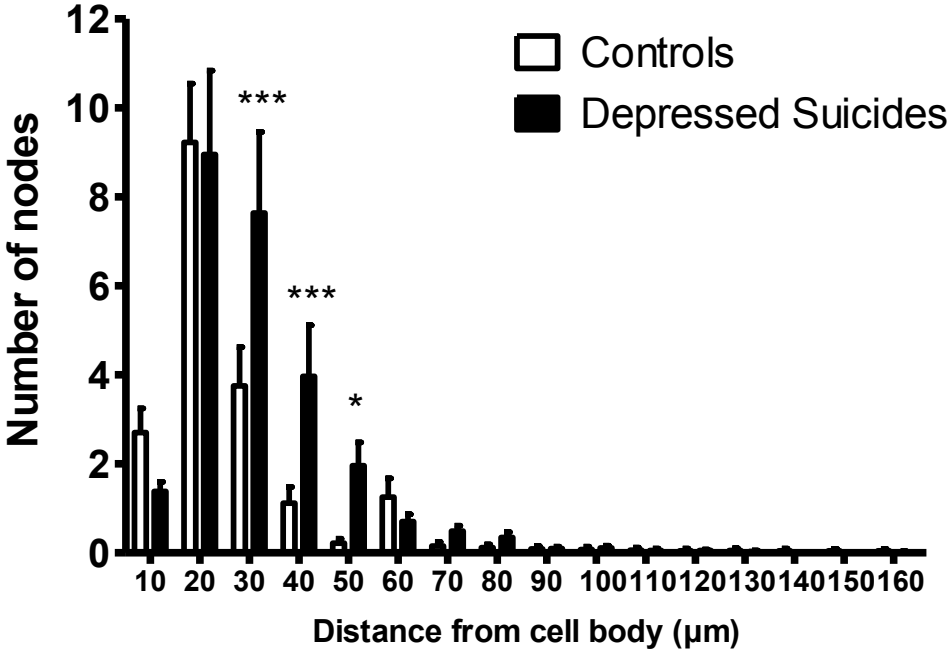

Fig. 9. *Sholl analysis of branching points displayed by fibrous astrocytic processes.* Sholl analysis revealed that the higher number of nodes displayed by fibrous astrocytes in depressed suicides compared to controls was mainly localized at 30, 40 and 50 µm away from the cell body. * $p < 0.05$, *** $p < 0.0005$.

Figure 10

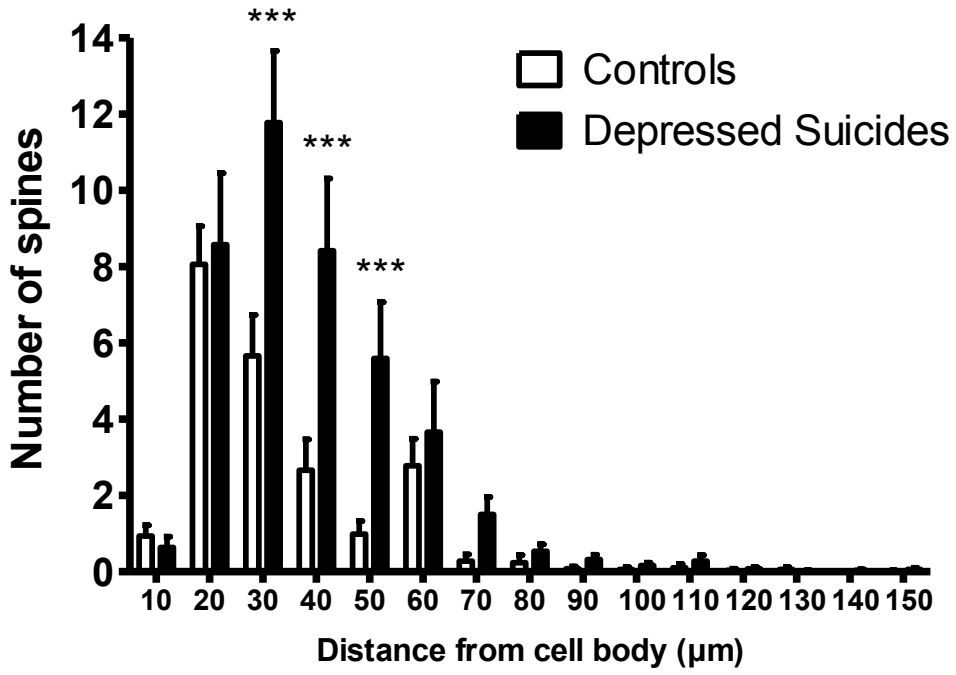

Fig. 10. *Sholl analysis of spine numbers displayed by fibrous astrocytic processes.* Sholl analysis revealed that the higher number of spines displayed by fibrous astrocytes in depressed suicides compared to controls was mainly localized between 30 and 60 µm away from the cell body. * $p < 0.05$, *** $p < 0.0005$.

Figure 11

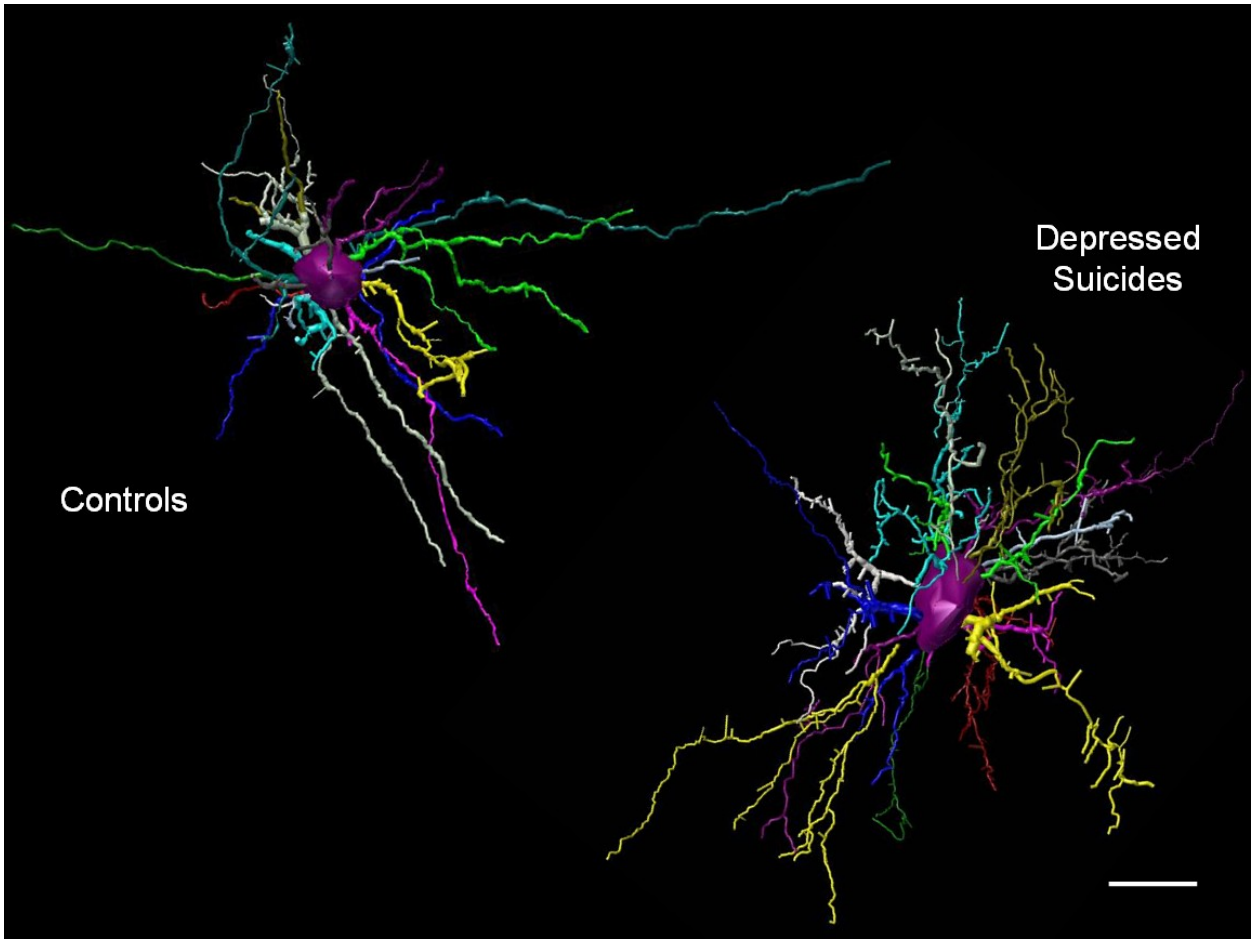

Fig. 11. *Fibrous astrocytes are hypertrophic in BA24 white matter of depressed suicides.* Three-dimensional reconstructions of representative BA24 fibrous astrocytes in controls and depressed suicides. As illustrated, fibrous astrocytes in the latter group display larger cell bodies and increased branching and length of their processes. Scale bar: 25 µm.

CHAPTER 3.0: PREFACE

Before our previous study, neuroanatomical investigations suggested altered densities and distribution patterns of glial cells (Ongur et al., 1998, Rajkowska et al., 1999) in fronto-limbic cortical circuitries including the ACC. However, all of these studies were limited by the fact that they are based on a simple discrimination of neurons and glia according to differences in cresyl violet staining of the cytoplasm and nucleus, and thus cannot account for changes in specific glial cell subpopulations. Some other studies using specific astrocytic markers such as glial fibrillary acidic protein (GFAP) (Miguel-Hidalgo et al., 2000, Si et al., 2004) and on the excitatory amino acid transporters (EAAT1-2) (Miguel-Hidalgo *et al*, 2010) suggested that astrocytic function may be perturbed in depression. Thus, suggesting that astrocytic function may be perturbed in depression. The study conducted by our group (Torres-Platas et al., 2011), represents the first postmortem study to provide a detailed fine neuroanatomical assessment of cortical astrocytes in both healthy and psychiatric subjects. But more importantly, this is the first report of selective cellular changes occurring in the white matter in depression and suicide.

Among the many functions displayed by astrocytes in a normal CNS, it is well known that astrocytes play an important role in brain immune system. In face of a stimulus, astrocytes display a graded response to immune challenges that includes changes in gene expression such as up-regulation of GFAP, progressive cellular hypertrophy and, in more severe reactions, cell proliferation and scar formation (John et al., 2003, Sofroniew, 2005, 2009, Sofroniew and Vinters, 2010). Astrogliosis, is the term generally used to designate this phenomenon. The results of the present study show a selective astrocytic hypertrophy in the dACC white matter of depressed suicides. This is suggestive of an astrocytic activation that may reflect a state of chronic inflammation affecting the white matter compartment of this limbic region in depression and suicide.

Thus, we hypothesize that this hypertrophy is result of an activation of the immune system.

Astrogliosis is elicited in response to wide variety of signalling molecules often secreted by microglial cells that are the main immune resident cells in the CNS and are, temporally, more sensitive than astrocytes. As mainly described in rodent studies of CNS injuries, microglial activation is characterized by physiological and morphological changes, that allow the migration of this cells to the site of injury. These morphological changes are characterized by the retraction of processes to the cell body until they reach an ameboid-like morphology. These alternative forms of activation represent the extreme of a spectrum rather than discrete status and represent the initiation, development and cessation of the inflammation.

Microglial morphology has been largely used to determine the “status” of the brain inflammatory response. Therefore, by following this approach, we sought to assess microglial morphology in the gray and white matter dACC of depressed suicides. However, we found that there is a current lack of detailed knowledge on the fine properties of microglial phenotypes in the human brain, and how these properties may generally compare to those of rodents, which are commonly used as models in biomedical research. Therefore, we performed a morphological characterization of microglial phenotypes in the gray and white matter dACC of control subjects, to have an accurate measurement their morphometric features for an accurate quantification of each phenotype.

MORPHOMETRIC CHARACTERIZATION OF MICROGLIAL PHENOTYPES IN HUMAN NEOCORTEX

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3.1. ABSTRACT

Background: Microglia can adopt different morphologies, ranging from a highly ramified to an amoeboid-like phenotype. Although morphological properties of microglia have been described in rodents, little is known about their fine features in humans. The aim of this study was to characterize the morphometric properties of human microglia in gray and white matter of dorsal anterior cingulate cortex (dACC), a region implicated in behavioral adaptation to neuroinflammation. These properties were compared to those of murine microglia in order to gain a better appreciation of the differences displayed by these cells across species. **Methods:** Postmortem dACC samples were analyzed from 11 individuals having died suddenly without any history of neuroinflammatory, neurodegenerative, nor psychiatric illness. Tissues were sectioned and immunostained for the macrophage marker Ionized calcium binding adaptor molecule 1 (IBA1). Randomly selected IBA1-immunoreactive (IBA1-IR) cells displaying features corresponding to commonly accepted microglial phenotypes (ramified, primed, reactive, amoeboid) were reconstructed in 3D and all aspects of their morphologies quantified using the *Neurolucida* software. The relative abundance of each morphological

phenotype was also assessed. Furthermore, adult mouse brains were similarly immunostained, and IBA1-IR cells in cingulate cortex were compared to those scrutinized in human dACC. **Results:** In human neocortical gray and white matter, all microglial phenotypes were observed in significant proportions. Compared to resting, primed microglia presented an average 2.5 fold increase in cell body size, with almost no differences in branching patterns. When compared to the primed microglia, which projected an average of six primary processes, the reactive and amoeboid phenotypes displayed fewer processes and branching points, or no processes at all. In contrast, the majority of microglial cells in adult mouse neocortex were highly ramified. This was also the case following a postmortem interval of 43 hours. Interestingly, the morphology of ramified microglia was strikingly similar between species. **Conclusions:** This study provides fundamental information on the morphological features of microglia in the normal adult human neocortex. These morphometric data will be useful for future studies of microglial morphology in various illnesses. Furthermore, this first direct comparison of human and mouse microglia reveals that these brain cells are morphologically similar across species, suggesting highly conserved functions. **Key words:** Human, Microglia, Morphology, IBA1, Anterior cingulate cortex

3.2 INTRODUCTION

Microglia have traditionally been recognized as the innate immune cells mediating inflammatory responses in the central nervous system (CNS). In recent years, however, it has become increasingly clear that ramified ("resting") microglia also participate actively in fundamental aspects of neuronal activity, including structural and functional plasticity (Butovsky et al., 2006, Sierra et al., 2010, Tremblay et al., 2010). Ramified microglia can respond to subtle microenvironmental changes arising from a wide variety of factors such as pathogens (Dellacasa-Lindberg et al., 2011, Ding et al., 2013, Garvey et al., 2013), stress (Sugama et al., 2012, Kopp et al., 2013), and injury, (Gulyas et al., 2012, Smith et al., 2012, Morrison and Filosa, 2013) with what is commonly referred to as

microglial activation. As mainly described in rodent studies of CNS injuries, this involves a rapid alteration of cell metabolism and function (Stence et al., 2001, Graeber and Streit, 2010, Kettenmann et al., 2011) which can be accompanied by a graded spectrum of morphological changes that transform highly ramified microglia into amoeboid-phagocytic microglia (Soltys et al., 2001, Stence et al., 2001, Kettenmann et al., 2011, Karperien et al., 2013). Following cell activation, highly branched microglia can reabsorb stochastically (and reversibly) into the cell body before transitioning to a dynamic motility stage with cycles of extension and retraction of new processes. Fully activated microglia then initiate a locomotor stage, by which they migrate throughout the tissue (Stence et al., 2001, Hung et al., 2010). Ramified microglia can also reach intermediate phenotypes before returning to a ramified morphology, without ever becoming amoeboid-like. Along the complete activation sequence described above, four major phenotypes are usually distinguished in rodents based on distinct morphological (Soltys et al., 2001, Stence et al., 2001) and molecular criteria (Raivich et al., 1999, Rock et al., 2005, Glanzer et al., 2007, Selenica et al., 2013): ramified, primed, reactive, and amoeboid.

Analyzing microglial morphology and function in human brains is obviously more challenging. Postmortem studies have confirmed the existence of various morphological phenotypes (Sheng et al., 1997, Graeber and Streit, 2010, Norden and Godbout, 2013) that had been previously described in rodents. Furthermore, some of the morphological and molecular mechanisms underlying human microglial reactivity have been described during development, (Andjelkovic et al., 1998, Rezaie et al., 1999, Rezaie et al., 2002), as well as in pathological conditions such as Creutzfeldt-Jakob (Szpak et al., 2006, Wojtera et al., 2012), Parkinson's disease (McGeer et al., 1988, Croisier et al., 2005, Sanchez-Guajardo et al., 2013) Alzheimer`s disease (Sheng et al., 1997, Xiang et al., 2006, Streit et al., 2009) and multiple sclerosis (Ulvestad et al., 1994, van Noort et al., 2012, Singh et al., 2013). Despite these advances, there is a current lack of detailed knowledge on the fine properties of microglia in the human brain, and how these properties may generally

compare to those of rodents, which are commonly used as models in biomedical research. Here, we report the first comprehensive morphometric analysis of the different microglial phenotypes found in postmortem samples of dorsal anterior cingulate cortex (dACC), an area that has been associated with the behavioral response to neuroinflammation (Miller et al., 2013), from adult individuals having died with no history of inflammatory, neurological nor psychiatric illness. Tissue sections were immunostained for Ionized calcium binding adapter molecule 1 (IBA1), a calcium-binding protein specifically expressed in macrophages and microglial cells (Ito et al., 1998), and the morphological features (cell body shape and size, length and branching of processes) of randomly selected IBA1-immunoreactive cells corresponding to each major morphological phenotype were measured following their 3D reconstruction. In addition, similar analyses were carried out in mouse neocortex for comparative purposes. This study not only provides fundamental information on the fine characteristics of human microglia, but also highlights the morphological similarities between human and mouse neocortical microglia. Altogether, we propose measurable criteria for the differentiation of human microglial phenotypes that could be applied in future postmortem studies of pathological conditions.

3.3 MATERIALS AND METHODS

Human and Mouse Brain Tissue

This study was approved by the Douglas Hospital Research Ethics Board, and written informed consent from next-of-kin was obtained for each subject. Fresh-frozen postmortem brain samples from the right hemisphere of individuals having died accidentally without any psychiatric, neurological, nor inflammatory illnesses (n = 11) were provided by the Douglas-Bell Canada Brain Bank. The average age at death of these subjects (10 males, 1 female) was 48 ± 5.2 years old. The average postmortem interval (PMI) was of 57.5 ± 5.4 hours, the interval between death and storage of the

body at 4˚C (refrigeration delay) was of 5.3 ± 2.0 hours, and the average brain pH was 6.6 ± 0.08. All subjects had died suddenly, without agony, from cardiovascular conditions (n=6), road accidents (n=2), or intoxications (n=3). Brain samples were dissected from the dACC, adjacent to the dorsal part of the genu of the corpus callosum (BA24) (Vogt et al., 1995, Gittins and Harrison, 2004), as described previously (Hercher et al., 2009b). After fixation by immersion in 10% formalin, tissue blocks were cut into serial 50µm-thick coronal sections using a freezing microtome. Every 12th section collected was processed for free-floating IBA1 immunohistochemistry, as detailed below.

Adult male C57Bl6 mice (1.5-3 months-old; n=4) were used in this study. All procedures were approved by the Douglas and McGill animal care committees. Three mice were deeply anesthetized with a solution containing ketamine and xylazine (0.1 mg/g, i.p.) and perfused intra-cardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4, 50 ml per mouse). Brains were removed, post-fixed overnight by immersion in the PFA solution at 4°C, and washed in PBS. One mouse was used to evaluate the effects of PMI on IBA1-IR cell distribution and morphology. This animal was sacrificed by cervical dislocation and kept 11 hours at room temperature before being placed 32 hours at 4°C, to mimic human PMI conditions. The brain was then dissected and fixed by immersion in a 4% PFA solution for 48 hours at 4°C. All mouse brains were cut with a vibrating microtome into 50µm coronal sections (brain slices corresponding to plates from +2.34mm to -0.46mm) containing cingulate cortex [50], and kept in PBS until further use. Samples were washed PBS and processed for free-floating IBA1 immunohistochemistry, as detailed below.

IBA1 Immunohistochemistry

All incubations occurred at room temperature. Prior to immunohistochemical labelling, human tissues underwent antigen retrieval by incubating sections for 10 min in a

solution of Tris-buffered saline (TBS) containing 20 µg/ml proteinase K, followed by a 10 min incubation in distilled water containing 3% H_2O_2. Sections were then pre-incubated for 24h in TBS + 0.05% tween containing 2% normal goat serum, before being transferred for 48 hours in the same solution containing polyclonal rabbit anti-IBA1 (1:1000; WAKO Chemicals USA, Inc., Richmond, VA, USA). This was followed by 1h incubation in biotinylated goat anti-rabbit antibody (1:1000; Vector Laboratories Inc., Burlington, ON, Canada), and the avidin-biotin complex procedure (ABC Kit, Vectastain Elite, Vector Laboratories Inc., Burlington, ON, Canada) for 30 min. Labelling was revealed with a diaminobenzidine kit (Vector Laboratories Inc., Burlington, ON, Canada) and samples were counter-stained with cresyl violet to better differentiate gray and white matter (**Fig.1**). Sections were mounted on glass slides, dehydrated, and coverslipped with Permount (Fisher Scientific Inc., Pittsburgh, PA, USA). Mouse brain sections underwent the same procedures, with the exception of the antigen retrieval step.

Morphometric analyzes in Microglial Phenotypes in the Human dACC

A general assessment of IBA1-immunoreactive (-IR) cells was first conducted to evaluate the relative distribution and abundance of microglial phenotypes in dACC gray and white matter. In all subjects, microglia in the gray matter were generally randomly distributed across and within layers, whereas they seemed aligned with myelinated fibers in the adjacent white matter (**Fig. 1**). Four distinct morphological phenotypes were easily recognizable in both gray and white matter. These morphologies corresponded to the previously described microglial phenotypes classically associated with differing states of activation: ramified, primed, reactive, and amoeboid (Kreutzberg, 1996, Sheng et al., 1997, Soltys et al., 2001, Stence et al., 2001, Kettenmann et al., 2011). In human dACC, IBA1-IR cells were categorized using the following distinctive features: ramified microglia displayed a small but defined cell body that appeared spherical in the gray matter (**Fig.2a**) and ellipsoid in the white matter (**Fig.**

3a). In both cortical compartments, ramified microglia displayed several highly branched processes. Primed microglia in gray matter remained highly ramified, albeit with fewer higher-order branches, but presented a distinctive ellipsoid-like soma (**Fig. 2b**). In the white matter, primed microglia were also highly ramified, but displayed a noticeably wider cell body (**Fig. 3b**). Reactive and amoeboid microglia both presented amoeboid-shaped cell bodies. The processes extended by reactive microglia were less extensive and generally longer than the cell body diameter (**Figs. 2c & 3c**), whereas amoeboid microglia were either devoid of processes or had few unbranched processes seen to be within the length of the cell body diameter (**Figs. 2d & 3d**).

Having performed a preliminary assessment that revealed very little intra-phenotypic morphological variability between subjects, we proceeded by analyzing the first 10 IBA-IR cells that corresponded unambiguously to the above-described features corresponding to each phenotype. We analyzed a total 40 gray matter and 40 white matter microglia, with 10 cells/phenotype being randomly selected and reconstructed across subjects. On average, 7.4 ± 1.0 cells per subject were traced, reconstructed, and analyzed. Cells were sampled throughout the cortical thickness, but since no noticeable difference was seen between layers, laminar distributions were not recorded. Cells were traced, reconstructed, and their morphometric features characterized as previously described (Torres-Platas et al., 2011). In brief, under a 100X (NA 1.4) oil immersion objective (Olympus BX51 light microscope) processes were analyzed in three dimensions within single sections using a computer-based tracing system (*Neurolucida* v. 8.10.2, MBF Bioscience, Williston, VT, USA), whereas cell bodies were analyzed in two dimensions (area at its largest cross-sectional diameter). Cell body area, maximum and minimum feret, roundness as well as number, length, branching points (nodes and ends) and volume of processes were measured for each IBA1-IR cell. A spherical cell body is calculated by the ratio between feret diameters. Feret is defined as the distance between two parallel lines draw tangentially to the cell body; minimum feret is the shortest chord draw in the cell body and maximum feret is the longest, as shown in the

blue and purple lines respectively in **Fig. 4a**. In a spherical cell body, the difference between maximum and minimum ferets (max-min feret) tends to zero.

Quantitative Assessment of Microglial Phenotype Distribution in Human dACC

To assess the proportions of the different microglial phenotypes present in grey and white matter, we conducted a quantitative analysis in dACC sections from five individuals, using a semi-unbiased stereological approach using an optical fractionator probe allowing for 3D quantification with the light microscope connected to a stereology workstation (*Stereo Investigator*; MBF Bioscience). This method estimates the total number of cells in a unit of tissue volume with an optical probe providing counts through the z axis. The sampling process was performed by adding a grid of dimensions 3137 µm by 2651 over the section in the white matter. We examined a counting frame measuring 150 µm ~ 250 µm with a 60x objective (NA 1.35). Consistent with the stereological methods of the dissector probe, we counted only cells with a cell body that fell within the counting frame and that did not contact the exclusion lines when they came into focus within a 15 µm– thick optical dissector. To avoid counting cells in non-representative areas of the tissue, we set top and bottom guard zones at 1 µm of the section thickness. To control for the variation tissue processing, the volume of each individual counting frame was calculated with the area of the frame multiplied by the thickness of each counting site. A total of 3604 cells were counted (average of 720 ± 67 cells per subject). The relative proportion of each of the four morphological phenotypes was calculated, and the total number of cells of each phenotype divided by the total volume of the counting sites.

Morphometric Analyses of Microglial Phenotypes in Mouse Cingulate Cortex

In contrast to what was observed in human tissues, IBA1-IR cells present in cingulate cortex of young adult mice were overwhelmingly of the ramified phenotype. In order to

gain a general quantitative appreciation of the morphometric differences and similarities between human and murine microglia, and given that virtually no variability was observed between animals, we reconstructed and analyzed cells in a single mouse. Thus, a total of 20 ramified cells (ten per cortical compartment) were randomly selected in the cingulate cortex of a 1.5 month-old mouse and analyzed as mentioned above.

Statistical Analyses

Statistical analyses were performed using PASW Statistics 18 (Statistical Product and Service Solutions, Chicago, IL, USA) and Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). All measurements were expressed as mean ± standard error of the mean (SEM), and $p \leq 0.05$ was considered significant in all statistical tests. Normality was assessed using Shapiro-Wilk tests, and two-tailed t-tests were used for normally distributed data with a Welch correction in case of significant difference of variances. U-tests were used for non-normally distributed data. A Bonferroni correction was performed to each dependent variable to counteract for multiple comparisons type one error.

3.4 RESULTS

Qualitative and Quantitative Features of Microglial Cells in Human dACC

Gray matter

The ramified, primed, reactive and amoeboid microglial phenotypes were consistently observed in the gray matter of all dACC samples. IBA1-IR cells with highly branched processes represented the majority of cells observed in this region. Our stereological estimates indicate that nearly 16% of IBA1-IR cells were of the ramified phenotype, while about 34% were of the primed phenotype. In general, all phenotypes were seen to be evenly distributed throughout cortical layers, with no overlapping domains (**Fig. 1a**). However, the distribution and relative space between IBA-IR cells varied within and between subjects. Compared to the other phenotypes, ramified microglia displayed a characteristic small and spherical cell body extending a large number of primary and

higher order processes (**Fig. 2a**). All other phenotypes were clearly distinct from ramified microglia in that they had an amoeboid-like cell body **(Fig. 2**). Primed microglia projected similar numbers of primary and higher order processes than ramified microglia (**Fig. 4e**), but clearly displayed a cell body of greater area ($U_{(18)}$ *0 p<0.0001,* **Fig. 4b**) and of decreased roundness ($t_{welch\ (9)}$ *4.612 p=0.0013*; **Fig. 4c**). This was reflected by a significant increase in minimum feret ($t_{welch\ (11)}$ 6.46 *p< 0.0001*) and max-min feret ($U_{(18)}$ 0 *p=0.0002;* **Fig. 4d**) compared to the ramified phenotype. The reactive and amoeboid phenotypes in the gray matter represented 32% and 18% of the total number of IBA1-IR cells in dACC, respectively. Although the cell body morphology of reactive microglia, in comparison to primed microglia, was statistically similar in al measured parameters (area, roundness and max-min feret), the processes of reactive microglia displayed significantly fewer first order (and overall) branches ($U_{(18)} \leq 9.50\ p \leq 0.0025$, **Fig. 4e**), as well as significantly shorter total process length ($t_{welch\ (10)}$ *5.50 p=0.0003,* **Fig. 4h**) and volume ($U_{(18)}$ *5.00 p=0.0002,* **Fig. 4i**). Amoeboid microglia had an increase in cell body roundness ($t_{(18)}$ *2.49 p=0.022,* **Fig. 4c**), compared to reactive microglia, with no significant differences in area, min feret, max feret and max-min feret. What characterized this phenotype, however, was a significant decrease of primary ($U_{(18)}$ *9.50p=0.0015,* **Fig. 4e**) and higher order branches ($U_{(18)}$ *0 p=0.0002,* **Fig. 4f & 4g**), as well as a significant decrease in total process length ($U_{(18)}$ *0 p=0.0001,* **Fig. 4h**) and volume ($U_{(18)}$ *11.00 p=0.0021,* **Fig. 4i**) compared to reactive microglia. On occasion, a few IBA1-IR amoeboid-like cells were observed in close proximity of larger blood vessels (**Fig. 2**). All results for gray matter cells are summarized in Table 1.

White matter

Similar to gray matter, all microglial phenotypes were observed in the white matter. Again, the majority of IBA1-IR cells displayed extensively branched processes. However, contrary to their grey matter counterparts, these cells had an oblong cell body from which emerged a bipolar arborisation. Furthermore, ramified microglia in the white matter appeared aligned to myelinated fiber tracts (**Fig. 1b**). From the total number of

cells quantified in the white matter (n=1, 534), ramified microglia accounted for 43% of the total number of IBA1-IR cells, compared to 27% for the primed phenotype. The primed phenotype could be distinguished by the presence of a wider cell body and a significantly larger cell body area than ramified microglia ($t_{welch\ (11)}$ *6.89 p<0.0001,* **Fig. 5a**); a feature that was reflected by a significantly larger maximum ($U_{(18)}$ *10.50 p=0.0032*) and minimum feret ($t_{(18)}$ *6.65 p<0.0001,* **Fig. 5b**). Yet, the roundness (**Fig. 5d**) and the max-min feret of the cell body did not statistically differ between primed and resting microglia (**Fig. 5c**). Ramified and primed microglia both presented a similarly high number of primary processes (**Fig. 5e**). However, primed microglia presented fewer higher order branches, as reflected by numbers of ends and nodes ($U_{(18)}$ *≤13.00 p≤ 0.0057,* **Fig. 5f & 5g**), as well as significantly shorter processes compared to ramified microglia ($U_{(18)}$ *13.00 p=0.0039,* **Fig. 5h**).

As measured in the grey matter, reactive and amoeboid phenotypes in the white matter each represented slightly more than 11% and 19%, respectively, of the total number of IBA1-IR cells in dACC white matter. The cell body morphology of reactive and primed microglia was comparable in all points (area, maximum feret, minimum feret, max-min feret and roundness, **Fig. 5**). However, reactive microglia presented a readily observable decrease of primary ($U_{(18)}$ *13 p=0.0030,* **Fig. 5e**) and higher order branches ($U_{(18)}$ *≤ 9.50 p≤ 0.0054,* **Fig. 5f & 5g**), as well as shorter processes ($t_{welch\ (11)}$ *3.66 p=0.0037*, **Fig. 5h**) than primed microglia. The cell body morphology of amoeboid microglia was measured to be statistically comparable to that of reactive microglia (**Fig. 5**). However, the former showed a near absence of overall processes (primary and higher order) ($U_{(18)}$ *≤ 6.50 p≤0.0005,* **Fig. 5**) that were thus of significantly reduced length and volume compared to reactive microglia ($U_{(18)}$ *≤ 6 p ≤0.0003,* **Fig. 5h & 5i**). As in gray matter, a few IBA1-IR cells were observed to be closely associated with large blood vessels in the white matter. All results for white matter cells are summarized in Table 2.

Morphological Phenotypes in the Mouse Cingulate Cortex

As mentioned above, IBA1-IR cells present in the mouse cingulate cortex were generally highly branched, and their cell bodies were evenly distributed in the tissue. The densely packed arborizations extended by IBA1-IR cells in 3 month-old mice were organized into distinct and non-overlapping domains. Although the morphology of IBA-IR cells was similar in the younger adult mouse (1.5 month-old), highly branched arborizations of neighboring cells were often found to overlap at their extremities, suggesting a regional organization that is not yet fully mature at that age (**Fig. 6**). Reactive and amoeboid-like morphologies were rarely observed in adult mice. As opposed to what was observed in human tissues, highly ramified IBA1-IR cells in mice presented heterogeneous cell bodies, with varying shapes and sizes (**Fig. 6**). A proportion of these cells could also have been of the primed phenotype, but could not be distinguished from the ramified phenotype because of the inconsistent cell body morphology of highly branched cells in rodents. In consequence, if using the same criteria as those used for human samples, it remains possible that a subset of these ramified microglia observed in young adult mice were in fact of the primed phenotype. In the white matter, the cell body of ramified microglia were more uniform in terms of shape and size, and their processes displayed a bipolar organization that was aligned, in a non-overlapping fashion, to white matter tracts.

Comparisons of reconstructed mouse and human ramified IBA1-IR cells showed that despite a significant increase in cell body area ($t_{(18)}$ *4.02 p=0.0008*) and high order branches (ends and nodes $U_{(18)}$ *5 p ≤ 0.0008*) in the mouse white matter, the surface are and volume of processes in the gray and white matter were statistically similar. In the gray matter, the heterogeneity in cell body shape was reflected by a significant decreased roundness ($t_{welch\ (11)}$ *4.92 p=0.0005*) and max-min feret ($t_{welch\ (9)}$ *4.93 p=0.0008)* in the mouse microglial ramified phenotype. All other parameters were statistically comparable between groups (**Fig. 7**).

Effects of postmortem interval on microglial phenotypes in mouse cingulate cortex

As in brains harvested and fixed at time of death, brains fixed after a PMI of 43 hours dispalyed ramified IBA1-IR microglia as the predominant phenotype in both gray and white matter. Furthermore, these cells displayed no noticeable degradation of their processes (**Fig. 8**). Some reactive and amoeboid IBA1-IR cells were also observed following this PMI (≈6% and ≈4%, respectively). These scarce cells were found in both neocortical gray and white matter, but rarely in association with any particular structure such as blood vessels.

3.5 DISCUSSION

In this study, we describe the morphometric features of IBA1-IR microglial cells in adult human gray and white matter dACC from individuals having died without inflammatory, neurological, or psychiatric illness. In the gray matter, microglial cells were abundant and evenly distributed, with neither overlapping domains nor obvious differences between cortical layers. This uniform distribution is hypothesized to allow microglial cells to efficiently and constantly survey the microenvironment with their highly motile processes (Nimmerjahn et al., 2005). This dynamic phenomenon has been implicated in many physiological activities, such as maintaining the viability of synaptic contacts (Wake et al., 2009, Tremblay et al., 2010). In white matter, IBA1-IR microglial cells were aligned with myelinated tracts, which generally conferred an oblong shape to their cell bodies. Cells were more abundant in proximity to the gray matter compared to deeper white matter regions, which displayed much more discrete staining.

Based on clear morphological differences described previously in humans and rodents (Kreutzberg, 1996, Sheng et al., 1997, Stence et al., 2001), we confirm the existence of four major microglial phenotypes in adult human neocortical gray and white matter. In the gray matter, ramified microglia were characterized by very extensive branching patterns and small spherical cell bodies. Primed microglia presented similar arborisation

patterns compared to resting microglia, but displayed an overall increase in cell body area as well as a decrease in roundness. Reactive microglia featured an amoeboid-like cell body with few ramified processes, whereas amoeboid microglia extended either a single unramified process or no processes at all. It has to be acknowledged here that this nomenclature, which is derived from previous morpho-functional studies, may not always accurately reflect the functional states of microglial cells, nor whether a cell is transitioning towards increased activation or reverting back to a ramified morphology. Furthermore, it is impossible to determine in postmortem brain tissues, the proportion of IBA1-IR cells that are resident microglia vs infiltrated monocytes that have differentiated (Hickey, 1991).

Significant numbers of IBA1-IR cells displaying each of the 4 morphological phenotypes were observed in both cortical compartments of all subjects. Estimates showed that the majority of IBA1-IR microglia in the gray matter were of the primed phenotype (34%), followed by the reactive (32%), amoeboid (18%) and ramified (16%) phenotypes. The proportions were different in the white matter, with ramified cells representing the majority (43%), followed by the primed (27%), amoeboid (18%) and reactive (12%) phenotypes. Interestingly, amoeboid cells consistently accounted for nearly one-fifth of the total number of IBA1-IR cells in the dACC. These results contrast dramatically with those observed in the cingulate cortex of young adult mice, in which IBA1-IR cells were overwhelmingly highly branched, and most likely of the ramified phenotype. Other microglial phenotypes were very rarely observed in mice. In order to determine whether the presence of different microglial phenotypes in human tissues may have resulted at least partly from postmortem factors, we recreated similar PMI conditions in the mouse. Although we observed a slight increase in reactive IBA1-IR cells following a PMI of 43 hours compared to immediate harvesting of the brain following sacrifice, the great majority (≥90%) of microglial cells remained of the ramified phenotype. These observations strongly suggest that the proportions and distributions of the different microglial phenotypes in human ACC were mostly present at time of death in individuals

who did not suffer from inflammatory, neurological or psychiatric illness. This is further supported by previous reports demonstrating that microglial morphological changes require adenosine triphosphate (ATP) (Dibaj et al., 2010), oxygen and glucose supply (Eyo and Dailey, 2012), all of which cease shortly after death, leaving little opportunity for microglial cells to undergo phenotypical changes, let alone retract their processes and adopt an amoeboid phenotype (Dibaj et al., 2010).

Another noticeable difference between human and mouse microglia relates to the shape of cell bodies. Whereas human microglia displayed a characteristic round-(ramified phenotype) or amoeboid-shaped (primed, reactive, amoeboid phenotypes) cell bodies, mouse microglia displayed highly heterogeneous cell body shapes that prevented the distinction of ramified vs primed phenotypes. Despite this difference, the size and general morphology of microglia were found to be highly similar between species. Microglia are thus different from astrocytes, another glial cell type, which are several-fold larger as well as much more diverse and complex in the human than in the mouse neocortex (Oberheim et al., 2009). This morphological similarity between species may be related to the different developmental origin of microglia, and to highly conserved roles in mammalian evolution.

The observed variability of microglial distributions between human subjects seems due, within each sample, to variations in inter-cell spacing and cell densities. In rodents, it has been shown that age could influence the spacing between neighbouring microglia (Tremblay et al., 2012). Similarly, stress is another factor that can influence microglial morphology and distribution (Gomez-Gonzalez and Escobar, 2010). These (and probably other) factors may have contributed to the inter-individual variations in microglial distribution in human brain samples.

In conclusion, this study is the first to provide a morphometric characterization of microglial morphology in humans. Interestingly, we found that the general

morphological features of human microglia in the dACC was highly similar to those displyed by microglia in the mouse cingulate cortex. Thus, the study of microglial anatomy and physiology in rodent models seems more appropriate than that of other glial subtypes such as astrocytes, which display highly distinctive characteristics in humans. Our results indicate that four major microglial phenotypes co-exist in adult human neocortical tissues. Given that the average age of the subjects analyzed in this study was 48 years, it is possible that this phenotypic distribution is a normal consequence of the aging process. This hypothesis could be tested in future studies of postmortem cortical samples from adolescents or young adults. Finally, the quantified data generated in this study will be instrumental in future studies examining the implication of microglia in various conditions and illnesses thought to arise, at least in part, from abnormal immune activity in the brain. As suggested previously, comparing microglial phenotypic ratios in well-characterized brain samples may be particularly instructive to understand the state of inflammatory processes in a given brain circuitry (Schnieder and Dwork, 2011). Likewise, this approach would allow the precision required to identify subtle imbalances in microglial phenotypic distributions that may characterize illnesses associated with a mild inflammatory component.

3.6 AKNOWLEDGEMENTS

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3.7 TITLES AND LEGENDS TO FIGURES

Table 1. Quantitative features of human IBA1-IR phenotypes in the GM.

Cell body					
	Area (μm^2)	**Roundness**	**Min feret (μm)**	**Max feret (μm)**	**Max-Min feret (μm)**
Ramified	30.83 ± 0.97	0.81 ± 0.01	5.92 ± 0.11	6.96 ± 0.10	1.04 ± 0.06
Range	26.7 - 36.07	0.76 -0.87	5.3 -6.4	6.5 - 7.6	0.8 - 1.2
Primed	79.13 ± 9.69	0.54 ± 0.05	8.19 ± 0.33	14.58 ± 1.91	6.39 ± 1.78
Range	52. 44 -156.98	0.24 - 0.76	7.0 -10.01	9.3 -28.6	2.3 -18.5
Reactive	68.21 ± 6.12	0.44 ± 0.05	7.25 ± 0.59	14.63± 1.17	7.38 ± 1.30
Range	31. 85 - 95. 98	0.22 -0.64	5.0 - 10.80	8.1 - 21.70	3.1 - 10.9
Amoeboid	101.02 ± 18.55	0.59 ± 0.03	9.18 ± 0.64	14.61 ±1.58	5.43 ± 1.09
Range	41. 07 - 225.012	0.42 - 0.72	6.0 - 13.3	9.9 - 27.7	3.1 -14.4

Processes					
	Primary Processes	**Ends**	**Nodes**	**Total length (μm)**	**Volume (μm^3)**
Ramified	5.8 ± 0.59	63.6 ± 9.85	46 ± 7.79	590.64 ± 77.55 μm	274.14 ± 31.20
Range	3.0 - 9.0	25 - 104	16 - 78	294.5 - 979.4	105.97 - 407.44
Primed	5.5 ± 0.60	40.2 ± 7.78	28.6 ± 6.53	496.08 ± 60.16 μm	356.87 ± 67.45
Range	3.0 - 8.0	14- 91	9 - 66	287.3 -793.0	86.56 - 772.45
Reactive	3 ± 0.25	9.8 ± 1.80	6.1 ± 1.55	122.47 ± 18.68 μm	102.65 ± 20.85
Range	2.0 - 5.0	5.0- 20	2.0 - 15	50 - 215.9	36.68 - 224.39
Amoeboid	1.2 ± 0.35	1.3 ± 0.39	0.10 ± 0.10	8.58 ± 3.40 μm	23.65 ± 15.10
Range	0 - 2.0	0-3	0 - 1	0 - 31.2	0- 145.92

Table 2. Quantitative features of human IBA1-IR phentoypes in the WM.

Cell Body					
	Area (μm^2)	**Roundness**	**Min feret (μm)**	**Max feret (μm)**	**Max-Min feret (μm)**
Ramified	16.40 ± 1.28	0.53 ± 0.05	3.67 ± 0.19	6.55 ± 0.57	2.88 ± 0.60
Range	9.11 - 24.41	0.22 - 0.85	2.5 - 4.8	4.8 - 9.9	1.7 - 5.6
Primed	42.74 ± 3.63	0.54 ± 0.03	5.85 ± 0.26	10.18 ±0.72	4.33 ± 0.64
Range	25.27 - 64.29	0.40 - 0.70	4.7 - 7.3	6.8 - 14.3	2.1 - 7.0
Reactive	59.62 ± 6.42	0.62 ±0.03	7.44 ± 0.50	11.02 ± 087	3.58 ± 0.75
Range	18.30 - 89.95	0.44 - 0.73	3.9 - 9	6.0- 16.0	2.1 - 7
Amoeboid	70.23 ± 7.03	0.59 ± 0.03	8.22 ± 0.55	12.32 ± 0.73	4.1 ± 0.55
Range	37.39 - 111.93	0.40 - 0.75	5.8 - 11.2	8.7 - 16.2	2.9 - 4.9

Processes					
	Primary Processes	**Ends**	**Nodes**	**Total length (μm)**	**Volume (μm^3)**
Ramified	4.90 ± 0.6	23.11 ± 2.18	16.7 ± 1.75	436.05 ± 42.43	196.75 ± 21.44
Range	2.0 - 9.0	16.0 - 41.0	11.0 - 30.0	290.3 - 761.7	124.86 -193.15
Primed	5 ± 0.55	13.9 ± 2.38	9 ± 2.02	268.28 ± 44.31	187.26 ± 31.37
Range	2.0 - 8.0	7.0 - 33.0	3.0 - 25.0	123.6 - 594.5	62.17 - 398.20
Reactive	2.8 ± 0.29	6.3 ± 0.78	3.2 ± 0.61	107.73 ± 17.03	125.27 ± 30.53
Range	2.0 - 3.0	3.0 - 9.0	0.0 - 6.0	25.9 - 187.8	10.85 - 278.98
Amoeboid	0.60 ± 0.22	0.70 ± 0.26	0.10 ± 0.10	6.88 ± 2.53	5.96 ± 2.51
Range	0.0 - 2.0	0.0 - 2.0	0.0 - 1.0	0.0 - 19.5	0.0 - 20.23

Table 3. Quantitative features of mouse and human IBA1-IR ramified microglia

Parameter		Area	Human		Mouse		p
			Value	Range	Value	Range	
Cell Body	**Area (μm^2)**	GM	30.83 ± 0.97	26.7 - 36.07	36.83 ± 3.77	29.64 - 50.98	
		WM	16.40 ± 1.28	9.11 - 24.41	21.34 ± 1.30	18.41 -25.59	***
	Roundness	GM	0.81 ± 0.01	0.76 -0.87	0.56 ± 0.04	0.45 - 0.71	***
		WM	0.53 ± 0.05	0.22 - 0.85	0.52 ± 0.02	0.44 - 0.55	
	Min feret (µm)	GM	5.92 ± 0.11	5.3 -6.4	5.54 ±0.44	4.7- 6.9	
		WM	3.67 ± 0.19	2.5 - 4.8	4.32 ± 0.43	3.6 - 5.9	**
	Max feret (µm)	GM	6.96 ± 0.10	6.5 - 7.6	9.08 ± 0.44	8.2 - 10.6	**
		WM	6.55 ± 0.57	4.8 - 9.9	7.18 ± 0.17	6.6 - 7.7	
	Max-Min feret (µm)	GM	1.04 ± 0.06	0.8 - 1.2	3.54 ± 0.46	1.9 - 4.7	**
		WM	2.88 ± 0.60	1.7 - 5.6	2.86 ± 0.29	1.8 - 3.5	
Processes	**Primary Processes**	GM	5.8 ± 0.59	3.0 - 9.0	7.4 ± 0.80	5.0 - 9.0	
		WM	4.90 ± 0.6	2.0 - 9.0	5.2 ± 0.40	4.0 - 6.0	
	Ends	GM	63.6 ± 9.85	25 - 104	82.8 ± 22.2	32- 165	
		WM	23.11 ± 2.18	16.0 - 41.0	69.6 ± 5.26	53 - 83	***
	Nodes	GM	46 ± 7.79	16 - 78	69 ± 19.63	20 - 140	
		WM	16.7 ± 1.75	11.0 - 30.0	59.4 ± 3.66	48 - 68	***
	Total length (µm)	GM	590.64 ± 77.55	294.5 - 979.4	974.4 ± 196.92	531.5 - 1697.1	
		WM	436.05 ± 42.43	290.3 - 761.7	850.32 ± 43.47	719.1 - 990.2	*
	Volume (μm^3)	GM	274.14 ± 31.20	105.97 - 407.44	402.07 ± 71.89	247.39 - 598.49	
		WM	196.75 ± 21.44	124.86 -193.15	278.36 ± 20.41	226.36 - 338.98	
	Surface (μm^3)	GM	1337.47 ± 157.62	623.12 - 2101.74	2140.05 ± 390.31	1297.83 - 3446.54	
		WM	1011.94 ± 98.62	653.94 - 1721.43	1651.40 ± 42.58	1454.15 - 1987.64	

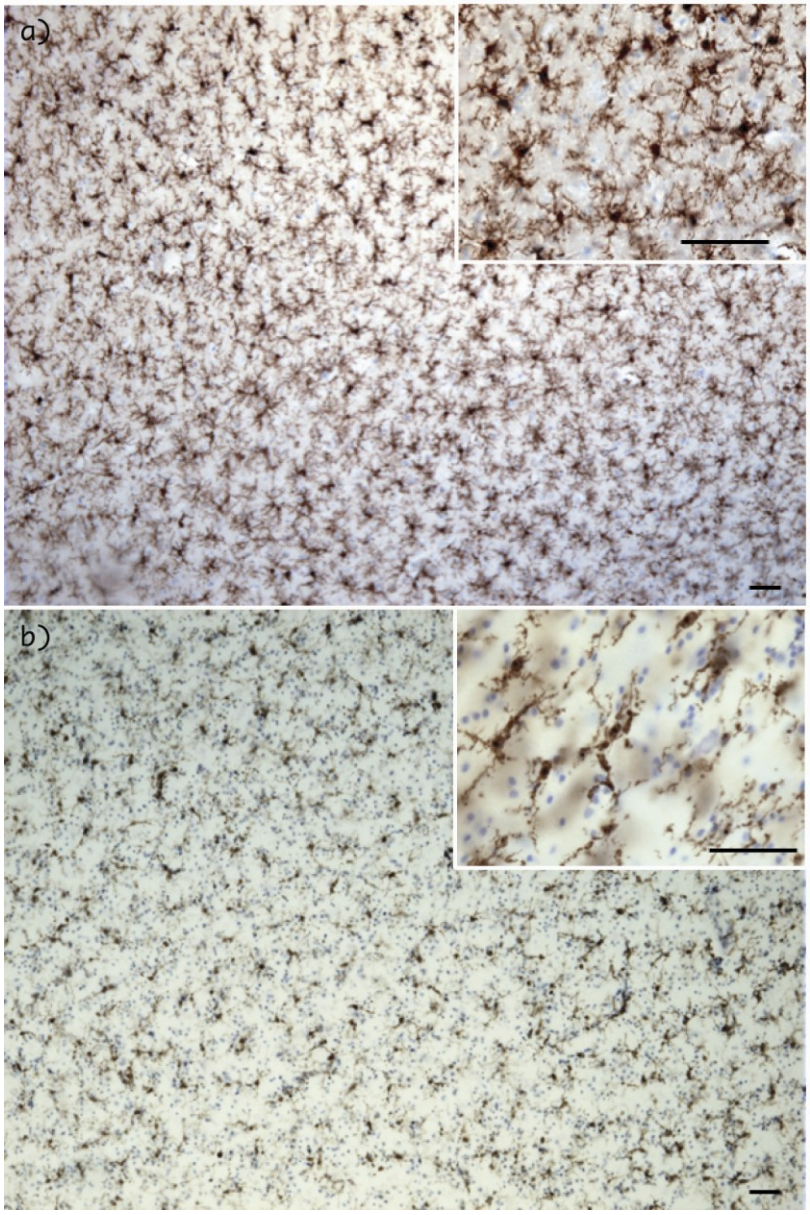

Figure 1. Distribution of IBA1-IR cells in the gray and white matter human dACC counterstained with cresyl violet. Human microglial cells in the dACC appear evenly distributed across the cortical layer in the gray matter (a) and aligned to myelinated tracts in the white matter (b). Scale bars: 50 µm.

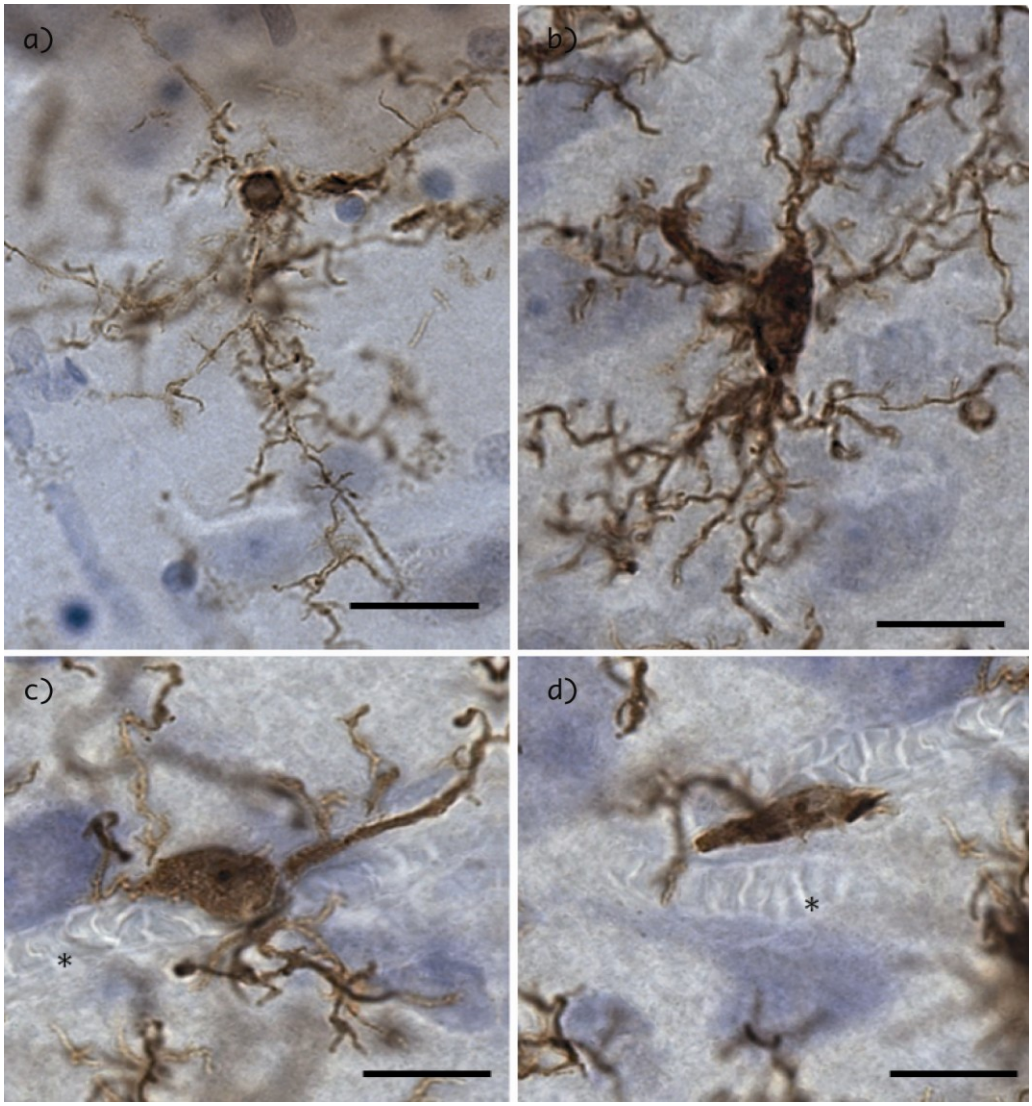

Figure 2. Four main phenotypes represent the population of resident IBA1-IR cells in the human dACC gray matter. Ramified microglia (a) display a small circular cell body with highly ramified processes. Primed microglia (b) present a bigger and less round cell body with similar ramification patterns when compared to the ramified phenotype. Reactive microglia (c) display an amoeboid cell body but still present a few ramified processes compared to amoeboid microglia (d), which can present, at most, two unramified processes or be completely devoid of them. These cells are occasionally observed to be associated with blood vessels (asterisks). Scale bar: 10 µm.

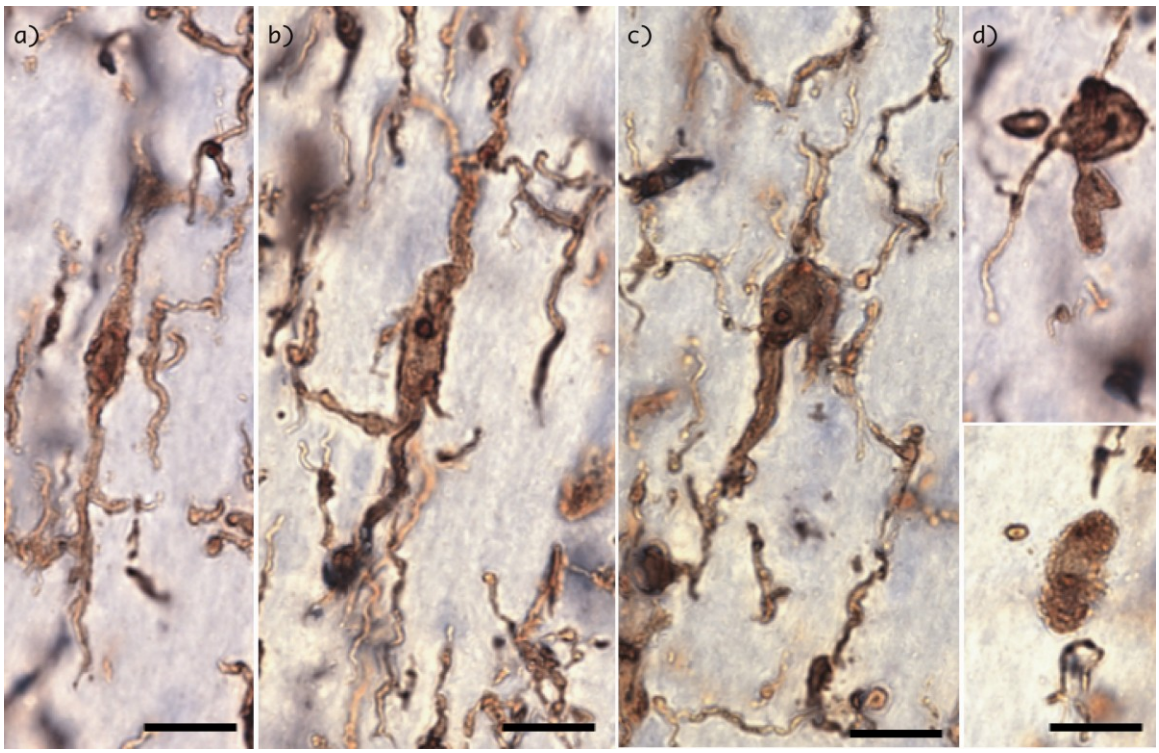

Figure 3. Four main phenotypes represent the population of resident IBA1-IR cells in the human dACC white matter. Ramified microglial cell body and highly ramified processes appear aligned to white matter tracts (a). Primed microglia display a wider cell body in the primed phenotype (b) compared to the ramified phenotype, but their processes and cell body retain a similar alignment. Reactive microglia present an amoeboid-shaped rounder cell body with a few ramified processes (c), whereas amoeboid microglia display a characteristic amoeboid-shaped cell body extending one or two unramified processes (top panel) or be completely devoid of processes (bottom panel) (d). Scale bar: 10 µm.

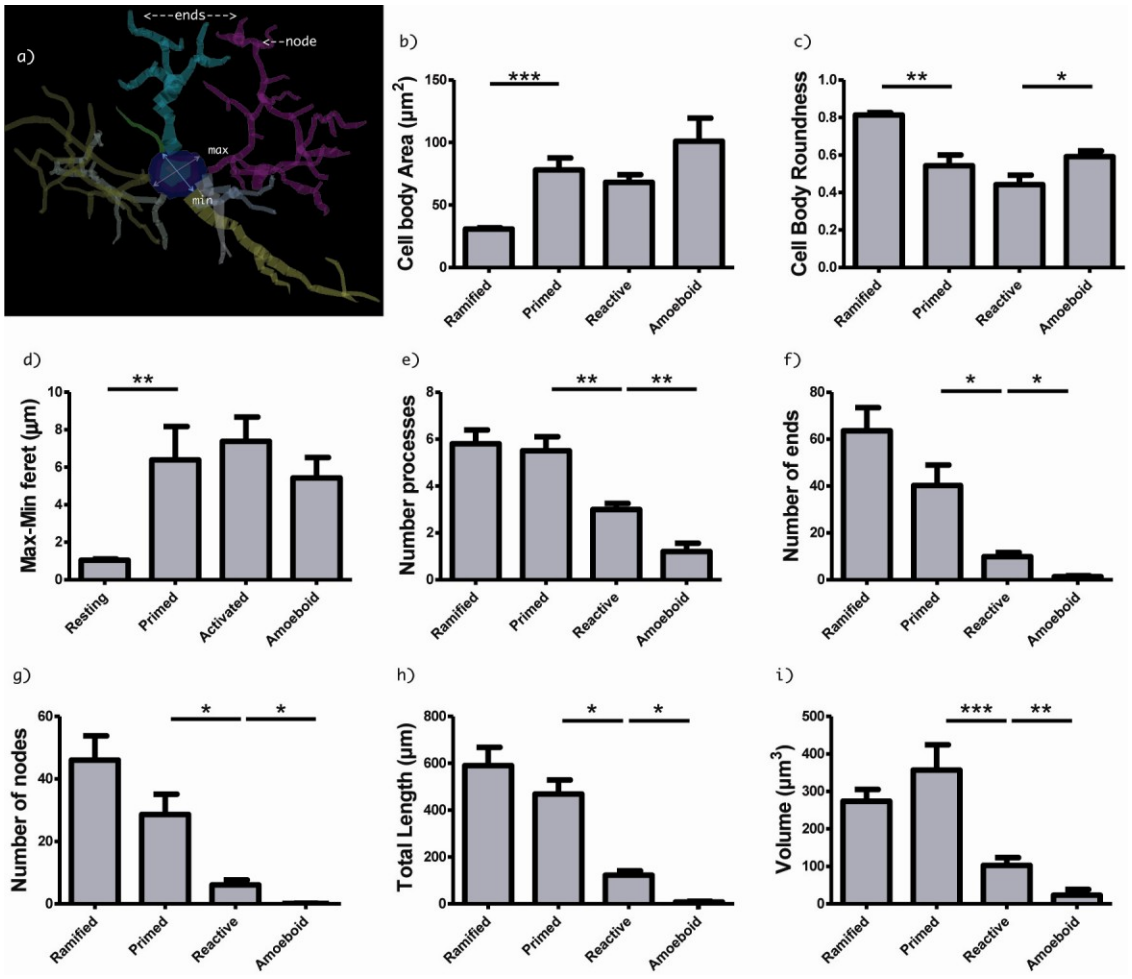

Figure 4. Microglial phenotypes in the human dACC gray matter are characterized by significant changes in the cell body and processes. Primed microglia display a cell body of greater area (a) and of decreased roundness (b), as reflected by a significant increase max-min feret (c) compared to the ramified phenotype. The cell body morphology of reactive microglia appears statistically similar in area (a), roundness (b) and max-min feret (c) as primed microglia, but presents a decrease in roundness (b) when compared with amoeboid microglia. A reconstruction of a primed microglia shows the shortest (blue arrow; min) and longest chord (violet arrow, max) representing the maximum and minimum feret, respectively, and the ramification patterns represented by the ends and nodes of the processes (d). Ramified and primed microglia project similar numbers of primary (e) and higher order processes (f-g). Reactive microglia display fewer first order (and overall) branches (e-g), as well as shorter total process length (h) and volume (i).

Amoeboid microglia have a significant decrease of primary and higher order branches (f-g), as well as a significant decrease in total process length (h) and volume (i) compared to reactive microglia *p< 0.01, **p< 0.001, ***p< 0.0001.

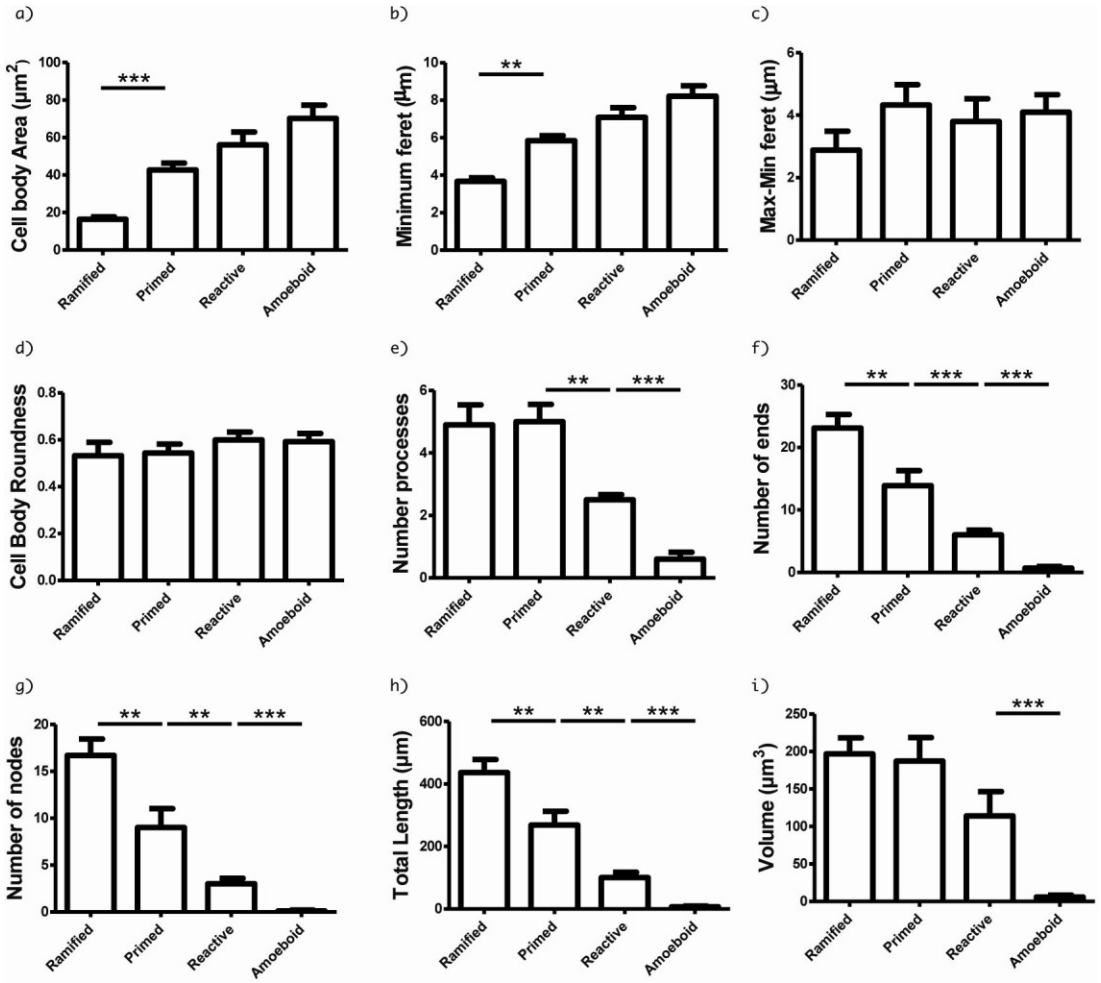

Figure 5. Microglial phenotypes display significant differences in cell body and ramification patterns. Compared to ramified, the primed phenotype displays a larger cell body area (a) as reflected by a significantly larger maximum (not shown) and minimum feret (b). Despite this significant increase in area, the max-min feret (c) and the roundness (d) of the cell body did not statistically differ between these or any of the other phenotypes. The cell body morphology of reactive, primed and amoeboid microglia appears comparable at all points (a-d). Ramified and primed microglia both extend a similarly numbers of primary processes (e), however, primed microglia hold fewer ends (f) and nodes, (g) as well as significantly shorter processes (h) with no significant changes on volume (i) compared to ramified microglia. Reactive microglia extend a significant decrease of primary (e) and higher order branches (f-g), as well as shorter processes than primed microglia (h) and amoeboid microglia present almost an

absence of overall processes (e-g) that were thus of significantly reduced length (h) and volume (i) compared to reactive microglia *p< 0.01, **p< 0.001, ***p< 0.0001.

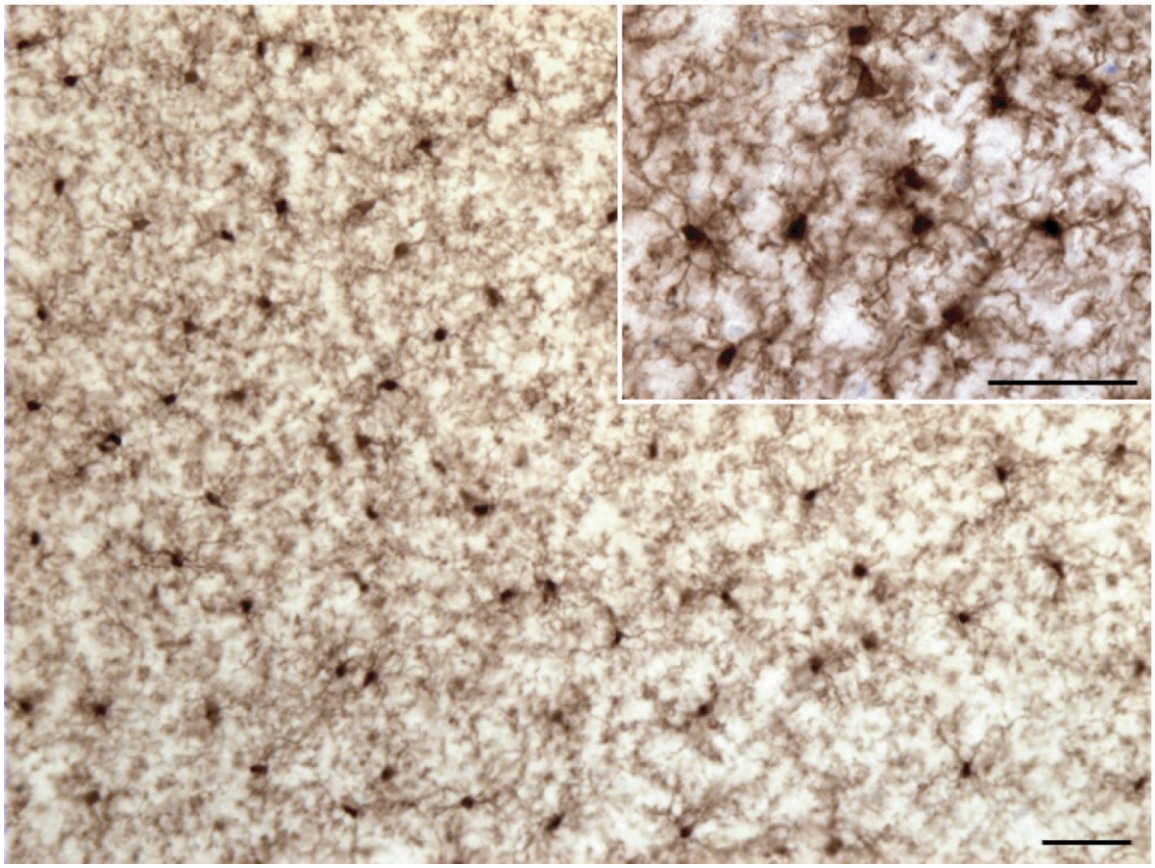

Figure 6. IBA1-IR cells in the cingulate cortex of a young adult mouse (1.5 month-old) display cell bodies of heterogeneous shapes and sizes, and highly ramified processes with overlapping domains. Scale bars: 50 µm.

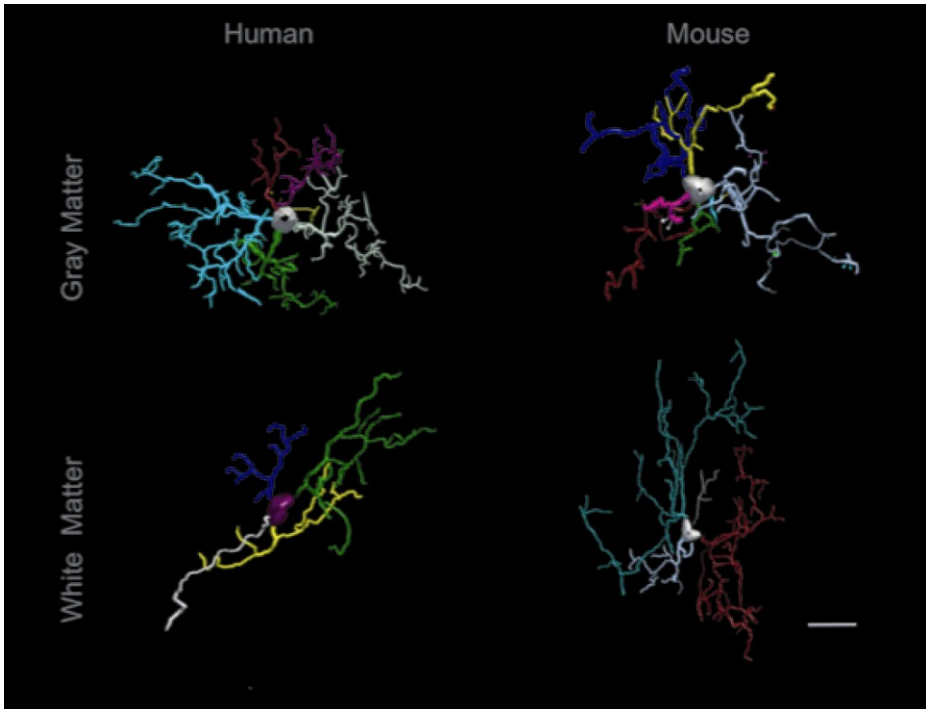

Figure 7. Representative reconstructions of gray matter (top row) and white matter (bottom row) ramified microglia in human dACC (left column) and mouse cingulate cortex (right column). Scale bar: 10 µm

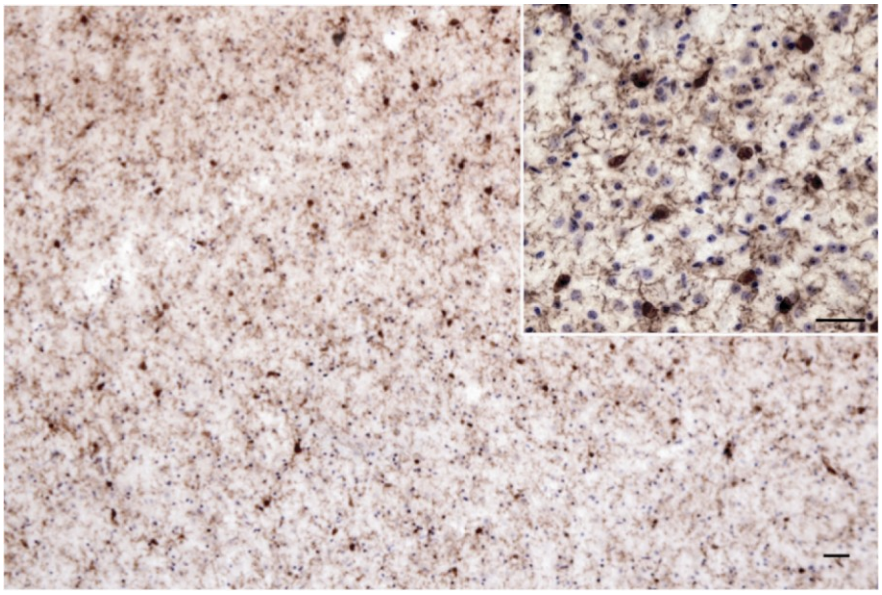

Figure 8. IBA1-IR cells in the non-perfused cingulate cortex of a of young adult mouse (3 month-old) following a PMI of 43 hours (11 hours at room temperature and 32 hours at 4˚C). The great majority of IBA1-IR cells observed remained ramified, and did not display noticeable signs of degradation. Sections were counterstained with cresyl violet. Scale bar: 25 µm.

CHAPTER 4.0: PREFACE

In our previous study, we conducted a detailed morphometric characterization of microglial phenotypes and assessed the distributions of each phenotype in gray and white matter or subjects devoid of any inflammatory, neurological or psychiatric condition (Torres-Platas et al., 2014a). Microglial morphological phenotypes reflect the brain inflammatory activity since microglial cells undergo a graded morphologically change ranging from a very ramified phenotype to an ameboid morphology that allows the rapid locomotion that allows migration to the site of lesion or infection through chemotactic signals (Kettenmann et al., 2013). These morphological changes are accompanied by changes in different molecules and receptors that confer particular physiological properties to microglial cells (for detailed review see chapter 1.2.4).

Our findings of hypertrophic astrocytes in the ACC white matter of depressed suicides (Chapter 2.0) led us to hypothesize that there is a chronic mild inflammatory state in the ACC of depressed suicides. Therefore, we aimed at assessing whether the distributions of microglial phenotypes were altered in the ACC white matter of depressed suicides. In the following study, we performed a stereological investigation of IBA1 immunostained cells in the white matter of depressed suicides compared them to matched non-psychiatric controls.

Depression has been associated with chronically elevated levels of circulating pro-inflammatory cytokines, which can impact brain physiology and stimulate microglia through direct and indirect pathways (Bay-Richter et al., 2011, Wohleb et al., 2012, Aguliar-Valles et al., 2013). We hypothesized that astrocytic hypertrophy and putative microglial activation may be caused or accompanied by a local increase of pro-inflammatory cytokines. In the following study, we measured the local expression of key pro-inflammatory and anti-inflammatory cytokines in ACC white matter samples from

depressed suicides and matched controls, and we assess the expression levels of molecules associated to microglial and astrocytic activation.

We explore the possibility that depressed suicides, which are more highly exposed to peripheral cytokines, may have abnormalities with the numbers of perivascular macrophages, a possible disruption of the integrity of the BBB. Additionally, we quantified, the degree of association of this population of IBA1+ cells to the blood vessels and the levels of the main chemokine MCP-1.

EVIDENCE FOR INCREASED MICROGLIAL PRIMING AND MACROPHAGE RECRUITMENT IN THE DORSAL ANTERIOR CINGULATE WHITE MATTER OF DEPRESSED SUICIDES

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4.1. ABSTRACT

Background: Despite increasing evidence supporting the neuroinflammatory theory of depression, little is known about cerebral macrophages in individuals suffering from major depression. In the present study, we investigated the morphology and distribution of cells immunostained for the macrophage-specific marker ionized calcium binding adaptor molecule 1 (IBA1) in the dorsal anterior cingulate cortex (dACC) white matter of middle-aged depressed suicides and matched non-psychiatric controls. This region is known for its implication in mood disorders, and its white matter compartment was previously found to display hypertrophic astrocytes in depressed suicides. **Methods:** Distributions of IBA1-immunoreactive (IBA-IR) microglial phenotypes were assessed using stereology and cell morphometry, and blood vessels were characterized as being intimately associated with either a high or a low density of IBA1-IR amoeboid-like cells. **Results:** Total densities of IBA1-IR microglia did not differ between depressed suicides and controls. However, a finer analysis examining relative proportions of microglial phenotypes revealed that the ratio of primed over ramified ("resting") microglia was significantly increased in depressed suicides. Strikingly, the proportion of blood vessels surrounded by a high density of macrophages was more than twice higher in depressed suicides than in controls (87% vs 42%, respectively), and this difference

was strongly significant. Consistent with these observations, gene expression of IBA1 and MCP-1, a chemokine involved in the recruitment of circulating monocytes, was significantly upregulated in depressed suicides. Furthermore, mRNA for CD45, a marker enriched in perivascular macrophages, was also significantly increased in samples from depressed suicides. An increase compared to controls was also observed in the proportion of blood vessels surrounded by a high density of CD45-IR cells, but this difference did not reach significance. These histological and molecular data suggest the recruitment of monocytes in dACC white matter of depressed suicides, although it cannot be excluded that other types of macrophages (including microglia) account for the observed accumulation of macrophages closely associated with blood vessels. **Conclusions:** Altogether, these findings suggest that the previously reported depression- and suicide-associated increases in circulating pro-inflammatory cytokines may be associated with low-grade cerebral neuroinflammation involving the recruitment of circulating monocytes. **Key words**: Depression, Suicide, Microglia, Perivascular Macrophages, Anterior Cingulate Cortex, Neuroinflammation, Human.

4.2 INTRODUCTION

Major depressive disorder (MDD) affects approximately 350 million people worldwide and is ranked as one of the main causes of disability (World Health Organization [WHO], 2012). This severe condition, characterized by depressed mood and/or loss of interest, too often results in suicide completion. Suicide ranks among the top ten causes of death for individuals of all ages and is the leading cause of death in most developed countries for subjects younger than 35 years (WHO, 2006). Psychological autopsy studies indicate that at least 50% of all adult suicides have had a previous diagnosis of depression (Kim et al., 2003). Furthermore, up to 15% of individuals with a lifetime diagnosis of MDD admit having attempted suicide at some point in their lives (Chen and Dilsaver, 1996). This serious societal problem has given rise to an increasingly multidisciplinary research field aimed at understanding the biological

causes underlying depression and suicide. The neuroinflammatory theory of depression stands as one of the main hypotheses having emerged from this research (Dantzer et al., 2008, Miller et al., 2009a). It has at its roots a number of independent clinical studies showing that the expression of some peripheral inflammatory markers is increased in depressed patients (Dowlati et al., 2010). In fact, increased expression of circulating pro-inflammatory cytokines has even been proposed as a biomarker of depression (Maes et al., 2009, Lichtblau et al., 2013). Further supporting this hypothesis, the peripheral administration of pro-inflammatory cytokines such as IFN-α for the treatment of hepatitis C leads to depressive symptoms in about half of the patients (Malaguarnera et al., 1998, Capuron and Miller, 2004). This treatment was also reported to activate the dorsal anterior cingulate cortex (dACC) (Capuron et al., 2005), a region that has been shown to display reduced volume and activity in magnetic resonance imaging studies of MDD patients (Ebert and Ebmeier, 1996, Chana et al., 2003) and associated with behavioral response to peripheral inflammation (Miller et al., 2013). These data strongly suggest that circulating pro-inflammatory cytokines may affect mood states through a functional alteration of limbic brain circuits. Further indication that immune activation may have a significant influence on mood comes from the increased incidence of depression in patients suffering from chronic inflammatory illnesses such as coronary artery disease (Frasure-Smith and Lesperance, 2006). Animal studies have also supported of this hypothesis, namely by providing strong evidence of a positive correlation between increased circulating pro-inflammatory cytokines and depressive-like behaviors (Merali et al., 2003, Goshen et al., 2008). Despite these converging lines of evidence, less than a handful of studies have examined the expression of cerebral cytokines in individuals having suffered from depression (Tonelli et al., 2008, Dean, 2011, Pandey et al., 2012).

At the cellular echelon, central immune responses are mainly modulated by microglia, but also by astrocytes, which generally play inflammatory and anti-inflammatory roles, respectively (McNally et al., 2008). Perivascular macrophages, which

share many features with microglia, are also implicated by mediating the brain's physiological responses to circulating pro-inflammatory cytokines (Serrats et al., 2010). In a murine model of social stress, Wohleb and colleagues recently described that social defeat is accompanied by the priming of monocytes and the recruitment of peripheral macrophages into cerebral perivascular space. This trafficking of myeloid cells in the brain coincides with the activation of resident microglia, the production of pro-inflammatory cytokines and the appearance of anxiety-like behaviours (Wohleb *et al*, 2012, Wohleb *et al*, 2013). There currently exists a knowledge gap with regards to these cells in regions implicated in depression and suicide. In fact, little is known about macrophages in the human brain (Guillemin and Brew, 2004), let alone in mood disorders. Interestingly, Steiner and colleagues have reported immunohistological evidence of increased cerebral gray matter HLA-DR-immunoreactive microglial densities in suicide victims, irrespective of psychiatric diagnosis (Steiner et al., 2008). However, the distribution and morphological phenotypes of white matter macrophages remains to be investigated, particularly in the dACC, a region that has been repeatedly implicated in mood disorders and in the behavioral response to inflammation (Capuron et al., 2005, Miller et al., 2013, Haroon et al., 2014c). Furthermore, white matter astrocytes in the dACC of depressed suicides display a hypertrophic phenotype suggestive of mild astrogliosis (Torres-Platas et al., 2011). The aim of the present study was to examine the distribution and morphology of macrophages in well-characterized dACC white matter samples from depressed suicides and matched psychiatrically healthy controls.

4.3 MATERIALS AND METHODS

Brain Samples

This study was approved by the Douglas Hospital Research Ethics Board, and informed consent from next-of-kin was obtained for each subject. Postmortem brain samples from depressed suicides and matched sudden-death controls were provided by

the Suicide section of the Douglas-Bell Canada Brain Bank. All psychiatric subjects committed suicide in the context of a major depressive episode, and controls died suddenly without psychiatric, neurological or inflammatory illnesses (Table 1). Seven controls died from sudden cardiac arrest, and one of the depressed suicides overdosed from a cocktail of drugs that included diphenhydramine, an anti-histaminergic compound. Fresh-frozen samples were obtained for messenger RNA (mRNA) experiments (16 cases and 14 controls) and fixed samples for the anatomical experiments (14 cases and 8 controls). For each individual, the cause of death was ascertained by the Quebec Coroner's office, and psychological autopsies were performed by proxy-based interviews, as described previously (Dumais et al., 2005). In brief, a trained interviewer conducted the *Structured Clinical Interview for DSM-IV* Psychiatric Disorders (SCID-I) with one or more informants of the deceased, after which a blind panel of clinicians reviewed SCID-I assessments, case reports, coroner's notes and medical records to obtain consensus psychiatric diagnosis. Groups were matched according to age, tissue pH, refrigeration delay, and postmortem interval. All tissue samples were dissected from the dACC adjacent to the dorsal part of the genu of the corpus callosum (Brodmann area 24 [BA24]) (Vogt et al., 1995, Gittins and Harrison, 2004), as described previously (Hercher et al., 2009b).

IBA1 & CD45 Immunohistochemistry

Fixed samples were processed as previously described (Torres-Platas et al., 2014a). In brief, tissue blocks were cut into 50 µm-thick serial coronal sections and every 12th section was processed for immunohistochemistry (IHC). Antigen retrieval was performed with proteinase K (20 µg/ml) for ionized calcium-binding adapter molecule (IBA1)-IHC and citrate buffer for cluster of differentiation (CD) 45 IHC, followed by an incubation in 3% H_2O_2. Sections were then pre-incubated respectively in 2% normal goat and horse serum for 24 hours before being transferred for 48 hours in the same solution containing polyclonal rabbit anti-IBA1 (1:1000; WAKO Chemicals USA, Inc., Richmond,

VA, USA) or overnight with monoclonal mouse anti-CD45 Leucocyte Common Antigen (1:100; Clones 2B11 & PD7/26; Dako Canada Inc. Burlington, ON, Canada). This was followed by 1h incubation in biotinylated goat anti-rabbit and anti-mouse antibody, respectively (1:1000; Vector Laboratories Inc., Burlington, ON, Canada). Labeling was revealed with a diaminobenzidine kit (Vector Laboratories Inc., Burlington, ON, Canada) and samples were counter-stained with cresyl violet. All slides were coded and analyzed by an experimenter blind to diagnosis.

Quantitative Assessment of Microglial Phenotypes

In a recent study, we performed a morphometric characterization of microglial cells in human dACC (Torres-Platas et al., 2014a), and generated quantitative parameters allowing us to accurately identify four main phenotypes, based strictly on morphology: ramified, primed, reactive and amoeboid (**Fig. 1**). IBA1-immunoreactive (IBA1-IR) microglial phenotypes were assessed as followed: Ramified microglia were characterized by a cell body ranging between 2.5- 4.9 µm in length in its shortest axis (minimum feret) and highly ramified processes displaying at least 16 ends. Primed microglia had a cell body ranging between 5 -7 µm in length in its shortest axis, and extended at least 2 ramified processes with a minimum of 7 ends. Reactive microglia displayed an amoeboid-like cell body with a diameter between 5-21 µm, at most 2 ramified processes longer than the cell body, and at least 3 ends. Finally, amoeboid microglia presented an amoeboid-like cell body with 6-27 µm in diameter and between 0-2 unramified processes that were shorter than the cell body's maximum feret, and were not closely associated with blood vessels. Densities of these phenotypes were quantified using an unbiased semi-stereological approach using an optical fractionator probe allowing for 3-D quantification with a light microscope (Olympus BX51) connected to a stereology workstation (Stereo Investigator; MBF Bioscience). This method estimates the total number of cells in a unit of tissue volume with an optical probe providing counts through the z-axis. The sampling process was performed by adding a 3137 µm x 2651 µm sampling grid over immunostained white matter immediately

adjacent to gray matter. Within this grid, the software randomly placed a counting frame (150 µm x 250 µm) within which cells were categorized and quantified using a 60x objective (NA 1.35). Consistent with the stereological methods of the dissector probe, only cells with a cell body that fell within the counting frame and that did not contact the exclusion lines when coming into focus within a 15 µm-thick optical dissector were considered. Top and bottom guard zones were set at 5 µm of the section thickness. To control for possible variations in tissue processing, the volume of each individual counting frame was calculated, with the area of the frame being multiplied by the thickness of each counting site. Densities were calculated for each of the four morphological phenotypes by dividing the total number of cells per phenotype by the calculated total sampling volume. Having first determined that the average density of ramified ("resting") microglia did not differ between groups (see Results below), each microglial phenotype was then expressed as the ratio of its density over that of ramified microglia.

Quantitative Assessment of IBA1-IR and CD45-IR Cells Surrounding Blood Vessels

In the same sections analyzed above for IBA1-immunoreactivity, as well as in sections immunostained for CD45, macrophages surrounding blood vessels were assessed in dACC white matter. Blood vessels were first imaged in bright field at a 10X magnification (N.A. 0.75) and classified as being surrounded either by a high or low density of IR cells at higher magnification, when necessary. In order to be considered, IR cell bodies had to contact directly the wall of a blood vessel. In each section, all blood vessels that could be observed were included for analysis, and the length of the shortest vessel corresponded to at least 10 macrophage cell body diameters. A high density of IR cells around blood vessels meant several cells that were on average separated by a space of approximately two or three amoeboid-like cell body diameters at most. Conversely, a low density was characterized by fewer IR cells that were on average separated by a space of approximately four or five amoeboid-like cell body diameters at least. Blood vessels were always surrounded by either a low- or a high-density of IR

cells. The proportion of total blood vessels that were surrounded by a high density of IR cells was determined for each subject.

Gene Expression

Total RNA was extracted from 50 mg of BA24 samples using the RNeasy Lipid Tissue Mini Kit (Qiagen Inc., Mississauga, Canada) with an additional DNase digestion following manufacturer's instructions. Total RNA content was quantified using a Nanodrop 1000 spectrophotometer (NanoDrop technologies, Rockland, DE, USA) and RNA integrity was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Samples with average RNA Integrity Numbers (RINs) below five were excluded. To quantify IBA1, CD68, CD45, monocyte chemoattractant protein-1 (MCP-1), Selectin-E, Catenin-cadherin-associated protein 102kDa alpha 1 (Catenin-α1), Zona Occludens-1 (ZO-1), interleukin (IL-) 1β, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts , cDNA was synthesized from 1 μg of total RNA using 400U M-MLV reverse transcriptase (Gibco BRL Life Technologies, Burlington, ON, Canada) and oligo-deoxythymidine (dT)-16 according to the instructions of M-MLV manufacturer. Given the low expression of cytokines IL-10, IL1-Ra and TNF-α in human brain, gene-specific reverse transcription was accomplished individually with the following oligonucleotides: CCCTGGTTTCTCTTCCTAAGAGTA for IL-10, GAATGCAGAGGCGACATGGAAGGGCT for IL-1RA and GAATCCCAGGTTTCGAAGTGGT for TNF-α. The following Taqman Gene Expression assays, labelled with the reporter dye FAM, were used: IBA1 (TaqMan assay id: Hs00610419_g1), CD68 (TaqMan assay id: Hs00154355_m1), CD45 (TaqMan assay id: Hs04189704_m1), MCP-1 (TaqMan assay id: Hs00234140_m1), Selectin E (TaqMan assay id: Hs00950401_m1), Catenin-α1 (TaqMan assay id: Hs00944794_m1), ZO-1 (TaqMan assay id: Hs01551861_m1), IL-1β (TaqMan assay id: Hs01555410_m1), TNF-α (TaqMan assay id: Hs00174128_m1), IL-1RA (TaqMan assay id: Hs00893626_m1), IL-10 (TaqMan assay id: Hs99999035_m1), and GAPDH (Life Technologies: 4310884E) were labeled with the fluorogenic reporter VIC-TAMRA. Real-

time PCR reactions were run in quadruplicate with 1µl of cDNA, 1 µl of 20X TaqMan Gene Expression Assay specific to each quantified gene, 10 µl TaqMan Mastermix (Applied Biosystems, Foster City, CA, USA) and water (Qsp 20µl). A standard curve was made of a pool of cDNA from all subjects. When cytokine expression was too low, a standard curve of cDNA from T-lymphocytes was created. All samples were analyzed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The thermal cycling conditions were set as follows: 50˚C for 2 min, 95°C for 10 min, 40 cycles each of 95˚C for 15 sec and 60˚C for 60 sec. The cycle threshold (CT) values for all replicates were pooled to obtain the mean value per subject. Samples with standard deviation of CT values above 0.3 were excluded from analysis to avoid excessive variability among replicates. Absolute quantification of expression of each gene was normalized by dividing it with the average of the housekeeping gene GAPDH, which was used as an endogenous control, and analyzed using SDS software version 2.4 (Applied Biosystems, Foster City, CA, USA).

Statistical Analyzes

Statistical analyses were performed using IBM SPSS Statistics 20 (Statistical Product and Service Solutions, Chicago, IL, USA). All measurements were expressed as mean ± standard error of the mean (SEM), and $p \leq 0.05$ was considered significant in all statistical tests. Normality was assessed using Shapiro-Wilk tests. Two-tailed U-tests were used for non-normally distributed data and t-tests otherwise. Welch correction was performed for significantly different variances between groups. Grubbs' tests were performed for all the expression data and significant outliers were removed. Multiple correlation analyses followed by analysis of covariance (ANCOVA) for significant results were used to examine the influence of potential confounders on measured variables.

4.4 RESULTS

Microglia

As described previously, four major microglial phenotypes were observed in IBA1-immunostained sections of human dACC white matter (Torres-Platas et al., 2014a). Total densities of IBA1-IR microglia (individual and combined phenotypes) did not differ between depressed suicides and controls. A finer analysis examining relative proportions of microglial phenotypes revealed that the ratio of primed over ramified microglia was significantly increased ($t_{(19)}$ *2.3 p=0.03*) in depressed suicides compared to controls (**Fig. 2a**). The ratios of reactive and amoeboid microglia over ramified microglia, however, remained statistically similar between groups (**Fig. 2b & 2c**).

Macrophages surrounding blood vessels

IBA1-immunostaining also revealed the presence of macrophages surrounding blood vessels in both groups (**Fig. 3**). Compared to controls (**Fig. 3a, b**), depressed suicides presented a readily noticeable increase in blood vessels that were associated with a high density of IBA1-IR cells (**Fig. 3c, d**). Quantification revealed that 42% and 87% of blood vessels in dACC white matter presented a high density of IBA1-IR cells in controls and depressed suicides, respectively. This increase was highly significant ($t_{(18)}$ *4.2 p=0.0005*; **Fig. 3e**).

CD45-IR cells were similarly found in low- (**Fig. 4a, b**) or high-density (**Fig. 4c, d**) around blood vessels. In addition, faintly CD45-IR cells that displayed the characteristic morphological features of microglia were observed throughout the tissue. In depressed suicides, 38% of the blood vessels in dACC white matter were associated with a high density of CD45-IR macrophages, compared to 30% in controls, but this increase was not significant (**Fig. 4e**).

Increased gene expression of microglial markers

IBA1 gene expression was significantly increased in depressed suicides compared to controls ($t_{welch\,(19)}$ *2.24 p=0.037*) (**Fig. 5a**). Similarly, CD45 mRNA, a marker enriched in perivascular macrophages, was significantly increased in depressed suicides ($U_{(30)}$ *70 p=0.031*). CD68 expression was also increased in depressed suicides, but this increase did not reach significance (**Fig. 5b**). The expression of ZO-1, catenin-α1, selectin-E, and MCP-1 was then measured to investigate whether factors involved in blood-brain barrier integrity and macrophage recruitment were differently regulated in dACC samples from cases. MCP-1 expression was significantly increased in depressed suicides compared to controls ($U_{(23)}$ *39 p=0.036*; **Fig. 6a**), whereas selectin-E expression was similar between groups (**Fig. 6b**). Before correcting for confounding factors (see below), ZO-1 (**Fig. 6c)** and catenin-1 (**Fig. 6d**) were both found to be significantly upregulated in depressed suicides compared to controls ($U_{(28)}$ *30 p=0.0004*, $U_{(28)}$ *53 p=0.0150*, respectively). Finally, the expression of cytokines IL-1β, IL-1Ra, TNF-α and IL-10 was found to be statistically similar between groups (**Fig. 7**).

Potential confounding factor

Multiple correlations were performed for all significant results to control for age, refrigeration delay, brain pH, medication use, alcohol dependence, substance abuse, antidepressants and smoking history as potential confounders. Spearman tests showed a significant positive correlation between IBA1 mRNA expression and age ($r_{(31)}$ *421 p=0.02*) as well as pH ($r_{(31)}$ *426 p=0.02*). However, analysis of covariance (ANCOVA) showed that the increased IBA1 mRNA in depressed suicides remained significant when controlled for both of these factors ($F_{(3,30)}$ *5.2 p<0.03*). Significant correlations were also found between pH and mRNA levels of ZO-1 ($r_{(31)}$ *0.484 p=0.005*) and catenin-α1 ($r_{(31)}$ *0.535 p=0.002*). After controlling for pH as a covariate, the results for ZO-1 remained significant ($F_{(2,30)}$ *4.04 p=0.05*), but not those for catenin-α1.

4.4 DISCUSSION

This study provides the first evidence of increased microglial priming in postmortem brain samples from individuals having suffered from MDD. Microglial cells can adopt different morphologies that are generally related to their functional status. Although intermediate stages are recognized, four main phenotypes have been described in rodents (Soltys et al., 2001, Stence et al., 2001, Kettenmann et al., 2011, Karperien et al., 2013) and in humans (Torres-Platas *et al*., 2014): ramified, primed, reactive, and amoeboid. Cells can rapidly and reversibly shift from one phenotype to another, but can also maintain the same morphological phenotype for extended periods (Karperien et al., 2013). In this study, we found evidence of microglial priming in the dACC white matter of depressed suicides. As discussed previously, it is highly unlikely that significant microglial morphological changes occurred post-mortem (Dibaj et al., 2010; Eyo and Dailey, 2012; Torres-Platas et al., 2014). We can thus speculate that the relative distribution of microglial phenotypes in postmortem brain tissues can inform on the local neuroimmune state at time of death, but that it can also reflect cumulative immune challenges during the lifetime of an individual. This is particularly relevant in the context of depression, which has been associated with chronically elevated levels of circulating pro-inflammatory cytokines, and hypothesized to be accompanied by chronic mild neuroinflammation in the brain (Miller et al., 2009a). Circulating cytokines can impact brain physiology and stimulate microglia through direct and indirect pathways, and the extent of cerebral reactions depends on the magnitude of the pro-inflammatory stimulation (Bay-Richter et al., 2011, Wohleb et al., 2012, Aguliar-Valles et al., 2013). An increase in microglial priming could thus reflect an increased immune "alertness" in dACC white matter of depressed suicides following chronic exposure to elevated levels of circulating pro-inflammatory cytokines. This would be consistent with our recent description of hypertrophic astrocytes in dACC white matter of depressed suicides (Torres-Platas et al., 2011), as this astrocytic phenotype is also suggestive of mild

neuroinflammation (Sofroniew and Vinters, 2010). It would also be consistent with accumulating evidence of altered microglial activity and metabolism in major depression and suicide (Steiner et al., 2008, Steiner et al., 2011, Erhardt et al., 2013). These investigations have further suggested that suicidal behavior may be associated with altered microglial activity and low-grade cerebral neuroinflammation, irrespective of psychiatric diagnosis. Although the present study is consistent with this interpretation, it was not possible to verify if this phenomenon is due solely to suicide since all case samples were from depressed suicides.

Further evidence of low-grade cerebral neuroinflammation in depression and suicide could potentially come from the demonstration that cerebral tissues presenting morphological signs of altered microglial activity also display altered cytokine expression. To investigate this possibility, we measured the local expression of key pro-inflammatory (IL-1β, TNF-α) and anti-inflammatory (IL-RA, IL-10) cytokines in dACC white matter samples from depressed suicides and matched controls, and found similar expression between groups for all these molecules. Only a few previous postmortem studies had investigated the cerebral (gray matter) expression of cytokines in depression and suicide (Tonelli et al., 2008, Dean, 2011, Pandey et al., 2012), with data generally suggesting increased cerebral expression of pro-inflammatory cytokines. The results of these investigations, however, are difficult to compare because of important differences in study design, namely with regards to the age of the subjects and brain regions analyzed. Our data do not preclude that the expression of other cytokines may have been altered in dACC white matter, or that the cytokines measured in these tissues were differentially expressed in other brain regions. However, the particular absence of group differences in the expression of IL-1β and TNF-α suggests that the increased microglial priming in the dACC white matter of depressed suicides observed here is indeed a mild phenomenon that is unaccompanied by major changes in the local cytokine expression, at least at the time of a depressive episode during which suicide was committed. Moreover, we cannot exclude that, compared to controls, brains from

depressed suicides are more highly exposed to peripheral cytokines crossing the blood-brain barrier (BBB). Although it is not specific to the BBB, the significant upregulation of ZO-1 in dACC of depressed suicides is consistent with such a view, and could result from a chronic exposure to elevated levels of circulating pro-inflammatory cytokines. Thus, the present data could also suggest that increased microglial priming constitutes part of the cerebral macrophage response to peripheral stimuli.

The increased IBA1 gene expression measured here in dACC white matter of depressed suicides could represent further evidence of increased microglial priming, as this protein is expressed more highly in microglial cells that are not in the ramified "resting" stage (Imai and Kohsaka, 2002). However, it seems more likely that this upregulation is associated with the important increase in proportion of blood vessels closely associated with high densities of IBA1-IR macrophages. In depressed suicides, this proportion more than doubled compared to matched controls. Interestingly, recent studies have revealed that repeated social stress in mice increases the recruitment of myeloid cells in the brain's perivascular space; a phenomenon that was further associated with microglial activation and anxiety-like behaviours (Wohleb et al, 2011, Wohleb et al, 2013). Elevated levels of IL-1□ and TNF-□ in the circulation, a consistent finding in studies of depressed patients (Dowlati et al., 2010), can increase the population of perivascular macrophages in the brain by stimulating the infiltration of circulating monocytes across the BBB (Audoy-Remus et al., 2008). Furthermore, Sawchenko and colleagues have provided elegant evidence that perivascular macrophages play a direct role in hypothalamic-pituitary-adrenal (HPA) axis activation following an immune challenge (Serrats et al., 2010). When directly stimulated by circulating IL-1□, perivascular macrophages release prostaglandin E2 (PGE2), which then stimulates medullary adrenergic and noradrenergic neurons that in turn excite the production of corticotropin-releasing factor by the hypothalamus (Serrats et al., 2010). In addition, these investigators demonstrated that eliminating perivascular macrophages abolishes HPA axis activation following an immune challenge.

The observations of increased IBA1 gene expression and of a greater proportion of blood vessels associated with high IBA1-IR cell densities are consistent with the notion of monocyte recruitment in dACC white matter of depressed suicides. This is further supported by evidence showing the significant upregulation of MCP-1, a chemokine involved in the recruitment of circulating monocytes, in depressed suicides compared to controls. Furthermore, CD45 mRNA, a marker enriched in perivascular macrophages, was also significantly increased in samples from depressed suicides. The proportion of blood vessels surrounded by high densities of CD45-IR cells was also increased in depressed suicides, but this did not reach statistical significance. The inability to detect a significant increase in CD45-IR cells surrounding blood vessels in depressed suicides may reflect a lower sensitivity of CD45-IHC compared to IBA1-IHC. However, we cannot exclude the possibility that other types of macrophages (including microglia), in addition to perivascular macrophages, account for the observed increase in IBA1-IR cells associated with blood vessels in case samples.

In conclusion, this study provides evidence of a higher proportion of microglial cells displaying a primed phenotype in dACC white matter of depressed suicides compared to matched controls, without any significant change in overall microglial densities between groups. Furthermore, blood vessels surrounded by a high-density of IBA1-IR macrophages were more than twice as frequent in depressed suicides as in matched controls. Consistent with these observations, IBA1, CD45 and MCP-1 gene expression in dACC white matter was significantly upregulated in depressed suicides. However, white matter expression of key pro- and anti-inflammatory cytokines (IL-1□, IL-Ra, TNF-□, IL-10) did not differ between groups. Altogether, these findings suggest that the previously reported depression- and suicide-associated increases in circulating pro-inflammatory cytokines may lead to low-grade neuroinflammation in dACC white matter. These and our previous findings on dACC astrocytes (Torres-Platas et al., 2011) may account for the altered white matter integrity evidenced by neuroimaging studies in depressed patients

(Bessette et al., 2013, Bracht et al., 2013), and further highlight the importance of considering white matter in the etiology and treatment of depression (Edgar and Sibille, 2012).

4.6 AKNOWLEDGEMENTS

We thank Quebec's coroner office as well as the next-of-kin of the deceased for their support. We also thank the expert staff of the Douglas-Bell Canada Brain Bank (Maâmar Bouchouka, Josée Prud'homme, Danielle Cécyre, Kirsten Humbert and Lucie Ratelle), Dr. Giamal Luheshi for helpful discussions, and Dr. Joseph Rochford for his assistance with the statistical analyses.

4.8 TITLES AND LEGENDS TO FIGURES

Table 1. Subject information

	Control (n=17) Mean ± SEM	**Depressed suicides (n=24)** Mean ± SEM
Age (years)	39 ± 4.53	46 ± 3.63
Gender	16M:1F	18M:6F
Tissue pH	6.57 ± 0.06	6.66 ± 0.04
Refrigeration Delay (hours)	11.51 ± 3.87	11.77 ± 1.79
Post-Mortem Interval	45.93 ± 4.5	42.72 ± 5.19
RIN	5.89 ± 0.18	6.17 ± 0.28
Cause of death	7 cardiovascular 6 road accident 1 accidental hanging 1 fall 2 intoxication	15 hanging 7 intoxication 1 jumping 1 drowning
Clinical information		
Unipolar depression	-	20
*Depression NOS**	-	4
Alcohol dependence	0	6
Smokers	4	6
Antidepressants	-	24 (6)**

* Depression not otherwise specified

** Presence of antidepressants was only detected by toxicological analysis in 6/24 subjects.

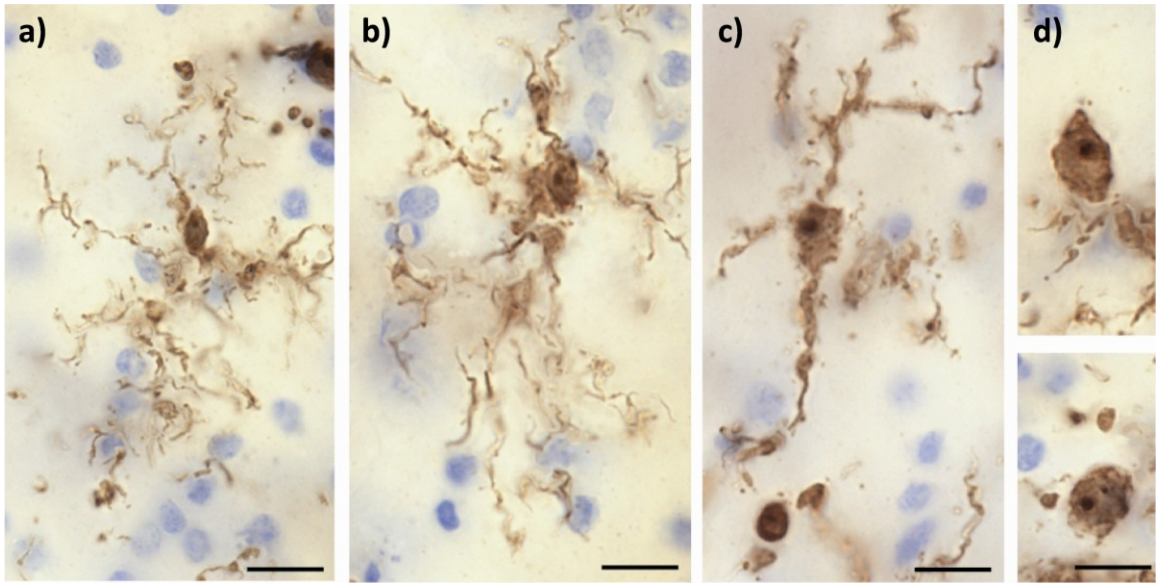

Figure 1. Four main IBA1-IR microglial phenotypes are observed in human dACC white matter. Ramified microglial cell body and highly ramified processes (a). Primed microglia display a wider cell body (b) compared to the ramified phenotype. Reactive microglia present an amoeboid-shaped rounder cell body with a few ramified processes (c), whereas amoeboid microglia display a characteristic amoeboid-shaped cell body extending one or two unramified processes (top panel) or are completely devoid of processes (bottom panel) (d). Scale bars: 10 µm.

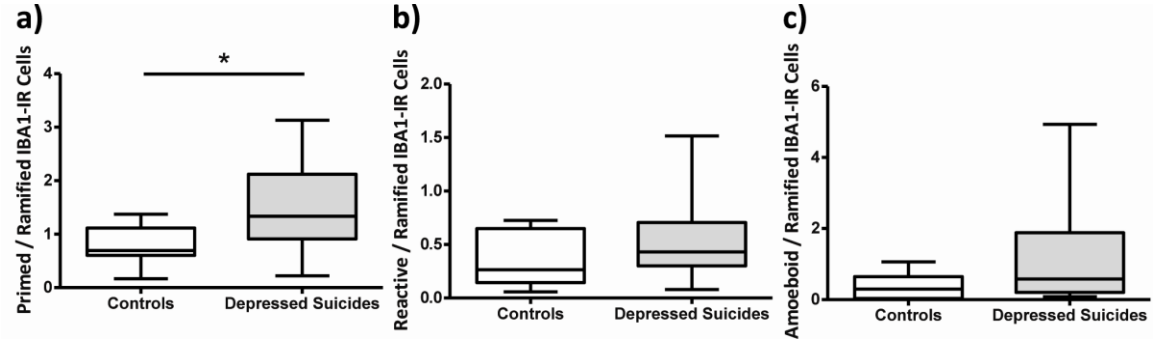

Figure 2. The ratio of primed over ramified microglia were significantly increased in depressed suicides compared to controls (a), whereas the ratios of reactive (b) and amoeboid microglia (c) remained similar between groups. (* p = 0.03)

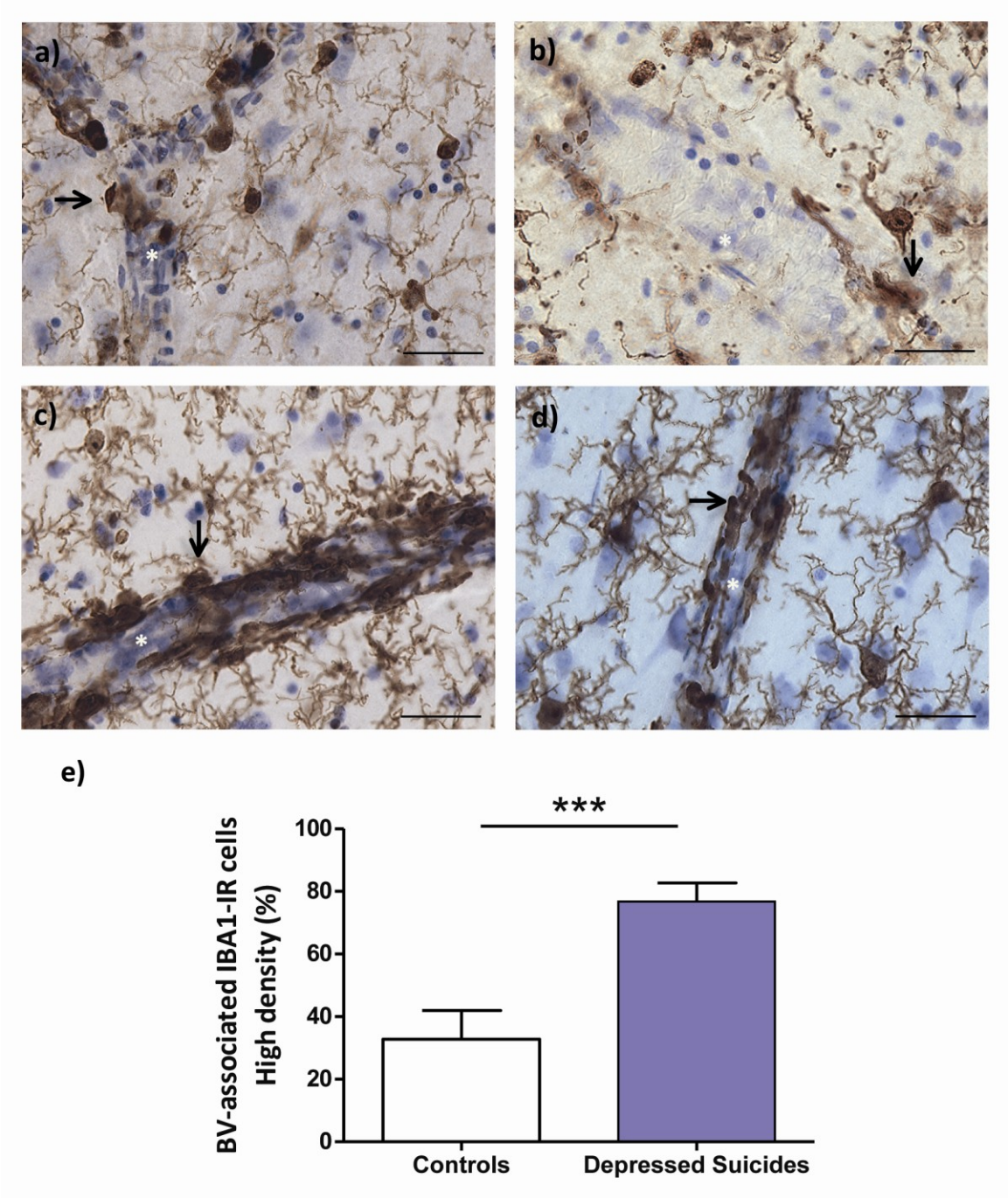

Figure 3. Representative micrographs of blood vessels (white asterisks) associated with low and high densities of IBA-IR macrophages in dACC white matter of controls (a, b) and depressed suicides (c, d). The percentage of blood vessels associated with a high density of IBA1-IR cells (black arrows) was significantly higher in depressed suicides than in controls (e). (*** $p = 0.0005$) Scale bars 25 μm.

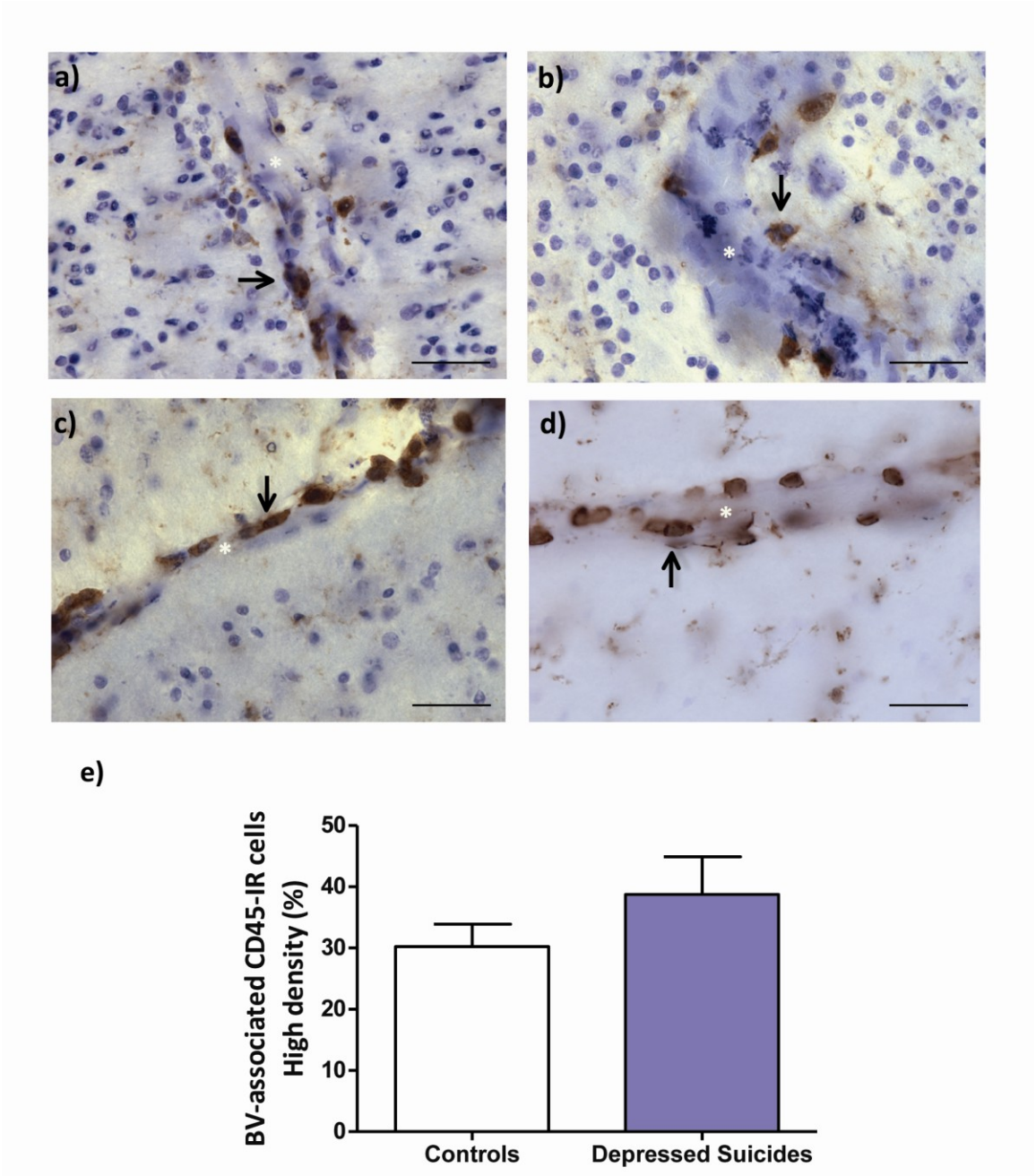

Figure 4. Representative micrographs of blood vessels (white asterisks) associated with high density (a, b) or low density (c, d) of CD45-IR macrophages in dACC white matter, irrespective of psychiatric diagnosis. The percentage of blood vessels associated with a high density of CD45-IR cells was non-significantly increased in depressed suicides (e). Scale bars 25 μm.

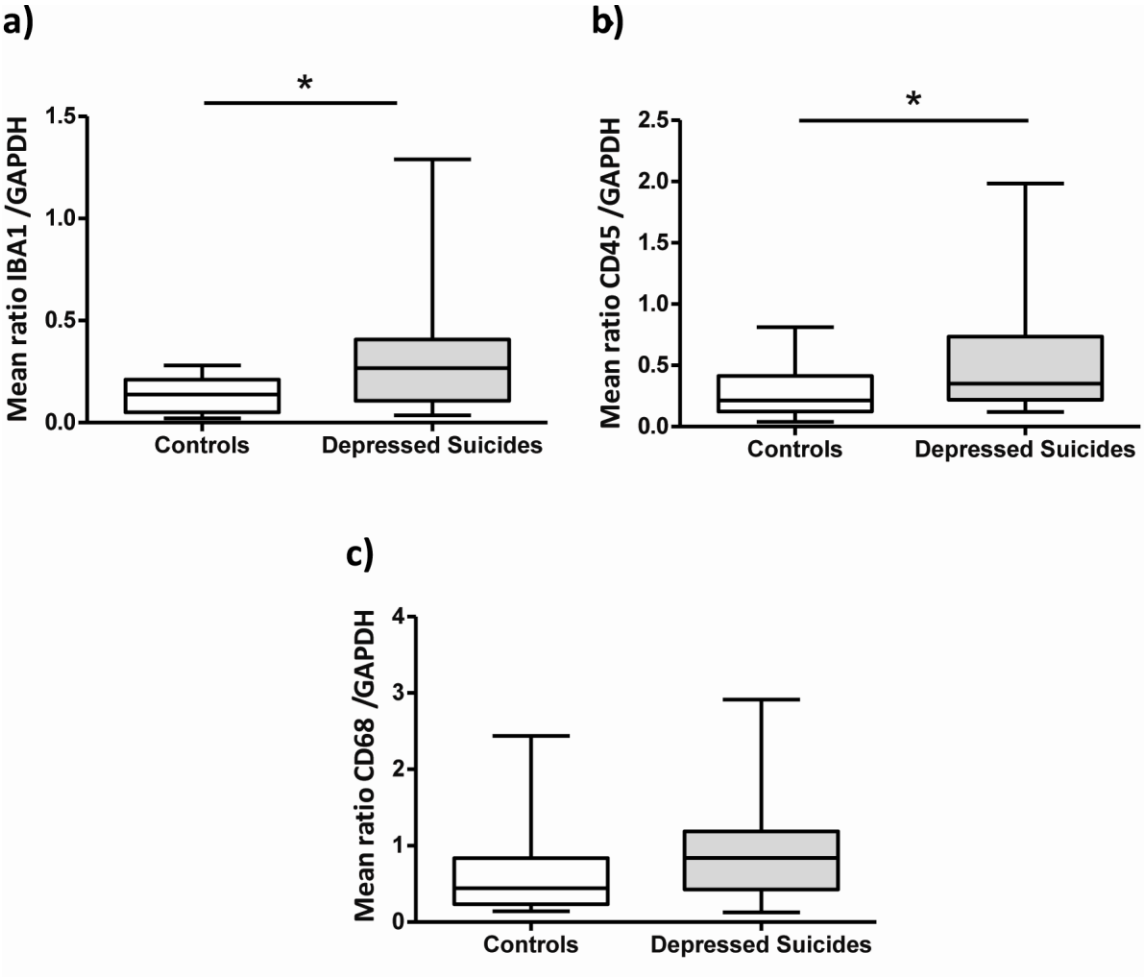

Figure 5. IBA1 (a) and CD45 (b) gene expression levels were significantly increased in depressed suicides compared to controls (* $p \leq 0.05$). CD68 expression was also increased in the same samples, but without reaching significance (c).

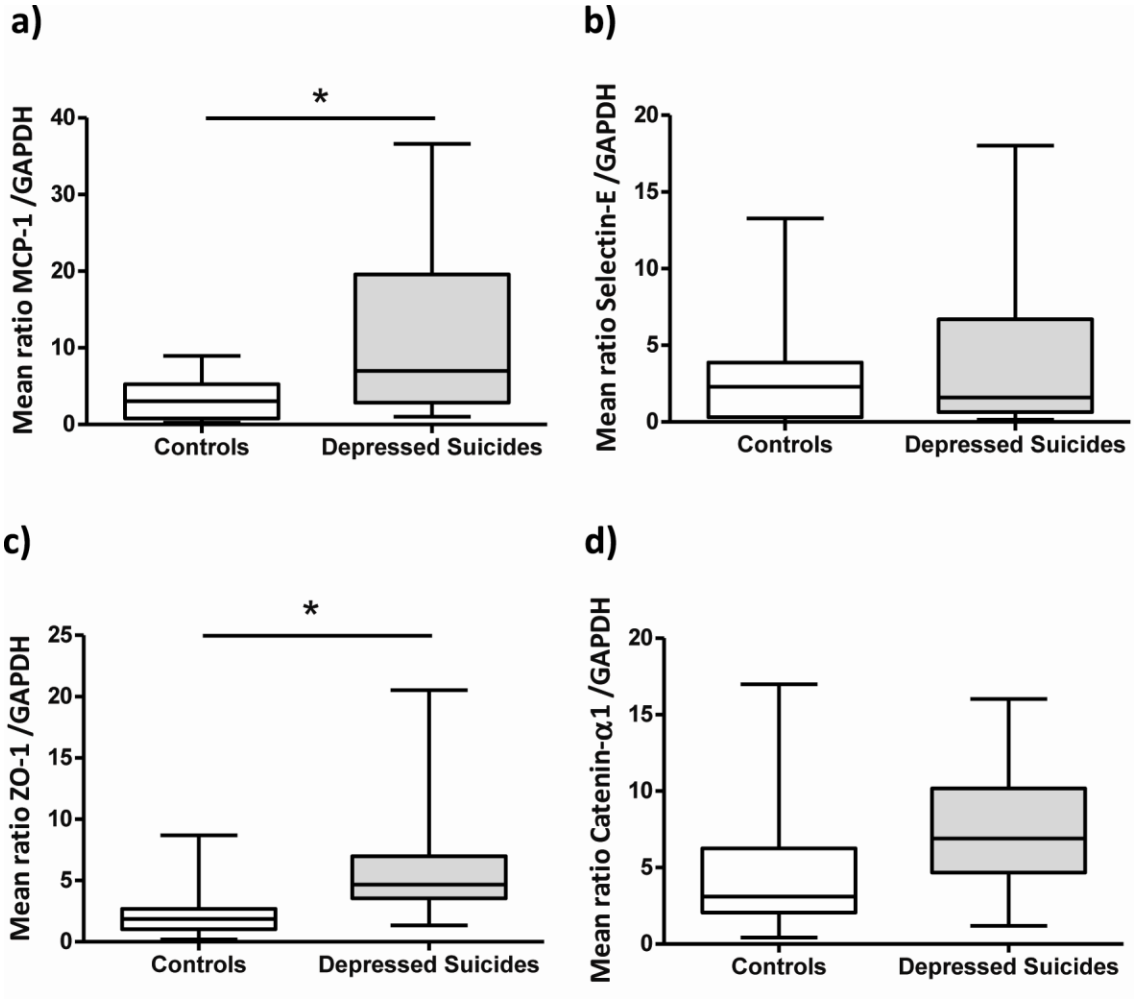

Figure 6. Expression of the monocyte-attracting chemokine MCP-1 was significantly increased in depressed suicides compared to controls (a) whereas expression of the adhesion molecule Selectin-E, also involved in the recruitment of monocytes, did not differ between groups (b). Zona occludens 1 (ZO-1) and catenin-1, proteins associated with blood-brain barrier permeability, were significantly upregulated (c) and unchanged (d), respectively, in depressed suicides compared to controls. (* $p \leq 0.05$)

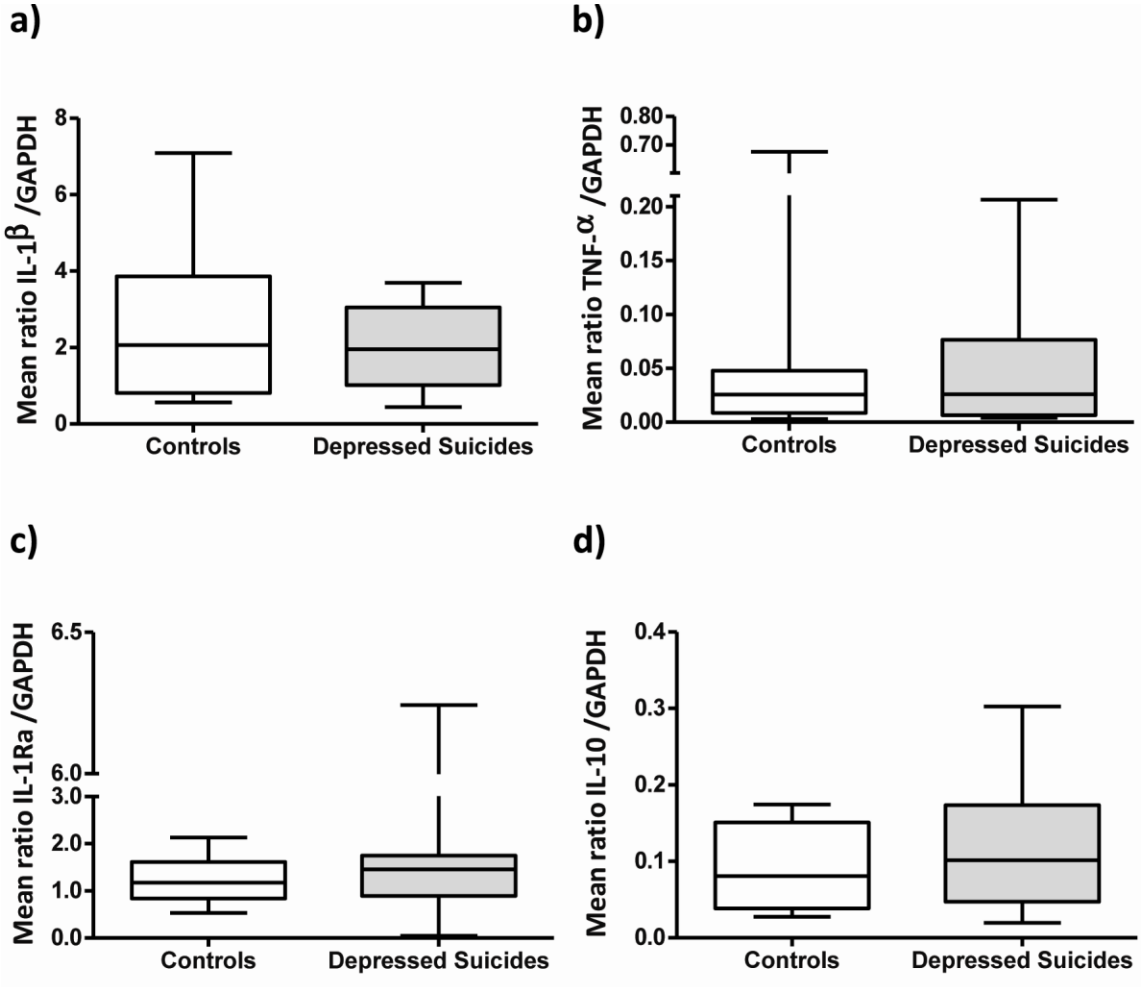

Figure 7. Gene expression of the pro-inflammatory cytokines IL-1β (a) and TNF-α (b) was statistically similar between groups. Similarly, the expression of anti-inflammatory cytokines IL-1Ra (c) and Il-10 (d) was comparable between controls and depressed suicides.

CHAPTER 5.0: GENERAL DISCUSSION

Before initiating the studies contained in this thesis, the literature covered limited information about neuroinflammation in depression and suicide. As detailed in chapter 2.0, evidence implicating glial cells in depression and suicide was restricted to studies using Nissl staining, which only provides information about cell subtypes (neurons vs glial cells). Specific evidence of astrocytic abnormalities in depression and suicide had been suggested by numerous independent studies showing decreased levels of GFAP (Miguel-Hidalgo et al., 2000, Webster et al., 2001, Davis et al., 2002, Fatemi et al., 2004, Si et al., 2004, Webster et al., 2005) and S100β (Hamidi et al., 2004) at the mRNA and protein levels in postmortem samples from patients suffering from depression. In 2008, evidence from a study using immunohistochemistry suggested that HLA-DR expressing microglia were dysregulated in depression and suicide, and that "microgliosis" was important to the underlying biology of these disorders (Steiner et al., 2008). However, detailed information concerning the neuroinflammatory environment in the brain of suicide completers, including evidence of astrocytic and microglial activation and the relative expression levels of pro- and anti-inflammatory cytokines, remained unknown. The work contained in this thesis aimed at exploring the inflammatory microenvironment in the brains of depressed suicide completers. Specifically, this work focused on the morphology and molecular activation of astrocytes and microglia, which are the primary components of the CNS immune system.

In this chapter, I will briefly review and summarize my findings, integrate them into the current body of literature, and discuss possible mechanisms relevant to the pathophysiology of depression and suicide. All of the studies contained within this thesis, were conducted in the white and/or grey matter of the dorsal anterior cingulate cortex (dACC; BA24) of depressed suicides and matched controls. This region was chosen given its implication in depression and other psychiatric disorders, and its

relevance to behavioral responses to peripheral inflammation (see chapter 1.5 for review).

5.1 OVERVIEW OF FINDINGS

The study described in Chapter 2.0 was designed to assess, through cell morphology, the possible involvement of astrocytes in depression and suicide. Our finding of hypertrophic astrocytes in the dACC white matter of depressed suicides compared to matched controls suggests that astrocytic function may be perturbed in these patients. This hypertrophy was absent in the protoplasmic astrocytes in the adjacent gray matter layer VI, which suggests that astrocytic remodelling occurs independently in these two cortical compartments, regardless of their proximity. Taken together, these results demonstrate the importance of white matter astrocytes in this psychiatric condition (Torres-Platas et al., 2011). Astrocyte hypertrophy is a hallmark of astrogliosis, a physiological response to immune challenge that is also accompanied by up-regulation of some genes and, in more severe cases, cell proliferation and scar formation (John et al., 2003, Sofroniew, 2005, 2009, Sofroniew and Vinters, 2010). Because we observed a hypertrophic phenotype in astrocytes that was not accompanied by proliferation nor scar formation, we hypothesized that this phenomenon reflects a chronic mild inflammatory state occurring in the dACC white matter (Torres-Platas et al., 2011).

Since astrocytic hypertrophy is often the consequence of an inflammatory stimulus and stands as one of the hallmarks of reactive gliosis (Wilhelmsson et al., 2004), we measured the expression of pro-inflammatory cytokines (IL-1β, TNF-α), anti-inflammatory cytokines (IL-10, IL-1Ra), cytokine receptors (IL-1R, TLR-2), astrocytic genes (GFAP, Cx30, Cx43) and microglial markers (IBA1, CD45) to investigate the inflammatory milieu in the dACC of depressed suicides. In Chapter 4.0, we presented quantitative PCR results showing no significant differences in the mRNA expression of

these genes between groups. Notably, expression of astrocytic genes were not significantly different between groups in neither the gray nor white matter. In contrast, we found that mRNA expression of the microglial markers IBA1 and CD68 was increased (CD68 results approached significance, while IBA1 showed a significant effect) specifically in the dACC white matter of depressed suicides when compared to healthy non-psychiatric controls (Torres-Platas et al., 2014b).

IBA1 is a calcium binding protein that is expressed in macrophages and microglial cells and is known to be upregulated during microglial activation, where it interacts with actin filaments (Ito et al., 1998). Therefore, IBA1 upregulation in our study suggests one of two things: (1) a significant increase in the total number of macrophages/microglial cells (proliferation) in the dACC white matter or (2) an activation of existing IBA1-expressing cells. To explore this further, we performed a stereological investigation to quantify the total number of microglial cells in the gray and white matter dACC of depressed suicides and controls. IBA1 is constitutively expressed throughout the cytoplasm of microglial cells and is an ideal marker to visualize whole cell morphology. Microglial activation includes a spectrum of morphological features, ranging from a very ramified phenotype to an amoeboid cell. In the literature, there were few reports describing these different microglial morphologies in rodents (Stence et al., 2001, Hung et al., 2010, Karperien et al., 2013) and just a few qualitative studies in humans (Sheng et al., 1997 reviewed in Graeber and Streit, 2010, Norden and Godbout, 2013). Therefore, due to a lack of information on the morphological properties of microglial cells in the human brain, I first conducted a morphometric study of IBA1-immunoreactive microglial cells in adult human and rodent gray and white matter dfrom individuals having died without inflammatory, neurological, or psychiatric illness (Torres-Platas et al., 2014a). As described in chapter 3.0, this quantitative morphometric analysis now allows to better classify the different microglial phenotypes (Torres-Platas et al., 2014a).

With this tool in hand, we performed stereological quantification of each of the microglial phenotypes in the gray and white matter dACC of depressed suicides and matched controls, and our results showed no significant differences of any microglial phenotypes between groups in the gray matter (unpublished data). However, we found that the primed phenotype was significantly enriched in the dACC white matter of depressed suicides compared to controls. The particular absence of group differences in the expression of IL-1β and TNF-α suggests and the increased microglial priming in the dACC white matter of depressed suicides observed here is part of a mild inflammatory phenomenon. This mild inflammation is unaccompanied by major changes in the local cytokine expression, at least at time of death. This study provides the first evidence of increased microglial priming in postmortem brain samples from individuals having suffered from MDD (Torres-Platas et al., 2014b).

When conducting these investigations, it became apparent that the walls of blood vessels in some samples were strongly enriched with surrounding IBA1-IR cells. We thus assessed the number of white matter blood vessels associated either with high or low density of IBA1-IR cells in all samples. Interestingly, our results showed that blood vessels surrounded by high density of IBA1-IR cells were significantly increased in depressed suicides (Torres-Platas et al., 2014b). This phenomenon could be the consequence of the (1) proliferation of perivascular macrophages, which also express IBA1, (2) Infiltration of monocytes from the blood to the brain through blood vessels walls, or (3) recruitment of microglial cells to the blood vessels. This represented a complex issue since there is currently no unique marker that can accurately differentiate between microglial cells, perivascular macrophages and infiltrating monocytes. However, it has been suggested that CD45 & CD163 are enriched in perivascular macrophages (Guillemin and Brew, 2004, Audoy-Remus et al., 2008). In order to further clarify the origin of these cells, the expression levels of CD45 was measured by real-time PCR. In parallel, white matter samples were immunostained for CD45, and blood vessels assessed in the same way as above. We measured a significant increase in mRNA levels

of CD45 in the white matter of depressed suicides but we did not find a statistical difference between the high or low density of CD45 cells associated to blood vessels (Torres-Platas et al., 2014b). This could mean that the resolution at the histological levels may not be sufficient to detect the significant increase in CD45 in cells expressing this marker. Furthermore, the population of perivascular macrophage progenitors expressing higher levels of CD45 residing in the luminal surface of cerebral capillaries expressing CD45 but not IBA1 (Audoy-Remus et al., 2008) can be a potential confounder. We cannot exclude the possibility that other types of macrophages (including microglia), in addition to perivascular macrophages, account for the observed increase in IBA1-IR cells associated with blood vessels in case samples. For purposes of this discussion we will refer to these IBA1 cells as perivascular macrophages given their location in the vasculature. However, a deeper analysis of the molecules expressed by these cells will allow us to better identify this population.

To further elucidate whether the origin of the IBA1 cells associated with blood vessels in high densities in depressed suicides was due to macrophage recruitment or infiltration from the periphery to the brain, we quantified the expression levels of MCP-1, one of the main chemokines involved in macrophage recruitment. We found a significant increase in MCP-1 expression in the dACC white matter of depressed suicides. Taken together, these results suggest a possible infiltration of peripheral monocytes. Infiltration of peripheral monocytes to the brain could be the result of a compromised BBB or a BBB breakdown. Therefore, we measured the expression of tight junctions and anchor proteins that join the endothelial cells together. We found a surprising upregulation of zona occludens 1, which is a protein that confers rigidity to the tight junctions. No significant differences were found in levels of catenin-α1 and selectin-E (Torres-Platas et al., 2014b).

5.2 POSSIBLE IMPLICATIONS

Several meta- analyses have been conducted to evaluate the literature showing evidence of increased levels of pro-inflammatory cytokines in patients with depression and/or suicides. The most recent ones taking suicide into account independently of psychiatric diagnosis showed that suicidal patients present significantly higher levels of IL-1β and IL-6 (Black and Miller, 2014), higher levels of TGF- β (Deborah et al., 2015) and lower levels of IL-4 and IL-2 (Black and Miller, 2014, Deborah et al., 2015) in blood. Moreover, CSF levels of IL-8 were significantly decreased in suicidal patients compared to control subjects (Black and Miller, 2014). When suicide was not taken into account, meta-analyses have shown that depressed patients present increased levels of the soluble IL-2 Receptor (sIL-2R), TNF-α and IL-6 (Dowlati et al., 2010, Liu et al., 2012). Therefore, taken together, these studies provide robust evidence suggesting that circulating pro-inflammatory cytokines are increased in the blood of depressed suicides. More specifically, increased levels of IL-6 and TNF-α are both a common denominator in blood of both cohorts of subjects with depression and suicide independently of psychiatric diagnosis.

Research showing evidence of increased levels of pro-inflammatory cytokines in the brain is scarce, inconclusive and has generally concerned gray matter only. In postmortem brain samples of teenage suicides, Pandey and colleagues found that the mRNA and protein expression of IL-1β, IL-6, and TNF-α was significantly increased in the prefrontal cortex (BA10) compared to controls (Pandey et al., 2012). Another study also analyzing the frontal cortex but in a different area (BA46), showed that the levels of trans-membrane TNF (tmTNF) were significantly increased in subjects with MDD. However, this study failed to find similar differences in BA24, and expression of the soluble form of TNF was not significantly different compared to controls in any of the areas analyzed (Dean et al., 2010). When separating subjects according to gender, another group studying the orbitofrontal cortex (BA11) found that the levels of IL-4 were significantly elevated in female suicides and IL-13 in male suicides when compared to controls (Dean et al., 2010). In our hands, mRNA levels of TNF-α in BA24 of middle-

aged subjects were not significantly different between depressed suicides and controls, in agreement with the study of Dean and colleagues (Dean et al., 2010). We did not measure group differences in cytokine expression neither. Altogether, these results suggest that, in depression and suicide, cytokines are differentially regulated and that the influence of increased circulating IL-6 and TNF-α levels may exert differential regional influences in the brain. Our data do not preclude that the expression of other cytokines may have been altered in the dACC white matter, or that the cytokines measured in these tissues were differentially expressed in other brain regions. Cytokines can be transported from the periphery to the brain across the BBB through transport systems and they can exert their effects through multiple pathways, or directly via the circumventricular organs. These molecules can thus influence, directly or indirectly, brain regions such as the ACC. Given the consistent finding of increased levels of pro-inflammatory cytokines in the blood of depressed and suicidal patients, it is likely that the subjects in our studies were more exposed to peripheral pro-inflammatory cytokines at the time of death and in every depressive episode when compared to controls. It is well known that pro-inflammatory cytokines can cause changes in mood and behavior (see below; reviewed in Dantzer and Kelley, 2007).

Several lines of evidence have shown that peripheral cytokines modulate mood by altering the activity in the ACC, which suggests a common pathophysiological basis for major depressive disorder and sickness-associated mood change (Harrison et al., 2009a). Peripheral increase of inflammatory markers naturally caused by infection or elicited experimentally by typhoid vaccination causes an inflammatory response characterized by increased circulating IL-6 and causes significant mood decrease in 3 hours post injection (Harrison et al., 2009a). This inflammation-associated mood decrease correlated with enhanced activity within subgenual ACC and its reduced connectivity to amygdala, medial prefrontal cortex, nucleus accumbens, and superior temporal sulcus (Harrison et al., 2009a). Moreover, it is suggested that personality traits and impulsivity phenotypes underlying suicidal behavior tend to be associated with

higher plasma IL-6 levels and influence the choice of a violent suicide attempt (Isung et al., 2014). IL-6 has been shown to significantly correlate with extraversion, impulsivity and monotony avoidance (Isung et al., 2014). Moreover, a vast amount of studies supports the evidence that suggests that pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6, induce behavioural symptoms referred to as the "sicknes behaviour", which is characterized by cognitive dysfunction, fatigue, anorexia, psychomotor slowing, sleep disturbances, and increased sensitivity to pain (Dantzer et al., 1999, Capuron et al., 2005). This sickness behaviour associated to animals or humans that suffer from a microbial infection can also be induced by the peripheral administration of pro-inflammatory cytokines. Administration of interferon (IFN)-α, a cytokine that activates a pro-inflammatory response, to treat infectious diseases, cancer or hepatitis C, causes neuropsychiatric symptoms such as alterations in mood, cognition, neurovegetative function (Capuron et al., 2002) and slow psychomotor activity and reaction time (Capuron et al., 2001). MRI studies have shown that treatment with IFN-α, causes a significant activation of the dACC that correlated with the number of task related errors (Capuron et al., 2005). These studies suggest that pro-inflammatory cytokines, namely IL-6, IFN –α, TNF –α and IL-1 cause behavioural changes, mood lowering and neural activation in the ACC (Harrison et al., 2009f, Engels et al., 2010, Haroon et al., 2014a).

In a different study assessing inflammatory conditions in the CSF, it was shown that suicide attempt was associated with increased levels of quinolinic acid, which is one of the main components of the kynurenine pathway and a product of inflammatory processes. These increased levels of quinolinic acid were associated with higher levels of IL-6 at the time of the suicide attempt and normalized 6 months after in a follow-up study (Erhardt et al., 2013). These results suggest that IL-6, which is systemically increased in suicide attempters and depressed subjects, is a good candidate to link the peripheral inflammation with changes in behaviour through the modulation of the ACC and may elicit the choice of a more violent suicide attempt. These systemic pro-inflammatory-induced changes in mood may result from changes in neural activity

through the production of quinolinic acid, an NMDA receptor agonist produced by microglial cells. Increased levels of quinolinic acid in the CSF of depressed suicides are likely to result from a depletion of the tryptophan available to produce serotonin. Moreover, studies have showed treatment with IFN-α for hepatitis C causes an increase in glutamate (normalized with creatine) in the ACC and the basal ganglia and depressive symptoms (Haroon et al., 2014a). Not surprisingly, novel antidepressant drugs such as ketamine work through normalizing glutamatergic neurotransmission and thus cause a rapid and clinically significant reduction in suicidal ideation and significantly ameliorates depressive episodes (Reinstatler and Youssef, 2015).

In addition to the behavioural changes that can be induced by systemic pro-inflammatory cytokine, increasing evidence suggests that peripheral inflammation causes activation of microglial cells and recruitment of monocytes in the brain. Animal models of peripheral inflammation such as hepatic failure cause increased levels of systemic TNF-α, activation of microglial cells and increased cerebral levels of MCP-1. These events are followed by increased levels of circulating monocytes expressing CCR2 and recruitment of monocytes into the brain parenchyma (D'Mello et al., 2009). Signalling of TNF-α to the brain was shown to be dependent on the TNF receptor 1 (TNFR1), which is necessary for the recruitment of monocytes (D'Mello et al., 2009) as this was abolished in the TNFR1 knockout mice. The recruitment of monocytes was correlated with the appearance of depressive-like behaviors (D'Mello et al., 2009) and abolished in inflamed mice that lacked MCP-1 or CCR2. These findings suggest a novel periphery-to-brain communication pathway following peripheral organ inflammation, and that the appearance of depressive-like behaviors is related to cerebral monocyte recruitment.

Using stress paradigms in mice, it was shown that repeated social defeat produced “priming” of CD11b+ peripheral myeloid cells (glucocorticoid insensitive) (Stark et al., 2001) and induced a hyper-inflammatory phenotype (higher production of

IL-1B and TNF-α when activated) (Bailey et al., 2009). This stress paradigm also caused a significant activation of microglial cells, significantly increased levels of IL-1β, and increased expression of CD14, CD86, and TLR4 in the microglial membranes. Moreover, this stress paradigm increased recruitment of macrophages to the brain and their presence within the perivascular space and parenchyma of the prefrontal cortex, amygdala, and hippocampus. Anxiety-like behaviours are dependent on the elevated numbers of circulating monocytes and brain macrophages. Repeated social defeat also reduced the levels of glucocorticoid responsive genes such as glucocorticoid-induced leucine zipper and FK506 binding protein-51 (Wohleb et al., 2011). Taken together, these studies suggest that these stress paradigms are sufficient to cause increased levels of pro-inflammatory cytokines such as TNF-α and IL-1 β and causes monocyte priming, microglial activation, increased MCP-1 expression and release, recruitment of monocytes in the brain parenchyma and reduced glucocorticoid responsiveness; all of which are consistent with our results and hypothesis of mild chronic inflammation in depressed suicides.

What is the mechanism whereby the brain senses peripheral inflammation to engage the HPA axis? Evidence indicates that non-neuronal cells in the cerebral vasculature, namely perivascular macrophages, can monitor cytokines and trigger the activation of the HPA axis. This HPA axis activation is triggered through the release of molecules such as prostaglandin E2 that act directly on brain stem catecholaminergic neurons that project to corticotropin-releasing factor expressing hypothalamic neurosecretory cells (Schiltz and Sawchenko, 2003). Monitoring circulating cytokines and modulating the HPA axis activation is suggested to be a result of a two-way interaction between perivascular macrophages and endothelial cells (Schiltz and Sawchenko, 2003). The perivascular macrophages are a subset of marrow-derived brain macrophages that are located between the endothelial cell basement membrane and the glia limitans in the perivascular space (Thomas, 1999; Williams et al., 2001). These perivascular macrophages have been suggested to play an important role in the

transduction of inflammatory signals from the periphery to the brain and thus, transduce the signal to engage the HAP axis (Schiltz and Sawchenko, 2002). A model of this transduction was proposed by Schiltz and colleagues, where increased of systemic levels of pro-inflammatory cytokines such as IL-1, are sensed by endothelial cells through the endothelial type 1 IL-1 receptors. The position of the endothelial cells is strategic for engaging the HPA axis, although their threshold to produce cyclooxygenase (COX-2) is very high. Nevertheless, perivascular macrophages are more sensitive to COX-2 induction (Serrats et al., 2010). Therefore, signalling through endothelial cells is necessary for upstream effects of the CNS but since they don´t express detectable levels of PGE2, they have to signal through perivascular macrophages. In this study, Serrats and colleagues showed that in face of a mild immune challenge such as injection of IL-1, only cells labeled with CD163, a marker thought to be exxpressed solely in perivascular macrophages, there is a significant increase on COX-2 in the cerebral vasculature and meninges (Serrats et al., 2010). After the elimination of PVM and after the challenge with IL-1, COX-2 was only detected in some neurons in cortical regions that express this enzyme constitutively. When PVM were selectively depleted and rats were then challenged with LPS, the following characteristics were observed compared to rats with non depleted PVM: (1) a massively increased 2.4-fold COX-2 response only in endothelial cells, (2) the levels of PGE2 were detected in endothelial cells and was also expressed in brain parenchyma, in microglial cells, (3) caused an increase in activated catecholamine-containing (majority adrenergic and noradrenergic) neurons in the caudal brainstem (nucleus of the solitary tract and ventrolateral medulla), (4) provoked a robust 31% increase in the number of labeled parvocellular cells in the paraventricular nucleus of the hypothalamus and (5) a greater increase in the CRF mRNA (2.2 fold) compared to the non PVM depleted (1.6 fold). Together, these findings suggest that perivascular macrophages exert a potent restrain on endothelial cell activity, and are required for the control of the HPA circuitry, which are sensitive to perivascular macrophage depletion at medullary and hypothalamic levels. This is positively correlated with vascular COX-2/PGE2 production. This extends to the exaggerated HPA-

regulatory responses seen in the LPS injection. Thus, these cells are important for the modulation and the transduction of signals from the periphery to the brain, since depletion of these cells resulted in an abrogation of normal HPA activity.

Consistent with these findings, we reported increased IBA1 gene expression and microglial priming and the proportion of blood vessels associated with high IBA1-IR cell densities were more than twice as frequent in the ACC white matter depressed suicides as in matched controls. This is consistent with the monocyte recruitment in the perivascular space of animal under an inflammatory condition or stress. This is further supported by evidence showing the significant upregulation of MCP-1, which is involved in the recruitment of circulating monocytes, in depressed suicides compared to controls and in these same animal models of stress and systemic inflammation, both conditions that are present in subjects with depression and commit suicide. Furthermore, CD45 mRNA, a marker enriched in perivascular macrophages, was also significantly increased in samples from depressed suicides. It is likely that the increased population of IBA1 cells associated with blood vessels belong to a mature population of perivascular macrophages (Audoy-Remus et al., 2008). These cells are likely to restrain the effect of the putative inflammatory environment in the blood of depressed suicides, and is tempting to speculate that these cells display an anti-inflammatory M2 phenotype and prevent an exacerbated immune response in the brain.

Our results were accompanied by astrocytic hypertrophy in the white matter of depressed suicides without a significant increase in GFAP at the time of death, which suggests a different temporal scale between these two events and, therefore, supports our hypothesis of a chronic effect. The differences between gray and white matter may have to do with an increased facility of cytokines to diffuse within the brain along white matter tracts (Konsman et al., 2000). Interestingly, white matter hyperintensities (WMHs) (Debette and Markus, 2010), which are thought to represent regions of acute astrocyte activation (Simpson et al., 2007) or astrogliosis (Fazekas et al., 1993) confer a

predisposition to developing MDD (de Groot et al., 2000, Bae et al., 2006, Iosifescu et al., 2007, Li et al., 2007) and are strongly associated with suicide (Grangeon et al., 2010). WMHs, have been proposed to arise from inflammation and oxidative stress (Xu et al., Wright et al., 2009 reviewed in Rosenberg, 2009) both of which are well documented to be increased in depression (Maes, 2008, Miller et al., 2009a). Additionally, we did not find a significant increase of local cytokines, which suggests a local mild inflammatory state, which is consistent with our hypothesis of chronic mild inflammation in the dACC white matter of depressed suicides.

Evidence of increased perivascular macrophages have been reported in the PFC, hypothalamus and hippocampus in animal exposed to chronic stress (Wohleb et al., 2013). Here, we provided evidence for increased numbers of IBA1 positive cells in dACC white matter of depressed suicides compared to matched controls. Interestingly, the dACC has been implicated in mediating the behavioral responses to inflammation (Conn, 2008). The immune and the stress system share common functional pathways and are closely related (see Chapter 1.1.5). Chronic stress, an important risk factor for depression and other neuropsychiatric disorders (Hall et al., 2015), can profoundly affect the function of immune cells such as T cells, which become glucocorticoid insensitive (Miller et al., 2008, O'Donovan et al., 2011). It can also be speculated that glial cells in the brain are also affected by chronic stress and increased peripheral inflammation, in particular through the stimulation of perivascular macrophages. This may in turn lead to the production of local pro-inflammatory cytokines which, chronically may skew the profile of the immune cells towards an inflammatory phenotype and significantly decrease tryptophan sources available for serotonin synthesis (McNally et al., 2008, Dantzer et al., 2011). An inflammatory phenotype implicating activated microglia would result in increased levels of quinolinic acid, an NMDA agonist that produces synaptosomal release of glutamate and inhibits glutamate reuptake in astrocytes (Tavares et al., 2002). Changes in the extracellular levels of glutamate and serotonin may alter the communication between brain regions and

within specific circuits that are implicated in mood regulation (Arango et al., 2002). This hypothesis, supported by an increasing number of independent studies, is consistent with the effectiveness, in a significant proportion of patients, of antidepressants aimed at increasing serotonergic tone or decreasing glutamatergic transmission. It is also consistent with the apparent anti-inflammatory effect of antidepressants (Hashioka et al., 2009), and with recent clinical trials indicating that combining classical antidepressants with anti-inflammatory compounds is a more efficacious treatment than antidepressants alone (Davis et al., 2010, Kohler et al., 2014). Altogether, these findings strengthen the notion that the immune system can be a major player in the biology of depression and suicide.

In conclusion, the previously reported depression- and suicide-associated increases in circulating pro-inflammatory cytokines may lead to low-grade neuroinflammation in dACC white matter. These findings may account for the altered white matter integrity evidenced by neuroimaging studies in depressed patients (Bessette et al., 2013; Brachtet al., 2013), and further highlight the importance of considering white matter in the etiology and treatment of depression (Edgar and Sibille, 2012).

5.3 LIMITATIONS

When interpreting results of this study, several limitations need to be kept in mind. Since all our investigations were performed in samples from depressed suicides, we cannot establish whether our observations are due to depression, suicide, or to a combination both. Future work could clarify this issue by including samples from non-depressed suicides and depressed non-suicides.

Since we are working with postmortem tissue, we are capturing a snapshot brain conditions at the time of death. The interpretations of our results are speculations of

possible mechanisms that are parallel to findings in the literature in animal models and, therefore, it is impossible to assume causality of any of the processes.

Multiple correlation analyses followed by ANCOVAs were performed to evaluate the influence of potential confounders such as alcohol dependence, smoking and medication. Thus, we cannot exclude the possibility that other factors during the lifetime of each individual can be influencing our results.

5.4 FUTURE DIRECTIONS

The results of the projects contained in this thesis formulate several hypotheses that need to be tested further. For instance, the fact that hypertrophic astrocytes were found in the white matter of depressed suicides and no differences were found in adjacent layer VI protoplasmic astrocytes (Torres-Platas et al., 2011), suggests that these two astrocytic subtypes display very different functions, react differently to injury (Sun et al., 2010), and display different sensitivity to ischemia (Shannon et al., 2007). Information of this kind is missing in human brain samples for obvious methodological reasons. Given that astrocytes in humans are overall more complex, they display 10-fold more GFAP primary processes, are 2.6-fold larger in diameter and propagate calcium waves four-fold faster than in rodents (Oberheim et al., 2009), results from these studies are unlikely to be comparable to those in humans. Astrocytes in humans are likely to play more important roles in the normal brain function and in psychiatric conditions. Therefore, studies assessing the functional differences between different astrocytic subtypes in human samples need to be investigated in the near future.

Studies characterizing the inflammatory phenotype of each of the cells studied in this thesis (i.e. perivascular macrophages, microglial cells, infiltrating monocytes, astrocytes) are crucial to unravel the inflammatory milieu in this psychiatric condition. In my view, this is the next fundamental step to understand what is the immune condition in the brain and thus, be able to target specific cells or pathways for possible

antidepressant treatments. More specifically, the activation phenotype of each of the inflammatory cells can be tested to determine whether they are M1 or M2 so we can have a better idea of the role they play in the dACC white matter of depressed suicides. This hypothesis can be tested by measuring the different membrane receptors that confer different functions and identities to these cells. Additionally, animal studies of chronic stress and measuring depressive like behaviours would be helpful to determine if any of the macrophages studied here may develop an anti-inflammatory phenotype in the brain parenchyma or in the perivascular space.

Along these lines, future studies should determine the origin of each cell population around blood vessels and whether they belong to blood monocytes, microglial cells, progenitors of perivascular macrophages or mature perivascular macrophages. Having at least 3 markers would help us to quasi differentiate the identity of each of these cells. Another logical follow-up of our investigations is to characterize the functional profile of microglial cells that accompanies each of the different states of activation and assesses how the primed phenotype is changed in the dACC of depressed suicides. Additionally, it would be interesting to know if this inflammatory profile we described in the dACC is a phenomenon happening in all brain regions or if it is selective to some brain regions relevant to depression and suicide.

Immediate studies can be performed using immunohistochemistry to determine the shape of the CD45 cells and the quantity of each in the perivascular space in depressed suicides and controls. The shape of the leukocytes rod or circles could indicate the migratory and infiltrating properties of these cells (Audoy-Remus et al., 2008).

Other studies that would highlight the relationship between periphery and central inflammatory dynamics and the relationship with the HPA axis are crucial to further understand the biology of depression and suicide. To address this, postmortem

studies need to be complemented with blood samples of each of the suicide brains and controls. This information is crucial to determine the interplay between these two systems and the dynamics between periphery and different brain regions relevant to these and other systems.

Science evolves at a fast pace and new technologies will be available to answer all these questions in an easier way. Our understanding of psychiatric disorders is limited by the access we have to the living brain. However, postmortem studies represent valuable information of the condition of the brain at the time of death. The postmortem studies contained in this thesis showed, for the first time, increased densities of perivascular macrophages and microglial priming in the dACC white matter of depressed suicides. Additionally, a significant hypertrophy of fibrous astrocytes was also described. These studies represent a valuable piece of evidence that suggests that the dACC (BA24) white matter of depressed suicides present traces of chronic mild inflammation. These studies also suggest that the dACC white matter is an important target for future antidepressant treatment that may act as anti-inflammatory agent.

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