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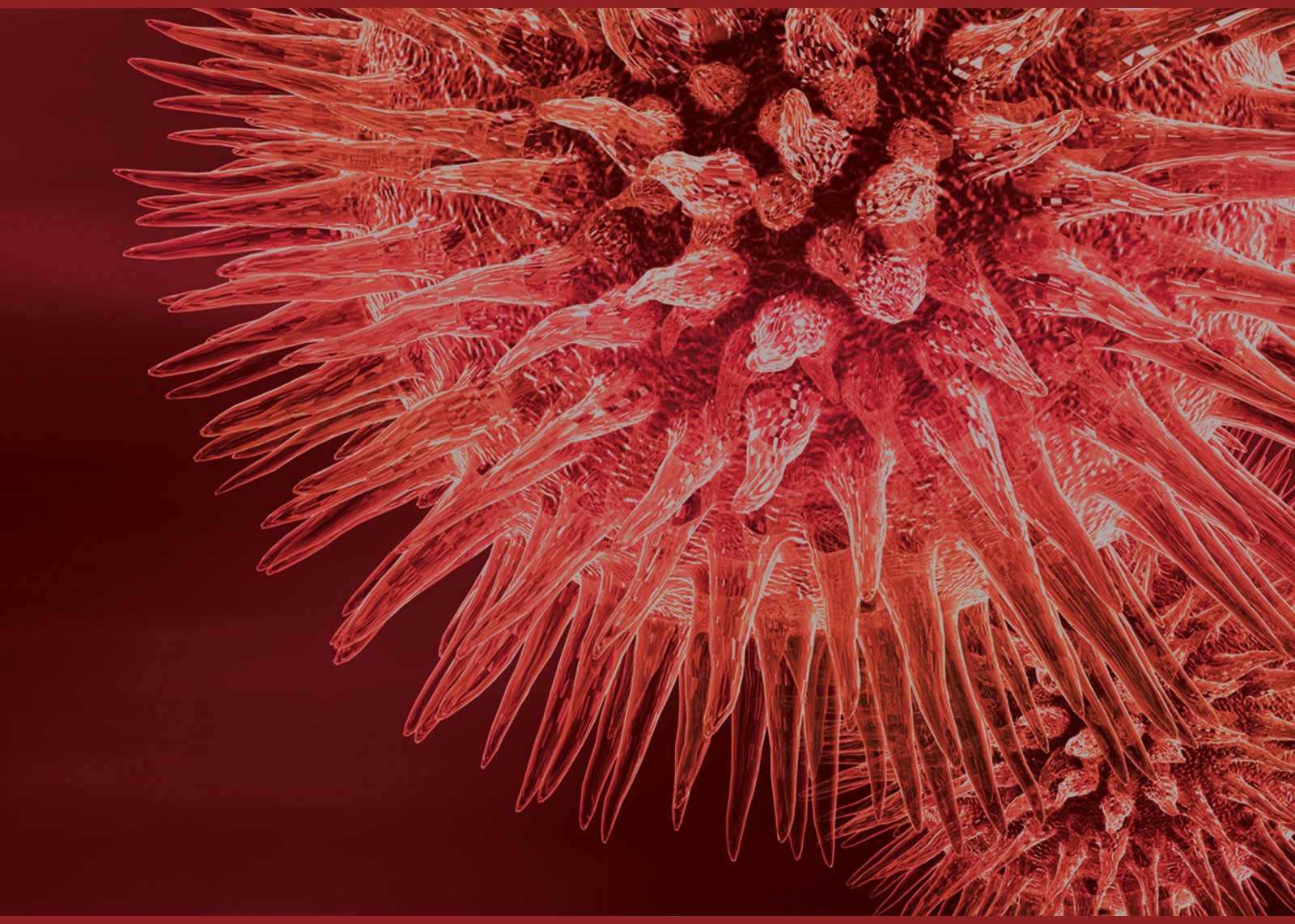
Neuroinflammation and Neurodegeneration: Pinpointing Pathological and Pharmacological Targets

Guest Editors: Antonio Carlos Pinheiro de Oliveira, Eduardo Candelario-Jalil, Bernd L. Fiebich, Magda da Silva Santos, András Palotás, and Helton José dos Reis



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Contents

Peripheral Administration of Tumor Necrosis Factor-Alpha Induces Neuroinflammation and Sickness but Not Depressive-Like Behavior in Mice, Steven Biesmans, Jan A. Bouwknecht, Luc Ver Donck, Xavier Langlois, Paul D. Acton, Patrick De Haes, Nima Davoodi, Theo F. Meert, Niels Hellings, and Rony Nuydens
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Editorial

Neuroinflammation and Neurodegeneration: Pinpointing Pathological and Pharmacological Targets

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For many years, the brain had been regarded as an immune-privileged organ because of an old tenet which stated that no classical immune activation or inflammation could take place intrathecally. However, this theory has quickly changed with the advent of several studies demonstrating that the central nervous system (CNS) is in fact immunologically specialized [1, 2].

Neuroinflammation has been viewed as a double-edged sword: it not only is essential for the recovery from a number of conditions, but also may play detrimental roles in neurodegenerative processes. In such disorders inflammation can be set off by versatile triggers: protein aggregates, mediators released from injured neurons, accumulation of abnormally modified cellular components, and suppression of mechanisms that would normally control inflammatory processes, just to mention a few [3].

Given the increased life-expectancy, the incidence of neurodegenerative diseases is steadily rising. In light of this, research into this large segment of neuropsychiatry is a top priority around the globe, and one of the main areas of focus is to understand neuroinflammation that underlies, at least in part, the most common degenerative conditions of the brain: Alzheimer's dementia, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's chorea, and many others [4–6]. By addressing intrathecal inflammation, some of these disorders could be prevented or even successfully treated.

This special issue compiles original articles and reviews dissecting various pharmacological targets of inflammation that may serve as a springboard for opening innovative therapeutic avenues and could be germane to advanced research in neurodegenerative disorders.

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Research Article

Cocaine Causes Apoptotic Death in Rat Mesencephalon and Striatum Primary Cultures

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To study cocaine's toxic effects *in vitro*, we have used primary mesencephalic and striatal cultures from rat embryonic brain. Treatment with cocaine causes a dramatic increase in DNA fragmentation in both primary cultures. The toxicity induced by cocaine was paralleled with a concomitant decrease in the microtubule associated protein 2 (MAP2) and/or neuronal nucleus protein (NeuN) staining. We also observed in both cultures that the cell death caused by cocaine was induced by an apoptotic mechanism, confirmed by TUNEL assay. Therefore, the present paper shows that cocaine causes apoptotic cell death and inhibition of the neurite prolongation in striatal and mesencephalic cell culture. These data suggest that if similar neuronal damage could be produced in the developing human brain, it could account for the qualitative or quantitative defects in neuronal pathways that cause a major handicap in brain function following prenatal exposure to cocaine.

1. Introduction

Drug abuse can have physiological, psychological, and social consequences [1]. Cocaine is a drug of abuse with reinforcing proprieties that can lead to the development of dependence. By binding to plasma membrane transporters, cocaine pre vents the uptake of extracellular monoamines, consequently enhancing their extracellular levels, including norepinephrine and dopamine [2–4].

The vast majority of developmental studies investigating cocaine effects have focused on the dopaminergic system, presumably as a result of dopamine's well-studied effects on reward and addiction [5]. The primary mesencephalic culture contains dopaminergic neurons from both the substantia nigra and ventral tegmental area, which expresses tyrosine hydroxylase (TH) [6, 7], the rate-limiting enzyme in dopamine synthesis. Dopaminergic afferents from substantia nigra pars compact provide dense innervations to the striatum [8, 9]. Given the reinforcing properties of cocaine such mesencephalic structures have been extensively investigated.

Besides its reinforcing properties, cocaine can cause damage to the CNS [10], being associated with cerebrovascular pathologies and convulsions that on occasion may be lethal [11]. More subtle functional and physical impairments may also be evident. Clinical and preclinical studies show learning and memory impairments, as well as the presence of movement disorders, following cocaine abuse, even after long periods of drug withdrawal [12, 13].

Cocaine can cross the placenta and accumulate in the fetus [14], with cocaine effects being especially evident in the newborns of females that abused cocaine during pregnancy. Maternal cocaine use during pregnancy is associated with significant impairment of cognitive development [15–17] that is detectable during the first two years of life and which may continue to contribute to learning difficulties and attentional dysfunction during later childhood [18]. In addition to the direct effects of cocaine, cocaine has a number of metabolites,

which will be present in the mother and fetus and which have a number of biological effects, including local anesthesia [19] and the inhibition of monoamine transporters [20], as well as vascular effects [21] and seizure induction [22].

Prior work on the effects of gestational cocaine has shown apoptosis in the fetal heart [23], decreased birth weight and head size, and deficits in cognition, attention, and language development in childhood [24, 25]. The prenatal cocaine exposure can result in molecular adaptations or anatomy changes in specific brains regions, including the hippocampus and cortex [26, 27]. The mechanisms underlying the damage caused by cocaine may involve a number of factors, including mitochondrial dysfunction, toxicity from dopamine metabolism, and/or reactive oxygen species (ROS) formation [28]. The nature of any subsequent cell death may be via either apoptotic or necrotic cell death processes.

The aim of this study was to determinate the toxicity of cocaine in two different types of primary culture, striatal and mesencephalic. To our knowledge, this is the first study showing cocaine to cause cell death in such cultures. It is of note that the cocaine concentrations in this study are comparable to those of previous investigators, although in different cell types [29–32], as well as in the plasma of human drug abusers, ranging between 0.3 μM and 1 mM [30].

2. Materials and Methods

2.1. Primary Mesencephalic/Striatal Cultures. Primary cultures were cultured as previously described [33]. In brief, the mesencephalon or striatum of Sprague-Dawley rat embryos on embryonic day 17 was isolated and digested with 0.5 mg/mL trypsin in Earle's Balanced Salt Solution (EBSS) (Life Scientific) for 2 hr at 37°C with 5% CO_2 and plated on poly-L-lysine (Sigma) coated glass coverslips on plastic culture dishes (MatTek), at a density of 1×10^6 cells/mL in high glucose Dulbecco's minimum essential medium (DMEM) supplemented with 10% bovine calf serum, 25 U/mL penicillin, 25 mg/mL streptomycin, and 2 mM glutamine (Invitrogen). These mixed neuronal/glial cultures were treated with cocaine hydrochloride (Sigma) 1.0 mM or phosphate saline buffer (PBS) as control, on day 9 *in vitro*, for 24 hours. The chosen cocaine concentration was based on previous studies [34].

2.2. Immunostaining. On day 10, after 24 hours *in vitro*, neurons were identified by staining with anti-MAP2 (1 : 100; Sigma) or anti-NeuN (1 : 100; Chemicon; MAB 377) [33, 35]. Unless otherwise stated, each experiment described below was repeated at least three times, and >100 neurons were scored for each condition on triplicate coverslips. After 24 hours of cocaine exposure, cultures were fixed with 4% paraformaldehyde (Sigma) in PBS and permeabilized with 0.1% Triton X-100. After blocking nonspecific binding with PBS plus 3% BSA and 3% fetal bovine serum, the cells were incubated with antibodies to identify neurons (anti-MAP2 or anti-NeuN) followed by secondary Alexa Fluor 594 goat anti-mouse antibodies (1 : 100; Molecular Probes). In the last wash step, Hoechst 33324 (1 g/mL) was added to assess nuclear morphology. Hoechst 33342 is a UV-excitable nucleic acid stain readily taken up by all cells. Its blue fluorescence is particularly bright in the condensed nuclei of apoptotic cells. Typically, several hundred cells were scored in each experiment using fluorescent microscopy.

2.3. TUNEL Assay. Cells with DNA fragmentation were detected by the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) method using the "*in situ* cell death detection-fluorescein kit" (Roche).

2.4. Statistical Analysis. Data were obtained from three independent experiments. In each experiment three replicate samples were quantified. Statistical comparisons were made by Student's *t-test* for single comparisons. All values of $P < 0.05$ were considered statistically significant.

3. Results

To characterize the primary striatum culture, on day 10 *in vitro*, we observed the expression of GABAergic neurons that were stained with anti-GAD65/67 [36]. Our results demonstrated that 90% of the neurons present in the culture were GABAergic neurons (data not shown). We also tested for the presence of dopaminergic neurons by antibody staining to identify tyrosine hydroxylase (TH), the rate-limiting enzyme in the dopamine synthetic pathway. The results showed that there was no sign of striatal neurons expressing TH (data not shown). The mesencephalic culture was positive for TH, indicating that our mesencephalic culture comprises 10% dopaminergic neurons, which is characteristic of mesencephalic cultures [6].

In the primary striatal culture, control neurons exhibited normal chromatin, showing only 3% cell death. In contrast, after cocaine treatment (1.0 mM, 24 hours), neurons manifested an increase in bright/condensed Hoechst 33342 fluorescence, with evidence of 10% cell death (Figures 1(a) and 1(b)). We also observed, as indicated by MAP2 and NeuN staining, that neurite extension was inhibited after cocaine treatment (Figure 1(a)).

Similarly, in the primary mesencephalic culture, treatment with cocaine (1.0 mM) for 24 hours caused a decrease in neuronal viability coupled to an inhibition of neurite prolongation (Figures 2(a) and 2(b)).

The TUNEL assay confirmed that cocaine caused apoptotic death in both striatal and mesencephalic cultures (Figures 3(a), 3(b), 4(a), and 4(b)).

4. Discussion

Cocaine abuse can lead to toxic effects, including causing damage in specific brain areas. Studies in humans [37, 38], animals [39], and cell cultures [40, 41] have shown the toxic effects of cocaine, which can lead to cell death. Neuronal death during CNS development can change the organization of synaptic connectivity, leading to developmental and behavioral abnormalities in the offspring. Previous work shows cocaine to modulate the development [42–44] and survival [43–45] of CNS cells.

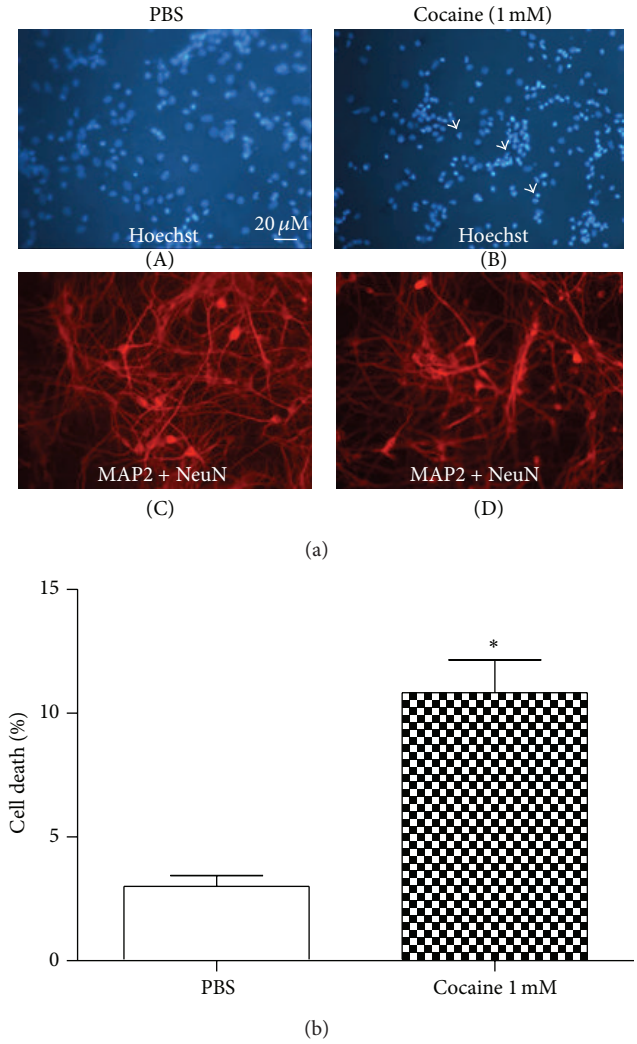

Figure 1: (a) Immunostaining of striatal primary cells treated with PBS (left panel) or treated with cocaine 1.0 mM (right panel) for 24 hours. Neurons were labeled with MAP2 and NeuN (red label). Hoechst 33342 (blue label) was added to monitor chromatin condensation. Arrows indicate dying neurons. Staining was observed under a fluorescent microscope. The treatment with cocaine caused a decrease in neuronal viability and an inhibition of neurite prolongation. (b) Percentage of cell death observed by immunostaining of striatal primary cultures treated with PBS and cocaine for 24 hours. Values are mean ± SEM from five independent experiments. The treatment with cocaine caused a decrease in the viability of the neurons. *Significantly different from the control (PBS) value: $^{*}P < 0.05$ by Student's *t-test*.

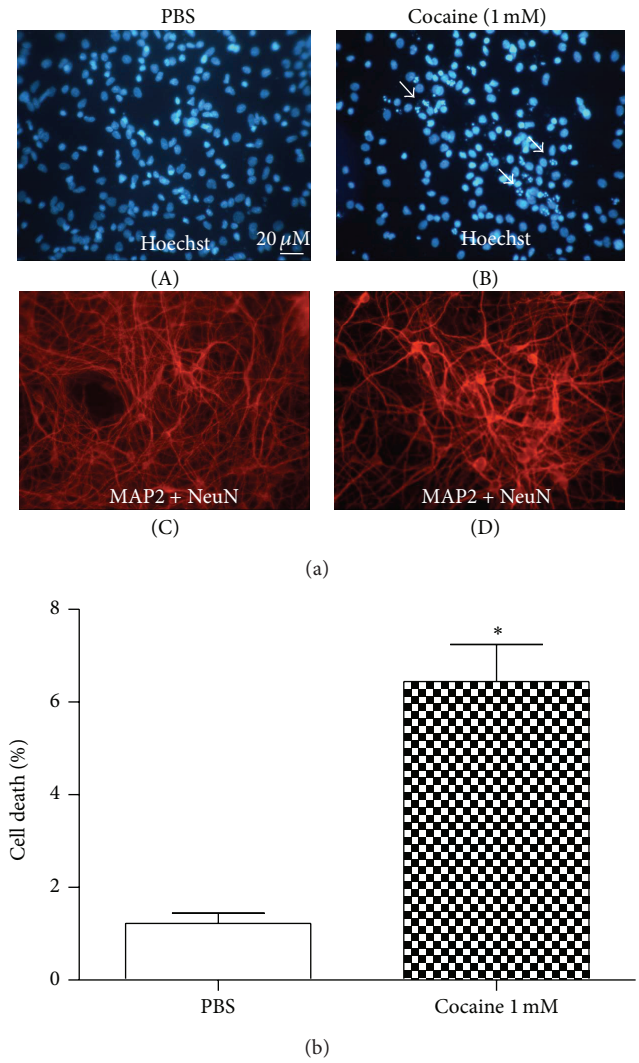

Figure 2: (a) Immunostaining of mesencephalic primary cells treated with PBS (left panel) or treated with cocaine 1.0 mM (right panel) for 24 hours. The neurons were labeled with MAP2 and NeuN (red labels). Hoechst 33342 (blue label) was added to monitor chromatin condensation. Arrows indicate dying neurons. Staining was observed under a fluorescent microscope. Cocaine treatment caused a decrease in neuronal viability. (b) Percentage of cell death observed by immunostaining of the mesencephalic primary culture treated with PBS and cocaine for 24 hours. Values are mean ± SEM from four independent experiments. Cocaine treatment decreased neuronal viability. *Significantly different from the control (PBS) value: $^{*}P < 0.05$ by Student's *t-test*.

The present study demonstrates that cocaine decreases neuronal survival in primary striatal and mesencephalic cultures, two different brain regions relevant to cocaine's mechanism of action. Most neurons in striatal culture are GABAergic, with some cholinergic neurons. Also, striatal cultures of primary neurons express functional D_1 and D_2 dopamine receptors [46, 47] as well as the dopamine transporter [7]. We also observed morphological changes in both cultures, characterized by chromatin condensation and DNA fragmentation, which indicates a process of apoptosis. In our model, striatal neurons in cell culture do not express the TH enzyme, the rate-limiting enzyme in dopamine synthesis, suggesting that this culture cannot produce dopamine. However, mesencephalic neurons in culture did express TH and therefore produce dopamine. Given the cocaine toxicity in both cultures, this suggests that cocaine's toxic effect may be regulated by dopamine, but also possibly by an array of signaling through multiple and diverse secondary messenger system(s).

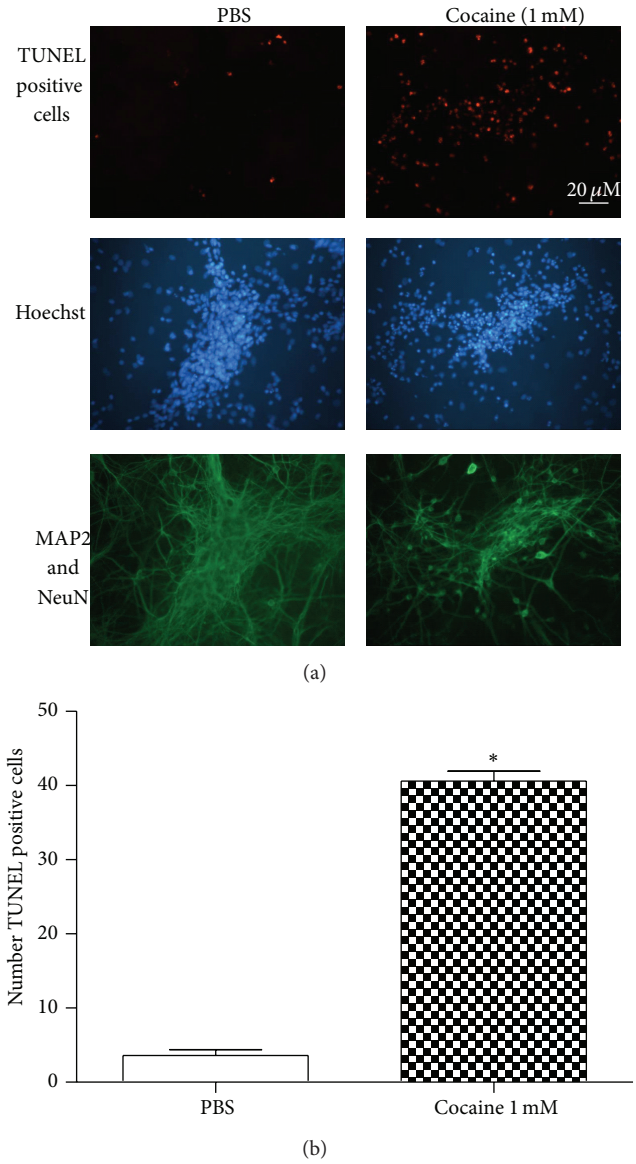

Figure 3: (a) *In situ* histochemical evidence of DNA fragmentation after cocaine exposure. Striatal cultures were first established for 7 days and incubated with cocaine (1.0 mM) for 24 hours. After cells were fixed, the TUNEL method was performed. Cultures were photographed at the level of the neuronal layer. Note the labeling in the vast majority of treated cells, in contrast with the labeling of a few control cells. TUNEL positive cells were dUTP labeled (brown label). The neurons were labeled with MAP2 and NeuN (green label) and Hoechst 33342 (blue label) was added to monitor chromatin condensation. (b) Number of TUNEL positive cells observed by immunostaining of the striatal primary culture treated with PBS or cocaine for 24 hours. Values are mean ± SEM from five independent experiments. Cocaine treatment decreased neuronal viability. *Significantly different from the control (PBS) value: $^{*}P < 0.05$ by Student's *t-test*.

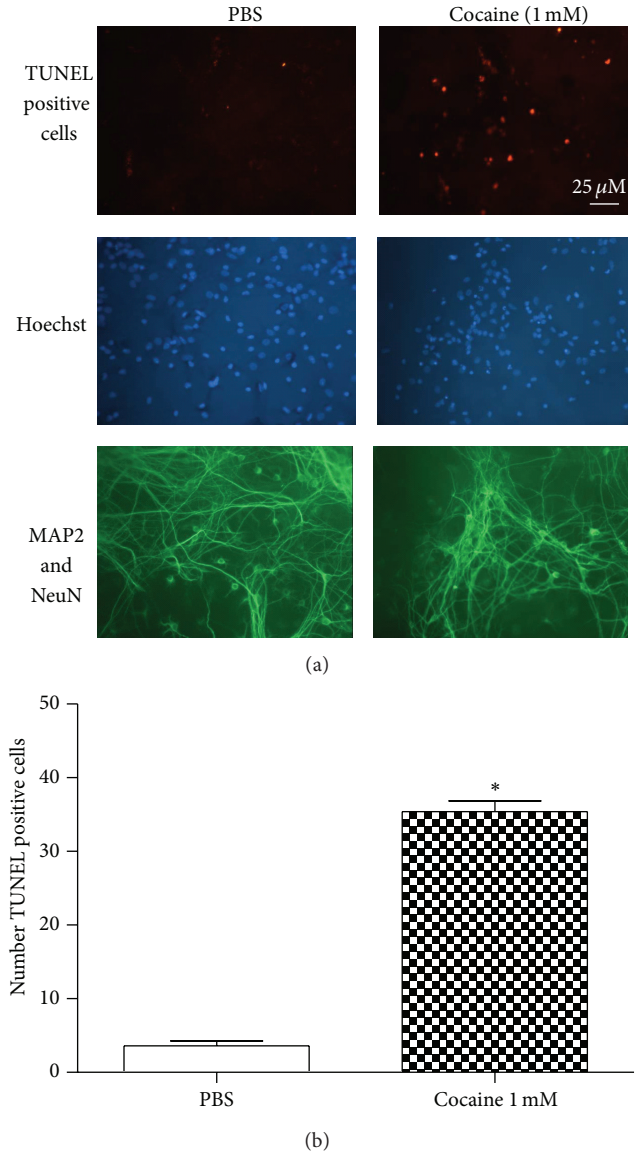

Figure 4: (a) Mesencephalic cultures were first established for 7 days and incubated with cocaine (1.0 mM) for 24 hours. After cells were fixed, the TUNEL method was used. Cultures were photographed at the level of the neuronal layer. Note the labeling in the vast majority of treated cells, in contrast with the labeling of a few control cells. TUNEL positive cells were dUTP labeled (brown label). The neurons were labeled with MAP2 and NeuN (green label) and Hoechst 33342 (blue label) was added to monitor chromatin condensation. (b) Number of TUNEL positive cells observed by immunostaining of the mesencephalic primary culture treated with PBS or cocaine for 24 hours. Values are mean ± SEM from five independent experiments. Cocaine treatment decreased neuronal viability. *Significantly different from the control (PBS) value: $^{*}P < 0.05$ by Student's *t-test*.

We showed that the exposure of both primary mesencephalic and striatal culture neurons to cocaine evoked an apoptotic process. Apoptosis has also been reported by some authors in other models but not always [48–50]. It might be that the great variability in the physiological and functional effects of cocaine on developing CNS is due to the multiple biochemical and pathophysiological routes of cocaine's actions. For example, dopamine and 5-hydroxytryptamine (serotonin) transporter knock-out mice

still continue to exhibit drug seeking behavior, suggesting the involvement of additional molecular pathway for cocaine action, besides blocking monoamine neurotransmitter transporters [4]. Studies demonstrated that GABA transmission in the nucleus accumbens is also altered after withdrawal from repeated cocaine [51]. At higher concentrations, cocaine can act as a local anesthetic, interacting with a variety of targets in both specific and nonspecific manners.

Chronic cocaine treatment can modulate voltage-gated Na^+ and Ca^{2+} channels activity via the production of cyclic AMP by DA_1 receptor stimulation [52, 53]. Also, direct modulation of ion channels can be responsible for some cocaine effects. It has been shown that cocaine can block voltage-dependent Na^+ channels [54] and Ca^{2+} channels [53]. Other ion channels are modified by cocaine including the K^+ channels activated by acetylcholine and adenosine [55]. Ca^{2+} and K^+ channels are involved in the repolarization and after-hyperpolarization phases of the action potential. The magnitude and duration of the after hyperpolarization phase determine the rate of neuronal firing. Blockade of the Ca^{2+}-activated-K^+ channels may facilitate repetitive neuronal firing that may enhance the propensity to induce seizures and neuronal function during cocaine overdose [56]. Blockade of K^+ could also underline a variety of effects mediated by cocaine, including increased Ca^{2+} influx at the presynaptic terminal, which can augment neurotransmitter and hormone release and can contribute to neurodegenerative processes. As such ionic regulation may be a significant mediator of cocaine's neurotoxicity. Although we have no evidence of the presence of cocaine metabolites (ecgonine, ethyl ecgonine, and ecgonine methyl ester) in these cultures, we cannot rule out that they could also be involved in the mechanism of cell death. However, previous work showed that only cocaine significantly decreased MAP2 content in cortical culture [57]. This could suggest that the apoptotic cascade might require the intracellular penetration of cocaine.

We also observed an inhibition of the neurite outgrowth in the cells exposed to cocaine. This could be due to the influence of cocaine on cytoplasmic calcium, thereby affecting the cytoskeletal network and altering neuronal regulation. Cocaine may target cytoskeleton proteins, particularly microtubule associated proteins (MAPs) [58] and actin filaments, altering the process of initiation, elongation, and turning of neuritic branches [59]. Cocaine can also act to modulate integrin structure and functions, thereby contributing to decreased neurite outgrowth. Nonintegrin ligands can alter neuronal integrin expression, with consequences for neurite outgrowth [60].

Maternal gestational cocaine abuse can cause damage to their offspring. Since the migration of neurons ultimately determines their connectivity, synaptic potential, and viability, altered neuronal migration may be a significant determinant of the consequences of maternal gestational cocaine use in the offspring. Here we demonstrate, for the first time, that an acute dose of cocaine can cause the apoptosis of primary striatal and mesencephalic culture cells after 24 hours. Further investigation as to the biological underpinnings of cocaine's effects is likely to contribute to the etiology, course, and treatment of the consequences of maternal gestational cocaine abuse in the offspring.

Conflict of Interests

The authors declare that there is no conflict of interests for any of the authors.

Acknowledgments

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Research Article

Intellectual Impairment in Patients with Newly Diagnosed HIV Infection in Southwestern Nigeria

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Neurocognitive impairment is a detrimental complication of HIV infection. Here, we characterized the intellectual performance of patients with newly diagnosed HIV infection in southwestern Nigeria. We conducted a prospective study at Owo Federal Medical Center by using the adapted Wechsler Adult Intelligence Scale (WAIS). The raw scores were converted to standardized scores (z-scores) and correlated with clinical and laboratory findings. Fifty-eight HIV positive patients were recruited; 72% were in WHO stages 3 and 4. We detected a high rate of intellectual impairment in HIV positive patients and controls (63.8% and 10%, resp.; $P < 0.001$). HIV positive patients performed worse throughout the subtests of both verbal and performance intelligence quotients. Presence of opportunistic infections was associated with worse performance in the similarities and digit symbol tests and performance and full scale scores. Lower body weight correlated with poor performance in different WAIS subtests. The high rate of advanced disease stage warrants measures aimed at earlier diagnosis and treatment. Assessment of neurocognitive performance at diagnosis may offer the opportunity to improve functioning in daily life and counteract disease progression.

1. Introduction

Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) continue to take their toll on the life of the patients and their caregivers. Sub-Saharan Africa is the most heavily affected region in the world with estimated 1.9 of the 2.5 million new HIV infections worldwide in 2011 [1]. Most of the fatal courses remain in the context of late diagnosis of HIV infection and delayed initiation of antiretroviral therapy (ART) [2, 3].

A major concern for public health is the occurrence of HIV-associated neurocognitive disorders (HAND) [4]. The manifestations range from asymptomatic or mild neurocognitive impairment to HIV-associated dementia. Intellectual impairment in HIV infection may cause unemployment, susceptibility for systemic disease, and impaired quality of life [5, 6]. Gender and drug-related risk behavior were identified as risk factors for HIV-associated neurocognitive impairment and an inverse correlation with adherence to ART was reported [7, 8]. This is detrimental, as compliance with ART can achieve almost a normal life span and physical and emotional health. In resource-poor settings, however, diagnosis as well as initiation and maintenance of ART have

been associated with major challenges. Notably, undetected cognitive impairment can increase the risk of poor medication adherence and impair social and academic functioning [9].

Nigeria has the second largest number of inhabitants living with HIV worldwide, and a prevalence rate of 3.6% was calculated for 2011 [2]. For Nigeria as for Sub-Saharan Africa, there is paucity of information on neurocognitive functioning in individuals with newly diagnosed HIV infection. The first step was done by Royal III and coworkers, who established a neuropsychological testing battery and reported a rate of 29% for abnormal performance among 60 HIV positive ART-naïve Nigerian patients [10]. None of the previous studies in the region used the Wechsler Adult Intelligence Scale (WAIS) so far. The test is designed to measure intelligence-related domains in adults and older adolescents [11]. The main advantage of the WAIS series is the comprehensive nature of the tests and includes different subtest of verbal intelligence quotient (IQ) and performance IQ. Results of both scores are summarized as full scale IQ.

The aim of this study was to determine pattern and severity of intellectual impairment as well as risk factors among patients with newly diagnosed HIV infection in southwestern Nigeria.

2. Material and Methods

2.1. Ethics Approval. The Ethical Committee of Owo Federal Medical Center gave approval for the study. Each participant signed the consent form and the study was conducted in accordance with the Declaration of Helsinki (1964).

2.2. Setting. The study was performed at Owo Federal Medical Centre. This tertiary care facility was appointed by the federal government as a HIV competence center for southwestern Nigeria. The major aim is the provision of free access to medical care and therapies including ART and laboratory investigations.

2.3. Study Population. This prospective trial recruited patients with newly diagnosed HIV infection. The patients either presented to the outpatient clinic or were newly admitted to the medical ward. The inclusion criteria were as follows: age greater than 16 years, completion of at least primary school education, as some of the test items require the patient to be literate and proficient in English language to understand the test items, and positive HIV serostatus. Patients already on ART were excluded. Further exclusion criteria were the presence of behavioral problems that would interfere with comprehension of the test items, current use of psychoactive drugs/alcohol abuse, and history of other neurological disorders not attributable to HIV infection such as stroke, Parkinson's disease, or epilepsy. A psychiatric disorder or current intake of antipsychotic drugs, anemia (hematocrit < 20%), severe functional impairment (Karnofsky performance score < 50), or severe medical illness that would interfere with the ability to undergo neuropsychological evaluation were further exclusion criteria.

The control group consisted of seronegative patients attending the outpatient clinic of the hospital. Applicable exclusion criteria were used for the seronegative controls.

2.4. Clinical Examination. The patients were staged for HIV infection using the World Health Organization (WHO) rating system. A questionnaire was used for sociodemographic data and medical history. We assessed the presence of opportunistic infections in HIV infected patients. Pulmonary tuberculosis was diagnosed with a chest X-ray and sputum examination. Empirical clinical response to antitoxoplasmosis drugs was taken as evidence of toxoplasmosis infection.

2.5. Laboratory Testing. Blood samples were collected for packed cell volume determination (hematocrit) and CD4 lymphocyte count was assessed using automated flow cytometry.

2.6. Adapted Wechsler Adult Intelligence Scale (WAIS). The intellectual performance was assessed in English with the adapted WAIS, version of 1955. This test was validated in a cohort of Nigeria Technical College students and was previously used to study Nigerian patients with epilepsy [12, 13]. The adaptation of the WAIS was based on previous work by D. E. Itsuokor and modified to suit the Nigerian population (unpublished Ph.D. thesis, University of Ife, Nigeria, 1981). The items modified were as follows: information items (1, 6, 7, 9, 10, 14, 17, 19, 20, 21, 24, 25, 27, 28), comprehension items (7, 11, 12, 13, 14), arithmetic items (3, 4, 5, 7, 9, 10, 13), vocabulary items (4, 17, 37, 38, 40), and a picture completion item (13). The most recent WAIS IV was not utilized because it has not been validated for the Nigerian population.

The subcategories of verbal IQ include the verbal comprehension index (CI) and the working memory index (WMI), whereas perceptual organization index (POI) and processing speed index (PSI) assess the performance IQ. Initially, the raw scores were recorded. The mean and standard deviation of the controls on each WAIS variable were calculated and this served as the basis for converting the raw scores of all the entire 108 participants to standard (z) scores for each of the WAIS subtests.

2.7. Statistical Analysis. Data were analyzed by SPSS version 21.0 software (SPSS Inc., Chicago, IL). Student's t-test was used to compare continuous variables and Fisher exact test (chi-square) to compare categorical variables. Linear regression was used to evaluate association of cognitive impairment with clinical and laboratory variables. Probability levels < 0.05 were taken as significant.

3. Results

3.1. Patients and Demographics. We enrolled 58 HIV positive patients (men 58.6%) and 50 controls (men 64%). Ethnical background was Yoruba in the majority (76%) and 9% were Igbos and 2% Hausa. The remainder were from minority tribes. Details of the sociodemographics, WHO stage, and comorbidities are shown in Tables 1 and 2. There was

Table 1: Sociodemographics.

Variables	HIV $N = 58$	Controls $N = 50$	P
Sex	N (%)	N (%)	
Men	34 (59)	32 (64)	ns
Women	24 (41)	18 (36)	
Duration of education			
Mean ± SD (years)	12.4 ± 3.3	13.3 ± 2.1	ns
Age (years)			
Mean ± SD	35.9 ± 8.0	35.4 ± 11.5	ns

ns = not significant.
SD = standard deviation.

Table 2: Clinical and laboratory finding in HIV positive patients.

	N	(%)
Packed cell volume (PCV)		
20–29	28	50.0
30–39	25	44.6
40–50	3	5.4
CD4 cell count (cells/μL)		
>200	35	60.3
200–349	14	24.1
350–499	5	8.6
≥500	4	6.9
Presence of opportunistic infections		
PTB	16	27.6
Oral candidiasis	4	6.9
CNS toxoplasmosis	2	3.5
PTB + oral candidiasis	6	10.3
Herpes skin infection + PTB	1	1.7
None	29	50.0
WHO HIV stage		
1	5	8.6
2	11	19.0
3	36	62.0
4	6	10.3
Weight (kg)		
30–39	6	11.1
40–49	16	29.6
50–59	21	38.9
60–69	9	16.7
≥70	2	3.7

a one-year higher average education in the HIV-control group. Statistical analysis did not reveal differences among the groups.

3.2. Laboratory Findings. Half of the HIV positive patients had mild to moderate anemia. Sixty % had a CD4 count <200 cells/UL. Opportunistic infections were present in WHO HIV stages 3 and 4 in 50% and 72%, respectively.

3.3. Intellectual Performance. Neurocognitive impairment as evidenced by a full scale z-score below one SD was present in 63.8% of the HIV infected patients and 10% of the controls ($P < 0.001$). Further details are shown in Table 4. HIV infected patients performed worse compared to the controls on WAIS in different domains including verbal and performance scores ($P < 0.001$). We found significantly lower mean z-scores for majority of the WAIS subtests. A large Cohen's effect size (d) was found in all analyses. Details are shown in Table 3.

3.4. Relationship of Gender and Intellectual Impairment. No gender-related neurocognitive differences were detected. Intellectual impairment was present in 100% of females and 97% of males in the HIV infected patients while intellectual dysfunction was present in the 61.1% of females and 59.4% of males in the control group.

3.5. Analysis of Intellectual Performance and Clinical/Laboratory Variables

Anemia. The PCV did not correlate with the verbal score, performance score, and full scale score. In the WAIS subtests, there was a correlation for the performance on the picture arrangement subtest and PCV ($P < 0.05$); further details are shown in supplements 1–4 in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/185891.

CD4+ Cell Count. There was no statistically significant association between the CD4 count and the patients' performance on the WAIS subtests, verbal scores, performance scores, and full scale scores.

Presence of Opportunistic Infections (OIs). HIV positive patients with opportunistic infections performed significantly worse than those without OIs on similarities and digit symbol test, which was reflected by lower performance score and full scale scores ($P < 0.05$). Subgroup analysis of the intellectual function of HIV positive patients with opportunistic infections showed that there was no significant difference among the various opportunistic infection categories ($P > 0.05$).

WHO HIV Stage. There were no significant associations between the WAIS subtests mean scores, verbal score, performance score, full scale score, and WHO HIV stage of the patients.

Weight. Weight was analyzed separately for men and women. Men in the weight range 60–69 kg performed better than in the other weight categories on similarities, pictures completion, performance score, and full scale score subtests of the WAIS ($P < 0.05$). Women in the weight range 60–69 kg performed significantly better on comprehension and similarities subtests compared to women in lower weight categories ($P < 0.05$). There was no significant association between the weight of patients with HIV infection and other WAIS subtests.

Table 3: Analysis of WAIS means z-scores in HIV positive patients and controls.

Tests	HIV+ ($N = 58$) Means ± SD	Controls ($N = 50$) Means ± SD	t	d	P
WAIS (verbal)					
Information	−0.77 ± 0.62	0.00 ± 1.00	−4.913	0.94	<0.001
Comprehension	−0.73 ± 0.90	0.00 ± 1.00	−4.010	0.77	<0.001
Arithmetic	−0.57 ± 0.59	0.00 ± 1.00	−3.634	0.71	0.001
Similarities	−0.85 ± 0.62	0.00 ± 1.00	−4.857	1.04	<0.001
Digit spam	−0.88 ± 0.82	0.00 ± 1.00	−5.666	0.94	<0.001
Vocabulary	−0.55 ± 0.61	0.00 ± 1.00	−3.529	0.68	0.001
WAIS (performance)					
Digit symbol	−1.32 ± 0.95	0.00 ± 1.00	−7.037	1.36	<0.001
Picture completion	−1.02 ± 0.47	0.00 ± 1.00	−6.962	1.34	<0.001
Block design	−1.50 ± 1.06	0.00 ± 1.00	−7.526	1.45	<0.001
Picture arrangement	−0.86 ± 1.67	−0.01 ± 1.00	−5.291	1.01	<0.001
Object assembly	−1.05 ± 0.69	0.00 ± 1.00	−6.422	1.24	<0.001
Verbal score	−0.74 ± 0.54	0.00 ± 1.00	−4.891	0.94	<0.001
Performance score	−1.57 ± 0.77	0.00 ± 1.00	−9.234	1.78	<0.001
Full scale score	−1.22 ± 0.60	−0.01 ± 1.00	−7.750	1.49	<0.001

t = Student's t-test value.
d = Cohen's effect sizes value.
0.2: small effect sizes.
0.5: medium effect sizes.
≥0.8: large effect sizes.

Table 4: Gender and the intellectual function in HIV positive patients and controls using the full scale (Fs) mean z-scores.

	Total number of subjects with Fs mean z-scores <1 SD	Men	Women
	N (%)	N (%)	N (%)
HIV+	37/58 (63.8)	22/34 (65)	15/24 (62.5)
Control	5/50 (10.0)	2/32 (6.3)	3/18 (16.7)

Fs mean z-score <0.00 = impaired Fs mean z-score.

4. Discussion

Here, we aimed to characterize intellectual functioning in yet ART-naïve adult patients with newly diagnosed HIV infection. The study is unique as the WAIS was used and the majority of the patients were in advanced WHO stages. We report a high prevalence of intellectual impairment in this cohort and confirm and propose several risk factors for impaired intellectual functioning but could not identify a typical neurocognitive domain altered with HIV infection.

Commonly used instruments for the assessment of neurocognitive impairment in patients with HIV infection include the HIV dementia scale (HDS), the International HIV Dementia Scale (IHDS), Community Screening Interview for Dementia, and Minimental State Examination (MMSE) [4, 14, 15]. Computer-delivered cognitive assessment batteries such as Cogstate (http://www.cogstate.com/) and Fepsy (http://www.fepsy.com/), as well as the Montreal Cognitive Assessment (MCA), are further test batteries that have been used [16]. In this context, the prevalence rate of neurocognitive impairment in HIV infected patient differs substantially among studies due to different methodological approach and study population [17]. Choice of normative data may also influence prevalence estimates. Here, we used sample standardized scores to reduce this bias. Yet, the high rate of intellectual impairment (63.8%) is more than that of a pivotal Nigeria study (54%) using IHDS [18]. In a South-Asian population, the prevalence of HAND was found to be 22.7% using MCA and IHDS [19]. A recent Ugandan study found severe NCI to be present in 27% of patients with HIV infection [20]. Using an abbreviated test battery, a prevalence rate of 19% for neurocognitive impairment was detected early in the course of HIV infection in the USA [21]. Of note, a recent survey in USA identified neurocognitive impairment in 47% even in the era of ART [22]. The high rate of intellectual impairment identified in our study can be mostly traced back to advanced disease stages in the majority of our patients. A predominant affection of the fronto-striato-thalamo-cortical systems was reported in neuropathological studies [23, 24]. Subsequently, the main impairment would be expected for executive functions (e.g., planning), memory, and psychomotor speed, with relative sparing of basic language and visuoconstructive skills. The WAIS is not focused on the assessment of cognitive functions including episodic memory and executive functions. However, our study did disclose reduced scores throughout the WAIS but did not identify such a pattern. Yet, a recent study from Cameroon revealed that AIDS patients performed worse than non-AIDS HIV infected patients on cognitive function test including performances on WAIS III symbol search tests [25].

There might be other factors apart from acquired brain injury from HIV infection and related conditions that might

have contributed to the poor intellectual performance. This may for instance be related to impaired premorbid intellectual functioning. There have been reports that baseline IQ and higher cognitive reserve provided more predictive information than the CD4 count and plasma HIV RNA levels [26]. Thus, the limitation that premorbid IQ levels were not available in our study needs to be taken into account. Another factor might be the age at which HIV was acquired. In this regard, a recent study showed that HIV infection during brain development in youth and adulthood has more profound effect on neurocognitive disturbances related to frontostriatal circuits [27]. Intrinsic factors related to test performance need to be considered when studying a Sub-Saharan population compared to European or American counterparts using the WAIS. Though English is the official language in Nigeria, the country is a multilingual society with different local languages being spoken. It is possible that variability in the degree to which these indigenous languages are spoken may affect the test result. Subsequently, we recruited participants that were able to read and write in English language and 91% of the participants had greater or equal to 11 years of education.

An increased rate of neurocognitive impairment is not only found with advanced stages of HIV infection. Previous studies had reported a variety of risk factors including alcohol and substance abuse, lower nadir CD4 counts, cardiovascular/metabolic diseases, psychiatric disorders, hepatitis C virus coinfection, host genetic factors, virus subtype, anemia, and opportunistic infections [6, 17]. Beforehand, viral load determination was not available in this study because of the costs. We focused on the association of CD4 counts, WHO stage, anemia, and body weight. The markers of immune compromise such as low CD4 cell counts and high HIV RNA levels are associated with diffuse cortical atrophy, disease progression, and higher risk of death [28]. In this study, there was no significant association between CD4 count and intellectual performance. This may be due to smaller proportion of patients with higher CD4 count. In analogy, the WHO clinical stages were not associated with the intellectual performances of the patients, most likely related to the preponderance for more advanced disease stages. Anemia was shown to be associated with impaired cognitive function and daily living activities in elderly people [28]. Anemia may not have influenced intellectual function in our cohort because patients with severe anemia and poor Karnofsky performance were excluded. Weight loss continues to be a common problem in the era of HAART and is associated with reduced quality of life. Notably, weight loss has also been associated with disturbed neurocognitive function even in non-HIV infected patients. A study showed a moderate impairment of memory and motor skills among HIV infection men with lower body weight [29]. This keeps with our finding that men with lower body weight performed worse on the picture completion subtest, performance score, and full scale. In this study, lower body weight in women was associated with poorer performance on verbal items tests such as comprehension and similarities subtests.

There is paucity of the literature on the effect of OIs on intellectual functions in patients with HIV infection. In this study, patients with OIs had poorer performance on similarities and digit symbol test, performance scores, and full scale scores when compared to the patients without OIs. We did not identify specific OIs to be associated with a specific pattern of impairment. A more pronounced effect of OIs was reported by Wang et al. where CNS toxoplasmosis and cryptococcal meningitis were associated with HIV-associated dementia [30].

5. Conclusions

Large scale campaigns have been launched to ensure early diagnosis and treatment of HIV infection and associated conditions as these measures were shown to improve mortality, morbidity, and subsequent quality of life. This study, however, demonstrated that many patients are still not diagnosed and treated at an early disease stage. A subsequent high rate of intellectual impairment and comorbidities is present in this cohort. The impact of psychiatric disease was not evaluated as only patients with minor psychiatric symptoms were included. Additional cause for structural brain damage and CNS infections could have been present in the cohort but was not evaluated due to lack of resources for brain imaging and laboratory testing. Further studies may also take the impact of HIV-associated cancer into account. Taken together, the findings of this study have potential implications for collaborative diagnostic and therapeutic efforts to be further undertaken in order to reduce the rate and consequences of unfavorable comorbidities associated with HIV infection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Taofiki A. Sunmonu and Johann Sellner contributed equally to this work.

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Research Article

S100B Inhibitor Pentamidine Attenuates Reactive Gliosis and Reduces Neuronal Loss in a Mouse Model of Alzheimer's Disease

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Among the different signaling molecules released during reactive gliosis occurring in Alzheimer's disease (AD), the astrocyte-derived S100B protein plays a key role in neuroinflammation, one of the hallmarks of the disease. The use of pharmacological tools targeting S100B may be crucial to embank its effects and some of the pathological features of AD. The antiprotozoal drug pentamidine is a good candidate since it directly blocks S100B activity by inhibiting its interaction with the tumor suppressor p53. We used a mouse model of amyloid beta- (Aβ-) induced AD, which is characterized by reactive gliosis and neuroinflammation in the brain, and we evaluated the effect of pentamidine on the main S100B-mediated events. Pentamidine caused the reduction of glial fibrillary acidic protein, S100B, and RAGE protein expression, which are signs of reactive gliosis, and induced p53 expression in astrocytes. Pentamidine also reduced the expression of proinflammatory mediators and markers, thus reducing neuroinflammation in AD brain. In parallel, we observed a significant neuroprotection exerted by pentamidine on CA1 pyramidal neurons. We demonstrated that pentamidine inhibits Aβ-induced gliosis and neuroinflammation in an animal model of AD, thus playing a role in slowing down the course of the disease.

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder [1], whose pathologic hallmarks are the deposit of neurofibrillary tangles and senile plaques (beta-amyloid protein deposits) in the brain [2, 3]. Increasing evidence demonstrates that inflammation in the brain, specifically neuroinflammation, plays a key role in the development of AD [4, 5]. This pathologic event is accompanied by the activation of glial cells in the brain, a phenomenon known as "reactive gliosis" [6]. In fact, it has been shown that amyloid-beta- (Aβ-) induced reactive gliosis and the consequent inflammatory responses with the release of neurotoxic cytokines are present in the AD brain and prominently contribute to the progression of the disease [7]. The two events are thus thoroughly linked and are object of the current research on AD pathophysiology.

The definition of "reactive gliosis" refers to the overexpression of glial-derived factors. Amongst all, one of the most interesting from a pharmacological point of view is the protein S100B [8, 9]. This small and soluble protein, belonging to the large family of EF-related Ca^{++}- and Zn^{++}-binding proteins, plays a dual effect. While at nanomolar concentrations S100B provides to a prosurvival effect on neurons and stimulates neurite outgrowth, at higher (micromolar) concentrations it promotes inflammation and neuronal apoptosis [10]. S100B overexpression has been linked to the typical features of reactive gliosis in AD [11, 12]. After release, and only when it reaches micromolar concentrations, the protein accumulates at the RAGE (receptor for advanced glycation end-products)

surface [13–15]. Such interaction leads to the phosphorylation of mitogen-activated protein kinase (MAPK) and the activation of nuclear factor-kappaB (NF-κB). This cascade, in turn, promotes the transcription of proinflammatory cytokines and inducible nitric oxide synthase (iNOS) protein [16]. The possibility of interfering with this harmful cycle, by directly targeting S100B, might therefore represent a novel approach to embank neurotoxicity in AD brain.

Pentamidine isethionate, discovered in 1938 as an antiprotozoal drug and approved in the United States for the treatment of *Pneumocystis carinii* pneumonia and other protozoal diseases [17], appears to be an intriguing candidate. In fact, in addition to the antiprotozoal activity, pentamidine also inhibits S100B-mediated effects because of its ability to block S100B/p53 interaction [18]. In spite of the several data showing the anti-inflammatory effect exerted by pentamidine due to S100B inhibition [19–22], no data on the possible effect of pentamidine on gliosis and neuroinflammation in AD models are available so far.

Based on this background, the present study was aimed at evaluating the effect of a daily intrahippocampal administration of pentamidine in a mouse model of AD characterized by Aβ-induced gliosis and neuroinflammation. Because of the capability to inhibit S100B protein, we investigated (1) the effect exerted by pentamidine on reactive gliosis, (2) the molecular mechanism by which pentamidine might interfere with reactive gliosis, and (3) whether pentamidine-mediated inhibition of reactive gliosis may result in the rescue of neuronal loss in AD brain.

2. Methods

2.1. Ethics Statement. All the experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and those of the Italian Ministry of Health (D.L. 116/92). The study was approved by the Institutional local Animal Care and Use Committees.

2.2. Animals. Experiments were performed in C57BL/6J mice (3–5 months old, weight range: 35–40 g; Harlan, Udine, Italy). Animals were housed under controlled illumination (12 h light/12 h dark cycle) and standard environmental conditions (room temperature 20–22°C, humidity 55–60%). Food and water were available *ad libitum*. All surgery and experimental procedures were performed during the light cycle. All efforts were made to reduce the number of animals used and the suffering during surgical experiments.

2.3. Surgical Preparation and Intrahippocampal Injection. Mice (total $n = 40$) were anesthetized i.p. with pentobarbital (40 mg/kg). They were then placed in a stereotaxic frame and injected in the hippocampi (CA1 area) with human Aβ (1–42) peptide (Tocris Cookson, UK). The coordinates for the injection were −1.58 mm posterior from bregma, ±1.2 mm lateral and 1.60 mm ventral to the skull surface. Aβ peptide was dissolved in ALZET artificial cerebrospinal fluid according to the manufacturer's instructions (ALZET-company, Cupertino, CA, USA). The final concentration was 10 μg/mL and a volume of 3 μL was injected using an ALZET microdialysis pump by keeping the flow at the constant speed of 0.5 mL/min. Control mice (vehicle-treated group, $n = 8$) were injected with an equivalent volume of artificial cerebrospinal fluid. Starting at the third day after surgery and using the previously implanted cannula, three groups of mice ($n = 8$ per group) received intrahippocampal infusion of pentamidine (0.05–5 μg/mL/day) for consecutive 7 days. At the end of treatments, the cannula was removed and the animals, to prevent damage to the scalp sutures, were kept in individual cages until they were killed for tissue processing.

2.4. Immunohistochemistry and Immunofluorescence Analyses. Immunohistochemistry analysis was performed on hippocampal coronal sections (adjacent to the site of the injection) obtained from the brains of vehicle-, Aβ-, and pentamidine-treated mice. Sections were incubated for 2 hours with blocking buffer (PBS containing 15 mM NaN_3, 10% albumin, and 0.25% Triton X-100) and then with mouse anti-GFAP antibody (1 : 400, Sigma-Aldrich, Milan, Italy) overnight at 4°C. Biotinylated secondary antibody (1 : 200; Vector Laboratories, Peterborough, UK) and the preformed avidin biotinylated peroxidase complex (VECTASTAIN ABC kit; Vector Laboratories) were then added and the reaction was revealed by 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Representative pictures were captured using a high-resolution digital camera (Nikon Digital Sight DS-U1).

For the immunofluorescence, hippocampal coronal sections obtained from the brains of vehicle-, Aβ-, and pentamidine-treated mice were blocked in 10% albumin bovine serum 0.1% Triton-PBS solution for 90 min and subsequently exposed for 1 h to rabbit anti-GFAP antibody (1 : 1000, Abcam, Cambridge, UK) and mouse anti-p53 antibody (1 : 500, Abcam). Sections were then incubated in the dark with the proper secondary antibody: fluorescein isothiocyanate-conjugated anti-rabbit (1 : 1000, Abcam) or Texas Red-conjugated anti-mouse (1 : 1000, Abcam), respectively. Immunofluorescence was analyzed with a Nikon Eclipse 80i microscope (Nikon Instruments Europe, Kingston upon Thames, UK) and images were captured by a high-resolution digital camera (Nikon Digital Sight DS-U1). The number of GFAP+ or p53+ cells was then calculated in every tenth coronal section spanning the ipsilateral hippocampus at the injection site using unbiased stereology (Stereo Investigator, MBF, Williston, VT, USA). According to the manufacturer's protocol, a counting frame ($15 \times 15 \times 20\,\mu$m) was placed at the intersection of a matrix ($200 \times 200\,\mu$m) randomly superimposed by the software onto the region of interest.

2.5. Nissl Staining. Hippocampal coronal sections ($n = 5$) obtained from the brains of vehicle-, Aβ-, and pentamidine-treated mice were sequentially dipped in different alcohol solutions of decreasing percentage to remove lipids from the tissue, stained with 2% cresyl violet solution for 5 minutes, and dehydrated with a series of baths with increasing alcohol percentage solutions. Sections were analyzed by a blind

observer through a Nikon Eclipse 80i microscope. Representative pictures were captured using a high-resolution digital camera (Nikon Digital Sight DS-U1) and analyzed using NIS-Elements software (Nikon Instruments Europe). The extent of neuronal damage was expressed as the ratio between the number of nonstained (death) neurons and the total number of neurons per mm of length of CA1 area in injected ipsilateral hippocampi, according with the following formula:

$$\frac{\text{death neurons per CA1 mm}^2 \text{ area}}{\text{total neurons per CA1 mm}^2 \text{ area}} = \text{extent of CA1 damage (\%)}. \quad (1)$$

2.6. Fluoro-Jade B Staining. To further evaluate neuronal loss/rescue in the hippocampus, Fluoro-Jade B (FJB) staining was performed on hippocampal coronal sections obtained from the brains of vehicle-, Aβ-, and pentamidine-treated mice. Sections were immersed in a basic alcoholic solution for 6 minutes and 0.06% $KMnO_4$ for 15 minutes. Sections were then incubated in 0.0004% FJB (Histo-Chem, Jefferson, AR, USA) for 20 minutes, washed in distilled water, and then dried. To quantify neuronal death, every tenth coronal section spanning hippocampus was analyzed from each animal ($n = 5$). A blinded observer counted the number of FJB-positive neurons in the hippocampal CA1 from ipsilateral hemispheres to the injection site. Mean counts of FJB-positive neurons from each region were used for the statistical analysis.

2.7. Immunoblot Analysis. Ipsilateral hippocampi to the injection site were dissected from frozen excised brains of vehicle-, Aβ-, and pentamidine-treated mice and lysed with ice-cold hypotonic lysis buffer (Tris/HCl pH 7.5 50 mM; NaCl 150 mM; EDTA 1 mM; Triton X-100 1%) supplemented with the proper protease inhibitor cocktail (Roche, Milan, Italy) and incubated on ice for additional 15 min. Total protein extracts were obtained by centrifugation at 13,000 g for 15 min at 4°C. Samples were subjected to SDS-polyacrylamide gel electrophoresis and proteins were transferred onto nitrocellulose membrane and incubated with one of the following antibodies: anti-GFAP (1 : 50000); anti-iNOS (1 : 200); anti-COX-2 (1 : 1000), anti-phospho(p)-p38 MAPK (1 : 400), anti-RAGE (1 : 1000), and anti-β-actin (1 : 2000) (all from Abcam). After wash in TBS 1X with 0.1% Tween 20, the membrane was incubated for 2 h at room temperature with the appropriate secondary HRP-conjugated antibodies anti-mouse (1 : 1000, Abcam) or anti-rabbit (1 : 1000, Abcam). Lastly, the membrane was exposed to the enhanced chemiluminescence substrate (ECL, Invitrogen, Milan, Italy), the immunoreactive bands were revealed through a Versadoc (Bio-Rad Laboratories, Milan, Italy) and the digital images were analyzed with the Quantity One Software (Bio-Rad Laboratories).

2.8. Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed to detect NF-κB activation in hippocampal homogenates obtained from the brains of vehicle-, Aβ-, and pentamidine-treated mice. Double stranded oligonucleotides containing NF-κB recognition sequence (5′AGTTGAGGGGACTTTCCCAGGC-3′) were end-labeled with ^{32}P-γ-ATP (Amersham, Milan, Italy). Nuclear extracts were incubated for 15 min with radiolabeled oligonucleotides (2.5–5.0 × 10^4 cpm) in 20 mL reaction buffer containing 2 mg poly dI-dC (Boehringer-Mannheim, Milan, Italy), 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dl-dithiothreitol, 1 mg/mL bovine serum albumin, and 10% glycerol. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 6% nondenaturing polyacrylamide gel in 1X Tris Borate EDTA buffer at 150 V for 2 hrs at 4°C. The gel was dried and autoradiographed with an intensifying screen at −80°C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning with Versadoc (Bio-Rad Laboratories) and computer software (Quantity One Software, Bio-Rad Laboratories). Oligonucleotide synthesis was performed to our specifications by Tib Molbiol (Boehringer-Mannheim, Ingelheim am Rhein, Germany).

2.9. Nitrite Assay. NO was measured as nitrite ($NaNO_2$) accumulation in mice hippocampal homogenates, obtained from the brains of vehicle-, Aβ-, and pentamidine-treated mice, by using the Griess method [23]. Briefly, Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in H_3PO_4) was added to an equal volume of tissue homogenate and the absorbance of the reaction product was measured at 550 nm. Nitrite concentration (nM) was thus determined using a standard curve of $NaNO_2$.

2.10. Lipid Peroxidation Assay. Malonyl dialdehyde (MDA) was measured by the thiobarbituric acid colorimetric assay in mice hippocampal homogenates obtained from the brains of vehicle-, Aβ-, and pentamidine-treated mice. Briefly, 1 mL trichloroacetic acid 10% was added to 450 μL of tissue lysate. After centrifugation, 1.3 mL thiobarbituric acid 0.5% was added and the mixture was heated at 80°C for 20 min. After cooling, MDA formation was recorded (absorbance 530 nm and absorbance 550 nm) in a PerkinElmer (Waltham, MA, USA) spectrofluorometer and the results were presented as ng MDA/mL.

2.11. Enzyme-Linked Immunosorbent Assay (ELISA) for Prostaglandin (PGE)2, S100B, and Interleukin- (IL-) 1beta (β). ELISA for PGE2, IL-1β, and S100B (all from R&D Systems, Minneapolis, Minnesota, USA) was carried out on mouse hippocampal homogenates, obtained from the brains of vehicle-, Aβ-, and pentamidine-treated mice, according to the manufacturer's protocol. Absorbance was measured on a microtiter plate reader. PGE2, IL-1β, and S100B levels were determined using standard curves method.

2.12. Statistical Analysis. Results were expressed as mean ± SEM of n experiments. Statistical analysis was performed using analysis of variance (ANOVA) and multiple comparisons were performed by Bonferroni's test, with $P < 0.05$ considered as significant.

3. Results

3.1. Pentamidine Attenuates Aβ-Induced Gliosis and Neuroinflammation in Hippocampi. Immunoblot analysis showed that Aβ injection significantly increased the expression of GFAP (34.0 ± 1.6 versus 11.0 ± 1.2, $P < 0.001$, Figures 1(a) and 1(b)), iNOS (8.1 ± 0.9 versus 1.6 ± 0.6, $P < 0.001$, Figures 1(a) and 1(c)), p-p38 MAP-kinase (8.6 ± 0.8 versus 1.2 ± 0.5, $P < 0.001$, Figures 1(a) and 1(d)), and COX-2 (9.0 ± 0.8 versus 1.0 ± 0.2, $P < 0.001$, Figures 1(a) and 1(e)) proteins in hippocampal homogenates, compared to vehicle-treated mice. In the same way, also extracellular RAGE protein expression was significantly increased (11.0 ± 0.8 versus 2.1 ± 0.6, $P < 0.001$, Figures 1(a) and 1(f)) in the hippocampi of Aβ-injected compared to vehicle-treated mice. Treatment with pentamidine (0.05–5 μg/mL/day) for 7 days, markedly and in dose-dependent manner blunted Aβ-induced overexpression of GFAP (24.0 ± 2.3, 20.0 ± 2.0 and 14.0 ± 2.0 versus 34.0.3 ± 1.6, $P < 0.05$, 0.01 and 0.001, resp., Figures 1(a) and 1(b)), iNOS (5.0 ± 0.5, 3.2 ± 0.3 and 2.0 ± 0.6 versus 8.1 ± 0.9, $P < 0.05$, 0.01 and 0.001, resp., Figures 1(a) and 1(c)), p-p38 MAPK (5.2 ± 1.0, 3.1 ± 0.7 and 2.0 ± 0.6 versus 8.5 ± 0.8, $P < 0.05$, 0.01 and 0.001, resp., Figures 1(a) and 1(d)), and COX-2 (6.0 ± 0.5, 4.0 ± 0.5 and 1.2 ± 0.4 versus 9.0 ± 0.8, $P < 0.05$, 0.01 and 0.001, resp., Figures 1(a) and 1(e)) in hippocampi homogenates, compared to Aβ-treated mice. The expression of the extracellular RAGE was also significantly and concentration-dependent reduced by pentamidine treatment (0.05–5 microg/mL/day) for 7 days (7.0 ± 1.0, 5.2 ± 1.0 and 3.1 ± 1.0 versus 11.0 ± 0.4, $P < 0.05$, 0.01 and 0.001, resp., Figures 1(a) and 1(f)).

At nuclear level, Aβ injection induced a significant upregulation of NF-κB (17.5 ± 1.9 versus 1.3 ± 0.8, $P < 0.001$, Figures 2(a) and 2(b)) compared to vehicle-treated mice, as demonstrated by EMSA analysis, indicating a marked Aβ-induced neuroinflammatory response in the hippocampi (Figures 2(a) and 2(b)). Pentamidine-mediated inhibitory effect was observed also for NF-κB in the nuclear extracts, which was significantly and dose dependently downregulated (10.6 ± 2.0, 8.4 ± 1.5 and 4.4 ± 1.4 versus 17.5 ± 1.9, $P < 0.05$, 0.01 and 0.001, resp., Figures 2(a) and 2(b)).

As expected, lipid peroxidation assay and Griess reaction showed that Aβ injection area caused a significant increase of nitrite (15.6 ± 1.9 versus 2.0 ± 0.8, $P < 0.001$, Figure 3(a)) and MDA (8.0 ± 0.8 versus 0.6 ± 0.1, $P < 0.001$, Figure 3(b)) in the hippocampi of Aβ-treated mice, as a sign of ongoing inflammation. ELISA also showed that PGE2 (8.7 ± 0.4 versus 0.7 ± 0.1, $P < 0.001$, Figure 3(c)), IL-1β (6.8 ± 1.0 versus 0.7 ± 0.1, $P < 0.001$, Figure 3(d)), and S100B (6.1 ± 1.2 versus 2.0 ± 1.0, $P < 0.001$, Figure 3(e)) released in the hippocampi of Aβ-injected mice were significantly increased compared to vehicle-treated mice. In line with immunoblot analysis, pentamidine treatment (0.05–5 μg/mL/day) for 7 days caused a marked and dose-dependent attenuation of all the abovementioned proinflammatory markers in the hippocampi: nitrite (11.3 ± 1.4, 8.1 ± 1.5 and 4.4 ± 1.4, $P < 0.05$, 0.01 and 0.001, resp., Figure 3(a)); MDA (4.7 ± 0.8, 2.0 ± 0.5 and 0.9 ± 0.3 versus 8.0 ± 0.8, $P < 0.05$, 0.01 and 0.001, resp., Figure 3(b)); PGE2 (6.0 ± 1.0, 2.8 ± 0.8 and 1.0 ± 0.4, $P < 0.05$, 0.01 and 0.001, resp., Figure 3(c)); and IL-1β (3.9 ± 0.8, 1.9 ± 0.8 and 1.2 ± 0.4, $P < 0.01$ and 0.001, resp., Figure 3(d)) compared to Aβ-treated mice. Only the release of S100B remained unaffected (5.8 ± 1.3, 5.8 ± 1.3 and 5.9 ± 1.2, resp., all $P > 0.05$, Figure 3(e)) in pentamidine-treated compared to Aβ-treated mice.

3.2. Pentamidine Inhibits Reactive Gliosis, Reduces Astrocyte Infiltration, and Rescues Neuronal Loss in Aβ-Injected Mice. Aβ injection caused a marked glia activation, as shown by the increased expression of the astrocytic marker GFAP (153 ± 25 versus 23 ± 5.8, $P < 0.001$, Figures 4(a) and 4(d)). In parallel, Nissl staining indicated that Aβ injection caused a severe neuronal loss, especially in the CA1 area (site of injection), compared to vehicle-treated mice (79 ± 5.0 versus 5.6 ± 2, $P < 0.001$, Figures 4(b) and 4(e)). Treatment with pentamidine (0.05–5 μg/mL/day) for 7 days concentration dependently rescued neurons integrity in the CA1 area (58 ± 4.1, 42 ± 7.0 and 22 ± 5.0 versus 79 ± 5.0, $P < 0.01$ and 0.001, resp., Figures 4(b) and 4(e)).

The neuroprotective effect exerted by pentamidine was confirmed by FJB analysis. Aβ injection caused a significant increase of FJB-positive cell number in CA1 area versus vehicle-treated mice (711 ± 102 versus 101 ± 52, $P < 0.001$ Figures 4(c) and 4(f)). Treatment with pentamidine (0.05–5 μg/mL/day) for 7 days reduced in a concentration-dependent way the number of dying neurons caused by Aβ injection in the same area (352 ± 100, 201 ± 95 and 141 ± 71 versus 101 ± 52, $P < 0.01$ and 0.001 resp., Figures 4(c) and 4(f)).

The neuroprotective effect of pentamidine was accompanied by a significant downregulation of gliosis grade, as shown by the concentration-dependent decrease of GFAP expression (84.6 ± 16, 53 ± 9.6 and 44.2 ± 15, $P < 0.01$ and 0.001, resp.), compared to the hippocampi of Aβ-treated mice (Figures 4(a) and 4(d)). According to the immunohistochemistry, immunofluorescence analysis of GFAP and p53 protein revealed that, after Aβ injection, GFAP+ cell number was significantly increased in the hippocampi of Aβ-compared to vehicle-treated mice (41 ± 6.0 versus 13.0 ± 3.0, $P < 0.01$, Figures 5(a) and 5(b)). Conversely, p53 expression in Aβ-treated mice was significantly reduced compared to vehicle-treated mice (3.2 ± 0.8 versus 9.0 ± 1, $P < 0.01$, Figures 5(a) and 5(b)), very likely as the consequence of astrocytes infiltration. Treatment with pentamidine (0.05–5 μg/mL/day) for 7 days caused a dose-dependent decrease of glial cells as indicated by GFAP positive cell infiltration in CA1 area (30 ± 6.0, 16.0 ± 6.0 and 12.0 ± 3.0 versus 41 ± 6.0, $P < 0.05$ and 0.001, resp., Figures 5(a) and 5(b)) and in the same time it resulted in a dose-dependent increase of nuclear p53 expression in GFAP expressing cells (16.0 ± 5.0, 24.0 ± 6.0 and 31.2 ± 3.0 versus 3.2 ± 0.8, $P < 0.01$ and 0.001 resp., Figures 5(a) and 5(b)).

4. Conclusions

Novel therapeutic approaches for the treatment of AD progression should direct towards the (re)discovery of new molecules able to have an impact on several pathological

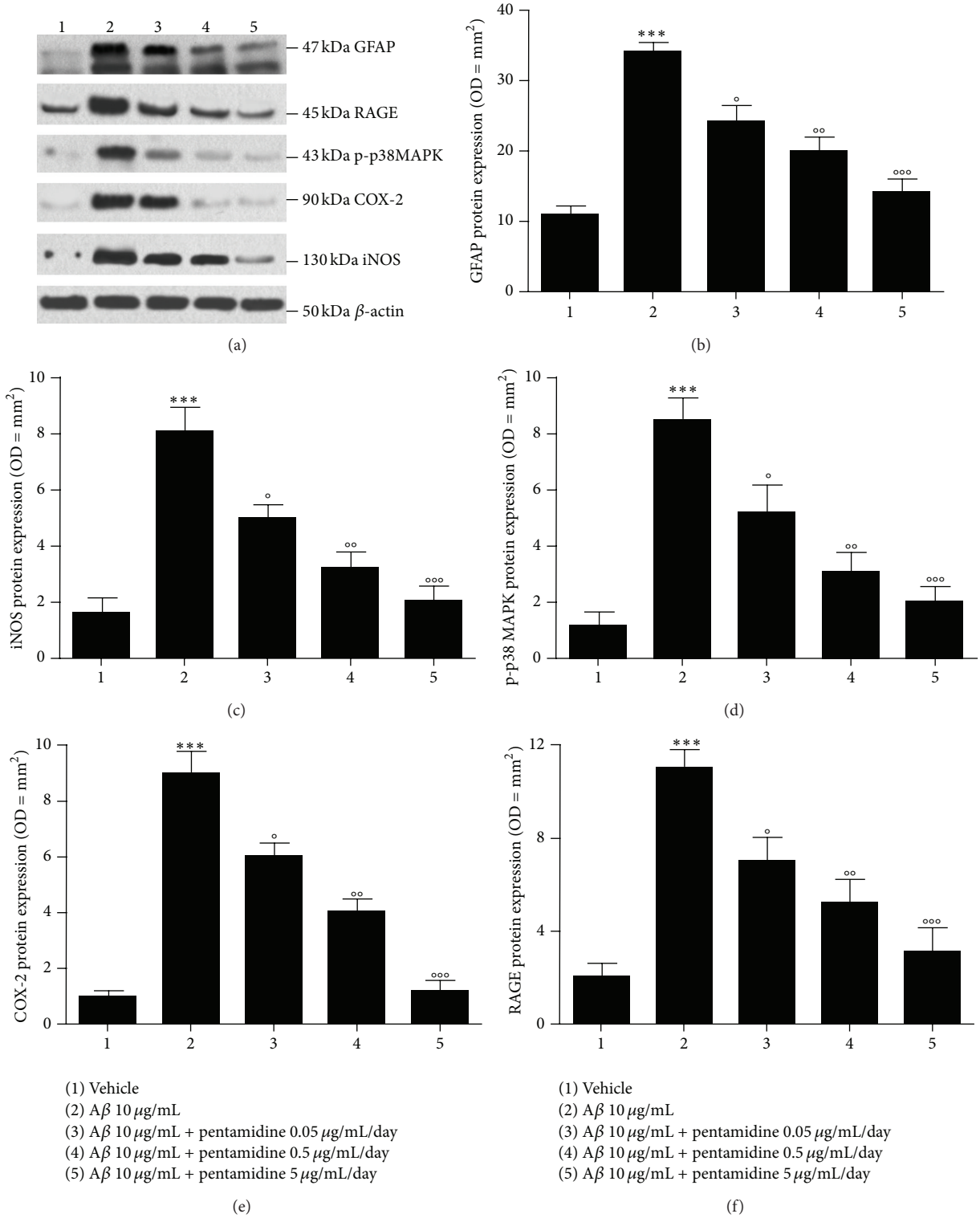

Figure 1: (a) Western blot and (b–f) densitometric analysis (arbitrary units normalized on the expression of the housekeeping protein β-actin) showing the effect of 7 days of intrahippocampal injection of pentamidine (0.05–5 μg/mL/day) on GFAP (b), iNOS (c), p-p38 MAPK (d), COX-2 (e), and RAGE (f) expression in Aβ-injected mice. Results are expressed as mean ± SEM of $n = 5$ experiments performed in triplicate. $^{***}P < 0.001$ versus vehicle-treated mice; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ and $^{\circ\circ\circ}P < 0.001$ versus Aβ-treated mice.

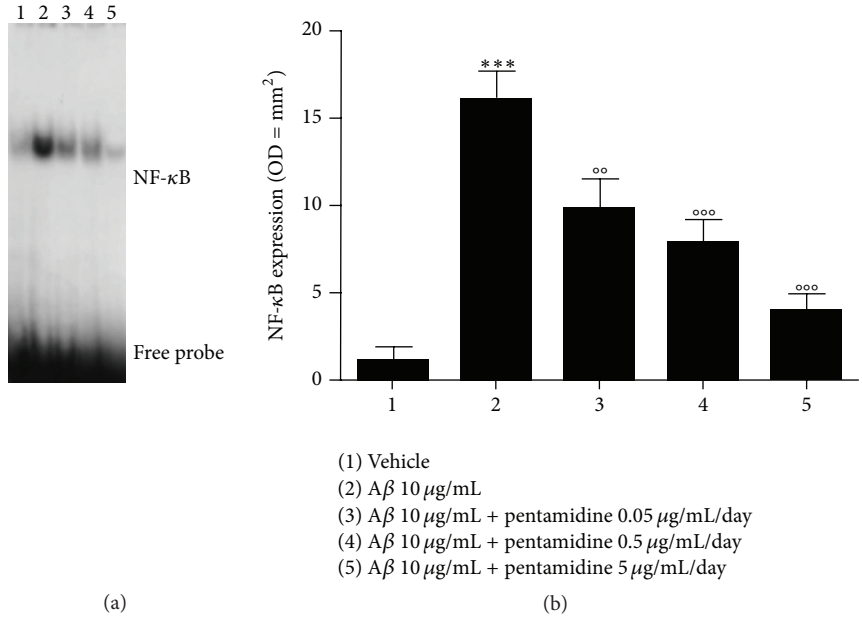

Figure 2: (a) Electrophoretic mobility shift assay (EMSA) and the relative (b) densitometric analysis showing the effect following 7 days of intrahippocampal injection of pentamidine (0.05–5 μg/mL/day) on the expression of NF-κB in Aβ-injected mice. Results are expressed as mean ± SEM of $n = 5$ experiments performed in triplicate. $^{***}P < 0.001$ versus vehicle-treated mice; $^{\circ\circ}P < 0.01$ and $^{\circ\circ\circ}P < 0.001$ versus Aβ-treated mice.

pathways that together converge to the progressive neurological decline characteristic of the disease. Inflammation, more specifically neuroinflammation, has been widely known as an accompanying and key feature in AD [24–26]. In fact, both *in vitro* and *in vivo* studies have shown that Aβ, the major constituent of the senile plaques in the AD brain, can directly or indirectly activate the secretion of proinflammatory cytokines [27]. Therefore, the search for new drugs should be based on diverse targets in the attempt to blunt the inflammatory scenario in the AD brain and not only to replace the neurotransmission failure.

Here we show that pentamidine, an ancient antiprotozoal drug that inhibits S100B protein, ameliorates gliosis and neuroinflammation in a mouse model of Aβ-induced AD. Many studies have been addressed in the attempt to enlarge the pharmacological knowledge on pentamidine and its novel therapeutic effects in disorders characterized by S100B upregulation, such as melanoma [28], glioblastoma [29], and colitis [19]. This has led to the discovery that, besides being an antiprotozoal drug, pentamidine also inhibits S100B activity by blocking the interaction at the Ca^{++}/p53 site of the protein. S100B is a unique glial-derived factor in the sense that it is responsible for the establishment of neuroinflammation and neurodegeneration [30]. In fact, in AD brains, S100B is released by reactive astrocytes, a phenomenon known as "reactive gliosis," and promotes the formation of neurofibrillary tangles in a RAGE-dependent manner [31]. Once released, S100B accumulates at the RAGE [15, 32] and this interaction leads to the induction of lipid peroxidation and to MAPK phosphorylation that in turn converge to NF-κB activation. By triggering this pathway, S100B induces the transcription of proinflammatory proteins and cytokines, such as iNOS protein, IL-1β, and TNFα [33, 34]. It is thus conceivable that, by specifically targeting the RAGE/S100B interaction in the brain, it would be possible to inhibit S100B-dependent neuroinflammation in AD. Different studies have suggested that a possible therapeutic approach might be the inhibition of the binding of S100B to the V domain of RAGE by using specific antibodies or small molecules [35]. However, since RAGE is not the sole receptor mediating S100B effects, it seems more logic to inhibit the protein itself before it binds to any target. The results of this study demonstrate that pentamidine, via direct inhibition of S100B protein, attenuates 1/reactive gliosis and neuroinflammation induced by Aβ in mouse hippocampi and 2/neuronal loss in the CA1 area of the brain. Specifically, pentamidine caused a dose-dependent decrease of GFAP protein expression, a sign of gliosis, in mice hippocampal homogenates. This was accompanied by the dose-dependent inhibition of iNOS, COX-2, and p-p38 MAPK protein expression. Consequently to S100B inhibition, pentamidine indirectly interferes with S100B-RAGE interaction, leading to a marked inhibition of RAGE protein expression, which was upregulated after Aβ injection. This result caused the interruption of the downstream RAGE-dependent effects such as NF-κB mobilization in the cytosol and the consequent induction of transcription of proinflammatory signaling molecules/cytokines. At confirmation of the amelioration of the inflammatory scenario, pentamidine was also able to reduce the release of proinflammatory cytokines, namely, PGE2 and IL-1β. Moreover, we demonstrated that pentamidine inhibited other proinflammatory events like lipid peroxidation and nitric oxide release. According to our

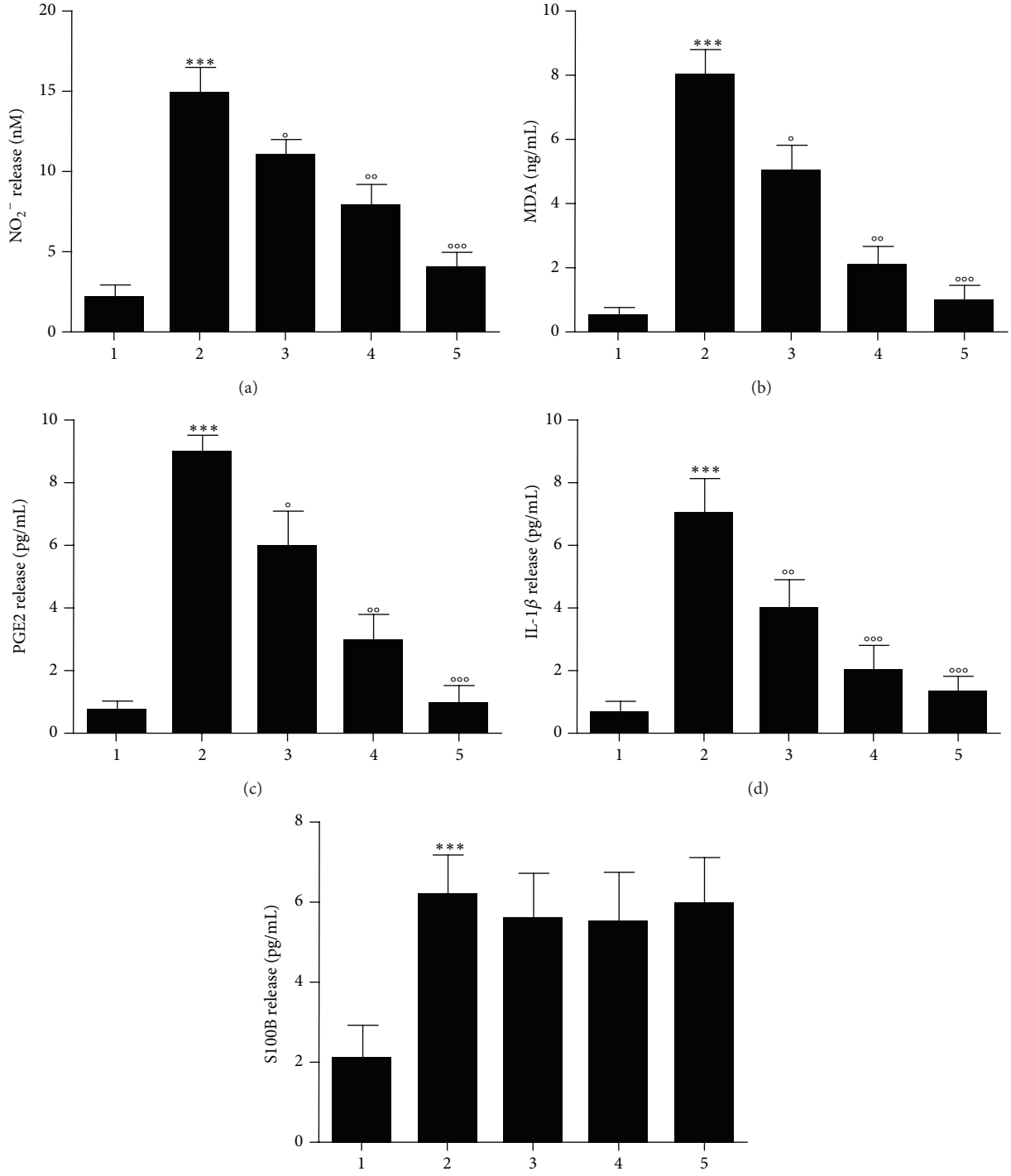

Figure 3: Effect of pentamidine on release of nitrites (a), MDA (b), PGE2 (c), IL-1β (d), and S100B (e) in hippocampal homogenates of Aβ-injected mice. Results are expressed as mean ± SEM of $n = 5$ experiments performed in triplicate. $^{***}P < 0.001$ versus vehicle-treated mice; $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ and $^{\circ\circ\circ}P < 0.001$ versus Aβ-treated mice.

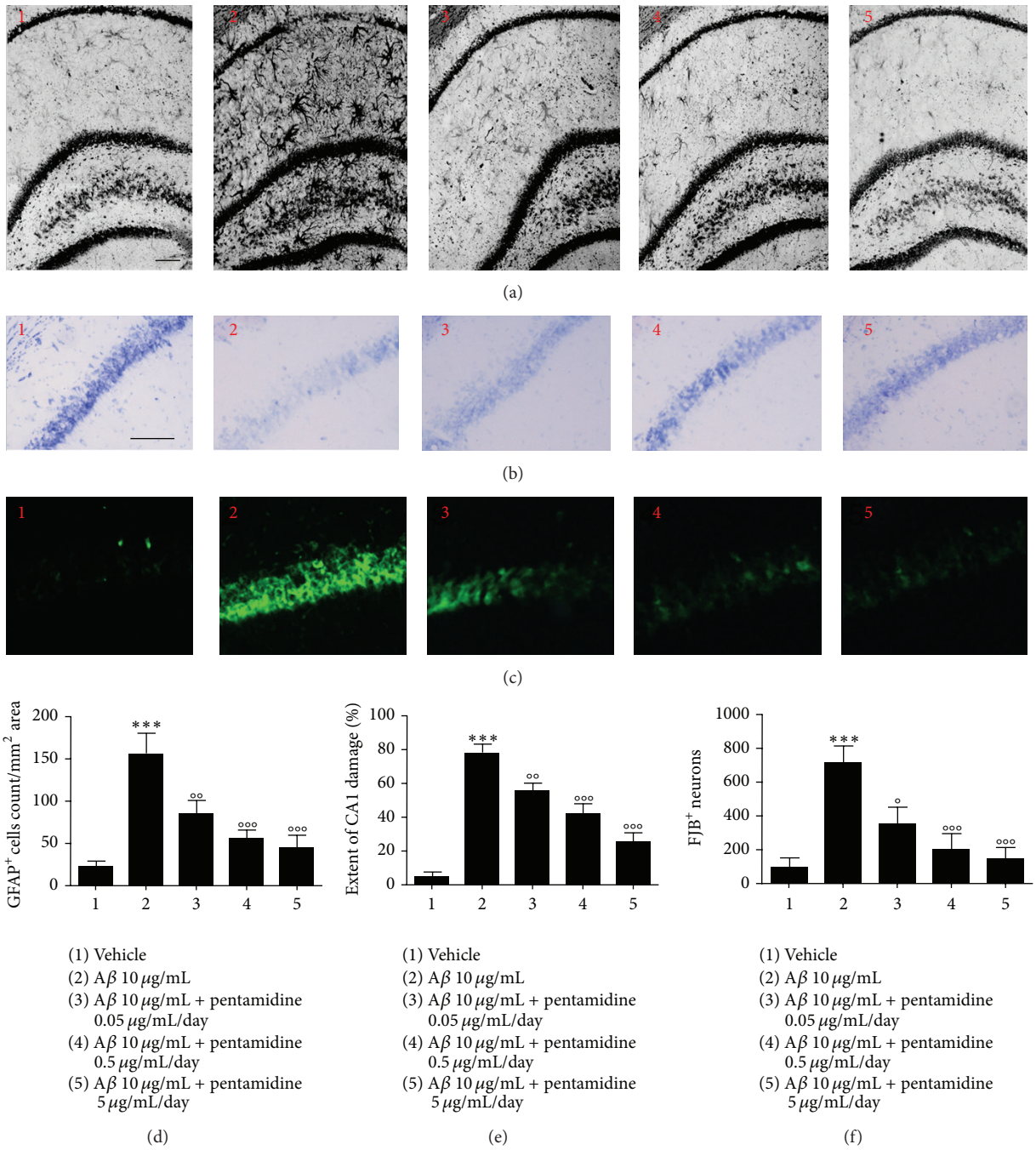

Figure 4: (a) Immunohistochemistry analysis showing the effect of pentamidine in hippocampal coronal sections after Aβ injection. The upper panel shows GFAP-positive cells (astrocytes) infiltrating the hippocampi. Note the increased number of GFAP-positive cells in Aβ-treated (2) compared to vehicle-treated mice (1) and the dose-dependent reduction after pentamidine treatment (3-4-5). Scale bar: 200 μm. (b) Nissl staining showing the effect of pentamidine on pyramidal neuron loss in the CA1 area after Aβ injection. Note the reduced number of neurons stained in Aβ-treated (2) compared to vehicle-treated mice (1) and the dose-dependent reduction of neuronal loss after pentamidine treatment (3-4-5). Scale bar: 200 μm. (c) Immunofluorescence analysis showing the effect of pentamidine in hippocampal coronal sections after Aβ injection. Note the reduced number of neurons after Aβ injection (2) compared to vehicle-treated mice (1) and the dose-dependent neuroprotection after pentamidine treatment (3-4-5). Scale bar: 200 μm. (d) Relative quantification of GFAP expression, (e) extent of CA1 damage measurement, and (f) number of neurons stained with Fluorojade B (FJB) in the hippocampi. Results are expressed as mean ± SEM of $n = 5$ experiments performed in triplicate. $^{***}P < 0.001$ versus vehicle-treated mice; $^{\circ\circ}P < 0.01$ and $^{\circ\circ\circ}P < 0.001$ versus Aβ-treated mice.

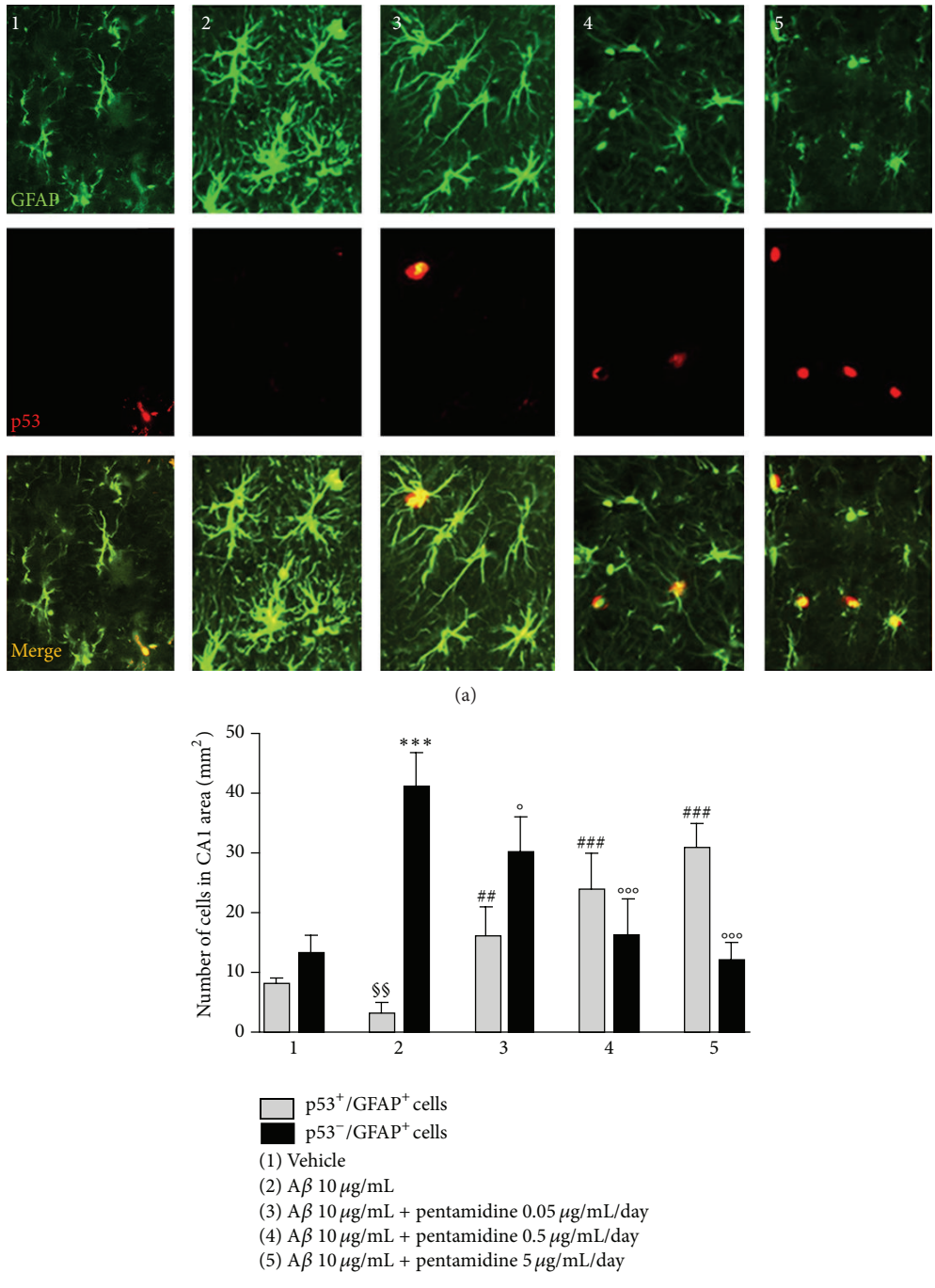

FIGURE 5: Effect of pentamidine (0.05–5 μg/mL/day) on GFAP and p53 expression in astrocyte in the hippocampi of Aβ-injected mice. (a) Immunofluorescence analysis of hippocampal coronal sections. Note the increased GFAP expression in hippocampal astrocytes of Aβ-treated (2) compared to vehicle-treated mice (1) and the dose-dependent reduction after pentamidine treatment (3-4-5). Scale bar: 50 μm. (b) Relative quantification of p53-positive/GFAP-positive (open bars) and p53-positive/GFAP-positive (filled bars) astrocytes in the CA1 area of the brain. Results are expressed as mean ± SEM of $n = 4$ experiments performed in triplicate. $^{***}P < 0.001$ versus vehicle-treated mice; $^{\circ}P < 0.05$ and $^{\circ\circ\circ}P < 0.001$ versus Aβ-treated mice. $^{\S\S}P < 0.01$ versus vehicle-treated mice; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Aβ-treated mice.

previous observations [29], S100B protein release was upregulated by Aβ injection but its level was not affected by pentamidine treatment. To prove that all the above discussed anti-inflammatory effects, together with the reduction of reactive gliosis, were due to the inhibition of S100B/p53 binding, we evaluated p53 expression in the different experimental conditions. We found that the treatment with pentamidine induced p53 expression on infiltrating astrocytes in mouse

hippocampi, as sign of enhanced apoptosis. Together with reduced gliosis, we also observed the rescue of neuronal loss in the damaged area of the brain.

Though preliminary, our data identifies in pentamidine a novel potential drug for the treatment of AD features. However, future studies are needed to investigate whether, together with its anti-inflammatory and neuroprotective activity, pentamidine may also improve mnemonic and cognitive performances in experimental models of AD. However, one of the limiting factors of pentamidine resides in its pharmacokinetic profile, which is characterized by low blood brain barrier crossing. Thus, new pharmacokinetic approaches aimed at increasing the delivery of pentamidine into the brain, in combination with a suitable compliance in terms of way of administration, look very intriguing.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Carla Cirillo and Elena Capoccia equally contributed to the paper.

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Review Article

An Overview of Potential Targets for Treating Amyotrophic Lateral Sclerosis and Huntington's Disease

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Neurodegenerative diseases affect millions of people worldwide. Progressive damage or loss of neurons, neurodegeneration, has severe consequences on the mental and physical health of a patient. Despite all efforts by scientific community, there is currently no cure or manner to slow degeneration progression. We review some treatments that attempt to prevent the progress of some of major neurodegenerative diseases: Amyotrophic Lateral Sclerosis and Huntington's disease.

1. Introduction

Neurodegenerative diseases are characterized by a slow and progressive loss of neurons in different areas of the central nervous system. Motor neurons are impaired in Amyotrophic Lateral Sclerosis (ALS) as well as thorny striatal neurons in Huntington's disease (HD) [1]. ALS symptoms include a progressive muscle phenotype of spasticity, hyperreflexia, fasciculations, atrophy, and paralysis. The disease is usually lethal within 3–5 years after the diagnosis and in most cases, patients die from respiratory failure [1]. The causes of the disease and the mechanisms causing premature death of motor neurons are not clear. In addition, two types of the disorder are recognized, familial ALS (fALS) and the most common sporadic ALS (sALS) [1].

Currently the only available treatment for ALS is Riluzole, approved for use in 1995. However, Riluzole only slows down the progression of the disease and gives the patient an average of 3 months of extended life span [2]. The exact mechanism of action of Riluzole is unknown; several papers have demonstrated its inhibition of sodium, calcium, potassium, and glutamate currents [3]. The coadministration of Riluzole with other potential therapeutic agents is constantly being assessed and is usually with no promising positive results [4, 5]. Therefore, the safety of the combination of Riluzole with a second study drug is unpredictable.

Huntington's disease (HD) is an inherited neurodegenerative disorder, characterized by progressive loss of neurons in the whole brain, notably in the basal ganglia and cerebral cortex, resulting in worsening chorea, cognitive and psychiatric disturbances [6]. In the United States, HD occurs in about 1 in 10,000 people. Currently about 30,000 people in the US have HD and up to 200,000 are at risk [7]. HD is caused by expanded polyglutamine cytosine-adenine-guanine (CAG) repeat sequence in the autosomal dominant gene that encodes huntingtin (Htt, 4p16.3) [8]. Mutant huntingtin protein accumulates and produces transcriptional dysregulation, proteasomal, autophagical, mitochondrial, and metabolic dysfunctions, oxidative stress, apoptosis, neuroinflammation, and consequent neurodegeneration, especially in the striatum [8, 9]. Unlike ALS, no major treatment has been developed specifically for Huntington's disease. Some symptomatic treatments are available for some motor or psychiatric features [10].

Since both ALS and HD are neurodegenerative disorders, the neuronal loss is progressive and leads to severe disability and reduced quality of life and life expectancy [11]. Although multiple factors are involved in the pathogenesis of these neurodegenerative diseases, which have heterogeneous etiology and background, the mechanisms underlying neuronal death are similar, enabling development of drugs that target many disorders [12]. Nervous system damage in response to

an insult may lead to acute or delayed neuronal death, apoptotic cell death, neuronal degeneration, injury and loss, and gliosis [11]. Thus, neuroprotection can be an important therapeutic intervention that prevents the death of vulnerable neurons and also retracts progression of the disease.

The aim of this review was to discuss possibilities of multimodal and neuroprotective therapies for ALS and HD, employing currently available drugs and potential targets that might be exploited in the future.

2. Amyotrophic Lateral Sclerosis (ALS)

Possibly because the pathogenesis of ALS remains mostly unknown, development of treatments that are effective across the spectrum of sporadic and familial ALS has not been achieved. Considerable evidence supports the involvement of mitochondrial dysfunction and oxidative stress, autophagy, apoptosis, and protein aggregation in the pathogenesis of ALS [13]. Several exciting potential targets for drug intervention to lessen neurodegeneration in ALS are in development [14–16]. We described some of the promising candidates for treating ALS.

2.1. Mitochondrial Function and Oxidative Stress Pathways. The involvement of mitochondria in oxidative damage, calcium buffering, and signaling of apoptotic pathways puts the organelle in perspective for therapeutic approaches. Questions such as whether mitochondrial dysfunctions are a trigger or a consequence in neurodegenerative disease dynamics are still in debate, and increasing interest and discoveries are in course regarding mitochondrial modulators [17]. Studies in ALS patients present strong evidences of mitochondrial dysfunction [18–20]. Moreover, mutant SOD1 mouse model for ALS shows morphological alterations in motor neurons and skeletal muscle tissue before onset of neurodegeneration symptoms, and similar abnormalities are found in sporadic ALS patients [21–23]. Furthermore mitochondrial alterations are not restricted to SOD1 mutants as demonstrated in TDP-43 mutant models [24].

MitoQ is a mitochondrial antioxidant that contains the antioxidant Quinone linked to a lipophilic triphenylphosphonium cation. Researchers showed that MitoQ prolonged life span and the pathology of SOD-knockout *Drosophila melanogaster*, the enzyme involved in ALS [25]. In addition, the compound has been shown to exert neuroprotective effects in SODG93A mice, slowing functional decline, decreasing oxidative damage and disease progression, and increasing survival [26]. Furthermore, other studies showed that MitoQ preincubation prevented the cell death observed in cultures of motor neurons + SOD-mutant astrocytes alone [27].

2.2. Melatonin. Melatonin is a natural hormone produced and secreted by the pineal gland. It is currently used to increase sleep efficiency and improve cardiovascular system and as an antiaging drug [28, 29]. Recent researches in experimental models showed positive effects of melatonin in major neurodegenerative disorders such as ALS, Parkinson's disease, Alzheimer's disease, and HD [30]. In ALS, the most promising effects of melatonin are those of blocking apoptotic pathways and reducing oxidative damage. The mechanisms of melatonin antiapoptotic effects are not completely clear, although the mitochondria have been identified as its target [31].

Oxidative damage caused by free-radicals is accepted as a common link between neurodegenerative diseases and melatonin acts scavenging these free-radicals showing antioxidant activity [32]. Experiments demonstrated attenuation of superoxide induced cell death and the modulation of glutamate toxicity by melatonin *in vitro* NSC-34 motor neurons culture [33].

Other recent study showed an inhibitory activity of melatonin over apoptotic caspase 1/cytochrome c/caspase 3 apoptotic pathway, with melatonin preventing cell death. In ALS SODG93A mice, melatonin inhibited motor neuron death in the ventral horn of spinal cord and delayed disease onset and mortality [34]. The hormone also inhibited Rip2/caspase 1 pathway. In addition, the work had novel findings in showing an association between disease progression and loss of melatonin and melatonin 1A receptors (MT1) in spinal cord of mice. These latter are interesting since MT1 receptors are linked to other major neurodegenerative diseases such as HD and Alzheimer's disease [35–37].

Melatonin clinical trials were so far aimed mostly at insomnia and sleep disturbances [38]. However more recently reduced oxidative damage was reported in patients treated with enteral melatonin at high-doses [33]. Melatonin showed well-toleration event at high-doses and crosses easily the blood-brain barrier further supporting its therapeutic potential.

2.3. Protein Aggregation, Altered Autophagy. One of the consequences of the genetic mutations involved in neurodegenerative diseases is the appearance of misfolded mutant protein aggregates [39]. These aggregates can be toxic and can affect organelles such as mitochondria by causing membrane disruption. In this way, some studies support that clearance of these aggregates could be a therapeutic intervention. In ALS, a link between the disease and protein aggregation altered autophagy was initially observed on morphological studies of spinal cord tissues of ALS patients and models, showing increased number of autophagosomes [40–42].

In this way, as evidences aroused that impaired autophagy may play a pathogenic role in neurodegeneration, compounds promoting autophagy started to be tested. Progesterone and trehalose, two autophagy stimulators, have showed promising results in the SOD model, delayed disease onset and prolonged survival, all related to an induction of the autophagic flux [43, 44].

Rapamycin, an FDA approved drug, that induces autophagy was tested in the SOD1-G93A model with negative results, enhancing the rate of disease progression and motor neuron death [45, 46]. Thus Rapamycin may be, in part, detrimental in ALS due to its immunosuppressive action [45]. However, there are recent data showing that Rapamycin enhances autophagy in immunodeficient mice avoiding the progression of ALS [47]. These authors used

a strategy to avoid the immunosuppressive effect of Rapamycin and so observed its action on increasing autophagy. Then in this case Rapamycin slowed down the progression of ALS [47].

In addition to the previously mentioned effects, the hormone melatonin has also demonstrated neuroprotective activity through enhanced autophagy [48, 49]. Melatonin protected against apoptosis via a mitochondrial pathway, reducing caspase 3 activity, cytochrome c release, and increasing LC3-II/LC3-I levels, an autophagy marker, as demonstrated by Chen et al. [48]. The granular corneal dystrophy type 2 hallmark is linked to impaired autophagic degradation of mutant protein deposits [49]. Choi et al. also demonstrated that melatonin activates autophagy via the mammalian target of Rapamycin pathway (mTOR). Melatonin has showed positive effects in autophagosome formation and maturation, and its cotreatment with Rapamycin had additive effects in autophagic clearance of mutant protein aggregates, in comparison to either drug alone [49]. Although Rapamycin effects of suppression of protective immune responses and enhancement of protective autophagy can cancel each other out in general health improvement [45], these findings suggest that more specific targeting of autophagy with drugs that produce less side effects holds potential for halting disease and neurodegeneration. For that matter melatonin treatment can be a more efficient option with fewer side effects. Moreover it could be possible that cotreatment with melatonin and lower dosages of Rapamycin would increase efficacy while maintaining Rapamycin-induced immunosuppression controlled.

2.4. Targeting the Endocannabinoid System. The endocannabinoid system is recognized as playing a major role in modulating various processes in the body. Increasing evidences suggest an antioxidant, anti-inflammatory, neuroprotective, and other activities such as improving appetite, anxiety, and depression related to endocannabinoids and cannabinoid therapies [50]. However, there are still major controversies regarding therapeutical applications of the endocannabinoid system, such as whether therapeutic effects are achieved by direct agonism/antagonism of cannabinoid receptors or by modulating the endocannabinoid system tonus, by reducing degradation of natural cannabinoids such as anandamide [50, 51].

In ALS, the antioxidant, anti-inflammatory, and neuroprotective activities of cannabinoids are expected to improve ALS symptoms. First surveys assessed marihuana usage in ALS patients and associated it with improvements of appetite, depression, pain, spasticity, and drooling [52].

In ALS SOD1-G93A mice, scientists assessed the effects of cannabinol, a nonpsychotropic compound of the plant. Positive effects regarding disease progression and delay of approximately 2 weeks of disease onset were observed, providing evidence of cannabinoid treatment for ALS [53]. Further evidence was found, showing that cannabinol, another phytocannabinoid, also exerts positive effects of normalizing mitochondrial dynamics associated with caspase 3, DNM1L, and synaptophysin levels in models [54]. More recently, a combination of phytocannabinoids called Sativex was tested in SOD1-G93A mice with moderately positive results [55]. All of these emerging findings point towards neuroprotective and antiapoptotic activities of the cannabinoids in neurodegenerative processes. Better results are expected by the design of more specific compounds, acting on specific populations of cannabinoid receptors, as suggested by discoveries of specific neuroprotective population of cannabinoid receptors [56].

3. Huntington's Disease

A neuropathological hallmark of Huntington's disease is the presence of neuronal nuclear inclusions and cytoplasmic aggregates of misfolded mutant huntingtin protein (mHtt) [9]. The mHtt accumulates and produces transcriptional dysregulation, proteasomal, autophageal, mitochondrial, and metabolic dysfunctions, oxidative stress, apoptosis, neuroinflammation, excitotoxicity, and consequent neurodegeneration [8, 9]. As HD is a genetic disease, affected patients have abnormal huntingtin from the very first moment of the protein's expression, which suggests that neuronal abnormalities might be present since the start. Targeting these pathways might be a cleaver strategy for treating HD.

3.1. Apoptotic Pathways. Neuronal cell death in HD is associated with neuronal apoptosis, particularly with the initiation of the intrinsic mitochondrial apoptotic pathway [12]. Markers for apoptotic cell death are activated in striatal neurons from both patients with HD and animals models [57]. Activation of caspases 3 and 9 and release of cytochrome c from the mitochondria into the cytosol are observed both in the brains of patients and animals with HD [58].

Minocycline is an antibiotic, reported to exert neuroprotective activities through caspase 1, caspase 3, inducible form of nitric oxide synthase, and p38 mitogen-activated kinase (MAPK). It has good oral bioavailability, tolerability, and crosses blood-brain barrier easily. In ALS models, minocycline delayed disease onset and extended survival. It also inhibited cytochrome c release, which was demonstrated both *in vivo* and in isolated mitochondria [59].

Chen et al. reported that the inhibition of caspases 1 and 3 expression by minocycline delayed mortality in R6/2 mice, a model of HD. In addition, minocycline attenuated dopaminergic cell loss and delayed mortality in MPTP-treated mice. Minocycline was taken to human trials for HD as a promising therapy but failed. Despite its promisor results, it has encountered limitations for its use due toxicity in pharmacological dosages [59]. Moreover, the currently available caspase inhibitors are toxic in pharmacological doses, precluding their immediate use in human studies [12].

3.2. Oxidative Stress. Oxidative stress is characterized by an imbalance between reactive oxygen species (ROS) and antioxidant systems [60]. Interestingly, Chen et al. described a correlation between lipid peroxidation products in plasma and degree of severity in patients with HD [59]. Klepac et al. also reported an occurrence of oxidative stress in HD [61].

Dimethyl fumarate (DMF), an essential member of fumaric acid ester (FAE) family, is the active ingredient of BG-12, which has been offered as an effective oral treatment option for patients with relapsing remitting multiple sclerosis (RRMS) [62]. DMF can activate transcription factor nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2). Nrf2 plays an important role of anti-oxidative pathways for tissue protection [63]. There is compelling evidence of the disruption of the Nrf2 system in HD and the contribution of Nrf2 activation to ameliorating oxidative stress and mitochondrial dysfunction in neuronal tissue damage in HD. Jin and colleagues have shown that mHtt disrupts Nrf2 signaling, which contributes to impaired mitochondrial dynamics and may enhance susceptibility to oxidative stress in STHdh (Q111/Q111) cells, striatal cells expressing mHtt [64]. Moreover, DMF treatment leads to an increase in Nrf2 staining in neuronal subpopulations relevant for motor functions, concomitant with elevated Nrf2 immunoreactivity in R6/2 mice, which mimic many aspects of HD. Additional studies in N171-82Q mice, another transgenic mouse model of HD, showed that 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid-ethyl amide (CDDO-EA) and 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid-trifluoroethyl amide (CDDO-TFEA) upregulate Nrf2/ARE induced genes in the brain and peripheral tissues and reduce oxidative stress, improving motor impairment and increasing longevity [65].

3.3. Neuroprotective Cell Signaling Pathways. The signaling pathways involved in HD are not yet clearly elucidated [66]. Thus, it is possible that alterations of receptor-mediated signaling pathways could contribute to protection or exacerbation of cell death cascades in the symptomatic and/or presymptomatic phases of HD [67]. Recently, Doria et al., 2015, described the effect of a positive allosteric modulator (PAM) for metabotropic glutamatergic receptor type 5 (mGluR5), named CDPPB (3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide) [68]. This drug was capable of delaying some symptoms related to HD [68, 69]. Chronic treatment of BACHD mice (model of HD) with CDPPB 1.5 mg/kg for 18 weeks increased the activation of cell signaling pathways, including increased AKT and ERK1/2 phosphorylation, and augmented the BDNF mRNA expression. CDPPB chronic treatment was also able to prevent the neuronal cell loss that takes place in the striatum of BACHD mice and decrease mutant huntingtin aggregate formation [68]. Moreover, CDPPB chronic treatment was efficient to partially ameliorate motor incoordination and to rescue the memory deficit exhibited by BACHD mice. Importantly, no toxic effects or stereotypical behavior was observed upon CDPPB chronic treatment; however the exact CDPPB mechanism of action and more safety tests need to be performed [68].

3.4. Excitotoxicity. Excitotoxicity is known to be an important piece in the development of HD. Thus, antiglutamatergic agents may, therefore, have beneficial neuroprotective effects [70]. One of these agents is tryptophan metabolite kynurenic acid (KYNA), which is an endogenous NMDA receptor antagonist [70]. Although the neuroprotective KYNA shows unaltered levels in mice models, the significant elevation in the concentrations of neurotoxic kynurenine pathway compounds leads to a shift in the metabolism resulting in relative KYNA deficiency [70]. These findings raise the possibility that increasing KYNA effect would be beneficial from a therapeutic aspect. However, higher doses of KYNA have low solubility and poor penetration to blood-brain barrier [71].

Memantine is a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist that stabilizes glutamatergic tone. Memantine can attenuate the excitotoxic mechanism [72]. One interesting study examined Memantine as a treatment for patients suffering from Huntington disease. The administration of 20 mg of Memantine/daily significantly improved motor symptoms and improved chorea but failed to improve patient's cognitive, behavioral, functional, or independence ratings [73].

4. Conclusion

Neurodegenerative diseases are multifactorial and despite recent progress, basic needs such as the definition of disease biomarkers and molecular mechanisms of neurodegeneration are still to be addressed. No major treatment has been developed specifically for ALS and HD. Some symptomatic treatments are available for some motor or psychiatric features. In the meantime, the revelation of new mechanisms involved in the disease onset and progression gives opportunity for novel approaches in symptomatic treatment. The development or improvement of disease models is also necessary for better assessment of drugs and pathological mechanisms. Multifunctional and multitarget approaches will probably be needed to restore neuronal health when cell dysfunction is present. We must have in mind that multitargeted and combined therapies may be an option in that regard.

Review Criteria

Articles were selected based on searches of PubMed using a number of different search terms, such as "Neurodegenerative diseases" "Huntington's disease," "Amyotrophic Lateral Sclerosis," "neuroprotection," "neurodegeneration," "pathogenesis," "mitochondria," "apoptosis," "huntingtin," and "autophagy." Only full-text papers written in English and its references were selected.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors exchanged ideas for the review. Caroline Zocatelli de Paula reviewed the references and prepared the first draft including the reference list. Bruno Daniel Correia

Gonçalves and Luciene Bruno Vieira reviewed the first draft and provided comments on the references. Luciene Bruno Vieira prepared the final draft. All authors approved the final paper.

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Research Article

Multicontrast MRI Quantification of Focal Inflammation and Degeneration in Multiple Sclerosis

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Introduction. Local microstructural pathology in multiple sclerosis patients might influence their clinical performance. This study applied multicontrast MRI to quantify inflammation and neurodegeneration in MS lesions. We explored the impact of MRI-based lesion pathology in cognition and disability. *Methods*. 36 relapsing-remitting MS subjects and 18 healthy controls underwent neurological, cognitive, behavioural examinations and 3 T MRI including (i) fluid attenuated inversion recovery, double inversion recovery, and magnetization-prepared gradient echo for lesion count; (ii) T1, T2, and $T2^*$ relaxometry and magnetisation transfer imaging for lesion tissue characterization. Lesions were classified according to the extent of inflammation/neurodegeneration. A generalized linear model assessed the contribution of lesion groups to clinical performances. *Results*. Four lesion groups were identified and characterized by (1) absence of significant alterations, (2) prevalent inflammation, (3) concomitant inflammation and microdegeneration, and (4) prevalent tissue loss. Groups 1, 3, 4 correlated with general disability (Adj-$R^2 = 0.6$; $P = 0.0005$), executive function (Adj-$R^2 = 0.5$; $P = 0.004$), verbal memory (Adj-$R^2 = 0.4$; $P = 0.02$), and attention (Adj-$R^2 = 0.5$; $P = 0.002$). *Conclusion*. Multicontrast MRI provides a new approach to infer *in vivo* histopathology of plaques. Our results support evidence that neurodegeneration is the major determinant of patients' disability and cognitive dysfunction.

1. Introduction

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disease affecting the brain and spinal cord. The hallmark of MS is the presence of multifocal lesions or "plaques," which are characterized by variable inflammatory, degenerative, and reparative processes [1, 2]. Plaques inflammation is widespread in the relapsing-remitting MS subtype, whereas important tissue loss is pronounced in progressive MS and in long-standing disease [3, 4]. In addition, new lesions are mostly characterized by inflammatory phenomena, leading to blood-brain barrier disruption, while older

lesions show a higher proportion of neurodegeneration and/or repair processes [4, 5].

Conventional magnetic resonance imaging (cMRI) is a valuable tool to provide information about the number, location, and inflammatory "activity" of focal lesions. Nevertheless cMRI offers only limited sensitivity to focal pathology in the cortex and little insight into the nature of local damage. Nonconventional MRI techniques such as double inversion recovery (DIR, Geurts Radiology 2005) and magnetization-prepared 2 rapid gradient echo (MP2RAGE, Marques Neuroimage 2010 and Kober 2012) have proven higher sensitivity to focal cortical pathology than cMRI. Similarly, the combination of multiple cMRI contrasts improved cortical lesions detection at all field strengths (1.5 T (B. Moraal), 3 T (M. Archambault-Wallenburg), and 7 T (W. L. De Graaf)). Besides, other advanced MRI techniques have shown to be sensitive to tissue pathology in lesions, such as axonal and myelin damage (diffusion tensor imaging (DTI) and magnetisation transfer imaging (MTI)) and axonal metabolic deficits (magnetic resonance spectroscopy) [2, 6–9]. MRI relaxometry has also been extensively used to study normal-appearing brain tissue in multiple sclerosis patients (for review see [9, 10]), but only few works focused on lesions properties and heterogeneity [11, 12]. Yet, some recent postmortem studies provided strong evidence of the value of MRI relaxometry techniques to study specific aspects of plaques pathology; Bagnato et al. showed that high $R2^*$ values in the periphery of white matter (WM) lesions correlated with iron accumulation in macrophages/microglia whereas high $R2^*$ inside the WM plaque had the appearance of iron aggregates typical of microbleeds [13]. Furthermore, Tardif et al. established that myelin loss within cortical lesions was associated with a concomitant increase of T1 and T2 relaxation times and a decrease of MTI measures [14].

In this work, we combined, *in vivo* in MS patients, three relaxometry techniques (T1, T2, and $T2^*$), and MTI. The aims of the study were (i) to classify MS cortical and white matter lesions according to the extent of inflammatory and neurodegenerative phenomena, as measured by unconventional MRI and (ii) to assess the clinical impact of MRI measures of lesion pathology in a cohort of relapsing-remitting multiple sclerosis patients.

2. Methods

2.1. Study Population. Thirty-six patients with relapsing-remitting MS (RRMS) and eighteen age-matched healthy controls (HC) were enrolled in this cross-sectional study between January and December 2012. The age of the patients was 34.8 ± 9.2 years (mean ± standard deviation (SD)) and gender ratio was 24/12, women/men. HC aged 33 ± 9.7 years and had a gender ratio of 9/9. The time elapsed since the first symptoms was 33.3 ± 21 months (range: 2–70 months) and the time since disease diagnosis was 27.1 ± 18 months (range 0–59 months). Immunomodulatory treatment, consisting in high dose interferon-beta (IFN-β) or fingolimod, was administered to thirty patients out of thirty-six patients (83%) for at least 3 months. No patient had received corticosteroid therapy within the three months preceding the study. The study was approved by the ethics committee of the Lausanne University Hospital (CHUV). Written, informed consent was obtained from each subject.

2.2. Clinical Assessment. Verbal and spatial memory, sustained attention, information processing speed, and verbal fluency on semantic cues were assessed at the time of MRI for each subject using the Brief Repeatable Battery of Neuropsychological Tests (BRB-N) [15]. Depression and fatigue were quantified using the Hospital Anxiety and Depression Scale (HAD) [16] and the Fatigue Scale for Motor and Cognitive Functions (FSMC) [17]. Finally, the Expanded Disability Status Scale (EDSS [18]) and the Multiple Sclerosis Functional Composite (MSFC [19]) scores were evaluated to quantify disability and motor performances.

2.3. MRI Acquisition. All subjects underwent MRI examinations on a 3 T Siemens Trio (Siemens, Erlangen, Germany) equipped with a 32-channel head coil. MRI protocol details were previously reported in [20] and summarized in Table 1s (supplementary data) (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/569123). In summary, a 3D magnetization-prepared acquisition with gradient echo (MPRAGE) was acquired for automatic brain tissue and atlas-based segmentation [21–23]; 3D fluid attenuated inversion recovery (3D FLAIR), 3D double inversion recovery (3D DIR), and 3D MP2RAGE [11] were acquired for lesion detection and segmentation. The MP2RAGE sequence additionally provided whole-brain T1 relaxometry [24]. $T2^*$ relaxometry maps were obtained using 32 echoes and a correction method based on an estimated B0 field map [25]. Magnetization transfer ratio (MTR) maps were derived from the $T2^*$ data, after registration of echoes with (MT) and without MT pulse (M0) (MT pulse flip angle: 220°; duration: 4000 ms; pulse offset: 2000 Hz; and spoiler moment: 25000 us∗mT/m). The magnetization transfer ratio MTR = (M0 − MT)/M0 was then computed for each echo and averaged over all echoes. For T2 relaxometry, we used a new nonlinear inverse reconstruction algorithm [26] that directly estimates a T2 and spin-density map from a train of undersampled spin echoes. The acquisition of T2 relaxometry maps was performed with a spatial resolution, which is lower than the one achieved for the other MRI contrasts and maps. Nevertheless, the current protocol appears to have quite similar resolution compared to recently published T2 mapping sequences [27] and was optimized to achieve the best T2 maps quality in clinically compatible scanning times.

Visual inspection of image quality was performed in all cases. An example of T1, T2, and $T2^*$ and MTR maps is reported in Figure 1.

The biological interpretation of changes in T1, T2, and $T2^*$ relaxation times (rt) and MTR was summarized in Figure 2 and previously reported in detail [20].

2.4. Image Analysis. Rigid registrations with BSpline interpolation were performed, using Elastix C++ [28], to register (i) the T2 maps to the T1 maps (MP2RAGE) and (ii) the

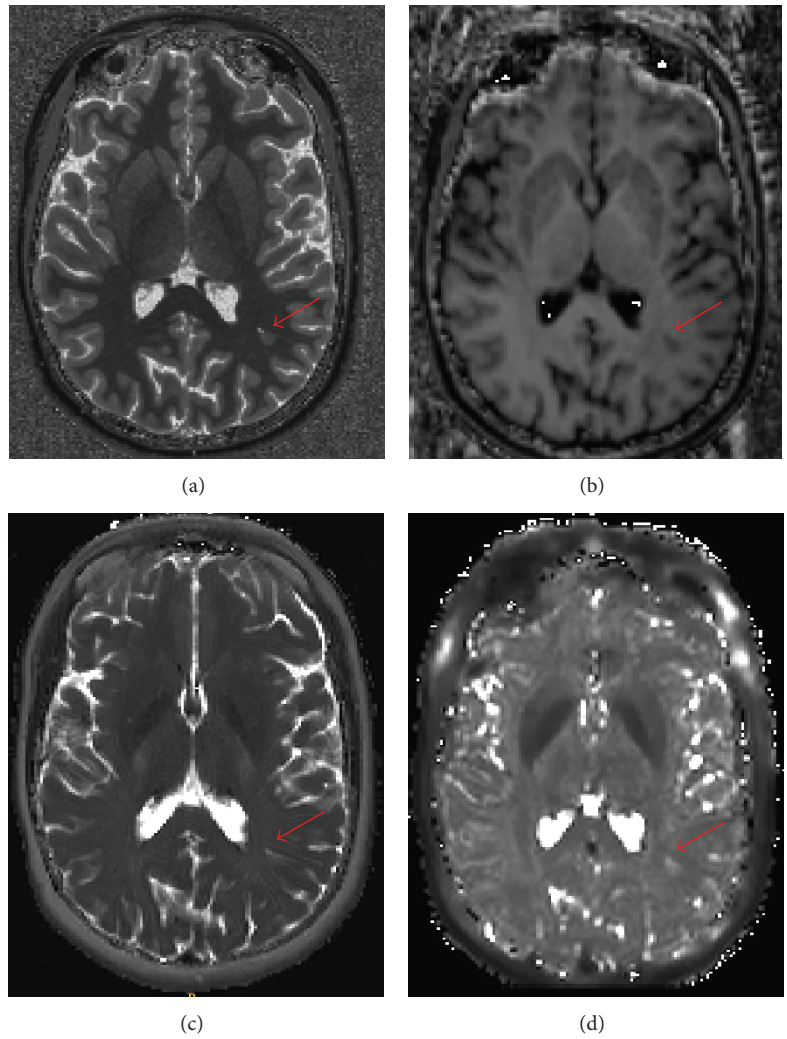

Figure 1: T1 map (a), MTR (b), T2 map (c), and T2* map (d) in one MS patient. An example of lesion is shown by a red arrow.

T2* maps, MPRAGE, FLAIR, and DIR images to one of the inverted contrasts of the MP2RAGE sequence.

Cortical and WM MS lesions were manually identified in patients by an experienced neurologist (CG) and a radiologist (DR) using 3D FLAIR, 3D DIR, and MP2RAGE images, as previously reported [20, 22, 24]. Manual contours were generated for each lesion by a trained technician for each contrast. As reported by [11, 20], we merged the lesions extracted from FLAIR, DIR, and MP2RAGE to obtain a final union lesion mask for each subject. Lesion volumes were computed and normalized by total intracranial volume as obtained using an in-house software [20, 29]. Only lesions with more than 10 voxels size were included in the analysis. Lesion masks were then registered to MP2RAGE space using the registration parameters described above and mean T1, T2* and MTR were calculated for each lesion.

In order to assess the mean distribution of T1, T2, and T2* rt and MTR in HC brain tissue, we segmented lobar WM and cortical GM (frontal, parietal, occipital, and temporal) as well as cerebellar WM/GM from the MPRAGE images using an in-house software based on variational expectation-maximization tissue classification [20, 29].

To compare lesion MRI properties in patients with the corresponding tissue in HC, we calculated a z-score for each contrast in each lesion (e.g., for T1 data):

$$z_{\mathrm{T1}} = \frac{1}{N} \sum_{v \in l} \frac{I_{\mathrm{T1}}(v) - \mu_{\mathrm{T1}}(L_l, T_l)}{\sigma_{\mathrm{T1}}(L_l, T_l)}, \quad (1)$$

where z_{T1} corresponds to the T1 lesion z-score (z), l to the lesion voxels, N to a normalisation term, I_{T1} to the T1 map, and $\mu_{\mathrm{T1}}(L_l, T_l)$ and $\sigma_{\mathrm{T1}}(L_l, T_l)$ to the mean and the standard deviation of the T1 map in the lobe L_l and tissue T_l (i.e., WM or GM) in the HC group, corresponding to the lesion location and type.

Considering the continuous distribution (without distinct cluster) of lesions z-scores in each contrast, we classified the lesions into 3 groups as follows: (i) z very low ($z < -2$), (ii) z very high ($z > 2$), and (iii) z close to the HC distribution ($-2 \leq z \leq 2$). The thresholds were chosen considering

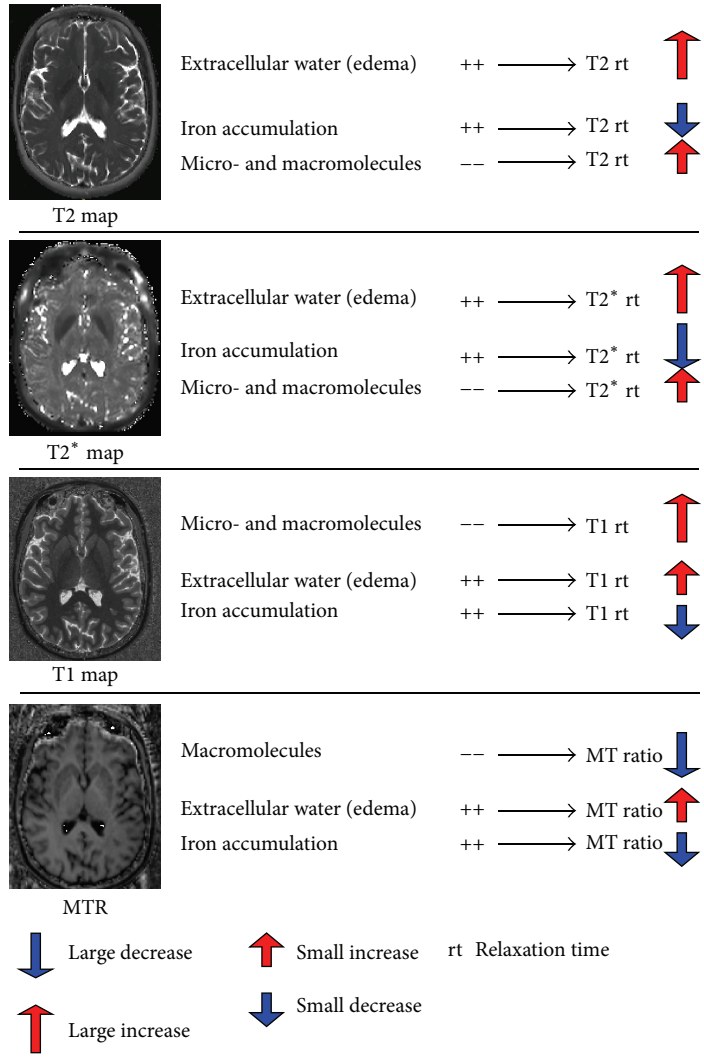

Figure 2: Biological interpretation of quantitative and semiquantitative MRI contrasts.

the fact that more than 95 percent of the z-scores belong to the interval $[-2, 2]$ in a normal distribution and that values beyond this interval reflect significant differences in patients compared to controls ($P < 0.05$).

Last, for each subject, all existing combinations (Figure 3) of z were computed for all contrasts (e.g., combination 1 = $z_{T1} > 2$, $z_{T2} > 2$, $z_{T2^*} > 2$, and $z_{MTR} < -2$; combination 2 = $z_{T1} > 2$, $z_{T2} > 2$, $-2 < z_{T2^*} < 2$, and $z_{MTR} < -2$, etc.) and mean lesion volume (MLV) was assessed for each combination (total normalized lesion volume/number of lesions).

2.5. Statistical Analysis

2.5.1. Between-Groups Comparisons of Subjects' Demographics and Clinical Scores. Differences in age, gender, education, and clinical performance were assessed using a nonparametric ANOVA (Kruskal-Wallis test) among HC and MS patients.

2.5.2. Multivariate Linear Regression of Clinical Scores in Patients with T1, T2, T2 and MTR in Lesions.* A multivariate linear regression of clinical scores was performed using a general linear model (GLM) applied to MLV in each combination of contrasts. Age, gender, educational years, anxiety, and depression scores (HAD) were considered as covariates, since they have been reported to be linked to functional performance in MS patients [30, 31]. Cognitive scores were adapted using Box-Cox transformation to satisfy the model assumption for normality [32].

We performed eight regressions and applied a backward stepwise approach to select the best prediction model for each dependent variable (clinical scores). Bonferroni correction was applied for multiple comparisons (seven tests). "Leave-one-out" (LOO) cross-validation was applied to assess the prediction quality and robustness of each model. A P value < 0.05 was considered statistically significant.

All regression analyses were performed using R software (http://www.R-project.org/).

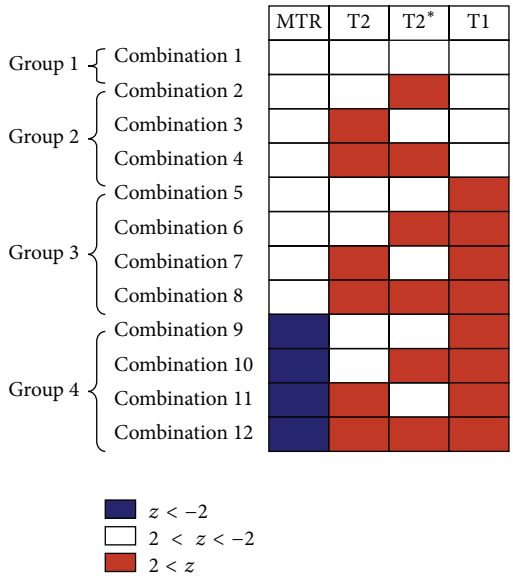

Figure 3: Groups and contrasts combinations of MS lesion z-scores for T1, T2, and T2* and MTR contrasts, as observed in our cohort of RRMS patients. Blue: parameter decrease; red: parameter increase. Group 1: lesions with no significant q/sq MRI contrasts changes; Group 2: lesions with prevalent inflammatory oedema; Group 3: lesions with prevalent tissue degeneration with or without inflammation; and Group 4: lesions with prevalent tissue loss.

3. Results

3.1. Between-Groups Comparisons of Subjects' Demographics and Clinical Scores. No significant differences were observed between HC and MS patients in terms of age ($P = 0.3$) or gender ($P = 0.8$); however, HC had slightly higher education levels (17 ± 4 years, mean ± standard deviation) than MS patients (15 ± 3 years; $P = 0.04$).

Mean EDSS in patients was 1.6 ± 0.3 (interval: 1-2). The FSMC motor score was significantly higher in MS patients (23.1 ± 10.5) than in HC (14.8 ± 5.8; $P < 0.02$). The FSMC cognitive scores, cognitive performance, MSFC scores, and anxiety and depression scores (HAD) were not significantly different between groups ($P > 0.1$).

3.2. Contrasts Combinations and Lesion Combination Distribution. We found 12 z-scores combinations in all MS lesions (1402 lesions, Figure 3). These combinations characterised plaques with no significant contrast changes (Group 1: combination 1, 54% cortical and 46% WM lesions), prevalent inflammatory edema (Group 2: isolated increase of T2 and/or T2* z-scores, combinations 2–4, 40% of cortical and 60% of WM lesions), microdegeneration, and/or inflammatory edema (Group 3: increase in T1 and/or increase in T2/T2*, combinations 5–8, 2% cortical and 98% WM lesions), and broad tissue loss (Group 4: strong increase in T1 and decrease in MTR z-scores, with or without increase in T2/T2*, combinations 9–12, 100% WM lesions) (Figure 3).

Most of the lesions (70%) showed a significantly high T1 z-score (Group 3 and 4) and only 27% of total number of lesions did not show any significant change in all contrasts (Group 1); 48% of lesions showed high T1 z-score only (Group 3), 32% exhibited high T1 z-score combined with high T2 or T2* (Group 3), and 18% were characterized by high T1 z-score combined with low MTR (Group 4). Group 2 containing lesions with high T2 and/or T2* and "nonsignificant" T1 and MTR counted less than 3% of the total number of lesions (Figure 4).

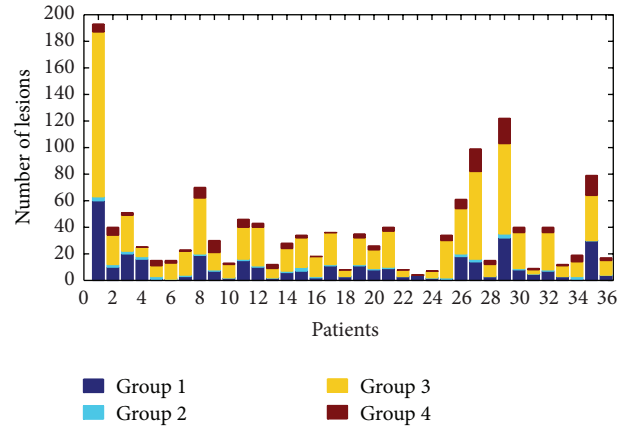

Figure 4: Lesion groups distribution in RRMS patients. Groups 1 and 3 account for more than 60% of all lesions and are the most represented groups in patients.

Table 1: Lesions count in brain hemispheres and cerebellum.

Combinations	Brain			Cerebellum		
	WM	Cortical		WM	Cortical	
		Type I	Type II		GM/WM	GM
1	161	186	7	10	11	0
2	7	8	4	0	1	0
3	10	3	0	0	1	0
4	438	7	6	24	0	0
5	3	1	0	0	0	0
6	216	0	0	1	0	0
7	50	1	1	3	0	0
8	89	0	0	1	0	0
9	51	0	0	0	0	0
10	8	0	0	0	0	0
11	34	0	0	0	0	0
12	58	0	0	1	0	0
Total no.	1125	206	18	40	13	0
%	80.24	14.69	1.28	2.85	0.93	0

The cortical lesions represented 17% of the total number of lesions; 90% were cortical lesions Type I (mixed GM/WM) and 10% Type II (GM only) (Table 1). They mainly belong to combination 1 (85%) and combinations 2 to 8. Most of the lesions were pure white matter lesions (83%, Table 1) and appeared in all combinations.

3.3. Multivariate Linear Regression of Clinical Scores in Patients with T1, T2, and T2 and MTR in Lesions.* GLM

Table 2: Multiple regression analysis between lesion combinations, covariates, and clinical scores.

(a)

Predictors (*P* value)	Clinical scores						
	MSFC	FV	SRT	SDMT	Tot10/36	FSMCCog	FSMCMot
Stepwise regression							
P value	0.00006*	0.00024*	0.00219#	0.00054*	0.03156†	0.03120†	0.03090†
Corrected *P* value	0.00045*	0.00166#	0.01536†	0.00379#	0.22092‡	0.21840‡	0.21630‡
Adjusted-*R*	0.55050	0.45350	0.38040	0.48960	0.20990	0.24770	0.2483
Cross-validation: leave-one-out							
P value	0.00001*	0.00004*	0.00097*	0.01300†			
Corrected *P* value	0.00005*	0.00030*	0.00677#	0.09100‡			
Adjusted-*R*	0.43660	0.37490	0.25620	0.14360			

(b)

Predictors (*P* value)	Clinical scores						
	MSFC	FV	SRT	SDMT	Tot10/36	FSMCCog	FSMCMot
z-scores combination							
Group 1							
Combination 1			0.0048#	0.0003*			
Group 2							
Combination 2							
Combination 3							
Combination 4							
Group 3							
Combination 5		0.0003*		0.0088#			0.0223†
Combination 6		0.0182†		0.0049#			
Combination 7							
Combination 8	0.0200†						
Group 4							
Combination 9	0.0011#	0.0256†	0.0175†	0.0001*		0.0331†	0.0168†
Combination 10			0.0057#				
Combination 11							
Combination 12							
Covariates							
Age				0.0056#			0.0144†
Gender	0.0007*	0.0004*					
Educational years							
HADA (anxiety)							0.0436†
HADD (depression)	0.0341†			0.0400†		0.0136†	

*$P < 0.001$.
#$P < 0.01$.
†$P < 0.05$.
Table 2(a): each line corresponds to the *P* values, corrected *P* values, and adjusted-*R* of each model ($n = 7$) subjected to regression and cross-validation analysis.
Table 2(b): each line corresponds to the *P* values of each predictor for every regression model performed.
The different symbols denote the difference in significance: *highest significance ($P < 0.001$), #middle range significance ($P < 0.01$), †low significance ($P < 0.05$), and ‡nonsignificant predictor ($P > 0.05$).

using stepwise regression revealed a highly significant association, confirmed by a cross-validation test, between lesions MRI characteristics of lesions and three clinical scores (Table 2).

(i) The MLV in combinations 8 and 9 (Group 3 and 4) together with age and depression score predicted the MSFC (general disability) score (Adj-R^2 = 0.6; P = 0.0005).

(ii) The MLV in combinations 5, 6, and 9 (Group 3 and 4) in conjunction with gender predicted the FV (execution) score (Adj-R^2 = 0.5; P = 0.002).

(iii) The MLV in combinations 1, 9, and 10 (Groups 1 and 4) predicted the SRT (verbal memory) score (Adj-R^2 = 0.4; P = 0.002).

(iv) MLV in combinations 1, 5, 6 and 9 (Groups 1, 3, and 4) with age and depression score predicted the SDMT (attention function) score (Adj-R^2 = 0.5; P = 0.004). Nevertheless, cross-validation test revealed a possible overfitting of the GLM (estimated score versus clinical score: Adj-R^2 = 0.1; P = 0.09).

4. Discussion

Current diagnostic and prognostic criteria in MS as well as clinical trials end-points are based on conventional MRI measures of lesions number, volume, and activity [33]. Nevertheless, these parameters provide only limited information about the nature and severity of tissue alterations in the central nervous system.

In fact, changes in conventional T1 and T2 signals are compatible with both inflammatory and degenerative phenomena [20]; moreover, the presence of "black holes," considered to be a marker of permanent axonal/myelin loss [34, 35], might be also due to inflammatory extracellular edema [35] and activated microglia [36, 37]. Furthermore, gadolinium (Gd) enhancement, a conventional marker of active inflammation, does not detect active lesions with mild changes in blood-brain barrier (BBB) permeability [38] and disseminated inflammation due to activated microglia [39]. In addition, the presence of Gd uptake might reveal incomplete restoration of tight junction integrity and BBB function in inactive, noninflamed, chronic lesions [40].

We recently showed the potential of advanced MRI techniques to unravel the nature of diffuse and focal tissue pathology in MS [20]. In this work, we aimed at investigating the influence of unconventional MRI metrics of lesion pathology on patients' disability and cognition.

In accordance with previous literature at 3 T [11] we found that the majority of lesions detected in our cohort of early MS patients were located in WM (83%), a moderate number were mixed WM/GM (cortical lesion Type I) (15%), and few were purely cortical and punctiform (cortical lesion Type II) (2%) (Table 1).

We identified twelve combinations of MRI contrasts in MS lesions, which we organized into four main groups according to the predominant underlying pathology (Figure 3). Group 1 was constituted by lesions that did not show any significant contrast change, possibly due to pathophysiological causes (i.e., presence of more efficient reparative processes in early stages of disease) and/or technical aspects (lack of sensitivity/spatial resolution). The other three groups were constituted by lesions exhibiting prevalent inflammation (Group 2), microdegeneration with/without inflammation (Group 3), or predominant tissue loss (Group 4). These four groups were consistent with those reported by the histopathological "Vienna Classification" of MS lesions (Group 1: Vienna lesion type VLT 6; Group 2: VLT 2; Group 3: VLT 2/5; and Group 4: VLT 5) [41].

Interestingly, we did not observe any T1/T2/T2* decrease in local plaques, suggesting that no significant iron accumulation occurs in our cohort of patients. However, since we performed an average lesion analysis, this observation does not exclude the presence of local iron increase, as previously reported [13, 42].

Last, we studied the relative impact of lesion combinations/groups on clinical performance in patients. And we found that lesions with concomitant microdegeneration/inflammation or important tissue loss had a greater impact on patients' disability, executive function, and verbal memory than prevalent inflammatory lesions. This result could be due to the presence of a minority of lesions in the purely inflammatory group (Group 2), which might be due to the fact that most of the patients were benefitting of immunomodulatory/immunodepressive therapy. In addition, lesions with no significant changes in multicontrast MRI (Group 1) played an important role in verbal memory and attention. This aspect is coherent with the fact that the majority of Group 1 lesions were located in the cortical layers; yet, it could be also due to the fact that a proportion of Group 1 lesions are located in eloquent areas. In order to elucidate this last point, an ongoing study is aiming at integrating the lesion location information in the current lesion classification.

In summary, our current work provides a new approach to infer histopathological information from MS plaques and supports evidence that MRI measures of lesion pathology are strong determinants of patients' clinical performance in our cohort.

A technical limitation of this study is the low in-plane resolution of the T2 relaxation maps, compared to the other applied maps and MRI contrasts. Though we tried to overcome this limit by setting a threshold to lesion size (>10 voxels), this aspect could impact the estimations of average T2 values in small lesions. Future hardware and software improvements are required to achieve higher spatial resolution in accelerated T2 relaxometry acquisitions. Another limitation of this method is the lack of sensitivity to repair/plasticity (i.e., gliosis, axonal remodeling, etc.) as well as to other inflammatory phenomena like lymphocytic/microglia infiltration and activation. Studies focusing on the longitudinal pattern of contrasts evolution in MS lesions and the combination with other MRI contrasts (i.e., diffusion imaging) or modalities (i.e., MRI-PET) might help to overcome these limits.

Conflict of Interests

Alexis Roche and Gunnar Krueger are Siemens AG employees. The other authors have nothing to disclose.

Authors' Contribution

Cristina Granziera and Gunnar Krueger carried out study design; Guillaume Bonnier, Alexis Roche, David Romascano, Samanta Simioni, Djalel Meskaldji, David Rotzinger, Ying-Chia Lin, Gloria Menegaz, Gunnar Krueger, and Cristina Granziera carried out collection, analysis, and interpretation of data; Guillaume Bonnier, Alexis Roche, David Romascano, Samanta Simioni, Djalel Meskaldji, David Rotzinger, Ying-Chia Lin, Gloria Menegaz, Myriam Schluep, Renaud Du Pasquier, Tilman Johannes Sumpf, Jens Frahm, Jean-Philippe Thiran, Gunnar Krueger, and Cristina Granziera carried out writing of the report and taking decision to submit the paper for publication.

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Research Article

CCL27: Novel Cytokine with Potential Role in Pathogenesis of Multiple Sclerosis

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Multiple sclerosis (MS) is an autoimmune and neurodegenerative disease of unknown etiology. Leukocyte infiltration of brain tissue and the subsequent inflammation, demyelination, axonal damage, and formation of sclerotic plaques is a hallmark of MS. Upregulation of proinflammatory cytokines has been suggested to play an essential role in regulating lymphocyte migration in MS. Here we present data on serum cytokine expression in MS cases. Increased serum levels of IL-17 and IL-23 were observed, suggesting activation of the Th17 population of immune effector cells. Additionally, increased levels of IL-22 were observed in the serum of those with acute phase MS. Unexpectedly, we observed an upregulation of the serum chemokine CCL27 in newly diagnosed and acute MS cases. CCL27 is an inflammatory chemokine associated with homing of memory T cells to sites of inflammation. Therefore, its upregulation in association with MS suggests a potential role in disease pathogenesis. Our data supports previous reports showing IL-17 and -23 upregulation in association with MS and potentially identify a previously unknown involvement for CCL27.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) with undefined etiology. The disease has a polysymptomatic onset and is usually first diagnosed between the ages of 20–40 years [1]. Although there are no clinical findings unique to MS, some symptoms are characteristic of the disease, such as sensory abnormalities and visual and motor impairment [2]. In 80–85% of cases, initial progression of MS is characterized by episodes of neurological disability and recovery. This clinical presentation is classified as remitting-relapsing MS (RRMS) [3, 4]. As the disease progresses, 60–70% of RRMS cases will gradually worsen with a steady progression of symptoms [5]. This pattern of disease is referred to as secondary RRMS. A small group of cases (approximately 10%) develop MS characterized with a steady progression of neurological symptoms without periods of recovery. This is classified as primary-progressive MS (PPMS) [6, 7].

Formation of multiple brain lesions is a typical characteristic of MS. Sclerotic plaques form at the sites of inflammation, demyelination, and axonal damage. It is believed that autoreactive T lymphocytes play a major role in initiating the immune assault against axonal myelin sheets in the CNS, which leads to demyelination and subsequent neuronal death [5, 8]. Increased permeability of the blood brain barrier

(BBB), as documented in MS cases [9–11], is essential for leukocyte migration into brain tissue. Accordingly, previous studies have shown substantial remodeling of the BBB in MS cases. For example, decreased expression of tight junction molecules in BBB endothelial cells has been observed in MS [11, 12]. Disruption of BBB integrity is further confirmed by the observation of elevated matrix metalloprotease (MMP) 2 and 9 expression in MS brain lesions [13–15]. Similarly, upregulation of MMP2 and 9 has been shown in astrocytes and neuroglia, implicating these cells in the pathogenesis of MS [13, 14].

Leukocyte infiltration is a common finding at the sites of MS brain lesions. Previous studies have reported high IFN-γ secretion by autoreactive T lymphocytes, suggesting a Th1 phenotype of myelin-specific T cells [16–18]. Recently, a Th17 lymphocyte subset was also shown to play a role in MS pathogenesis. In this study, Brucklacher-Waldert et al. reported an increase in the numbers of Th1 and Th17 cells in the blood and cerebrospinal fluid (CSF) of MS cases [19]. They also reported a significant increase in the number of Th17 lymphocytes during the relapse stage; however, Th1 counts remained unchanged [19]. Furthermore, they determined that Th17 cells from MS cases had a higher proliferative capacity and were less susceptible to suppression, as compared to Th1 cells. In another study, Kebir et al. demonstrated that, in MS, Th17 lymphocytes more readily cross the BBB as compared to Th1 cells [20]. Therefore, they suggested that Th17 lymphocytes might be more encephalitogenic than Th1 cells.

A compromised BBB and facilitated migration of autoreactive immune effector cells are essential for development of MS. Both BBB integrity and leukocyte trafficking are regulated by cytokines. Previous studies have shown upregulation of Th1 type cytokines, such as IL-2, IFNγ, and IL-12, in subjects with MS, while the Th2 cytokines, IL-4 and IL-10, were downregulated [21, 22]. Furthermore, administration of IFNγ exacerbated clinical and hematological symptoms of MS [23, 24], and higher levels of IL-2 and lower levels of IL-10 have been detected in relapsed MS cases [21]. Elevated levels of IL-17 have been observed in the CSF and serum of MS cases [25], and during clinical exacerbations, higher levels of IL-17 mRNA were also detected in the CSF relative to that in the blood [26]. Subsequently, Th17 transcripts were detected in MS lesions [25]. IL-17-producing leukocytes have been suggested to belong to a new subset of Th17 lymphocytes that is maintained and driven by IL-23 [27, 28]. Therefore, the current paradigm of MS pathogenesis indicates that Th17 lymphocytes together with Th1 cells are central to development of neuroinflammation, demyelination, and neural death [29, 30].

Although it is well established that cytokines play a central role in lymphocytes breaching the BBB, as well as their subsequent migration into neuronal tissue, our knowledge regarding cytokine activation and their involvement in MS is limited. Here we report data on serum levels of 57 cytokines in MS cases with different clinical presentations. Overall, our data support a role for mononuclear leukocytes in the pathogenesis of MS. Furthermore, our data support the previous observations of others regarding the upregulation of IL-17 group cytokines in MS, thus providing conformational evidence of their involvement in MS pathogenesis. We observed upregulated levels of serum IL-22 in acute stage RRMS, suggesting that Th22 lymphocytes may play a role during MS exacerbations. We also observed that serum CCL27 was upregulated in MS cases. To the best of our knowledge, this report is the first to describe a CCL27 involvement in association with MS.

2. Materials and Methods

2.1. Study Subjects and Samples. A total of 42 cases were admitted to the Department of Neurology, Neurosurgery and Medical Genetics of Kazan State Medical University, Russian Federation. A diagnosis of MS was established based upon clinical presentation and brain MRI scans. Serum from 20 healthy individuals was collected to serve as controls. Informed consent was obtained from each subject according to the clinical and experimental research protocol, approved by the Local Ethic Expert Committee of the Kazan State Medical University (number 196, 10 May 2010).

2.2. Cytokine Analysis. Serum cytokine levels were analyzed using Bio-Plex (Bio-Rad, Hercules, CA, USA) multiplex magnetic bead-based antibody detection kits following the manufacturer's instructions. Bio-Plex Pro Human Th17 Cytokine Panel, Bio-Plex Pro Human Cytokine 27-plex Panel, and Bio-Plex Human Cytokine 21-plex Panel were used for detection of a total of 57 analytes. Serum aliquots (50 μL) were collected from healthy donors and 42 MS cases. A minimum of 50 beads per analyte was acquired. Median fluorescence intensities were measured using a Luminex 200 analyzer. Data collected was analyzed with MasterPlex CT control software and MasterPlex QT analysis software (Hitachi Software San Bruno, CA, USA). Standard curves for each analyte were generated using standards provided by manufacturer.

2.3. Statistical Analysis. Statistical analysis was conducted using Statistica and XLSTAT software (StatsSoft, Tulsa, OK and Addinsoft, New York, NY, resp.). Differences between the means of compared groups were analyzed using the Mann-Whitney test for nonparametric data with significance at $P \leq 0.05$.

3. Results

3.1. Patients. Serum samples from 42 MS cases (40 female and 2 male) were analyzed. MS diagnosis was established according to the 2010 Revised Diagnostic Criteria for MS [31]. Thirty-two (76.2%) cases were diagnosed with RRMS, 7 (16.7%) were diagnosed with secondary RRMS, and 3 (7.1%) were newly diagnosed. The mean age for MS cases was 41.6 years (24–58 years) and mean duration of the disease was 12.4 years (1–32 years). Expanded Disability Status Scale (EDSS) score was 3.5 (0–10). MRI revealed multiple lesions in the subcortical region, corpus callosum, and pons. In some subjects, demyelination foci were detected in cervical and thoracic regions of the spinal cord. Five subjects received glatiramer, while the remaining 38 receive no treatment.

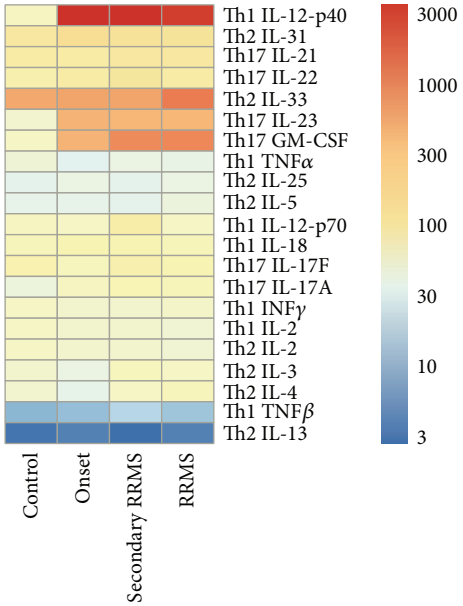

Figure 1: Heat map analysis of serum cytokine profile in MS cases. All MS cases were grouped based on presentation/stage of the disease. Serum cytokine profile in newly diagnosed cases (onset), secondary RRMS, and RRMS forms of MS were analyzed.

3.2. Cytokine Profile. A total of 57 cytokines were analyzed. Serum levels of 9 cytokines, IL12-p40, CCL27, M-CSF, MIF, IL-17A, IL-23, CCL2, CCL3, and IL-2Ra, differed significantly between the serum of all MS cases and the controls (Table 1). On average, serum levels of HGF, TRAIL, CXCL10, and CCL5 were elevated in MS cases; however, they did not reach statistical significance when compared to control sera. Serum levels of the remaining 42 cytokines were generally similar between cases and controls (Table 1).

To further elucidate the role of cytokines in MS, we organized the respective cytokines by functionality. Three categories of cytokines were investigated based on their putative role in immune activation. Cytokines that activate Th1 immune effector cells made of the first group and included IL-2, IL-12, and IFNγ, while Th2 cytokines, which included IL-4, IL-5, IL-9, IL-10, and IL-13, made up the second group. The final group of cytokines included those that activate Th17 lymphocytes and were IL-6, IL-23, IL-1β, IL-17a, IL-21, and IL-22. Among the Th1 cytokines, only serum IL-12p40 was significantly upregulated in MS cases when compared to controls (Table 1). Similarly, heat map analysis revealed upregulation of IL-12p40 in all MS cases as compared to healthy controls (Figure 1). Serum levels of IL-2 and IFNγ remained unchanged in MS cases and no differences were observed in the levels of Th2 cytokines between MS cases and controls (Table 1, Figure 1). However, serum levels of the Th17 cytokines IL-17a and IL-23 were significantly higher in MS cases as compared to controls (Table 1). Likewise, heat map analysis has shown increased serum concentration of IL-23 and IL-17a (Figure 1).

Increased IL-12p40, CCL2, and M-CSF in the serum of MS cases suggest activation of mononuclear immune effector cells. Additionally, serum levels of CCL27 were significantly

Table 1: Serum cytokine levels in MS cases.

Analyte	MS (ng/mL)	Control (ng/mL)
IL-1α	6.6 ± 0.8	5.3 ± 2.2
IL1b	8.8 ± 0.9	6.6 ± 0.4
IL-1ra	23 ± 7.3	38 ± 17.2
IL-2Ra	320.3 ± 51.9	36.3 ± 4.1
	$P <$ **0.04**	
IL-2	56.23 ± 32	78 ± 27
IL-3	82.0 ± 12.9	67.2 ± 13.4
[1pt] IL4	87.8 ± 15.4	63.0 ± 18.6
IL-5	24.12 ± 17	33 ± 23
IL-7	5.2 ± 0.3	6.2 ± 0.8
IL-8	4.7 ± 0.5	6.6 ± 1.6
IL-9	9.7 ± 1.7	7.3 ± 1.4
IL10	73.6 ± 6.2	68.5 ± 10.2
IL-12p40	4005.7 ± 327.1	86.0 ± 6.1
	$P <$ **0.00015**	
IL-12(p70)	90.2 ± 17.9	84.1 ± 8.6
IL-13	2.7 ± 0.3	2.1 ± 0.2
IL-15	85.0 ± 3.4	68.6 ± 4.6
IL-16	119.7 ± 33.9	241.1 ± 67.9
IL17A	103.79 ± 6.5	44.74 ± 6.3
	$P =$ **0.001**	
IL17F	115.2 ± 8.3	44.7 ± 6.3
IL-18	118.3 ± 12.0	101.9 ± 24.3
IL21	184.3 ± 6.5	162.005 ± 16.1
IL22	163.5 ± 7.7	149.358 ± 18.7
IL23	481.4 ± 29.8	62.1517 ± 8.9
	$P <$ **0.000003**	
IL25	36.8 ± 1.9	32.4 ± 1.7
IL31	230.5 ± 14.2	195.055 ± 29.8
IL33	1050.4 ± 291.0	683.6 ± 158.2
CCL2	915.9 ± 46.9	145.6 ± 23.0
	$P =$ **0.0000001**	
CCL3	73.8 ± 7.1	35.7 ± 4.1
	$P <$ **0.05**	
CCL4	64.2 ± 8.9	47.5 ± 13.2
CCL5	8743.6 ± 2219.2	2589.5 ± 248.3
CCL7	337.4 ± 55.2	119.8 ± 6.3
CCL11	220.3 ± 25.0	113.5 ± 28.8
CCL27	4674.9 ± 643.3	854.6 ± 51.0
	$P <$ **0.05**	
CXCL1	342.9 ± 46.2	135.6 ± 40.6
CXCL9	2307.1 ± 338.7	2461.8 ± 301.6
CXCL10	1804.3 ± 264.6	732.2 ± 111.5
CXCL12	2575.6 ± 355.1	2582.4 ± 166.5
IFN-α2	59.8 ± 6.7	33.5 ± 8.0
INFγ	74.1 ± 4.1	77.3 ± 8.9
FGF basic	34 ± 24	56 ± 26
G-CSF	26.3 ± 2.9	19.7 ± 4.3
GM-CSF	45 ± 21	76 ± 34

Table 1: Continued.

Analyte	MS (ng/mL)	Control (ng/mL)
HGF	1206.7 ± 164.1	931.7 ± 287.7
LIF	44.0 ± 7.9	29.2 ± 14.5
M-CSF	430.2 ± 41.7 P = **0.002**	37.3 ± 7.0
MIF	401.1 ± 32.9 P = **0.01**	148.6 ± 38.9
b-NGF	6.6 ± 0.4	6.7 ± 0.8
PDGF-bb	1256 ± 235	1462 ± 562
sCD40L	3592.9 ± 620.7	2756.0 ± 1007.3
SCF	230.5 ± 25.8	142.7 ± 18.9
SCGF-b	51782.6 ± 8900.2	46927.3 ± 7531.6
TNFα	39.8 ± 3.5	55.0 ± 5.3
TNF-β	10.4 ± 1.4	7.0 ± 1.5
TRAIL	252.3 ± 41.7	46.2 ± 10.0
VEGF	114.6 ± 12	74.4 ± 10.4

increased in MS cases when compared to healthy controls. CCL27 is a well-known chemoattractant for mononuclear leukocytes and has been shown to attract memory T cells to the site of cutaneous lesions [32]. Therefore, when considered together, our data suggests that the serum cytokine profile of subjects with MS is characteristic of a classic Th17/Th1 shift which promotes mononuclear leukocytes infiltration of inflamed tissue.

Finally, we analyzed the serum cytokine profile of MS cases with respect to the different clinical presentations. Cases were organized into 3 groups: those with secondary RRMS acute phase, those with RRMS in remission, and those who were newly diagnosed with MS. Acute phase secondary RRMS was characterized by significant changes in the serum levels of 13 cytokines, while changes in smaller number of cytokines were detected in the serum of RRMS cases in remission and newly diagnosed MS (Table 2, Figure 2). Further analysis revealed that serum IL-12(p40), M-CSF, CCL2, and IL-23 were significantly upregulated in all MS cases, regardless of the stage or phase of the disease (Table 3, Figure 2). All MS cases were characterized by an increase in serum levels of GROa (CXCL1), CCL7, and IL-22; however, these changes were only significant for acute phase secondary RRMS cases. Interestingly, subjects with acute phase secondary RRMS had significantly higher level of serum CCL27 and TRAIL, similar to that observed in newly diagnosed MS cases. However, newly diagnosed MS cases had significantly lower levels of serum XCL9, CXCL12, and CCL3, while the levels of these cytokines in all RRMS cases did not differ from controls. Upregulation of IL-2Ra, MIF, and IL-17A was observed for all RRMS cases; however, these cytokines did not significantly differ between newly diagnosed MS cases and controls (Table 2, Figure 2).

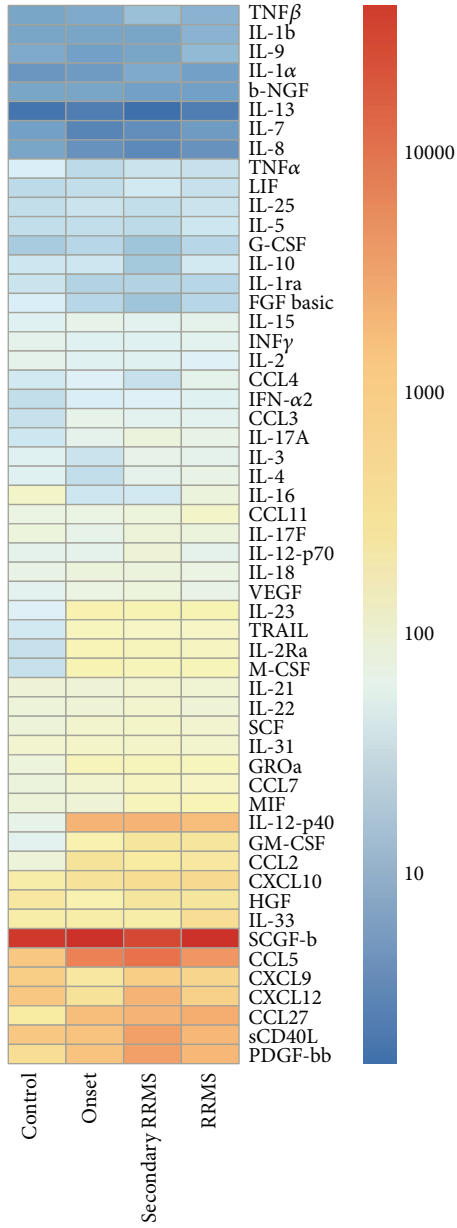

Figure 2: Heat map analysis of serum cytokines in MS cases with different presentation/stage of the disease. All MS cases were grouped based on presentation/stage of the disease. Serum cytokine profile in newly diagnosed cases (onset), secondary RRMS, and RRMS forms of MS were analyzed.

4. Discussion

Lymphocyte infiltration of brain tissue, demyelination, and gliosis are hallmarks of MS. It is believed that brain infiltrating autoreactive lymphocytes promote axonal demyelination leading to neuronal death and sclerotic plaque formation. A subset of Th17 lymphocytes has been implicated in the pathogenesis of MS. For example, high levels of IL-17 cytokine have been observed in serum and CSF of MS cases [25]. Also, adoptive transfer of IL-17-producing T lymphocytes induced experimental autoimmune encephalitis (EAE), an experimental model of MS [28], and administration of anti-IL17 antibodies prevented development of EAE and delayed the onset of symptoms [28, 33]. Clinical studies have reported the presence of IL-17 and IFN-γ expressing T lymphocytes in

Table 2: Cytokines and disease progression/stage.

Analyte	Secondary RRMS acute (ng/mL)	RRMS remission (ng/mL)	MS onset (ng/mL)	Control (ng/mL)
IL-1α	8.0 ± 1.8	6.09 ± 1.0	5.9 ± 1.0	5.3 ± 1.1
IL1β	6.8 ± 1.4	9.2 ± 1.2	7.1 ± 0.3	6.6 ± 0.4
IL-1ra	24.6 ± 12.5	27.3 ± 9.4	24.5 ± 12	38 ± 17.2
IL-2Ra	376.1 ± 71.7 P = **0.001**	301.5 ± 61.2 P < **0.04**	481.0 ± 303.5	36.3 ± 4.1
IL-2	67.3 ± 12.4	59.3 ± 23	67.3 ± 12	78 ± 27
IL-3	93.8 ± 15.8	78.7 ± 15.0	39.6 ± 9.6	67.2 ± 13.5
IL4	80.6 ± 25.9	95.9 ± 23.5	33.2 ± 5.0	63.0 ± 18.6
IL-5	30.5 ± 12.5	42.4 ± 23	33.2 ± 16.2	33 ± 23
IL-7	4.2 ± 0.6	5.5 ± 0.4	3.5 ± 0.03	6.2 ± 0.8
IL-8	3.6 ± 0.6	0.4 ± 0.7	4.7 ± 0.3	6.6 ± 1.6
IL-9	6.9 ± 1.4	11.0 ± 2.9	6.2 ± 0.6	7.3 ± 1.4
IL10	18.1 ± 4.2	49.8 ± 9.5	42.3 ± 16.6	45.4 ± 10.2
IL-12p40	3892.5 ± 821.9 P = **0.003**	3248.8 ± 327.7 P = **0.00014**	4016.3 ± 571.7 P = **0.00008**	86.0 ± 6.1
IL-12 (p70)	159.7 ± 103.2	78.0 ± 9.4	77.1 ± 0.7	84.1 ± 8.6
IL-13	1.9 ± 0.3	2.6 ± 0.3	2.7 ± 0.8	2.1 ± 0.2
IL-15	75.0 ± 6.5	80.4 ± 2.7	86.9 ± 11.2	68.6 ± 4.6
IL-16	51.0 ± 10.8 P = **0.01**	123.6 ± 51.7	42.0 ± 13.1	241.1 ± 67.9
IL17A	119.0 ± 12.0 P < **0.0003**	104.3 ± 8.9 P = **0.0023**	82.7 ± 28.0	44.7 ± 6.3
IL17F	145.7 ± 26.7	119.8 ± 10.7	88.5 ± 9.9	137.9 ± 30.4
IL-18	124.4 ± 21.5	104.9 ± 8.5	126.4 ± 38.0	101.9 ± 24.3
IL21	194.5 ± 17.0	191.1 ± 8.9	169.5 ± 24.6	162.0 ± 16.1
IL22	216.9 ± 15.0 P < **0.02** $^{*}P$ = **0.007**	162.6 ± 9.4	173.1 ± 28.8	149.4 ± 18.7
IL23	530.2 ± 84.5 P <**0.0004**	527.3 ± 27.2 P = **0.000000002**	585.3 ± 180.9 P = **0.003**	62.2 ± 8.9
IL25	31.9 ± 4.9	39.1 ± 2.8	40.7 ± 7.0	32.4 ± 1.7
IL31	253.8 ± 49.9	234.9 ± 19.5	262.5 ± 14.2	195.1 ± 29.8
IL33	717.2 ± 192.9	1370.7 ± 501.6	720.0 ± 221.0	683.6 ± 158.1
CCL2	821.1 ± 80.5 P < **0.00002**	918.3 ± 54.8 P = **0.00000001**	1152.5 ± 192.5 P < **0.005**	145.6 ± 23.0
CCL3	72.3 ± 18.2	73.1 ± 10.7	89.4 ± 10.8 P = **0.0002**	35.7 ± 4.1
CCL4	37.5 ± 10.7	82.7 ± 13.9	57.7 ± 25.9	47.5 ± 13.2
CCL5	15926.6 ± 11653.2	7726.7 ± 1699.9	11287.8 ± 4843.4	2589.5 ± 248.3
CCL7	317.0 ± 65.4 P = **0.03**	286.7 ± 63.6	211.2 ± 93.5	119.8 ± 6.3
CCL11	128.6 ± 31.3	246.7 ± 29.7	107.3 ± 14.6	113.5 ± 28.8
CCL27	4192.9 ± 773.6 P < **0.005**	4282.5 ± 912.8	3152.7 ± 582.9 P < **0.002**	854.6 ± 51.0

Table 2: Continued.

Analyte	Secondary RRMS acute (ng/mL)	RRMS remission (ng/mL)	MS onset (ng/mL)	Control (ng/mL)
CXCL1	362.7 ± 64.1 P = **0.02**	332.0 ± 48.7	379.9 ± 194.6	135.6 ± 40.6
CXCL9	2319.4 ± 687.2	1878.1 ± 314.4	967.2 ± 536.4 P = **0.04**	2461.8 ± 301.6
CXCL10	1655.1 ± 80.5	1754.4 ± 384.4	1308.3 ± 500.6	732.2 ± 111.5
CXCL12	59.3 ± 13.3	65.4 ± 9.0	51.3 ± 15.9	33.5 ± 8.0
IFN-α2	68.8 ± 23.3	46.3 ± 12	79 ± 12	40.9 ± 16.4
INFγ	48.3 ± 12.3	67.3 ± 23	67.2 ± 12	56 ± 26
FGF basic	16.5 ± 4.0	27.5 ± 3.8	26.5 ± 6.9	19.7 ± 4.3
G-CSF	56.3 ± 12.3	66.4 ± 24	78.3 ± 6	76 ± 34
GM-CSF	1085.5 ± 299.7	1101.2 ± 149.4	570.5 ± 117.4	931.7 ± 287.7
HGF	48.3 ± 21.7	37.3 ± 5.0	32.0 ± 10.0	29.2 ± 14.5
LIF	438.7 ± 75.4 P = **0.001**	401.1 ± 43.3 P = **0.00065**	495.6 ± 142.0 P < **0.005**	37.3 ± 7.0
M-CSF	368.3 ± 53.2 P = **0.01**	462.2 ± 43.2 P < **0.003**	173.5 ± 15.8	148.6 ± 38.9
MIF	6.3 ± 0.7	6.3 ± 0.4	7.1 ± 1.6	6.7 ± 0.8
b-NGF	1324.5 ± 256.3	1526 ± 329.3	1134 ± 213	1462 ± 562
PDGF-bb	6012.3 ± 2966.4	3606.5 ± 592.3	2962.4 ± 906.1	2756.0 ± 1007.3
sCD40L	251.2 ± 51.1	206.9 ± 16.3	222.4 ± 65.9	142.7 ± 18.9
SCF	33385.6 ± 4425.1	54557.7 ± 13801.1	49672.2 ± 13153.3	46927.3 ± 7531.6
SCGF-b	4089.7 ± 1952.1	2174.5 ± 188.5	1254.8 ± 22.6 P < **0.001**	2582.4 ± 166.5
TNFα	39.4 ± 8.9	36.0 ± 4.6	28.6 ± 14.1	55.0 ± 5.3
TNF-β	13.8 ± 3.2	8.9 ± 1.5	7.9 ± 1.4	7.0 ± 1.5
TRAIL	283.1 ± 22.7 P = **0.000002**	271.1 ± 72.0	332.5 ± 29.2 P = **0.000006**	46.2 ± 1.5
VEGF	135.9 ± 43.4	97.4 ± 13.4	111.5 ± 39.1	74.4 ± 10.5

P: to control.
* P: to RRMS remission.

the brain tissue of RRMS cases [20]. Additionally, high levels of IL-17 mRNA have been detected in blood and CSF of MS cases, with the highest levels observed during exacerbations [26]. Our data supports a role for IL-17 in the pathogenesis of MS. We have observed significantly increased levels of IL-17 in serum of RRMS cases. Interestingly, levels of IL-17 in serum of newly diagnosed MS were also elevated, although differences were not significant compared to controls. These data suggest that Th17 activation occurs early during MS and increases with progression of the disease.

IL-23 has been shown to play role in the activation of Th17 type immunity. Current evidence suggests that IL-23 has an essential function in the differentiation and expansion of Th17 T lymphocytes from naïve CD4+ T cells [27, 33, 34]. Therefore it is believed that IL23-IL17 axis plays an important role in developing autoimmunity [35, 36]. IL-23 shares the p40 subunit with IL-12, another cytokine implicated in MS pathogenesis [37, 38]. Furthermore, it has been shown that IL-23, rather than IL-12, is required for the development of the EAE [39–41]. Another important function of IL-23, as demonstrated by Langrish et al., is to promote the expansion of encephalitogenic T cells that drive the production of IL-17A and IL-17F [28]. Our data suggests that IL-23 is upregulated in the serum of all MS cases, regardless of the stage of the disease. The fact that increased serum IL-23 levels did not depend of the progression of the disease suggests an essential role of IL-23 in establishing and maintaining autoimmunity.

We observed increased levels of serum CCL27 for all MS cases; however, the differences reached statistical significant only for the RRMS cases. CCL27 upregulation is primarily associated with the pathogenesis of atopic dermatitis [42]; however, it is likely that its role is not exclusively restricted to skin inflammation. For instance, the enhancement of mucosal immunity has been demonstrated in animals immunized with plasmids containing HIV gag

Table 3: MS serum cytokines differ from healthy controls.

Analyte	Secondary RRMS acute (ng/mL)	RRMS remission (ng/mL)	MS onset (ng/mL)	Control (ng/mL)
IL-2Ra	376.1 ± 71.7 P = **0.001**	301.5 ± 61.2 P < **0.04**	481.0 ± 303.5	36.3 ± 4.1
IL-12p40	3892.5 ± 821.9 P = **0.003**	3248.8 ± 327.7 P = **0.00014**	4016.3 ± 571.7 P = **0.00008**	86.0 ± 6.1
IL-16	51.0 ± 10.8 P = **0.01**	123.6 ± 51.7	42.0 ± 13.1	241.1 ± 67.9
IL17A	119.0 ± 12.0 P < **0.0003**	104.3 ± 8.9 P = **0.0023**	82.7 ± 28.0	44.7 ± 6.3
IL22	216.9 ± 15.0 P < **0.02** $^{*}P$ = **0.007**	162.6 ± 9.4	173.1 ± 28.8	149.4 ± 18.7
IL23	530.2 ± 84.5 P < **0.0004**	527.3 ± 27.2 P = **0.000000002**	585.3 ± 180.9 P = **0.003**	62.2 ± 8.9
CCL2	821.1 ± 80.5 P < **0.00002**	918.3 ± 54.8 P = **0.00000001**	1152.5 ± 192.5 P < **0.005**	145.6 ± 23.0
CCL3	72.3 ± 18.2	73.1 ± 10.7	89.4 ± 10.8 P = **0.0002**	35.7 ± 4.1
CCL7	317.0 ± 65.4 P = **0.03**	286.7 ± 63.6	211.2 ± 93.5	119.8 ± 6.3
CCL27	4192.9 ± 773.6 P < **0.005**	4282.5 ± 912.8	3152.7 ± 582.9 P < **0.002**	854.6 ± 51.0
CXCL1	362.7 ± 64.1 P = **0.02**	332.0 ± 48.7	379.9 ± 194.6	135.6 ± 40.6
CXCL9	2319.4 ± 687.2	1878.1 ± 314.4	967.2 ± 536.4 P = **0.04**	2461.8 ± 301.6
M-CSF	438.7 ± 75.4 P = **0.001**	401.1 ± 43.3 P = **0.00065**	495.6 ± 142.0 P < **0.005**	37.3 ± 7.0
MIF	368.3 ± 53.2 P = **0.01**	462.2 ± 43.2 P < **0.003**	173.5 ± 15.8	148.6 ± 38.9
CXCL12	4089.7 ± 1952.1	2174.5 ± 188.5	1254.8 ± 22.6 P < **0.001**	2582.4 ± 166.5
TRAIL	283.1 ± 22.7 P = **0.000002**	271.1 ± 72.0	332.5 ± 29.2 P = **0.000006**	46.2 ± 1.5

and CCL27 [43]. Furthermore, expression of CCL27 mRNA has been detected in brain tissue, particularly the cerebral cortex and limbic structures [44]. Expression of CCR10, the receptor for CCL27, has been confirmed in astrocytes and neurons of the hippocampus [44–46]. Additionally, CCL27 acts as chemoattractant for antigen-specific T lymphocytes [47]. This suggests that CCL27 may facilitate autoreactive T lymphocyte migration into brain tissue of MS cases, thus promoting brain inflammation.

Our data suggest that IL-22 is upregulated in the serum of acute secondary RRMS. Current knowledge regarding IL-22 expression in association with MS is limited as only a small number of reports address its role in the pathogenesis of autoimmune demyelinating diseases. Almolda and coworkers reported that changes in serum IL-22 levels correlate with the development of EAE [48]. They further reported that serum IL-22 levels increase during the acute phase, peak at the height of clinical presentation, and decrease during recovery. Recently, increased plasma and CSF levels of IL-22 have been reported for subjects diagnosed with Guillain-Barré syndrome, another acute autoimmune-mediated inflammatory demyelinating disease [49]. Li et al. reported that IL-17 and IL-22 levels in CSF correlate with disease disability [49]. Our data suggest that serum levels of IL-22 are significantly elevated during the acute phase of secondary RRMS when compared to healthy controls. Although it is slightly elevated during the remission phase of RRMS, serum IL-22 did not significantly differ from controls. These data are consistent

with the observation of Almolda et al. whereby changes in serum IL-22 reflect the stage of the disease [48]. IL-22 is produced by variety of leukocytes including Th17 cells [50]. Although IL-22 and IL-17 are often simultaneously present at high levels in inflamed tissues, their biological effects differ. For example, IL-22 primarily activates an innate immune response, while IL-17 typically acts as a proinflammatory mediator [51–54]. This suggests that the MS cytokine milieu promotes chronic inflammation and activation of immune effector cells.

In summary, our data provide evidence that mononuclear leukocytes play a role in the pathogenesis of MS. The serum cytokine profiles observed in our MS study subjects suggest Th17 activation, consistent with the previous reports of others [25, 28, 33]. Furthermore and adding to the present body of knowledge, we observed an increase in serum IL-22 during the acute phase of MS. We also observed a general upregulation of CCL27 in association with all presentations of MS. Previous studies regarding the role of CCL27 in human pathology are primarily limited to atopic dermatitis [55]; however, our data support a broader function involving MS inflammation and lymphocyte activation. Future studies will be required to delineate the role of CCL27 in MS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Insights into Neuroinflammation in Parkinson's Disease: From Biomarkers to Anti-Inflammatory Based Therapies

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Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide, being characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta. Among several putative factors that may contribute to PD pathogenesis, inflammatory mechanisms may play a pivotal role. The involvement of microglial activation as well as of brain and peripheral immune mediators in PD pathophysiology has been reported by clinical and experimental studies. These inflammatory biomarkers evaluated by imaging techniques and/or by biological sample analysis have become valuable tools for PD diagnosis and prognosis. Regardless of the significant increase in the number of people suffering from PD, there are still no established disease-modifying or neuroprotective therapies for it. There is growing evidence of protective effect of anti-inflammatory drugs on PD development. Herein, we reviewed the current literature regarding the central nervous system and peripheral immune biomarkers in PD and advances in diagnostic and prognostic tools as well as the neuroprotective effects of anti-inflammatory therapies.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide. The major pathological findings in PD are the progressive loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of intraneuronal inclusions of the protein α-synuclein (known as Lewy bodies) [1]. Neuronal death in the substantia nigra results in dopamine deficit at the striatum and, as an outcome, the clinical hallmarks of Parkinsonism: bradykinesia, rigidity, resting tremor, and postural instability. PD diagnosis, which is essentially clinical, is based on the diagnosis of Parkinsonian syndrome and the exclusion of other causes of Parkinsonism [2]. Good response to levodopa and asymmetry of motor symptoms support the diagnosis. Although PD is traditionally regarded as a movement disorder, motor symptoms may be heralded or accompanied by several nonmotor symptoms, such as hyposmia, constipation, neuropsychiatric, and sleep disorders [3].

PD was first described in 1817 [4], and despite the well-characterized pathological features, the cause of neuronal death in PD remains a matter of debate. Among several putative factors that may contribute to PD pathogenesis, inflammatory mechanisms may play an important role. For instance, microglial activation is associated with dopaminergic neuronal loss, which suggests that neuroinflammatory reaction may contribute to the progressive degenerative process. Moreover, it has been reported that the protein α-synuclein has an important role in the initiation and maintenance of inflammation in PD (see Figure 1) [5].

A recent meta-analysis revealed an overall prevalence of PD of 315 per 100,000 individuals. Prevalence of PD increases steadily with age, raising from 428 per 100,000 in individuals for the age group of 60 to 69 years, to 1,903 per 100,000 individuals for the group of 80 years or older [6]. Overall worldwide incidence of PD is estimated in 36.5 per 100,000 person-years for females and 65.5 per 100,000 person-years among males [7]. Most countries are facing marked demographic changes, with progressively larger proportion of their populations entering old age. PD affects predominantly the elderly, being a disease worthy of concern, since the causes are still unknown and the treatment is palliative and merely symptomatic. Levodopa, the first breakthrough in the treatment of PD, is still the most effective drug for

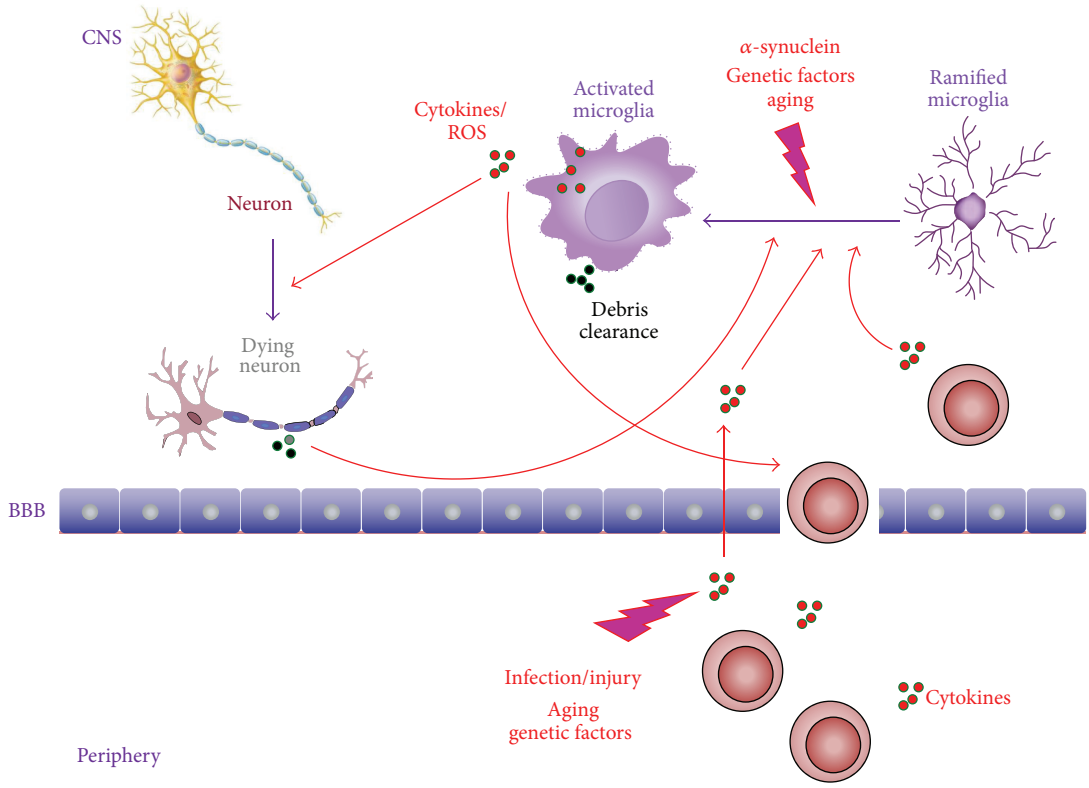

Figure 1: Inflammatory pathways in Parkinson's disease. An acute insult to CNS (e.g., α-synuclein aggregates) triggers the activation of microglia with changes in their morphofunctional characteristics, increased proliferation and release of inflammatory mediators (e.g., cytokines and ROS). Inflammatory molecules can induce the recruitment of peripheral leukocytes into the CNS. This neuroinflammatory process can be regarded as beneficial for neuronal tissue since it promotes clearance of cell debris. Conversely, inflammatory mediators do not modulate only immune cells but also act on neurons, contributing to neurodegeneration. Neuronal death further activates inflammatory mechanisms, resulting in a vicious cycle of inflammation and neuronal death. Systemic inflammation due to infection or peripheral injury can exacerbate symptoms and promote neuronal damage in PD. Leukocytes secrete proinflammatory cytokines which can affect the brain by several routes, including action on endothelial cells and leakage through damaged BBB. These cytokines induce self-synthesis and the synthesis of other cytokines, which can then stimulate microglia to secrete chronically inflammatory mediators, maintaining neuroinflammation and, as a consequence, slow and progressive neuronal death. Genetic and aging factors might contribute to this process. BBB: blood-brain barrier; CNS: central nervous system; PD: Parkinson's disease, ROS: reactive oxygen species.

the motor symptoms of the disease. In certain instances, other medications such as monoamine oxidase type B inhibitors, anticholinergics, and dopamine agonists may be initiated first to prevent levodopa-related motor complications [8]. Although the number of people suffering from PD rises significantly year by year, there are no established disease-modifying or neuroprotective therapies for PD. In this scenario, in the present review, we discuss current evidence regarding the contribution of immune dysfunction and/or inflammation in PD, advances in recent image techniques as valuable tolls for PD diagnosis and progression, and the perspectives of anti-inflammatory based therapies (data are summarized in Table 1).

2. Neuroinflammation in PD: Lessons from *Post-Mortem* and Neuroimaging Studies

2.1. Microglial Activation Role in PD. The first evidence of inflammation involvement in PD was derived from James Parkinson's report on the first clinical and pathological description of the disease in the early nineteenth century [4]. More direct evidence was provided much later in the twentieth century from systematic *post-mortem* analysis of the brain of PD patients [9]. Based on morphological features and immunohistochemical staining against HLA-DR, human glycoprotein of the MHC-II group expressed on the surface of immunocompetent cells, a significant increase in the number of reactive microglia was found in the substantia nigra of PD patients. Interestingly, reactive microglia was also found to be enhanced in the hippocampus of PD patients who also presented dementia [9].

Neuronal death in PD precedes the development of motor symptoms by many years. The mechanisms underlying the progressive neurodegeneration in PD are still elusive and the discovery of the active or main driving force is of paramount importance in the search of effective therapeutic strategies. Neuroinflammation has been proposed to actively participate in PD onset and progression. An acute insult to the central nervous system (CNS) triggers microglial activation, leading to a series of changes in microglia, notably in shape, increased

Table 1: Evidence regarding the contribution of immune dysfunction and/or inflammation in Parkinson's disease.

Evidence	Source	Results	Reference
CNS inflammation	Human brain	Significant increase in the number of reactive microglia in the substantia nigra of PD patients.	[9, 12]
		Coexistence of α-synuclein and activated microglia.	[15]
		Higher expression/increased levels of inflammatory mediators in PD brains.	[13, 16–18]
	Human CSF samples	Increased levels of IL-1β, IL-2, IL-4, IL-6, TGF-α, free TGF-β1, and total TGF-β2 in the CSF of PD patients.	[30–32]
Peripheral inflammation	Serum/plasma samples	Increased levels of IFN-γ, IL-1β, IL-2, IL-3, IL-10, MIF, TNF-α, and its soluble receptors sTNFR1 and sTNFR2 in PD patients samples.	[48–58]
	Supernatants from cell cultures	MCP-1, MIP-1α, IL-8, IFN-γ, IL-1β, and TNF-α levels were significantly higher in PD patients.	[65]
	Blood leukocytes	PD patients exhibited lower total lymphocyte counts; decrease in the percentage of T (CD3+) and B (CD19+) cells and reduction in T helper (Th, CD4+) lymphocytes; higher percentage of NK cells.	[52, 61, 71–74, 77]
Genetic evidence	DNA extracted from brain, blood, or buccal samples	Enhancement in IL-1β 511, IL-6, and TNF-α polymorphisms.	[21–24]
Epidemiological evidence	Clinical and population-based studies	NSAIDs use was associated with a lower risk for PD.	[91, 92, 94, 95, 104].
		IFN-α-induced Parkinsonism in chronic hepatitis [67–69].	[67–69]
		The relationship between PD and systemic infections (severe influenza).	[70]

CSF: cerebrospinal fluid; CNS: central nervous system; IFN: interferon, IL: interleukin; MIF: migration inhibitory factor; MCP: monocyte chemotactic protein; MIP: macrophage inflammatory protein; NSAIDs: nonsteroidal anti-inflammatory drugs; PD: Parkinson's disease; TGF: transforming growth factor; TNF: tumor necrosis factor; sTNFR: TNF soluble receptor.

proliferation, and production of inflammatory mediators that can stimulate the recruitment of peripheral leukocytes to the CNS. This inflammatory process can be regarded as beneficial for neuronal tissue, since it promotes clearance of cell debris and secretion of neurotrophic factors. Conversely, inflammatory mediators do not only modulate immune cells but also act on neurons and contributing to neurodegeneration. Neuronal death further activates inflammatory mechanisms, resulting in a vicious cycle of inflammation and neuronal death. Therefore, inflammatory responses, although essential for tissue homeostasis, can contribute to neuronal injury when it is not controlled and/or chronic (Figure 1). As neural tissues have a restricted cell renewal and regenerative capacity, CNS is extremely vulnerable to uncontrolled immune and inflammatory processes [10]. Dopaminergic neurons from substantia nigra are particularly vulnerable to microglial-mediated neurotoxicity [11].

Banati et al. demonstrated higher microglial activation in the substantia nigra of patients with PD as indicated by increased expression of CR3/43 and EBM11, markers for activated microglia [12]. The number of activated microglia (MHC-II, ICAM-1, and LFA-1 positive cells) in the substantia nigra and putamen of PD patients also increased in parallel with neuronal degeneration in those regions. Moreover, microglial activation persisted regardless of the presence or absence of Lewy bodies and was frequently associated with damaged neurons and neuritis [13]. The lack of reactive astrocytes in autopsies of the substantia nigra and putamen from PD patients contrasts with the response (with reactive astrocytes and microglia) typically found in other neurological disorders (e.g., seizures), supporting the hypothesis that the inflammatory process in PD is a unique phenomenon [14]. Autopsy brain tissue acquired from substantia nigra and basal ganglia of PD patients demonstrated that α-synuclein is present in regions of brain where microglial activation is known to be also present. Furthermore, an *in vitro* stimulation of murine microglia with aggregated and nitrated α-synuclein shift microglial morphology to an amoeboid shape

and elicited dopaminergic neurotoxicity. The mechanism by which α-synuclein activates and alters the function of microglia in PD is not yet clear, although evidence from genomic and proteomic assays has supported a role for the transcript factor nuclear factor-kappa B [15]. Taken together these studies provide evidence supporting CNS immune resident cells role in PD. Whether microglia activation is a secondary event following the ongoing neurodegeneration or a primary inducer of the disease remains to be defined.

2.2. Central Nervous System Inflammatory Mediators in PD. Over the past decades, apart from microglia activation, a growing body of clinical and experimental research has been supporting a role for oxidative stress and inflammatory mediators (cytokines and chemokines), events potentially associated with microglial reaction, in PD [13, 16–18]. For instance, higher expression of the chemokine receptor CXCR4 and of its natural ligand CXCL12 was found in dopaminergic neurons of the substantia nigra of patients with PD, and this was associated with an increase in microglial activation [18]. CXCL12/CXCR4 signaling can induce neurotoxic events, including activation of caspase-3, leading to neuronal death by apoptosis. Negative effects on the CNS mediated by CXCL12 could be induced through a direct action on dopaminergic neurons expressing CXCR4 or the release of cytokines from microglia [18, 19]. A direct link between CXCL12/CXCR4 upregulation and loss of dopaminergic neurons was provided in an animal model of degeneration of the nigrostriatal system following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration, a well-recognized model of PD [18]. The presence of activated microglia expressing the inflammatory cytokines interleukin- (IL-) 6 and tumor necrosis factor- (TNF-) α, as well as enzymes associated with inflammation, such as inducible isoform of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) was also evidenced by immunohistochemistry assays in *post-mortem* brain tissue from PD patients [13, 16, 20]. A previous study demonstrated an enhancement in the inflammatory cytokine IL-1β 511 polymorphism from DNA extracted from brain tissues of PD patients [21]. Similar findings were reported for IL-6 and TNF-α using peripheral tissue samples (i.e., blood or buccal samples), indicating polymorphisms in these cytokines as risk factors of PD [22–24].

Upregulation of inflammatory mediators involved in apoptotic cell death through TNF-α-induced signaling pathway, including caspase-1, caspase-3, and TNF receptor R1 (TNF-R1 or p55), was identified in the substantia nigra from Parkinsonian patients, indicating the occurrence of a proapoptotic environment in PD [25]. Neutralization of soluble TNF signaling *in vivo* with dominant-negative TNF inhibitor XENP345 (a PEGylated version of the TNF variant A145R/I97T) abrogated in 50% the dopaminergic neuronal degeneration in an experimental model of PD induced by striatal injection of the oxidative neurotoxin 6-hydroxydopamine (6-OHDA) [17]. A more recent study demonstrated that long-lasting TNF-α expression induced by the injection of an adenovector expressing soluble mouse TNF-α (AdTNFα) directly in the substantia nigra of adult rats leads to dopaminergic neuronal death, motor symptoms, and microglia activation associated with recruitment of peripheral monocytes [26]. Similar findings were reported following chronic expression of IL-1β induced by 60 days administration of a recombinant adenovirus expressing IL-1β in the substantia nigra of adult rats [27]. Interestingly, alterations in mRNA expression of mediators of the immune response during PD, including members of the complement system, colony stimulating factors, Toll family, and cytokines, seem to occur in a brain region-dependent manner. For instance, a downregulation in the mRNA expression of tumor necrosis factor related protein 7 (C1QTNF7), a member of the complement system, was found in the substantia nigra whereas an upregulation was observed in the putamen of PD patients at the same stage of the disease. Immunohistochemistry also reveals the expression of cytokines, including IL-6 and TNF-α, by microglia and neurons in the PD substantia nigra and frontal cortex [28]. Active NFκB is localized in the nucleus of subpopulations of neurons and glial cells mainly in substantia nigra and less frequently in putamen and cerebral cortex [28]. Altogether, these studies suggest an involvement of inflammation, in particular related to CNS resident immune cells activation, in the degeneration of dopaminergic neurons associated with PD.

Cerebrospinal fluid (CSF) mirrors metabolic and pathological states of the CNS more directly than any other body fluid. Therefore, CSF is a good source for neuroinflammation evaluation and PD biomarker discovery since it is more accessible than brain tissue and less costly than imaging [29]. In this regard, studies have evaluated levels of inflammatory markers in the CSF of PD patients. Increased levels of IL-1β and IL-6 were found in the CSF of PD patients [30]. Corroborating these findings, concentrations of IL-2 and IL-6 were higher in ventricular CSF from PD patients in comparison with control subjects. In addition, concentrations of IL-1β, IL-2, IL-4, and transforming growth factor- (TGF-) α in ventricular CSF were higher in juvenile PD patients (PD manifesting clinically bellow the age of 40) than those in controls [31]. Free TGF-β1 and total TGF-β2 levels were elevated in *post-mortem* ventricular CSF of patients with PD in comparison with age and gender-matched controls [32]. However, one study failed to find significant differences in CSF levels of the inflammatory markers C-reactive protein (CRP), IL-6, TNF-α, eotaxin, interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein- (MIP-) 1β from PD patients in comparison with a reference group [33].

Using a highly sensitive Luminex assay, one study assessed a series of CSF molecules in PD, Alzheimer's disease (AD), multiple system atrophy (MSA) patients, and healthy controls: total tau, phosphorylated tau, amyloid beta peptide 1–42 [Aβ(1–42)], Flt3 ligand, and fractalkine. CSF levels of Flt3 clearly differentiated PD from MSA, a disease that clinically overlaps with PD, with excellent sensitivity (99%) and specificity (95%). In addition, CSF fractalkine/Aβ(1–42) ratio positively correlated with PD severity and PD progression. Flt3 ligand and fractalkine are inflammatory markers possibly related to PD [29].

2.3. Insights from the Genetic Leucine-Rich Repeat Kinase 2 (LRRK2) Model of Neuroinflammation Associated with PD. Animal models of PD have become valuable tools to the understanding of its pathophysiology, regardless of their limitations in mimicking all features of the human disease. Neurotoxin-based animal models (6-OHDA and MPTP), referred to as pathogenic models, have largely been used to induce selective neuronal death in both *in vitro* and *in vivo* studies. Currently, genetic-based models (or etiologic models), such as those related to mutations in the Leucine-rich repeat kinase 2 (LRRK2) gene, have opened new directions of investigation of molecular and cellular mechanisms underlying PD pathogenesis [34, 35].

Fine-mapping, gene expression, and splicing analysis from human *post-mortem* brain tissues have supported a role for LRRK2 gene in PD. There is convincing evidence for a common variant PD association located outside of the LRRK2 protein coding region (rs117762348) [36]. In this scenario, it has been shown that activated myeloid lineage cells, including macrophages and microglia, presented high levels of LRRK2, suggesting an involvement of this gene in the neuroinflammation associated with PD [37, 38]. An elegant study demonstrated that α-synuclein overexpression in rats' substantia nigra induced LRRK2 expression in activated microglial cells, and this correlated with a high expression of iNOS, known to be involved in PD [39]. LRRK2 knockout rats are protected from dopaminergic neurodegeneration elicited by α-synuclein overexpression or intracranial administration of lipopolysaccharide (LPS). Neuroprotection observed in the absence of LRRK2 was associated with reduction in proinflammatory CD68-positive myeloid cells in the substantia nigra, indicating an involvement of LRRK2 in conditions where neuroinflammation may underlie neuronal dysfunction and degeneration such as PD [39].

2.4. Positron Emission Tomography (PET) as a Diagnostic Tool for Neuroinflammation Related to PD. Positron emission tomography (PET) is a noninvasive functional imaging technique that detects gamma rays emitted by a positron-emitting radionuclide (tracer) which is introduced into the body on a biologically active molecule [40].

The isoquinoline carboxamide PK11195 is currently the most widely used ligand for the translocator protein 18 kDa (TSPO, also known as peripheral benzodiazepine receptor). TSPO is a marker of microglial activation and has been used to assess and quantify the dynamics of activated microglia in neurodegenerative diseases, including PD. [^{11}C]PK11195 is used in PET studies for imaging brain inflammation *in vivo* [41]. PET studies using [^{11}C]PK11195 demonstrated increased binding potential values (parameter that mixes receptor density with ligand affinity) in the midbrain as well as in the pons, basal ganglia, and frontal and temporal cortices in PD, indicating an anatomically widespread distribution of microglial activation, possibly associated with the pathological process of PD [42, 43]. Longitudinal analysis of these patients revealed stable [^{11}C]PK11195 binding potential values, indicative of early activation of microglia in PD pathogenesis [43]. However, [^{11}C]PK11195 tracer cannot distinguish between microglial protective or damaging profile. To overcome this, a PET tracer for the dopamine-transporter (DAT), [^{11}C]CFT, has been used in conjunction with [^{11}C]PK11195 in order to further investigate microglial activation in parallel with the viability of the presynaptic dopaminergic neurons. Midbrain [^{11}C]PK11195 binding potential levels were inversely correlated with [^{11}C]CFT binding potential values in the putamen and positively correlated with the severity of motor symptoms, suggesting that neuroinflammation associated with microglial activation might contribute to the progression of the disease [44]. PET imaging has also been employed to investigate *in vivo* potential therapeutic strategies for PD. For instance, [^{11}C]PK11195 PET was used to evaluate the ability of COX-2 inhibition with celecoxib to reduce neuroinflammation in PD patients. Patients showed higher putamen and midbrain binding potential in comparison with controls, but considerable overlap was seen between groups, and differences were not statistically significant. This prevented reliable assessment of the changes in the [^{11}C]PK11195 uptake by celecoxib treatment [45]. In a rat model of PD induced by intrastriatal administration of 6-OHDA, PET imaging revealed that the COX-2 inhibitor celecoxib decreased microglial activation and prevented dopaminergic neuron degeneration [46]. A study conducted by Edison et al. demonstrated by PET analysis that both PD patients with or without dementia presented significant microglial activation in cortical brain regions, suggesting that neuroinflammation could be an early phenomenon in PD, persisting as the disease progress [47].

3. Peripheral Immune Response in PD

3.1. Peripheral Immune Biomarkers. A great body of evidence regarding peripheral inflammatory/immune markers has supported the hypothesis of inflammation involvement in PD. Studies of cytokines in serum or plasma have revealed increased levels of proinflammatory cytokines such as TNF-α [48, 49] and its soluble receptors sTNFR1 [50, 51] and sTNFR2 [51] and IL-1β [52] in PD patients in comparison with matched controls. Increased serum levels of macrophage migration inhibitory factor (MIF) were found in PD patients in comparison with healthy subjects [53]. Also the levels of IL-2 [54, 55], interferon (IFN)-γ [54], IL-6 [49, 54, 56, 57], and the anti-inflammatory cytokine IL-10 were described to be increased in PD [54, 58]. IL-6 plasma concentration was prospectively associated with an increased risk of developing PD [58]. In contrast, some authors failed to show significant alterations in cytokine levels in PD. Peripheral levels of the cytokines IL1-α, IL-6, TNF-α [50, 52, 59, 60], IFN-γ, IL-2, IL-4, IL-10 [61], and IL-12 [62] were similar in PD patients and age- and gender-matched controls. Circulating levels of the chemokines MIP-1α, IL-8 [63], eotaxin, eotaxin-2, IP-10 [63, 64], and MCP-1 [64] did not differ between PD patients and controls. These controversial findings could be explained, at least in part, by methodological differences among the studies, including heterogeneous PD samples and different techniques to measure the molecules.

Apart from serum/plasma studies, the concentration of cytokines produced by peripheral cells *in vitro* has been

assessed in PD. Both basal production and LPS-induced production of MCP-1, MIP-1α, IL-8, IFN-γ, IL-1β, and TNF-α were significantly higher in PD patients compared with control subjects [65]. Conversely, the secretion of IL-2 by peripheral blood mononuclear cells (PBMC) after mitogenic stimulation was decreased in PD patients in comparison with controls, whereas IL-6, IFN-α, IFN-γ, and sIL-2R levels were comparable in both groups [66].

Several case reports of IFN-α-induced Parkinsonism in chronic hepatitis patients further corroborate the hypothesis of the role played by peripheral inflammation in PD pathogenesis [67–69]. The relationship between PD and systemic infections also supports this hypothesis. For instance, in a population-based case-control study in British Columbia, Canada, severe influenza infection was associated with PD, although this effect was attenuated when cases were restricted to those occurring ten or more years before diagnosis (Figure 1) [70].

3.2. Peripheral Immune Cells. Studies have also described changes in the percentage of peripheral blood immune cells in PD, such as lower total lymphocyte counts in comparison with controls [71–73]. Reduction in the total number of lymphocytes may result from the decrease in the percentage of T (CD3+) and B (CD19+) cells in PD patients. Changes in CD3+ cells were associated with a reduction in T helper (Th, CD4+) lymphocytes, while T cytotoxic (CD8+) cells increased or remained unchanged [71–74]. Lower number of CD4+ cells could be explained by the fact that in PD these cells presented both increased spontaneous apoptosis and activation-induced apoptosis [75].

Not only the percentage of circulating immune cells but also their activation profile must be taken into account when evaluating immune parameters. One study showed that the number of "naïve" (CD4+CD45RA+) and memory helper (CD4+CD29+) T cells was decreased, while the number of activated (CD4+CD25+) T cells was increased in PD [71]. In addition, impaired ability of regulatory T cells (Treg) to suppress effector T cell function has been described in PD patients [73]. Increased oxidative stress may also be associated with changes in lymphocyte profile in PD, since both whole cell and mitochondrial reactive oxygen species (ROS) in peripheral blood mononuclear cells are increased in PD [76].

Some studies have reported similar percentages of CD3+ lymphocytes in PD patients and control subjects [52, 72]. T helper lymphocytes (CD4+) were decreased, while CD8+ cell counting increased in PD [72].

There is evidence of higher percentage of natural killer (NK) cells in peripheral blood of PD patients compared to controls, and this increase has been associated with disease severity and progression [52, 61, 77]. Despite increased number of NK cells in PD, their activity seems to be unchanged in PD [61, 77].

3.3. The Concomitant Effect of Inflammaging. PD is unequivocally an age-related disorder. Aging is a complex process accompanied by many physiological changes, notably in the immune system. Aging results in an increase in systemic levels of inflammatory markers, indicating the presence of subtle chronic inflammation, a phenomenon known as inflammaging. Chronic inflammation damages cells of the brain, heart, arterial walls, and other body structures, contributing to the onset and progression of a broad spectrum of degenerative diseases of aging, including heart disease, rheumatoid arthritis, AD, and PD. Inflammation generates oxidative stress, which might contribute to neuronal death in diseases such as AD, PD, and amyotrophic lateral sclerosis (ALS) (Figure 1) [78].

4. GWAS Studies: Further Evidence for a Role of Inflammation in PD

Genome-wide association studies (GWAS) have also identified genetic markers that link PD and inflammation. Hamza et al. detected an association between PD and the human leukocyte antigen (HLA) region (chromosome 6p21.3), finding replicated in two datasets with Caucasians (North-American of European ancestry). Associations were particularly strong for individuals with sporadic and late-onset PD and men. The variant most strongly associated with PD was rs3129882 in intron 1 of HLA-DRA [79]. The protein chains are encoded by the closely linked HLA-DRA and HLA-DRB form the class II HLA-DR antigens that are expressed by antigen-presenting cells, including microglia in the brain, and interact with T-cell receptors [79]. This result is in line with PD specific overexpression of HLA-DR antigens in substantia nigra [9]. One study has also confirmed HLA region as PD risk locus among the Dutch population [80].

One GWAS was conducted to identify common genetic variants associated with motor and cognitive outcomes in PD. The single nucleotide polymorphisms (SNP) rs10958605 (C8orf4 gene) and rs6482992 (CLRN3 gene) were associated with motor and cognitive outcomes, respectively. The encoded protein by C8orf4 gene may play a role in the NF-κB and ERK1/2 signaling pathways, highlighting inflammation as a possible pathogenesis mechanism for progression in PD [81].

A recent meta-analysis has identified four loci, including the HLA region, that contain a secondary independent risk variant for PD that exerts an effect independently of the primary risk allele [82].

Genetic factors may also be essential in determining an individual's susceptibility to inflammation-induced nigral dopaminergic neuronal cell death (Figure 1) [83].

5. Immune Changes Induced by Antiparkinsonian Drugs

Long-term treatment with antiparkinsonian drugs may result in changes in immune system. For example, treatment with amantadine, originally established as an antiviral drug, was associated with an increase of the CD4 : CD8 ratio [84]. Treatment with amantadine has been described to increase IL-2 levels [85, 86]. The same was not observed in patients in use of levodopa as monotherapy [85]. Levodopa therapy induced changes in T lymphocytes proteome [87]. Levodopa-treated

patients showed significantly higher IL-15 and RANTES circulating levels in comparison with healthy controls and higher, but not statistically significant levels, with respect to untreated patients [88].

In order to evaluate a putative immunomodulatory role of levodopa, PBMC of PD patients and controls were incubated *in vitro* with the drug. Levodopa caused an inhibition of mitogen-induced proliferation, stimulation of IL-6, and TNF-α production, whereas the secretion of IL-1β and IL-2 was not affected in both groups [89].

6. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) Use and Risk of PD

Based on the hypothesis that neuroinflammation is involved in PD pathophysiology, epidemiological studies have evaluated nonsteroidal anti-inflammatory drugs (NSAIDs) use and risk of PD. The first study conducted with this purpose was a prospective cohort in which the regular use of NSAIDs, but not aspirin, was associated with a delay or prevention of PD onset [90]. The same research group later investigated whether NSAIDs use was associated with a lower risk for PD in a large cohort with more detailed information on different types of NSAIDs. They found no association between the use of aspirin, other NSAIDs, or acetaminophen and PD risk. Interestingly, PD risk was lower among ibuprofen users than nonusers, suggesting that ibuprofen use may delay or prevent the onset of PD [91]. In line with these results, a prospective study revealed that ibuprofen users had a significantly lower PD risk than nonusers, even when adjusting for age, smoking, caffeine consumption, and other covariates. The same effect was not observed for aspirin, other NSAIDs, or acetaminophen [92]. Since only the use of ibuprofen, but not other NSAIDs, was associated with lower PD risk, some specific effects of ibuprofen may be important. In fact, an earlier study examined the effects of NSAIDs drugs on cultured primary rat embryonic neurons from mesencephalon, the area primarily affected in PD. Ibuprofen protected both dopaminergic neurons and other neurons against glutamate toxicity. In addition, ibuprofen alone increased the relative number of dopaminergic neurons by 47% [93].

In contrast with the above mentioned studies, a population-based study described a decreased risk of PD among regular aspirin users. A stronger protective effect was observed for regular nonaspirin NSAIDs users. It is noteworthy that the aspirin effect differed by gender, showing a protective effect only in women, especially among long-term regular users [94]. The most recent study supporting the association between NSAIDs and reduced PD risk was conducted in 2008. NSAIDs use was described to significantly reduce PD risk in 20% to 30%. The effect of the combination of NSAIDs use and smoking and coffee consumption was also evaluated. People who were at the highest exposure to smoking and coffee and used NSAIDs had an estimated 87% reduction in PD risk. As properly stated by the authors, whether this finding reflects true biological protection needs to be further investigated [95].

There are studies that failed to show any association between NSAIDs use and PD [96–102]. The discrepant results may be due to different methods used to conduct the investigations, especially how authors collected data about NSAID use (medical records, self-report, pharmacy databases, etc.) and the evaluated population.

Several case-control studies have been performed to examine the association between NSAIDs use and PD risk. Given the discrepancy in results, meta-analysis is of great value to better define this association. A meta-analysis with this purpose concluded that NSAIDs do not seem to modify the risk of PD. However, ibuprofen may have a mild protective effect in lowering the risk of PD [103]. Another meta-analysis estimated an overall reduction in 15% in PD incidence among users of nonaspirin NSAIDS, with a similar effect observed for ibuprofen use. The protective effect of nonaspirin NSAIDs was more pronounced among regular and long-term users. No protective effect was observed for aspirin or acetaminophen [104]. In conclusion, there is evidence for a protective effect of nonaspirin NSAIDs use in relation to PD, which is consistent with the neuroinflammatory hypothesis for PD pathogenesis.

In this scenario, among several studies evaluating anti-inflammatory strategies in animal models of PD, one is noteworthy. The nitric oxide (NO)-NSAID HCT1026 [2-fluoro-α-methyl(1,1′-biphenyl)-4-acetic-4-(nitrooxy)butyl ester], NO-donating flurbiprofen, is an anti-inflammatory agent obtained by derivatization of conventional NSAIDs with a NO-donating moiety which strongly reduces their untoward side effects without altering the anti-inflammatory effectiveness. Oral treatment with HCT1026 showed a safe profile and a significant efficacy in counteracting MPTP-induced dopaminergic neurotoxicity, motor impairment, and microglia activation in aging mice [105], providing a promising approach towards the development of effective pharmacological neuroprotective strategies against PD.

7. Autoimmunity and Immune-Based Therapies in PD

PD has been associated with autoimmunity. Juvenile Parkinsonism has been reported as a manifestation of systemic lupus erythematosus [106]. Anecdotal reports tried to establish an association between PD and rheumatoid arthritis [107, 108]. Antibodies against dopaminergic neurons were demonstrated in the serum of a patient with a complex autoimmune disorder and rapidly progressing PD [109]. One study reported significantly higher antibody levels towards monomeric α-synuclein in the sera of PD patients compared to controls, and their levels decreased with PD progression. According to these authors, this possibly indicates a protective role of autoimmunity in maintaining body homeostasis and clearing protein species whose imbalance may lead to misfolded protein aggregation [110].

All currently available treatments for PD are of only symptomatic benefit, and a pharmacological strategy with disease-modifying effect is highly needed. In this context, immune-based therapies have been proposed for PD

treatment. The first strategy was based on immunotherapy against aggregated forms of α-synuclein. Transgenic mice displaying abnormal accumulation of human α-synuclein and α-synuclein-immunoreactive inclusion-like structures in the brain were vaccinated with human α-synuclein. There was decreased accumulation of aggregated α-synuclein in neuronal cell bodies and synapses, and, as a consequence, reduced neurodegeneration. Similar effects were observed with an exogenously applied FITC-tagged α-synuclein antibody [111]. The same work group showed that passive immunization with a monoclonal α-synuclein antibody (9E4) against the C-terminus α-synuclein reduced the accumulation of calpain-cleaved α-synuclein in axons and synapses in the α-synuclein transgenic mice. In addition, 9E4 was able to cross the blood brain barrier into the CNS, to bind to cells displaying α-synuclein accumulation and to promote α-synuclein clearance via the lysosomal pathway [112].

Studies on AD have provided valuable information about immunotherapy in neurodegenerative disorders. Immunotherapy against the β-amyloid peptide in AD showed that approaches targeting cerebral proteins can be applied to humans with relative safety. Neuropathological examination showed the clearance of amyloid plaques in brains of AN1792-vaccinated AD patients. Nonetheless, relevant issues must be considered. For instance, T cell responses specific for cerebral antigens need to be avoided. Another important issue is to define which patient should be vaccinated. Disease-modifying approaches are more effective when applied in the early stage of the disease, when diagnosis is not established yet [113].

AFFITOPE PD01, the most promising vaccine developed for PD so far, entered clinical trials and therefore represents the first PD vaccine to be tested clinically. AFFITOPE PD01 has been developed to induce antibodies recognizing α-synuclein but sparing the family member β-synuclein, which has neuroprotective properties [113].

Immune stimulation in the periphery may also provide a new strategy to halt PD progression. In addition to studies on immunotherapy against aggregated forms of α-synuclein, one study described the neuroprotective effects of Bacillus Calmette-Guérin (BCG) vaccination in the MPTP mouse model of PD. BCG vaccination had a significant beneficial effect on both striatal dopamine content and DAT ligand binding levels. BCG vaccination prevented the increase in the number of activated microglia in the substantia nigra induced by the MPTP, suggesting that general immune stimulation in the periphery can limit CNS microglia response to a neuronal insult [114].

8. Conclusion

We reviewed the evidence regarding the contribution of immune dysfunction and/or inflammation in PD, including microglial activation and brain and peripheral levels of immune mediators. Assessment of these biomarkers may contribute to the development of diagnostic and prognostic tools in PD. In addition, the protective role of NSAIDs further supports the neuroinflammation hypothesis in PD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Natália Pessoa Rocha and Aline Silva de Miranda contributed equally to the study.

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Research Article

Oligoclonal Bands in Cerebrospinal Fluid of Black Patients with Multiple Sclerosis

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Genetic susceptibility is a well-recognized factor in the onset of multiple sclerosis (MS). The objective of this study was to determine the frequency of oligoclonal bands (OCB) restricted to the cerebrospinal fluid, in an ethnically mixed group of MS patients in the city of São Paulo, Brazil. Techniques used to detect OCB consisted of isoelectric focusing followed by immunoblotting. OCB were found in 49 (54.4%) out of 90 patients with clinically definite MS; out of the 23 brown/black patients, 17 (73.9%) were OCB+; out of the 66 white patients, 32 (48.5%) were OCB+; and the only patient yellow was OCB+ ($p = 0.05$). Analysis of the IgG index was also consistent with the findings, but with lower statistical significance. The data presented in our study show that the ethnic differences in MS extend to the immune response.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system. MS is thought to develop in genetically susceptible individuals when one or more environmental factors trigger a cascade of events leading to disease manifestation [1]. Genetic susceptibility is a well-recognized factor in the onset of MS [1]. Individuals of African origin appear to have a "genetic protection" against MS, as reflected by the low incidence of the disease among Africans and Afrodescendants [1, 2]. This holds true not only for people living in Africa, but also for those living in Europe, North America, and South America who have an African background [3–5]. Since this ethnic factor seems to influence the physiopathogenic mechanisms of MS, it is plausible to expect that inflammatory markers of the disease might also be influenced by ethnicity. Here, we describe the frequency of oligoclonal bands (OCB) and IgG index status in cerebrospinal fluid (CSF) in an ethnically mixed group of MS patients in the city of São Paulo, Brazil.

2. Methods

The present study was approved by the Research Ethics Committee of the University of São Paulo, under the number 800/05. All patients or their legal guardians signed

the informed consent agreement prior to enrolment in this study. The MS patients' histories were taken, physical examinations were conducted, and MRI analysis was done immediately after the patients had signed their consent. A second medical doctor reevaluated these parameters independently. Both doctors were neurologists with expertise in MS diagnosis (authors PDG and DC). Subsequently, the patients underwent sampling of CSF as part of the laboratory investigation of MS.

The diagnosis of MS was established in accordance with McDonald's International Criteria, as revised in 2005 [6]. None of the patients had previously undergone a CSF exam and, therefore, the results from the CSF analysis were not used to establish the diagnosis of MS, excluding thereby a diagnostic interpretation bias. Patients who failed to fulfill the clinical and radiological criteria for diagnosing MS were excluded from the present study. In accordance with ethical considerations, they were guaranteed full follow-up at the institution's department of neurology, irrespective of whether they were participating in this study.

A group of 39 patients with a variety of chronic inflammatory disorders of the CNS and 19 subjects with neither neurological complaints nor infectious conditions, whose CSF sample was collected during anesthetic procedure for minor surgery, were used as controls.

The criteria used for ethnic origin were the same as used by the Brazilian research institute, the IBGE (Brazilian Institute of Geography and Statistics). Because of the high degree of miscegenation in Brazil, the IBGE classifies races through self-declared skin color, grouped as white, black, brown (mulatto/mixed), "yellow" (East Asian), and indigenous Indian.

Patients with MS and both control groups were recruited consecutively, all during the same period, from August 2005 to January 2008.

Blood serum and CSF analysis were carried out simultaneously. The CSF analysis included the classical routine tests (cytomorphological profile, determination of total protein content, and assaying of glucose and chlorides). Upon suspicion of involvement of infectious processes, specific immunological reactions were performed and, possibly, analysis on antigenic material and PCR.

Isoelectric focusing (IF) on polyacrylamide gel, followed by immunoblotting [7], was used to assess OCB (ETC Elektrophorese Technik, Westermeier & Schickle GmbH, Bahnhofstrasse 26, 72138 Kirchentellinsfurt, Germany). Each sample of CSF and blood serum was subjected to IF (CWP-400 Isolab Inc.), always with parallel samples. In each of the procedures, a positive and a negative control sample were used. After IF, the proteins were transferred to a nitrocellulose membrane (Bio Agency) for immunoblotting method entailing a primary antibody (goat anti-human IgG, Sigma) and a peroxidase-labeled secondary antibody (polyclonal rabbit anti-goat immunoglobulins, Dako Cytomation). OCB were considered positive when two or more bands were found in the CSF, but absent in the serum. For greater reliability, the results from these tests were examined by two specialists (authors JAL and HRG) who were not aware of the group to which the samples belonged (MS or controls). If there was any doubt or difference of opinion regarding the interpretation of a particular result, the sample in question was processed again.

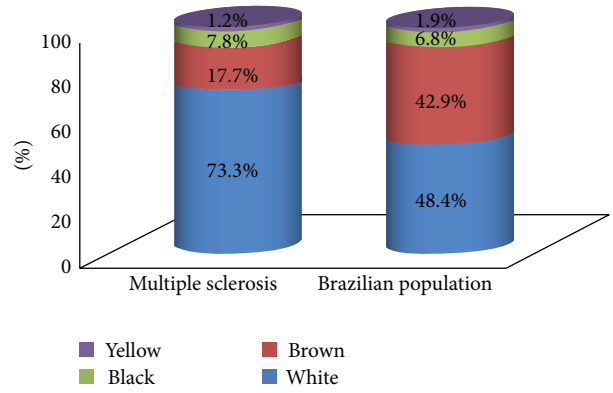

Figure 1: Comparison of self-declared skin color between the study population with multiple sclerosis and the general Brazilian population.

Quantitative intrathecal immunoproduction of IgG was carried out in parallel. Concentrations of IgG and albumin both in the serum and CSF were measured by means of nephelometry. This made it possible to determine the IgG index, which was considered to be greater than normal for values ≥ 0.8.

Tests aimed at ruling out diseases that might have differential diagnoses with MS in specific cases were performed when deemed necessary [8].

3. Results

Preliminary results were presented earlier [9, 10].

The cohort comprised 90 subjects with MS who were attending the Demyelinating Diseases Reference Center at Hospital das Clinicas, University of São Paulo Medical School.

Figure 1 shows the demographic characteristics of the population studied. From this group, of 90 patients with MS, 66 patients self-declared themselves as white, 23 as brown or black, and one as yellow. The designations of black and brown (mulatto/mixed) both refer to individuals of African origin and therefore were grouped as a single element in subsequent analysis.

Out of the 90 patients with MS, 49 (54.4%) presented OCB restricted to the CSF. The specificity of OCB in the CSF was 100% when patients without neurological disease were used as controls and was 82.1% when patients with inflammatory diseases of the CNS were used as the control.

When we compared the self-declared ethnicity (skin color) with the results from the OCB analysis, it was observed that (1) out of the 23 brown/black patients 17 (73.9%) were OCB+; (2) out of the 66 white patients 32 (48.5%) were OCB+; and (3) the only patient self-declared as yellow was OCB+. There was a positive correlation between African origin and OCB presence in the CSF ($X^2 = 3.54$; g.l. = 1; $p = 0.051$; $R = 0.69$; 95% CI = 0.48–0.98; Figure 2).

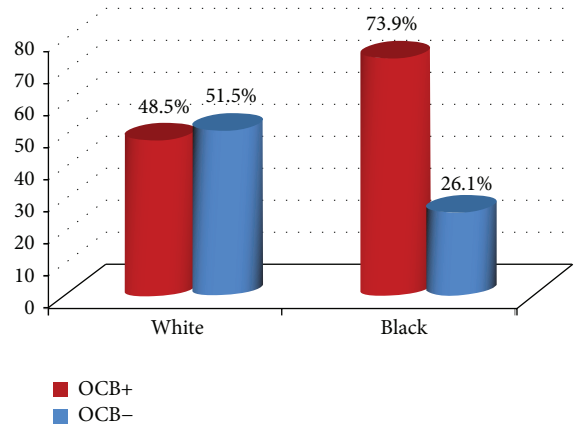

Figure 2: OCB (oligoclonal bands). The test of nonassociation between color and presence of OCB gave the following results: X^2 = 3.54; g.l. = 1; p = 0.051.

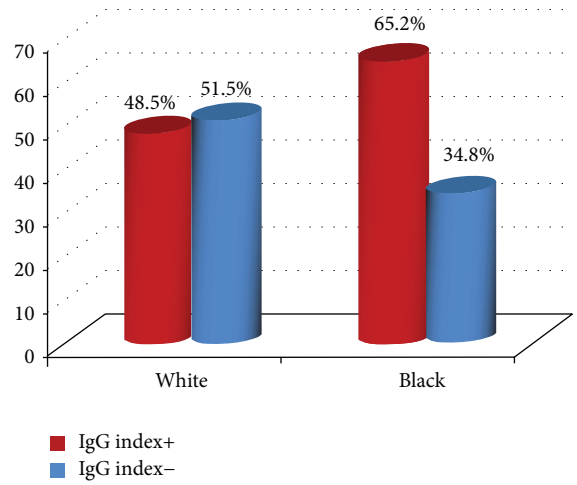

Figure 3: IgG index+ (≥0.8). The test of nonassociation between color and IgG+ gave the following results: X^2 = 1.39; g.l. = 1; p = 0.24.

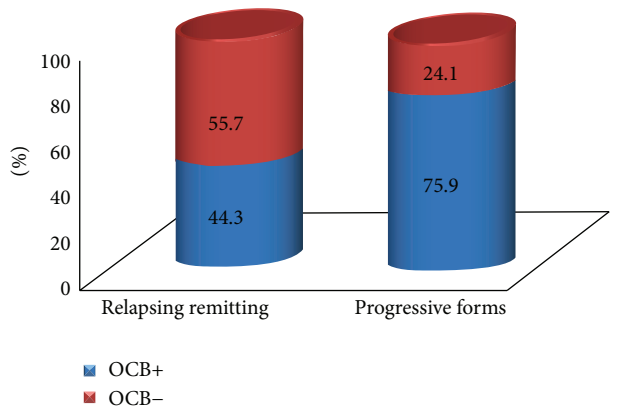

Figure 4: Distribution of the presence of oligoclonal bands (OCB) according to the clinical forms of multiple sclerosis. Test of homogeneity: X^2 = 7.913; g.l. = 1; p = 0.0049; relative risk R = 0.58; 95% CI = 0.41–0.83.

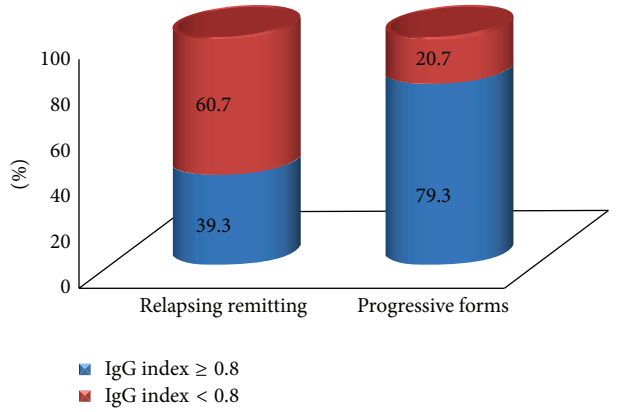

Figure 5: Distribution of the IgG index status according to the clinical forms of multiple sclerosis. Test of homogeneity: X^2 = 12.58; g.l. = 1; p = 0.0004; relative risk R = 0.50; 95% CI = 0.35–0.71.

The IgG index was abnormal in 47 of the 90 patients with MS (52.2%), with a median of 0.8 and mean of 1.02 (SD 0.67). The sensitivity of the IgG index for diagnosing MS was 52.2%; the specificity was 94.8% when patients without neurological disease were used as controls and was 64.2% when patients with inflammatory diseases of the CNS were used as controls.

When we compared the self-declared ethnicity with the results from the IgG index analysis, it was observed that (1) out of the 23 brown/black patients 15 (65.2%) had an abnormally high IgG index; (2) out of the 66 white patients 32 (48.5%) had an abnormally high IgG index; and (3) the only patient who was self-designated as yellow had a normal IgG index (X^2 = 1.39; g.l. = 1; p = 0.24; Figure 3).

When OCB were analyzed in relation to MS disease clinical forms, it was observed that progressive forms of MS were significantly related to OCB and IgG index status in CSF (Figures 4 and 5). This result was remarkable when adjusted for ethnicity. From the group of patients with the relapsing-remitting form of MS, 16 white patients (out of 46; 34.8%) were OCB+, while 17 brown/black patients (out of 23; 73.3%) were OCB+. When progressive forms of MS were taken into consideration, 16 white patients (out of 20; 80%) were OCB+, while seven black patients (out of eight; 87.5%) were OCB+. This result showed that there was a positive correlation between the presence of OCB and the progressive course of MS when adjusted for ethnicity (X^2 = 7.40; g.l. = 1; p = 0.006; Figure 6).

From the group of patients with the relapsing-remitting form of MS, 16 white patients (out of 46; 34.8%) had an abnormally high IgG index, while 16 brown/black patients (out of 23; 69.6%) had an abnormally high IgG index. When progressive forms of MS were taken into consideration, 18 white patients (out of 20; 90%) had an abnormally high IgG index, while eight black patients (out of nine; 88.8%) had an abnormally high IgG index. This result showed that there was also a positive correlation between the presence of an abnormally high IgG index and a progressive course of MS

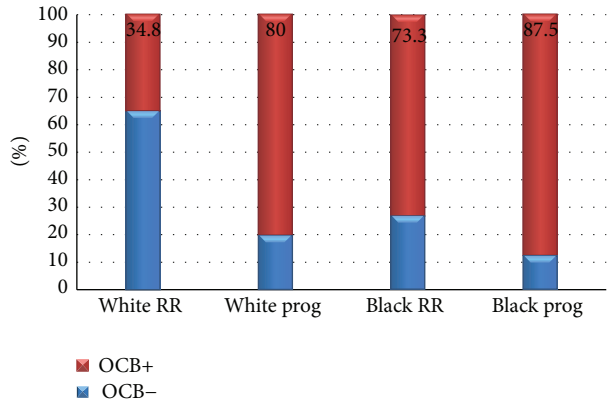

Figure 6: RR (relapsing remitting); prog (secondary-progressive, primary-progressive, and progressive relapsing forms of multiple sclerosis); OCB (oligoclonal bands). Association between OCB and clinical forms of multiple sclerosis adjusted according to patients' self-declared color. Test of nonassociation: $X^2 = 7.40$; g.l. = 1; $p = 0.0065$.

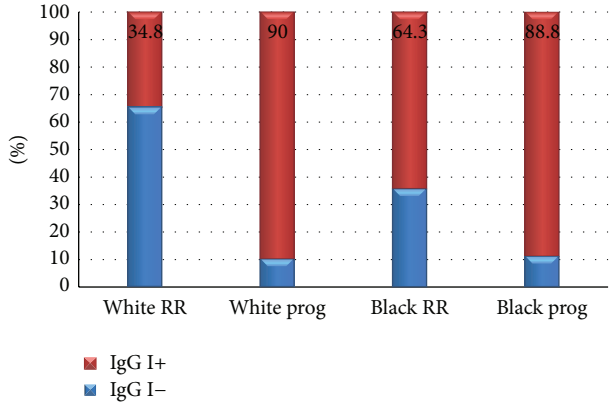

Figure 7: RR (relapsing remitting); prog (secondary-progressive, primary-progressive, and progressive relapsing forms of multiple sclerosis); IgG I (IgG index). Association between IgG index status and clinical forms of multiple sclerosis adjusted according to patients' self-declared color. Test of nonassociation: $X^2 = 7.7$; g.l. = 1; $p = 0.0055$.

when adjusted for ethnicity ($X^2 = 7.70$; g.l. = 1; $p = 0.0055$; Figure 7).

Other correlations, such as disease duration and disease progression and/or relapse rate, were not assessed due to the very wide range of these parameters in this group of 90 patients.

4. Discussion

MS is widely considered to be an autoimmune disease due primarily to CD4+ T-cell mediated immune responses to the major myelin proteins, myelin basic proteins (MBP) and proteolipid proteins (PLP) [1]. Humoral immune responses are also believed to contribute to the immunopathology of MS, and the presence of OCB and/or increased IgG index in CSF directly reflects a high humoral inflammatory response in the patient [11]. In the present study, the presence of OCB in CSF was significantly associated with African origin among the patients and with progressive forms of MS in all ethnicities. The presence of OCB in CSF has been correlated with more aggressive forms of MS [11] and with higher risks of conversion to MS from clinically isolated demyelinating syndrome [12, 13]. Furthermore, African ethnicity has been correlated with a more severe course of MS [3, 4, 14–16] and higher humoral activity [17].

Large studies that investigated genetic differences and clinical and demographic characteristics in relation to OCB status and IgG index have strongly supported the idea that MS patients with and without OCB and/or abnormally high IgG index are genetically distinct [18, 19]. This differentiation may extend to races, as demonstrated through our data.

The aim of the present study was not to assess the correlation between OCB and disease severity or progression in patients of different ethnicities but rather to investigate whether there would be any difference in the immune response in CSF depending on ethnicity. However, the significantly higher humoral activity in MS among patients with an African background indicates possible (and still unknown) biological differences in MS depending on the ethnic background.

The data presented in our study show that the ethnic differences in MS extend to the immune response and may add another measurable immunological phenomenon to the list of differences between ethnic groups.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Transdermal Nicotine Application Attenuates Cardiac Dysfunction after Severe Thermal Injury

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Background. Severe burn trauma leads to an immediate and strong inflammatory response inciting cardiac dysfunction that is associated with high morbidity and mortality. The aim of this study was to determine whether transdermal application of nicotine could influence the burn-induced cardiac dysfunction via its known immunomodulatory effects. *Material and Methods*. A standardized rat burn model was used in 35 male Sprague Dawley rats. The experimental animals were divided into a control group, a burn trauma group, a burn trauma group with additional nicotine treatment, and a sham group with five experimental animals per group. The latter two groups received nicotine administration. Using microtip catheterization, functional parameters of the heart were assessed 12 or 24 hours after infliction of burn trauma. *Results*. Burn trauma led to significantly decreased blood pressure (BP) values whereas nicotine administration normalized BP. As expected, burn trauma also induced a significant deterioration of myocardial contractility and relaxation parameters. After application of nicotine these adverse effects were attenuated. *Conclusion*. The present study showed that transdermal nicotine administration has normalizing effects on burn-induced myocardial dysfunction parameters. Further research is warranted to gain insight in molecular mechanisms and pathways and to evaluate potential treatment options in humans.

1. Introduction

Severe burn injury activates a multitude of immunologic defense mechanisms, one of these being the massive production of proinflammatory mediators [1, 2]. Looking at the organ level, one pivotal consequence of burn trauma is cardiac dysfunction [3, 4]. For its pathogenesis the focus of scientific interest has been on proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, which have been shown to either alone or in concert exert cardiodepressive effects [1, 3, 5]. Elevated levels of IL-1β, IL-6, and TNF-α corresponded to increased levels of creatinine kinase [5]. Various proinflammatory cytokines demonstrate peak concentrations 12 to 24 hours after trauma [1].

There is a link between the central nervous system and immunologic mechanisms [6]. The vagus nerve axis of parasympathetic activity represents one of the mechanisms that can induce anti-inflammatory effects. Its activation may contribute to preventing a hyperactivation of immune subsystems and reaction overshoot [7, 8]. Macrophages have been identified to be the key target cells for the parasympathetic anti-inflammatory effect of vagus nerve activation. A reason for this is the vagus nerve innervation of most internal organs [6, 9]. Also in other organs such as the heart, the release of TNF-α and IL-1β was inhibited directly via stimulation of the vagus nerve [10]. Tracey and colleagues identified this signal transduction pathway and named it

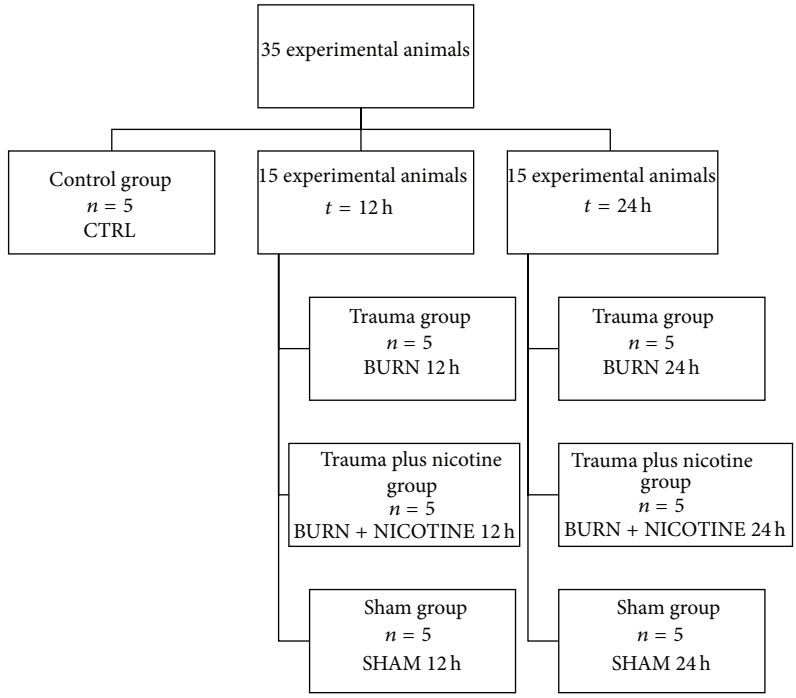

Figure 1: Experimental groups. The classification of 35 experimental animals into seven experimental groups with five experimental animals each is illustrated. Catheter results 12 h and 24 h after experimental burn trauma are compared to control group.

"cholinergic anti-inflammatory pathway" because of its primary neurotransmitter acetylcholine [6, 11]. Mechanistic studies have shown that the molecular basis for this pathway is the nicotinergic acetylcholine receptor (nAChR), which is also present in the sympathetic part of the autonomic nervous system [6, 12]. Nicotine, a receptor agonist, can stimulate the parasympathetic anti-inflammatory mechanisms via the nAChR [12, 13]. Nicotine is well known as one of hundreds of components of tobacco smoke [14, 15]. However, it has also been used pharmacologically. Transdermal application and absorption of nicotine have been used as a noninvasive application method in a plethora of studies, verifying its anti-inflammatory potential [16–18]. Additionally nicotine application in experimental animal models of rheumatoid arthritis and autoimmune myocarditis, other pathologies based on an overwhelming inflammation, provided evidence for a potential clinical relevance of the immunomodulatory effect of nicotine [19, 20].

The present experimental study was designed to evaluate whether the transdermal application of nicotine is feasible and to evaluate potential beneficial effects on myocardial function after severe thermal injury.

2. Material and Methods

The university committee for the use and protection of animals and the Lower Saxony State Office for Consumer Protection and Food Safety approved the present study (study protocol # 05/1052). We created seven study groups with five animals each. This included one control group (CTRL). Microtip catheterization was done after 12 h or 24 h according to the respective treatment. That resulted in the additional six groups BURN 12 h and BURN 24 h, BURN + NICOTINE 12 h, and BURN + NICOTINE 24 h and SHAM 12 h and SHAM 24 h (Figure 1). The study design, the burn trauma, and nicotine application were previously described [21].

2.1. Myocardial Function. Microtip catheterization of the left ventricle was carried out in experimental animals of the CTRL group without any previous treatment and in the BURN groups, BURN + NICOTINE groups, and SHAM groups 12 or 24 hours after experimental burn injury or sham trauma, respectively. The right carotid artery was surgically exposed by midline sternotomy. Using microsurgical techniques, a vascular incision large enough to induce the microtip catheter was created (Fa. Millar Instruments, Houston, TX, USA). The correct catheter position was confirmed by the appearance of the characteristic pressure curves and fluoroscopy imaging (Figure 2). Cardiac actions were then recorded for 5 minutes using commercially available analysis software (Chart 5, AD Instruments GmbH, Spechbach, Germany).

2.2. Statistical Analysis. We used Prism 5 software for statistical analysis (GraphPad Inc., La Jolla, CA) performing Analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$. The results are illustrated as means ± standard deviation (SD).

3. Results

3.1. Blood Pressure. The results of the microtip catheter for systolic and end-diastolic blood pressure measured 12 and 24

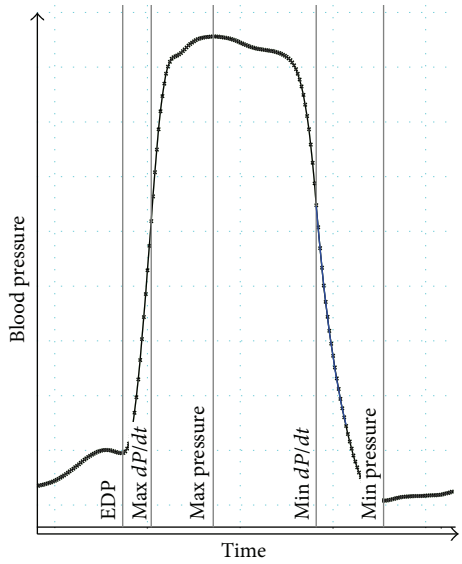

Figure 2: Representative blood pressure curve to illustrate measured parameters. Relevant values are marked. From left to right: end-diastolic pressure (EDP) and maximum blood pressure rise per time (Max dP/dt), systolic blood pressure (Max pressure) and maximum blood pressure drop per time (Min dP/dt). Additionally the minimum blood pressure is illustrated (Min pressure).

hours after burn trauma showed significantly decreased values for the BURN groups compared to the respective CTRL groups. For both physiological parameters analyzed, nicotine administration increased BP levels significantly compared to the untreated BURN groups except for end-diastolic blood pressure after 24 h. However, the baseline BP value of the control group was not completely reestablished by nicotine application.

After time $t = 12$ h post burn injury, the mean values of the BURN group for the systolic blood pressure were significantly decreased compared with values measured in the CTRL group (Figure 3(a), 91.1 ± 3.8 mmHg versus 113.4 ± 10.3 mmHg, black versus white bar, $p < 0.001$). Nicotine partially normalized the effect of the burn trauma and lead to a systolic blood pressure of 106.3 ± 5.5 mmHg (Figure 3(a), striped bar, $p < 0.001$). The same trend was found at $t = 24$ h after experimental burn procedure. The average systolic BP level of BURN 24 h group of 93.6 ± 5.1 mmHg was restored by nicotine application to 101.2 ± 8.6 mmHg (Figure 3(b), black versus striped bar, $p < 0.001$).

The results of the end-diastolic blood pressure are shown in Figures 3(c) and 3(d). Rats subjected to burn trauma developed significantly lower end-diastolic blood pressure than their CTRL counterparts ($p < 0.001$). The nicotine administration resulted in a significantly higher end-diastolic pressure 12 h after burn trauma whereas the difference after 24 h was statistically not relevant (Figures 3(c) and 3(d), striped versus black bars, $p < 0.001$ after 12 h and not significant after 24 h).

3.2. Systole. For the assessment of cardiac function during systole the duration of systole and contractility were measured in terms of maximum blood pressure rise per second.

The duration of the systole was on average 0.083 s in CTRL group (Figures 3(a) and 3(b), white bar). For duration of systole of BURN groups we found mean values of 0.096 s after 12 h and 0.098 s after 24 h, whereas the values of BURN + NICOTINE groups were significantly decreased (Figures 4(a) and 4(b), black versus striped bars, $p < 0.001$).

A significant increase of maximum blood pressure rise per second was observed when results of BURN groups were compared to BURN + NICOTINE groups (Figures 4(c) and 4(d), black versus striped bars, $p < 0.001$). Interestingly, SHAM and BURN + NICOTINE groups displayed slightly higher values for cardiac contractility than CTRL animals (Figures 4(c) and 4(d), grey and striped bars versus white bar). The BURN 12 h group demonstrated a value for maximum blood pressure rise per seconds of 4586 ± 859.7 mmHg/s and the BURN 24 h remained at a low level with 4653 ± 451.4 mmHg/s. In contrast, the BURN + NICOTINE groups revealed 5878 ± 576.3 mmHg/s and 6316 ± 649.9 mmHg/s, respectively, (Figures 4(c) and 4(d), striped bars versus black bars, $p < 0.05$).

3.3. Diastole. Based on the assessment of cardiac function during systole we determined the duration of diastole and the maximum blood pressure drop per second (relaxation) for the assessment of cardiac function during the diastolic phase of the cardiac cycle. With regard to the relaxation it is important that a higher negative value indicates a faster relaxation.

The duration of diastole after burn injury was on average 0.96 after 24 h and 0.99 s after 12 h (Figures 5(a) and 5(b), black bars). When burn-injured animals underwent nicotine treatment, duration of diastole was slightly increased compared to burn alone animals (Figures 5(a) and 5(b), striped versus black bars, not significant).

Animals subjected to burn trauma showed a markedly slower relaxation compared to their SHAM and CTRL counterparts (Figures 5(c) and 5(d), black versus grey and white bars, $p < 0.001$). Nicotine treatment induced a relaxation similar to sham animals (Figures 5(c) and 5(d), black versus striped bars, $p < 0.001$).

3.4. Heart Rate. Concerning the heart rate no significant differences were found 12 and 24 h after burn injury. The heart rate values ranged from 309.3 ± 3.2 beats/min (Figure 6(b), black bar) up to 315.4 ± 23.4 beats/min (Figure 6(b), striped bar).

4. Discussion

Intense burn trauma imposes life-threatening consequences to the victim. The multifactorial pathogenesis of burn-induced cardiac dysfunction has been extensively evaluated and described. This study was conducted to evaluate whether the known immunomodulatory effect of nicotine can improve cardiac function after burn trauma.

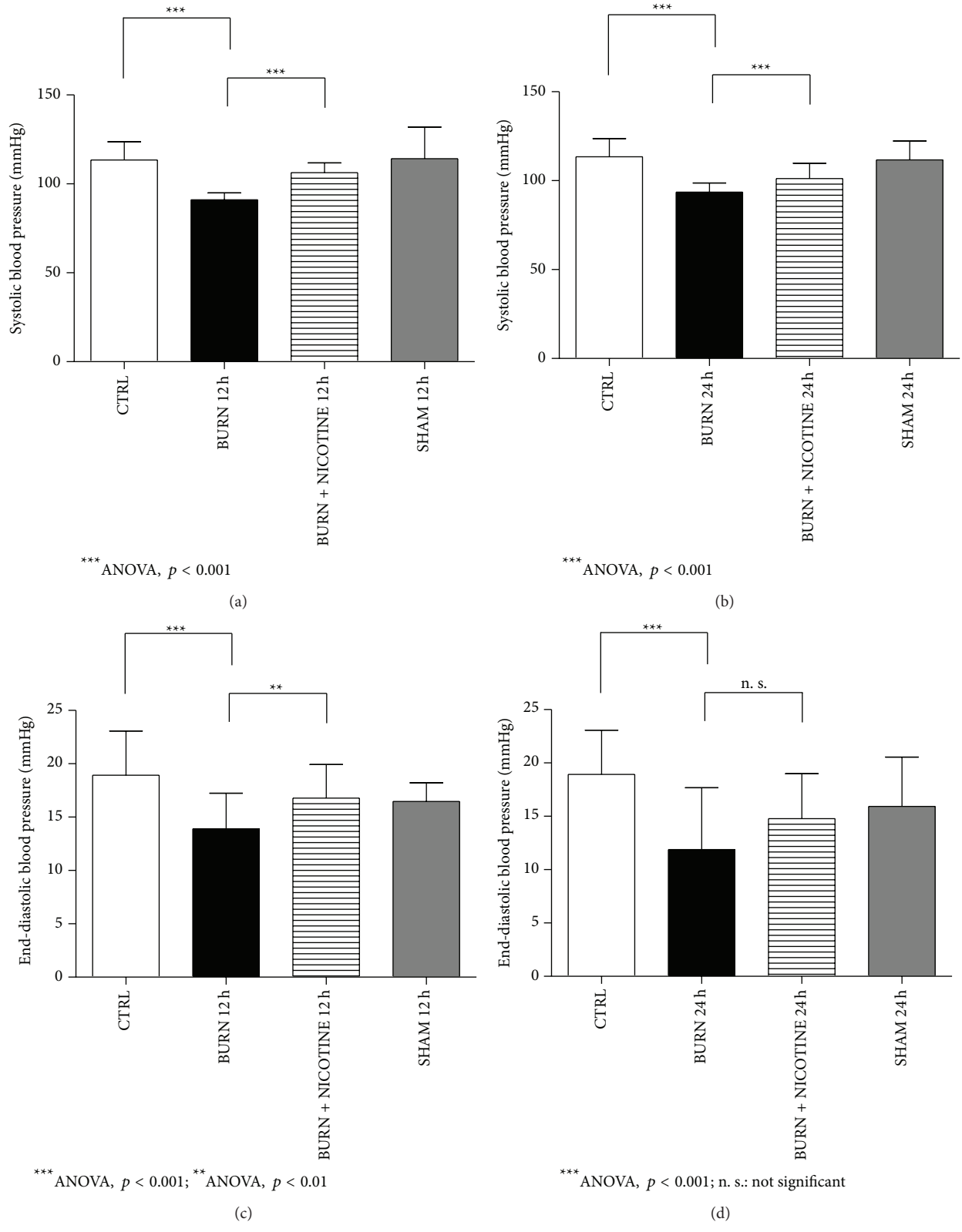

Figure 3: Blood pressure parameters. (a) and (b): systolic blood pressure. (c) and (d): end-diastolic blood pressure. Concerning the experimental design, note decreased BP levels in the BURN groups in contrast to the CTRL group ($p \leq 0.05$). Transdermal nicotine application at least partially restored this effect. BP values are expressed as means ± SD. Each bar represents $n = 5$ experiments.

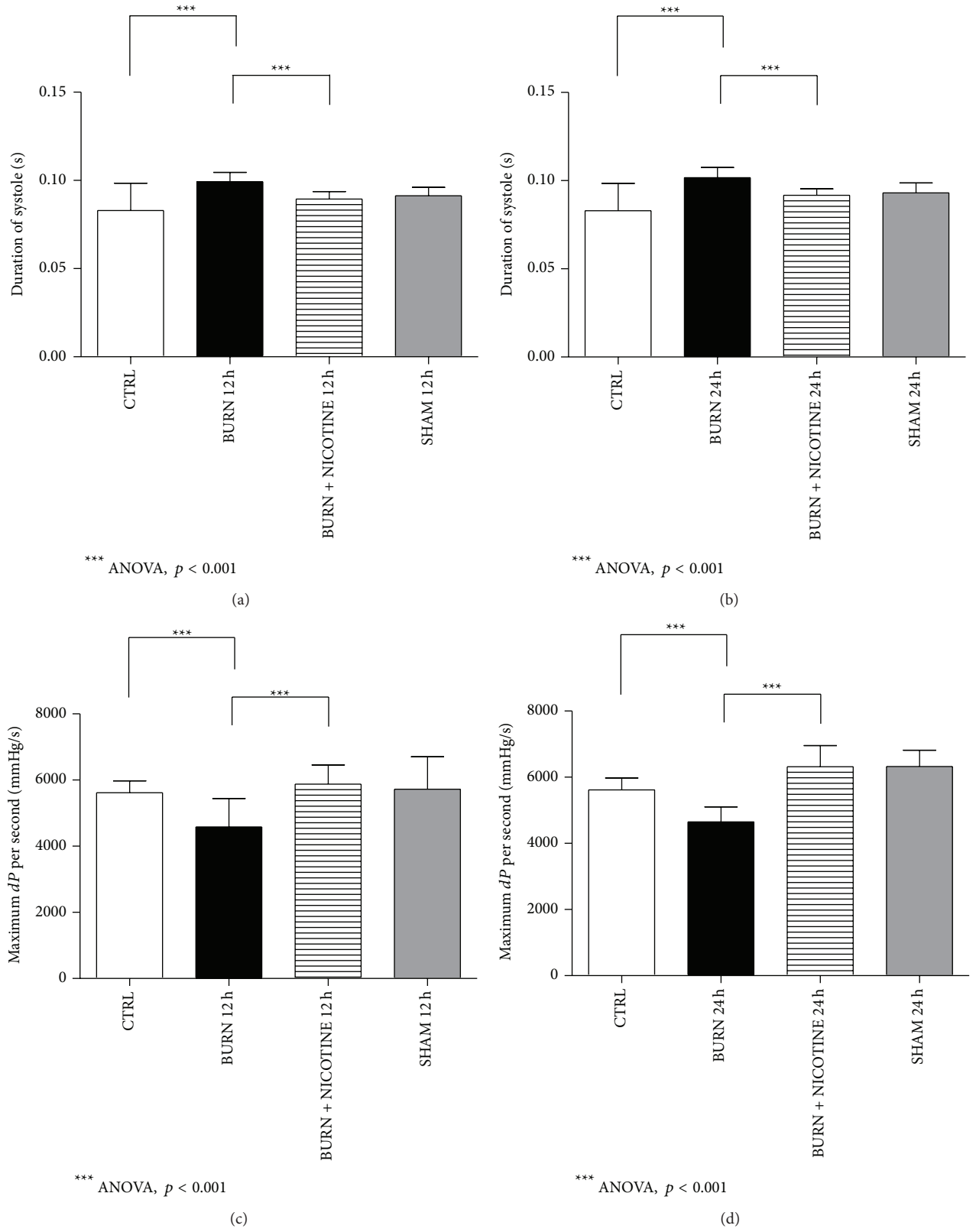

FIGURE 4: Systole. (a) and (b): duration of systole. The systole duration increased in experimental burn group (black versus white bar, $p < 0.05$). The BURN + NICOTINE groups revealed significantly lower duration of systole values than their burn alone counterparts (striped versus black bar, $p < 0.05$). (c) and (d): maximum increase of blood pressure per second (contractility). Reduced contractility was found for the BURN group (black versus white bar, $p < 0.05$). Nicotine administration showed restored contractility (striped versus black bar, $p < 0.05$). All values are expressed as mean + SD. Each bar represents $n = 5$ experiments.

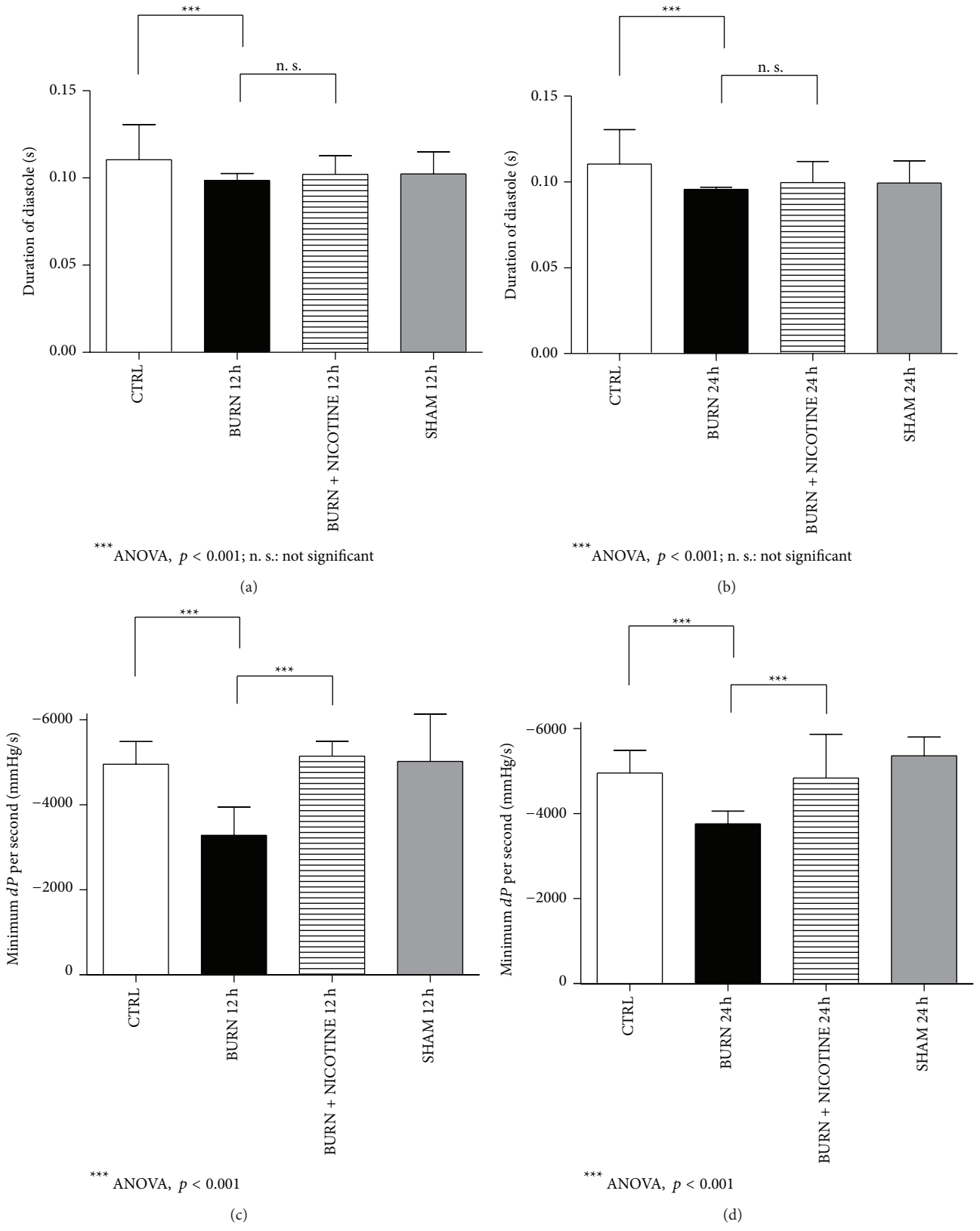

FIGURE 5: Diastole. (a) and (b): duration of diastole. The BURN group revealed significantly shorter diastole duration than the CTRL group (black versus white bar, $p < 0.05$). After posttraumatic nicotine application this effect is slightly reduced although differences were not statistically relevant (striped versus black bar, *not significant*). (c) and (d): minimum blood pressure difference per second (relaxation). Note that the scale is negative. The difference between the BURN and BURN + NICOTINE animals is marked (black versus striped bars, $p < 0.05$). Interestingly, both SHAM and BURN + NICOTINE groups display higher values than the control group. All results are expressed as mean + SD. Each bar represents $n = 5$ experiments.

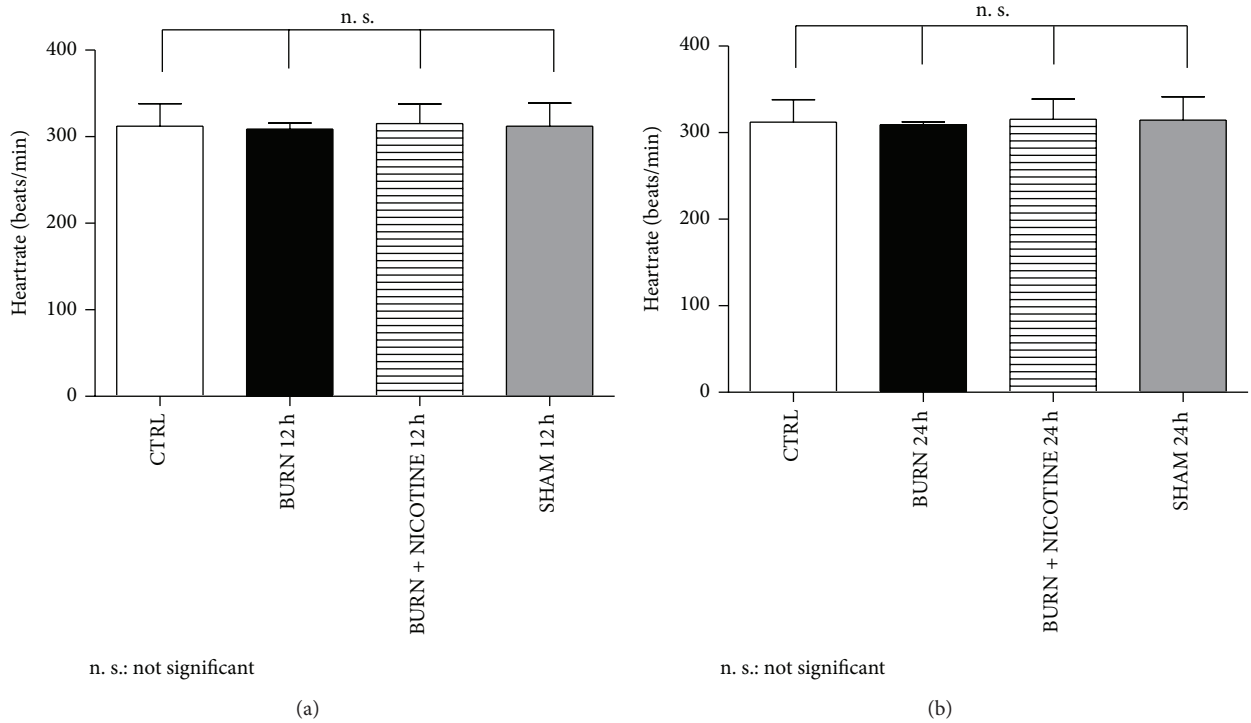

FIGURE 6: Heart rate. (a) and (b): heart rate. In the analysis of heart rate, no significant differences between the groups of experimental animals were found. The values are expressed as mean + SD. Each bar represents $n = 5$ experiments.

Previous experiments of our research group were looking at direct electrical stimulation of the vagus nerve and we could prove attenuation of proinflammatory cytokine production after electrostimulation of the parasympathetic axis [10, 22]. Additionally several studies used transdermal nicotine application to stimulate the parasympathetic axis because nicotine administration has been shown to induce anti-inflammatory effects [16, 17, 21, 23]. Clinically, this has been demonstrated in patients suffering from colitis ulcerosa [17, 23]. But in contrast, nicotine has adverse effect on Crohn's disease [23]. The reason for this ambiguous reaction in inflammatory bowel diseases is still not understood in entirety [23]. Still nicotine itself is seen doubtfully as an additive aspect in the treatment after burn trauma especially due to described adverse effects on wound healing [24, 25]. This points up the importance of further experiments to distinguish the positive anti-inflammatory effects of nicotine from adverse local effects on wound healing.

The present study showed that the administration of nicotine partially restores or normalizes decreased systolic blood pressure following burn injury. The results published by the groups of Sambol and Adams also revealed decreased systolic blood pressure at 24 hours after burn trauma [4, 26]. However decreasing the burn-induced increased activity of cardiodepressive cytokines like TNF-α and IL-1 thereby leading to normalization of blood pressure after a burn trauma [4, 26]. Thus, there is a negative correlation between circulating proinflammatory cytokine concentrations of TNF-α and IL-1 and the systolic blood pressure [5].

The left ventricular systolic blood pressure has a direct relationship to the arterial blood pressure that is based on the intravascular blood volume, stroke volume, and the total peripheral resistance. Burn trauma affects all of these three parameters. Capillary leakage results in severe fluid loss and decreased intravascular blood volume; the decreased contractility of the ventricle generates reduced stroke volume and circulating proinflammatory cytokines such as IL-1β result in total peripheral resistance loss [3, 27]. Nicotine effectively reduces the concentration of vasodilatory cytokines such as IL-1β and thereby leads to an increased total peripheral resistance [12]. In addition, the concentrations of TNF-α, IL-1β, and IL-6 are reduced which diminishes and, at best, abolishes their negative impact on cardiac contractility [3]. Stimulation of sympathetic neurons via nicotine application might support the restoration of systolic blood pressure. Additionally the volume resuscitation after burn trauma could contribute to the normalization of systolic blood pressure levels. However, sole fluid therapy is insufficient to abolish burn-induced cardiac dysfunction [28], as also seen in our BURN group.

For end-diastolic blood pressure levels burn injury also resulted in decreased levels. This parameter is essentially determined by the intravascular volume and contractile force of the left ventricle. So it may be deduced that the increase of the end-diastolic blood pressure of BURN + NICOTINE groups compared to BURN groups is a result of an impact on left ventricular function. This would be contradictory to the measurements of the other parameters

of myocardial function in the present study, indicating a positive effect of nicotine administration on left ventricular function. A second explanation for the increase in end-diastolic blood pressure according to nicotine application is based on the posttraumatic intravascular volume. The large burn-induced volume loss followed to the capillary leak results in a decreased intravascular volume and consecutively in a decreased end-diastolic blood pressure. In this context, Ipaktchi et al. were able to show that immunosuppressive therapy after burn trauma reduces the capillary leakage significantly [29]. This group used the specific inhibition of mitogen-activated protein kinases that has been shown to be a signal transduction element of the proinflammatory signaling cascade. This pathway is also inhibited by nicotine, which was shown by Oke and Tracey [30]. The improved end-diastolic blood pressure may result secondarily via the Frank-Starling mechanism.

The BURN groups showed significantly higher values for duration of systole compared to the control group. This was accompanied by significantly decreased values for the maximum blood pressure rise per second and a shorter duration of diastole. Previous studies also revealed a burn-induced contractile dysfunction [4, 26]. Additionally burn trauma affected diastolic cardiac function. Again, nicotine could attenuate the burn-induced negative effect: The BURN + NICOTINE and SHAM groups revealed increased levels compared to the BURN group and even to control group. Comparative data for this purpose does merely exist to our knowledge. Only the work of Adams and colleagues described the same effect of burn trauma [4]. Their and our results reveal that thermal trauma leads to systolic and diastolic cardiac dysfunction. As a result of our experiments, the application of nicotine has demonstrated, with few exceptions without significant differences, a positive effect on systolic and diastolic function after burn trauma.

Heart rate showed no significant differences. This is consistent with the results of several authors [5, 10, 26, 31]. Adams and colleagues found a steady heart rate in a burn model 24 h after trauma [4]. Similarly, Sambol, Maass, and our own results of burn models showed no changes in heart rate [5, 10, 26]. It is noteworthy that nicotine administration does not induce bradycardia. Bradycardia would be expected from stimulation of the vagal nerve. Interestingly however, in the literature there are data that nicotine has a more tachycardic effect [15, 17]. However, an additional effect of nicotine on sympathetic neurons cannot be excluded and could be an explanation for this aspect.

5. Conclusions

In the present study, we found that in a rat model of severe burn injury the trauma causes decreased blood pressure and a decrease in contraction and relaxation velocity of the heart. Adding on to the current knowledge base, further experiments will be done to explore the potent anti-inflammatory mechanisms of activation of the parasympathetic neural system. Our encouraging results that led to restoration of nearly normal myocardial function parameters have already prompted us to follow up on this path.

Whether nicotine may also affect the lethality of severe burn trauma was not the subject of these investigations. This would be of great importance for further studies.

Conflict of Interests

The authors declare no conflict of interests or financial incentives in the production of this paper.

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Research Article

Peripheral Administration of Tumor Necrosis Factor-Alpha Induces Neuroinflammation and Sickness but Not Depressive-Like Behavior in Mice

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Clinical observations indicate that activation of the TNF-α system may contribute to the development of inflammation-associated depression. Here, we tested the hypothesis that systemic upregulation of TNF-α induces neuroinflammation and behavioral changes relevant to depression. We report that a single intraperitoneal injection of TNF-α in mice increased serum and brain levels of the proinflammatory mediators TNF-α, IL-6, and MCP-1, in a dose- and time-dependent manner, but not IL-1β. Protein levels of the anti-inflammatory cytokine IL-10 increased in serum but not in the brain. The transient release of immune molecules was followed by glial cell activation as indicated by increased astrocyte activation in bioluminescent Gfap-luc mice and elevated immunoreactivity against the microglial marker Iba1 in the dentate gyrus of TNF-α-challenged mice. Additionally, TNF-α-injected mice were evaluated in a panel of behavioral tests commonly used to study sickness and depressive-like behavior in rodents. Our behavioral data imply that systemic administration of TNF-α induces a strong sickness response characterized by reduced locomotor activity, decreased fluid intake, and body weight loss. Depressive-like behavior could not be separated from sickness at any of the time points studied. Together, these results demonstrate that peripheral TNF-α affects the central nervous system at a neuroimmune and behavioral level.

1. Introduction

Clinical depression is a chronic, disabling psychiatric condition that affects over 120 million people worldwide [1]. It is predicted that, by 2030, depression will be the second leading cause of disability in the world [2]. Although its etiology remains poorly understood, it is generally accepted that depression is a multifactorial disorder with numerous interacting systems underlying its pathogenesis. A number of clinical observations suggest that dysregulation of the immune system might also play a role in the development of depression, at least in a subset of susceptible individuals. For example, depression frequently occurs as a comorbidity of medical conditions characterized by a chronic inflammatory component including rheumatoid arthritis [3], cancer [4], type 2 diabetes [5], stroke [6], obesity [7], and coronary artery disease [8]. Even in absence of other medical illnesses, depressed patients often show elevated circulating levels of inflammatory mediators such as proinflammatory cytokines and the acute-phase C-reactive protein [9, 10]. Moreover, up to half of cancer and hepatitis C patients that receive therapeutic administration of proinflammatory cytokines eventually develop depressive symptoms [11–13].

There are several indications that tumor necrosis factor-α (TNF-α) is one of the key cytokines involved in the pathogenesis of inflammation-associated depression. Recent meta-analyses confirmed that circulating levels of TNF-α are significantly higher in depressed patients compared with

healthy control subjects [10, 14]. Moreover, elevated plasma levels of TNF-α are associated with treatment resistance to conventional antidepressants [15]. In hepatitis C patients that are chronically treated with interferon-α, increased blood levels of TNF-α correlate with the development of depressive symptoms [16]. Furthermore, peripheral administration of anti-TNF-α antibodies improves depressed mood in patients suffering from psoriasis [17], Crohn's disease [18], and rheumatoid arthritis [19]. TNF-α antagonism has also been shown to improve treatment resistant depression in a subgroup of patients with high baseline inflammatory biomarkers [20].

Inflammation-associated depression is often studied in rodents by peripheral administration of immunostimulants such as bacterial lipopolysaccharide (LPS). It is known that systemic injection of LPS elicits a widespread immune response, characterized by the release of numerous immune mediators and the occurrence of sickness, a behavioral state comprised of symptoms such as malaise, lethargy, decreased motor activity and appetite, sleep disturbances, and increased sensitivity to pain [21, 22]. There are some indications that this sickness response is followed by a phase of depressive-like behavior [23–25]. However, the characteristics of sickness can substantially confound the evaluation of depressive-like behavior in behavioral tests. For example, sick animals display reduced exploration, which can potentially interfere with measurements of immobility used to estimate behavioral despair in paradigms such as the forced swim (FST) and tail suspension test (TST) [26]. Moreover, sick animals eat and drink less, which can bias measures of sweetened fluid intake in assays designed to evaluate anhedonia (the inability to experience pleasure from naturally rewarding activities). Using a panel of behavioral paradigms in mice, we recently demonstrated that it is difficult to separate depressive-like behavior from sickness following acute peripheral LPS administration [27].

Based on the fact that systemic LPS administration induces a broad immune response and the clinical data linking TNF-α to human inflammation-associated depression, we hypothesized that peripheral administration of TNF-α itself may provide a more specific approach to study depressive-like behavior in mice. Indeed, systemic administration of TNF-α has already been shown to have central effects as indicated by increased proinflammatory gene expression in the brain and the development of sickness [28, 29]. Moreover, intracerebroventricular (i.c.v.) injection of TNF-α was shown to lead to depressive-like behavior in mice [30, 31]. However, to our knowledge, no study has systematically assessed the effect of peripheral TNF-α administration on neuroinflammation and depressive-like behavior over time. Therefore, the present series of experiments aimed at characterizing the central effects of systemic TNF-α injection by combining multiple techniques to quantify neuroinflammation and behavioral changes. First, serum and brain levels of immune mediators were quantified at several time points after systemic TNF-α administration. Next, transgenic bioluminescent Gfap-luc mice were used to evaluate the time course of TNF-α-induced astrocyte activation, as a marker of glial cell activation *in vivo*. Then, the occurrence of glial cell activation was confirmed by immunohistochemistry using the microglial marker ionized calcium-binding adapter molecule 1 (Iba1). Finally, TNF-α-injected mice were tested in a panel of behavioral paradigms to assess whether depressive-like behavior could be separated over time from sickness.

2. Material and Methods

2.1. Animals and TNF-α. All animal care and use were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NRC) and experimental protocols were approved by the Institutional Ethical Committee on Animal Experimentation, according to applicable regional law. Male NMRI mice were purchased from Charles River Laboratories (France), male wild-type FVB mice were purchased from Janvier (France), and male transgenic Gfap-luc mice (FVB/N-Tg(Gfap-luc)-Xen) were obtained from Taconic Laboratories (USA). The latter animals express luciferase under the transcriptional control of the glial fibrillary acidic protein (Gfap) promoter [32] and are commonly used as a model system for noninvasive quantification of astrocyte activation in living animals over time [27, 33, 34]. Unless mentioned otherwise, animals were housed in groups of 4 per cage under a normal 12:12 h light-dark cycle (lights on at 06:00 a.m. with a 30 min dim and rise phase). Food and water were available ad libitum.

Recombinant mouse TNF-α was purchased from Biolegend (product ID 575208) and dissolved in sterile phosphate buffered saline prior to injection.

2.2. Cytokine Measurements. 10-week-old male NMRI mice were injected intraperitoneally (i.p.) with 0, 63, 125, or 250 μg/kg TNF-α (n = 6-7 per group) and sacrificed by decapitation at 2 h, 6 h, or 24 h. This dose range was based on results from the open field test (OFT) (see Section 2.5). Serum and whole brain samples were collected and processed as previously described [27]. Concentrations of interferon-γ (IFN-γ), interleukin- (IL-) 1β, IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1), and TNF-α were determined in each sample using a mouse cytokine/chemokine magnetic bead panel kit from Merck Millipore. All steps in the assay were conducted according to the manufacturer's instructions. Cytokine levels below detection limit were assigned a value equal to the lowest detectable value of that cytokine.

2.3. In Vivo Bioluminescence Imaging. Astrocyte activation in 10-week-old male Gfap-luc mice was quantified before (baseline) and at 2 h, 6 h, 24 h, 48 h, 72 h, and 96 h after i.p. administration of either 0, 63, or 250 μg/kg TNF-α (n = 7 per group). Brain bioluminescence was detected as described previously [27, 35]. Briefly, Gfap-luc mice were anesthetized by inhalation of 2% isoflurane in 1 L/min oxygen, shaved on the head, and injected with 126 mg/kg D-luciferin (Promega, product ID E1601) in the tail vein. Three minutes later the animals were scanned with a charge-coupled device camera (IVIS Imaging System 200 Series, PerkinElmer) mounted on a dark box. Photon emission from the whole brain was measured using Living Image 3.2 software (PerkinElmer) in

a region of interest (ROI) that was kept constant across mice. Bioluminescence coming from the ears was considered to be basal Gfap activity and was excluded from the ROI. Imaging signals were measured in physical units of surface radiance (photons/s/cm^2/steradian [sr]).

2.4. Immunohistochemistry. 10-week-old male FVB mice were injected i.p. with vehicle or 250 μg/kg TNF-α ($n = 8$ per group) and tissue was collected 24 h later. Immunohistochemical staining of Iba1 protein in the dentate gyrus of the hippocampus was performed using a rabbit polyclonal anti-Iba1 primary antibody (1 : 500, Wako Chemicals) and a fluorescent Alexa 555 goat anti-rabbit secondary antibody (1 : 500, Invitrogen), as previously described [27].

2.5. Behavioral Tests. All behavioral tests were performed on separate groups of 10-week-old male NMRI mice. The OFT and FST setups were custom-made and were described in detail previously [27].

Two independent OFTs were performed in this study. In the first OFT, mice were injected with 0, 63, 250, or 1000 μg/kg TNF-α ($n = 10$ per group) and repeatedly tested at 2 h, 6 h, 24 h, and 48 h after administration. Two of the 10 mice that received 1000 μg/kg TNF-α died during the first 24 h after injection. Therefore, it was decided to take 250 μg/kg TNF-α as the highest test dose for all experiments and to repeat the OFT with a vehicle and 125 μg/kg TNF-α group.

In the FST, mice ($n = 10$ per group) were injected with 0, 63, 125, or 250 μg/kg TNF-α and tested at 2 h, 6 h, 24 h, and 48 h after administration.

The sucrose preference test (SPT) started by single-housing the animals in individually ventilated cages ($L \times W \times H$: 35 × 31 × 16 cm; Tecniplast, Italy) fitted with two 250 mL drinking bottles and ad libitum access to food. Each bottle contained either filtered tap water or a 5% sucrose solution. The location of the bottles on the cage was randomized during every exposure session with half of the animals receiving sucrose on the left and half on the right. The SPT protocol lasted for 5 days and consisted of a familiarization and a test phase. The familiarization phase started on day 1 by exposing all mice to one water- and one 5% sucrose-filled bottle (W/S) for 24 h. On day 2, the animals had free access to two water-filled bottles (W/W) until 4:00 p.m., after which they were fluid-deprived overnight. The test phase started on day 3 by injecting mice i.p. with 0, 63, 125, or 250 μg/kg TNF-α ($n = 10$ per group). To test the effects of TNF-α at 2 h, 6 h, 24 h, and 48 h, the animals were presented with W/S during a 1 h exposure period at these time points. Mice were fluid-deprived in between exposure periods. In order to avoid a protracted deprivation period between the 24 h and 48 h time points, mice were given access to W/W from 4:00 to 5:00 p.m. on day 4.

In the SPT study using a within-subject design it became clear that exposing the thirsty animals to W/S at 2 h affected the total volume intake at 6 h (less thirsty). To exclude that the effects of TNF-α were confounded by retesting the same animals over time, the SPT study was repeated in an independent between-subjects design study using separate groups of TNF-α challenged mice that were tested at either 6 h or 24 h. These mice underwent the same familiarization phase as described above. At the beginning of the test phase, the animals were injected with 0, 63, 125, or 250 μg/kg i.p. TNF-α ($n = 10$ per group). At 6 h after TNF-α, mice from the 6 h time point group were exposed to W/S for a 1 h period. Animals from the 24 h time point were allowed to drink W/W for 1 h at 6 h after TNF-α in order to avoid a protracted deprivation period between TNF-α administration and the 24 h time point. At 24 h, mice from the 24 h group were presented with W/S during a 1 h exposure period.

In both SPT studies, the amount drunk by a mouse was determined by subtracting the weight of a bottle at the start of an exposure period and at the end (taking fluid density as 1 g/mL). Total fluid intake was calculated as the total change in volume from both bottles combined. A fluid intake that was greater than the mean +2x standard deviation was considered to be an invalid measure that probably resulted from leaking bottles. Invalid measures were replaced by the group mean of the relevant solution (water or sucrose). This occurred for less than 4% of all bottle measurements. Sucrose preference was calculated as the percentage of consumed sucrose solution of the total fluid intake.

2.6. Statistical Analysis. SPSS Statistics software version 20 (IBM Inc.) was used for data analysis. Analysis of variance (ANOVA) or repeated measures ANOVA (rmANOVA) was performed to determine the statistical significance of differences between treatment groups. To correct for potential violation of the sphericity assumption, a Greenhouse-Geisser correction epsilon (ε) was used for repeated measures analysis [36]. This correction multiplies both the numerator and the denominator degrees of freedom by ε and the significance of the F-ratio is evaluated with the new degrees of freedom, resulting in a more conservative statistical test. To account for the skewness of the data distribution, bioluminescence measurements and cytokine concentrations were log-transformed prior to analysis. ANOVAs and rmANOVAs were considered statistically significant if $P < 0.05$. When appropriate, post hoc comparisons were made by using an independent samples t-test with a Bonferroni-corrected P value. For consistency between the analysis and the visualization of bioluminescence measurements and cytokine concentrations, the group means and its standard error of the mean (SEM) were back-transformed and visually presented on a logarithmic scale. All other data are expressed as mean ± SEM on a linear scale.

3. Results

3.1. TNF-α Increases Immune Mediator Levels in Serum and Brain. To characterize the immunological response to peripheral TNF-α injection, serum and brain levels of several immune factors were quantified at 2 h, 6 h, and 24 h after administration. Factorial ANOVA showed a significant time × dose interaction on serum levels of IL-6, TNF-α, and MCP-1 (IL-6: $F(6, 68) = 13.4$, $P < 0.001$; TNF-α: $F(6, 66) = 15.7$, $P < 0.001$; MCP-1: $F(6, 68) = 7.2$, $P < 0.001$), a main effect of time and dose on serum levels of IL-10 (time: $F(2, 68) = 5.3$, $P < 0.01$; dose: $F(3, 68) = 4.8$, $P < 0.01$), and a main

effect of dose on serum levels of IL-1β ($F(3, 68) = 3.2$, $P < 0.05$) and IFN-γ ($F(3, 68) = 5.8$, $P < 0.01$). Post hoc analysis demonstrated that serum levels of IL-6, TNF-α, and MCP-1 peaked at 2 h after systemic injection of TNF-α and then gradually waned over time (Figure 1, left). The TNF-α-induced release of IL-10 followed a different time course as serum levels of this cytokine were only elevated at 6 h after TNF-α. At 24 h, the serum concentrations of IL-6, TNF-α, and IL-10 had returned to baseline values, while MCP-1 levels remained significantly elevated in mice that were injected with 250 μg/kg TNF-α. Serum concentrations of IFN-γ were higher across time points in animals that received 250 μg/kg TNF-α, while IL-1β in serum was not significantly different at the post hoc level.

For brain tissue, a significant time × dose interaction was found on protein levels of IL-6, TNF-α, and MCP-1 (IL-6: $F(6, 67) = 6.4$, $P < 0.001$; TNF-α: $F(6, 67) = 70.2$, $P < 0.001$; MCP-1: $F(6, 67) = 15.4$, $P < 0.001$) and a main effect of dose on IFN-γ levels ($F(3, 67) = 4.0$, $P < 0.05$). No significant effect of time or dose could be detected on brain levels of IL-1β or IL-10. Post hoc analysis revealed that brain levels of IL-6 and TNF-α peaked at 2 h and had dissipated by 6 h (Figure 1, right). However, at 6 h there was still a trend for elevated IL-6 levels in mice that had received 250 μg/kg TNF-α. Comparable to the time course of its release in serum, brain levels of MCP-1 remained strongly elevated from 2 h until 6 h after treatment. At 24 h, there was still a trend for increased MCP-1 levels in mice injected with 250 μg/kg TNF-α. Brain concentrations of IFN-γ were decreased across time points in animals from the 63 and 125 μg/kg TNF-α group, but not in mice that received 250 μg/kg TNF-α group.

3.2. TNF-α Induces Glial Cell Activation. To quantify the effects of systemic TNF-α administration on astrocyte activation over time, Gfap-luc mice were injected i.p. with different doses of TNF-α and bioluminescence was measured at specific time points. Factorial rmANOVA revealed a significant time × dose interaction ($F(12, 84) = 5.8$; $P < 0.001$; $\varepsilon = 0.53$) for photons emitted per second in the brain ROI. Post hoc analysis demonstrated that, at 6 h after administration, a strong bioluminescent signal was present in the brain of TNF-α-injected mice (Figure 2). This signal was higher in mice injected with 250 μg/kg TNF-α as compared to mice that received 63 μg/kg TNF-α. Brain bioluminescence in mice treated with 63 μg/kg TNF-α reached control levels at 24 h, while it took up to 72 h to normalize for animals injected with 250 μg/kg TNF-α.

In order to confirm TNF-α-induced activation of glial cells by using a different technique and focusing on another cell type, immunohistochemistry was performed using a microglial activation marker. The expression of Iba1 was quantified in the hippocampal dentate gyrus at 24 h after systemic injection of vehicle or 250 μg/kg TNF-α. This brain structure was chosen based on its association with stress and depression [37–39]. Pairwise comparison demonstrated that immunoreactivity against Iba1 in the dentate gyrus at 24 h was significantly higher in TNF-α-injected mice when compared to mice that received vehicle ($F(1, 13) = 7.3$, $P < 0.05$) (Figure 3).

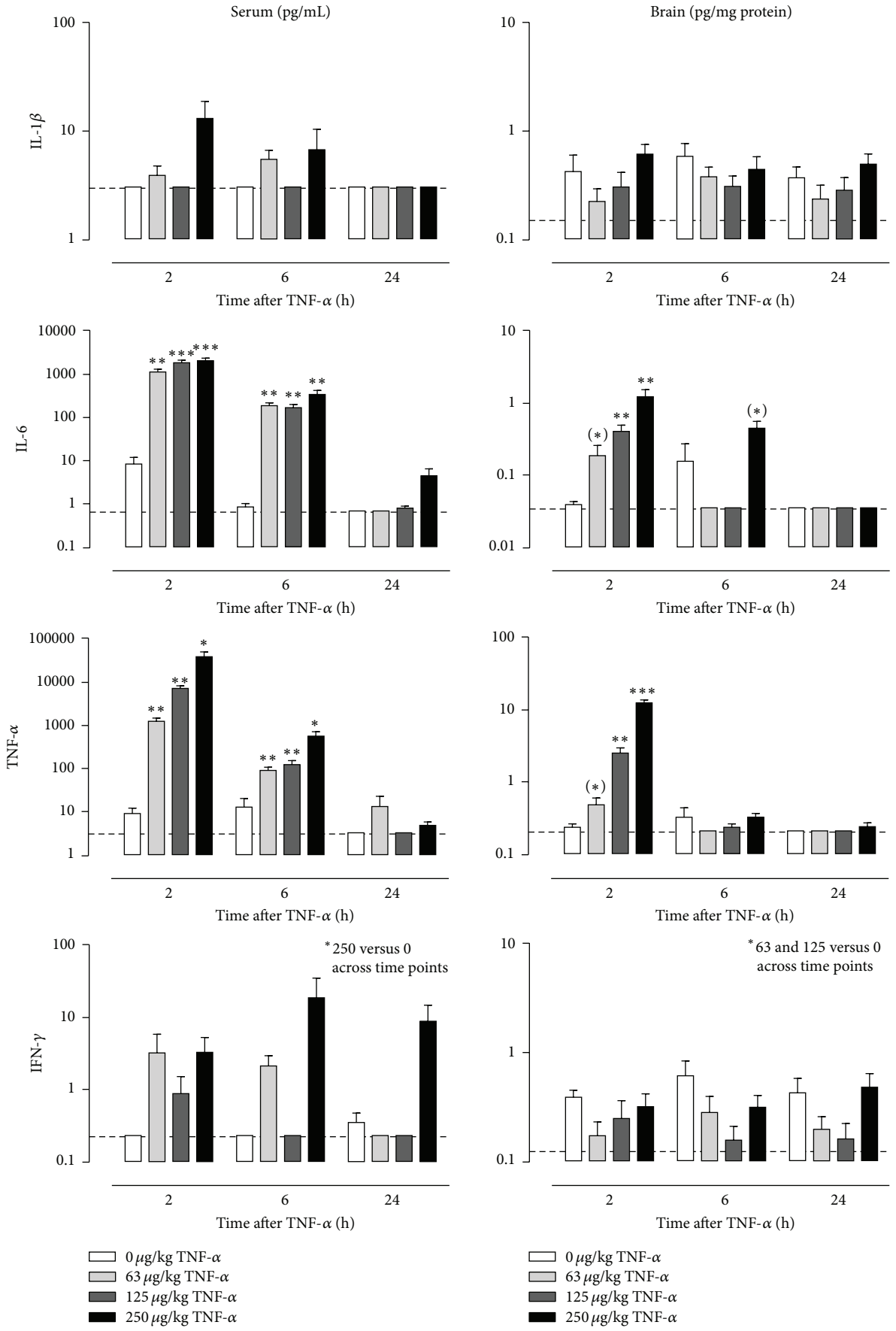
3.3. TNF-α Causes Sickness but No Depressive-Like Behavior. Sickness behavior in rodents is commonly evaluated by measuring changes in body weight and by assessing their locomotor activity in the OFT. Unfortunately, 2 out of 10 mice that were injected with 1000 μg/kg TNF-α died within 24 h after administration. These subjects were removed from the analyses, resulting in a group size of $n = 8$ for this dose. rmANOVA showed a time × dose interaction for change in body weight ($F(6, 68) = 24.9$; $P < 0.001$; $\varepsilon = 0.95$). Post hoc analysis demonstrated that there was a dose-dependent weight reduction at 24 h and 48 h after TNF-α administration (Figure 4(a)). Mice that were injected with 63 μg/kg and 250 μg/kg TNF-α started to gain weight at 48 h, while mice in the 1000 μg/kg TNF-α group continued to lose weight.

A significant time × dose interaction was found for total distance travelled in the OFT ($F(9, 102) = 10.2$; $P < 0.001$; $\varepsilon = 0.70$). At 2 h after systemic application, TNF-α reduced locomotor activity in a dose-dependent manner (Figure 4(c)). By 6 h, the total distance travelled by mice administered with 63 μg/kg TNF-α had normalized to control levels, while it further declined in animals from the 250 μg/kg and 1000 μg/kg group. At 24 h, animals from the 250 μg/kg group had recovered whereas this took up to 48 h for mice injected with 1000 μg/kg TNF-α.

Based on the mortality rate of 20% in mice that received 1000 μg/kg TNF-α, it was decided to take 250 μg/kg TNF-α as the highest dose and to introduce a 125 μg/kg TNF-α group in all of the behavioral experiments that followed. To test the effect of this additional dose on body weight and locomotor activity, a second, independent OFT was performed. rmANOVA showed a time × dose interaction for change in body weight ($F(2, 36) = 6.0$; $P < 0.05$; $\varepsilon = 0.74$) and a main effect of time ($F(3, 54) = 14.2$; $P < 0.001$; $\varepsilon = 0.66$) and dose ($F(1, 18) = 7.0$; $P < 0.05$; $\varepsilon = 0.66$) for distance travelled in the OFT. Post hoc analysis revealed that the weight of mice injected with 125 μg/kg TNF-α was reduced at 24 h, but not anymore at 48 h (Figure 4(b)). Moreover, systemic administration of 125 μg/kg TNF-α decreased the distance travelled at 6 h, but not at any of the other time points measured (Figure 4(d)).

In the FST an animal is placed in a water-filled cylinder from which it cannot escape. Behavioral despair can be evaluated in this paradigm by quantifying duration of immobility, which can be confirmed by measuring the total distance the animal swims. rmANOVA revealed a significant effect of time, but not of dose, for total distance ($F(3, 108) = 20.4$; $P < 0.001$; $\varepsilon = 0.77$) and immobility time ($F(3, 108) = 38.0$; $P < 0.001$; $\varepsilon = 0.75$). Post hoc analysis showed that compared to the 2 h time point all animals swam less and remained immobile longer at 6 h, 24 h, and 48 h after TNF-α (Figures 4(e) and 4(f)). This happened independently of the TNF-α dose given and indicates habituation to the experimental procedure during retesting.

In the SPT an animal's preference for a sweetened solution versus water is measured. This paradigm allows evaluating sickness by assessing total volume intake while reductions in sucrose preference can be used as a measure for anhedonia, which is a key symptom of depression. rmANOVA demonstrated a significant effect of time ($F(3, 108) = 26.0$;



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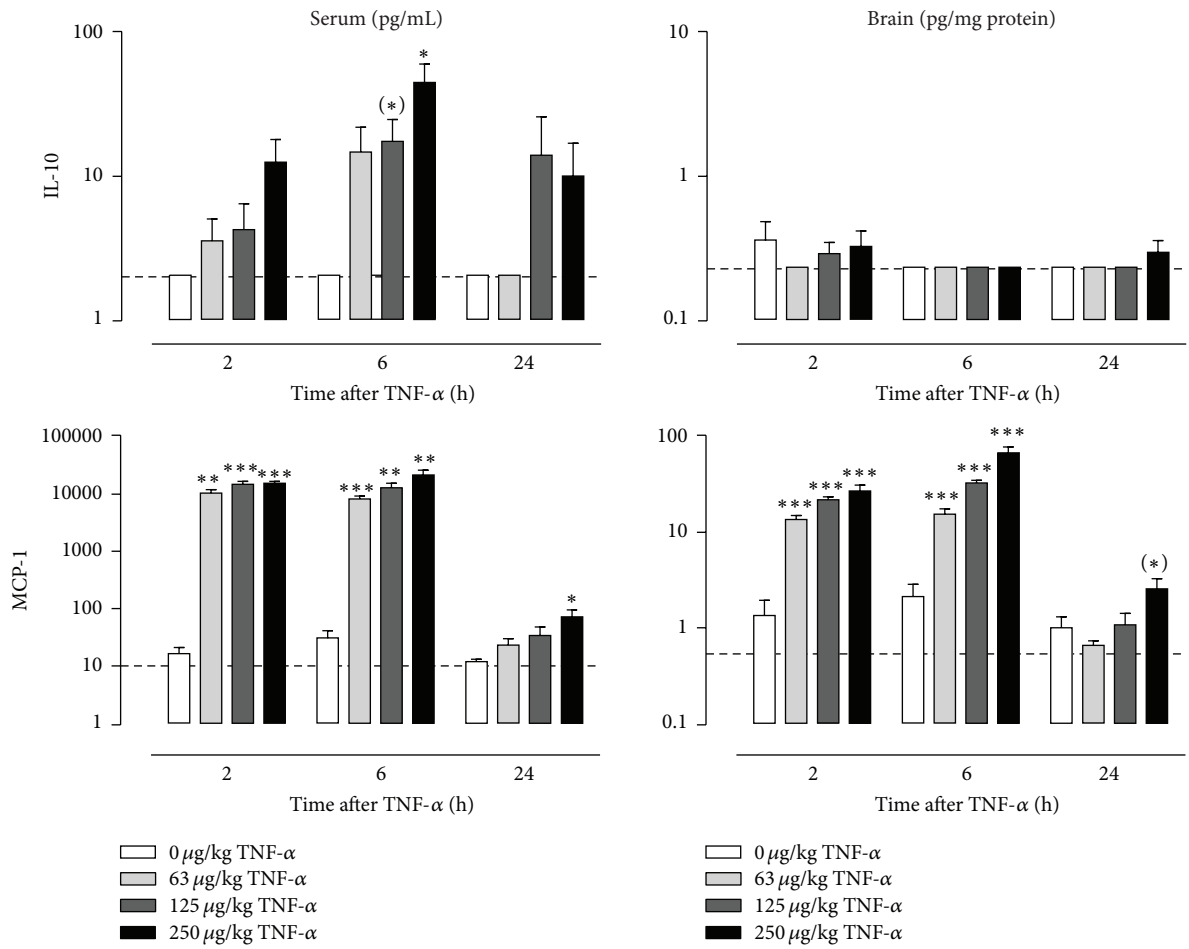


Figure 1: Peripheral TNF-α administration induces the release of immune mediators in serum and brain. Time course of serum (left) and brain concentrations (right) of interleukin- (IL-) 1β, IL-6, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-10, and monocyte chemoattractant protein-1 (MCP-1) as measured at 2 h, 6 h, and 24 h after i.p. TNF-α injection. Note that serum concentrations are shown as pg/mL while brain levels are expressed in pg/mg protein. Dashed lines indicate the detection limit of the measured analyte. Graphs are plotted as mean + SEM (n = 6-7 per group). Data were analyzed by ANOVA followed by independent samples t-test. $(*)$ $0.1 < P < 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to 0 μg/kg TNF-α.

$P < 0.001$; $\varepsilon = 0.98$) and dose ($F(3, 36) = 4.0$; $P < 0.05$; $\varepsilon = 0.98$) for total volume intake. Moreover, there was an effect of time ($F(3, 108) = 23.5$; $P < 0.001$; $\varepsilon = 0.96$) and a trend for dose ($F(3, 36) = 2.6$; $P = 0.07$; $\varepsilon = 0.96$) for sucrose preference. At 2 h and 6 h after administration, animals that were injected with 250 μg/kg TNF-α drank significantly less than vehicle-treated controls, while mice that received 125 μg/kg TNF-α only showed reduced fluid intake at 6 h (Figure 4(g)). Sucrose preference was lower at 2 h and 6 h in animals injected with 250 μg/kg TNF-α but not at lower doses (Figure 4(h)).

All animals including the vehicle-injected controls showed reduced total volume intake at 6 h when compared to the other time points. This probably resulted from the fact that the fluid-deprived mice were allowed to drink at 2 h and hence were less thirsty at 6 h. To exclude that the effects of TNF-α were confounded by retesting the same animals over time, the SPT study was repeated for the 6 h and 24 h time points using separate groups of TNF-α challenged mice for each time point. In this second SPT study there was a main effect of time ($F(1, 72) = 5.7$, $P < 0.05$) and dose ($F(3, 72) = 9.0$, $P < 0.001$) for total volume intake and a main effect of dose ($F(3, 72) = 3.3$, $P < 0.05$), but not time, for sucrose preference. Post hoc analysis revealed that using naive animals for each time point stabilized total volume intake in mice injected with vehicle (Figure 5(a)). At 6 h after administration, all TNF-α-treated mice drank less than their vehicle-injected controls. Volume intake at 24 h was only significantly reduced in mice administered with 250 μg/kg TNF-α. Sucrose preference across both time points was reduced in mice that received 125 μg/kg TNF-α, but not at any of the other doses (Figure 5(b)).

4. Discussion

A substantial set of literature data indicates a link between activation of the immune system and depression, at least in subpopulations of patients. Several clinical observations

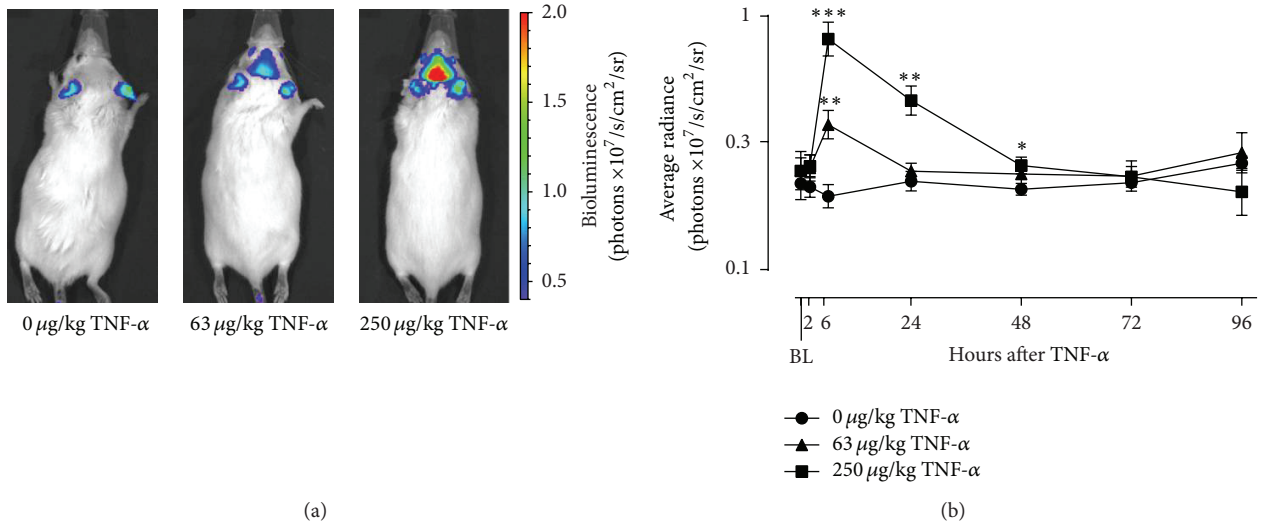


FIGURE 2: TNF-α activates astrocytes in a dose- and time-dependent manner. Intraperitoneal injection of TNF-α caused a clear bioluminescent signal in the brain of Gfap-luc mice, as shown in representative images taken at 6 h after injection (a). This signal peaked at 6 h and then gradually waned over time (b). The color scale indicates the number of photons emitted from the animal per second. The graph is plotted as mean ± SEM ($n = 7$ per group). Data were analyzed by rmANOVA followed by independent samples t-test. BL: baseline. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to 0 μg/kg TNF-α.

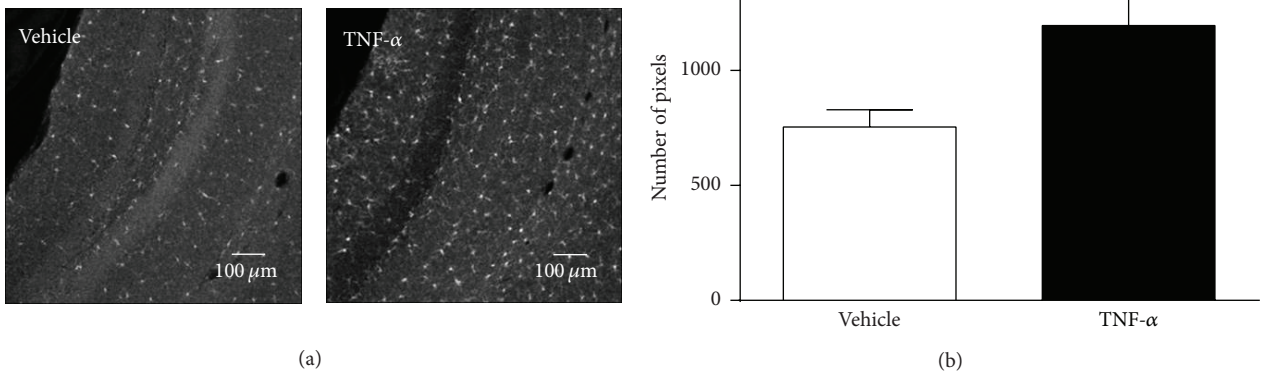
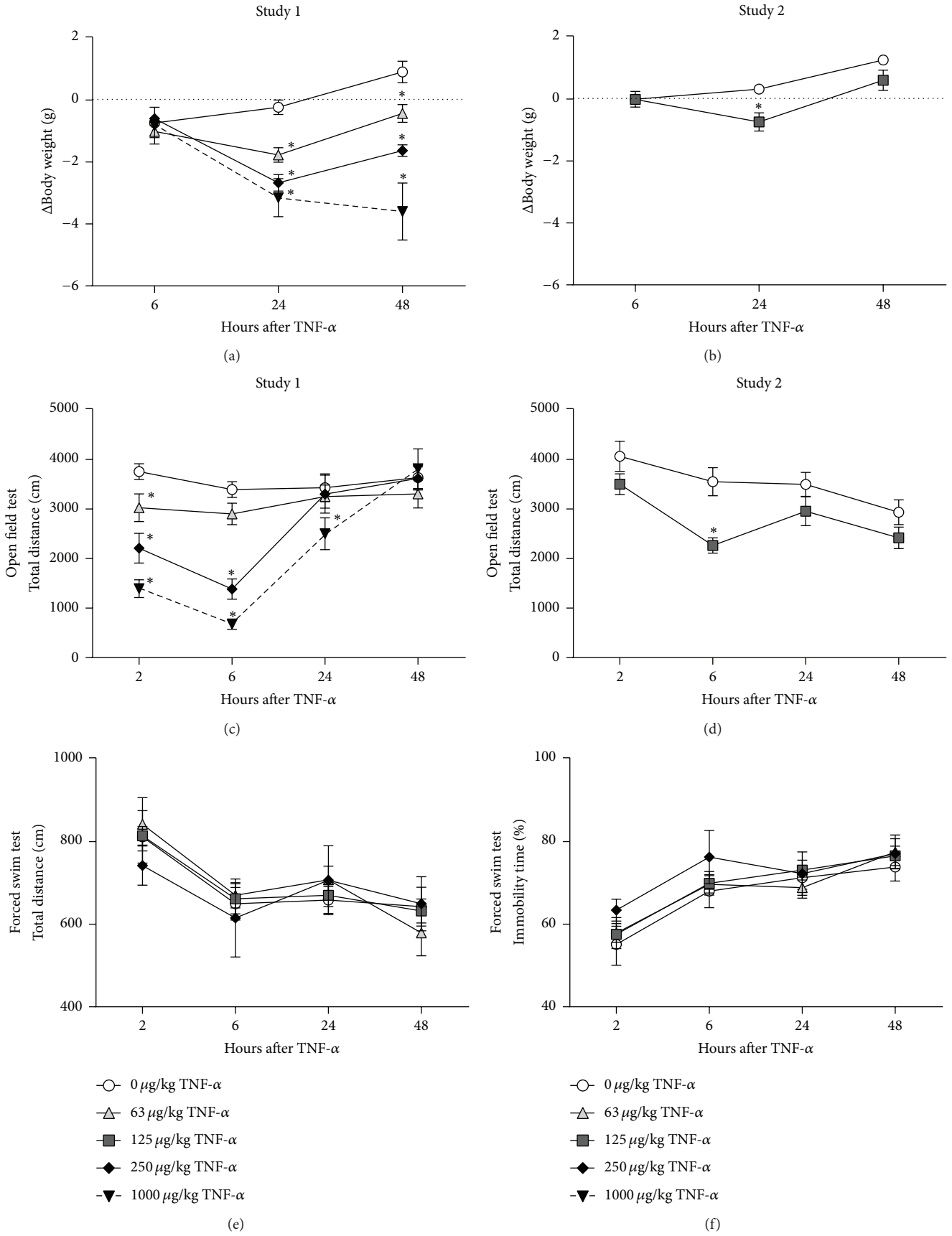


FIGURE 3: TNF-α increases Iba1 immunoreactivity in the dentate gyrus. TNF-α (250 μg/kg, i.p.) caused a strong upregulation of the microglial activation marker Iba1 in the hippocampal dentate gyrus at 24 h after administration. Representative images (10x) (a) and image quantifications of $n = 8$ per group (b). Graph is plotted as mean + SEM. Data were analyzed by ANOVA followed by independent samples t-test. $^{*}P < 0.05$ compared to vehicle.

suggest that TNF-α is one of the key cytokines contributing to the development of inflammation-associated depression. In this series of experiments, we tested whether peripheral administration of TNF-α in mice is able to induce neuroinflammation as well as behavioral changes relevant to human depression.

TNF-α is a pleiotrophic cytokine that plays an important role in the early stages of inflammatory responses and in triggering the release of downstream immune molecules [40–42]. To assess the effect of peripheral TNF-α administration on immune activation in mice, we measured serum and brain levels of a selection of immune mediators. As expected, systemic injection of TNF-α caused a robust dose-dependent increase in circulating levels of TNF-α. Due to the fact that recombinant mouse TNF-α was administered, it was not possible to discriminate injected from endogenously produced TNF-α. Previous studies have shown that systemic injection of TNF-α upregulates cytokine gene expression in the liver [29, 43], thereby indicating that TNF-α is capable of eliciting a broad immunological response. In line with these findings, we found that peripheral TNF-α administration increased circulating levels of the proinflammatory immune mediators IL-6 and MCP-1. Moreover, the peak release of these factors was followed by an increase in the serum concentration of IL-10. This cytokine is a potent anti-inflammatory mediator that plays a role in attenuating inflammatory responses and



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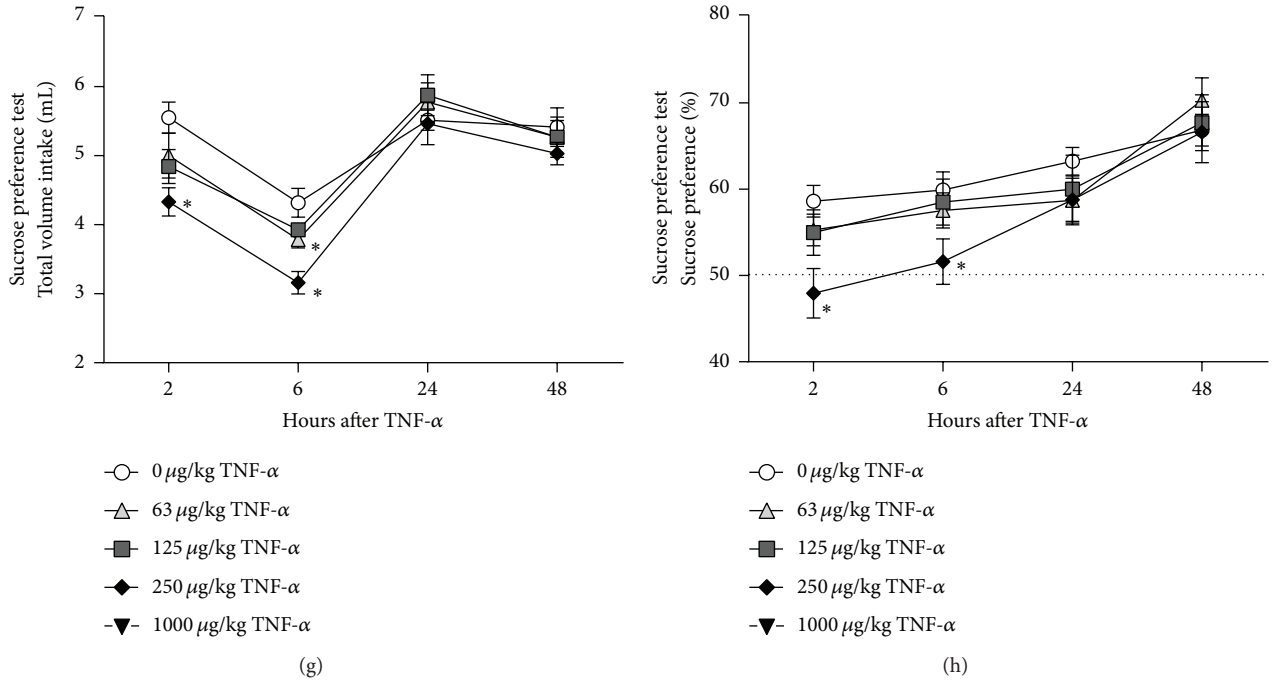

Figure 4: TNF-α causes sickness, but no clear depressive-like behavior. Systemic injection of TNF-α caused body weight loss ((a)-(b)), reduced locomotor activity in the open field test ((c)-(d)), and decreased total fluid intake in the sucrose preference test (g). Measures of behavioral despair in the forced swim test were not affected by administration of TNF-α ((e)-(f)). A high dose of TNF-α did decrease sucrose preference in the SPT (h) but this can be considered biologically irrelevant due to the overlapping time course of sickness. The dashed line in the sucrose preference test indicates the chance level (50%) for sucrose preference. Please note that the y-axis does not start at 0 for the forced swim and sucrose preference test data. Graphs are plotted as mean ± SEM (n = 10 per group, except n = 8 for 1000 μg/kg TNF-α). Data were analyzed by rmANOVA followed by independent samples t-test. $^{*}P < 0.05$ compared to 0 μg/kg TNF-α.

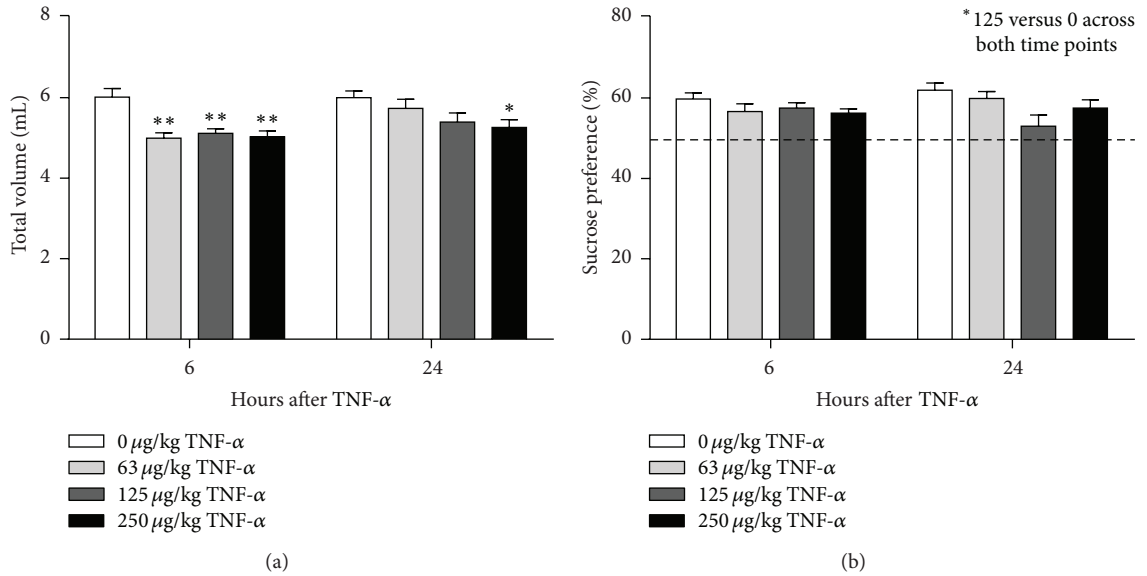

Figure 5: Peripheral TNF-α administration reduces total fluid intake (a) but does not induce clear anhedonia (b) in the sucrose preference test. Separate groups of naive animals were injected i.p. with TNF-α and tested in the SPT at either 6 h or 24 h. Dashed line indicates chance level for sucrose preference. Graphs are plotted as mean + SEM (n = 10 per group). Data were analyzed by ANOVA followed by independent samples t-test. $^{*}P < 0.05$; $^{**}P < 0.01$ compared to 0 μg/kg TNF-α at the same time point.

suppressing the expression of proinflammatory cytokines [44]. Apart from MCP-1 levels in mice treated with the highest dose of TNF-α, the concentration of all cytokines had returned to baseline values at 24 h. This indicates that the inflammatory response to a single injection of TNF-α is short-lasting. Our cytokine data corroborates with findings from a recent study where systemic TNF-α was also reported to increase circulating levels of pro- and anti-inflammatory mediators [29]. However, not all of our findings are in line with those described by Skelly et al. In our study, for example, TNF-α-induced increases in IL-6 were of a higher magnitude than the ones previously described [29]. Moreover, in contrast to Skelly's data, we were not able to detect statistically significant increases in IL-1β levels. This was unexpected as TNF-α is known to induce the expression and release of IL-1β [29, 43]. These discrepancies may in part result from differences in experimental protocols, including the use of dissimilar recombinant TNF-α, mouse strains and gender, blood sampling methods, and potentially the sensitivity of the techniques used to quantify cytokine levels.

Several *in vitro* studies indicate that cross-regulation occurs between TNF-α and interferons [45–49]. However, results from these studies are often contradictory and the effect of TNF-α on interferon synthesis seems to depend on the inflammatory condition, the type of interferon (type I or type II), and the cell type studied [50]. Effects of TNF-α on IFN-γ levels *in vivo* are poorly described in literature. Our study did not detect a robust effect of TNF-α administration on IFN-γ levels in either serum or brain.

Cytokines from the periphery can pass the BBB through various mechanisms and access the brain [51]. TNF-α influences these processes in several ways. For example, TNF-α increases the permeability of the BBB [52], thereby facilitating the passage of relatively large molecules such as cytokines from the blood into the brain. Moreover, TNF-α stimulates the release of the chemokine MCP-1, which increases BBB permeability even further and subsequently drives the infiltration of leukocytes into the brain [53]. Accordingly, we found that brain levels of TNF-α, IL-6, and MCP-1 transiently increased in response to peripheral TNF-α administration. As we did not assess the integrity of the BBB, it is not clear whether these immune mediators entered the brain through a leaky BBB and/or if they were actively produced and released locally in the brain. It is possible that a fraction of the measured brain cytokine levels originated in the periphery. However, several studies described that bolus injected TNF-α is rapidly cleared and has a short half-life of up to 20 minutes [54–56]. These findings together with reports of cytokine gene expression in the brain following peripheral TNF-α administration [29] indicate that de novo transcription of these molecules does occur within the brain.

Besides elevated concentrations of proinflammatory cytokines, depressed patients frequently display increased circulating levels of MCP-1 [57, 58]. This chemokine is an important regulator of brain inflammation following a peripheral immune challenge [59, 60]. Additionally, MCP-1 has been suggested to act as a modulator of neuronal activity and neuroendocrine functions [61, 62]. In our study, peripheral TNF-α caused a robust release of MCP-1 both in serum and in whole brain tissue. As MCP-1 is preferentially expressed in the hippocampus and other neuroanatomical regions linked to depressive symptoms [62], it may play an important role in the development of inflammation-associated depression.

The neuroinflammatory response to peripheral TNF-α was further characterized using a transgenic mouse line that expresses luciferase under the transcriptional control of the Gfap promoter. GFAP is an intermediate filament protein that is predominantly expressed by astrocytes, and its expression is upregulated when astrocytes are activated [63]. These Gfap-luc mice thus allow noninvasive quantification of Gfap mRNA expression, as a marker of astrocyte activation, in living mice over time. We found that systemic administration of TNF-α caused a strong dose- and time-dependent activation of astrocytes. This TNF-α-induced astrocyte activation occurred after the peak release of proinflammatory cytokines and lasted for 2 days, thereby suggesting that the brain sequelae to a peripheral immune challenge may propagate in absence of the initial stimulus.

Although quantification of glial cell activation using bioluminescence imaging offers numerous advantages, this technique does not allow for spatial discrimination of specific brain regions. However, previous work has shown that neuroinflammatory responses to a peripheral immune challenge are brain region specific [64–66]. To confirm glial activation at a cellular level, focusing on another cell type and a specific brain area, we quantified the expression of Iba1 in the hippocampus. This brain structure is associated with depression and has previously been shown to display immune cell activation following a peripheral immune challenge [27, 67, 68]. In the brain, Iba1 is primarily expressed by microglia and its expression is upregulated upon microglial activation [69]. Consistent with measures of astrocyte activation in Gfap-luc mice, TNF-α injection increased Iba1 immunoreactivity in the hippocampus of FVB wild-type mice. This indicates that, in addition to astrocytes, microglia also show signs of activation following peripheral TNF-α administration.

Activated microglia are known to release proinflammatory cytokines, particularly TNF-α and IL-1β, but brain levels of these cytokines were not elevated at the time point at which we observed microglial activation. This may partly be explained by the fact that we quantified cytokine levels in the whole brain and not in specific brain regions. Moreover, assessing protein levels of cytokines in the brain is hampered by the limited sensitivity of available quantification techniques. This problem could be overcome by quantifying transcript expression using quantitative PCR, which is more sensitive approach compared to measuring protein levels of immune mediators in brain tissue. Cytokine as well as chemokine activity, however, is not only limited by gene expression, but also regulated at the posttranscriptional and posttranslational level [44, 70, 71]. Therefore, assessing protein levels of cytokines is suggested to be a more accurate indicator of cytokine activity [72]. Irrespective of differences in assay sensitivity, our data align with results from previous

studies showing that cytokine expression in the hippocampus and hypothalamus was no longer elevated at 24 h after peripheral TNF-α administration [29].

To our knowledge, no study has systematically assessed the time course of sickness and depressive-like behavior following systemic TNF-α administration. After confirming that peripheral injection of TNF-α induces a central inflammatory response, we evaluated the time course of TNF-α-induced behavioral changes across a panel of assays commonly used to study sickness and depressive-like behavior in rodents. Our behavioral data demonstrate that TNF-α dose dependently induces sickness during the first 24 h after systemic administration. This could be seen as a decrease in body weight, reduced exploration in the OFT, and suppressed drinking in the SPT. In contrast to i.c.v. administration [30, 31], peripherally injected TNF-α did not affect measures of behavioral despair in the FST. Moreover, mild signs of anhedonia observed in the SPT overlapped with the time course of sickness and can therefore be considered biologically irrelevant. One limitation in our study is the within-subject design for the individual behavioral paradigms. This approach allowed reduction of animal numbers but also led to habituation of the mice to some of the experimental paradigms. Such habituation effects were observed in vehicle-injected control animals upon retesting in the FST (i.e., less swimming and longer immobility time) and in the SPT (i.e., less drinking at 6 h than at 2 h). To rule out that effects of TNF-α were missed because of habituation during retesting, the SPT study was repeated using separate groups of naive animals for the 6 h and 24 h time points. From this experiment it also became clear that peripheral TNF-α administration induced sickness, but not anhedonia. Taken together, the behavioral data indicate that acute systemic injection of TNF-α is not a reliable model to induce depressive-like behavior in mice. Based on the strong but short-lasting effects of TNF-α on neuroinflammation and behavior, it may be possible that prolonged or intermittent administration of TNF-α, leading to chronic upregulation of cytokines, offers a more valid approach to study depressive-like behavior in rodents. Such chronic TNF-α administration would mimic the human situation where inflammation-associated depression is believed to develop on a background of sustained, low-grade inflammation.

5. Conclusions

The present set of experiments using a variety of techniques and readouts showed that systemically administered TNF-α induced a strong but temporal release of immune mediators in the circulation and the brain. This release of inflammatory factors was followed by glial cell activation, as measured by astrocyte activation in the Gfap-luc mouse and increased Iba1 immunoreactivity in the hippocampus of FVB wild-type mice. Additionally, systemic administration of TNF-α led to a strong sickness response and mild signs of anhedonia. Due to the overlapping time course of these behavioral states it was not possible to unambiguously distinguish depressive-like behavior from sickness. Taken together, these results demonstrate that TNF-α in the periphery affects the central nervous system by inducing neuroinflammatory processes and behavioral changes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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