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Mood Disorders and Inflammatory Markers Pathophysiology and Implications for Treatment

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ABSTRACT

INTRODUCTION

Mood disorders are severe, chronic disorders, responsible for relevant disability worldwide. To date, their etiology is not completely understood, yet, in recent years a growing body of literature has proposed a psycho-neuro-immunological hypothesis, according to which inflammation may play an important role in the development and response to treatment of such diseases. Both Major Depressive Disorder and Bipolar Disorder have been associated with peculiar patterns of serum cytokine alterations and, on the other hand, some psychotropic drugs showed the ability to affect cytokines production, making an immunomodulatory action of these drugs plausible.

METHODS

The PubMed database was searched for relevant studies.

Our research also included a comparison of Neurokinin-1Receptor (NK-1R) expression and Substance P (SP) ability to induce NF-κB activation in monocytes from BD patients and healthy donors (HD).

A further study was conducted on human monocytes, which were used as such or differentiated into M1 and M2 macrophages. Cells were treated with vortioxetine before or after differentiation, and their responsiveness, in terms of included oxy-radical and TNFα production, TNFα and PPARγ gene expression, and NF-κB translocation was evaluated.

RESULTS

An increase in pro-inflammatory cytokines such as IL-4, TNF-α, soluble interleukin-2receptor (sIL2-R), IL-1β, IL-6, soluble receptor of TNF-alpha type1 (STNFR1) and C-reactive protein (CRP) is reported in BD patients, during all phases of the disease. IL-1β, IL-6, and TNF-α serum levels are elevated and an increased microglial activation can be observed in some brain regions in MDD patients.

Most antidepressant drugs seem to affect cytokine production and the relative selection of T helper (Th)1 or Th2 cytokine responses.

NK-1R expression showed relevant alterations in BD patients and SP involvement appeared plausible.

Vortioxetine showed anti-inflammatory effects, which are most likely due to an activation of the peroxisome proliferator-activated receptor-gamma (PPARγ) signaling, via a serotoninergic mechanism.

CONCLUSIONS

Neuro-immunomodulation must be taken into consideration when dealing with the pathophysiology of psychiatric disorders and in the choice of antidepressants; the effect of medications affecting the serotoninergic pathway on the innate immune system should be further investigated, also in a disease-specific context.

INTRODUZIONE

I disturbi dell'umore sono disordini gravi e cronici, che determinano una quota rilevante di disabilità in tutto il mondo. Sebbene ad oggi la loro eziologia non sia del tutto chiara, una quota importante della Letteratura scientifica ha recentemente proposto una ipotesi psico-neuro-immunologica, secondo la quale l'infiammazione rivestirebbe un ruolo importante nello sviluppo e nella risposta al trattamento di queste patologie. Sia il Disturbo Depressivo Maggiore che il Disturbo Bipolare sono stati associati a pattern specifici di alterazioni delle citochine sieriche e, d'altro canto, alcuni psicofarmaci si sono dimostrati in grado di agire sulla produzione di citochine, rendendo l'ipotesi di un'azione immunomodulante di questi farmaci plausibile.

METODI

Si sono ricercati studi rilevanti tramite il database PubMed.

La nostra ricerca ha incluso anche un confronto sull'espressione del Recettore per Neurochinina (NK-1R) e sulla capacità della Sostanza P (SP) di indurre l'attivazione di NF-κB nei monociti di pazienti affetti da BD e in controlli sani (HD).

E' stato condotto un ulteriore studio su monociti umani, utilizzati come tali o differenziati in macrofagi M1 o M2. Le cellule sono state trattate con vortioxetina prima o dopo la differenziazione, e ne è stata valutata la responsività in termini di produzione di radicali dell'ossigeno e di TNFα, espressione dei geni codificanti TNFα e PPARγ, e traslocazione di NF-κB.

RISULTATI

Nei pazienti BD, in tutte le fasi di malattia, si osserva un incremento di citochine proinfiammatorie quali IL-4, TNF-α, recettore solubile per interleuchina-2 (sIL2-R), IL-1β, IL-6, recettore solubile per TNF-alfa tipo1 (STNFR1) e proteina C reattiva (CRP). In pazienti MDD, i livelli sierici di IL-1β, IL-6, e TNF-α risultano elevati e si osserva un'aumentata attivazione della microglia in alcune regioni cerebrali.

La gran parte dei farmaci antidepressivi sembra influenzare la produzione di citochine e la conseguente selezione della risposta T helper (Th)1 o Th2.

L'espressione di NK-1R ha mostrato alterazioni rilevanti nei pazienti BD, con un possibile interessamento di SP.

Vortioxetina ha mostrato effetti anti-infiammatori, verosimilmente dovuti all'attivazione del signaling del recettore perossisomale gamma attivato dal proliferatore (PPARγ), tramite un meccanismo serotoninergico.

CONCLUSIONI

Il ruolo della neuro-immunomodulazione andrebbe preso in considerazione quando si tratta di fisiopatologia dei disturbi psichiatrici e di scelta di farmaci antidepressivi; sono necessari studi ulteriori sugli effetti dei farmaci che agiscono sul sistema immunitario innato tramite un meccanismo serotoninergico, anche in un contesto patologia-specifico.

INTRODUCTION

The inter-communicative nature of the brain and the immune system has been known for decades, actually dating back to 1927, when the psychiatrist Wagner-Jauregg was awarded the Noble prize for his work on malaria inoculation in dementia paralytica [1].

In 1980, Robert Ader used the term '*psychoneuroimmunology'* (PNI) to define a new field of research trying to describe processes by which mental events modulate immune functions and, in turn, mechanisms allowing the immune system to interfere with the function of the mind [2].

Recently, plenty of studies have described the role of inflammation and innate immune processes in many psychiatric and neurological disorders, such as Alzheimer's Disease (AD) [3], schizophrenia [4], mood and anxiety disorders [5].

Inflammation can be measured via cytokines, which are small soluble proteins crucial as inflammatory markers. Some of them can promote the immune response, while some others have an inhibitory role. Among the pro-inflammatory cytokines, Tumor Necrosis Factor alpha (TNF-α), interleukin (IL)-1β and IL-6 are responsible for acute local inflammatory response. Interferon gamma (IFN-γ) and IL-2 provide a cellular immune response, while IL-4 and IL-10 are responsible for the humoral immune response; the negative feedback they exert on immune cell activation determines an anti-inflammatory effect [6, 7].

Pro-inflammatory cytokines are mainly mediated by T-helper 1 (Th1) cells, while anti-inflammatory cytokines are mediated by T-helper 2 cells (Th2). Th1 and Th2 responses are mutually inhibitory, and the homeostasis between Th1 and Th2 activity directs different immune response pathways: Th1 cells lead the cell-mediated immunity to fight intracellular pathogens such as viruses and eliminate cancerous cells, while Th2 cells lead the humoral activity to fight extracellular organisms [8].

Even though the brain has been substantially considered and immunologically privileged site, due to the presence of the blood-brain barrier (BBB), it is now ascertained that a peripheral inflammation may reach the brain through leaky regions or pathogen-associated molecular patterns affecting afferent nerves such as the vagus nerve [9]. As a result, brain resident

macrophages, known as microglia, may be activated and release pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α, thus determining a brain inflammation [10].

Moreover, cytokine-releasing cells express functional receptors for neurotransmitters, glucocorticoids and cytokines which include the NE and 5-HT receptors such as β2 [11], 5-HT1A, 5-HT2A, 5-HT1B, 5-HT3 and 5-HT transporter [12], and the glucocorticoid and mineral corticoid receptors [13].

We can thereby argue the existence of a bidirectional communication pathway between the immune and nervous systems, playing an important role in the development and response to treatment of some psychiatric diseases.

AIM OF THE THESIS

The general aim of this thesis is to briefly review current Literature describing the imbalance between pro- and anti- inflammatory cytokines in patients suffering from mood disorders, namely MDD and BD, to evaluate existing data about the immunomodulatory effects of antidepressant medications, finally focusing on the anti-inflammatory activity of vortioxetine, a novel antidepressant with multimodal activity currently approved for the treatment of major depressive disorder.

MOOD DISORDERS AND IMMUNE DISTURBANCES

Major depressive disorder (MDD)

Major depressive disorder (MDD) is a common and debilitating illness, responsible for relevant disability worldwide. Its lifetime prevalence is approximately 16% [14], it is more common in women and its etiology has not been fully elucidated yet.

Major depressive episodes are associated with a number of physical, emotional and cognitive symptoms, such as poor sleep or appetite (both of which can be increased in atypical presentations), fatigue, low mood, anxiety, feelings of worthlessness and guilt, psychomotor retardation, suicidal thoughts; symptoms should last 2 weeks at least. Episodes might be recurrent or isolated, generally followed by residual symptoms and signs [15], which can obviously have a negative impact on occupational and social life.

As far as etiology is concerned, a well established theory is the monoamine hypothesis, according to which depression is secondary to a reduced activity of the catecholaminergic and/or serotoninergic systems [16]. Such theory is supported by the evidence of anti-depressive action of drugs increasing DA, 5HT and/or NA in the synaptic cleft via reuptake blockage. Yet, such medications reduce symptoms in less than 50% of patients, and require 3 to 6 weeks to induce changes in mood [17].

Non-monoamine neurotransmitters also show alterations in MDD: elevated levels of glutamate and an altered expression and function of N-methyl-D-aspartate (NMDA) receptor were observed [18]. Ketamine, an NMDA antagonist primarily used as an anaesthetic agent, has actually proved effective in inducing relief of depressive symptoms in treatment-resistant patients.

Moreover, in recent years several studies reported that inflammation and cytokine changes contribute to the pathophysiology of depression: IL-1β, IL-6, and TNF-α serum levels are elevated and an increased microglial activation can be observed in some regions of cortex and insula [19]. Some studies reported that medication-free MDD patients present a shift in cytokine balance towards a Th2 profile [20],[21], together with altered antibody levels and complement deficiencies [22] and altered sub-sets of lymphocytes [23]. Nevertheless, other studies suggest a

Th1 shift [24, 25], so that melancholic and atypical depression appear to be associated with peculiar cytokine profiles: the first one seems to be characterized by a Th2 shift, via a chronic hyperadrenergic state leading to an increase in systemic IL-6 levels [26]. On the other hand, a Th1 shift seems to occur in atypical depression, via a central hypo-noradrenergic state associated with a hypofunction of central CRH pathway [27].

As previously stated, MDD shares neuroinflammation with neurodegenerative diseases, such as AD, moreover AD patients are at higher risk of developing MDD and vice-versa. Increased pro-inflammatory cytokines were observed in the CSF of AD patients with depressive symptoms, and the Aβ40/Aβ42 ratio in serum was increased in MDD patients in comparison with healthy controls [28].

Finally, older adults are more vulnerable to MDD: in such patients, NE and DA are reduced, cortisol levels are increased, glial activation and augmented expression of pro‐inflammatory cytokines that affect the synaptic activity can be observed. All these features can also be found in MDD patients [29].

The role of inflammation as a neurobiological substrate of cognitive impairment, which can be observed during mood episodes, will be discussed in the following section.

Bipolar disorder (BD)

Bipolar disorder (BD) is a severe, chronic multisystem disorder characterized by episodic recurrent mania (type 1, BDI) or hypomania type 2; BDII), usually interspersed amongst episodes of major depression. The presence of some mixed episodes with those two phases is also a possibility. This disorder affects mood, cognition, behavior, and social functioning [30]: during a manic episode the individual is euphoric or irritable in terms of mood, and usually describes a decreased need for sleep, along with increased energy and “racing” thoughts. The patients’ judgment is often impaired, leading to risk-taking behaviour, substance misuse and self harm. It is not unusual to observe grandiose and delusional ideas, often melding into psychosis. The prevalence of BD is approximately 1% [31]: this low rate can be explained by the fact that it is particularly hard to

diagnose both hypomania and mixed states. Sub-threshold cases are thought to contribute an additional 2 to 5% and therefore collectively as much as 4 to 7% of individuals may qualifiy as having a disorder that falls within the “bipolar spectrum” [32].

Bipolar disorder (BD) is associated with significant morbidity and mortality with an estimated reduction in life expectancy of 11–20 years compared to the general population [33], mainly due to cardiovascular diseases, along with autoimmune disorders, multiple sclerosis (MS), migraine, obesity, and diabetes.

Currently, several aspects of the pathophysiology of BD remain poorly understood, and possible reasons for the high prevalence of general medical conditions are largely unknown, yet several studies suggest that immune dysfunction may be an important mechanistic link between BD and metabolic/ inflammatory comorbidities [34]. Such findings include increased inflammatory cytokines levels in the serum [35] and neuroinflammatory markers in the cerebral spinal fluid (CSF) in BD patients [36] compared to healthy control as well as pathological evidence of inflammation in the frontal cortex in post-mortem samples of patients with BD [37].

Besides medical comorbidities, other factors commonly reported in BD patients may be responsible for inflammation, namely history of early childhood adversity, chronic oxidative stress and a dysfunctional gut-microbiota [19].

Existing literature reports elevated pro-inflammatory cytokines during all the phases of the disease, in particular, IL-4, TNF-α, soluble interleukin-2receptor (sIL2-R), IL-1β, IL-6, soluble receptor of TNF-alpha type1 (STNFR1) and C-reactive protein (CRP) are increased in BD patients when compared to healthy controls [38]. Similarly to MDD, BD also shows different cytokines patterns according to the features of the episode: euthymia is characterized by an isolated increase of sTNFR1 [39]; manic episodes by elevated IL-6, TNF-α, sTNFR1, CXCL10, CXCL11, IL-4 [38, 40]; depressive periods by increased sTNFR1 and CXCL10 [41].

Inflammation can subsequently modulate neurotransmitters, such as 5-HT, whose levels are tipically low both in BD and MDD, arguably due to IL-2, TNF-α and IL-6 action. IL-2 increases the activity of indoleamine 2,3-dioxygenase (IDO), thus increasing the breakdown of tryptophan to tryptophan catabolites (TRYCATs), namely kynurenine, kynurenic acid and quinolinic acid; the increades cleavage of tryptophan, in turn, leads to a decrease in 5-HT levels. TNF-α and IL-6

increase the metabolization of 5-HT to 5-hydroxyindoleacetic acid (5-HIAA) that also leads to low 5-HT levels [42, 43].

It has also been proposed that cytokines may have a direct effect on neuroplasticity, due to the presence of cytokine receptors on the neuronal cell surface: at physiologic levels, cytokines favour the formation of new synapses, facilitating long term potentiation (LTP) in the hippocampus, while elevated IL-1 and TNF-α levels show the opposite effect [44].

Another detrimental effect that serum TNF-α and IL-1β may operate on mood and cognition is that of an overactivation of microglial cells, which in turn causes increased apoptosis, decreased BDNF levels, increased oxidative stress and inappropriate synaptic pruning involving key neural circuits. The final result is that of a neuronal dysfunction, leading to impaired cognitive, affective and behavioral function [45]. Postmortem brains of BD patients revealed elevated levels of IL-1β, MyD88 (key factor in TRL4 signaling), NF-κB subunits (p65 and p50), and activation glial markers in the prefrontal cortex, which is an important region for cognitive control and affective regulation [37]. An over-activation of microglia in the anterior cingulate cortex (ACC), prefrontal cortex (PFC) and insula, key brain regions for mood and cognition, supporting the microglial hypothesis of mood disorders, was also detected by positron emission tomography (PET) imaging using a marker of microglial activation [46, 47].

In BD patients, the chronic inflammatory state may over-activate the hypothalamic–pituitary–adrenal (HPA) axis [48], yet the simultaneous sustained production of pro-inflammatory cytokines leads to decreased levels and sensitivity of glucocorticoid receptors (GR), so that the negative feedback of the immune response is prevented [49]. As a result, chronic hypercortisolemia is determined, leading to impaired neuroplasticity with negative effects on mood and cognition [50]. Moreover, both the over-activated HPA axis and the chronic inflammatory condition can cause metabolic dysfunction with insulin resistance, which has been strongly associated with cognitive impairment in diabetic patients. The affected domains include executive function, attention and memory [51, 52].

THE IMMUNOMODULATORY EFFECTS OF ANTIDEPRESSANTS

Inflammation may play an important role in the pathophysiology of depression, and monitoring the therapeutic efficacy of drugs used to treat depression at the level of the immune system and immune-targeted therapies may be helpful for identifying unique patient populations.
The effects of antidepressants on Th1/Th2 balance is summarized in Table 1 [53].

Antidepressant	Effect on Cytokines	Th1/Th2 balance	References
SSRIs: paroxetine, sertraline, fluoxetine, escitalopram	↓IL-4, IL-10, IL-13, IL-6; ↑IL-1β, IFN-γ, ↑↓ IL-2 (conflicting data)	Th1 shift	Hernandez et al., 2008
SNRIs: venlafaxine	↓IL-12, TNF-α, IFN-γ; ↑IL-10, TGF-1β; ↓IL-6 (low dose); ↑IL-6 (high dose)	Th1 shift (low dose) Th2 shift (high dose)	Kubera et al., 2001 Vollmar et al., 2008 Kubera et al., 2004
SNRIs: duloxetine	↑IL-6	Th2 shift	Fornaro et al., 2011
TCAs: impiramine	↓IL-1β, IL-6, TNF-α	Th2 shift	Ramirez et al., 2016
NDRIs: buproprion	↑IL-10; ↓TNF-α, IFN-γ, IL-1β	Th2 shift	Brustolim et al., 2006
Atypical: mirtazapine	↑TNF-α, ↓IL-6; ↓IFN-γ, ↑IL-4	Th1 shift	Kraus et al., 2002 Kubera et al., 2006

Table 1 The table summarizes mechanisms of action, neuromodulatory effects, cytokines modulation and hypothetical influence on Th1/Th2 balance for each class of antidepressants. (SSRIs: Selective Serotonin Reuptake Inhibitors; SNRIs: Serotonin Norepinephrine Reuptake Inhibitors; TCAs: Tricyclic Antidepressants; NRIs: Norepinephrine Reuptake Inhibitors; NDRIs: Norepinephrine Dopamine Reuptake Inhibitors; IL: Interleukin; IFN: Interferon; TNF: Tumor Necrosis Factor; TGF; Tumor Growth Factor)

Several clinical studies have reported the effects of SSRI on the immune and neuro-endocrine response. SSRIs mainly increase circulating serotonin (5-HT) levels, while at the endocrine level they produce a decrease in circulating cortisol by reestablishing the down-regulated

glucocorticoid receptor sensitivity [13]. As far as immunity is concerned, most studies examined the effects of these medications over a short period of treatment, reporting a dose-dependent decrease in IL-1β, IL-6, IL-10, IFN-γ and TNF-α [54, 55]. On the other hand, Hernandez et al. [56] reported the long-term effects of a chronic SSRI treatment on the cortisol levels and pro-/anti-inflammatory cytokine profile in a group of 31 MDD patients: before treatment high levels of cortisol, IL-4, IL-13 (Th2) and IL-10 were observed in MDD patients when compared with healthy volunteers. After 20 weeks of treatment, the depressive episode was remitting, along with an increase of IL-2 and IL-1β but with no changes in cortisol levels. By week 52, the authors observed a significant reduction in cortisol levels, with increased IL-1β and IFN-γ and decreased Th2 cytokines.

SSRI administration thus enhances 5-HT activity, which in turn exerts immunostimulatory effects on Th1 cytokines and immunoinhibitory effects on Th2 cytokines.

Chen et al. [57] compared the immunomodulatory effects of an eight-week treatment with paroxetine or venlafaxine in order to clarify the relationships between plasma cytokine levels and the therapeutic effectiveness of these drugs. The results suggest that venlafaxine has a greater anti-inflammatory action than paroxetine: paroxetine increased the levels of proinflammatory cytokines IFN-γ, TNF-α, and IL-6 and decreased Th2 cytokine levels, with IL-6 increasing more in the non-remitter group than in the remitter group. After venlafaxine treatment, both the remitter and non-remitter groups showed decreased IL-1β levels, to values seen in the healthy controls. As far as IL-6 is concerned, venlafaxine seems to exert a dose-dependent effect, apparently due to its peculiar pharmacodynamics: at a low dose (when its action does not differ much from that of an SSRI) IL-6 serum levels are reduced [58], while at a higher dose, when venlafaxine acts as a proper SNRI, IL-6 increases [59].

Duloxetine, another SNRI, is characterized by a greater affinity to the NE transporter when compared to venlafaxine, and it determines an increase in IL-6, regardless of the dosage. Such data apparently supports the hypothesis that IL-6 levels depend on the 5HT/NE balance [60].

Ramirez et al. [61] tried to characterize the effects of imipramine on stress induced neuroimmune dysregulation in a murine model, by administrating the tricyclic antidepressant in the drinking water while exposing the mice to repeated social defeat. Imipramine was effective in attenuating

the production of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in microglia. Interestingly, there was a significant decrease of monocytes in the spleen after stress exposure in mice that were treated with the antidepressant. Imipramine also blocked neuroinflammatory signaling and prevented stress-related anxiety- and depressive-like behaviors. These data support the notion that pharmacomodulation of the monoaminergic system, besides exerting anxiolytic and antidepressant effects, may have therapeutic effects as a neuroimmunomodulator during stress.

There is some evidence that bupropion can reduce TNF-α, IFN-γ and IL-1β circulating levels, while increasing IL-8 and IL-10, thus shifting the Th1/Th2 balance towards Th2 response [62]. Starting from the usual clinical practice to combine two or more drugs for the treatment of major depression, Maciel et al. [63] observed depression-like behavior in chronically inflamed mice: the co-treatment with bupropion and celecoxib significantly inhibited both inflammation and depression, along with decreased brain levels of IL-1β.

Mirtazapine also seems to influence cytokines production, by increasing TNF-α and IL-6 while reducing IL-6 and IFN-γ [64, 65]; it also decreases cortisol levels, thus favouring a shift in balance towards a Th1 response [66]. Yet, the immunomodulating effects of non selective reuptake inhibitors are more difficult to characterize, due to their more complex action on neurotransmitters.

The anti-inflammatory properties of effects of vortioxetine, a novel antidepressant endowed of a multimodal pharmacodynamic profile, were the object of our research and will be illustrated in a further chapter.

The impact of mood stabilizing drugs on cytokine levels should also be mentioned: an 8 week monotherapy with lithium induced and increase in TNF-α and IL-4 levels in a BD group when compared to both a medication- free BD group and healthy controls [67].

Leu et al. [68] evaluated the immunomodulatory effects of lithium and valproic acid (VPA) in a mice model of collagen-induced arthritis (CIA): VPA and lithium both reduced the secretion of IL-6 and TNF-α. However, only lithium significantly increased the production of IL-10, while VPA increased the production of IL-8 but substantially reduced the secretion of IL-10 and IL-23. The

authors reported that treatment with VPA acted on dendritic cells (DCs), which are the most important antigen-presenting cells in the body, capable of stimulating naïve T cells and initiating an immune response to foreign antigens. VPA reduced the capacity of DCs to promote the differentiation of T helper 17 cells that are critical in the promotion of inflammatory responses. Such results suggest that VPA and lithium may differentially modulate inflammation by regulating the capacity of DCs to mediate distinct T cell responses.

Yet, existing data concerning VPA are conflicting: in a a double-blind, placebo-controlled study of add-on low-dose memantine (5 mg/d) plus valproic acid (VPA) treatment in patients with BP-II, the effects on IL-1β, IL-6, and IL-8 were not significant in any of the groups; TNF-α levels were significantly lower in the VPA + memantine group than in the VPA + placebo group, regardless of therapeutic effects [69].

Studies reporting immunomodulatory properties of other mood stabilizers such as carbamazepine or lamotrigine are lacking [70] .

SELECTED PUBLICATIONS

NEUROKININ (NK)-1 RECEPTOR EXPRESSION IN MONOCYTES FROM BIPOLAR DISORDER PATIENTS: A PILOT STUDY

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Abstract

BACKGROUND:

Neurokinin 1 receptors (NK-1R) have been involved in several psychiatric disorders including major depression, but less is known for bipolar disorder (BD).

METHOD:

We compared NK-1R expression and Substance P (SP) ability to induce NF-κB activation in monocytes from BD patients and healthy donors (HD), also looking for the effects of tobacco smoke. After informed written consent, 20 euthymic BD patients, either bipolar type 1 (BDI) or type 2 (BDII), and 14 age-matched healthy donors (HD) were enrolled. NK-1R expression in monocytes was evaluated by Western blot and expressed as the ratio between NK-1R and

Na(+)/K(+)-ATPase protein expressions. NF-κB activation was assessed by measuring the nuclear content of the p50 subunit (ELISA kit).

RESULTS:

NK-1R expression was significantly reduced ($P<0.001$) in monocytes from BD patients as compared to HD, with no major differences between BDI and BDII patients. Tobacco smoke enhanced NK-1R expression in HD, but not in BD patients. Un-stimulated monocytes from BD patients presented a constitutively higher ($P<0.05$) content of nuclear p50 subunit as compared to HD. SP and an NK-1R agonist induced NF-κB activation, with a higher effect in HD: this effect was receptor-mediated as it was abrogated by an NK-1R antagonist.

LIMITATIONS:

As a pilot study enrolling 20 BD patients, an obvious limitation is the sample size.

CONCLUSIONS:

Our results show the existence of a relevant alteration in NK-1R expression in BD patients and further suggest SP involvement in BD, so improving our understanding of the underlying mechanisms of this disease.

1. Introduction

Bipolar disorder (BD) is a severe, chronic, recurring mental disorder characterized by episodes of major depression and mania (type1; BDI) or hypomania (type 2; BDII), progressive deterioration and cognitive deficits, often associated with co-morbidities (Perugi et al., 2015). Its pathophysiology is really complex, neurobiological bases and how genetic and environmental influences predispose and/or precipitate the symptoms being not fully understood. Genome-wide association and linkage studies have been performed but the overall results are inconclusive (Nurnberg et al., 2014; Ogden et al., 2004). Activation of the immune system plays a key role in the BD pathogenesis, mononuclear cells and pro-inflammatory cytokines being particularly investigated (Beumer et al., 2012; Drexhage et al., 2010a, 2011; Haarman et al., 2014). Indeed, higher plasma levels of pro-inflammatory cytokines (TNF-α, mainly) were described in BD patients during both manic and depressive episodes (Brietzke and Kapczinski, 2008; Kim et al., 2007; Munkholm et al., 2013) and mood stabilizers, as well as some anti-depressants, were shown to

reduce cytokine level and/or modulate neuro-inflammation (Leonard, 2014; Rao et al., 2007). Regarding mononuclear cells, the "monocyte/macrophage-T cell theory", initially proposed for schizophrenia (Smith, 1992) and depression (Maes et al., 1995), was recently extended to BD, a specific "signature" for monocytes being identified (Drexhage et al., 2010b, 2011; Haarman et al., 2014; Padmos et al., 2008).

Others and we previously demonstrated that substance P (SP) triggers inflammatory-immune responses in monocytes, macrophages, microglial cells and lymphocytes, acting preferentially on neurokinin-1 receptors (NK-1R) (Amoruso et al., 2008; Lai et al., 1998, 2006; Rasley et al., 2002). Besides being expressed in immune cells, NK-1R are distributed in different areas of mammalian brain, including the cingulated and frontal cortex (Burnet and Harrison, 2000; Nagano et al., 2006).

We recently reported that, in monocytes isolated from patients with major depressive disorder, NK-1R expression was reduced as compared to healthy volunteers, whereas NK-2R (the receptor that preferentially binds neurokinin A, NKA) expression was augmented (Bardelli et al., 2013). Moreover, the tachykinin receptor 1 gene (TACR1, which encodes SP and NKA) and other SP-related genes have been associated with BD (Mendlewicz et al., 2005; Ogden et al., 2004; Sharp et al., 2014).

Based on these evidences, we hypothesized that NK-1R expression could be altered in BD. Therefore, this pilot study was aimed to evaluate the constitutive expression of NK-1R protein in monocytes isolated from BDI and BDII patients in comparison to age-matched healthy volunteers. We also evaluated the ability of SP and [$Sar^{9}Met(O_2)^{11}$]SP (selective NK-1R agonist) to modulate NF-κB activation in both BD patients and healthy volunteers.

2. Materials and methods

2.1. Patients

This observational study enrolled 20 consecutive BD patients (13 females and 7 males; mean age 55.3+2.8 years) at the Outpatient Centre of Borgomanero and 14 healthy volunteers (8 females and 6 males; age-matched to BD patients, with no family history of psychiatric disorders, drug-

free at the time of the study). This study was approved by the Ethic Committee of the Novara Hospital; informed written consent was obtained by each participant. Inclusion criteria for BD patients were: stable pharmacological therapy from at least two months, euthymia or mild depression, informed written consent. Diagnosis of BDI or BDII was assessed by two expert trained psychiatrists by a structured clinical interview based on the DSM-IV-TR defined criteria (First et al., 1994). Clinical assessment included the collection of demographic and clinical variables, as well as the score values obtained in the Hamilton Rating Scale for Depression (HAM-D; Hamilton, 1960) and the Young Mania Rating Scale (YMRS; Young et al., 1978). Eight participants (5 females, 3 males) were diagnosed as BDI patients, while 12 (8 females, 4 males) were BDII; 8 BD patients and 7 healthy volunteers were smokers. BD patients had a long-lasting disease (>26 years) and usually employed two drugs: a mood stabilizer (*n*=20; lithium/valproate/lamotrigine: 10/6/4) and an antidepressant (*n*=14; SSRI/SNRI: 8/6) or atypical antipsychotic (*n*=4). The HAM-D score was 6.9+0.8 (range 1–12), suggestive for euthymia; the YMRS score was 3.3+0.4 (range 0–7).

2.2. Monocyte isolation from BD patients and healthy volunteers

Monocytes were isolated from venous blood (20 ml, obtained at fasting) by standard technique (dextran sedimentation and Hystopaque gradient centrifugation; 400*g*, 30 min, room temperature), and obtained by adhesion (90 min, 37 °C, 5% CO_2), as described (Amoruso et al., 2008). Monocyte viability (trypan blue dye exclusion) and purity (flow cytometry analysis of CD14) were>96%.

2.3. Western blotting for NK-1R in monocytes

Monocytes (3×10^6) were scraped in ice-cold phosphate buffered saline containing protease inhibitors and centrifuged (14,000*g*; 30 s, 4 °C). The pellet was re-suspended in 10 mM Tris–HCl (containing EDTA and protease inhibitors), subjected to freezing and thawing and centrifuged again (Amoruso et al., 2008); the pellet so obtained was used for the experiments. Na^+/K^+-ATPase was selected as reference house-keeping membrane enzyme. Immunoblots were performed as described (Bardelli et al., 2013), using a polyclonal NK-1R antibody (ab466; Abcam) and a

monoclonal anti-Na^+/K^+-ATPase (α subunit) antibody (clone M7-PB-E9; Sigma). Western blots were quantified by densitometry (Bio-Rad software) and expressed as the ratio between NK-1R and Na^+/K^+-ATPase protein expression (Bardelli et al., 2013).

2.4. Monocyte stimulation and evaluation of NF-κB p50 subunit

NF-κB activation was evaluated by measuring the nuclear content of p50 subunit. Monocytes were challenged for 2 h with 10^{-6} M phorbol 12-mirystate 13-acetate (PMA, used as standard stimulus), SP or the selective agonist [$Sar^9Met(O_2)^{11}$]SP: these conditions were previously demonstrated as the optimal ones (Bardelli et al., 2005). In some cases, monocytes were pre-treated for 30 min with the antagonist GR 71251 ([D-Pro^9(spiro-gamma-lactam)Leu^{10},Trp^{11}]SP) and then challenged with SP. Nuclear extracts were evaluated using Trans AM™ NF-κB p50 Chemi Transcription Factor Assay kit (Active Motif Europe), according to Bardelli et al. (2013). The p50 subunit activity was measured by a luminometer and results are presented as RLU (Relative Luminescence Unit).

2.5. Statistical analysis

Statistical analyses were performed using Graph Pad Prism 5. Data are presented as mean+SEM of '*n*' independent experiments on monocytes from different BD patients or healthy donors. Differences between groups were analyzed using non-parametric Mann Whitney test. $P<0.05$ was considered statistically significant.

3. Results

3.1. NK-1R expression in monocytes from BD patients and healthy subjects

NK-1R expression was significantly lower ($P<0.001$) in monocytes from BD patients as compared to healthy subjects (Fig. 1A), with no major differences between BDI and BDII (Fig. 1B), in spite of a higher NK-1R/Na^+/K^+-ATPase ratio (not reaching statistical significance) in BDII patients. Tobacco smoke significantly increased NK-1R in monocytes from healthy volunteers, smokers presenting a 3-fold higher constitutive expression, while NK-1R expression in monocytes isolated from BD

patients was unaffected by tobacco smoke (Fig. 1C). Representative Western blots of NK-1R expression are shown in Fig. 1D.

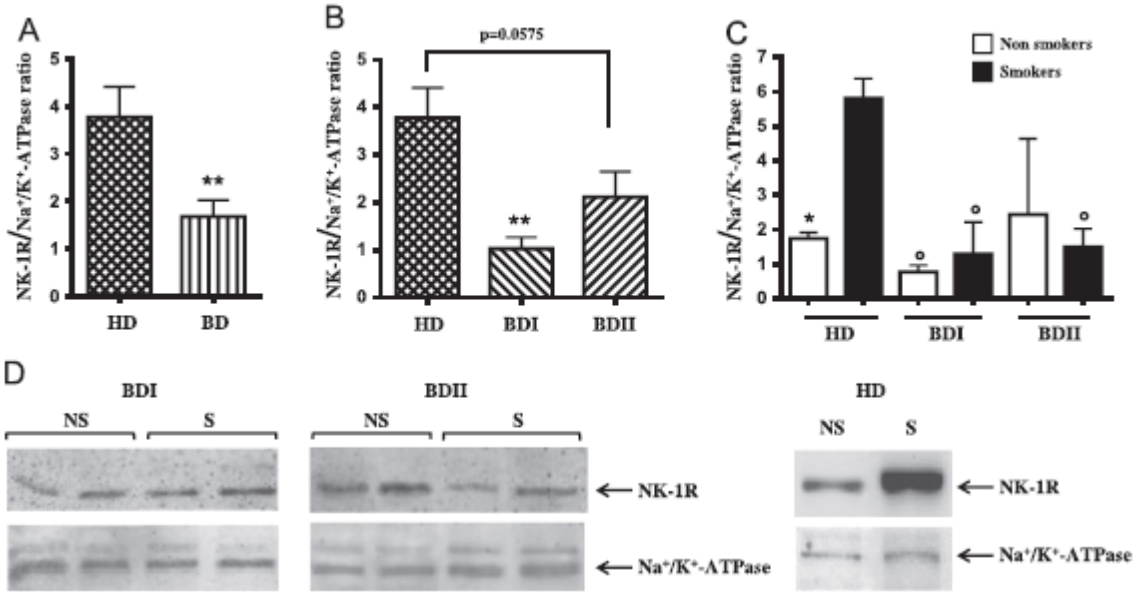

Fig. 1. NK-1R constitutive expression in monocytes from healthy subjects (HD) and bipolar disorder (BD) patients and effects of tobacco smoke.

(A) Comparison between healthy donors (HD; *n*=14) and bipolar disorder (BD) patients (*n*=20). (B) Comparison among healthy donors (HD; *n*=14), bipolar disorder type 1 (BDI) patients (*n*=8) and bipolar disorder type 2 (BDII) patients (*n*=12). (C) NK-1R expression in healthy subjects (HD; smokers: *n*=7, non-smokers: *n*=7), bipolar disorder type 1 patients (BDI; smokers: *n*=4, non-smokers: *n*=4) and bipolar disorder type 2 patients (BDII; smokers: *n*=4, non-smokers: *n*=8). NK-1R expression is evaluated as the ratio between NK-1R and Na^+/K^+-ATPase expression; data are mean+SEM. $^{\square\square}P<0.001$ *vs* HD, $^{\square}P<0.01$ *vs* smoker HD, $^{\circ}P<0.05$ *vs* the respective HD sub-group. (D) Representative Western blots of NK-1R expression and Na^+/K^+-ATPase expression in monocytes from smokers (S) and non-smokers (NS).

3.2. Nuclear translocation of NF-κB p50 subunit in BD patients and healthy subjects

To ensure a quantitative evaluation of NF-κB activation, we measured nuclear translocation of the p50 subunit in monocytes from 5 individuals (2 non-smokers and 3 smokers) for each group (Fig. 2). BD patients presented significantly higher amounts of nuclear p50 subunit than healthy

volunteers: 2230+88, 6030+615 and 5652+570 RLU for healthy donors, BDI and BDII patients, respectively (P<0.05; Fig. 2A). PMA, SP and the NK-1R agonist enhanced about 5-fold p50 nuclear translocation in healthy donors, with lower efficacy in BDI and BDII patients (Fig. 2B). Interestingly, the effects exerted by SP are receptor-mediated, as they were completely reversed in the presence of GR71251, an NK-1R selective antagonist (Fig. 2B).

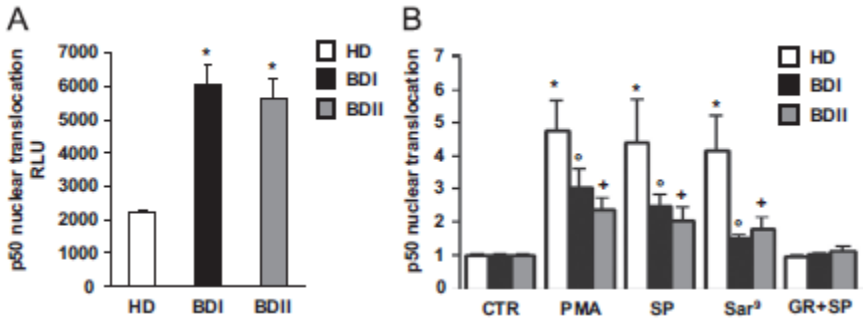

Fig. 2. Nuclear translocation of the p50 NF-κB subunit in monocytes from healthy subjects (HD), bipolar disorder type 1 (BDI) and bipolar disorder type 2 (BDII) patients.

(A) Nuclear translocation of p50 NF-κB subunit in monocytes from healthy subjects, BDI and BDII patients. Results are expressed as RLU; n=5 (2 non-smokers and 3 smokers) for each group; P<0.05 *vs* healthy subjects. See text for further details.

(B) Effects of SP and other stimuli on p50 subunit translocation in monocytes from healthy subjects, BDI and BDII patients. The p50 nuclear content of control (CTR), unstimulated monocytes of each group was taken as 1; phorbol 12-mirystate 13-acetate (PMA) was used as standard stimulus. PMA, substance P (SP), [$Sar^9Met(O_2)^{11}$]SP (Sar^9) and GR71251 (GR) were all used at 10^{-6} M. Data are mean+SEM; n=5 (2 non-smokers and 3 smokers) for each group. $^{*,\circ,+}P$<0.05 *vs* the respective control.

4. Discussion, limitations and conclusions

These results demonstrate a reduced NK-1R expression in monocytes from BD patients as compared to age- and gender-matched volunteers, and further support circulating monocytes as a suitable and easily accessible cell model to assess major mental illnesses (Bardelli et al., 2013; Drexhage et al., 2010a, 2011). Several preclinical and clinical studies have investigated the

pathogenic role of SP in mood disorders, demonstrating that SP and NK-1R can directly interact with the monoaminergic system in brain areas involved in the modulation of anxiety, stress, mood and behavior; alterations in this system have been proposed to explain BD (Ebner et al., 2009; Machado-Vieira and Zarate, 2011; Nagano et al., 2006). Moreover, pharmacological or genetic inactivation of NK-1R reduced aggressive responses and depressed behavior in different animal models (Ebner et al., 2009). The use of an NK-1R antagonist to treat depression was first reported in 1998, MK-869 being as effective as paroxetine in patients with moderate-severe anxiety (Kramer et al., 1998); however, subsequent larger trials failed to confirm these findings (Keller et al., 2006). In monocytes isolated from depressed patients we evidenced an imbalance of NK-1R and NK-2R expression, the former being significantly reduced while the latter enhanced, as compared to healthy volunteers (Bardelli et al., 2013). Interestingly, a good correlation was documented between NK-1R expression and HAM-D score, patients with more severe disease presenting the lowest level of receptor expression (Bardelli et al., 2013).

As far as BD is concerned, several reports suggested the involvement of SP and SP-related genes in its pathogenesis and some polymorphisms of TACR1 gene were shown to increase susceptibility to BD (Perlis et al., 2008; Sharp et al., 2014). Our present results indicate that NK-1R protein expression in monocytes from BD patients is only 50% of the one observed in age-matched healthy volunteers; this reduced NK-1R expression could be related to an enhanced SP release (as documented in depressed patients; Bondy et al., 2003) that in turn causes receptor internalization and/or down-regulation (Smith et al., (1999)), or, alternatively, could depend on drug therapy. Unfortunately, we cannot provide evidence for these hypotheses, as we neither measured SP serum levels nor enrolled naïve BD patients.

Our previous results indicate that monocytes possess functional nicotinic receptors on their surface (Amoruso et al., 2007) and that cigarette smoke enhances NK-1R expression in monocytes from healthy volunteers, but not in patients with major depressive disorder (Amoruso et al., 2008; Bardelli et al., 2013). In BD patients, too, monocyte NK-1R expression was similar in both smokers and non-smokers. As BD patients usually smoke more than the general population, this fact deserves further investigations. Indeed, in BD patients, the expression of the nicotinic acetylcholine receptor α7 was significantly reduced (Benes, 2012; Thomsen et al., 2011);

therefore, it is tempting to speculate that, due to the reduction of nicotinic receptors, monocytes from smoker and non-smoker BD patients have the same NK-1R expression.

Furthermore, in monocyte/macrophages from healthy donors and depressed patients, SP induced NF-κB activity (Bardelli et al., 2005, 2013); we now demonstrate that SP is more effective in healthy subjects than in BD patients, in line with the reduced level of NK-1R expression in patients. Moreover, as compared to healthy volunteers, monocytes from BD patients presented a constitutive higher content of nuclear p50 subunit. This is in good agreement with the findings by Barbosa et al. (2013), who reported increased phosphorylation levels of p65 NF-κB subunit in mononuclear cells from BDI patients, and with the results of a post-mortem study showing increased protein and mRNA levels of p50 and p65 NF-κB subunits in the frontal cortex of BD patients (Rao et al., 2010).

Some limitations affected this pilot study. First, the small sample size may have weakened the statistical power; second, drug therapy and smoking habit can behave as confounding factors. To minimize these facts, we included only BD patients on stable therapy and remittent phase, and evaluated separately smokers and non-smokers; however, we cannot exclude that the observed decrease in NK-1R expression could be ascribed to drug therapy. Larger studies are needed to further address these points.

The results of this study represent a novel finding that might contribute to better appreciate BD pathophysiology and to improve strategies for BD treatment.

VORTIOXETINE EXERTS ANTI-INFLAMMATORY AND IMMUNO-MODULATORY EFFECTS ON HUMAN MONOCYTES/ MACROPHAGES

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Abstract

BACKGROUND AND PURPOSE:

A crosstalk between the immune system and depression has been postulated, with a key role played by monocytes/macrophages and cytokines. In this paper we examined whether vortioxetine, a multimodal anti-depressive drug, was endowed of antiinflammatory and anti-oxidative activity, leading to immunomodulatory effects on human monocytes and macrophages.

EXPERIMENTAL APPROACHES:

Human monocytes were isolated from buffy coats and used as such or differentiated into M1 and M2 macrophages. Cells were treated with vortioxetine before or after differentiation, and their responsiveness was evaluated. This included oxy-radical and TNFα production, TNFα and PPARγ gene expression, and NF-κB translocation.

KEY RESULTS:

Vortioxetine significantly reduced the PMA-induced oxidative burst in monocytes and in macrophages (M1 and M2), causing a concomitant shift of macrophages from the M1 to the M2 phenotype, demonstrated by a significant decrease of the expression of the surface marker CD86 and an increase of CD206. Moreover, treatment of monocytes with vortioxetine rendered macrophages derived from this population less sensitive to PMA, as it reduced the oxidative burst, NF-kB translocation, TNFα release and expression, while inducing PPARγ gene expression. FACS analysis showed a significant decrease of the $CD14^+$ /$CD16^+$ /$CD86^+$ M1 population.

CONCLUSION AND IMPLICATIONS:

These results demonstrate that in human monocytes/macrophages vortioxetine is endowed of an anti-oxidant activity and antiinflammatory effects driving polarization of macrophages toward the alternative phenotype. Our findings therefore suggest that vortioxetine, alongside its antidepressive effect, may have immunomodulatory properties.

ABBREVIATIONS

BD, bipolar disorder; CytC, cytochrome C; GM-CSF, granulocyte macrophage colony-stimulating factor; 5-HT, 5-hydroxytryptamine (serotonin); LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MDD, major depressive disorder; MFI, median fluorescence intensity; NK, neurokinin; PMA, phorbol 12-myristate 13-acetate; PPARγ, Peroxisome Proliferator-Activated Receptor γ PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; SERT, serotonin transporter; SOD, superoxide dismutase.

INTRODUCTION

Several evidence point to an association between the immune system and depression, and this may be driven by high levels of proinflammatory cytokines and by increased oxidative stress, both arising from active monocytes/macrophages. An involvement of cytokines in psychiatric diseases was first postulated through the observation that patients treated with the proinflammatory cytokine interferon-α (IFN-α) developed several depressive symptoms that disappeared when treatment was interrupted, leading to the formulation of “the monocyte/macrophage theory” of depression and schizophrenia (Maes, 1995, Smith, 1991). Since then, more evidences have emerged that support the involvement of proinflammatory markers and immune cells in the pathogenesis of depression (Dantzer et al., 2008, Miller and Raison, 2016). Elevated levels of cytokines in plasma of patients affected by major depressive disorders (MDD) have been confirmed in a number of meta-analyses (Goldsmith et al., 2016, Haapakoski et al., 2015). Similarly, a recent meta-analyses has shown that anti-cytokine drugs (e.g. adalimumab, tocilizumab, etc) are associated with an antidepressant effect and increase the response to antidepressants (Kappelmann et al., 2016). This is true also for antiinflammatory agents

(Rosenblat et al., 2016). Hence, monocytes/macrophages together with their counterpart in the central nervous system, microglia, can be considered the bridge between inflammation and depression (Nazimek et al., 2016b). In fact, expression of genes involved in inflammation and immunity are up-regulated in monocytes from MDD patients compared to healthy volunteers (Grosse et al., 2015), while glucocorticoid receptor expression is reduced (Carvalho et al., 2014). Moreover, we have described a significant imbalance of neurokinin (NK)-1 and NK-2 receptor expression in monocytes isolated from MDD patients under stable therapy (Bardelli et al., 2013) and in patients affected by bipolar disorders (BD) (Amoruso et al., 2015) compared to monocytes from healthy subjects.

Peripheral monocytes can differentiate into macrophages and dendritic cells (Tacke and Randolph, 2006) that share similarities with microglia. Macrophages and microglia are characterised by a distinct plasticity that leads to the identification of two functional states, depending on the extracellular environment: M1 or M2. M1 macrophages, which in vitro can be induced by the exposure to lipopolysaccharide (LPS), GM-CSF and interferon (IFN)-γ express and release pro-inflammatory cytokines/mediators and reactive oxygen/nitrogen species (ROS/RNS). M2 macrophages, that in vitro can be induced by the exposure to M-CSF, IL-4 and IL-13 express and release antiinflammatory cytokines that are involved in tissue repair (Murray et al., 2014, Sica and Mantovani, 2012). The two phenotypes can be distinguished by the different expression of surface markers (e.g. CD86/CD80 for M1 and CD206 for M2). Macrophages can continuously switch from one state to the other according to the environmental cues (Porcheray et al., 2005), and the possibility that microglia M1/M2 polarization contribute to relapse and remission, respectively, of MDD has been recently suggested (Nakagawa and Chiba, 2014). Moreover, Torres-Platas et al. (2014) showed an increased number of microglia cells/macrophages in post-mortem brain samples from suicide patients, and a dysregulation of the microglia transcriptome has been demonstrated in a mice model of inflammation-associated depressive behaviour (Gonzalez-Pena et al., 2016) and in a mouse microglia cell line stimulated with Toll-like Receptors ligands (Das et al., 2015). Interestingly, various antidepressants have shown immunomodulatory effects on peritoneal mice macrophages (Nazimek et al., 2016a) and on microglia polarization (Kalkman and Feuerbach, 2016).

Vortioxetine is a novel antidepressant agent approved by FDA and EMA for treatment of MDD (Frampton, 2016), endowed of a multimodal pharmacodynamic profile, that combines inhibition of the serotonin transporter (SERT) with direct effects on serotonin receptors (Sanchez et al., 2015). Serotonin, alongside a CNS neurotransmitter, is an important peripheral mediator (Shajib and Khan, 2015), that for example drives human macrophage polarization skewing M2 phenotype via 5-HT2B and 5-HT7 receptors, contributing to the maintenance of an antiinflammatory state (de Las Casas-Engel and Corbi, 2014). Yet, Soga et al. (2007) demonstrated that serotonin activates monocytes inducing the expression of CD80/86 surface markers (characteristic of the M1 pro-inflammatory phenotype), potentiates LPS-cytokine release, and rescues monocytes from apoptosis, leading to the amplification and chronicity of an inflammatory state.

Due to its unique activity, we used vortioxetine to investigate the crosstalk between immune system and depression, evaluating human monocytes/macrophages from healthy donors. We now demonstrate the ability of vortioxetine to direct monocytes/macrophages toward an antiinflammatory phenotype.

METHODS

Monocytes isolation and differentiation

Human monocytes were isolated from 20 healthy anonymous human buffy coats (provided by the Transfusion Service of Ospedale Maggiore della Carità, Novara, Italy) by standard technique of dextran sedimentation and Histopaque (density=1.077 g cm^{-3}, Sigma-Aldrich) gradient centrifugation (400xg, 30 min, room temperature) and recovered by thin suction at the interface, as described previously (Lavagno et al., 2004). Purified monocytes populations were obtained by adhesion (90 min, 37 °C, 5% CO_2) in serum free RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM glutamine and antibiotics. Cell viability (trypan blue dye exclusion) was usually >98%. To differentiate monocytes into M1 macrophages, cells were cultured in 10% FBS-enriched medium with hrGM-CSF (50 $ngmL^{-1}$) for 5 days, and then IFN-γ (20 $ngmL^{-1}$) and LPS (50 $ngmL^{-1}$) were added for additional 24 h. To obtain M2 macrophages, monocytes were cultured in 10% FBS-enriched medium added by hrM-CSF (50 $ngmL^{-1}$) for 5 days, and then hrIL4, hrIL13 and hrIL10 (20 $ngmL^{-1}$) were added for additional 24 h. Cell phenotype characterization was evaluated by the

expression of specific surface marker CD86 (M1) and CD206 (M2). Cells were treated with vortioxetine at 2.5, 5 and 7.5 nM, concentrations equivalent to those found in plasma of treated patients 10, 20 and 30 ng/ml, respectively. Moreover, cells were always treated with serotonin (1µM), rosiglitazone (1 µM) and 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2) (10 µM), used as reference drugs. Rosiglitazone (an antidiabetic drug) and PGJ2 are both agonists of PPARγ whose antiinflammatory effect is well recognised. Serotonin was used because directly involved in the pharmacodynamic action of vortioxetine. Monocytes were treated and analysed as such, or treated as requested by each specific assay, washed and left to differentiate into macrophages, and then analysed. Macrophages M1 and M2 were treated and analysed.

All the experiments were performed in triplicate using cells isolated (and consequently differentiated into macrophages) from each single donor.

Cell viability

To assess potential drugs toxicity in monocytes and MDM, cell viability was evaluated using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Cells (1×10^4 cells) were challenged for 1 and 24 h with vortioxetine in a concentrations range between 1 nM to 10 µM, and serotonin 1µM; then, the medium was replaced by the MTT assay solution (1 $mgml^{-1}$; 2 h, 37 °C 5% CO_2; Sigma-Aldrich). Supernatant was removed and DMSO (Sigma-Aldrich) was added in order to dissolve the purple formazan; the absorbance was read at 580 and 675 nm.

Superoxide anion ($O2^-$) production

Cells (1×10^6 cells/plate) were treated for 1 h with drugs and then stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) 1µM for 30 min. PMA is a well known stimulus to induce a strong and significant respiratory burst via PKC activation (Myers et al. 1985). Therefore, it can be used to explore the antiinflammatory efficacy of any substance. Superoxide anion production was then evaluated by the superoxide dismutase (SOD)-sensitive cytochrome C reduction assay and expressed as nmoles cytochrome C reduced/10^6cells/30 min, using an extinction coefficient of 21.1 mM. To avoid interference with spectrophotometrical recordings, cells were incubated with RPMI 1640 without phenol red, antibiotics and FBS.

Moreover, we evaluated the percentage of cells producing $O2^-$ using the Cellular ROS/Superoxide Detection Assay Kit (AbCam) according to the manufacturer's instructions. Results were analyzed

by Windows Multiple Document Interface for Flow Cytometry (winMDI, v. 2.9; Joseph Trotter, The Scripps Institute) and expressed as percentage of cells positive to $O2^{-}$ staining.

Quantitative real-time RT-PCR

Cells were evaluated for PPARγ and TNFα expression after 6 h challenging with vortioxetine at 2.5, 5 and 7.5 nM, serotonin 1 µM rosiglitazione 1 µM and PGJ2 10 µM. Total RNA was isolated by Tri-Reagent (Sigma-Aldrich). The amount and purity of total RNA were spectrophotometrically quantified by measuring the optical density at 260 and 280 nm. cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was carried out in a volume of 10 µl per well in a 96-well optical reaction plate (Biorad) containing 0,5 µl of TaqMan Expression Assay (PPARγ or TNFα; Applied Biosystems, USA), 2,5 µl of RNase-free water, 5 µl of TaqMan Universal PCR MasterMix (2×) (without AmpErase UNG; Applied Biosystem) and 2 µl of cDNA template, as described (Amoruso et al., 2009a). The plate was run on the 7000 ABI Prism system (Applied Biosystems). To compensate for variations in cDNA concentrations and PCR efficiency between tubes, an endogenous gene control (β-glucuronidase) was included for each sample and used for normalization. The relative quantification was determined by the ΔΔCT method (Livak and Schmittgen, 2001).

ELISA

Commercially available kits were used to test the presence of cytokine and transcription factors. Cell supernatants were tested for the presence of TNFα(Human TNFα-Elisa Kit- Ready-SET-Gol eBioscience Bender); cell lysates were tested for the presence of activated NF-kB p65 (NF-kB p65 - total/phospho – InstantOne, eBioscience Bender).

Flow Cytometry analysis

Measurement of surface markers expression was performed by multiparametric analysis by flow cytometry (FACS Calibur, BD) and analyzed by Windows Multiple Document Interface for Flow Cytometry (winMDI, v. 2.9; Joseph Trotter, The Scripps Institute). The following antibody panels were used: FITC anti-CD16, FITC anti-CD36, PE anti-CD86, PE anti-CD163, PerCp anti-CD206, and APC anti-CD14. The monocytes and macrophage populations were defined as CD14+ cells. Data were therefore expressed as the number of CD16+, CD86+, CD36+, CD163+ or CD206+ cells over

the number of CD14+ cells. CD16 and CD86 are M1-like markers, while CD36, CD163 and CD206 are M2-like markers. Comparison between treated and no-treated cells was performed and data were expressed as percentage of positive events.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. Data are expressed as mean ± S.E.M. of 'n' independent experiments performed in triplicate. Statistical significance among different cell treatments was assessed by Student's paired t -test, or one-way repeated measures ANOVA with Kruskal-Wallis multiple comparisons test if more than two treatment groups were compared. Statistical significance was defined as $P < 0.05$.

Antibodies and reagents

Vortioxetine was kindly provided by Lundbeck, Italy. Rosiglitazone and 15-deoxy-Δ12,14-prostaglandin J2 were purchased from Cayman Chemicals (Milan). Hystopaque, PBS, RPMI 1640 medium (with or without phenol red), glutamine, Hepes, streptomycin, penicillin, PMA, SOD, cytochrome C, RIPA buffer, LPS, protease inhibitor cocktail and phosphatase inhibitor cocktail were obtained from Sigma (Milan, Italy); all cytokines were purchased by Immunotools; FACS antibodies were purchased by eBioscience.

RESULTS

Effects of vortioxetine on cell viability

In order to avoid confounding effects attributable to cell toxicity, we evaluated the effects of vortioxetine and of serotonin on cell viability via the MTT assay. No significant differences compared to control untreated cells were found in the viability of monocytes or macrophages after treatment for 6 or 24 h with vortioxetine (2.5-10 nM) or serotonin (1µM; data not shown). Similarly, rosiglitazone or PGJ2, used as controls in the present experiments, had no effect on cell viability as previously reported (Amoruso et al., 2009a, Amoruso et al., 2009b).

Effect of vortioxetine on monocytes, M1 and M2 macrophages

Vortioxetine reduced the PMA-induced burst in monocytes, M1 and M2 macrophages

Monocytes and macrophages, as major phagocytes, release basal amounts of O2- that increase after stimulation. We therefore analysed the effect of vortioxetine on this parameter evaluating the nmoles of reduced cytochrome C (CytC).

Basal $O2^-$ production from unstimulated monocytes (ctrl) was unaffected by vortioxetine at the highest concentration tested (10 nM) (data not shown). PMA 1µM treatment for 30 min (Fig 1A) led to a 10-fold increase. Pre-treatment of cells with vortioxetine dose-dependently decreased this response, with a significant reduction already at 0.1 nM; at a concentration of 2.5 nM the effect was comparable to that of the PPARγ agonist rosiglitazone and to serotonin, while treatment with PGJ^2 at 10 µM resulted in greater inhibition. Vortioxetine was very efficacious at inhibiting PMA-induced bursts in monocytes achieving about 90% inhibition at the maximal concentration used, with an IC^{50} of 0.033 + 0.009 nM. Moreover, we evaluated by flow cytometry analysis the percentage of cells producing $O2^-$. As shown (Fig 1B), monocytes producing superoxide anion significantly increased after PMA treatment, and vortioxetine significantly reduced the percentage of responsive cells, as did rosiglitazone.

We then tested macrophages differentiated into M1 or M2 phenotypes. As expected, M1 macrophages (Fig 2A) had a higher level of basal superoxide anions (Fig 2B) compared to M2 macrophages. PMA 1µM induced superoxide anion production in both phenotypes and vortioxetine potently reduced the responsiveness of both M1 and M2 macrophages, totally reverting the effect of PMA with an IC^{50} of 2.27 + 0.37 nM in M1 and 0.37 + 0.10 nM in M2. While these IC^{50}s were fitted considering a single component, visual inspection of results suggests that two inhibitory components exist, one in the pM and one in the nM range. Inhibition of PMA-induced bursts was seen also with the PPARγ agonists and serotonin on both cell populations.

We then evaluated the PMA-induced burst-responsiveness (Fig 3) in the M1 and M2 populations via FACS analysis. As shown in Fig 3A, the median fluorescence intensity (MFI) of untreated $O2^-$-labelled M1 cells was significantly higher than M2, as expected, and the M1 responsiveness to PMA was also higher. Interestingly, vortioxetine was able to completely abolish the PMA effect restoring the basal intensity of $O2^-$ in both macrophage populations, already at a concentration of 1 nM.

Moreover, as shown in Fig 3B, we found that in the M1 population the staining for CD86 and $O2^-$ were overlaid both at rest and after PMA, while vortioxetine was able to revert the pro-oxidant stimulus and, surprisingly, to reduce the expression of CD86 skewing the phenotype versus M2, as demonstrated by the increased expression of the CD206 marker (Fig 3B). The effect was bell-shaped, with a peak at 5 nM. Viceversa, in the M2 resting population (Fig 3C) we found a very low percentage of CD206+/O2- stained cells but after PMA stimulation these cells switched versus an M1 phenotype, demonstrated by an increase of CD86 and the contemporary decrease of CD206. Vortioxetine again re-addressed macrophage polarization toward the M2 phenotype.

Effects of drugs on basal PPARγ and TNFα gene expression

We then analysed whether vortioxetine was able to modulate gene expression of PPARγ, a recognized antiinflammatory factor, and TNFα, a recognized inflammatory cytokine. As expected, resting cells were characterised by different expression of PPARγ: monocytes and M1 macrophages expressed approx. three-fold lower levels of PPARγ compared to M2 macrophages (data not shown). TNFα expression, on the contrary, was significantly higher in M1 macrophages and lowest in M2 macrophages (data not shown). As shown in Fig 4, PPARγ mRNA expression was increased by vortioxetine already at the lowest concentration both in monocytes (Fig 4A) and M2 macrophages (Fig 4C), while TNFα was not modified. In the M1 phenotype (Fig 4B), PPARγ and TNFα mRNA expression were less affected by vortioxetine. Interestingly, in monocytes, serotonin and PGJ2 were unable to modulate either PPARγ or TNFα. In M1 macrophages, serotonin decreased TNFα and increased PPARγ while in M2 macrophages a significant decrease of TNFα was evident while the effect on PPARγ was blunted.

Effect of vortioxetine on monocyte differentiation to M1 or M2 macrophages

We then felt it was noteworthy to evaluate the effect of vortioxetine on monocyte differentiation toward M1 or M2 macrophages. We therefore treated monocytes for 6 h with vortioxetine (0.01 nM - 10 nM), serotonin 1 μM, rosiglitazone 1 μM or PGJ_2 10 μM. Cells were then differentiated versus M1 (GM-CSF, IFNγ and LPS) or M2 (M-CSF plus IL4, IL10 and IL13) phenotypes for 6 days in

the absence of vortioxetine. Macrophages were then analysed under basal conditions or after stimulation with PMA 1µM for 30 min.

Vortioxetine inhibited PMA-induced oxidative burst.

PMA induced a significant response in control M1 (Fig 5A) and M2 (Fig 5B) macrophages, as expected. Interestingly, both M1 and M2 macrophages derived from vortioxetine-treated monocytes were resistant in a dose-dependent manner to the PMA-oxidative burst induction (Fig 5). The IC^{50}s for inhibition were in the nM range for both cell types (0.44 + 0.16 nM for M1 and 1.71 + 0.51 nM for M2).

Vortioxetine modulated PPARγ and TNFα expression

To evaluate the possibility that vortioxetine drives macrophage polarization toward an antiinflammatory state, we analysed by RT-PCR the expression of the antiinflammatory nuclear factor PPARγ in M1 and M2 macrophages derived from monocyte pre-treated with vortioxetine. As shown in Fig 6, vortioxetine pre-treatment was able to significantly induce PPARγ mRNA expression in both M1 (Fig 6A) and M2 populations (Fig 6B). Concomitantly, vortioxetine was able to significantly reduce the gene expression of the strong pro-inflammatory cytokine TNFα both in M1 (Fig 6A) and in M2 (Fig 6B) populations at the maximal concentration.

Effects of vortioxetine on TNFα release and NF-κB activity

We next used these cells differentiated after vortioxetine treatment to evaluate PMA-induced NF-κB activity and TNFα release. As depicted in Fig 7A (M1) and 7B (M2), PMA-treatment of macrophages led to a significant increase of NF-κB activity (left y axis) which was significantly reduced by pre-treatment with vortioxetine in M1 macrophages, and by vortioxetine and rosiglitazone in M2 macrophages. In our experimental conditions, resting M1 macrophages released very high levels of TNFα (Fig 7A, right y axis), therefore their responsiveness to PMA was quite bland and slightly modulated by vortioxetine, by serotonin and PPARγ agonists. Viceversa, PMA-treatment of M2 induced a strong TNFα release (Fig 7B, right y axis) that vortioxetine significantly reduced with an inverse bell shaped curve. PPARγ agonists and 5HT also significantly reduced PMA-induced TNFα release.

Vortioxetine inhibited M1 polarization of macrophages induced by M-CSF/LPS/ IFNγ.

To confirm our data reporting a trend of vortioxetine to prevent M1 polarization, we analysed the expression of specific surface marker such as CD86 for M1, and CD206 for M2 macrophages. As shown in Fig 8, vortioxetine at 7.5 nM, and serotonin as well, significantly decreased the % of CD14+/CD16+/CD86+ cells in the M1 population, demonstrating its ability to contrast the effect of the cytokine cocktail used to differentiate vortioxetine-treated monocytes. On the contrary, vortioxetine and serotonin did not influence the CD14+/CD163+/CD206+ M2 population.

DISCUSSION

Vortioxetine is an antidepressant with a unique activity on serotoninergic transmission: alongside being a potent 5-HT transporter (SERT) inhibitor, it acts as a 5-HT3, 5-HT1D and 5-HT7 receptor antagonist, a 5-HT1A agonist, and as a 5-HT1B partial agonist (Sanchez et al., 2015). The IC^{50} and EC^{50} for these actions are in the low nanomolar range (D'Agostino et al., 2015). Human monocytes/macrophages express SERT as well as the following serotoninergic receptors: 5-HT1E, 5-HT2A, 5-HT3A, 5-HT4, 5-HT7 (Durk et al., 2005, Fiebich et al., 2004) through which 5-HT exerts its immunomodulatory effects (Arreola et al., 2015).

In our study, ex vivo experiments were carried out to evaluate the possible immunomodulatory and antiinflammatory effects of vortioxetine in human monocytes/macrophages. We used two different experimental approaches: we evaluated the effect of vortioxetine in modulating responsiveness of monocytes/macrophages and in reverting macrophage polarization, as well as evaluated the effect of vortioxetine in modulating monocyte differentiation induced by cytokine cocktails. Alongside, we used rosiglitazone and/or PGJ_2 as positive controls, and serotonin.

We firstly demonstrated that vortioxetine is endowed with the ability to reduce PMA-induced oxidative bursts in monocytes and M1 and M2 macrophages, with a potency thousand-time superior to 5-HT and rosiglitazone. Under our experimental conditions, we could also demonstrate that both PMA-treated M1 and M2 macrophages undergo phenotypic adaptations (i.e. an increase of the surface marker CD86 and a decrease of CD206), and vortioxetine restored the basal conditions toward the M2 phenotype. Moreover, vortioxetine induced gene expression of PPARγ in resting monocytes and in both macrophage populations as well as induced a negative trend in the expression of TNFα. It is well known that PPARγ, whose expression increases during

differentiation from monocytes to macrophages (Chinetti et al., 1998), exerts important antiinflammatory (Chinetti et al., 2000) and immunomodulatory effects (Chinetti et al., 2003), and its activation is able to prime monocytes into M2 phenotype (Bouhlel et al., 2015). Hence our results demonstrated that vortioxetine was able to program monocytes toward an antiinflammatory state inducing PPARγ gene expression while 5-HT and PGJ_2 did not modify the low physiological level of the receptor. To further understand the immunomodulatory potential of vortioxetine we next evaluated its ability to prime resting monocytes towards differentiation by cytokine cocktails. Also in this case, treatment of monocytes with vortioxetine strongly reduced the PMA-induced burst in the resulting M1 and M2 cells, even 6 days after treatment. This suggests that pre-challenge of monocytes with vortioxetine made the resulting M1 cells resistant to PMA stimulation preventing therefore the induction of the pro-inflammatory phenotype, meanwhile supporting the differentiation toward the M2 population. The predisposition of vortioxetine-challenged monocytes to acquire an antiinflammatory phenotype was demonstrated by the significant increase of PPARγ gene expression in the M2 population as well as, with surprise, in M1 cells. Indeed, PPARγ activation induces repression of the inflammatory genes by interfering with different signal transduction pathways, such as the NF-κB, STAT, and AP1 pathways, in turn directly involved in the expression and release of TNFα in activated monocytes/macrophages (Chinetti et al., 1998, Delerive et al., 1999, Ricote et al., 1998, Chinetti et al., 2000).

All the results highlighted above were paralleled, with minor exceptions, by 5-HT, suggesting that (i) the effects observed are unlikely to result from an off-target effect of vortioxetine (i.e. the serotoninergic system is involved); (ii) the effects observed are either the result of an increase in 5-HT (due to the inhibition of SERT), a direct agonistic effect at one of its receptors, or a mixed effect due to vortioxetine's multi-modal action. Given that 5-HT alone was able to induce PPARγ gene expression and reduce NFκB activation and TNFα release, this would draw a signalling cascade of event. Indeed, these observations are in keeping with previous works, which demonstrated that the levels of proinflammatory cytokines were reduced by inhibition of the 5HT-3 receptor (Fiebich et al., 2004, Stratz et al., 2012) or of SERT (Kagaya et al., 2001, Lanquillon et al., 2000), two targets inhibited by vortioxetine. An effect of vortioxetine mediated in part by

5-HT3 is also in line with Gupta et al. (2016) that demonstrated that antagonism of 5-HT3 could have a neuroprotective effect against oxidative stress. This explanation may disagree with Durk et al. (2005) that demonstrated that serotonin was able to modulate inflammation and cytokine release via activation of subtype receptor 3, 4 and 7. The possibility that vortioxetine, in this respect, is different from other SSRIs is supported by the atypical nature of the dose-response curves observed in the present investigation. For example, when investigating PMA-induced oxidative bursts (Fig 2), a reproducible plateau after low doses was observed with higher doses eliciting further effects, and when investigating CD86/CD206 ratios in M1 macrophages (Fig 3B): a reproducible bell-shaped curve was observable that peaked around 5 nM. We hypothesize that these can be attributed to the sequential recruitment of the diverse mechanisms that characterize this antidepressant.

This working hypothesis postulates that vortioxetine, via a serotoninergic mechanism, activates PPARγ signalling, which in turn is responsible for the anti-inflammatory effects observed. This would be consistent with the parallel effects seen with serotonin and PPARγ agonists. It is also possible nonetheless, that vortioxetine activates directly PPARγ in serotonin-independent fashion. This alternative hypothesis would be consistent with reports suggesting that PPARγ agonists may have antidepressant activity via their ability to reduce inflammatory cytokines such as IL-6 and TNFα both in depressed patients and murine models of depression (Kemp et al., 2014, Colle et al., 2016, Colle et al., 2017, Liao et al., 2017).

Similar effects of fluoxetine and citalopram as antiinflammatory and immunomodulatory drugs on microglia *in vitro* have been shown previously (Su et al., 2015) as well as an effect of 5-HT in inducing M2 polarization (de Las Casas-Engel and Corbi, 2014). The present contribution shows for the first time that priming of monocytes before differentiation with serotonin or vortioxetine is able to skew polarization towards an M2 phenotype.

We recognize that the present investigation was performed in monocytes from healthy volunteers. Given that an impairment of the serotoninergic transmission is at the basis of depression, the effect of antidepressants in this population might be different. Future studies should therefore aim at replicating these results in a clinical trial employing monocytes/macrophages from patients treated with vortioxetine. Furthermore, we have

evaluated solely the effect of vortioxetine on human monocytes/macrophages and are at present unable to determine whether the effect observed participates in the drug action. Last, we investigated solely vortioxetine, a serotoninergic reuptake inhibitor with a peculiar pharmacodynamic profile and are unable to determine whether traditional SSRIs act differently in our models.

Overall, therefore the effect of vortioxetine is antioxidative and antiinflammatory and is long-lasting in directing macrophages towards the alternative phenotype. The present contribution therefore suggests that the effect of the serotoninergic pathway and of drugs that act on it used in the clinic in the innate immune system should be further investigated, also in a disease-specific context.

Fresu_fig1

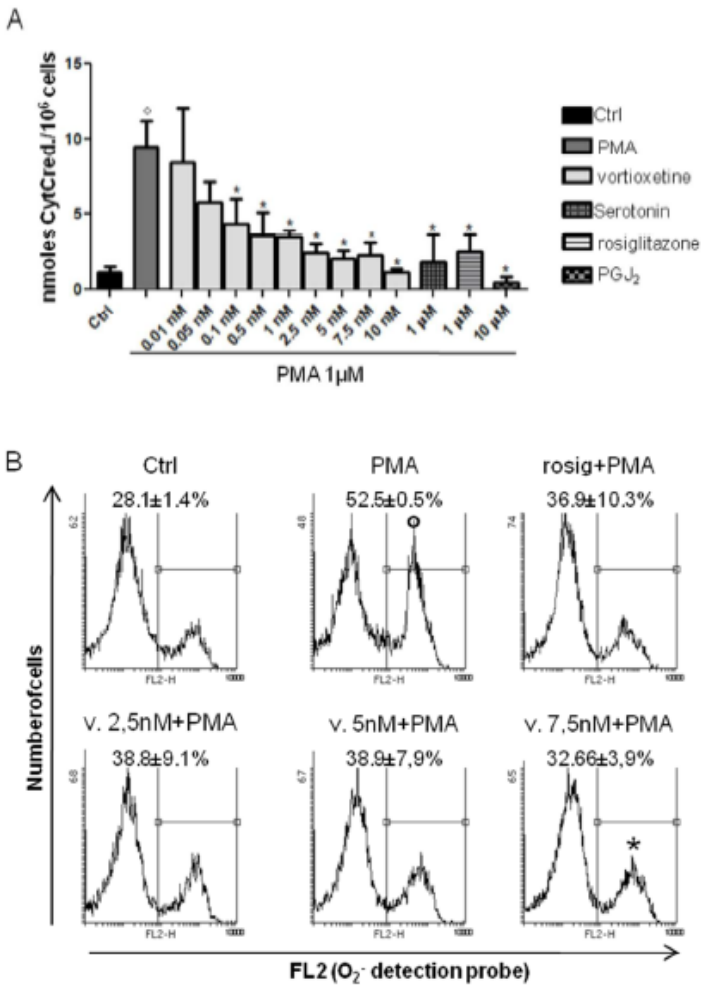

Figure 1. **Effect of vortioxetine on superoxide anion production in monocytes**. Human monocytes were pre-incubated for 1 h with the indicated drugs (Ctrl, control untreated cell) and then stimulated with PMA 1 µM for 30 min. (A) Dose-dependent decrease of the PMA-oxidative burst by vortioxetine in stimulated cells. Data are means ± S.E.M. of 6 independent experiments from distinct donors, analysed by one-way ANOVA with Kruskal Wallis test for multiple comparison. (B) FACS analysis of O2- positive cells in the presence or absence of the indicated drugs (vortioxetine, v; rosiglitazone, rosig). Data are means ± S.E.M. of **6** independent experiments from distinct donors, analysed by one-way ANOVA with Kruskal Wallis test for multiple comparison. Significance levels: °P < 0.05 *vs* Ctrl; *P < 0.05 *vs* PMA.

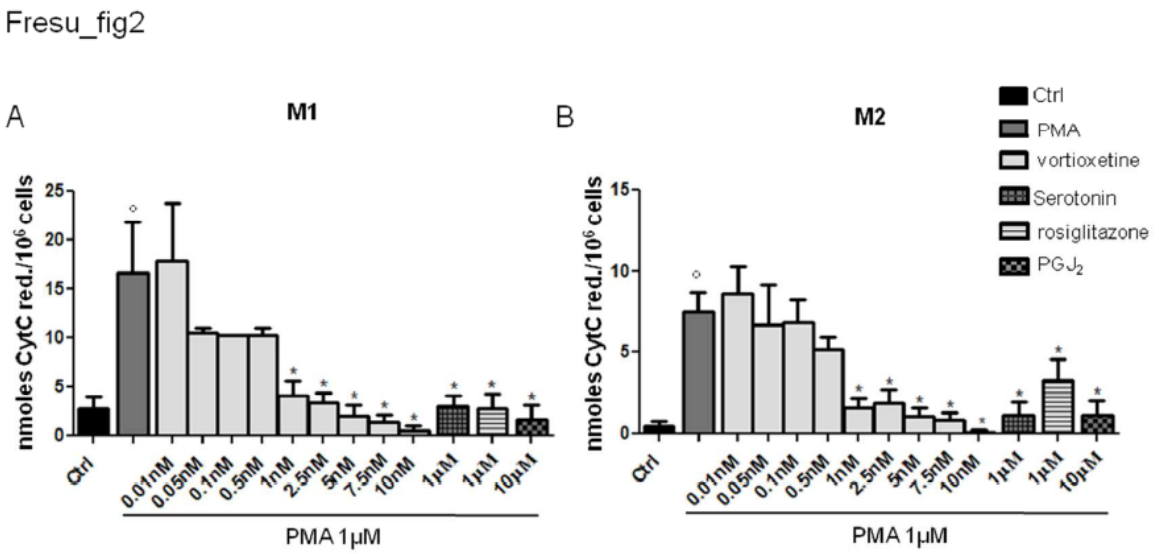

Figure 2. **Effect of vortioxetine on superoxide anion production in M1 and M2 macrophages**. Macrophages were pre-incubated for 1 h with the indicated drugs (Ctrl, control untreated cell) and then stimulated with PMA 1 µM for 30 min. Vortioxetine decreased the level of reduced cytC on PMA stimulated M1 (A) and M2 (B) macrophages in a dose-dependent manner. Significance levels: °P < 0.05 *vs* Ctrl; *P < 0.05 *vs* respective PMA. Data are means ± S.E.M. of 5 independent experiments from distinct donors, analysed by analysed by one-way ANOVA with Kruskal Wallis test for multiple comparison.

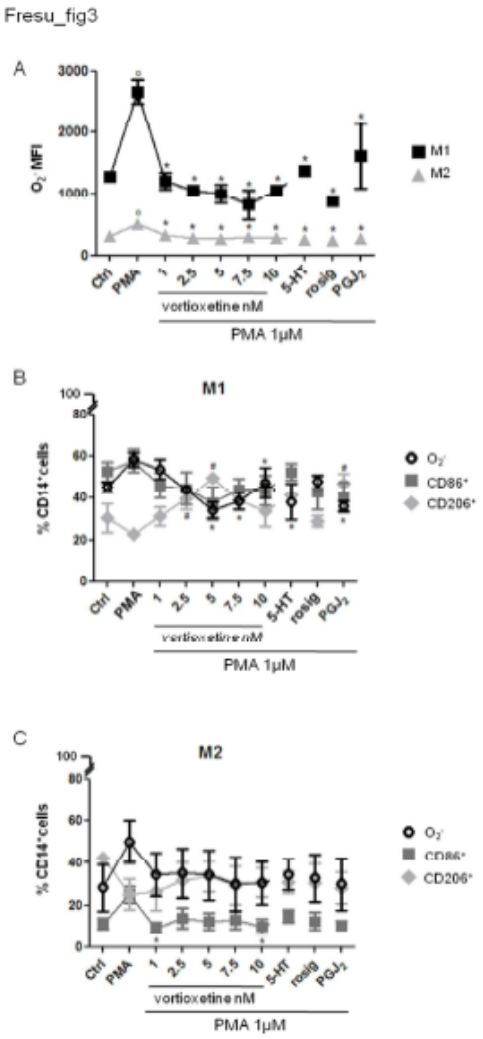

Figure 3. **FACS analysis of phenotype/superoxide anion production in M1 and M2 macrophages.** Macrophages differentiated from human monocytes were pre-incubated with the indicated drugs [Ctrl, (control) untreated cells; serotonin, 5-HT, 1 µM; rosiglitazone (rosig), 1 M; 15-deoxy- D-prostaglandin J2 (PGJ2,)10 M] and then stimulated with PMA 1 µM for 30 min. Cells were then stained with antibodies anti-CD86, anti-CD206 and anti-O2-. (A) Mean fluorescence intensity (MFI) of cells stained with the O2- detection probe. Significance level: °P < 0.05 *vs* Ctrl; *P < 0.05 *vs* PMA. (B) Percentage of M1 macrophages positive to the O2- staining correlated to CD86 and CD206 expression. Significance level: *P < 0.05 *vs* PMA, referred to O2-. #P < 0.05 *vs* PMA, referred to CD206. (C) Percentage of M2 macrophages positive to the O2- staining correlated to the CD86 and CD206 expression. Significance level: *P < 0.05 *vs* PMA, referred to CD86 curve. All data are

expressed as means ± S.E.M. of 5 independent experiments from distinct donors analysed by one-way ANOVA with Kruskal Wallis test for multiple comparison.

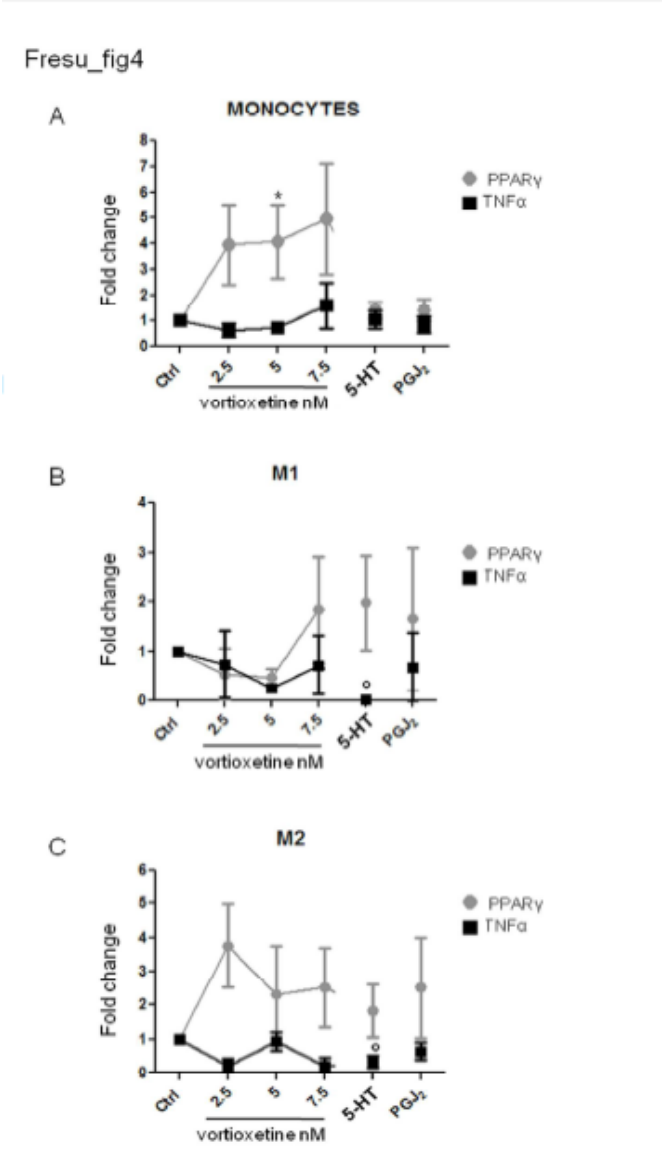

Figure 4. **Real-time analysis of PPARγ and TNFα expression**. PPARγ and TNFα gene expression in (A) monocytes, (B) M1 and (C) M2 macrophages, challenged for 6 h with vortioxetine, serotonin (5-HT) or PGJ2, 15-deoxy- D-prostaglandin J2 (PGJ2) 10 µM. Data are means ± S.E.M. of 8 independent experiments from distinct donors analysed by one-way ANOVA with Kruskal Wallis test for multiple comparison. Significance level: °P < 0.05 *vs* respective **control**; *P < 0.05 *vs* respective control.

Fresu_fig5

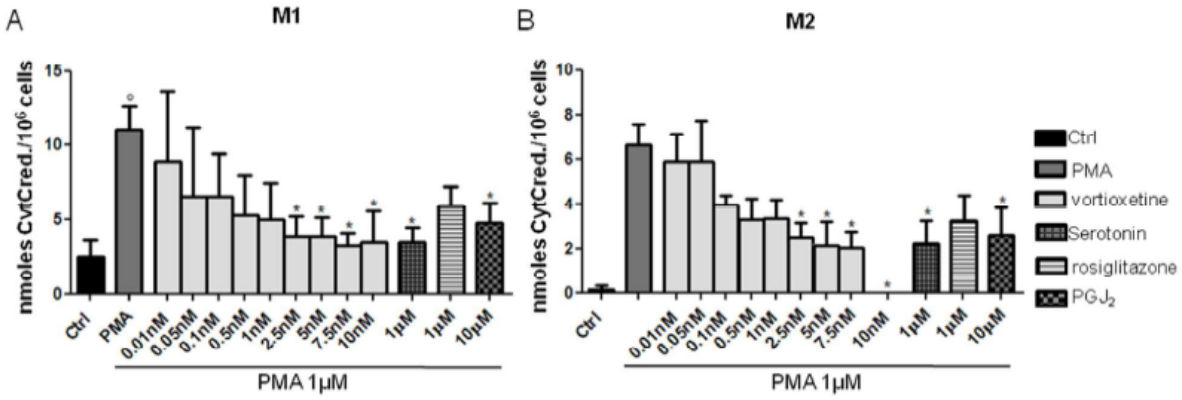

Figure 5. **Superoxide anion production in M1 and M2 macrophages differentiated from vortioxetine-treated monocytes**. M1 (A) and M2 (B) macrophages were differentiated for 6 days, in the presence of the specific cytokine cocktail, from monocytes treated for 6 h with vortioxetine, serotonin (5-HT) 1µM, rosiglitazone 1 µM or 15-deoxy- D-prostaglandin J2 (PGJ_2) 10 µM. The day of the experiment, macrophages were stimulated with PMA 1 µM for 30 min. Results are expressed as nmoles of reduced cytochrome C *vs* PMA. Significance level: °P < 0.05 *vs* Ctrl; *P < 0.05 *vs* PMA. Data are means ± S.E.M. of 5 independent experiments from distinct donors, analysed by one-way ANOVA with Kruskal Wallis test for multiple comparison.

Fresu_fig6

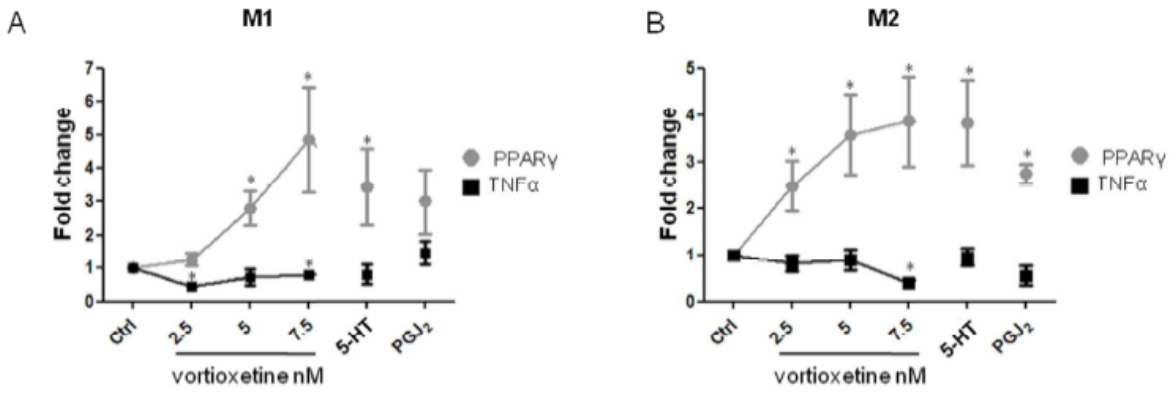

Figure 6. **Real-time analysis of PPARγ and TNFα expression**. M1 (A) and M2 (B) macrophages were differentiated for 6 days, in the presence of the specific cytokine cocktail, from monocytes treated for 6 h with vortioxetine, serotonin (5-HT) or 15-deoxy- D-prostaglandin J2 (PGJ_2) 10 µM. Data are means ± S.E.M. of 6 independent experiments from distinct donors, analysed by one-way ANOVA with Kruskal Wallis test for multiple comparison. Significance levels: *P < 0.05 *vs* Ctrl.

Fresu_fig7

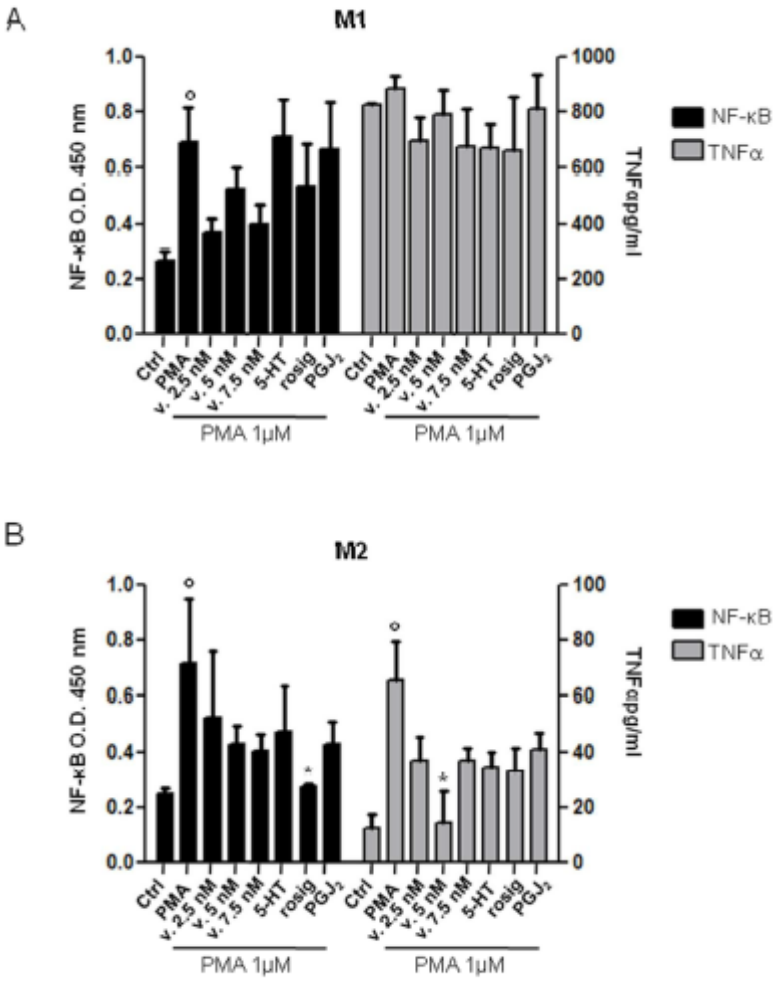

Figure 7. **Effect of vortioxetine on NF-κB activity and TNFα secretion in PMA-stimulated cells.** ELISAs were performed in M1 (A) and M2 (B) macrophages differentiated for 6 days, in the presence of the specific cytokine cocktail, from monocytes treated for 6 h with vortioxetine (v), serotonin (5-HT) 1 µM, rosiglitazone (rosig) 1 µM and 15-deoxy- D-prostaglandin J2 (PGJ2) 10 µM. The day of the experiment, macrophages were stimulated with PMA 1 µM for 30 min. NF-κB activity is expressed as O.D. at 450 nm and secreted TNFα as pg/ml. Data are means ± S.E.M. of **6** independent experiments from distinct donors analysed by one-way ANOVA with Kruskal Wallis test for multiple comparison. Significance levels: °P < 0.05; *P < 0.05 *vs* respective control.

Fresu_fig8

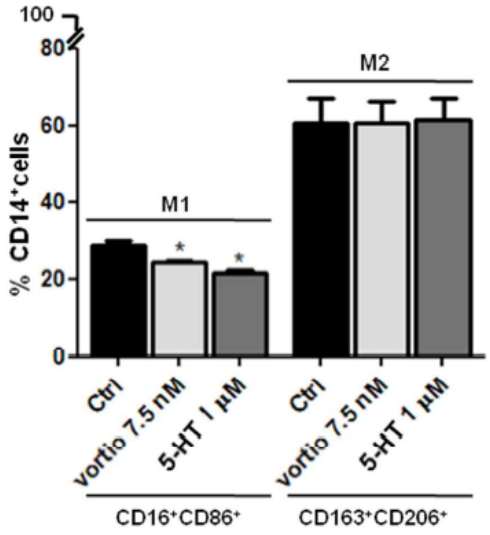

Figure 8. **Effect of vortioxetine on surface marker expression**. M1 and M2 macrophages were obtained differentiating monocytes treated for 6h with vortioxetine (vortio) 7.5 nM or serotonin (5-HT) 1 M. Cells were stained with the indicated antibodies and the co-expression of CD14/CD16/CD86 and CD14/CD163/CD206 in the two populations was analysed. Vortioxetine significantly reduced the M1 population (CD14+/CD16+/CD86+ cells), while the M2 population (CD14+/CD163+/CD206+) was not influenced by drug treatment. Data are expressed as mean ± SEM of 6 independent experiments from distinct donors, analysed by one-way ANOVA with Kruskal Wallis test for multiple comparison. Significance level: *P < 0.05 vs macrophages differentiated from untreated monocytes (Ctrl).

CONCLUSIONS AND FUTURE PERSPECTIVES

A growing body of Literature suggests that MDD and BD are associated with an up regulation of pro-inflammatory cytokines, which apparently follows a state-specific pattern.
The studies we examined have demonstrated that most antidepressants and possibly some mood stabilizers can induce an anti-inflammatory shift of the immune response, pointing out that reducing inflammation might contribute to treatment response.

The main results from the publications selected for this thesis are summarized below:

1. Circulating monocytes can be considered a suitable and easily accessible cell model to assess major mental illnesses: a reduced NK-1R expression was observed in monocytes from BD patients as compared to age- and gender- matched volunteers. The more severe was the disease, as evaluated by HAM-D, the lower the level of receptor expression. Moreover, several studies showed that NK-1R and also SP can interact with the monoaminergic system in brain areas involved in the modulation of anxiety, stress, mood and behavior. Yet, we could not measure SP levels, nor we examined drug-naïve patients, which is a limitation of our study.
2. Vortioxetine showed immune-modulatory and anti-inflammatory effects on human monocytes/macrophages: it reduced the PMA-induced oxidative burst in monocytes and in macrophages (M1 and M2), causing a concomitant shift of macrophages from the M1 to the M2 phenotype. Moreover, treatment of monocytes with vortioxetine rendered macrophages less sensitive to PMA, as it reduced the oxidative burst, NF-kB translocation, TNFα release and expression, while inducing PPARγ gene expression.

To our knowledge, ours is the first study assessing the immunomodulatory properties of vortioxetine. However, it was performed on monocytes isolated from healthy volounteers, and only vortioxetine, with its peculiar pharmaco-dynamic profile was examined.

Further research is obviously needed to establish the exact influence of both vortioxetine and other antidepressants on the immune system either on unipolar depressed or bipolar depressed patients. A new study project regarding depressed patients has been planned in next year. Surely, clarification of these questions will provide a deeper understanding of the pathophysiology of mood disorders and could contribute to the development of more effective antidepressant medications.

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