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Effects of Gc-Macrophage Activating Factor in Human Neurons; Implications for Treatment of Chronic Fatigue Syndrome

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ABSTRACT

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a debilitating disease of multifactorial aetiology characterized by immune system dysfunction, widespread inflammation, multisystemic neuropathology and persistent pain. Given the central role of the immune system in the pathogenesis of the syndrome, we studied the effects of a potent modulator of the immune system in in vitro and in vivo models that could help clarifying its role and indications in ME/CFS treatment. To this end, we studied the effects of vitamin D-binding protein-derived macrophage activating factor (also designated as Gc-Macrophage Activating Factor or (GcMAF)) on human neuronal cells (SH-SY5Y) and on the persistent pain induced by osteoarticular damage in rats. GcMAF at pM concentration increased neuronal cell viability and metabolism through increased mitochondrial enzyme activity. These effects were accompanied by cAMP formation and by morphological changes that were representative of neuronal differentiation. We hypothesize that these effects are to be ascribed to the interconnection between the GcMAF and Vitamin D Receptor (VDR) signalling pathways. The results presented here confirm at the experimental level the therapeutic effects of GcMAF in ME/CFS and elucidate the mechanisms of action through which GcMAF might be responsible for such therapeutic effects.

Keywords: Chronic Fatigue Syndrome, Immunotherapy, Vitamin D, Macrophage Activating Factor, GcMAF

1. INTRODUCTION

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a complex disorder characterized by immune system dysfunction, widespread inflammation and multisystemic neuropathology (Carruthers *et al*., 2011; De Meirleir *et al*., 2013). Dysfunction of the immune system involves abnormal functions and distributions of T lymphocytes, B lymphocytes, natural killer cells and monocyte/macrophages (Uchida, 1992; Bansal *et al*., 2012). The aetiology of ME/CFS has still to be clearly defined and multiple factors may be responsible for its onset and progression, thus lending credit to the hypothesis that both aetiology and pathogenesis are multifactorial (Carruthers *et al*., 2011). Heavy metal exposure and viral infections are among the factors that contribute to ME/CFS aetiology and pathogenesis and a role for human endogenous retroviruses has been recently hypothesized (De Meirleir *et al*., 2013). Both

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chronic heavy metal exposure and viral infections are considered responsible for the immune system dysfunction and neuropathology that are typical of ME/CFS (Pacini *et al*., 2012a; De Meirleir *et al*., 2013).

Among the cells of the immune system that play a role in the pathogenesis of ME/CFS, macrophages are considered of primary importance since the nervous and immune systems mutually cooperate via release of mediators of both neurological and immunological derivation (Covelli *et al*., 2005). Macrophages, originating from the migration and differentiation of circulating monocytes into virtually all tissues, are extremely flexible and plastic cells. They play vital homeostatic roles both in the nervous and immune systems, i.e., in those systems that are altered in ME/CFS (Castagna *et al*., 2012).

One of the most prominent regulators of macrophage function is the vitamin D-binding protein-derived macrophage activating factor, also designated as Gc-macrophage activating factor or GcMAF (Nagasawa *et al*., 2005). Macrophage activation by GcMAF induces a significant variation of surface receptors that in turn recognize abnormality in malignant cell surface and induce the apoptosis of cancerous cells as well as virus-infected cells (Yamamoto *et al*., 2009; Thyer *et al*., 2013a).

The interest for clinical use of GcMAF in a variety of conditions derives from the observation that different diseases involving the immune system such as cancer, viral infections and autoimmune diseases, show elevated level of serum alpha-N-acetylgalactosaminidase (Nagalase) an enzyme that deglycosylates vitamin D-binding protein. This results in the loss of GcMAF precursor activity and consequent dysfunction of the immune system (Yamamoto *et al*., 2008a; 1997). Thus, serum Nagalase activity has been used as diagnostic indicator for a variety of conditions ranging from cancer to autism (Greco *et al*., 2009; Bradstreet *et al*., 2012). In this latter study it was demonstrated that GcMAF treatment of autistic children with elevated levels of Nagalase was associated with a significant improvement of autism symptoms, thus further stressing the interconnection between the immune and the nervous system.

It should be noticed, however, that GcMAF, in addition to stimulating macrophages, shows a number of other biological properties that contribute to its therapeutic effects. These range from inhibition of angiogenesis (Pacini *et al*., 2011) to direct inhibition of human cancer cell proliferation and metastatic potential (Gregory *et al*., 2010; Pacini *et al*., 2012b) to increased production of energy at the mitochondrial level (Pacini *et al*., 2013). We recently demonstrated that these multifaceted effects of GcMAF can be interpreted considering the interconnection between the GcMAF and the Vitamin D Receptor (VDR) signalling pathways (Thyer *et al*., 2013b).

Therefore, based on these premises, there is a solid scientific rationale to propose the use of GcMAF in ME/CFS, a syndrome characterized by alterations that can be targeted by this protein. In this study we describe in vitro and in vivo results that are consistent with a direct effect of GcMAF on human neuronal viability and metabolic activity and on inflammation-associated pain.

2. MATERIALS AND METHODS

2.1. Reagents

Commercially available GcMAF was obtained from Immuno Biotech Ltd, Guernsey, Channel Isles. GcMAF was purified according to the procedure previously described (Yamamoto *et al*., 2008b). Briefly, vitamin D-binding protein (Gc-protein) was isolated from purified human serum obtained from the American Red Cross, using either 25-hydroxyvitamin D3-Sepharose high affinity chromatography or actin-agarose affinity chromatography. The bound material was eluted and then further processed by incubation with three immobilized enzymes as described (Bradstreet *et al*., 2012). The resulting GcMAF was filter sterilized. Protein content and concentration were assayed using standard Bradford protein assay methods (Bradford, 1976). Purity was assessed by SDS-PAGE and Western Blot analysis performed after each step of the preparation procedure; at the end of the procedure, only one band could be evidenced (**Fig. 1A**). At the end of the production process, GcMAF was checked for sterility in-house and externally by independent laboratories. Its safety and biological activity were tested in monocytes, human breast cancer cells and chick embryos (Pacini *et al*., 2011; 2012a; Thyer *et al*., 2013a). When not indicated otherwise, common reagents were from Sigma Aldrich, Milano, Italy.

2.2. Cell Cultures

Human SH-SY5Y neuronal cells were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia, Italy. Cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO2 in Eagle's minimum essential medium in Earle's Balanced salt solution (45%), Ham's F12 medium (45%), foetal calf serum 10%. In the experiments described in **Fig. 2 and 3 and Table 1**, the cells were starved for 24h prior to the experiment in serum-free medium and the experiments were conducted in serum-free medium.

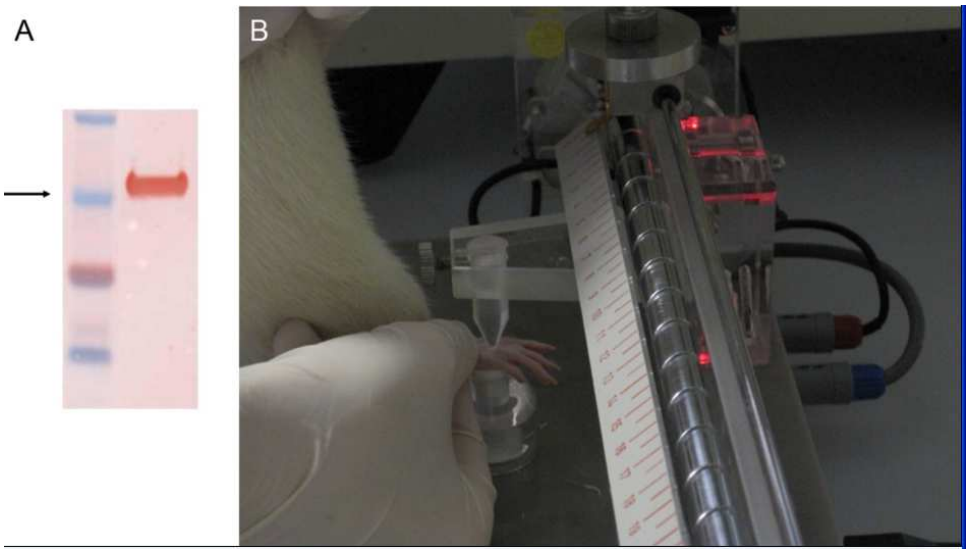

Fig. 1. (A) Western blot analysis of highly purified GcMAF. The presence of alpha-*N*-acetylgalactosamine was evidenced by blotting against biotin-labelled peanut lectin. Streptavidin labelled with horseradish peroxidase (Vector Labs, UK) was applied to the membrane and then stained with AEC (3-Amino-9-ethylcarbazole) staining kit (Sigma-Aldrich, UK). 15µL of 100 µg mL^{-1} GcMAF by total protein assay were applied and the electrophoresis was performed in MES running buffer. The molecular weight markers are SeeBlue® Plus2 Pre-Stained Standard (Invitrogen Life Technologies, UK). Left lane: molecular weight markers. Arrow indicates glutamic dehydrogenase standard. Right lane: GcMAF. (B) The analgesimeter to determine pain threshold. A constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe. Pressure was increased until a vocalization or a withdrawal reflex occurred. The picture was taken before beginning to apply the pressure and the circular weight is on the far left of the scale that is at the top of the picture

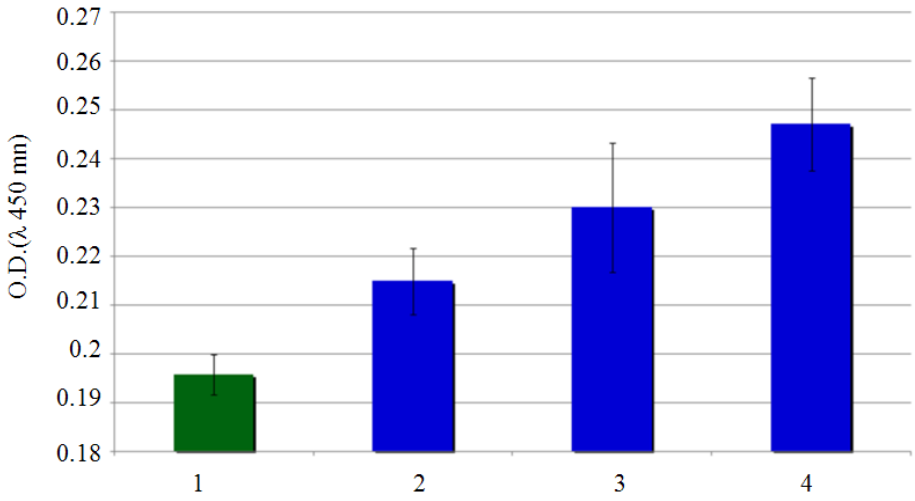

Fig. 2. Effects of GcMAF on human neuronal cell viability and metabolism. Human SH-SY5Y neuronal cell viability and metabolism were determined as described in Materials and Methods. GcMAF (8-800 pM) was added to cell cultures for 24h in serum-free medium. Column 1: control (saline containing 800 pM Gc-protein). Columns 2, 3, 4: GcMAF 8, 80, 800 pM. Results were expressed as optical density at 450 nm wavelength and are means±S.E.M (n=12). All the data referring to GcMAF stimulation were statistically different from control ($p<0.02$)

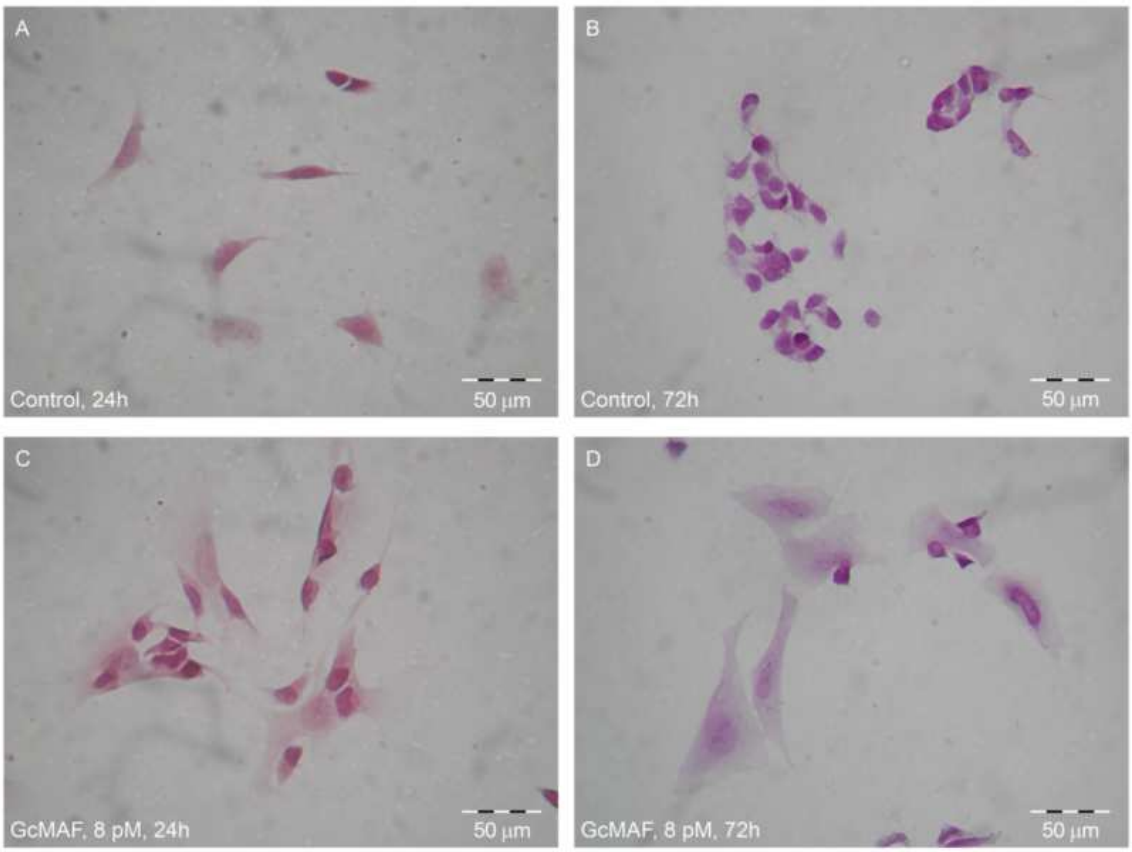

Fig. 3. Effects of GcMAF on human neuronal morphology. Human SH-SY5Y neuronal cells were stained with haematoxylin-eosin after 24 h or 72h incubation with saline containing 8 pM Gc-protein (control), or with 8 pM GcMAF. A: control, 24 h. B: control, 72 h. C: GcMAF, 8 pM, 24 h. D: GcMAF, 8 pM, 72 h

Table 1. cAMP concentration in human neuronal cells stimulated with GcMAF

Treatment	cAMP
Control	10.5±1.2
GcMAF 8 pM	12.0±1.1
GcMAF 80 pM	19.8±1.2*

2.3. Study of Cell Viability

Assessment of cell viability and metabolic activity were determined by Cell Counting Kit-8 for quantitation of viable cell number (Sigma Aldrich). According to the Manufacturer, this assay measures active mitochondrial dehydrogenases of living cells. Each condition was replicated with quadruplicate samples and each experiment was replicated three times. Results are expressed as optical density (absorbance) at 450 nm. Absorbance is directly proportional to cell viability and metabolic activity. Differences between experimental values were evaluated by the Student's t-test. In order to avoid biases, the experiments were performed in blind and the experimenters were not aware of the treatment.

2.4. cAMP Assay

3'-5'-cyclic Adenosine Monophosphate (cAMP) levels were measured by "cAMP Direct Immunoassay Kit" obtained from Abnova, Heidelberg, Germany. The kit utilizes recombinant Protein G coated 96-well plate to efficiently anchor cAMP polyclonal antibody onto the plate. cAMP-HRP conjugate directly competes with cAMP from sample binding to the cAMP antibody on the plate. After incubation and washing, the amount of cAMP-HRP bound to the plate can easily be determined by reading HRP activity at O.D. 450 nm. The intensity of O.D. 450 nm is inversely proportional to the cAMP concentration in samples expressed as nM. Each condition was replicated in quadruplicate samples

and each experiment was replicated three times. Differences between experimental points were evaluated by the Student's t-test.

2.5. Studies on Animals

Male Sprague-Dawley rats (Harlan, Varese, Italy) weighing approximately 200-250 g at the beginning of the experimental procedure were used for the experiments. The Institutional Animal Care and Use Committee (IACUC) or ethics committee that approved this study is the structure of the University of Firenze where the experiments with animals where performed that is the Centro Stabulazione Animali di Laboratorio (Ce.S.A.L., Centre for Stabulation of Laboratory Animals), Viale Pieraccini 6 50139 Firenze. Tel. +39.055.4271308. Fax. +39.055.4271203. e-mail: cesal@unifi.it. The Centre complies with all the E.U. laws concerning experiments on animals. Four animals per cage were housed at 23±1°C under a 12 h light/dark cycle; they were fed with standard laboratory diet and tap water ad libitum and used at least one week after their arrival. The experimental protocol complied with the European Community guidelines for animal care (DL 116/92, the European Communities Council Directive of 24 November 1986: 86/609/EEC). The ethical policy of the University of Firenze complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication no. 85-23, revised 1996; University of Firenze assurance number: A5278-01). Animals were anesthetized with 2% isoflurane before the surgical procedures and sacrifice, which was performed by cervical dislocation. All efforts were made to minimize suffering and reduce the number of animals used. Rats were randomly assigned to each experimental group and individually habituated to handling before testing. Unilateral osteoarthritis was induced by injection of Monoiodoacetate (MIA, Sigma Aldrich) into the tibiotarsal joint. On day 0, rats were slightly anesthetized by 2% isoflurane, the left leg skin was sterilized with 75% ethyl alcohol and the lateral malleolus located by palpation; then, a 28-gauge needle was inserted vertically to penetrate the skin and turned distally for insertion into the articular cavity at the gap between the tibiofibular and tarsal bone until a distinct loss of resistance was felt. MIA dose of 2 mg in 25 µL saline was delivered into the left articular cavity (Mannelli *et al*., 2013). The paw pressure tests (see below) were performed at day 14. GcMAF (25 ng) was i.p. administered. Control rats received 25 µL of saline solution (day 0) in the tibiotarsal joint and saline containing 25 ng of Gc-protein i.p. at day 14. The pain threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy) as described (Mannelli *et al*., 2013). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe (**Fig. 1B**). Pressure was increased until a vocalization or a withdrawal reflex occurred. The withdrawal threshold was expressed in grams, the test was repeated twice and the mean was considered as the value for each paw. In order to avoid biases, the experiments were performed in a blinded manner where the experimenters were not aware of the treatment. It was ascertained that recorded pressure values did not vary when repetitively measured during the experimental session.

3. RESULTS

3.1. Effects of GcMAF on Human Neurons

It is well established that significant neuroanatomical changes occur in ME/CFS and they are consistent with the neuropathologic symptoms that are characteristic of this syndrome. As recently demonstrated, the most evident neuroanatomical alteration in the brain of ME/CFS patients is the reduction of the grey and white matter volume in several anatomical areas of the brain (Puri *et al*., 2012). It can be hypothesized that reduction of grey and white matter volume corresponds to decreased connectivity between neurons with consequent impairment of function as it has been observed in autism (Zikopoulos *et al*., 2013). Therefore, it can be proposed that factors stimulating neuronal viability and metabolism might counteract such a reduction of grey matter volume and connectivity thereby improving clinical symptoms in ME/CFS patients. Having noticed a significant improvement of neurological symptoms in ME/CFS patients (see below), we decided to study the effects of GcMAF on human neurons using a well established in vitro experimental system. To this end we used human SH-SY5Y neuronal cells, a cellular in vitro model considered useful in many areas of neuroscience research (Agholme *et al*., 2010). In fact, these cells represent a model system to study the neurobiology of neurodegenerative diseases (Xie *et al*., 2010) and they are used to evaluate the neuroprotective effects of a variety of substances (Thapa *et al*., 2013). In addition, these cells express the VDR receptor, thus making them suitable to study those effects of GcMAF that

are interconnected with VDR receptor signalling (Celli *et al*., 1999; Thyer *et al*., 2013b). **Figure 2**, shows that GcMAF (8-800 pM) significantly increased neuronal cell viability and metabolic activity in a dose-dependent manner, with significant effects observed even at the lowest pM concentration. These effects on cell viability were associated with dose-dependent intracellular cAMP production (**Table 1**). The results shown in **Fig. 2 and Table 1** as well as those presented in **Fig. 3** were obtained in serum-free medium, thus ruling out the effects of possible confounding factors present in serum (Inui *et al*., 2013).

Increased viability and metabolic activity following GcMAF stimulation as well as cAMP formation were consistent with the morphological changes induced by GcMAF in human neurons and shown in **Fig. 3**. In the absence of GcMAF, SH-SY5Y neuronal cells, stained with haematoxylin-eosin, appeared as small, relatively undifferentiated cells with large nuclei (**Fig. 3A and 3B**). After 24h stimulation with 8 pM GcMAF, several cells showed a significant change in morphology that was consistent with the induction of neuronal differentiation and increased connectivity (**Fig. 3C**). The cytoplasm was enlarged and several cytoplasmic elongations could be observed. After 72 h incubation with 8 pM GcMAF, these morphological changes were more evident and well differentiated cells could be observed (**Fig. 3D**). After incubation with GcMAF, the cells appeared to establish contacts with each other. Taken together these results indicate that GcMAF at pM concentration increases neuronal cell viability, metabolic activity and differentiation, with the first effects being observed at 24 h. Considering the well demonstrated role of cAMP in the induction of SH-SY5Y differentiation (Kume *et al*., 2008), it can be hypothesized that these effects of GcMAF are mediated by the cAMP signalling pathway involving the Extracellular signal-Regulated protein Kinase (ERK) and the nuclear factor kappa B (NF-κB) pathways (Sun *et al*., 2012). As it was recently demonstrated, this latter signalling pathway is strictly interconnected with the VDR signalling pathway in human mononuclear cells (Nakou *et al*., 2010), thus lending credit to the hypothesis that the effects of GcMAF are due to a complex, interconnected, network of intracellular signalling. It is worth noticing that the assay that we used to determine cell viability measures mitochondrial activity and mitochondrial dysfunction is known to be hallmark of ME/CFS (Booth *et al*., 2012). Therefore, the effects of GcMAF described here might directly counteract one of the basic alterations of ME/CFS at the sub-cellular level.

3.2. Effects of GcMAF on Inflammatory Pain

There exist several animal models to study the neurobiology of fatigue and many of them are centered on immunologically induced fatigue (Harrington, 2012). However, it is well assessed that, in addition to fatigue, persistent pain and systemic inflammation are the most debilitating symptoms of ME/CFS. Because of this consideration, we decided to evaluate the effects of GcMAF on an experimental model of persistent pain instead of using a model of fatigue.

In fact, it has been hypothesized that mitochondrial dysfunction is involved in muscle pain and central sensitization is typically observed in these patients (Meeus *et al*., 2013). Therefore, having observed a significant stimulatory effects of GcMAF on mitochondrial activity in human neurons (**Fig. 2**), we decided to study its effects in a well-established model of inflammatory pain that is the osteoarthritic pain induced by MIA (Mannelli *et al*., 2013). It is well assessed that morphological alterations are associated with a persistent inflammatory pain which, starting from the 14th day after MIA injection, possesses a neuropathic component (Ivanavicius *et al*., 2007). Nonsteroidal anti-inflammatory drugs such as diclofenac can reduce MIA-dependent pain during the first inflammatory phase, but they are ineffective in the degenerative neuropathic phase (Fernihough *et al*., 2004), while gabapentin, an antiepileptic molecule widely used to treat neuropathic pain in adult patients (Davis and Srivastava, 2003), is effective (Ivanavicius *et al*., 2007).

The effectiveness of GcMAF was evaluated after acute i.p. administration (25 ng, 15 min before the test). As expected, fourteen days after MIA, the weight tolerated on the ipsilateral paw (MIA), was significantly reduced as compared to the contralateral paw and control animals. The weight tolerated on the ipsilateral paw was 30±4 g, whereas the weight tolerated on the contralateral healthy paw and in control animals was 60±5 g. GcMAF, 15 min after i.p. administration, increased the withdrawal threshold and it was still effective after 24 h. Thus, the weight tolerated on the ipsilateral paw was 55±6 g, that is almost identical to the weight tolerated on the contralateral paw and in control animals. It is worth noticing that GcMAF did not modify the weight tolerated in the contralateral healthy paw, that remained 60±5 g. This demonstrates that the effects of GcMAF were not to be attributed to non-specific

analgesia. In analogy with the results presented in (Ivanavicius *et al*., 2007), it can be concluded that GcMAF counteracts the degenerative neuropathic phase that is responsible for the neuropathic pain.

4. DISCUSSION

ME/CFS is a debilitating disease of multifactorial aetiology with infectious as well as non-infectious factors possibly involved. In fact, it has been observed that symptoms often follow a viral infection or a period of stress even though a causal relationship with any particular type of infectious agent has yet to be defined (Bansal *et al*., 2012). If the aetiology is still ambiguous, the pathogenetic mechanisms, however, appear to be more defined and include immune system dysfunction as well as anatomical changes in certain areas of the brain that control the neurological functions impaired in ME/CFS patients (Puri *et al*., 2012).

GcMAF is a protein that controls a number of events at the molecular and cellular level resulting in a variety of biological effects that have been exploited to treat several clinical conditions, ranging from cancer to autism (Yamamoto *et al*., 2008c; Bradstreet *et al*., 2012). The rationale for using GcMAF in such diverse clinical conditions stems from the observation that in those conditions endogenous production of GcMAF is impaired by elevated serum levels of Nagalase. Nagalase is an enzyme whose activity is elevated in the serum of cancer patients as well as in the serum of patients with viral infections or autoimmune diseases (Yamamoto *et al*., 2009). Nagalase impairs endogenous production of GcMAF because it deglycosylates vitamin D-binding protein, the precursor of GcMAF, thus preventing the conversion of vitamin D-binding protein to active GcMAF (Yamamoto *et al*., 1997). Recent evidence, however, suggests that the therapeutic results observed with GcMAF are due not only to macrophage activation, but also to other direct effects of GcMAF on cell signalling and metabolism. We recently proposed that these effects may be explained considering the interconnection between GcMAF and VDR signalling pathways (Thyer *et al*., 2013b). The results presented in this study suggest a further interconnection with other signalling pathways that include ERK and NF-κB. This complex web of intracellular signalling could then be responsible for the diversified effects of GcMAF in different cell types and therefore, in different diseases.

In this study we demonstrate for the first time a direct effect of GcMAF on human neurons. In fact, GcMAF significantly improves human neuronal cell viability and metabolism and efficiently counteracts inflammatory/neuropathic pain in experimental animals. Considering that neurological symptoms, inflammation and persistent pain are the most debilitating features of ME/CFS, the results reported here explain the therapeutic effects reported by clinicians and patients using GcMAF in ME/CFS. In fact, several clinical observations on the efficacy of GcMAF in ME/CFS have been presented at international congresses. Among these, the results presented at the biennial meeting of the International Association for CFS and ME, held in Ottawa, Ontario, in 2011 (CFS, 2011) and those presented by researchers and patients at the GcMAF Immunology Conference, 2013 in Frankfurt, Germany. Consistent with these congress presentations, a very recent peer-reviewed paper reported the therapeutic effects of GcMAF on a variety of neurological diseases, including ME/CFS (Thyer *et al*., 2013a).

The results described in the present study suggest that the therapeutic efficacy of GcMAF in ME/CFS as well as in other cognate conditions such as autism (Bradstreet *et al*., 2012) in addition to immuno-modulation, may be ascribed also to direct effects on neurons and neuropathic pain.

The results described in this study and the clinical observations quoted above raise two questions:

- Is GcMAF able to cross the blood brain barrier and act directly on central nervous system neurons increasing their metabolic activity and restoring connectivity?
- Which is the mechanism underlying the rapid effects of GcMAF on inflammatory pain?

Although we have no experimental data to answer the first question, it could be hypothesized that the interconnection between GcMAF and VDR signaling could favor transport through the blood brain barrier. In fact, transport of macromolecules across the blood brain barrier requires both specific and nonspecific interactions between macromolecules and proteins/receptors expressed on the luminal and/or the abluminal surfaces of the brain capillary endothelial cells (Xiao and Gan, 2013). Since VDR signaling appears to enhance brain to blood transport at the blood brain barrier through both genomic and non-genomic actions, it can be hypothesized that the

interaction between GcMAF and VDR (Thyer *et al*., 2013a) may favor GcMAF transport into the brain.

The interconnection between GcMAF and VDR signaling pathways might also be responsible for the rapid effects of GcMAF on inflammatory neuropathic pain observed in rats with MIA-induced osteoarthritis. In fact, a recent report demonstrated that activation of VDR was associated with significant reduction of musculoskeletal pain associated with acute phase inflammatory response in women (Catalano *et al*., 2012).

5. CONCLUSION

The results presented here confirm at the experimental level the therapeutic effects of GcMAF in ME/CFS and elucidate the molecular mechanisms of action through which GcMAF might be responsible for such therapeutic effects.

6. ACKNOWLEDGMENT

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6.1. Potential Conflicts of Interests

David J. Noakes is the CEO of Immuno Biotech, Ltd (the company isolating and purifying the GcMAF protein). In order to avoid biases, the experiments were performed in blind and the experimenters, including DN, were not aware of the treatment.

Since November 1st, 2013, Marco Ruggiero is consulting scientific director of Immuno Biotech Ltd.

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